Cotton Technical Assistance Programme for Africa

Fellowship training programme on

"Applied Cotton Biotechnology"

Central Institute for Cotton Research – Nagpur

21 October to 20 December 2013

Compendium of Lectures / Training Manual

INDIA-AFRICA FORUM SUMMIT

ENHANCING PARTNERSHIP. SHARED VISION.

सत्यमेव जयते

Programme Implementing Agencies

Department of Agriculture & Cooperation
Ministry of Agriculture
Government of India

DOCD | CICR | CIRCOT | ILFS

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Strengthening the Value Chain

Fellowship training programme on

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Ministry of commerce and Industry (MoC)
Government of India

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Ministry of External Affairs (MEA)
Government of India

Programme Implementing Agencies

Directorate of Cotton Development (DOCD)

Central Institute for Cotton Research (CICR)

Central Institute for Research on Cotton Technology (CIRCOT)

IL&FS Cluster Development Initiative Limited

Preface

Fellowship Training programme on "Applied Cotton Biotechnology" for strengthening the Value Chain is an International capacity building program under Cotton Technical Assistance Programme for Africa with IL&FS Clusters, New Delhi as the Project Implementing Agency. This programme was launched by the Hon'ble Minister for Commerce, Industry and Textile, Government of India. Ministry of Commerce has identified CICR, Nagpur as premier ICAR institute and implementing agency to undertake the specific activities related to cotton cultivation. Twelve delegates from six countries of Africa including C-4 countries (Benin, Burkina Faso and Chad), Nigeria, Uganda and Malawi are participating in this training programme at Central Institute for Cotton Research, Nagpur from October 21 to December 20, 2013.

This programme was formulated to fulfil Prime Minister of India's commitment to share its scientific and technical expertise with Nigeria, Uganda, Malawi and C-4 countries (Benin, Burkina Faso and Chad). This comprehensive 'Technical Assistance Program' was conceived to provide necessary soft skills to the staff and professionals of these countries that would act as catalyst to improve existing value chain. This initiative has set its goal as 'Strengthening Competitiveness of cotton Value chain in Africa' through enhanced capabilities of engaged stakeholders.

The main objectives of the training are to enhance basic and applied knowledge in research for the improvement of cotton yield, fibre quality, development of new varieties to resist biotic and abiotic stresses, exposure to molecular breeding, DNA fingerprinting and transgenic cotton development. Training modules are designed to cover basic biotechnology techniques like gene isolation, gene cloning, bacterial transformation, gene construct preparation, tissue culture, plant genetic transformation, biosafety, molecular breeding, DNA Fingerprinting, Bioinformatics, IPR (intellectual property rights) etc. This program would provide hands-on training to participants on current techniques of cotton biotechnology with latest equipments used in the field of molecular biology. All our faculties are well trained in field and laboratory techniques and have years of experience in R&D. All trainees would get transformed with advanced techniques, aptitude, confident and enhanced knowledge on molecular breeding, genetic engineering & biotechnology. The programme is also designed to have theory sessions along with hands on training, study tours, field visit etc., to provide participants confidence to carry out current molecular biology techniques. For any further queries or clarification the participants are invited to get in touch with the Director or Coordinators. I am confident that with well trained faculty at CICR, Nagpur, the training programme would fulfil its objectives as well as expectations envisaged at the inception of programme.

Dr. K.R. Kranthi
Director, CICR, Nagpur

ACKNOWLEDGEMENTS

We thankfully acknowledge the guidance, help and encouragement received from Dr. S. Ayyappan, DG, ICAR, Dr. S. K. Datta, DDG (CS) and Dr. N. Gopalakrishnan, ADG (CS), ICAR, New Delhi. Thanks are also due to Heads of the Divisions, CICR, Nagpur Dr. Suman Bala Singh, Dr. Sandhya Kranthi and Dr. S. B. Nandeshwar. Inputs and Cooperation rendered by all the contributors and committee members deserved heartfelt appreciation. We would also thank one and all at CICR, Nagpur who have directly or indirectly assisted for conducting this training programme successfully.

Course Director	*Nodal Co-ordinator*	*Course Co-ordinators*
Dr. K R Kranthi	*Dr. Blaise Desouza*	*Dr. G Balasubramani & Dr. K P Raghavendra*

BROCHURE
Training programme on "Applied Cotton Biotechnology" under Cotton TAP for Africa

Introduction:

Cotton is a natural cellulose fiber grows around seeds. The fiber is almost pure cellolose, which is spun into yarn or thread and used to make soft clothes. It also provide oil for human consumption, feed for livestock, biomass for particle board, corrugated box, paper etc. The cotton plant is a shrub native to tropical and subtropical regions around the world, including the Americas, Africa, and India. Cotton is sustainable, renewable and biodegradable products grown in >75 countries in 32 million hectares but it faces a lot of challenges for successful cultivation. With a view to develop a Centre of excellence for carrying out basic and strategic research to improve yield, fibre quality, by-product, create new genetic variability, assist in the transfer of modern cotton production technology to various user and extend consultancy and linkages with international agencies, the ICAR (Indian Council of Agricultural Research) has established the Central Institute for Cotton Research at Nagpur in April, 1976.

The institute has been recognized all over the world for its outstanding work on cotton breeding, development of Bt-cotton, development of immunological diagnostic kits, basic research on insect resistance to insecticides Cry toxins and xenobiotics, development and dissemination of IRM (Insecticide Resistance Management), INM (Integrated Nutrient Management) and IPM (Integrated Pest Management) technologies for conventional and Bt-cotton. The institute has developed several transgenic events including *G. arboreum* and *G. hirsutum* genotypes incorporating *cry1Ac*, *cry1F* and multigenes (*cry1Ac & cry2Ab).* The institute played a major role in supporting research for the introduction and popularization of Bt-cotton in India. By developing package of practices and appropriate strategies to optimize input use and maximize benefits from Bt-cotton. The institute has the world's largest germplasm collection (10,375) and a large collection of wild species (26). Thirty-six (36) genetic stocks (*G. hirsutum* – 26 and *G. arboreum* – 10) have been registered for their unique, novel and distinct characteristics.

CICR has developed and released twelve varieties of *Gossypium hirsutum,* three varieties of *G. arboreum* and one variety of *G. barbadense* and nine intra-*hirsutum* hybrids, two interspecific (*G. hirsutum* x *G. barbadense*) hybrids and one intra-*arboreum* hybrid including both conventional and male sterility based. Several other innovative aspects of useful research include the discovery of apomixes, cleistigamy, temperature sensitive male sterility and five-loculed genotypes.

The institute have developed immunological diagnostic kits (Bt-express, Bt-Quant and gus-test) for quick detection of Bt and non-Bt cotton hybrids which have been patented in South Africa, China Uzbekistan, Mexico and South Korea thus far. These kits became very popular all over the country and are being used by farmers' seed testing labs and extension workers. The Ministry of Agriculture declared CICR as 'National Bt-Referral laboratory' to provide expertise for regulatory purposes to maintain the seed purity and seed quality of Bt-cotton. The institute has won several awards for its outstanding work in development and dissemination of IRM strategies.

Objectives:

The main objectives of the training are to enhance basic and applied knowledge in research for the improvement of cotton yield, fibre quality, development of new varieties to resist biotic and abiotic stresses, exposure to molecular breeding, DNA fingerprinting and transgenic cotton development.

Contents:

Training modules are designed to cover basic biotechnology techniques like gene isolation, gene cloning, bacterial transformation, gene construct preparation, tissue culture, plant genetic transformation, biosafety, molecular breeding, DNA Fingerprinting, Bioinformatics, IPR (intellectual property rights) etc. This program would provide hands-on training to participants on current techniques of cotton biotechnology with latest equipments used in the field of molecular biology. All our faculties are well trained in field and laboratory techniques and have years of experience in R&D. All trainees would get transformed with advanced techniques, aptitude, confident and enhanced knowledge on molecular breeding, genetic engineering & biotechnology.

Durations and dates

The duration of training program would be two months from 21st October to 20th of December 2013. There are 5-modules are prepared to cover all the basic and applied research of biotechnology and molecular breeding. As the duration of program is quite long, all the participants would be given chance to get acquainted with all the techniques to carryout independently.

Venue

The training program will conducted in Biotechnology laboratories of Central Institute for Cotton Research, Panjari Farm, Nagpur, India.

The training course will cover:

1. General Techniques in Biotechnology

Biotechnology techniques available to plant researchers allow for the identification, isolation and alteration of genes and their reintroduction into target organisms in order to produce transgenic varieties.

- Introduction to basic of biotechnology
- Good Laboratory Practices (GLP)
- Sterilization technique
- Stock and reagent preparation- calculations
- Lab Equipments principles and applications
- Plant genomic DNA isolation
- Agarose gel electrophoresis
- Polymerase chain reaction (PCR)
- Basics of Bioinformatics & Primer designing

2. Techniques in Isolation and cloning of gene

This module is to demonstrate how the DNA of a particular gene in an organism's genome isolated and cloned into a plasmid vector i.e. recombinant DNA (rDNA). Then, the cloned gene can be used for many things and can also be sequenced and studied. This experiment will illustrate some of the basic techniques often found today in laboratories that use recombinant DNA technology. The basics of the experiment will be demonstrated by cloning gene isolated through PCR based method. Techniques

- Gene isolation by PCR
- Methods of gene cloning-TA & Directional cloning
- Gel elution and ligation
- Bacterial Transformation
- Screening of transformants
- Plasmid isolation for sequencing
- Total RNA isolation &cDNAsynthesis
- RT-PCR

3. Transgenic cotton development and molecular characterisation

Plant tissue culture or cell culture is an important tool in the development of transgenic plants, since a single transformed cell would get regenerated into a complete plant through these techniques. After successful transformation and regeneration, the foreign gene integration and its expression must be confirmed by defined molecular techniques. This module would cover all those techniques and hand on training.

- Overview of cotton transgenic research
- Plant transformation methods
- *Agrobacterium* mediated Transformation
- Direct gene transfer through Biolistic gene gun
- Molecular characterisation of putative transformants
- Gene integration analysis- Gene/Selection marker based PCR
- Southern blot technique
- Gene expression analysis of using semi quantitative and real time PCR
- Protein expression analysis- ELISA, Immuonological dip sticks
- Event identification
- Insect Bioassay
- Biosafety on GM plants
- RNA interference for Insect control
- Physiology and molecular basis of drought tolerance
- Cloning and characterization of insecticidal toxin genes from entomopathogenic nematode-bacterial system

4. Molecular cotton breeding

Application molecular markers for cotton improvement will be covered

- Plant variety Purity and Protection
- Molecular marker –Basic Principles
- DNA finger printing
- Genetic diversity analysis
- Software for genetic diversity analysis- Darwin
- Molecular phylogenetics in cotton & Software
- Mapping population
- Marker Assisted Breeding in Cotton
- MAS for disease resistance in cotton
- QTL & Association mapping

5. Biosafety issues and IPR

Dr. Norman Borlaug said that **"Transgenic plants have been hailed as a powerful way to increase agricultural productivity and to help alleviate hunger in underdeveloped nations"**, but also have raised concern over possible health, bio safety and environmental risks, pointing to the need for risk assessment and management. An overview of biosafety guidelines in India Risk assessment of growing transgenic cotton on soil biological attributes and Intellectual property rights will be given

Field visit and Study tour:

- Visit to GTC CIRCOT, NAGPUR
- Visit to Breeding support centre, Ankur seeds Nagpur
- Visit to Morarjee textile Mills, Nagpur
- Visit to Dr. PDKV University, Akola
- Visit to Mahyco Seeds, Jalna
- Visit to historical places (Ellora caves, Sevagram-Gandhi Ashram)

Contacts:

<table>
<tr><td colspan="2">Course Director:
Dr. K. R. Kranthi, Director,
Central Institute for Cotton Research(CICR),
Nagpur, Maharashtra, India</td></tr>
<tr><td colspan="2">Nodal Co-ordinator:
Dr. Blaise Desouza
Head, Crop Production Division
CICR,Nagpur</td></tr>
<tr><td>Course Co-ordinators:
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Principal Scientist, Biotechnology Section,
CICR, Nagpur
Email: bala77bio@gmail.com</td><td>
Dr. K. P. Raghavendra
Scientist, Biotechnology Section,
CICR, Nagpur
Email: kpraghavendra@gmail.com</td></tr>
</table>

Committee's for Training Programme

	Committee's	Members
1	**Organizing Committee**	The Director, (Chairman) HOD, Crop Improvement HOD, Crop Production HOD, Crop Protection Head, Biotechnology Section
2	**Purchase Committee (Tea/Coffee Vending Machine, Consumables, Stationary, Books, Training Kit (Bag, Pen, Note Book, Pendrive, Epron, Marker Pen, Caps), Momento etc...**	Dr. S.B.Nandeshwar (Chairman) Dr. PunitMohan Dr. G.Balasubramani Er. Gautam Majumdhar Dr. J.Amudha
3	**Publication (Editing and compilation)**	Dr. Sandhya Kranthi (Chairman) Dr. S.B. Nandeshwar Dr. G. Balasubramani Dr. K.P. Raghavendra Dr. K. Velmourougane Dr. J. Annie Sheeba Dr. M.S.Yadav
4	**French translation of lecture notes**	Dr. A.Sampath kumar Dr. H.B.Santosh
5	**Printing of certificates/Banner etc.**	Smt.Vandana Shri. Ghanshyam V. Deogirkar
6	**Allowance for participants**	Shri. Deepak Maheshwari, FACO (Chairman) Shri U M Narkhede Shri Vinod Bhende
7	**Arrangement of visits**	Dr. P.R.Vijayakumari (Chairman) Dr. V.Chinna Babu Naik Dr. A.Sampath Kumar Dr. H.B.Santosh
8	**Transportation**	Shri. R M Lokande
9	**Nodal Coordinators**	Dr. Blaise Desouza
10	**Course coordinators**	Dr.K.P.Raghavendra Dr.G.Balasubramani

Programme schedule for TAP training on Cotton Biotechnology at CICR – Nagpur India

Date	Day	Time	Theory Session	Speaker(s)	Practical Session	Instructor(s)
21-10-2013	Mon	1.30 pm	Arrival of 5-participants			
22-10-2013	Tue	1.30 pm	Arrival of 2-participants Biotechnology Lab visits	Dr. Nandeshwar Dr. G.Balasubramani Dr. J. Amudha Dr.KP Raghavendra		
23-10-2013	Wed	1.30 pm	Arrival of 3-participants			
24-10-2013	Thu	10.00 -1.00	Arrival of 2 participants Field visit interaction with scientists	Dr. Vinita Gotmare Dr. Sampath Kumar		
					Library Visit	Dr.KP Raghavendra Mr. Katare
Module: 1 General Techniques in Biotechnology						
25-10-2013	Fri	10.00 -11.00	Introduction to basic of biotechnology	Dr. G. Balasubramani		
		11.30-1.00	RNAi mediated gene silencing for pest management	Dr. K. R. Kranthi		
		2.00- 5.00			Lab visits and interaction with scientists	Dr. K.Velmourougane Dr.Chinnababu Naik
26-10-2013	Sat	10.00 -1.00	Pre training assessment Good Laboratory Practices (GLP)	Dr.G.Balasubramani Dr.J.Amudha Dr. Raghavendra Dr.K.Velmourougane		
		2.00- 5.00			Sterilization technique	Dr KVelmourougane
27-10-2013	Sun					

28-10-2013	Mon	10.00 -1.00	Stock and reagent preparation / calculations	Dr. J. Annie Sheeba Dr. A. Sampath Kumar Dr.KP Raghavendra		
		2.00- 5.00			Preparation of Plant DNA isolation reagents	Dr. J. Amudha
29-10-2013	Tue	10.00 -11.30	Sterilization of reagents and material	Dr. J. Amudha		
		11.30- 1.00	Lab Equipments Principles and applications	Dr. G. Balasubramani		
		2.00-5.00			Plant genomic DNA isolation	Dr. J. Amudha
30-10-2013	Wed	10.00-1.00	Inauguration of training programme			
31-10-2013	Thu	10.00 -12.00	Plant genomic DNA isolation (cont...) Agarose gel electrophoresis	Dr. J. Amudha		
		12- 1.00			Quantification of nucleic cid	Dr. J. Amudha
		2.00- 5.00	Types of Polymerase chain reaction (PCR)	Dr. G. Balasubramani		
01-11-2013	Fri	10.00 -1.00	Standardization of PCR conditions and gene isolation by PCR	Dr. KP Raghavendra		
		2.00- 5.00			Primer designing	Dr. KP Raghavendra
02-11-2013	Sat	10.00 -1.00	Gel electrophoresis and interpretation	Dr. KP Raghavendra		
		2.00- 5.00			Gel elution	Dr. KP Raghavendra
03-11-2013	**Sun**	**Holiday**				
Module 2: Techniques in Isolation and cloning of gene						

			Theory session		Practical	
04-11-2013	Mon	10.00 -1.00	Central Dogma of Molecular Biology	Dr. J. Annie Sheeba		
		2.00- 5.00			Ligation for TA cloning	Dr.KP Raghavendra
05-11-2013	Tue	10.00 -11.30	Bacterial transformation	Dr. K.P. Raghavendra		
		2.00-5.00			Bacterial transformation	Dr.KP Raghavendra
06-11-2013	Wed	10.00 -1.00	Screening transformants Chemical competent cell preparation	Dr.KP Raghavendra		
		2.00- 5.00			Bacterial Transformation	Dr.KP Raghavendra
07-11-2013	Thu	10.00 -1.00 2.00- 5.00	Directional cloning Restriction digestion for cloning	Dr.KP Raghavendra	Gel elution and ligation	Dr.KP Raghavendra
08-11-2013	Fri	10.00 -1.00 2.00- 5.00	Total RNA isolation	Dr.KP Raghavendra	cDNA synthesis	Dr.KP Raghavendra
09-11-2013	Sat	Local visit				
10-11-2013	Sun	Holiday				
11-11-2013	**Mon**	**10.00 -1.00**	RT-PCR	Dr.KP Raghavendra		Dr.KP Raghavendra
Module 3 :	**Transgenic cotton development and molecular characterisation**					
12-11-2013	Tue	10.00 -5.00	Visit to Ankur seeds	Dr.Balasubramani Dr. Santosh H.B.		
13-11-2013	Wed	10.00 -1.00	Overview of cotton transgenic research	Dr. G.Balasubramani		
		2.00- 5.00			Preparation of ½ MS media	Dr.G.Balasubramani

					and explants	
14-11-2013	**Thu**	10.00 – 1.00	Closed holiday: Local visit			
15-11-2013	Fri	10.00 -1.00	Basics of GM crops development	Dr.G.Balasubramani		
		2.00- 5.00	Cotton fiber development	Dr.G.Balasubramani	Callus induction from cotton explants-hypocotyls	Dr.G.Balasubramani
16-11-2013	Sat	10.00 -11.00	Molecular characterisation of putative transformants (a) Gene integration analysis	Dr. G. Balasubramani		
		11.30-1.00	(b)Gene expression analysis of using semi quantitative and real time PCR	Dr.J.Amudha		
		2.00- 5.00			Gene based PCR/ Selection marker based PCR /Construct specific PCR	Dr.J.Amudha
17-11-2013	**Sun**					
18-11-2013	Mon		Visit to Dr. PDKV University, Akola	Dr.G.Balasubramani Dr..HB.Santosh Mr. Ghanshyam		
19-11-2013	Tue		Visit to Mahyco Seeds Laboratory, Jalna			
20-11-2013	Wed		Visit cotton fields of Mahyco seeds, Jalna and Aurangabad			
21-11-2013	Thu		Site seeing & Return to Nagpur			
22-11-2013	Fri	10.00 -1.00	Plant tissue culture techniques	Dr.S.B.Nandeshwar		
		2.00- 5.00			Practical: MS Stock preparation & Sterilization	Dr.S.B.Nandeshwar Dr.J. Amudha
23-11-2013	Sat	10.00 -1.00	Application of tissue culture techniques	Dr.S.B.Nandeshwar		
		2.00- 5.00			Practical's contd. Media preparation (½ MS/Full MS)	Dr.S.B.Nandeshwar Dr.J. Amudha

24-11-2013	**Sun**	**Holiday**				
25-11-2013	Mon	10.00 -1.00	Development of Bt cotton	Dr.S.B.Nandeshwar		
		2.00- 5.00			Hands on training: Explants preparation-Seeds inoculation	Dr.S.B.Nandeshwar Dr. G. Balasubramani
26-11-2013	Tue	10.00 -1.00	Shoot tip culture	Dr.S.B.Nandeshwar		
		2.00- 5.00			Hands on training: Contd., *Agrobacterium* culture	Dr.S.B.Nandeshwar Dr. G. Balasubramani
27-11-2013	Wed	10.00 -1.00	Plant transformation methods	Dr. G. Balasubramani		
		2.00- 5.00			Hands on training: Co-cultivation of *Agrobacterium* + cotton explants	Dr.S.B.Nandeshwar Dr. G. Balasubramani
28-11-2013	Thu	10.00 -1.00	Gene transfer by Biolistic Gene gun	Dr. G. Balasubramani		
		2.00- 5.00			Hands on training: Contd., *Agrobacterium* mediated Transformation	Dr.S.B.Nandeshwar Dr. G. Balasubramani
29-11-2013	Fri	10.00 -1.00	Molecular characterization of “transgene”	Dr. G. Balasubramani		
		2.00- 5.00			Hands on training: Callus culture and sub-culture	Dr.S.B.Nandeshwar Dr. J. Amudha
30-11-2013	Sat	10.00 -1.00	Confirmation of gene integration by PCR and Southern blot	Dr. J. Amudha		
		2.00- 5.00			PCR amplification of transgene	Dr. J. Amudha
01-12-2013	Sun	**Holiday**				

02-12-2013	Mon	10.00 -1.00	Biosafety on GM plants in India	Dr. G. Balasubramani		
		2.00- 5.00			Confirmation of gene integration by Southern blot	Dr. J. Amudha
03-12-2013	Tue	10.00 -1.00	Development cotton leaf curl virus (CLCuV) resistant and transgenic cotton.	Dr. J. Amudha		
		2.00- 5.00			Southern blot: Contd.	Dr. J. Amudha
04-12-2013	Wed	10.00 -1.00	Development drought resistant and transgenic cotton. Gene expression study by Real Time-PCR	Dr. J. Amudha		
		2.00- 5.00			Practical: Real time PCR	Dr. J. Amudha
05.12.2013	Thu	10.00 -1.00	(c) ELISA Immunogenic dip sticks	Dr. Sandhya Kranthi		
		2.00- 5.00			ELISA Immunogenic dip sticks	Dr. Sandhya Kranthi
06.12.2013	Fri	10.00 -1.00	(d)Event identification	Dr. Sandhya Kranthi		
		2.00- 5.00			Insect Bioassay	Dr.Sandhya Kranthi
Module 4: Molecular cotton breeding						
07.12.2013	Sat	10.00 -11.00	Plant variety Purity and Protection	Dr. V. Santhy		
		2.00- 5.00			Physiology and molecular basis of drought tolerance	Dr.J. Annie Sheeba
08.12.2013	Sun	Holiday				
09.12.2013	Mon	10.00 -1.00	Molecular Phylogenetics in cotton	Dr.M.Saravanan		
		2.00- 5.00			Phylogentic software	Dr.M.Saravanan

10.12.2013	Tue	10.00 -1.00	Visit to Centre Point School & interaction with students			
		2.00- 5.00			PCR-SSR marker	Dr.V.Santhy Dr.H.B.Santosh
11.12.2013	Wed	10.00 -11.00	Molecular marker –Basic Principles	Dr. H.B. Santosh		
		11.15.-1.00	Marker assisted breeding for BLB in cotton	Dr. A. Sampath Kumar		
		2.00- 5.00			DNA finger printing and Genetic Diversity and Software	Dr.H.B.Santosh
12.12.2013	Thu	10.00 -11.30	Mapping Population	Dr.H.B.Santosh		
		2.00 – 5.00			Construction of Genetic linkage Map	Dr.H.B.Santosh
13.12.2013	Fri	10.00- 11.30	Insecticidal toxins from entomopathogenic (epn) nematodes	Dr. Nandini Gokte Narkhedkher		
		11.30- 1.00	Association Mapping	Dr.H.B.Santosh		
		2.00-5.00			QTL Mapping	Dr.H.B.Santosh
14.12.2013	Sat		Visit to Morarjee Textiles Mill	Dr.Vijaya Kumari Dr.Chinnababu Naik Dr.Sampath Kumar		
15.12.2013	**Sun**					
16-12-2013	Mon	10.00 -11.00	Determining fiber quality-Physiological Perspectives	Dr. Jayant Meshram		
		11.30- 1.00	Molecular marker and application in cotton improvement	Dr. Manoj Palak (Guest Speaker from Ankur seeds, Nagpur)		

		2-00 - 5.00			Marker assisted Breeding in Cotton	Dr.VN Waghmare
17.12.2013	Tue	10.00- 11.00	Association mapping for crop improvement Software analysis	Dr.S.Sentilvel (Guest speaker from DOR Hyderabad)		
					Association mapping for crop improvement Software analysis (Contd.	Dr.S.Sentilvel (Guest speaker from DOR Hyderabad)
18.12.2013		10.00- 11.00	Benefits of Bt cotton in India	Dr. C. D. Mayee		
		11.30-1.30	FISH & GISH Techniques	Dr. Vinita Gotmare		
		2.00-5.00			IPR (Intellectual Property Rights)	Dr. G. Balasubramani
19-12-2013	Thu	10.00 -1.00	Briefing of training program and feed back from participants			
		2.00- 5.00			Valedictory Function	
20-12-2013	Fri	10.00- 1.00	Thanks giving meeting with participants and all staff of CICR involved in the training program		Departure of participants to their respective countries via Mumbai	

Contents – Lectures

Contents – Training Manual

PART-I

LECTURES

Introduction to Basic of Biotechnology

G. Balasubramani

Principal Scientist (Biotechnology)
Central Institute for Cotton Research
Post Box No. 2, Shankar Nagar Post, Nagpur - 440 010

Introduction:

All living organisms are made up of one or more cells and cell products. The essence of a cell is its ability to grow and divide to produce progeny. All metabolic reactions in unicellular and multicellular organisms take place in cells. There are hundreds of different chemical reactions taking place within the cells of organisms. Many of these reactions are involved in providing energy for the cells. With the progress of biochemistry, it was shown that there were fundamental similarities in the chemical composition and metabolic activities of all cells. In higher organisms there is a distinction between physiology and biochemistry. But single-celled organisms like bacteria have no distinct organs or tissues there is no valid distinction between anatomy, physiology, and biochemistry. The cell wall of most bacteria consists of a single peptidoglycan molecule and bacterial nutrition involves uptake of discrete molecules, not large lumps of food.

Prokaryotes versus Eukaryotes:

Living cells are divided into two major types - **prokaryotes** and **eukaryotes**, on the basis of their genetic organization. Bacteria (including the blue green "algae") are prokaryotes. Higher organisms together with microorganisms such as fungi, algae and protozoa are eukaryotes. In eukaryotes the genetic information (DNA) is separated from the rest of the cell in a membrane bound compartment - the nucleus. In prokaryotes the DNA is free in the cytoplasm. The presence or absence of a separate nucleus defines the difference between the prokaryotic and eukaryotic cell. The presence of a separate nucleus allows eukaryotes to accumulate much more DNA per cell than prokaryotes. Consequently eukaryotic cells are usually much larger than prokaryotes. In addition the DNA of eukaryotes is generally partitioned among several chromosomes. The greater genetic complexity of eukaryotes facilitates the differentiation of cells into the complex tissues and organs seen in higher organisms. Eukaryotic cells contain several membrane-bound organelles and membrane systems. The two most prominent organelles of eukaryotes are the mitochondria and chloroplasts. Both possess their own DNA, their own ribosomes. New mitochondria and chloroplasts arise only by the division of pre-existing mitochondria or

chloroplasts. Thus these organelles are in some ways equivalent to prokaryotic cells. The endosymbiont theory proposes that mitochondria and chloroplasts evolved from symbiotic bacteria which were trapped inside larger eukaryotic cells and lost their independence.

Endosymbiont theory:

The endosymbiotic theory is the idea that a long time ago, prokaryotic cells engulfed other prokaryotic cells by endocytosis. This resulted in the development of first eukaryotic cells. How did this theory get its name? Symbiosis occurs when two different species benefit from living and working together. When one organism actually lives inside the other it's called endosymbiosis. The endosymbiotic theory describes how a large host cell and ingested bacteria could easily become dependent on one another for survival, resulting in a permanent relationship. Over millions of years of evolution, mitochondria and chloroplasts have become more specialized and today they cannot live outside the cell. This theory was proposed by Lynn Margulis (1970). Although, people accepted as fact but this theory needs scientific substantiation. Furthermore, using the DNA sequences of modern organisms, biologists have tentatively traced the most recent common ancestor of all life to an aquatic microorganism that lived in extremely high temperatures. The investigation continues and may eventually point towards a different site for the origin of life.

Important Era:

Years ago	Episode
4.5 Billion	Origin of Earth
3.5 Billion	Prokaryote Bacteria Dominate
2.5 Billion	Oxygen accumulates in Atmosphere
1.5 Billion	Eukaryotes – First nucleated cell
0.5 Billion	Cambrian Explosion of multicellular Eukaryotic organisms

Virus:

A virus is neither an organism nor a cell, yet it consists of a core of nucleic acid (DNA or RNA) enclosed in an external mantle of protein. In the free-state viruses are quite inert. They become activated only when they infect a living host cell and in the process only the nucleic acid core enter the host's cell. The nucleic acid which is the genetic substance takes over the metabolic activity of the host cell and utilises the cell machinery for the formation of more viruses, ultimately killing the host cell. In a way, thus, viruses are cellular parasites that cannot reproduce

by it. But, because viruses are primitive and simpler units of life, therefore, one should know about virus prior to other cells.

The Cell:

The cell is the basic structure of the living organism. For example, human body is built of billions and trillions of cells. Cells of different organs vary according to their function. Each cell contains the hereditary material and can make copies of themselves by reproducing and multiplying. The major components of the advanced eukaryotic cells mentioned below.

Nucleus – This is central part of the cell that carries the blue print for the cell functioning and tells the cell when to grow, reproduce and die. It also houses DNA (deoxyribonucleic acid).

Mitochondria – These are the powerhouses of the cell and produce energy for the various activities of the cell.

Cytoplasm – This is a jelly like fluid within the cell in which the other organelles float.

Endoplasmic reticulum (ER) – This helps in processing the molecules (e.g. proteins) created by the cell.

Ribosomes – These lie over the ER and process the genetic instructions or the blueprints within the DNA and create new proteins. These can also float freely in the cytoplasm.

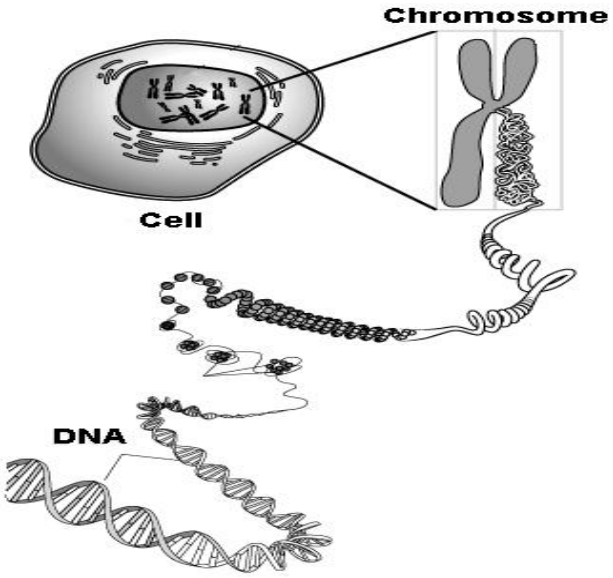
Lysosomes and peroxisomes – These help in digesting foreign bacteria that invade the cell, rid the cell of toxic substances.

Cell membrane – This is the outer lining of the cell.

The Chromosomes:

Chromosomes are thread-like structures located inside the nucleus of animal and plant cells. The term chromosome comes from the Greek words for color (chroma) and body (soma). Each chromosome is made of protein and molecule of DNA which is passed from parents to offspring, DNA contains the specific instructions that make each type of living creature

unique. Eukaryotes (cells with nuclei such as those found in plants, yeast, and animals) possess multiple large linear chromosomes contained in the cell's nucleus. Each chromosome has one centromere, with one or two arms projecting from the centromere, although, under most circumstances, these arms are not visible as such. Prokaryotes do not possess nuclei. Instead, their DNA is organized into a structure called the nucleoid. The nucleoid is a distinct structure and occupies a defined region of the bacterial cell. This structure is, however, dynamic and is maintained and remodelled by the actions of a range of histone-like proteins, which associate with the bacterial chromosome. In archaea, the DNA in chromosomes is even more organized, with the DNA packaged within structures similar to eukaryotic nucleosomes. Prokaryotic chromosomes have less sequence-based structure than eukaryotes. Bacteria typically have a single point (the origin of replication) from which replication starts, whereas some archaea contain multiple replication origins. The genes in prokaryotes are often organized in operons, and do not usually contain introns, unlike eukaryotes.



The discovery of DNA:

DNA was first discovered in 1869 by Friedrich Miescher but its structure was not determined. After 84 years the breakthrough was made by James Watson and Francis Crick in 1953 (Watson and Crick, 1953). In the early 1950s, the race to discover DNA was on. At Cambridge University, graduate student Francis Crick and research fellow James Watson had become interested. Meanwhile at King's College in London, Maurice Wilkins and Rosalind Franklin were also studying DNA. The Cambridge team's approach was to make physical models to narrow down the possibilities and eventually create an accurate picture of the molecule. The

King's team took an experimental approach, looking particularly at x-ray diffraction images of DNA. In 1951, Watson attended a lecture by Franklin on her work and she had found that DNA can exist in two forms, depending on the relative humidity in the surrounding air. This had helped her deduce that the phosphate part of the molecule was on the outside. Franklin, working mostly alone, found that her x-ray diffractions showed that the "wet" form of DNA (in the higher humidity) had all the characteristics of a helix. She suspected that all DNA was helical but did not want to announce this finding until she had sufficient evidence on the other form as well.

Wilkins was frustrated. In January, 1953, he showed Franklin's results to Watson, apparently without her knowledge or consent. Watson and Crick took a crucial conceptual step, suggesting the molecule was made of two chains of nucleotides, each in a helix as Franklin had found, but one going up and the other going down. Crick had just learned of Chargaff's findings about base pairs in the summer of 1952. He added that to the model, so that matching base pairs interlocked in the middle of the double helix to keep the distance between the chains constant. Watson and Crick showed that each strand of the DNA molecule was a template for the other. During cell division the two strands separate and on each strand a new "other half" is built, just like the one before. This way DNA can reproduce itself without changing its structure except for occasional errors, or mutations.

The structure so perfectly fit the experimental data that it was almost immediately accepted. DNA's discovery has been called the most important biological work of the last 100 years, and the field it opened may be the scientific frontier for the next 100. By 1962, when Watson, Crick, and Wilkins won the Nobel Prize for physiology/medicine, unfortunately, Franklin died. The Nobel Prize only goes to living recipients, and can only be shared among three winners.

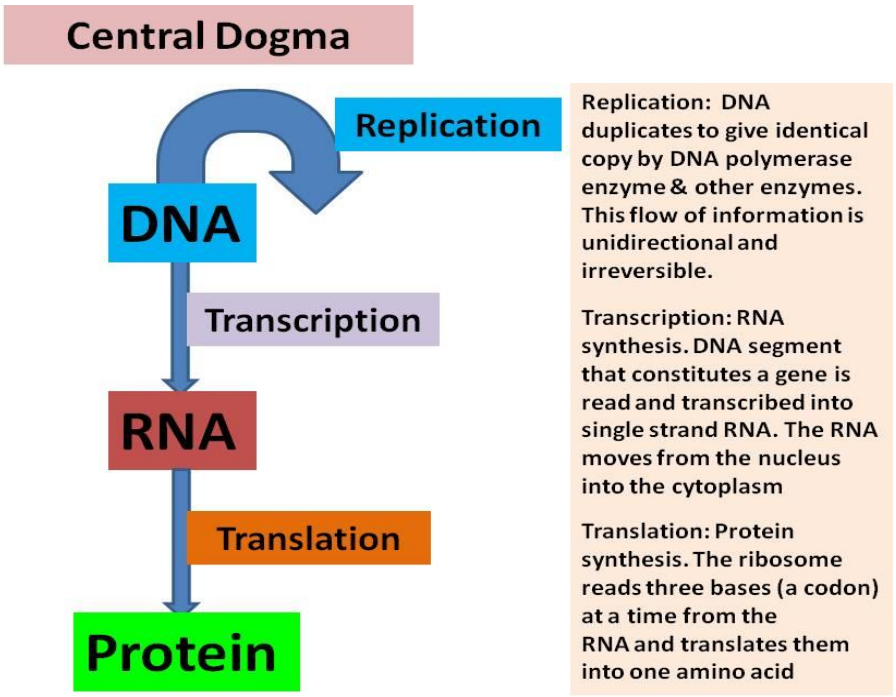
Central Dogma of molecular biology:

The central dogma of molecular biology is a framework which explains the flow of genetic information from DNA to RNA, RNA to proteins. There are 3 main processes in involved: DNA Replication, Transcription and Translation.

DNA Replication: Basically, the DNA in cells undergoes replication to make more DNA so that when cells divide, they will each have the same amount of DNA which carries the same genetic information.

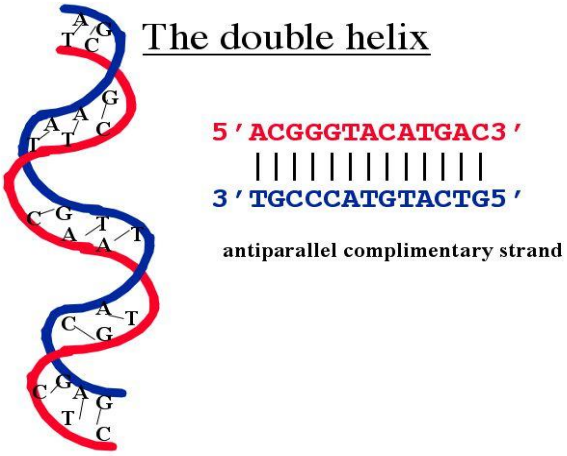
Transcription: The info in the DNA molecule is copied onto a messenger RNA molecule.

Translation: The mRNA molecule leaves the nucleus, carrying the genetic info with it. It attaches to a ribosome and translation begins. The info on the mRNA is translated into amino acids to form one long polypeptide protein. (3 mRNA bases code for one amino acid)



Deoxyribonucleic acid (**DNA**):

DNA is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms. Chemically, DNA is a long polymer of simple units called nucleotides, with a backbone made of sugars and phosphate groups joined by ester bonds. The double helical structure of DNA looks like a twisted ladder and the base pairs form the rungs of the ladder and the sugar and phosphate molecules form the sides of the ladder. This arrangement of DNA strands is called antiparallel. The asymmetric ends of DNA strands are referred to as the 5′ (five prime) and 3′ (three prime) ends. The four bases found in DNA are adenine (abbreviated A), cytosine (C), guanine (G) and thymine (T). A fifth pyrimidine base, called uracil (U), usually takes the place of thymine in RNA. The discovery of double helix DNA by Watson and Crick in 1953 enlightened a revolution in our understanding of living organism.



Types of DNA:

DNA types are differentiated by their formation and helix structure. The primary difference between the forms is the direction that the helix spirals.

B-DNA is the form commonly observed in chromosomes and Watson and Crick described this B form. B-DNA is a right-handed helix with 10 base pairs per turn. B-DNA is replicated and used in the transcription and translation of RNA, which is the molecule used for protein synthesis. B-DNA can be denatured, which means the hydrogen bonds are removed. This is essentially the first step in replicating DNA in the cell.

A-**DNA** is also a right-handed helix. However, there are more base pairs per turn. A-DNA has 11 base pairs per turn. Other than the more compact structure, A-DNA is similar to B-DNA. It is biologically active in the cell, and it forms crystallized structures in lab experiments.

Z-DNA is the type of DNA that is a left-handed helix. It is also known to be biologically active in zigzag formations of repeating base pair sequences. Z-DNA has 12 base pairs per turn, so it carries the most genes between each turn. Z-DNA plays a role in RNA transcription, which is the protein synthesis process of creating mRNA from a strand of DNA.

Ribonucleic acid (RNA):

DNA is transcribed into RNA which is synthesis protein. RNA comes in variety of shape and size. RNA is single strand. RNA contains ribose sugar and DNA contains deoxyribose sugar (which lacks one oxygen atom). There are three types of RNA such as mRNA (messenger RNA), tRNA (transfer RNA) and rRNA (ribosomal RNA). The genome of an organism is inscribed in

DNA (in some viruses RNA). The portion of the genome that codes for a protein or RNA is referred to as a gene. In RNA, thymine (T) is replaced by uracil (U).

Messenger RNA (mRNA):

Each protein-coding gene or information is transcribed or copied into a template molecule called RNA, known as messenger RNA or mRNA. This in turn is translated on the ribosome into an amino acid chain or polypeptide. The process of translation requires transfer RNAs specific for individual amino acids.

Transfer RNA (tRNA):

tRNA contains about 75 nucleotides, three of which are called anticodons, and one amino acid. The tRNA reads the code and carries the amino acid to be incorporated into the developing protein. There are at least 20 different tRNA's - one for each amino acid. The basic structure of a tRNA is shown in the left graphic. Part of the tRNA doubles back upon itself to form several double helical sections. On one end, the amino acid, phenylalanine, is attached. On the opposite end, a specific base triplet, called the **anticodon**, is used to actually "read" the codons on the mRNA. The tRNA "reads" the mRNA codon by using its own anticodon. The actual "reading" is done by matching the base pairs through hydrogen bonding following the base pairing principle. Each codon is "read" by various tRNA's until the appropriate match of the anticodon with the codon occurs. In this example, the tRNA anticodon (AAG) reads the codon (UUC) on the mRNA. The UUC codon codes for phenylalanine which is attached to the tRNA. Remember that the codons read from the mRNA make up the genetic code as read by humans.

Ribosomal RNA (rRNA):

In the cytoplasm, ribosomal RNA and protein combine to form a nucleoprotein called a ribosome. The ribosome serves as the site and carries the enzymes necessary for protein synthesis. The ribosome is made from two sub units, 50S and 30 S. There are about equal parts rRNA and protein. The ribosome attaches itself to mRNA and provides the stabilizing structure to hold all substances in position as the protein is synthesized. Several ribosomes may be attached to a single RNA at any time.

Translation:

Protein building blocks are amino acids. Amino acids are joined between the carboxyl ends of one amino acid to the amino group of the other by the enzyme called peptidyl transferase. Before the genetic code was cracked, it was a mystery as how four nucleotides could encode for 20 amino acids. Discovery of mRNA as an intermediate molecule helped crack the genetic code. Scientists developed cell extracts which when mixed with mRNA, could synthesize polypeptide chains (***in vitro* translation**). Scientists were also able to synthesize short segments of mRNA of defined sequence. Using defined mRNA sequences, they cracked the genetic code in a systematic fashion: For instance,

– UUUUUU--- = Phe-Phe---etc.
– UCUCUC--- = Ser-Leu----etc.

Some ambiguities were resolved by further experimentation. Finally it was concluded that most amino acids are encoded by multiple codons. There are potentially 64 codons, and only 20 amino acids. If each amino acid is encoded by only one codon, there would be 44 codons which would not code for any amino acid. This would imply that more than 50% of the time, a frame shift would result in a codon that would be a 'nonsense' codon. Conclusion was drawn that the genetic code is degenerate and more than one codon can code for each amino acid. Each amino acid can be coded for by more than one codon and each codon only codes for one amino acid.

Characteristics of the Genetic Code:

- It is a triplet code.
- Each three-nucleotide codon in the mRNA specifies one amino acid
- Bases are read continuously.
- mRNA is read three bases at a time without skipping any bases.
- It is non-overlapping/non-ambiguous.
- Each nucleotide is part of only one codon and is read only once during translation.
- It is almost universal.
- In nearly all organisms, most codons have the same amino acid meaning.
- It is degenerate.
- Of 20 amino acids, 18 are encoded by 2 or more codons.
- The code has start and stop signals.

- AUG is the usual start signal and defines the open reading frame.
- Stop signals are codons with no corresponding tRNA
- the nonsense or chain-terminating codons.
- generally three stop codons: UAG, UAA, and UGA.

Amino acids Symbols, codes and codons:

Amino acid	Letter symbol	3-Letter symbol	Codons
Alanine	A	Ala	GCA, GCC, GCG, GCU
Arginine	R	Arg	AGA, AGG, CGA, CGC, CGG, CGU
Asparagine	N	Asn	AAC, AAU
Aspartic acid	D	Asp	GAC, GAU
Cysteine	C	Cys	UGC, UGU
Glutamic acid	E	Glu	GAA, GAG
Glutamine	Q	Gln	CAA, CAG
Glycine	G	Gly	GGA, GGC, GGG, GGU
Histidine	H	His	CAC, CAU
Isoleucine	I	Ile	AUA, AUC, AUU
Leucine	L	Leu	CUA, CUC, CUG, CUU, UUA, UUG
Lysine	K	Lys	AAA, AAG
Methionine	M	Met	AUG
Phenylalanine	F	Phe	UUC, UUU
Proline	P	Pro	CCA, CCC, CCG, CCU
Serine	S	Ser	AGC, AGU, UCA, UCC, UCG, UCU
Threonine	T	Trp	ACA, ACC, ACG, ACU
Tryptophan	W	Trp	UGG
Tyrosine	Y	Tyr	UAC, UAU
Valine	V	Val	GUA, GUC, GUG, GUU

Protein synthesis:

Protein synthesis is simply the "making of proteins." Although the term itself is easy to understand, the multiple steps that a cell in a plant or animal must go through are not. In order to make even one protein, the body must seek the aid of messenger RNA, transfer RNA, DNA, amino acids, ribosomes, and multiple enzymes. A protein is simply a long chain of amino acids linked together by bonds. The backbone of amino acids form strong covalent bonds and the actual amino acids form temporary weak bonds. These weak bonds allow the amino acids to change shape, remain mobile, and attain flexibility. The most important quality to understand about proteins is that the position of their amino acids determines their function. During translation of

mRNA into protein, an amino acid activating enzyme attaches an amino acid to one end of transfer RNA (tRNA), also called an adaptor molecule. On the other end of the adaptor is a condon (a specific three-nucleotide code) which will be used when the adaptor reaches the mRNA. Once the condon is retrieved, the mRNA continues to the ribosome. So basically, in this stage, an adaptor is connected to an amino acid so it can be energized and have enough strength to continue its journey. A number of steps are involved during translation.

Step 1: An ATP molecule lands on the activating enzyme in a place that is prepared for it (think of this space as a reserved parking place).
Step 2: An amino acid then parks in the next space which was also prepared just for it. Step 3: The ATP molecule and the amino acid drift closer together until they bond, which releases two phosphates. At this point, the amino acid is energized.
Step 4: The adaptor then comes and parks in its reserved space next to the amino acid
Step 5: The adaptor comes closer to the amino acid until they bond.
Step 6: Energy from the ATP molecule is transferred into this bond and the ATP molecule is released.
Step 7: The activating enzyme finally releases the adaptor with the amino acid attached to one end.

Remember that it takes many amino acids to make up one protein. Therefore, there must be a way to link these amino acids into a single protein in order to complete protein synthesis. This is where the ribosome comes in which is so good at producing proteins that is often termed a "protein factory." The ribosome "reads" the start condon (AUG) and associates it with the proper amino acid. Once all the amino acids have been linked, mRNA signals STOP. The ribosome releases the mRNA and the amino acid, and a protein has been made. This stage is divided into three parts: Initiation, Elongation, and Termination. During the initiation phase, a ribosome attaches to the mRNA and reads the condon (AUG) During the elongation phase, tRNA bring the corresponding amino acids to each codon, as the ribosome moves down the mRNA strand. During the last phase, termination, the molecule is read, and the synthesis ends and releases the protein.

Milestones or discoveries in Genetics:

1866 - Mendel first published his discoveries.
1900 - Mendel's observations were rediscovered and confirmed by others.

1910 - Morgan presented experimental evidence that genes are carried on chromosomes.

1903 - Chromosomes are hereditary units

1913 - Chromosomes contain linear arrays of genes

1927 - Mutations are physical changes in genes

1931 - Recombination is caused by crossing over

1944 - Avery *et al.* demonstrated that DNA was genetic material

1945 - A gene codes for a protein

1953 - Watson and Crick determined the double helical nature of DNA.

1958 - DNA replicates semi conservatively

1961 -- Nienberg *et al*. and Ochoa *et al*. deciphered the genetic code.

1969 - First gene isolated (sugar metabolism)

1973- Beginning of genetic engineering (toad gene into bacteria)

1977 -- Sanger et al. published a simple and rapid method for DNA sequencing

1982- Insulin

1983- PCR, Kary Mullis

1984- DNA fingerprinting

1990- Human Genome Project, $3 billion

1990- Jurassic Park

1993- Rough map of human chromosomes

2003 - Sequencing of entire human genome is completed (24^{th} April DNA DAY).

Recombinant DNA (rDNA):

In 1972, American biochemist Paul Berg and his colleagues at Stanford University synthesized the first recombinant DNA (rDNA), and he subsequently led the international community of rDNA researchers in their efforts to address the potential physical and ethical hazards posed by that revolutionary technology. Dr. Berg was investigating how mammalian viruses might pick up genes and transfer them to new cells as bacterial viruses often did. Tumor viruses were too small to act as vectors, so Berg and his colleagues engineered a virus for the purpose. The technique they developed in 1971-72 for splicing two DNA molecules, one from a tumor virus and one from a plasmid carrying *E. coli* genes marked the beginning of recombinant DNA (rDNA) technology. He received the 1980 Nobel Prize in Chemistry for his protein synthesis and rDNA work, and has continued to elucidate genetic mechanisms, using specially designed recombinant organisms. Dr Berg had wrote a to the journal Science, insisted on the National Institutes of Health (NIH) to regulate the use of rDNA technology and urged scientists to

halt most rDNA experiments until they better understood whether the technique is safe to human and to the environment. However, the newly created DNA molecules heralded a new era in the manipulation, analysis and exploitation of biological molecules. This process termed gene cloning has enabled numerous discoveries and insights into gene structure and regulation. Since their initial use methods for the production of gene libraries have been steadily refined and developed. Although the polymerase chain reaction (PCR) has provided shortcuts to gene analysis and there are still many cases where gene cloning methods are not only useful but are an absolute requirement.

Gene cloning:

Cloning technology involves the construction of novel DNA molecules by joining DNA sequences from different sources. Any DNA cloning procedure has four essential steps.

1. A method for generating DNA fragments
2. Joining foreign DNA fragments into the suitable vectors
3. Transformation of recombinant plasmids into a host cells in which it can replicate
4. A method for selecting or screening a clone, which carries the recombinant.

cDNA is copy DNA or complementary DNA made from RNA using reverse transcriptase. This is another gene cloning technology found by Craig Venter in 1991. There are two potential reasons for wanting to do this. First, the DNA gene itself may be unknown. In this case, the cDNA is made from mRNA those codes for a known protein. Then the DNA gene can be found using the cDNA as a probe. Secondly, the cDNA is a better choice for expression of eukaryotic gene in a bacterium, since it is free from introns and it can be directly inserted into an expression vector and the gene product can be studied.

Following the production of recombinant molecules, the so-called constructs are subsequently introduced into cells to enable it to be copied a large number of times as the cells replicate. Initial recombinant DNA experiments were performed by bacterial cells because of the latter's ease of growth and short doubling time. Gram - negative bacteria such as *E. coli* can be made "competent" for the introduction of recombinant DNA. Bacteriophage has been well exploited for introduction of DNA into bacterial system and 10 - 100 fold higher efficiency of transformation with compare to bacteria with plasmids. The delivery of cloning vectors into eukaryotic cells is, however, not as straightforward as that for the prokaryote *E. coli*.

It is possible to deliver recombinant molecules into animal and plant cells by different means, broadly classified as direct and indirect methods. The transformants are selected with help of marker gene especially antibiotic resistance gene. Alternatively, plant and animal cells, the foreign DNA can be introduced directly by electroporation, biolistic gene gun where the marker gene can be avoided. In case of plant system indirect method like *Agrobacterium* - mediated gene transfer was found to be best method using tissue culture techniques. The cell culture plays a crucial role for the development of transgenic plants, since each cell has the capacity to regenerate a complete plant. Tremendous progress has been made for the improvement of animal, plant and microbe system with useful genes.

Biotechnology:

Biotechnology can be simply defined as an applied science. With help of genetic engineering tools, we can improve the existing system by adding / deleting genes or creating new organism or products. It solves perceived specific problems and enhance yield. Molecular biology helps to understand the nature or biology also to manipulate the genetic material which would ultimately help to increase the yield as per the requirement. Biotechnology tender quick solutions to problems thus, future agricultural success will come only through Biotechnology to increased yield long term sustainability (The detailed biotechnology principle and applications are dealt in other lecture notes).

Interesting Milestones of Biotech:

Test tube baby:

British professor Dr. Robert Edwards was first one to develop and succeeded the test tube baby in human in 1978 by *in vitro* fertilization. He was awarded the Nobel Prize for Medicine in 2010. The test tube baby is now 35 years old and her name is Louise Brown. Now more than 4 millions babies are produced by *in vitro* fertilization (IVF) technique.

Dolly:

The first female sheep "Dolly" was cloned from an adult somatic cell using the process of nuclear transfer and is by far the world's most famous clone. She was cloned by Ian Wilmut, Keith Campbell and Colleagues at Roslin Institute, part of University Edinburgh, and the biotechnology company PPL Therapeutics near Edinburgh in Scotland, the United

Kingdom. Dolly was born on 5 July 1996 and had three mothers (one provided the egg, another DNA and a third carried the cloned embryo to term).To clone Dolly, researchers took a cell from the udder of a six year old Finn Dorset sheep, and kept in a nutritional solution. They then deprived the cell of the nutrients which made the cell's DNA go into a 'sleeping' state. Then they injected the cell into an unfertilized egg cell which had had its nucleus removed, and made the cells fuse by using electrical pulses. The combined cells acted like a fertilized egg cell and starting to divide and grow, thus starting the development of Dolly. The egg was then implanted into a third ewe and a few months later, Dolly was born. She was genetically an **exact copy** using the udder cells. Cloning may have uses in preserving endangered species and may become a viable tool for reviving extinct species. Cloning of domesticated animals could be important in the future production of transgenic livestock. The development of **cloning technology** has led to new ways to produce medicines and is improving our understanding of development and genetics.

ANDi (inserted DNA)

The first genetically modified rhesus monkey, at the Oregon regional primate research centre in Oregon, USA. The birth of ANDi, the first rhesus monkey cloned by embryo splitting, is another incremental step toward designing and perfecting new treatments for human genetic disorders.

Pigs:

Five female piglets were cloned; they were born on Christmas Day 2001. Developed by the Scottish-based firm called PPL Virginia, USA on March 5th 2000. Therapeutics says is a major step towards successfully producing animal organs and cells for use in human transplants. The pigs lack a gene to which the human immune system reacts aggressively. When an all-male litter is born and bred with the females, a true knock-out pig will be created.

Pomato:

The concept of grafting different plant species belonging to same family was originally developed in 1977 at the Max Planck Institute for Developmental Biology in Tubingen, Germany. They grafted *Solanaceae* family potatoes and tomatoes to produce a hybrid plant "*pomato*" to have both on the same plant. Like most standard types of plant grafting, a small incision is made in the stem of both plants and they are strapped together. Once the cuts have healed and the plants are joined, the leafy top of the potato plant can be cut away and the roots of the tomato can be

removed, leaving the leaves of the tomato plant to nourish the roots of the potato plant. The rootstock (potato) acts as a stable and healthy root system and the scions (tomato) are chosen for their fruit, flowers or leaves. The tomatoes should be ready to harvest after about 12 weeks during the summer months; the potatoes should be ready after the tomato leaves begin to die back, normally in early autumn.

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Central Dogma of Molecular Biology

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Introduction

Cells pack their DNA not only to protect it, but also regulate which genes are accessed and Cells package their DNA not only to protect it, but also to regulate which genes are accessed and when. DNA packaging helps conserve space in cells. Packaging is the reason why the approximately two meters of human DNA can fit into a cell that is only a few micrometers wide.

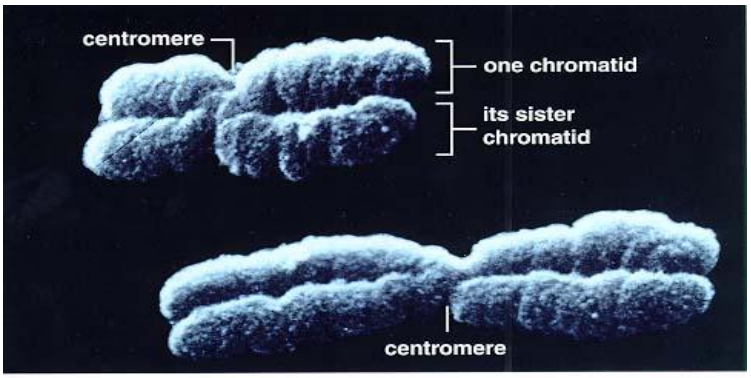


Image source: http://www.uic.edu/classes/bios/bios100/lectures/mitosis.htm

Cellular DNA forms a complex with various protein partners that help package it into such a tiny space. This DNA-protein complex is called chromatin, wherein the mass of protein and nucleic acid is nearly equal. Within cells, chromatin usually folds into characteristic formations called chromosomes. Each chromosome contains a single double-stranded piece of DNA along with the packaging proteins. Eukaryotic chromosomes consist of repeated units of chromatin called **nucleosomes**. Nucleosomes are made up of double-stranded DNA that has complexed with small proteins called histones. The core particle of each nucleosome consists of eight histone molecules, two each of four different histone types: H2A, H2B, H3, and H4. The structure of histones has been strongly conserved across evolution, suggesting that their DNA packaging function is crucially important to all eukaryotic cells.

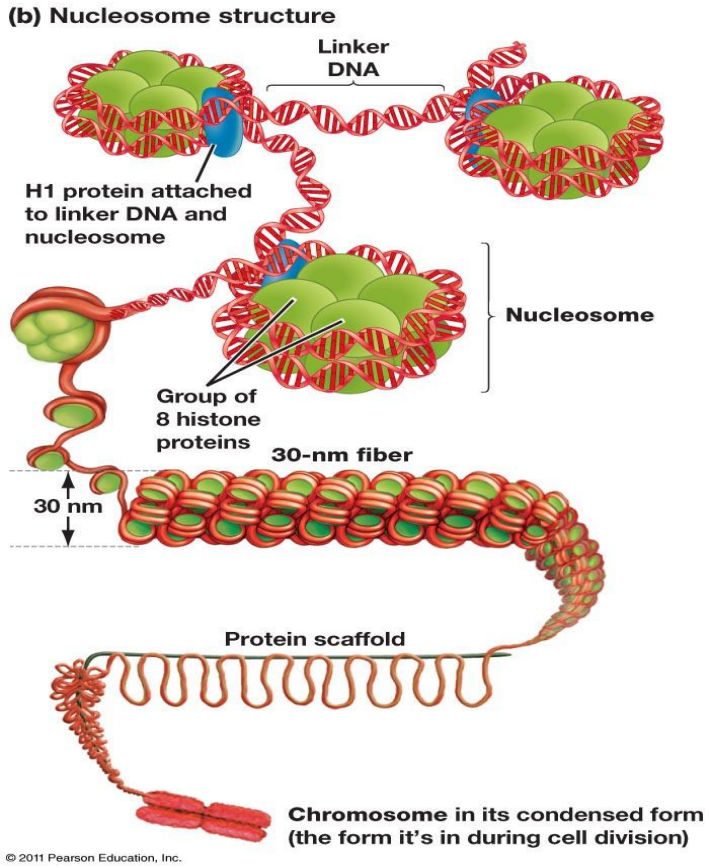


Image source: http://www.uic.edu/classes/bios/bios100/lectures/mitosis.htm

Histones carry positive charges and bind negatively charged DNA in a specific conformation The exact length of the DNA segment associated with each histone core varies from species to species, but most such segments are approximately 150 base pairs in length. Furthermore, each histone molecule within the core particle has one end that sticks out from the particle. These ends are called N-terminal tails, and they play an important role in higher-order chromatin structure and gene expression.

Chromatin packing also offers an additional mechanism for controlling gene expression. Specifically, cells can control access to their DNA by modifying the structure of their chromatin. Highly compacted chromatin simply isn't accessible to the enzymes involved in DNA transcription, replication, or repair. Thus, regions of chromatin where active transcription is taking place (called euchromatin) are less condensed than regions where transcription is inactive or is being actively inhibited or repressed (called heterochromatin).

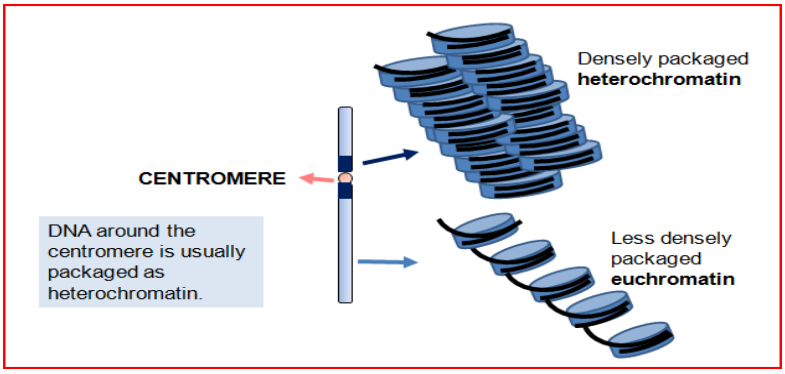


Image source: http://www.plantcell.org/site/teachingtools/

Central Dogma of Life

The central dogma of molecular biology is an explanation of the flow of genetic information within a biological system. The general transfers describe the normal flow of biological information: DNA can be copied to DNA (DNA replication), DNA information can be copied into mRNA (transcription), and proteins can be synthesized using the information in mRNA as a template (translation). Special transfers of biological sequential information include reverse transcription and RNA replication.

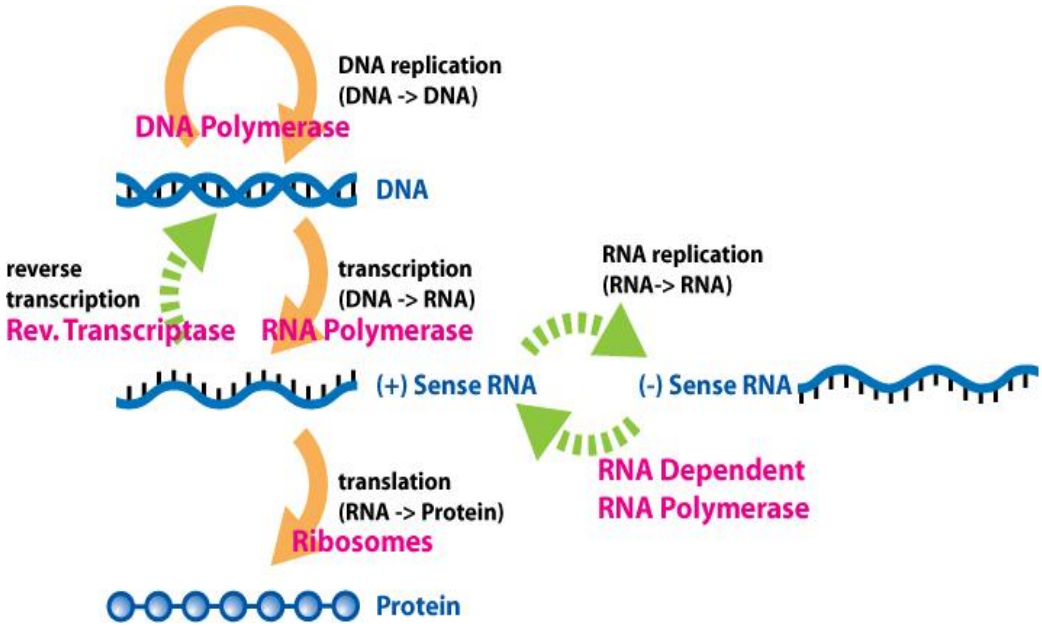


Image source: http://en.wikipedia.org/wiki/File:Extended_Central_Dogma_with_Enzymes.jpg

DNA replication

Replication involves use of an existing strand of DNA as a template for the synthesis of a new, identical strand. DNA replication is a complex process as described below.

DNA topoisomerase

DNA topoisomerase enzymes facilitate DNA helix unwinding. DNA Helicase Unzips the double-stranded DNA for replication making a forked structure.

DNA polymerases

Two DNA polymerase molecules are active at the fork at any one time. One moves continuously to produce the new daughter DNA molecule on the leading strand, whereas the other produces a long series of short Okazaki DNA fragments on the lagging strand (as it can work only in 5' to 3' direction).

DNA Primase

In addition to the template, DNA polymerases need a pre-existing DNA or RNA chain end (a primer) onto which to add each nucleotide. For this reason, the lagging strand polymerase requires the action of a DNA primase enzyme before it can start each Okazaki fragment. The primase produces a very short RNA molecule (an RNA primer) at the 5' end of each Okazaki fragment onto which the DNA polymerase adds nucleotides.

Single stranded DNA binding protein

Finally, the single-stranded regions of DNA at the fork are covered by multiple copies of a single-strand DNA-binding protein, which hold the DNA template strands open with their bases exposed. The lagging-strand DNA polymerase remains tied to the leading-strand DNA polymerase. This allows the lagging-strand polymerase to remain at the fork after it finishes the synthesis of each Okazaki fragment. As a result, this polymerase can be used over and over again to synthesize the large number of Okazaki fragments that are needed to produce a new DNA chain on the lagging strand. Okazaki fragments of bacterial DNA are typically between 1,000 and 2,000 nucleotides long, whereas in eukaryotic cells, they are only about 100 to 200 nucleotides long.

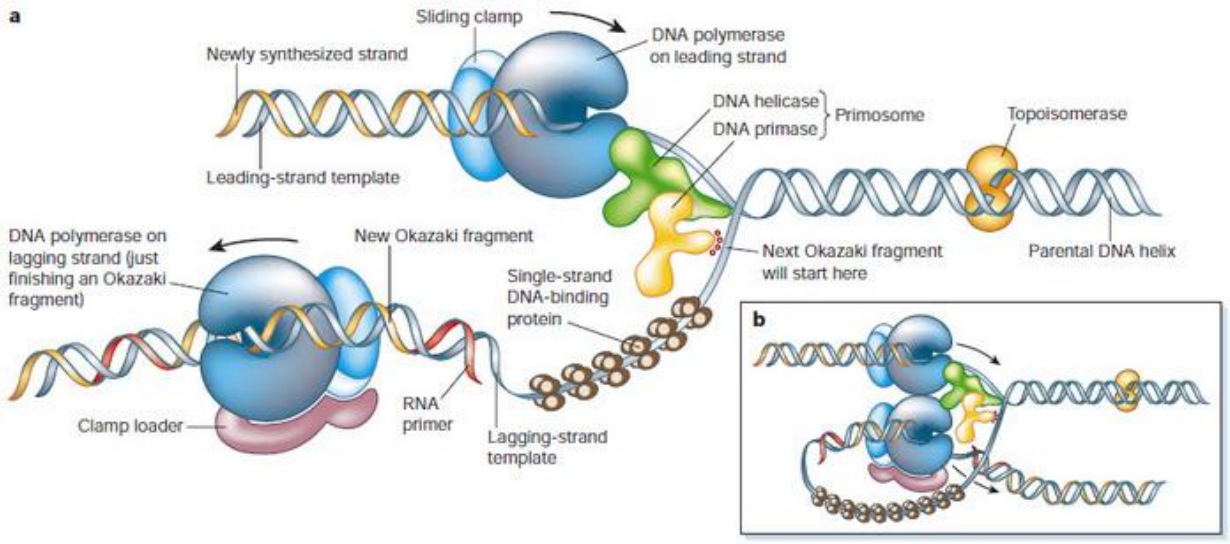


Image source:© 2008 Nature Publishing Group Cimprich, K. A. & Cortez, D. ATR: an essential regulator of genome integrity. *Nature Reviews* 9, 616–627 (2008). All rights reserved

Transcription

Transcription is the process by which the information in a strand of DNA is copied into a new molecule of messenger RNA (mRNA). Transcription is carried out by an enzyme called RNA polymerase and a number of accessory proteins called transcription factors.

Three different types of RNA polymerase exist in eukaryotic cells, whereas bacteria have only one. In eukaryotes, RNA pol I transcribes the genes that encode most of the ribosomal RNAs (rRNAs), and RNA pol III transcribes the genes for one small rRNA, plus the transfer RNAs that play a key role in the translation process, as well as other small regulatory RNA molecules. Thus, it is RNA pol II that transcribes the messenger RNAs, which serve as the templates for production of protein molecules.

Initiation

The first step in transcription is initiation, when the RNA pol binds to the DNA upstream (5′) of the gene at a specialized sequence called a promoter. Eukaryotic promoters are more complex than their prokaryotic counterparts. Many eukaryotic genes also possess enhancer sequences, which can be found at considerable distances from the genes they affect. Enhancer sequences control gene activation by binding with activator proteins and altering the 3-D structure of the DNA to help "attract" RNA pol II, thus regulating transcription. Because eukaryotic DNA is tightly packaged as chromatin, transcription also requires a number of specialized proteins that help make the coding strand accessible. In eukaryotes, the "core"

promoter for a gene transcribed by pol II is most often found immediately upstream (5′) of the start site of the gene. Most pol II genes have a TATA box (consensus sequence TATTAA) 25 to 35 bases upstream of the initiation site, which affects the transcription rate and determines location of the start site.

Eukaryotic RNA polymerases use a number of essential cofactors (collectively called general transcription factors), and one of these, TFIID, recognizes the TATA box and ensures that the correct start site is used. Another cofactor, TFIIB, recognizes a different common consensus sequence, G/C G/C G/C G C C C, approximately 38 to 32 bases upstream.

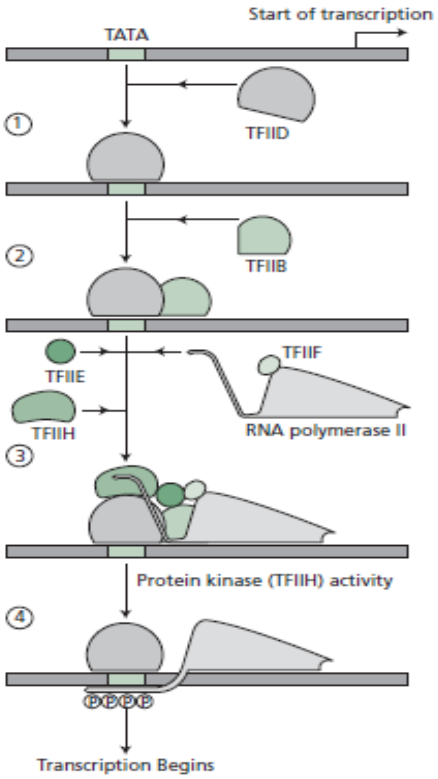


Image source: Plant Physiology by Taiz and Zeiger 3/e

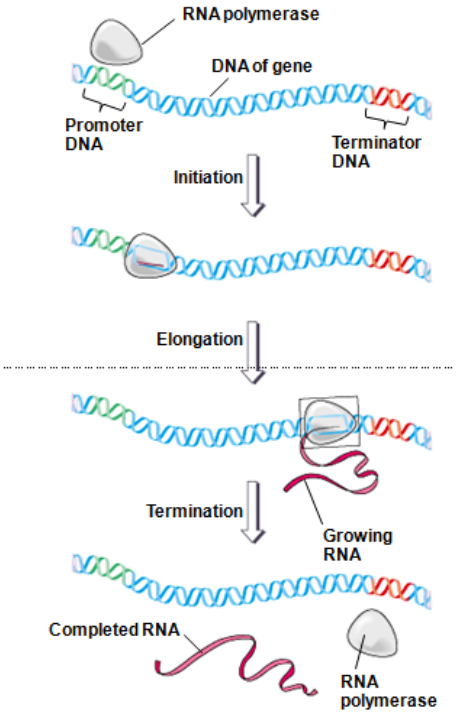
1.TFIID binds to TATA box ; 2. TFIIB joins TFIID; 3. TFIIF bound to RNA polymerase II Joins with TFIIE and TFII H; 4. TFIIH phosphorylates RNA polymerase II releasing other transcription factors and initiates transcription

Transcription factors can bind to specific DNA sequences called enhancer and promoter sequences in order to recruit RNA polymerase to an appropriate transcription site. Together, the transcription factors and RNA polymerase form a complex called the transcription initiation complex.

Elongation

Once transcription is initiated, the DNA double helix unwinds and RNA polymerase reads the template strand, adding nucleotides to the 3′ end of the growing chain. At a

temperature of 37 degrees Celsius, new nucleotides are added at the rate of about 15-20 amino acids per second in bacteria, while eukaryotes proceed at a much slower pace of approximately five to eight amino acids per second.



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Termination

In eukaryotes, termination of transcription occurs by different processes, depending upon the exact polymerase utilized. For pol I genes, transcription is stopped using a termination factor, through a mechanism similar to rho-dependent termination in bacteria. Transcription of pol III genes ends after transcribing a termination sequence that includes a polyuracil stretch, by a mechanism resembling rho-independent prokaryotic termination.

Post- transcriptional Changes

In eukaryotes, the primary RNA transcript of a gene needs further processing before it can be translated. This step is called "RNA processing". Also, it needs to be transported out of the nucleus into the cytoplasm.

Steps in RNA processing:

- Capping at the 5' end
- Addition of poly-A tail to the 3' end
- Splicing out introns (non coding sequences)

RNA is inherently unstable, especially at the ends. The ends are modified to protect it. At the 5' end, a slightly modified guanine (7-methyl G) is attached "backwards", by a 5' to 5' linkage, to the triphosphates of the first transcribed base. At the 3' end, the primary transcript RNA is cut at a specific site and 100-200 adenine nucleotides are attached, the poly-A tail.

Translation *(Clancy and Brown, 2008)*

During translation, the mRNA is "read" according to the genetic code, which relates the DNA sequence to the amino acid sequence in proteins. Each group of three base pairs in mRNA constitutes a codon, and each codon specifies a particular amino acid (hence, it is a triplet code). The mRNA sequence is thus used as a template to assemble—in order—the chain of amino acids that form a protein. Within all cells, the translation machinery resides within a specialized organelle called the ribosome. In eukaryotes, mature mRNA molecules must leave the nucleus and travel to the cytoplasm, where the ribosomes are located.

In all types of cells, the ribosome is composed of two subunits: the large (50S) subunit and the small (30S) subunit (S, for svedberg unit, is a measure of sedimentation velocity and, therefore, mass). Each subunit exists separately in the cytoplasm, but the two join together on the mRNA molecule. The ribosomal subunits contain proteins and specialized RNA molecules—specifically, ribosomal RNA (rRNA) and transfer RNA (tRNA). The tRNA molecules are adaptor molecules—they have one end that can read the triplet code in the mRNA through complementary base-pairing, and another end that attaches to a specific amino acid.

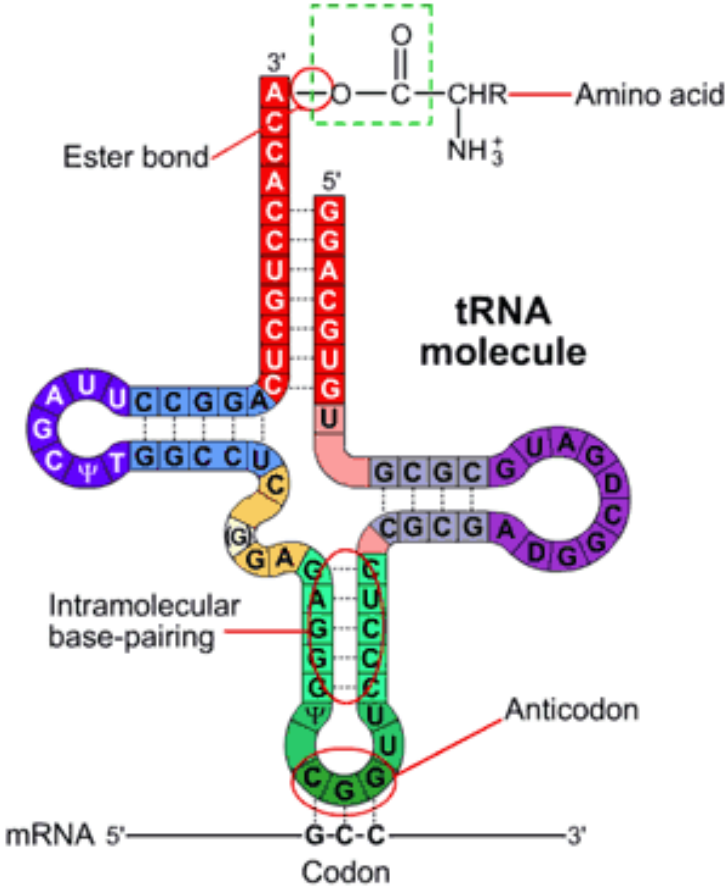


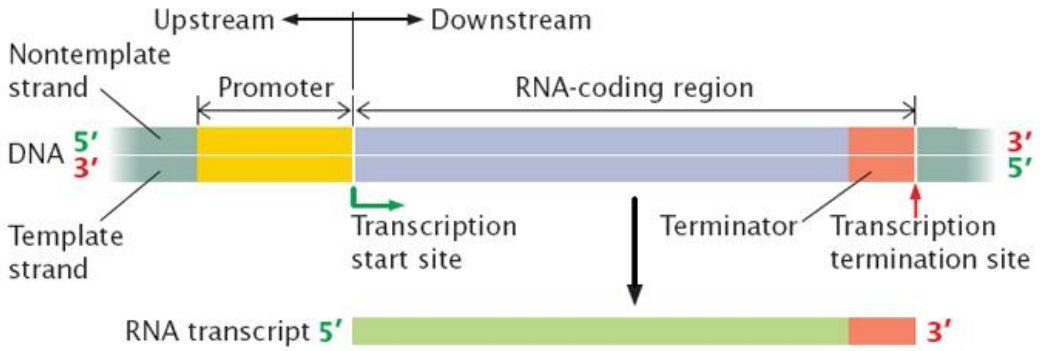
Image source: http://www.wiley.com/college/boyer/0470003790/structure/tRNA/trna_intro.htm

Within the ribosome, the mRNA and aminoacyl-tRNA complexes are held together closely, which facilitates base-pairing. The rRNA catalyzes the attachment of each new amino acid to the growing chain.

First base	Second base: U	Second base: C	Second base: A	Second base: G	Third base
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

© 2005 W. H. Freeman Pierce, Benjamin. Genetics: A Conceptual Approach, 2nd ed. (New York: W. H. Freeman and Company), 412.

Interestingly, not all regions of an mRNA molecule correspond to particular amino acids. In particular, there is an area near the 5' end of the molecule that is known as the untranslated region (UTR) or leader sequence. This portion of mRNA is located between the first nucleotide that is transcribed and the start codon (AUG) of the coding region, and it does not affect the sequence of amino acids in a protein.



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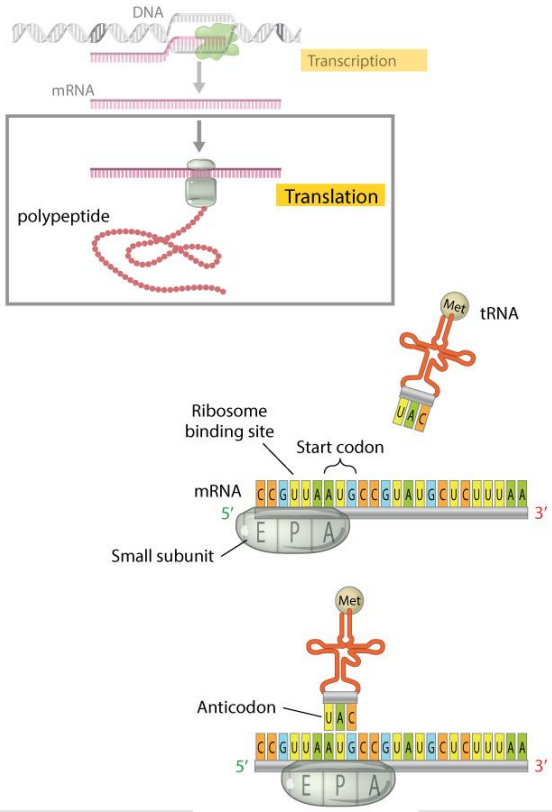
It turns out that the leader sequence is important because it contains a ribosome-binding site. In bacteria, this site is known as the Shine-Dalgarno box (AGGAGG), after scientists John Shine and Lynn Dalgarno, who first characterized it. A similar site in vertebrates was characterized by Marilyn Kozak and is thus known as the Kozak box. In bacterial mRNA, the 5' UTR is normally short; in human mRNA, the median length of the 5' UTR is about 170 nucleotides. If the leader is long, it may contain regulatory sequences, including binding sites for proteins, that can affect the stability of the mRNA or the efficiency of its translation.

Initiation

When translation begins, the small subunit of the ribosome and an initiator tRNA molecule assemble on the mRNA transcript. The small subunit of the ribosome has three binding sites: an amino acid site (A), a polypeptide site (P), and an exit site (E). The initiator tRNA molecule carrying the amino acid methionine binds to the AUG start codon of the mRNA transcript at the ribosome's P site where it will become the first amino acid incorporated into the growing polypeptide chain. Here, the initiator tRNA molecule is shown

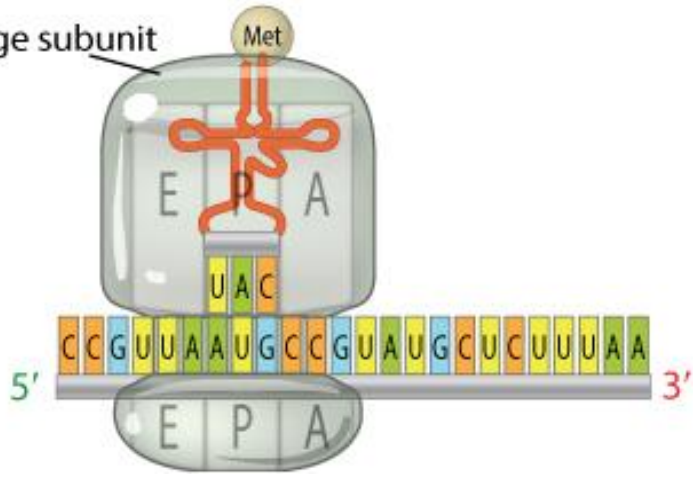
binding after the small ribosomal subunit has assembled on the mRNA; the order in which this occurs is unique to prokaryotic cells. In eukaryotes, the free initiator tRNA first binds the small ribosomal subunit to form a complex. The complex then binds the mRNA transcript, so that the tRNA and the small ribosomal subunit bind the mRNA simultaneously.

First, three initiation factor proteins (known as IF1, IF2, and IF3) bind to the small subunit of the ribosome. This preinitiation complex and a methionine-carrying tRNA then bind to the mRNA, near the AUG start codon, forming the initiation complex.



Once the initiation complex is formed on the mRNA, the large ribosomal subunit binds to this complex, which causes the release of IFs (initiation factors). The large subunit of the ribosome has three sites at which tRNA molecules can bind. The A (amino acid) site is the location at which the aminoacyl-tRNA anticodon base pairs up with the mRNA codon, ensuring that correct amino acid is added to the growing polypeptide chain. The P (polypeptide) site is the location at which the amino acid is transferred from its tRNA to the growing polypeptide chain. Finally, the E (exit) site is the location at which the "empty" tRNA sites before being released back into the cytoplasm to bind another amino acid and repeat the process. The initiator methionine tRNA is the only aminoacyl-tRNA that can bind

in the P site of the ribosome, and the A site is aligned with the second mRNA codon. The ribosome is thus ready to bind the second aminoacyl-tRNA at the A site, which will be joined to the initiator methionine by the first peptide bond.



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Elongation

The next phase in translation is known as the elongation phase. First, the ribosome moves along the mRNA in the 5'-to-3'direction, which requires the elongation factor G, in a process called translocation. The tRNA that corresponds to the second codon can then bind to the A site, a step that requires elongation factors as well as guanosine triphosphate (GTP) as an energy source for the process. Next, peptide bonds between the now-adjacent first and second amino acids are formed through a peptidyl transferase activity. The transferase activity is a catalytic function of rRNA (Pierce, 2000). After the peptide bond is formed, the ribosome shifts, or translocates, again, thus causing the tRNA to occupy the E site. The tRNA is then released to the cytoplasm to pick up another amino acid. In addition, the A site is now empty and ready to receive the tRNA for the next codon. This process is repeated until all the codons in the mRNA have been read by tRNA molecules, and the amino acids attached to the tRNAs have been linked together in the growing polypeptide chain in the appropriate order. At this point, translation must be terminated, and the nascent protein must be released from the mRNA and ribosome.

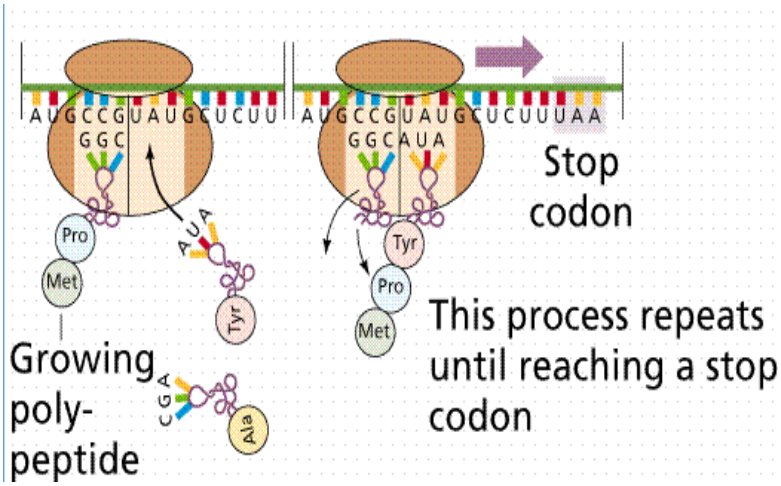


Image source: Purves et.al., Life: The Science of Biology, 4/e,by Sinauer associates and W.H. Freeman

Termination of Translation

There are three termination codons that are employed at the end of a protein-coding sequence in mRNA: UAA, UAG, and UGA. No tRNAs recognize these codons. Thus, in the place of these tRNAs, one of several proteins, called release factors, binds and facilitates release of the mRNA from the ribosome and subsequent dissociation of the ribosome.

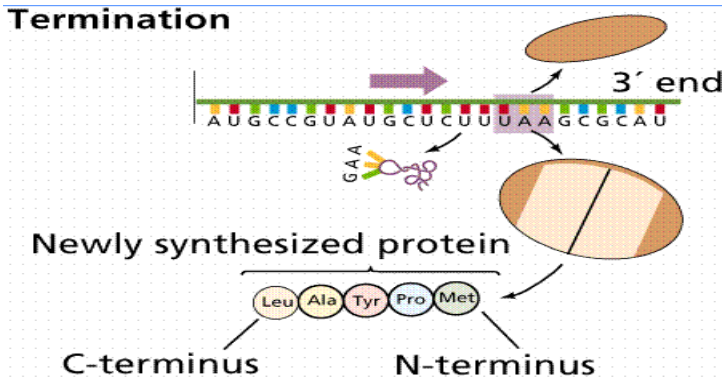
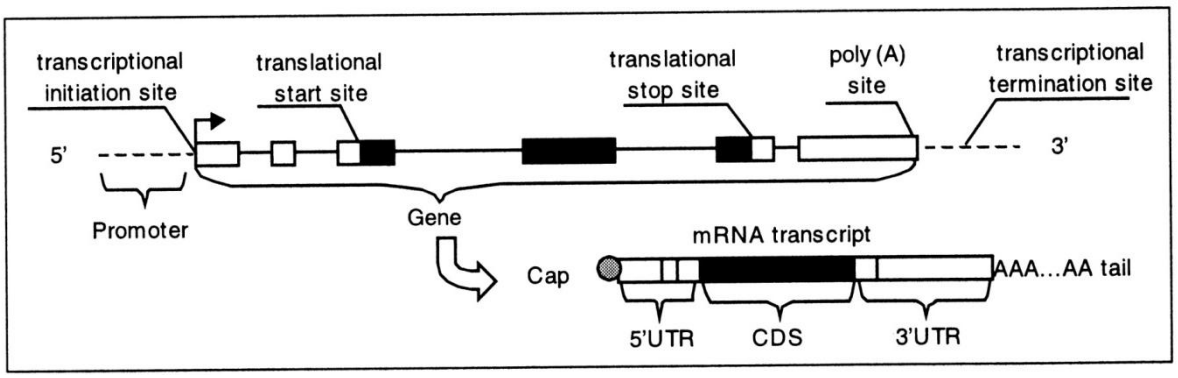


Image source: Purves et.al., Life: The Science of Biology, 4/e,by Sinauer associates and W.H. Freeman

Regulation of gene Expression

Gene

The entire nucleic acid sequence that is necessary for the synthesis of a functional polypeptide or RNA molecule



Levels of gene expression (Becker *et.al.*, 1997)

Eukaryotic gene expression can be regulated at multiple levels. (1) genomic regulation, by gene amplification, DNA rearrangements, chromatin decondensation or condensation, or DNA methylation; (2) transcriptional regulation;(3) RNA processing, and RNA turnover in the nucleusand translocation out of the nucleus; (4) translational control (including binding to ER in some cases); (5) posttranslational control, including mRNA turnover in the cytosol, and the folding, assembly, modification, and import of proteins into organelles

Eukaryotic promoter

- **Core or minimum promoter, consisting of the minimum upstream** sequence required for gene expression .The minimum promoter for genes transcribed by RNA polymerase II typically extends about 100 bp upstream of the transcription initiation site and includes several sequence elements referred to as **proximal promoter sequences.** About 25 to 35 bp upstream of the transcriptional start site is a short sequence called the **TATA box,** consisting of the sequence TATAAA(A). TATA box is the site of assembly of the transcription initiation complex. Approximately 85% of the plant genes sequenced thus far contain TATA boxes.
- Eukaryotes contain two additional regulatory sequences , the CAAT box and the GC box which are major sites of binding of transcription factors

Transcription factors

- Proteins that enhance the rate of transcription by facilitating the assembly of the initiation complex are called transcription factors. The DNA sequences themselves are called cis-acting sequences since they are adjacent to the transcription units they are

regulating. The transcription factors that bind to the cis-acting sequences are called trans-acting factors since the genes that encode them are located elsewhere in the genome.

- *cis-acting sequences located farther* upstream of the proximal promoter sequences can exert either positive or negative control over eukaryotic promoters **distal regulatory sequences .** They are located **within 1000** bp of the transcription initiation site .The positively acting transcription factors are called **activators** and those which inhibit transcription are called **repressors**.

Regulation of transcription by distal regulatory sequences and trans- acting factors

- Trans –acting factors bound to distal regulatory sequences can act in concert to activate transcription by making direct physical contact with the transcription initiation complex. (Alberts *et. al*., 1994)

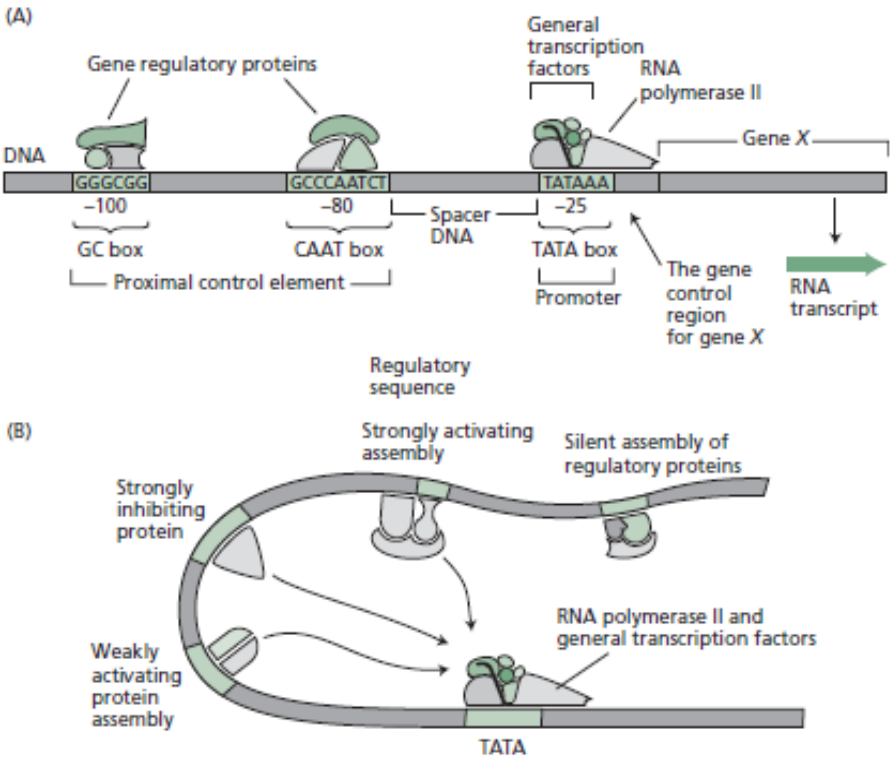


Figure 14.7 Organization and regulation of a typical eukaryotic gene. (A) Features of a typical eukaryotic RNA polymerase II minimum promoter and proteins that regulate gene expression. RNA polymerase II is situated at the TATA box in association with the general transcription factors about 25 bp upstream of the transcription start site. Two *cis*-acting regulatory sequences that enhance the activity of RNA polymerase II are the CAAT box

Image source: Plant Physiology, Taiz and Zeiger, 3/e

During formation of the initiation complex, the DNA between the core promoter and the most distally located control elements loops out in such a way as to allow all of the transcription factors bound to that segment of DNA to make physical contact with the initiation complex. Through this physical contact the transcription factor exerts its control, either positive or negative, over transcription. Given the large number of control elements that can modify the activity of a single promoter, the possibilities for differential gene regulation in eukaryotes are nearly infinite.

Reverse Transcription

Generation of complementary DNA (cDNA) from an RNA template is termed as reverse transcription. Reverse Transcriptase is the enzyme catalyzing this process. Self-replicating stretches of eukaryotic genomes known as retrotransposons utilize reverse transcriptase to move from one position in the genome to another via an RNA intermediate. They are found abundantly in the genomes of plants and animals. Telomerase is another reverse transcriptase found in many eukaryotes, including humans, which carries its own RNA template; this RNA is used as a template for DNA replication. Reverse transcriptase enzymes include an RNA-dependent DNA polymerase and a DNA-dependent DNA polymerase, which work together to perform transcription. In addition to the transcription function, retroviral reverse transcriptases have a domain belonging to the RNase H family which is vital to their replication.

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Principles, Types and Applications of Polymerase Chain Reaction

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Introduction

PCR has revolutionized scientists approach to **basic** and **applied** research. It provides an extremely flexible tool for the research scientist, and every molecular biology research laboratory now uses PCR routinely and often adapting and tailoring the basic procedures to meet their own special needs. Using PCR we can now isolate essentially any gene from any organism. It has become a foundation stone of genome sequencing projects, used both for determining DNA sequence data and for the subsequent study of putative genes and their products by high throughput screening methodologies.

Basic Principles

PCR copies DNA in the test-tube and uses the basic elements of the natural DNA synthesis and replication processes. In a living cell a highly complex system involving many different proteins is necessary to replicate the complete genome. In simplistic terms, the DNA is unwound and each strand of the parent molecule is used as a template to produce a complementary 'daughter' strand. This copying relies on the ability of nucleotides to base pair according to the well-known Watson and Crick rules such as A always pairs with T and G always pairs with C. The template strand therefore specifies the base sequence of the new complementary DNA strand. A large number of proteins and other molecules, such as RNA primers, are required to ensure that the process of DNA replication occurs efficiently with high fidelity, which means with few mistakes, and in a tightly regulated manner. DNA synthesis by a DNA polymerase must be 'primed', meaning we need to supply a short DNA sequence called a **primer** that is complementary to a template sequence. Primers are synthetically produced DNA sequences usually around 20 nucleotides long. The DNA polymerase will add nucleotides to the free 3-OH of this primer according to the normal base pairing rules PCR requires only some of the components of the complex replication machinery to copy short fragments of DNA in a simple buffer system in a test tube.

PCR proceeds in three distinct steps governed by temperature.

1) **Denaturation:** the double-stranded template DNA is denatured by heating, typically to 94°C, to separate the complementary single strands.
2) **Annealing:** the reaction is rapidly cooled to an annealing temperature to allow the oligonucleotide primers to hybridize to the template. The single strands of the template are too long and complex to be able to reanneal during this rapid cooling phase. During this annealing step the thermostable DNA polymerase will be active to some extent and will begin to extend the primers as soon as they anneal to the template. This can lead to specificity problems if the annealing temperature is too low.
3) **DNA synthesis:** the reaction is heated to a temperature, typically 72°C for efficient DNA synthesis by the thermostable DNA polymerase.

In the first cycle of PCR each template strand gives rise to a new duplex, doubling the number of copies of the target region. Likewise at each subsequent cycle of denaturation, annealing and extension, there is a theoretical doubling of the number of copies of the target DNA. If PCR achieved 100% efficiency then 35 cycles would yield a 34 billion copied. Of course PCR is not 100% efficient for a variety of reasons that we will consider shortly, but by increasing the number of cycles and optimizing conditions amplification exponential greater is routinely achievable. One of the great advantages of PCR is its ability to amplify a defined region of DNA from a very complex starting template such as genomic DNA. It is therefore worth dissecting what is happening during PCR amplification from a genomic DNA template as this will provide a better understanding of the reaction process.

History of PCR

During in 1971, H.L.Khorana and colleagues described an approach for replicating a region of duplex DNA by using two DNA synthesis primers designed so that their 3-ends pointed towards each other. However, the concept of using such an approach repeatedly in an amplification format was not conceived for another 12 years. Kary Mullis, the inventor of PCR, starts an account in *Scientific American* of how, during a night drive through the mountains of Northern California in Spring 1983, he had a revelation that led him to develop PCR (Mullis, 1990). Mullis was awarded the 1993 Nobel Prize for Chemistry for his achievement.

The DNA polymerase originally used for the PCR was extracted from the bacterium *Escherichia coli*. Although this enzyme had been a valuable tool for a wide range of applications and had allowed the explosion in DNA sequencing technologies in the preceding decade, it had distinct disadvantages in PCR. For PCR, the reaction must be heated to denature the double-stranded DNA product after each round of synthesis. Unfortunately, heating also irreversibly inactivated the *E. coli* DNA polymerase and therefore fresh aliquots of enzyme had to be added by hand at the start of each cycle. What was required was a DNA polymerase that remained stable during the DNA denaturation step performed at around 95°C. The solution was found when the bacterium *Thermophilus aquaticus* was isolated from hot springs, the DNA polymerase of these bacteria called *Taq* survived and proliferated at extremely high temperatures, and amplified the target DNA. *Taq* was discovered by T. Brock in Yellowstone National Park, USA. PCR and the thermostable polymerase responsible for the process were named as the first 'Molecule of the Year' in 1989 by the international journal *Science*.

PCR is covered by patents, granted to Hoffman La-Roche and Roche Molecular Systems, and these have been vigorously enforced to prevent unlicensed use of the method. Some of these patents terminated on 28 March 2005. From this date it has been possible to perform basic PCR in the US without a license, although some other patents still apply to instruments and specific applications. Outside the US in countries covered by the equivalent patents, there is a further year of patent protection to run.

PCR Reagents and other parameters

PCR is a technically simple operation nowadays in which reagents are mixed and incubated in a thermal cycler that automatically regulates the temperature of the reaction cycles according to a pre-programmed set of instructions. The DNA polymerase, being thermostable, need only be added at the start of the reaction so once you have started your PCRs you can get on with another experiment!

Magnesium chloride:

PCR buffers are generally supplied by the manufacturer when you purchase a thermostable DNA polymerase. Check the composition of the buffer and specifically whether it contains MgCl2. Magnesium ions are critical for DNA synthesis. Some buffers will contain MgCl2, typically designed to give a final concentration of 1.5 mM in the final PCR. Other buffers

will not contain any MgCl2, but a stock solution will usually be supplied by the manufacturer to allow you to determine the optimal MgCl2 concentration.

dNTPs:

dNTPs can be purchased from many commercial sources and it is recommended that you use such ready prepared solutions, as these are quality assured. Stock solutions (100–300 mM) should be stored at –70°C and working solutions should be prepared by diluting stocks to between 50 μM and 200 μM of each dNTP in sterile double-distilled water. Because these working solutions should ideally only be stored for 2–3 weeks at –20°C it is recommended that relatively small volumes of working solutions are made. It is important for successful PCR that the four dNTPs are present in equimolar concentrations otherwise the fidelity of PCR can be affected. Similarly, the concentration of dNTPs should be around 50–200 μM. If the concentration is higher the fidelity of the process will be adversely affected by driving *Taq* DNA polymerase to misincorporate at a higher rate than normal, while if the concentration is lower it may affect the efficiency of PCR. Protocols often suggest using 200 μM of each dNTP. This amount would be sufficient to synthesize about 10μg of product although the most you are likely to achieve is 2–3μg. Reducing the concentration of dNTPs below 200μM each is not recommended when proofreading polymerases are being used as they have a 3→5 exonuclease activity that will degrade single-stranded DNA molecules such as the primers. This activity increases as nucleotide concentration decreases. *Taq* and other thermostable DNA polymerases will usually incorporate modified nucleotides into DNA.

Primers

Oligonucleotides are widely available and there are many companies that offer low-cost custom synthesis and purification of your primer sequences within a few days of ordering. For most PCRs (with the exception of some genomic mapping approaches, such as RAPD analysis) you will need two primers of different sequence that anneal to complementary strands of the template DNA. When you know the DNA sequence of your template it is quite easy to design suitable primers to amplify any segment that you require. There are several computer programs that can be used to assist primer design.

Thermostable DNA polymerases

Taq DNA polymerase, from the thermophilic bacterium *Thermus aquaticus*, was first described by Brock and Freeze in 1969, but was not widely used until the need for such an enzyme

arose for PCR. The stability of *Taq* DNA polymerase at the high temperatures (97°C) used in PCR allowed repeated amplification cycles following the single addition of enzyme at the start of the reaction. The enzyme displays an optimum temperature for DNA synthesis of around 72–75°C. As high temperatures (55–72°C) can be used during the primer annealing steps the added bonus is improved specificity of primer annealing leading to greater amplification of target sequences and less amplification of non-target sequences.

A mixture of *Taq* DNA polymerase and a proofreading enzyme allows efficient repair of errors during PCR. If *Taq* DNA polymerase introduces a mismatched nucleotide it will stall and will dissociate from the template. The error can be corrected by the proofreading DNA polymerase that will continue to synthesize DNA until it also dissociates from the template allowing *Taq* DNA polymerase to reinitiate DNA synthesis. The use of a mixture containing predominantly *Taq* DNA polymerase with a low concentration of a proofreading enzyme improves the fidelity over *Taq* DNA polymerase alone, but allows high levels of product to be synthesized.

Pfu DNA polymerase

Pfu DNA polymerase is from the hyperthermophilic archaebacterium *Pyrococcus furiosus* and is available from various manufacturers. It has both 3→5 exonuclease proofreading activity and 5→3 exonuclease activity. The fidelity of the enzyme is some 12-fold higher than for *Taq* DNA polymerase and probably has the highest fidelity of any of the currently used thermostable DNA polymerases. *Pfu* has also been combined with a thermostable factor in *PfuTurbo*™ DNA polymerase (Stratagene), which is reported to increase product yield, without affecting the high fidelity of the enzyme, allowing increased product from complex targets, fewer cycles of PCR and increased sensitivity of amplification from low quantities of template.

Template DNA

A wide range of DNA and RNA samples can be used as templates for PCR, including genomic DNAs, mRNAs, cDNAs, libraries, plasmid, phage, cosmid, BAC and YAC clones. In addition to preparing the template yourself there are an increasing number of commercial suppliers providing genomic DNAs, genomic libraries (in various vectors), cDNA libraries, total RNAs and poly(A)+ RNAs. These are available from several animal and plant species, tissues and cell types. The amount of template required is likely to require optimization, but generally less than a nanogram of cloned template and up to a microgram of genomic DNA is used. For high molecular weight genomic samples PCR should prove successful, but it may be useful to do a partial

digestion of the DNA with rare-cutting restriction enzymes such as *Not*I or *Sfi*I. PCR can be used to amplify from single cells or even a single template molecule although it is critical in such cases to avoid contamination. It is also possible to perform some PCR analyses *in situ* within a fixed sample of tissue to explore the expression of genes at the level of tissue or cells or to identify the presence of viral transcripts associated with disease.

Essentially any method can be used to prepare genomic DNA from bacteria, fungi, plants and animal. For the amplification of large fragments (>1000bp) purer genomic DNA will mean better amplification and there are a number of very good commercially available DNA purification kits. However, when dealing with a large number of samples, preparation of highly purified DNA can become very time consuming. Crude sample preparations will often suffice for successful amplification of small products (200–1000 bp). The amount of template DNA required for a PCR varies according to the application and the source of the template.

Types of PCR

1. Inverse PCR

Inverse PCR also called IPCR, and was first described by Ochman *et al.* in 1988. Inverse polymerase chain reaction is a variant of PCR, and is used when only one internal sequence of the target DNA is known. It is therefore very useful in identifying flanking DNA sequences of genomic inserts. Similar to other PCR methods, inverse PCR amplifies target DNA using DNA polymerase. Inverse PCR uses standard PCR; however it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been ligated upon it to form a circle. The inverse PCR method includes a series of digestions and self-ligations with the DNA being cut by a restriction endonuclease. This cut results in a known sequence at either end of unknown sequences. Target DNA is lightly cut into smaller fragments of several kilobases by restriction endonuclease digestion. Self-ligation is induced under low concentrations causing the phosphate backbone to reform. This gives a circular DNA ligation product. Target DNA is then restriction digested with a known endonuclease. This generates a cut within the known internal sequence generating a linear product with known terminal sequences. This can now be used for PCR. Standard PCR is conducted with primers complementary to the now known internal sequences. Inverse PCR has numerous applications in molecular biology including the amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

2. Touchdown PCR

Touchdown PCR starts initially with an annealing temperature higher than the Tm of the primers and then at each of the earlier cycles of the PCR the annealing temperature is lowered gradually to below the Tm. This ensures that only specific annealing of the primers to their correct target sequence takes place before any nonspecific annealing events. A good rule of thumb, described by Don *et al*. (1991) when using primers about 20 nucleotides in length, is to reduce the annealing temperature by 1°C every 2 cycles moving from 65°C to 55°C over the first 20 cycles. The reaction should then be completed by another 10 cycles at a 55°C annealing temperature. Since the first products to be made are specific products, this increases the concentration of true target sequences in the early stages of the PCR thereby enhancing the accumulation of true product as the amplification continues at a less specific annealing temperature.

3. Hot Start PCR

Probably the most common approach used for hot start is DNA polymerase whose polymerase and in some cases 3→5 exonuclease activity has been inhibited by the physical binding of inactivating monoclonal antibodies that prevent it reacting with substrates. This allows all the reaction components to be mixed together in the absence of any polymerization. When the reaction reaches a high temperature the antibody(s) denatures thereby releasing the thermostable DNA polymerase in an active form, allowing polymerization. There are many DNA polymerases of this type sold by a range of companies. These require sufficient time during the initial denaturing step to inactivate the antibodies, but usually this is achieved by a 5 min soak at 94°C. Even if it is not fully activated during this step, it will activate during thermal cycling at each denaturation step during the early cycles of a PCR. Hot-start procedures are most useful when low concentrations of a complex template, such as genomic DNA, are being used. However, artefactual amplifications can occur in any reaction and it is generally recommended that all PCRs should be performed under a hot-start procedure.

4. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is based on the polymerase chain reaction (PCR). More importantly it is based on the process of reverse transcription, which reverse transcribes RNA into DNA and was initially isolated from retroviruses. The techniques of RT-PCR allows the formation of cDNA (complementary or copy DNA) from RNA, which stores the sequence of RNA (such as messenger RNA, mRNA) in the more stable form of nucleic acid,

DNA. This reverse transcription from RNA into its reverse complement DNA (cDNA) is the first step of a usually two-step process of RT-PCR. Furthermore, by copying the RNA into DNA, one can then amplify the cDNA sequence by using primers specific for the DNA sequence. This amplification is the final second major step of the two-step process of RT-PCR.

The Process of RT-PCR

The First step of RT-PCR is referred to as the "first strand reaction". In the first-strand reaction, complementary DNA also termed cDNA, is made from the messenger RNA template of interest using oligo dT (oligonucleotide poly-dTs act similar to primers and bind to the 3' polyA sequence located at the 3' UTR - untranslated region, which are present in most mRNAs), dNTPs, and an RNA-dependent DNA polymerase, reverse transcriptase, through the process of reverse transcription. These factors are combined in a reverse transcriptase buffer for 1 hour at 37°C. After reverse transcriptase reaction is complete, and the cDNA has been synthesized, RNaseH is added (an RNA digestion enzyme) which digests the RNA away from the RNA-cDNA hybrid. After incubation with RNaseH, standard PCR or polymerase chain reaction is conducted using DNA oligo primers specific for the sequence of interest. This second step is referred to as the "second strand reaction".

Thus by adding the thermostable DNA polymerase, upstream and downstream DNA primers, the single stranded DNA becomes double stranded and is amplified, allowing the detection of even rare or low copy mRNA sequences by amplifying its complementary DNA. The exponential amplification of complementary sequence of mRNA or RNA sequences via reverse transcription polymerase chain reaction allow for a high sensitivity detection technique, where low copy number or less abundant RNA molecules can be detected. It is also used to clone mRNA sequences in the form of complementary DNA, allowing libraries of cDNA (cDNA libraries) to be created which contain all the mRNA sequences of genes expressed in a cell. Furthermore, it allows the creation of cDNA constructs which were cloned by RT-PCR and allow the expression of genes at the RNA and protein levels for further study.

5. Real-Time PCR

Although the traditional methods of quantitating mRNA are fairly good such as northern blotting and in situ hybridization, they do not approach the ease and speed of Real Time PCR. RT-PCR or reverse transcriptase PCR is semi-quantitative due to need to load samples on a gel and the insensitivity of ethidium bromide. Thus, real time PCR was developed out of the need to quantitate

differences in mRNA expression in a easy and quick manner, and due to the need to use of small amounts of mRNA such as those obtained by small tissue samples, and LCM (laser capture microdissection) isolated cells. Real-time reverse-transcriptase (RT) PCR is different from other quantitative PCR as it quantitates the initial amount of the template instead of detecting the amount of final amplified product (Freeman, 1999; Raeymaekers, 2000).

Real Time PCR is characterized by the **point in time during cycling when amplification of the PCR product of interest is first detected** rather than the amount of the PCR product of interest which is accumulated at the end-point after PCR which contained a large number of cycles. Real Time PCR does this by monitoring the amount of fluorescence emitted during the PCR reaction, and this acts as an indicator of the amount of PCR amplification that occurs during each PCR cycle. Thus, in newer Real Time PCR machines, one can visually see the progress of the reaction in "real time". Real Time PCR also has a much wider dynamic range of up to 107-fold (compared to 1000-fold in conventional RT-PCR). The dynamic range of an assay determines how much the target concentration can vary and yet still be quantified. This wide dynamic range also results in a more accurate quantitation.

6. Nested PCR

Nested PCR provides a tool for increasing sensitivity allowing you to 'fish out' the specific amplification product from the 'sea' of nonspecific products. Even if PCR primers have amplified nonspecific sequences, making it impossible to identify the desired product, it is highly unlikely that these nonspecific products will also have sites for a further pair of 'nested' specific primers. These nested primers are designed to anneal to sequences that will be present within the correct target PCR product. The true product will possess such nested primer target sequences while non-target sequences will not. So, second PCR using nested primers, designed to amplify an internal region of the original amplified product, should lead to a 104 enhancement of the true product over nonspecific products. Quite simply, a small aliquot of the first PCR, perhaps 1 µl of a 1-in-10 or 1-in-100 dilution can be used as the template for the nested PCR.

7. Allele-Specific PCR

Allele-specific PCR is a variation of the polymerase chain reaction which is used as a diagnostic or cloning technique, to identify or utilize single-nucleotide polymorphisms (SNPs) (single base differences in DNA). Allele-specific PCR does require the sequence of the target DNA sequence, including differences between the alleles.

Primers for Allele-Specific PCR

The allele-specific PCR uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with a SNP-specific primer signals presence of the specific SNP in a sequence.

8. *In Situ* PCR

In Situ PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell on a slide. *In situ* PCR amplification can be performed on fixed tissue or cells. During the initiation and progression of disease, minute quantities of a product in small populations of cells or tissues may be vital for the pathogenesis of the disease. In many slowly-evolving diseases which require months or even years to manifest themselves clinically, it has been shown that the majority of the affected cell population is in a transcriptionally inactive state, and at a level of one gene per host cell. Nucleic acid hybridization methods and the polymerase chain reaction (PCR) have both been employed to examine the expression and detection of such affected genes during pathogenesis. While both these techniques are quite useful, the disadvantage of these techniques is that they are essentially conducting cell expression and population studies. Nucleic acids are isolated from a population of cells which contains either a sufficient number of molecules to detect directly by standard hybridization techniques, or, when a subpopulation contains as little as a single copy of nucleic acid, that molecule amplified by the PCR, and detected after amplification.

In situ hybridization (ISH) applies the methodology of the nucleic acid hybridization technique to the cellular level. Combining cytochemistry and immunocytochemistry, In Situ PCR allows the identification of cellular markers to be identified and further permits the localization of to cell specific sequences within cell populations, such as tissues and blood samples. *In Situ PCR* is limited to the detection of non-genomic material such as RNA, genes or genomes, as the

detection limit in most conditions is several copies of the target nucleic acid per cell. Therefore, due to copy number limitations, hybridization of RNA is more sensitive than DNA detection.

9. Intersequence-specific PCR or ISSR

Intersequence-specific PCR or ISSR PCR is a method based on the polymerase chain reaction. The ISSR PCR method is used for DNA fingerprinting by amplifying regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths. AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions. Amplified Fragment Length Polymorphism PCR, also called AFLP PCR was originally described by Zabeau *et al.,* 1993.

AFLP is composed of 3 steps:

1) Cellular DNA is digested with one or more restriction enzymes. Typically this involves a combination of two restriction enzymes: a 4 base cutter (MseI) and a 6 base cutter (EcoRI). Ligation of linkers (restriction half-site specific adaptors) to all restriction fragments.

2) Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences.

3) Electrophoretic separation and amplicons on a gel matrix, followed by visualisation of the band pattern. The aim of this tool is to perform a theoretical AFLP-PCR experiment by using the same principles, and to suggest the adaptors and primers needed in the experiment.

AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions. Asymmetric PCR is used to preferentially amplify one strand of the target DNA more than the other.

10. Asymmetric PCR Method

The asymmetric PCR method is conducted as the standard PCR protocol, however a great excess of the primers for the chosen strand is used. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A-PCR in which the predominant product is a single-stranded DNA, as a result of

unequal primer concentrations. As asymmetric PCR proceeds, the lower concentration primer is quantitatively incorporated into double-stranded DNA. The higher concentration primer continues to primer synthesis, but only of its strand.

Applications of Asymmetric PCR

The Asymmetric PCR is useful in some sequencing and hybridization probing applications where having only one of the two complementary stands is sufficient or required.

11. Multiplex

Multiplex PCR, in which several primer sets amplify several amplicons in the same reaction, add a degree of complexity to designing optimal primers. The additional issues to consider are those of possible heterodimer formation between all of the candidate primers and possible alternate hybridization sites within any of the target sequences. Some of the available primer design software provides functions for these types of designs. Several programs are available for PCR primer design. HYBsimulator is the most powerful such program and does provide PCR primer selection based on all criteria. Other popular programs are Oligo™ and Primer Premier™, which provide a subset of these functions but are slightly easier to use.

Multiplexing is very useful for Genotyping SNPs and STRs, Genotyping AFLPs (Indels, Alu polymorphisms), Quantitative PCR (QPCR), Limited template amplifications, aDNA (Ancient DNA). Some of the applications of multiplex PCR include: Pathogen Identification, High Throughput SNP Genotyping, Mutation Analysis, Gene Deletion Analysis, Template Quantitation, Linkage Analysis, RNA Detection, and Forensic Studies.

12. Quantitative PCR

Quantitative PCR (Q-PCR) is used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative real-time PCR has a very high degree of precision. QRT-PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (Real Time PCR) or RQ-PCR. QRT-PCR or RTQ-PCR is more appropriate contractions, since RT-PCR commonly refers to reverse transcription PCR, often used in conjunction with Q-PCR.

13. Methylation-specific PCR

Methylation-specific PCR (MSP) was developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.

14. Rapid amplification of cDNA Ends (RACE)

RACE is a procedure for amplification of cDNA regions corresponding to the 5' or 3'end of the mRNAand it has been used successfully to isolate rare transcripts. The gene-specific primer may be derived from sequence data from a partial cDNA, genomic ex on or peptide. In 3' RACE the polyA tail of mRNA molecules is exploited as a priming site for PCR amplification. mRNAs are converted into cDNA using reverse transcriptase and an oligo-dT primer. The generated cDNA can then be directly PCR amplified using a gene-specific primer and a primer that anneals to the polyA region. The same principle as above applies for 5' end, but there is of course no polyA tail. First-strand cDNA synthesis extends from an antisense primer, which anneals to a known region at the 5' end of the mRNA. However, there is no known priming site available for the subsequent PCR amplification. The trick is to add a known sequence to the 3' end of the first-strand cDNA Molecule. Terminal transferase, a templateindependent polymerase, will catalyse the addition of a homopolymeric tail, such as poly-dC, to the 3' end of each cDNA molecule. PCR amplification can now be performed using a nested internal antisense primer together with an oligo-dG primer. This will allow the specific amplification of unknown 5' ends of the mRNA molecule.

Introduction to Basics of GM crop development

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Introduction

Over the past 30 years our ability to alter life-forms has been revolutionized by modern biotechnology. We have learned how to modify and transfer strands of DNA, which contain the instructions for specific traits. Genetically modified crops, popularly called GM crops refer to crop plants created with genetic engineering techniques for the benefit of mankind and the environment. In this extremely elegant method the genetic material called DNA (deoxyribonucleic acid) coding for a desirable trait is identified from a donor organism (any living organism), isolated, characterized and modified for better expression. Then a small piece of well characterized DNA (gene) codes for a trait is introduced into the recipient cells by different methods.

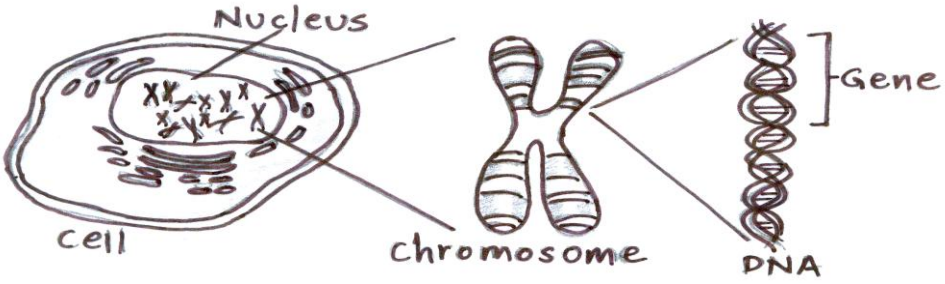


Fig 1: The gene (expressing unit) is coiled into chromosome and present in the nucleus, which is responsible for all traits. DNA is the blue print of life for the entire living organism (from bacteria to man), only the arrangements and information varies like alphabets

The transformed cells are then regenerated into a complete plant by tissue culture techniques. This technique is a primary requirement for the development of GM crops, because a single somatic cell would act as embryo to develop into a complete plant. Thus a number of plants have been modified to express desired traits like insect, disease and herbicide resistance and some plants with improved quality traits.

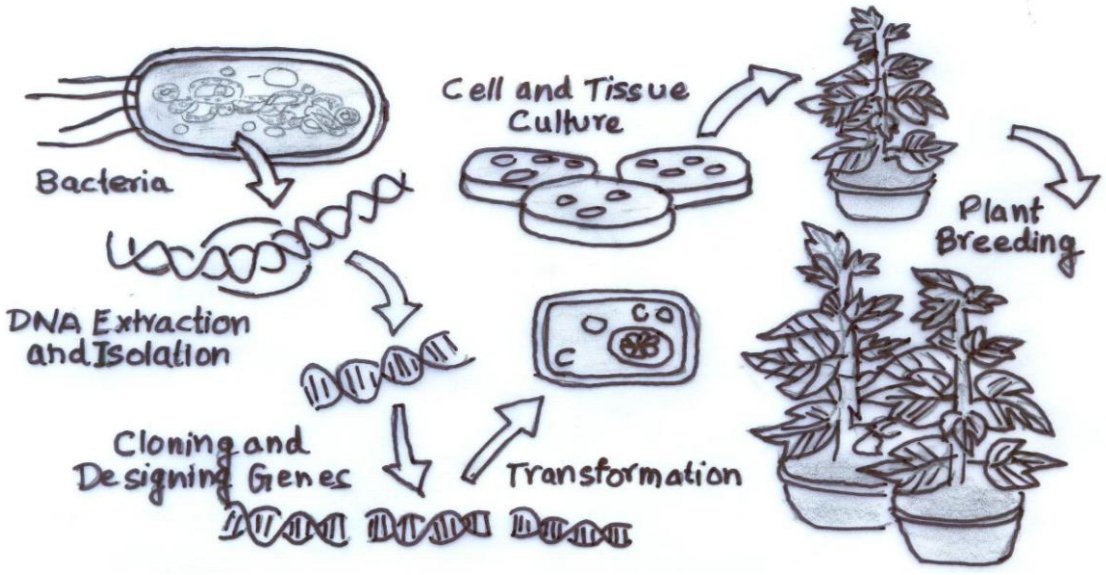


Fig 2: Overview of GM crop development (e.g. Bt gene transfer into cotton plant)

Worldwide 12 major crops such as soybean, maize, cotton, canola, potato, sugar beet, alfalfa, papaya, squash, tomato, poplar and sweet pepper have been genetically modified, commercially cultivated (James 2011). Soybean is leading GM crop occupying 75.4 Mha, followed by maize (51.00 Mha), cotton (24.7 Mha) and canola (8.2 Mha). Initially GM crops cultivation started in six countries namely USA, China, Canada, and Mexico, Australia and Argentina and reached 29 countries in 2011. Growing awareness of GM crops and acceptance by farmers resulted in increased global area under GM crops from 1.7 Mha in 1996 to 160 Mha in 2011 (James 2011).

GM Cotton

Cotton is long duration crop and is attacked by large number of insect pests throughout its growth and development. The cotton bollworm complex is a major and serious threat to the cotton, causing potential yield losses across the world and reported that the annual loss of at least US $ 300 million. High level of insecticide resistance in bollworms necessitates repeated application of insecticides, thereby aggravating the problem of resistance and also leading to heavy expenditure on cultivation and crop failures. Therefore it was important to initiate the development of alternative technologies such as genetic modification to enable plants to resist against the insect attack.

Genetic Engineering

With advent of molecular biology and genetic engineering technology, we can transfer the gene(s) of interest across the genome and express the traits as per the requirement and develop unique and new plants in the laboratory condition. DNA cloning involves separating a specific gene or DNA segment from a chromosome, attaching it to a DNA carrier molecule called vector, and replicating the modified DNA into thousands or millions of copies. The result is selective purification and amplification of a particular target gene or DNA segment from a complex mixture of DNA molecules by number of ways, but currently by PCR amplification. The methods used to accomplish these and related tasks are collectively referred to as recombinant DNA technology or genetic engineering.

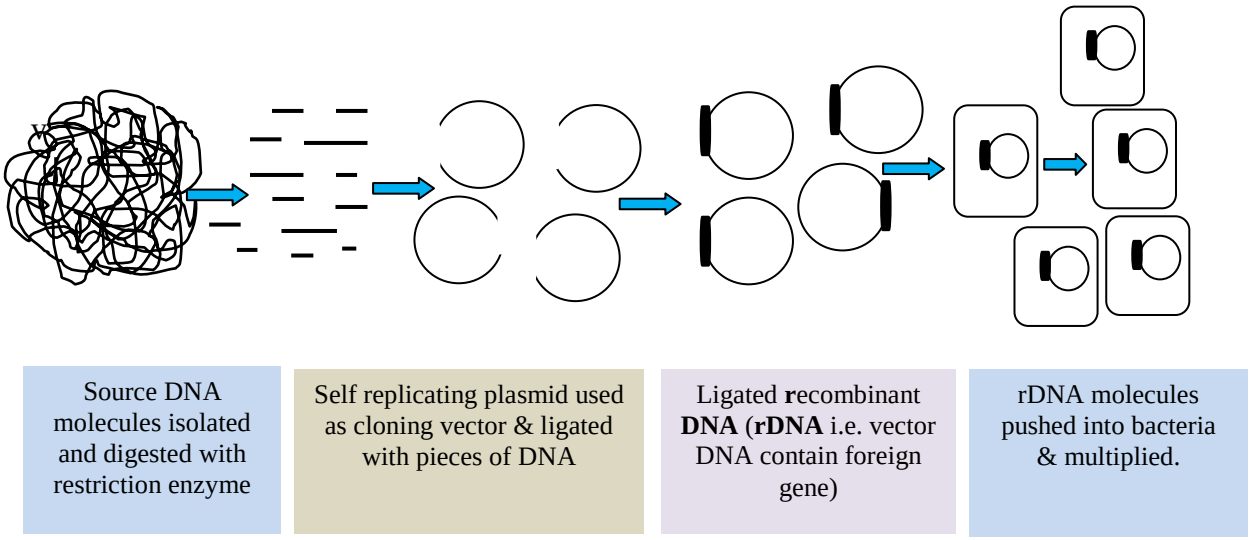


Fig-3: Over view of Cloning: To make identical copies

Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plants. Number of crops have been transformed with useful genes especially insect, disease and herbicide resistance, among them Bt cotton for insect resistance become very popular in India and other countries.

What is Bt?

"Bt" is short for *Bacillus thuringiensis*, it is a soil bacterium occurring naturally. *Bt* was first discovered in 1901 by Shigetane Ishiwatari. In 1911, *B. thuringiensis* was rediscovered in Germany by Ernst Berliner. He isolated the cause of a disease called *Schlaffsucht* in flour moth caterpillars. A unique feature is its produce *cry*stal proteins called as "*CRY* proteins" or "Insecticidal *Cry*stal Protein" (ICP) that selectively kill specific groups

of insects for example Lepidopteran caterpillars (moth and butterflies), Diptera (mosquitoes and black flies), Coleoptera (beetles), Diptera (flies and mosquitoes), and nematodes.

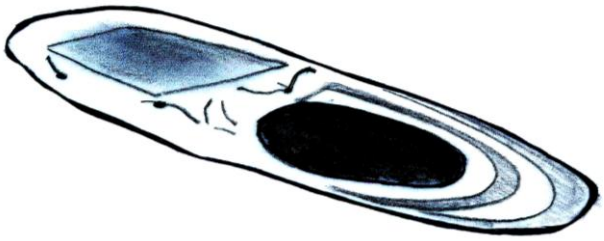


Fig 4: Longitudinal view of Bacillus thuringiensis towards the end of sporulation, showing the spore (black ovoid structure) and the toxins, with insecticidal properties, that accumulates to form a large bipyramidal crystal inclusion.

Mode of action by ICP

The target organ for Bt toxins is the insect larvae's midgut. The midgut of the larvae is a simple, tubular epithelium that dominates the internal architecture of the insects. After ingestion by insect's larvae, the *Bt* δ-endotoxin disrupt of the epithelium in the insect midgut. The alkalinity of insect midgut (pH 12) dissolves the *Cry*stals, releasing the *Cry* protoxin where it is cleaved by insect proteases to generate the trypsin resistant core of the active δ-endotoxin. The active toxin traverses the peritrophic membrane to bind receptor of brush border cells of the insect midgut. Integration of the toxin into the epithelial membrane, resulted in osmolysis of the cells, and paralysis occurred and dies within 2 days. Different *Bt* strains produce different *CRY* proteins, and there are hundreds of known strains which have identified more than 60 types of *Cry*-proteins that affect a wide variety of insects.

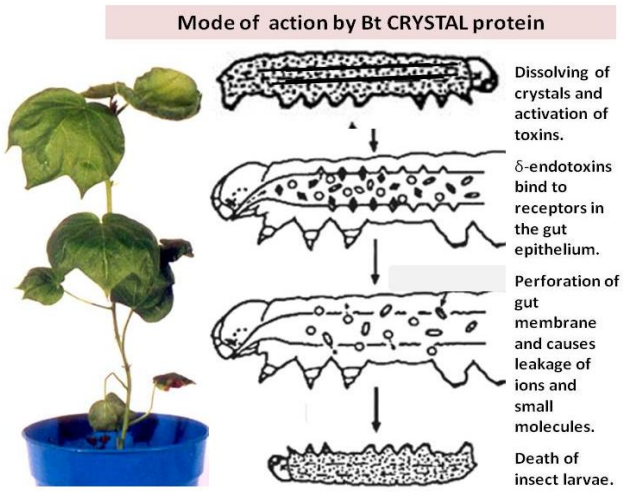


Fig 5: mode of action by Bt crystal protein

Genetic engineering provides us to isolate, characterize, modify and sequence the gene of interest and transfer into target tissue. Bt gene can be introduced into crop plants with great

precision, without losing their insecticidal property and yet being safe to human and other organisms. Once they are integrated into plant chromosome, they are inherited and expressed like any other plant genes.

There are three primary requirements for the development of transgenic plants. First, the target gene (foreign gene or transgene), virulent vector molecule, promoter (constitutive or transient and tissue specific or universal), strong plant selection marker gene and terminator (collectively called as gene construct), secondly simple and reliable method of gene transfer and finally most importantly tissue culture protocol for regeneration of plantlets through somatic embryos (Somatic Embryogenesis).

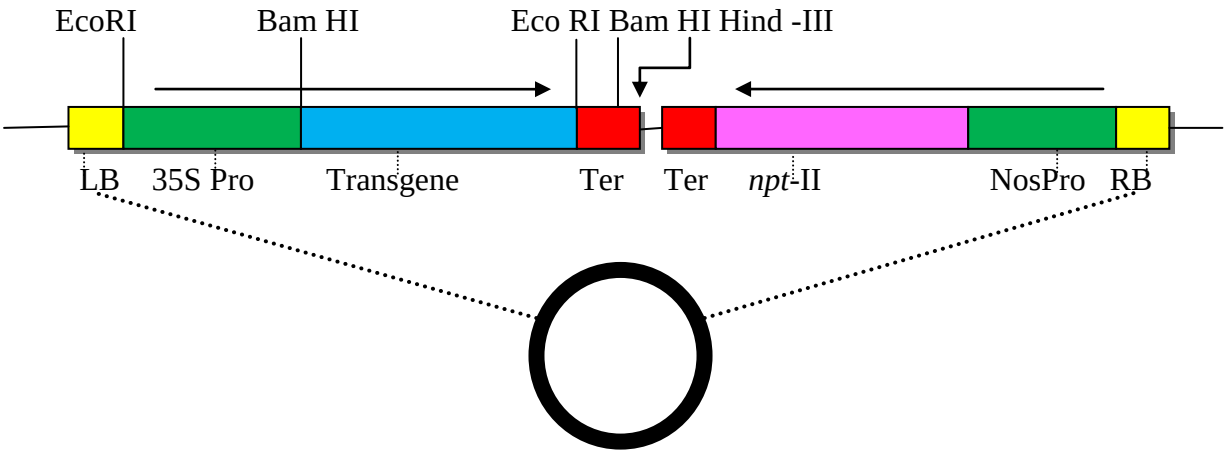
Gene constructs:

Gene construct can be defined as engineered DNA fragment to be transferred, integrated and expressed in the genome of target plant**.** Apart from the gene of interest itself, a so-called promoter ("starter") and a terminator ("stop signal") are required for expression. In most cases, additional sequences are included, e.g. marker genes, which are essential for selection of transformants and this gene also accompanied by a promoter and a terminator. In case of *Agrobacterium* gene construct the left and right border sequences are essential unit flanking the above mentioned genes and these are collectively called as gene construct. The name **"construct"** is used because the sequences normally do not exist in this combination, but must be "put together" (constructed).

Vector

Vector is a DNA molecule used as a vehicle to transfer foreign gene into recipient cell. It consist of insert (*transgene*), origin of replication (at particular sequence in a genome at which replication is initiated), and a larger sequence that serves as the "backbone" of the vector. There are a number of vectors are available, however, for genetic transformation in plant system plasmid vectors are commonly used. Many plasmids are commercially available for such uses. Plasmids serve as important tools in development of transgenics in many labs, where they are commonly used to multiply and express particular genes. The transgene to be inserted is cloned along with other selection marker gene (antibiotics) at multiple cloning site (MCS, or polylinker), which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location. All these details are

depicted below in a schematic diagram (Fig-6) showing physical position of genes, direction of transcription, location of transgene, and restriction site etc.



(LB: Left Border of T-DNA, 35S Pro: CaMV Promoter (Constitutive), Ter: Terminator, NosPro: Nopaline Promoter of *Agrobacterium*, *npt*-II: Neomycin Phosphotransferase – II gene, RB: Right border of T-DNA)

Fig-6. A typical plant transformation vector map for *Agrobacterium*-mediated gene transfer:

Border sequences

Transfer DNA also called T-DNA, which left and right border repeat sequences are essential genetic element that define and delimit T-DNA unit. T-DNA border repeat sequences contain 25 bp that are highly conserved in all Ti- and Ri-plasmids (Waters *et al.*, 1991). Transfer is initiated at the left border and terminated at the right border with help of *virulence* (*vir*) genes of the Ti plasmid.

Promoter

The upstream of each gene contains regulatory information about how and when the gene is to be expressed. The area binds to proteins (RNA polymerase) that are needed for gene expression (transcription). All genes must have a promoter in order to be expressed. Genes transferred by genetic engineering must be accompanied by a promoter. Different promoters can give genes different expression patterns. Some promoters are active in all cells at all times called constitutive promoter (e.g. 35S CAMV promoter); while others are specific to different organisms or tissue types (e.g. seed specific). Others are sensitive to external signals such as temperature or the presence of a certain chemical. Such promoters can be used as controllable on/off switches for genes. 35S CaMV promoter is very strong well known

constitutive promoter and widely used across the World for the plant transformation. It was discovered at the beginning of the 1980s, by Chua and collaborators at the Rockefeller University.

Terminator

In genetics, a **terminator**, or **transcription terminator** is a section of genetic sequence that marks the end of gene or operon on genomic DNA for transcription. In eukaryotes, terminators are recognized by protein factors that co-transcriptionally cleave the nascent RNA at a polyadenylation signal, halting further elongation of the transcript by RNA polymerase. Terminator sequences are distinct from termination codons that occur in the mRNA and are the stopping signal for translation, which may also be called nonsense codons.

Selection marker gene

Marker gene is used in the transgenic development to determine if a piece of foreign DNA has been successfully inserted into the host organism and select a viable putative transformants at early stage of regeneration process. There are two types of marker genes are used: (i) selectable markers and (ii) screening markers. Once a selectable marker is introduced into a cell, especially a bacterium or to plant cells, which confers a trait suitable for artificial selection. Selection marker gene will protect the organism from a selective agent that would normally kill or prevent its growth of non-transformants. In most applications, only one in a several million or billion cells will take up DNA. Rather than checking every single cell, we use a selective agent to kill all cells that do not contain the foreign DNA, leaving only the transformed or desired ones. They are a number of selection marker genes used in genetic engineering and transgenic development to indicate the success of a transformation or other procedure meant to introduce foreign DNA into a cell. Selectable markers are often antibiotic resistance genes; bacteria or plant cell that have been subjected to a procedure to introduce foreign DNA are grown on a medium containing an antibiotic, and those bacterial or plant cells that can grow have successfully taken up and expressed the introduced genetic material.

The most commonly used plant selection marker genes are *npt-II* (neomycin phosphotransferase-II) and *hpt* (hygromycin phosphotransferase). Kanamycin sulfate and hygromycin B are aminoglycoside antibiotics used as a selective agent to isolate the transformants which have taken up the transgenes. A large number of plants have been transformed with the npt-*II* and *hpt* genes. However, *hpt* gene and hygromycin B has proved

very effective in the selection of a wide range of plants, including monocotyledonous. Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals. The sequence of the *hpt* gene has been modified for its use in plant transformation. Deletions and substitutions of amino acid residues close to the carboxy (C)-terminus of the enzyme have increased the level of resistance in certain plants, such as tobacco. At the same time, the hydrophilic C-terminus of the enzyme has been maintained and may be essential for the strong activity of HPT.

Npt-II gene is successfully used in plants such as maize, cotton, tobacco, *Arabidopsis*, flax, soybean and many other crops but ineffective for several legumes and gramineae crops. The effective concentrations differ with plant species ranging from 50 to 500 mg/l. Some plant species have very low-level tolerance to kanamycin or hygromycin B and care should be taken to determine the minimum concentration of antibiotic that will completely kill non-transformed tissues.

The other selection systems such as herbicide resistance (phosphinothricin / gluphosinate), salt and growth-inhibiting hormones are directly used as selective agent to score transformants and some are used as metabolic (phospho-mannose isomerase,) markers. (Todd and Tague 2001).

Gene of interest

As a starting point in any transformation system, appropriate gene of interest (GOI) should be cloned to solve specific perceived problem for crop improvement whereby "viable" transgenic plants for commercial production can be generated. Much effort in recent years has been devoted to identifying potential target genes for use in genetic engineering for economically important traits especially biotic and abiotic stress resistance and improved product quality. The process has been accelerated by reference to the rapidly expanding bioinformatics data bases by progress in elucidating the plant and bacterial genomes. There is no doubt that the use of herbicide and pest resistance genes (Bt), singly and in combination, has been successful in practice, aside from social and environmental concerns. But attempts to confer oxidative and specific stress resistance through single gene transformation events are coming up gradually. In many cases, only an incremental improvement in tolerance was reported. Gene pyramiding or stacking appears to confer relatively greater benefit as does the reported case of increased expression of a biotic stress regulatory gene (Cassells and Doyle,

2003). The gene of interest should be free from technical complexity, issues of food / feed safety and consumer health risk.

Transform and Regenerate

The development and commercial release of GM crop or transgenic crop relies exclusively on two basic requirements such as "transform" and "regenerate". The first being the ability to "transform" a plant by introducing a foreign gene, or genes, into the plant genome that are stably transmitted and expressed in the progeny of subsequent generations. There are two gene delivery methods are used successfully. The widely used *Agrobacterium*-mediated transformation method, and particle gun bombardment. The second requirement is the need to "regenerate" fertile plants derived from individual cells i.e. somatic cells, first the foreign gene is transferred to a single cell and that transformed cell is regenerated into a complete plant, so that all the plant cells would carry a copy of foreign gene and express, and this technique proven to be the much more difficult in many crops including cotton. To overcome this problem another method called "direct shoot organogenesis" is being followed in cotton.

Types of gene transfer techniques:

The precise gene transfer method is very essential for successful development of transgenic plants. In general, there are two types of gene transfer methods are used in plants such as direct method (vector-less) and indirect method (vector-mediated).

***Direct gene transfer or Vector-less*:**

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector such as **chemical mediated** gene transfe**r** using polyethylene glycol (**PEG**), **microinjection** into plant protoplast (cells without cell wall), e**lectroporation** using electric pulse, p**article gun/biolistic/gene gun, lipofection (**circular lipid molecules**)** etc**.** Among them biolistic method is used successfully in many plants including cotton.

***Particle gun/biolistic/gene gun*:**

An alternative route to getting foreign DNA into plant cells is called biolistic. In this method genetically manipulated DNA is coated onto small (gold) particles and these are fired

into plant cells by high pressure helium gas in the device. The concept of particle bombardment has also been known as biolistics, microprojectile bombardment, gene gun, etc. Following these experiments, the technique was shown to be a versatile and effective way for the creation of transgenic plants (***www.bio-rad.com***).

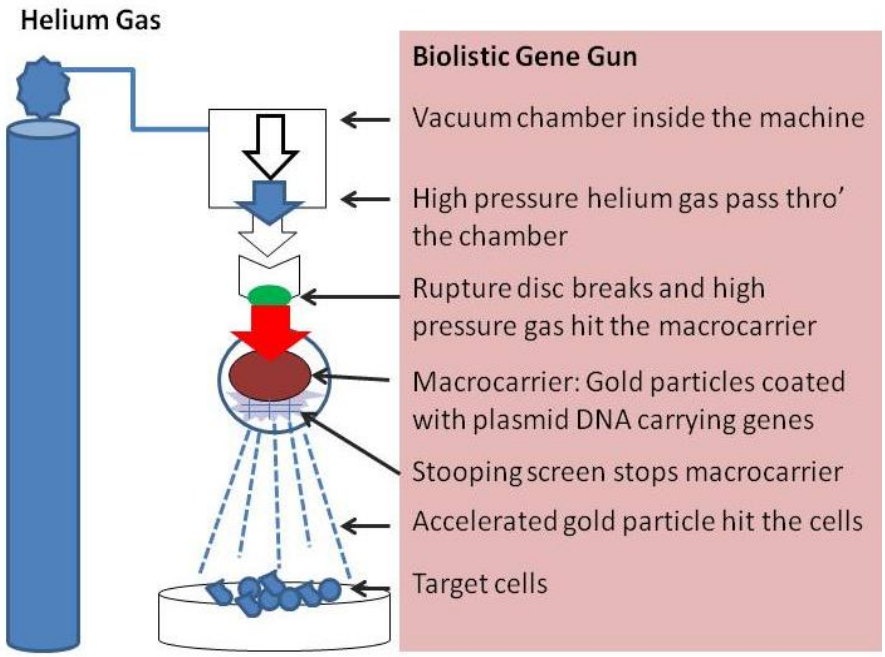


Fig 7: Functioning of the gene gun

Vector-mediated or indirect gene transfer:

Agrobacterium tumefaciens has been widely used for gene transfer in plants. This bacterium is known as "**natural genetic engineer**" of plants because these bacteria have natural ability to transfer its small piece of DNA called **T-DNA** from the Ti (tumour inducing) plasmids into plant genome at the wound site/cell and cause an unorganized growth of a cell mass known as crown gall, this is due to presence auxin and cytokinin genes. Therefore, these genes i.e. growth hormone producing genes are removed and cloned useful foreign genes and allowed to transfer T-DNA into target plant cells and tissues.

To transform plants, leaf discs or embryogenic callus are collected and infected with ***Agrobacterium*** carrying economically important foreign gene. The infected tissue is then cultured (co-cultivation) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. After this, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic to selectively eliminate non-transformed

tissues. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root-inducing medium, and after another 3-4 weeks, complete plants are transferred to soil following the hardening (acclimatization) of regenerated plants. The molecular techniques like PCR and Southern hybridization are used to detect the presence of foreign genes in the transgenic plants.

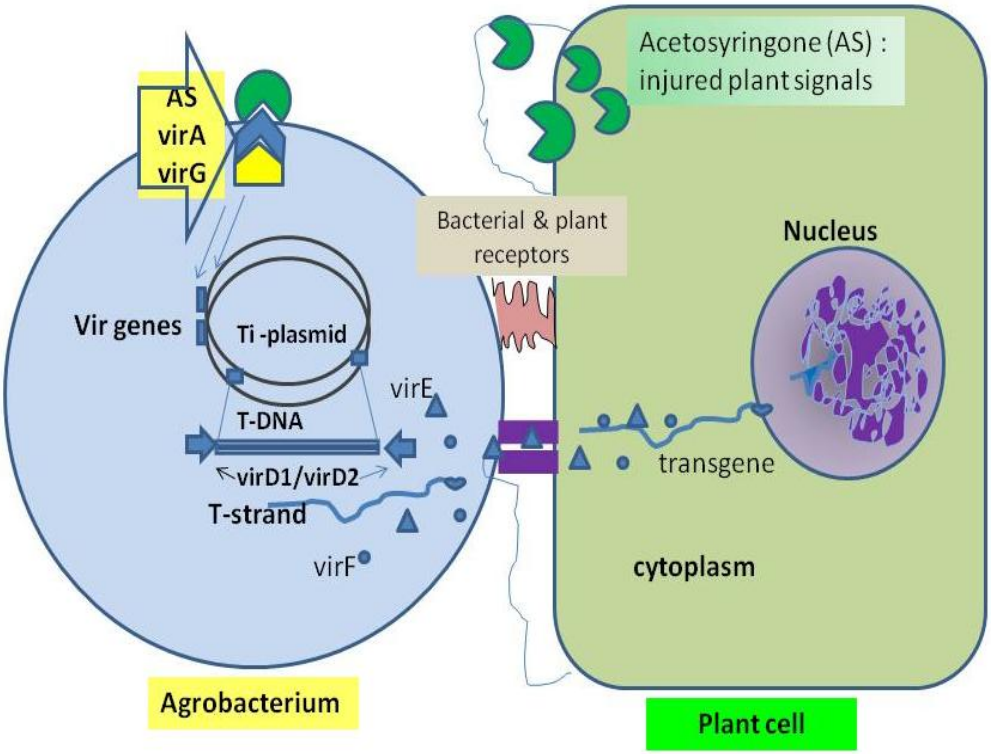


Fig 8: The basic principles of *Agrobacterium* – mediated gene transfer in plants

Somatic Embryogenesis

Plant tissue culture or the aseptic culture of cells, tissues and organs, is an important tool in both basic and applied studies. It is founded upon the research of Haberlandt in 1902, who introduced the concept of totipotency, that all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant. That is a somatic cell would act as embryo and regenerate into complete plant, which is called somatic embryogenesis, however this capacity is absent in animal cells. The somatic cell undergoes four stages such as globular, heart shape, torpedo and cotyledanary and finally regenerates into shoots. For many crops, an efficient tissue culture procedure has been developed, e.g. tobacco, rice and some horticultural crops. In comparison with other crops, successes in cotton tissue culture lag behind those in other crops.

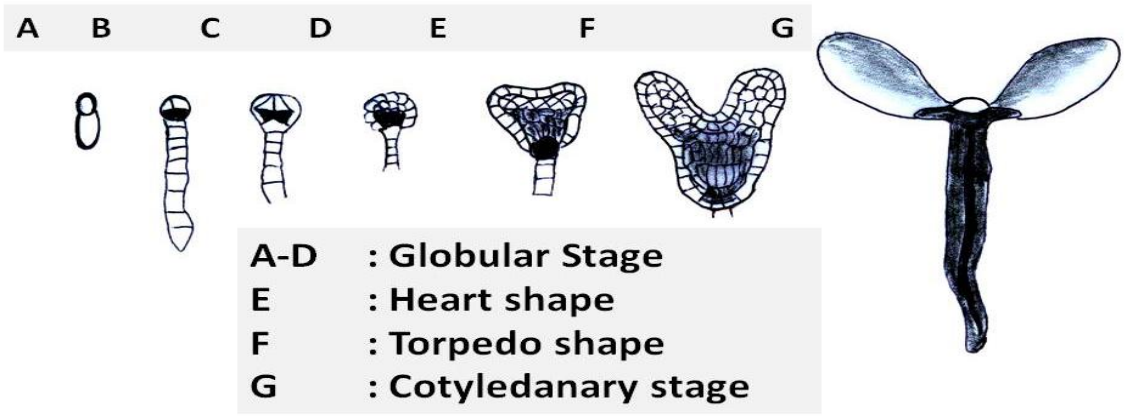


Fig 6: The somatic cells under goes different stages of embryo and regenerate into a complete plant (somatic embryogenesis)

Direct shoot organogenesis:

Direct shoot organogenesis is another method to regenerate cotton or any recalcitrant plants from the shoot apical meristem of young seedling. Tissues containing the apical meristematic region of a plant seedling are isolated and chilled to stop cell divisions. The tissue is then exposed to a transforming agent such as a biolistic gene gun or an *Agrobacterium* carrying a plasmid vector which contains a DNA encoding a gene of interest. After 24 hours the treatment is reversed, so that the metabolic activity of the tissue returns to normal, allowing the meristematic cells to enter mitosis synchronously and rapidly. The transformed cells are then regenerated into plantlets in MS medium (Murashige and Skoog 1962) containing growth hormone cytokinin such as BAP (Benzylaminopurine), 2ip (N6-(2-isopentenyl)-adenosine), kinetin etc., and evade callus phase.

Molecular confirmation of transgene integration and its expression

A wide variety of crops have been genetically engineered to contain beneficial traits such as insect resistance, herbicide resistance and disease resistance. Accurate and reliable detection of genetically modified organisms (GMOs) through molecular characterization is becoming increasingly important. After successful transformation and regeneration of transformants, the confirmation of transgene integration and its expression in the host cells should be analysed with great precision. In brief, the transgene integration is confirmed by PCR, Southern blot and RT-PCR. The expression of transgene may be detected by the

specific phenotype produced or by assaying the enzyme/protein produced using various assays, including ELISA test and Western blot.

PCR/ RT PCR

The polymerase chain reaction (PCR) is a technique for rapid amplifying a particular piece of DNA in the test tube. PCR invention has revolutionized molecular genetics. The use of this technique has generated unprecedented advances in gene discovery, diagnostics, and gene expression analysis. In addition, new techniques that build on PCR have further expanded its range of scientific applications. PCR is carried out in most of the laboratory to confirm the gene integration in the putative transformants using marker gene (*npt*-II) or target gene with genes specific primers. These are typically short, single stranded oligonucleotides which are complementary to the outer regions of known sequence. More care should be taken while carry out PCR experiment especially preparation of sample (devoid of contamination), appropriate concentration of master mix, primers, cycling, additives and more. Perhaps the most critical parameter for successful PCR is the design of primers especially primer length, melting temperature (Tm), specificity, complementary primer sequences, G/C content, and polypyrimidine (T, C) or polypurine (A, G) stretches and 3-end sequence.

Real-time PCR is a powerful advancement of the basic PCR technique. Through the use of appropriate fluorescent detection strategies in conjunction with proper instrumentation, the starting amount of nucleic acid in the reaction can be quantified. Quantification is achieved by measuring the increase in fluorescence during the exponential phase of PCR. Applications of real-time PCR include measurements of viral load, gene expression studies, clinical diagnostics, and pathogen detection. Real-time quantitative PCR (qPCR) protocols are extensively used for the detection and quantification of transgene expression in the genetically modified crops and copy number can be detected. The assay utilizes SYBR Green I (SGI) detection of amplicons of the genetically modified organism (GMO) specific sequences as well as a common known gene as a reference. The assay can detect GMO materials down to 0.5% (w/w) concentration in seed. The use of SGI for detection makes this protocol readily adaptable for the analysis of other non-GM crops for the presence of GMO material.

Southern blot analysis

Southern blot is one of the commonly used methods to detect specific DNA. This method is highly reliable to confirm the transgene integration in the GM crop with labeled

probe. To detect the inserted gene in the host plant and transgene copy number. Southern hybridization was developed and published by Edwin Southern in 1975. The method he devised is still used in laboratories today and bears his name, the Southern blot. This technology opened the way for later methods focused on RNA and for protein. In this procedure, DNA fragments are digested into small fragments using restriction enzyme. The mixture of DNA fragments is then separated according to size by way of a technique called gel electrophoresis. Following separation, the double-stranded pieces of DNA are denatured, or separated, into single strands within the gel. Next, the DNA is transferred from the gel onto a blotting membrane. Once the transfer is complete, the membrane carries all of the bands originally on the gel. The membrane is then treated with a small piece of DNA or RNA called a probe, which has been designed to have a sequence that is complementary to a particular DNA sequence in the sample; this allows the probe to hybridize, or bind, to a specific DNA fragment on the membrane. In addition, the probe has a label, which is typically a radioactive atom or a fluorescent dye. Thus, following hybridization, the probe permits the DNA fragment of interest to be detected from among the many different DNA fragments on the membrane. Southern blot hybridization is the most common method for copy number determination in the transgenic plant. However, it is labour intensive and time consuming. RT-PCR techniques are also being used to identify copy number. The most accurate method, TaqMan, has made a fluorogenic 5'- nuclease assays in RT PCR to determine transgene copy number (Ingham *et al*., 2001).

ELISA test

ELISA (Enzyme Linked Immunosorbent Assay) is one of the most widely used biochemical method in laboratory analysis and diagnostics. This antibody-antigen reaction is used in the ELISA test. Analytes such as peptides, proteins, antibodies and hormones can be detected selectively and quantified in low concentrations among a multitude of other substances. Additionally, ELISAs are rapid, sensitive, and cost effective, can be performed in a high-throughput manner. An ELISA is used in a vast variety of different types of assays (e.g. direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA etc). Nevertheless, all ELISA variants are based on the same principle, the binding of one assay component – antigen or specific antibody – to a solid surface and the selective interaction between both assay components. Molecules not specifically interacting with the assay component bound to the solid surface are washed away during the assay. For the detection of the interaction the antibody or antigen is labelled or linked to an enzyme (direct ELISA). Alternatively, a

secondary antibody conjugate can be used (indirect ELISA). The assay is developed by adding an enzymatic substrate to produce a measurable signal (colorimetric, fluorescent or luminescent). The strength of the signal indicates the quantity of analytes in the sample. The protein quantitative data should be generated with transformed plants and documented. This would assist to select the best event based on transgene expression.

Western blot

The Western blot (protein immunoblot) is an analytical technique commonly used to detect immunologically specific proteins in a GM sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native / non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or polyvinylidene difluoride (PVDF), where they are probed (detected) using antibodies specific to the target protein. That is, antibodies directed against a particular protein or set of proteins are used to selectively detect those, and only those, proteins. (Hempelmann *et al*.1987). There are now many reagent companies that specialize in providing antibodies (both monoclonal and polyclonal antibodies) against tens of thousands of different proteins. The method originated from the laboratory of George Stark at Stanford. The name **Western blot** was given to the technique by W. Neal Burnette (1981).

The two main advantages of electrophoresis are:

(i) sensitivity - silver staining can detect 10 ng whilst western blotting can detect 0.1 ng
(ii) specificity - only the protein(s) of interest will be detected.

Bt - Cotton Technology

The era of transgenic cotton began when Perlak *et al*. (1990) introduced *cry* 1A(b) and *cry* 1A(c) genes into cotton (*G. hirsutum*) plants and transformed plants showed a high level of resistance to *Helicoverpa*. During the field and laboratory tests, it was demonstrated that transgenic cotton is highly effective against neonate larvae of *H. armigera* (cotton bollworm) *H. virescens* (Tobacco budworm), *Pectinophora gossypiella* (pink bollworm). The Bt gene from the original genetically engineered mother plant was Coker – 312. Transferred to advanced cotton cultivars through backcrossing. Later Gene stacking, involving the introduction of more than one gene of similar effects is becoming an attractive alternative for

developing durable resistance and for simultaneous and effective control of more than one insect together. For instance, in cotton, Monsanto transgenic event 'Bollgard -II' carries two genes *viz. cry* 1Ac (against American Blllworm and *cry* 2Ab (Against tobacco bud worm).

Indigenous Bt Cotton

Cotton is recalcitrant to somatic embryogenesis, thus alternative and genotype independent methods are being used, i.e. direct shoot organogenesis, in this method the meristems are used to transform and regenerate directly from meristem cells without callus phase. This method has the advantage of being genotype independent; almost all cultivars can be regenerated from shoot tips. The Central Institute for Cotton Research, Nagpur is working on development of transgenic cotton both in G. *hirsutum* and *G. arboretum* genotypes to transfer Bt *cry* genes such as *cry*1Ac, *cry*1F, *cry*1Aa3 by *Agrobacterium* mediation. These transgenic are developed with straight varieties; thus farmers can reuse Bt seeds for consecutive generation, further these genotypes are being utilized in backcross breeding program for development of straight varieties with other elite cultivars as well as suitable hybrids.

Advantage of Bt – cotton

- *Effective control of burrowing insects (difficult to reach with sprays)*
- *Control all stages of insects' development*
- *Independent of weather condition (season long protection)*
- *Economic to the producer by avoiding chemical sprays*
- *Environmentally safe by avoiding chemical pollution*
- *Reduced input cost to the farmer*
- *Qualitative insect control and*
- *It breaks resistance development by the insects at neonate larval stage.*

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Development of Bt cotton

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Introduction

Deliberate alteration of the genome of an organism by introduction of one or a few specific foreign genes, is referred as "Genetic engineering" or "Genetic transformation". The modified organism is generally described as "transgenic organism" or "genetically modified organism". In this highly sophisticated method of genetic modification of plants the genetic material or DNA sequence coding for a desirable trait is identified from a donor organism by a variety of molecular techniques and then cut out from the parental DNA using the restriction endonucleases. The relatively small piece of DNA is then introduced into the recipient plant cells by one of several possible methods and plants are regenerated from them.

Genetic transformation of plants has become an indispensable aid to plant physiologists and biochemists in enabling them to understand the role of individual genes in plant. On the practical side, recombinant DNA technology is ushering in the era of molecular breeding of plants which offers many advantages over the established methods of foreign gene transfer in plants by sexual or for that matter, somatic hybridization (Logemann and Scheel, 1993a). The traditional breeding methods allow movements of genes only between closely related plants. But through genetic engineering techniques, sources of new genetic material to be introduced, is unlimited. For example, genes from viruses, bacteria, yeast, animals and completely unrelated species of plants to that of the recipient species, can be introduced in a functional form, into elite plant cultivars. Secondly, the addition of a useful trait by molecular breeding would not disrupt an elite phenotype which is often a problem with conventional breeding. In the latter case, segments of the whole genomes are transferred from the donor organism to the recipient organism and it is often difficult to separate beneficial genes from deleterious genes which are closely linked. Consequently, the process of plant breeding is slow, and takes up to at least 15 years to produce a new variety. By the introduction of a single well defined gene that usually does not disrupt economically important phenotypic traits of the recipient elite cultivar, crop improvement can be

considerably accelerated. Thirdly, once a particular gene has been restructured, it can be cloned.

Cotton (*Gossypium* spp.) belonging to the genus *Gossypium* in the family Malvaceae is an important fibre crop of global importance. Cotton is grown in tropical and subtropical regions of more than 80 countries with an annual production of 20 million tonnes (Li et al 2004). It is an important source of oil and high quality protein and plays a significant role in the national economy. Besides being the backbone of the textile industry, cotton and its byproducts are also part of the livestock feed, seed-oil, fertilizers, papers and other consumer products. Handling, processing and production of various consumer based products of cotton also play an important role in the social and industrial structure.

Biotic stresses such as insects and pathogens are the most important factors that severely affect the productivity in cotton. More than 162 species of insect pests including sucking pests, tissue borers and defoliators have been reported to infest cotton at various stages of growth, causing losses up to 60%. Cotton is vulnerable to a number of insect species, especially to the larvae of lepidopteran pests. This group of insects seriously damages the crop causing annual losses of atleast US $ 300 million.

During the past few decades, plant breeders have been successful in developing varieties/hybrids with high yield, fibre quality and pest and disease resistance by exploiting the naturally available variability, however it appears that a plateau has almost been reached and routine breeding methods may not be useful to break the impasse. Development of new genetic material possessing resistance to insects, especially for bollworm has not made the required impact in achieving complete resistance due to lack of genetic variation for resistance. Ploidy differences limit the utilization of wild species also.

As biotechnology tools have become reality in the last two decades, transgenic technology has become a popular option for the development of bollworm resistant cotton (Sawahel, 2001). *Bt*, in short a terminology for *Bacillus thuringiensis*, refers to a naturally occurring soil bacterium used by farmers to control Lepidopteran insects owing to a toxin it produces inside the insect guts. Through genetic engineering, scientists have introduced the gene responsible for making the toxin into cotton. *Bt* crops express the insecticidal gene throughout their entire growing cycle. *Bt* cotton, with its promise of increased yield and reduced insecticide spraying, is now being adopted in many developing countries as a more powerful environmentally- safe and cost effective alternative to conventional cotton farming. Transgenic cotton varieties with multiple traits (*Bt* or herbicide tolerance) are grown on 9 million hectares spread over commercial cultivation since 2002. The area under transgenic

cotton has increased from 86,240 ha in 2003 to 6.2 million ha in 2007 (Source; ISAAA, 2008). A nationwide survey carried out in 2003 indicated that the *Bt* cotton growers in India were able to obtain on an average, a yield increase of about 29% and a reduction in chemical sprays by 60% and an increase in net profit by 78% as compared to their non Bt counterparts (Bennett et al., 2004).

In the past decade extensive research efforts have been focused on cotton, and a number of genes conferring agronomic advantages have been introduced through *Agrobacterium* (Perlak et al., 1990, Bayley et al., 1992; Thomas et al., 1995, Leeelavathi et al., 2004; wu et al., 2005 and zhao et al., 2006) or through particle bombardment (Finer and Mcmullen, 1990; Rajashekaran et al., 2000) or by a combination of both methods (Majeed et al., 2000).

However, the introduction of foreign genes into cotton through either *Agrobacterium* or biolistic transformation involves the development of an efficient regeneration system. Regeneration system through somatic embryogenesis is preferred over organogenesis because of a probable single cell origin of the somatic embryos (Merkle et al, 1995). Bt technology is the only insect technology that has been commercially exploited so far (Wilkins et al., 2000). Cotton has been a difficult crop to manipulate with high efficiency through somatic embryogenesis. As only Coker varieties were found to respond better for gene transfer, most of the desirable genes are introduced initially into Coker which in turn is backcrossed into other adapted varieties later. Several years of backcrossing and selection are required to identify lines suitable for commercialization. With the exception of Coker lines, cotton remains to be the one of the recalcitrant species to manipulate in vitro culture (Wilkins et al 2004). Genetic analysis revealed that the embryogenic potential in these generable lines is a heritable trait with low heritability.(Kumar et al., 1998)

The regeneration and transformation of cotton pose problem in the development of transgenic cotton as regeneration in cotton is genotype dependent, and regeneration protocol so far standardized are not reproducible. The majority of the cotton varieties are recalcitrant to regeneration and therefore not amenable to genetic manipulations. The introgression of the transgenes in to the elite cultivars is time consuming and always leads to the transfer of certain undesirable traits from the regenerable cultivars. On the other hand, germline transformation protocols have very low transformation efficiency, requiring large number of transformation to obtain a reasonable number of transgenic. Secondly screening of T1 plants are necessary in order to eliminate chimeric plants.

Cotton Regeneration

In-vitro regeneration of cotton is most difficult because of its genotype specificity. Many cultivars of cotton are recalcitrant to regeneration especially by somatic embryogenesis, which is preferred method over organogenesis. The advantage of somatic embryogenesis is that it is the single cell origin and somatic embryos have no vascular connections with the callus tissue, indicating that they are more amenable to *in-vitro* manipulations (Shoemaker et al,1986). The in-vitro culture involves intact tissue and organs and therefore, requires that the structural integrity of the tissue is maintained. A variety of genotypes as well as culture media have been screened for cotton by various workers to search for reasonably morphogenic cultivars in Coker series, which would give rise to embryogenic calli in the presence of an auxin. There is lot of variation between cultivars as well as within a cultivar for callus initiation, proliferation and regeneration potential. Though media and hormonal manipulations can be attempted to induce regeneration in a cultivar of interest, the inherent variation amongst seedlings of the same cultivar also needs to be addressed. Kumar et al (1998) developed a pure line of Coker 310 for high regeneration potential and the trait was further introduced in the F1 hybrids involving other recalcitrant varieties. Similarly highly regenerable lines of the elite Acala cotton have also been developed by successively selecting for the high regeneration potential (Mishra et al 2003) .Beside screening of genotype for the identification of regenerable cultivar for somatic embryogenesis, the manipulation of other techniques was thought to provide answer to the cotton regeneration. Cotton protoplast was found to form microcalli but no regeneration was observed (Bhojwani et al, 1977; Finner and Smith 1982; Firozabady and DeBoei 1986; Saka et al 1987). Protoplast isolated from hypocotyl derived embryogenic cell lines of Coker 312 was found to regenerate in to plants (Peters et al 1994).

Somatic Embryogenesis – Callus induction

Schenk and Hildebrandt (1972) initiated the tissue culture work in cotton and described a culture medium for obtaining soft friable callus from *G.hirsutum* var.Auburn-56 out of mesocotyl explants. They reported fast growing callus which can be obtained after three weeks of culture on a basic medium containing 2,4-D (0.5 mg/L), p-Chlorophenoxy acetic acid (pCPA) (2 mg/L).myoinositol (1 mg/l)and low levels (0.1mg/L) of kinetin. This was followed by Devis et al (1974), Smith et al (1977) Price et al (1977) , Nandeshwar and Dongre (1993) who studied callus proliferation under high light condition alone, low light vs

high light and variable glucose concentration. Hypocotyl was found to be the best explants from *in-vitro* seedling for vigorous callus growth.

Differentiation of callus into somatic embryos or shoot bud (organogenesis) is a pre-requisite for regeneration of complete plants. Early studies indicate differentiation of roots (Smith et al 1977, Nandeshwar, 1993) and shoot buds (Nandwshwar, 1995) from callus of *G.hirsutum* and *G.thurberii* but somatic embryos were not obtained. Leaching of phenolic compound into the culture media leading to a browing of callus tissue was attributed as main cause for non-differentiation in cotton tissue culture. Sucrose as carbohydrate source was observed to enhance browning of callus tissue (Beasley and Ting, (1971); Sandstedt, (1975); Satyanarayana (1985) and Nandeshwar and Dongre, (1993), Sandhya Kranthi et al, (1997).To overcome this problem anti-oxidants such as PVP and ascorbic acid at 0.1 % and 0.01 % individually or in combination were incorporated in the media. These antioxidants appeared to decrease the phenolic content in the callus while no definite relationship was observed with the extent of phenolic leaching in the medium.

Somatic Embryogenesis and plant regeneration in Coker genotypes

Regeneration via Somatic embryogenesis was first reported in *G .hirsutum* cv Coker 310 from 2 years old callus on modified LS medium containing 2 mg/L NAA + 1 mg/L kin (Davidonis and Hamilton,1983).They reported increase in percentage of embryoids by prolonged culture adding double the standard of KNO3 and omitting NH4NO3 from the medium. Later somatic embryogenic response in cotton was observed to be genotype specific (Gavel et al 1986); Shoemaker et al 1986).Trolinder and Goodin (1987) selected Coker 312 as a model system for regeneration.They found hypocotyl explants as the best for callus induction and Somatic embryogenesis. They determined optimal media for induction of somatic embryogenesis from mature and immature tissue of *G. hirsutum* cultivars. Trolinder and Xhixian (1989) screened 38 cotton genotypes belonging to *G .hirsutum* and four classes of responses were identified viz. high moderate, low and non-embryogenic. Requirement of hormonal combinations and kind of explants for efficient somatic embryogenesis and plant regeneration was optimized in the Coker 312 (Trolinder and Goodin 1988). Highly embryogenic individual were selected from cv Coker 312 and Paymaster 303 for use as germplasm source for transfer of the embryogenic trait. Even embryogenic trait identified in the cv T-25 and Coker 312 was utilized in crossing in diallel fashion with non-embryogenic cultivar like Acala SJ-S and Paymaster 303, T1 and T-169 and backcross populations were

raised and tested for embryogenesis on MS medium containing 4.0 mg/L NAA,1.0 mg/L kin,100 mg/L, myo-inositol and 30 g/L glucose (Gawel and Robacker,1990). Rangan (1993) and Mittan (1985) also described somatic embryogesis and plant regeneration in cotton. This procedure involved a lengthy culture period and thus was difficult to repeat.

Somatic embryogenesis and plant regeneration in other genotypes

Investigation on regeneration response in Indian cultivar belonging to *G. hirsutum* like PKV081, LRA5166, LRK516 (Anjali) and Bikaneri Narma were evaluated for their capacity of callus formation and induction of somatic embryogenesis (Nandeshwar et al 1994 and Dongre et al 1994).Three explants viz hypocotyls, mesocotyl and cotyledonary leaves were evaluated against eight different media combination. Three different cell lines were isolated from hypocotyls derived callus of which one was highly embryogenic. Several other investigators have worked extensively on plant regeneration through somatic embryogenesis from not only Coker (Finer 1988, Firozabady and DeBoer 1993, Trolinder and Xhixian 1989 and Chaudhary et al 2003) but also in Sicala, Siokara (Cousins et al 1991; Rangan and Rajsekharan, 1996), Chinese cotton Simian-3 (Zhang et al, 2001) and Acala varieties (Rangan 1993, Rangan and Rajsekaran 1996). Although regeneration efficiency via somatic embryogenesis has been improved, there are still some problems associated with cotton regeneration. These are, variation in the genotype dependent response, prolonged culture period, a high frequency of abnormal somatic embryo development, low conversion rate of somatic embryos into plantlets and lack of shoot elongation. In long term cultures of cotton, regeneration of morphologically abnormal and sterile plants are produced. These changes are due to chromosome aberration in plant cells. The most common are chromosome breakage and rearrangement, chromosome losses and additions. These phenomenon create genetic variability and are useful for obtaining somaclonal variants.

Variation in chromosome number during cell culture

Barrow et al (1978) produced haploid and diploid callus from anthers of *G.barbadense* and *G.hirsutum* genotypes. The cytological examinations of anther derived suspension culture revealed that the frequency of haploid cells in culture was only 2-3%.Bajaj and Gill (1985) raised callus from embryos, ovules, anther and hypocotyls segments of *G arboreum* and *G.herbeceum* genotypes. The extent of variability in terms of chromosomes number ranged from haploid (13) chromosomes to a highly polyploids (>104). In callus derived from ovules,

the range of genetic variability was greater in callus of *G.herbeceum* in which haploid, triploid, pentaploid and hexaploid were present in high frequency, where as in *G.arboreum* callus, higher number of diploid and tetraploid cells was present. Cytological abnormalities in the form of chromosome number and bridges during meiosis were observed in cell culture of *G.hirsutum* (Li et al ,1989)and cotton somaclone callus culture (Stelly et al, 1989). Somatic variation observed during long term cultures of cotton can be overcome up to certain extent by the use of freshly initiated or cryo-preseved calli for regeneration. (Rajesekaram, 1996). An embryonic callus line with a high regeneration potential can be cryo preserved and can be used from time to time for transformation and regeneration. Kumaria et al (2003) have reported a high frequency of accelerated production and development of somatic embryos that grew into the normal plantlet within five to six months after callus initiation through manipulation of nutrition and microenvironment condition.

Shoot tip and meristem culture

Shoot tip and meristem culture is another method of plant regeneration .This method of regeneration has been utilized in many crops especially those which are recalcitrant to regeneration by somatic embryogenesis. Stewart (1986) described the result of organ and tissue culture of cotton. Bajaj and Gill (1986) explored the possibility of micro propagation and germplasam preservation of cotton through shoot tip and meristem culture. They used 1 mm long shoot tip of *G.arboreum* and *G.herbeceum* and three interspecific hybrids cultured on MS+NAA and kin or BAP. The explants formed callus, adventitious buds and multiple shoots. The formation of multiple shoots was observed on the medium containing NAA (0.5 mg/L) and BA (2 mg/L) or kinetin (6 mg/L). In general, they found better responses of *G. hirsutum* compared to *G.arboreum* .Similarly method of regenerating cotton plants from shoot apical meristem of seedlings was developed for use with particle gun and *Agrobacterium* mediated transformation in *G.hirsutum* and *G.barbadense* genotype. (Gould et al 1991). Shoot tips cultured from variable age group of seedlings of cvs belonging to *G.hirsutum* such as Coker 417-68, Coker 310, Coker 100 st, LRA 5166, PKV081 and one intra-hirsutum hybrids, showed varied responses to shoot and root development (Nandeshwar, 1995) in 15 and 45 days old shoot apices consisting of apical meristem , 2-3 partially expanded leaves and cotyledonary node. Murashige and Skoog basal salts supplemented with different combinations of BAP and kin induce multiple shoot buds (Nandeshwar et al 1997, 2002).The response of multiple shoots was 100 and 77 % from 6 to 14 days old explants. Hemphill et al

(1977) also developed a rapid, clonal propagation procedure to regenerate cotton plants from pre-existing meristem excised from *in-vitro* grown tissue. Screening of BA concentrations demonstrate that shoot apices, secondary leaf node, primary leaf node and cotyledonary leaf node derived from *in-vitro* grown 28 day old seedling (Paymaster HS 26) varied in their ability to form elongated shoots depending on the level of BA. Using 2-3 mm long shoot apices containing meristem and unexpanded leaves and a small portion of cotyledons isolated from seedling of three cultivars developed successfully into plantlets without callus formation and no significant differences among cultivars were found.

Multiple shoot induction

Shoot proliferation from coty node of eight cultivars of *G.hirsutum* and *G.arboreum* produced multiple shoots on modified MS nutrient agar supplemented with cytokinin, benzyl adenine (Gupta et al, 1997). They reported benzyl adenine to be most effective in inducing organogenic masses that differentiated in to secondary shoots on repeated subculture. *G.hirsutum* cv Khandwa 2 produced higher number of primary shoots under *in-vitro* condition and 60% them were rooted. The similar responses were also observed by Nandeshwar et al (1998, 2002) in *G.hirsutum* cultivars involving Khandwa 2, Bikaneri Narma, MCU 10, MCU 5, Indore 2 and Coker 312. This protocol was also extended to an intra-hirsutum hybrid by the manipulation variable of age group of seedling .Shoots were harvested in three successive cycle producing 12-22 shoots/explants. The R1 plants were also evaluated for their performance .A modified *in-vitro* method using a sprouted shoots from nodal segment of field grown plants was reported by Hazra et al (2001) in *G.hirsutum* cv DCH 32 and NHH44.Pruning of the sprouted shoots followed by re-culturing of the explants on MS medium supplemented with Benzyl adenine triggered the dormant 'accessary bud' followed by proliferation and differentiation of multiple shoots. Rooted shoots were obtained in the MS basal medium devoid of growth regulators .Multiple shoot formation was also observed using embryo axis explants by treating them with cytokinin BA (Morre et al (1998; Banerjee et al 2003). An average of 3.4 shoots per embryonic axis was obtained in 3 mg/L concentration growth regulator. With the decotyledonated and split embryo axis from six Indian cotton cultivars had synergistic effect on the frequency of shoot and root formation was observed (Hazra et al 2002).

Transformation of cotton

The genus *Agrobacterium*

Agrobacterium tumefaciens causes crown gall diseases of a wide range of plant, especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. The discovery of the bacterial origin of crown gall diseases (Smith and Townsend 1907) sparked a number of studies with understanding the mechanism of oncogenesis in general and applied it to study of cancer disease in animal and humans as objectives. The elegant work of Binns and Thomashaw (1988) which revealed that *A.tumefeciens* is capable of transferring a particular DNA segment transfer (T) DNA of the tumor inducing (Ti) plasmid in to the nucleus of infected cells where it is subsequently integrated in to the host genome,changed the objectives of research on *A. tumefaciens* to transformation of plants. Early realization of this goal was brighten with the report that the T-DNA contains two types of genes,the oncogenic genes ,encoding for enzymes involved in the synthesi of auxins and cytokinins and responsilble for tumor formation and the genes encoding for the synthesis of opines , a product resulted from condensation between amino acids and sugars, which are produced and excreted by the crown gall cells and consume by *A.tumfaciens* as carbon and nitrogen sources.

Virulent strains of *A. tumefaciens* contain a large megaplasmid (>200 kb) that plays a key role in tumor induction and for this reason, it was named Ti plasmid. The transfer is mediated by the cooperative actions of proteins encoded by genes determined in the Ti plasmid virulence region (vir region) and in the bacterial chromosome. The 30 kb virulence (vir region) is a region organized in six operons that are essential for the T-DNA transfer (Vir A, Vir B, Vir D and Vir G) or for the increasing of transfer efficiency (Vir C and Vir E) (Zupan and Zambrysky 1995; Jeon et al 1998). The initial result of the studies of T- DNA transfer process to plant cell demonstrate three important facts for the practical use of the process in plant transformation . Firstly, the tumor formation is a transformation process of plant cell resulted from transfer and integration of T-DNA and the subsequent expression of the T-DNA genes. Secondly the T-DNA genes are transcribed only in the plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cell, no matter where it comes from. These well established facts, allowed the construction of the first vector and bacterial strains system for plant transformation (Riva et al 1998; Opabode 2002 and 2006).

Bacillus thuringenesis

Bacillus thuringiensis (*Bt*) is a gram positive, aerobic, endospore forming bacterium belonging to morphological group I along with *Bacillus cereus, Bacillus anthracis* and *Bacillus laterosporus* (Parry et al 1983). These bacteria all have oval endospore which do not distend the sporangium and are positive for lecithinase production .Its parasporal body known as crystals, which is clearly visible in th sporangium using a phase contrast microscope recognize Bt confirmation that the organism is *Bt*. It is known that the genes encoding the crystal named the δ endotoxin (Heimpel, 1967) are on plasmid.

Although *Bt* was described by the Berliner 1915 from the province of Thuringia in Germany in 1915, the earliest description was actually that of Ishiwata in 1902, from Japan. The name *Bacillus Sotto* was given to the bacteria, which caused a wilt disease of silkworm. Aoki and Chigasaki (1915) experimentally infected silkworm with B. sotto and thus reproduced the symptom of the disease. Interest were rekindled by Mattes (1927) who was able to reisolate a strain of *Bt* from Ephestia and subsequent field tests with this isolate on the European corn borer gave promising results (Husz,1928). This work eventually led to the first commercial product, sporcine which was produced in France in 1938 (Luthy, et al 1982).

Modern History

The modern history of *Bt* starts with the pioneering research work of Steinhaus in California (Steinhaus, 1951). He reported reduction in caterpillar population below the economic level within a few days and the dead caterpillars exhibited the symptom similar to those artificially infected in the laboratory. Angus working in Canada on *B. satto* from Japan showed that the toxicity which led to paralysis and the death was associated with crystals that could be activated by the gut juice of silkworm (Angus, 1954, 1956). At the same time, Hannay (1953) and Hannay and Fitz James (1955) produced a superb electron micrograph of the crystal showing its lattice structure. They found that the crystal was composed of 175 nitrogen and contained 17 amino acids

Structure and Function of Cry proteins

Most plasmid borne Cry genes direct the synthesis of protoxins of approximately 130 KDa that are deposited as insoluble precipitates as paracrystalline bodies during sporulation and hence the designation of the Bt endotoxins as crystal (Cry) proteins. The toxic moity of Cry proteins is localized to a 60KDa trypsin resistant core that resides with the N-terminal

portion of the Cry protoxin. While the C-terminal region plays a role in the crystallization process. Amino acids homology comparisons, coupled with deletion studies, revealed a number of conserved domains in both the N-and C terminals regions that are potentially of functional significance. The three dimensional structure of the Cry proteins revealed a tripartite structure in which each subdomain provides an essential component to the active endotoxin conferring a insecticidal activity (Perfereon,1992).Domain 1 of the N-terminal region is compromised of α-helices that facilitate membrane insertion of the toxin. The β-sheets of domain –II operate in determining in target specificity via recognition and binding to receptors in the insect midgut.

Mode of action

After ingestion by susceptible insects, the *Bt* δ-endotoxin result in the larval mortality via disruption of the epithelium in the insect midgut (Gill et al 1992,) (Knowles and Dow 1993, Schnepf et al 1998). The alkaline milieu of the insect midgut dissolves the crystals, releasing the *Cry* protoxin where it is thereby cleaved by insect proteases to generate the trypsin resistant core of the active δ-endotoxin. The active toxin traverses the peritrophic membrane to bind receptor of brush border cells of the insect midgut. Integration of the toxin into the epithelial membrane, resulting in osmotic lysis of the cells, and paralysis occured within minutes. A number of factors can easily influence the mode of action and thus regulate the activity and specificity of a particular *Cry* protein. The specificity of the protease itself could moreover play a pivotal role in determining insecticidal activity. The toxic effect of Cry protein is also a function of the number of receptor present in the membrane and the binding affinity of the toxin for the receptor.

Bacillus thuringenesis: **From soil to spray**

The publication in scientific American of a popular article entitled "Living insecticide by Steinhaus in 1956 stimulated a lot of commercial interest in USA. However the early products were not successful to measure results being the poor formulations, which led to the blockage of nozzles, and lack of any understanding of sterilization. At this time, spore forms were mainly used to determine the activity and thus the rate of application. The isolation of the highly potent Kurstaki by Kurstak in 1962 from Anagasta (Ethestia) Kuhniella in France (Dulmage and Aizawa, 1982) and then from *Pectinophora gossypilla* (SB1) in the USA.Dulmag (1970) provided a much needed boost to the commercialization of *Bt*. Perhaps

the most well known and widely used biopesticides come from *Bacillus thuringiensis* (*Bt)*. This new isolate is still active in gradient in most *Bt* product it was subsequently found that thousands of starins of *Bacillus thuringiensis* exist (Lereclus et al, 1993). Each strain produces its own unique insectidal crystal protein or δ-endotoxin, which is encoded by a single gene on a plasmid (Whalom and Mc Gaughey, 1998)..Nevetheless the set of *Bt* δ-endotoxin affected variety of species from the order Coleopetra (Beetels), Lepedopetera (Moths and Butterflies), and Dipeera (Flies and Mosquitoes) (Gould and Keeton, 1996).Some *Bt* δ-endotoxin has toxicity at par with that of widely used organophosphate pesticide. Unlike organophosphate, which are quite general in their effect, *Bt* toxins are very specific to certain harmful insects and are safe to most beneficial insects and other animals. Additionally *Bt* toxins are biodegradable and do not persist in the environment (Van Frankenhuyxen, 1993).

Bt directly causes mortality in insects and isolates of the toxin from different strains follow similar mode of action. After delta endotoxin crystals are ingested, they are proteolytically processed into fragments, one of which binds to the midgut epithelium cells. The activated proteins disrupt the osmotic balance of these cells by forming pores in the cell membrane causing the cell to lyse (Van Rie, et al 1992). The gut becomes paralysed and the insect stops feeding, most insects will die within a few hours of ingestion (Marrone and Macintosh, 1993). The binding affinity of these toxin fragments is often directly related to the toxicity, though binding does not assure toxicity.

Transformation of C*ry* genes in cotton

Agrobacterium mediated transformation is the most widely used method to transfer genes into plants. Transformation is typically done on a small excised portion of a plant known as explant. The small piece of transformed plant tissue is then regenerated into mature plant through tissue culture techniques. The first reported plant transformation by *Agrobacterium* was in Tobacco in 1983 (Fraley et al 1983).

In cotton, Firoozabady et al (1987) first reported successful transformation in cotton *G. hirsutum* producing transgenic cotton in cv Coker 312. Cotyledonary leaves excised from 12 day old aseptically germinated seedling were inoculated with *A. tumeficiens* strain LBA 4404 containing virulent Ti plasmid with chimeric gene encoding kanamycin resistance. More than 80% of calli were induced to form somatic embryos which were germinated and plants established. Transformation was confirmed by opine production, kanamycin resistance

immunoassay and DNA blot hybridization. This was followed by the development of transgenics in *G.hirsutum* cv Coker 312 using hypocotyl explants (Umbeck, et al, 1987). They used *A. tumeficiens* strain LBA 4404, nptII and Cat genes, regulated by the nopaline synthase promoter. Molecular evaluation confirms that the genes were present in the primary plants. Using octapine *A. tumeficiens* strain 1058 carrying binary vector Bin 19. Makarora, et al (1989) successfully transformed leaf explants of *Nicotiana tabacum*, cotyledonary leaf explants of *G. arboreum* and tuber explants of *S. tuberosum*. Expression of the bacterial gene for nptII was confirmed in the transformed callus. The earlier transformation experiments were not thoroughly characterized and were difficult to repeat in other laboratories. Umbeck, et al (1989) was the first who repeated progeny analysis of the transgenic cotton containing foreign genes. Segregation ratios of 3:1(self) and 1:1(backcrossed) were reported. The first agronomically important gene into cotton cv Coker 312 were inserted by using *Agrobacterium* strain A208. The truncated form of insecticidal protein gene of *B. thuringiensis* var Kurstaki HD-1(*Cry* I Ab) and HD-73(*Cry* IAc) which controlled lepidopteron insect pest was transferred (Perlock, et al (1990).Protection from insect damage of leaf tissue from transformed plants were observed in the laboratory assay by larval feeding. Whole plant assay under conditions of high insect pressure with *Heliothis zea* showed Effective Square and boll protection. Immunological analysis of cotton indicated that the insect coat protein represented 0.05% to 0.1% of total soluble protein. Chair, et al (1997) carried out genetic transformation of cotton cv Sr Smrong 60 with a synthetic cry I Ac gene, using *Agro bacterium*. Addition of 100mM acetosyringone to the culture medium during inoculation and co-cultivation period enhanced transformation frequency. Jiao, et al (1997) used six day old seedling of *G. hirsutum* cotton and transformed with *Agro bacterium* harboring *Bt* gene, Gus (vidA) reporter gene and nptII as selectable markers. The Agro infection was done for 48 hours. The 30% of the regenerated plants were transformed. Integration of the *Bt* gene was confirmed by DNA hybridization studies.

Steinitz, et al (2002) investigated the insecticidal effectiveness of a δ endotoxin *Cry* protein from *B. thuringiensis* in non regenerable callus of a commercial *G. hirsutum* cv Hazera 23. Two callus types were generated. The first one harboured the *Cry* I Ac gene and the glucorunidase (GUS) and npt II. The growth and the survival rates of three major cotton moth species, *Pectinophora gossypiella*, *H.armigera* and *Spodoptera literalis* were examined with neonatal reared on callus. Normal larval development occurred in all species supplied with non transgenic callus, but insect died, or their growth was severely restricted when

reared on transgenic callus harvested from hygromycin β supplemented medium. The development of larvae on transgenic control and on non transgenic callus became very much alike after the transgenic control tissue had been sub cultured on a hygromycin β free medium for about 100 days prior to the insect callus bioassay. Accordingly, for the detection of Bt toxin activity without the interference of the influence of hygromycin β on insects, *Cry* I Ac callus was infested with insects after it had been propagated for more than 100 days on a medium free of the antibiotic. Under these conditions all the neonatal died, and the growth (weight increment) of *S. Littoratis* survivors was markedly impeded by *Cry* I Ac callus subsequently.

Leelavati et al (2004) reported transformation and regeneration of cotton. Embryogenic calli co-cultivated with *Agrobacterium* carrying cryIIA5 gene was cultured under dehydration stress and antibiotic selection for 3-6 weeks to generate several transgenic embryos. About 83% plants were confirmed transgenic by southern blot analysis. In order to improve transformation efficiency Sunilkumar and Rathore (2001) examined various aspects of transformation and regeneration in *G.hirsutum* cvs Coker 312, TAM-941, and TAM-94WE 37s. GFP proved to be a valuable tool in elucidating the timing and localization of transient gene expression and in visualizing conversion of transient events to stable transformation events. Huang et al (2001) used *Agrobacterium tumefaciens* strain LBA4404 in pollen tube pathway studies using GFP gene under the control of 35s CaMV promoter produced bright green colored fluorescence that can be detected and screened easily in cotton tissue without destroying the material.

Agro bacterium mediated transformation of shoot tip

Although cotton has been transformed via *Agrobacterium* and plants have been subsequently regenerated, commercially important cultivars have proven very difficult to regenerate from embryogenic cells. To circumvent the problem of genotype dependent regeneration of cotton, shoot tip was used as explants in many reports. Gould et al(1991,1997) suggested a method based on inoculation of isolated seedlings shoots with *Agrobacterium t*o generate putative transgenic plants of *G.hirsutum* cv Acala SJ2, Tarncot H495, Tarncot-sphinx, Stovepipe, CA 3076 etc which were fertile and produced R1, R2 and R3 progeny carrying the transferred reporter gene. The overall transformation efficiency was around 8-12%. Later Hamphill et al (1997); Gould and Magalanes – Cedeno (1998) reported procedure of co-cultivation of pre existing cotton meristem *in vitro* with *Agrobacterium* which resulted

into the regeneration of transgenic plant, which was confirmed by GUS activity assay. Gould and Magalanes –cedeno (1998) suggested a method of mild kanamycin selection after co cultivation and directly regenerated as shoots *in vitro*. Zapata et al (1999) however used 100mg/L kanamycin for selection of transformed shoots. Progenies obtained by selfing were grown in green houses and selected for expression of the T-DNA marker gene encoding nptII by painting kanamycin (2%) on the leaves. Luo et al (2000) used *G. hirsutum* cotton cultivars by using two vectors which contained genes associated with plants pathogenesis (PR genes), osmotin (PR-5). After transformation and kanamycin selection, shoots were grafted onto in vitro grown seedling. Regenerated plants were normal fertile and produce normal progeny.

Kategari et al (2007) developed transgenic cotton of an elite Indian genotype (Bikaneri nerma) of cotton (*G.hirsutum*) by *Agrobacterium*. They used shoot apical meristem isolated from seedling as explants and a synthetic gene encoding *Cry* IAc delta-endotoxin of *B.thuringiensis.* Regeneration of shoots was carried in selection medium containing kanamycin (100 mg/L) after co-cultivation of the explants with *Agrobacterium tumefaciens (*strain EHA 105). Rooting was accomplished on a medium containing naphthalene acetic acid and kanamycin. Progeny obtained by selfing was grown in the green house and screened for the presence of neomycin phosphotransferase (nptII) and *Cry* IAc genes by polymerase chain reaction (PCR) and southern hybridization. Expression of *Cry* IAc protein in the leaves of the transgenic plants was detected by express strips and quantified by Quant ELISA kits (Desigen). Insect bioassay was performed under contained conditions. Results of the field tests showed considerable potential of the transgenic cotton for resistance against cotton bollworm. The DB has approved it as new event and released for commercial cultivation.

Nandeshwar et al (2009) developed an *Agrobacterium* mediated gene transfer protocol for the diploid cotton *Gossypium arboretum* using meristematic cells of shoot tips followed by direct shoot organogenesis or multiple shoot induction of putative transformants. Seven day old shoot tips of in vitro germinated seedlings of *G.arboreum* cv RG8 were excised by removing cotyledonary leaves and providing "V" shaped oblique cuts on either sides of explants.Excised explants were inoculated with an overnight grown culture of *Agrobacterium tumefaciens* carrying a plant cloning vector harboring the *Cry IAc* gene. The explants were co cultivated in Murashige and Skoog medium supplemented with 30mg/l myoinositol, 10mg/l thiamine and 30mg/l glucose for three days in the dark. Following co cultivation, explants were incubated on the same medium supplemented with 20mg/L kanamycin for the first three

passages of 10-12 days each and subsequently on 50mg/l kanamycin to facilitate stable expression of transgene. Explants were then transferred to a fresh MS medium supplemented with either kinetin (0.1mg/L) or benzyladenine, BA (2mg/L)+ kinetin (1mg/L) to induce either single or multiple putative transformants shoots, respectively. Following 6 weeks, shoots were transferred to a rooting medium consisting of liquid MS medium supplemented with 0.05-0.1mg/l NAA and 15mg/l glucose. Rooted plantlets were first acclimatized in liquid MS with 0.05mg/l NAA and 15g/l glucose, transferred to plastic pots containing soilrite Mix-TC(a mixture of Irish peat moss and horticultural grade expanded perlite, 75:25), and grown under controlled temperature and humidity conditions in a growth chamber. Acclimatized plants were then transferred to clay pots and grown in the green house. These plants were confirmed as transgenic for cryIAc gene using polymerase chain reaction, enzyme linked immunosorbent assay, and southern blot analysis.

Useful genes introduced via Agrobacterium mediated transformation in cotton

Variety	Explants	Gene	Traits	Reference
Coker 312	Seedling explants	Cry I Ac	Resistance to lepidoteran insect	Perlak et al 1990
Coker 312	Seedling explants	2,4-D monooxygenase	Herbicide resistance	Bayley et al 1992, Lyon et al 1993
Coker 312	Seedling explants	Protease inhibitor	Resistance to insects	Thomas et al 1995
Coker 312	Seedling explants	EPSP synthase	G. M phosate tolerance	Nida et al 1996
Acala- GC 510, B3991, CSC28, Royale	Embryonic cell suspension	Mutant acetohydoxy acid (AHAS)	Tolerance to sulphonylurea and imidazolinones	Rajasekaran et al 1996
Coker 315	Seediling explants	Bromxynil specific nitrilase	Bromoxynil tolerance	Stalker et al 1988
Coker315	Seediling explants	Mutant acetoacetate synthase	Tolerance to sulphonyl urea	Sauri and Mauvais, 1996
Coker315	Seediling explants	Superoxide dismutase	Tolerance to oxidative stress and freezing	Allen 1995, Allen and Trolinder 1995
Coker312	Seedling explants	Mn superoxide dismutase	Oxidative stress tolerance	Payton et al, 1997
Coker 315	Seedling explants	Glucose oxidase	Fungal resistance	Murray et al 1999
Coker 310	Embryonic calli	*cry* II A5	Resistance to lepidopteron	Leelavathi et al 2003

			insect	
RG8	Seedling explants	*cry*I Ac	Resistance to lepidopteron insect	Nandeshwar et al 2009

VIP Cotton: A new type of transgenic cotton

The Bt gene contained in Bollgard (*cry I Ac)* is most effective against tobacco budworm *Heliothis virescens* and cotton bollworm *Helicoverpa zea,* among all lepidopteran controlled by the Cryi Ac protein. However, most cotton fields around the world are attacked not only by these two species, but also by a variety of other lepidoterans, including spodopterans species, such as armyworm and beet armyworm. Also, there is a need to avoid the problem od development of resistance to the c*ry I Ac* toxin. On a long term basis, the objectives of broad spectrum insect control and resistance management can only be achieved by exploring and utilizing new generations of transgenics significantly different from *cry* I Ac. While Monsanto has come up with a second generation *Bt* gene in Bollgard II expressing *cry* II Ab, the syngenta Group Company has developed an entirely new generation of the Bt gene with a broad spectrum effect on insects threatening cotton production. The Bt gene in the embedded form has overcome the following limitations faced during commercial use of Bt as an insecticide formulation.

- The *Bt* insecticides must be sprayed so that all plant parts eaten by the target insects are covered by a minimum quantity of the target insects are covered by a minimum quantity of the Bt insecticide. If a plant or part of a plant is missed during the Bt foliar application, the target insects will survive on those plants/parts.
- The *Bt* toxins is rapidly degraded by ultraviolet light, heat, high leaf pH or desiccation. The toxin can be degraded before it is actually consumed by the target insects.
- A specific dose of the insecticide is required to kill insects. Insects must eat enough of the treated plant part to accumulate a lethal dose.
- Experience shows that c*ry* proteins are less toxic to older larvae.
- Bt insecticides must be sprayed, which is an additional expenditure and also demands that all precautions/requirements for chemical insecticide applications be followed, including equipment, uniform, complete coverage, drift etc. the Bt gene within the plant changes the toxin delivery system to a more effective method.
- VIP is a protein that is secreted by *Bacillus thuringiensis* it grows, and thus it is classified as an exotoxin.bollgard and BollgardII genes are found during the sporulation phase and thus are classified as δ endotoxins.

- *cry* I Ac and *cry* II Ab are found in a crystalline phase and require solubilization before they can be activated by midgut proteases. On the contrary, VIP is already in a soluble state and readily available.
- Both technologies, Bollgard and Bollgard II and VIP, target the midgut receptors in target species.
- Structurally, both types of proteins are different from each other.

VIP Effects on the Midgut Cells

It is known that ingestion of endotoxins cause swelling and disruption of the midgut epithelial cells by osmotic lysis in the target insects. The following four factors play an important role for any insecticidal protein to be effective against its targets.

- Presence of specific binding sites on susceptible cells.
- Insertion of the bound toxin into the membranes.
- Solubilization of the crystal protein.
- Processing of the midgut proteases

Ingestion of the insecticidal protein and target binding sites, where the protein will be bonded, are the most important factors without which the biology of insecticidal protein fails to work. How quickly the insecticidal protein is solubilised into the target cells/receptors will determine the duration of the effects of the insecticidal protein on the target insect. If the protein is easily solubilized, the effects on target insects will become visible faster in the form of the slower feeding and slower movements. If the protein has been ingested but it is sitting there until it is properly absorbed into the epithelial cells, the insects will continue to feed and damage the crops.

The VIP gene shows insecticidal activity against a variety of lepidopterons and exhibits severe bioactivity towards *Agrotis ipsilon*(black cutworm), *Spodoptera frugiperda*(fall armyworm) and *Spodoptera exigua*(beet armyworm). A detailed study on the effect of VIP3A on the midgut epithelium cells of susceptible insects was published by Yu et al (1997). They determined the VIP mode of action and examined the proteins effect on target cells in susceptible and non-suseptible insects. They used two highly susceptible insects and one non

susceptible insect in the same order i.e black cutworm and fall armyworm and European corn borer(*Ostrinia nubilanis*), respectively.

Stability of VIP3A in the Midgut and Insecticidal activity

Fresh gut juices were extracted from the third and the fourth instar larvae of the three insects by dissecting them and extracting their gut contents by pipette. Gut juices were centrifuged and protein concentration was measured in the gut juices. Proteolytic reactions were also performed using different amounts of VIP3A containing supernatants and gut juices. The results showed that when equal amounts of VIP3a were incubated with black cutworm, fall armyworm and European corn borer gut fluids, four major VIP3A proteolysis products were identified. However, whole insects and midgut tissue extracts from both susceptible insects, probed with the same purified anti-VIP3A antibody, revealed no background bands. The relationship between the proteolytic processing and insecticidal activity of VIP3A was studied by Yu et al(1997) from the bioassays conducted, following the incubation of VIP3A with gut fluids isolated from three insects. VIP3A processed by black cutworm and fall armyworm gut fluids was active against the black cutworm and fall worm. VIP3A processed by the European corn borer was found active against black cut worm and fall armyworm. None of the VIP3A processed show acute activity against the European corn borer, a non susceptible insect.

Future perspectives:

Cotton is an important renewable resource that shall continue to play an important role in the future world economy. Some major issues in cotton management are tremendous losses incurred by pest infestation and the expensive and environmentally harmful chemical control of these pests. Therefore, the thrust at present is on bioengineering insect resistant cotton varieties. In addition, there are several other traits that need to be addressed to further improve the cotton crop for enhancing its role in the future economies. Some of these traits are: lint yield and fiber quality with respect to color, length, and elasticity, dye retention, spinning quality, naturally colored fibers or fibers with antimicrobial and heat resistant properties. The improvement of the seed oil content, reduction in gossypol content and bioengineering resistance to fungal and viral infection as well as tolerance to low water conditions also are priority. In the field of insect resistant cotton too, better strategies have to be evolved to avoid

the development of resistance in the insect population. Some of the suggested steps are: the introduction of multiple cry genes, mutated *cry* genes, along with non *Bt* insecticidal genes; tissue specific expression; enhanced expression or organelle specific expression like chloroplast expression, where the expression levels are high. This will totally eliminate the use of pesticides, besides *Bt* crops management by crop rotation and maintenance of refuge plants.

Several molecular markers linked to the important traits have been developed for cotton. Furthermore, the molecular regulation of various vital processes is also under investigation through identification of differentially expressed genes. Some of these genes have been identified and characterized. Production of colored fibre is another area of interest in transgenic cotton. The up regulation or down regulation of these genes can be engineered to achieve some of these goals. Engineering multi gene regulated traits that are essential to improve yields and other qualities of cotton would require the isolation and characterization of tissue specific promoters for regulated transgene expression. As for the transformation of cotton, such that a high expression and maternal inheritance of the genes of choice is achieved. Varieties other than Coker have also been successfully transformed but most of the elite commercial varieties remain recalcitrant to tissue culture. It is of utmost importance that the elite varieties are transformed, so that the delay due to backcrossing and the retention of the undesirable traits from the regenerable cultivars can be avoided. Though the basic engineering tools are available for this crop, cotton biotechnologists would have to face many more challenges before this crop could be bioengineered for planting all over the subtropical world under diverse ecological conditions related to both biotic and a biotic stresses.

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Physiological and molecular basis of drought resistance in cotton

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Introduction

Plants, as they are sessile, cannot escape harsh environments and are left with no option but to endure the extreme environmental conditions. They acclimate themselves to extreme environments by various morphological and metabolic adaptations. Drought is one of the major stresses affecting plant growth, development and yield of cotton. Even though cotton is a drought-tolerant crop, it responds well to sufficient water by producing lint proportional to amounts of rainfall or water supplied. The three critical periods of cotton growth that should be supplemented with moisture occur at stand establishment, pre-bloom and boll formation stages. Severe drought on cotton plants will hamper plant development and cause small bolls and squares to shed. Water deprivation during boll development also affects lint quality. Drought may make cotton cut out early or may result in early growth cycles in plants, depending on the stress and timing of the dry periods.

Drought resistance mechanisms are of three types: escape mechanism in which the plants complete their life cycle before severe stress, desiccation postponement which maintains the tissue hydration level and desiccation tolerance mechanisms, which permit the plant to withstand drought stress. Morphologically the presence and/or intensity of trichomes, epicuticular wax, stomata and other parameters such as leaf orientation, reduction in leaf area, deep roots determine the rate of drought avoidance. Accumulation of compatible osmolytes like amino acids, sugars, sugar alcohols, polyamines and quaternary ammonium compounds form the basis of metabolic adaptations of plants to water stress. While the morphological adaptations are less expensive in terms of energy and help to maintain hydration level during drought, the metabolic adaptations are costly and help in drought tolerance (ability to function under dehydration).

Most of the drought breeding programs concentrate on selection of cotton genotypes that yield well under drought conditions. The selection is based on certain traits that can be used to screen the cotton genotypes for drought tolerance. Many of such attributes including

root traits and physiological traits such as gaseous exchange, osmotic adjustment, leaf water potential, relative water contents and cell membrane stability are important components of drought tolerance in cotton.

Morphological adaptations

Trichomes provide resistance to drought by reducing the absorption of solar radiation and increasing the leaf surface boundary layer by condensing the moisture in the air onto the leaf surface, making the leaf surface cool. **Epicuticular wax** deposition reduces cuticular transpiration and helps the plants to maintain high relative water content even under drought conditions. In addition it provides protection to plants from harmful UV-B radiation and sucking pests. **Stomatal** frequency, size and the rate of stomatal closure decide the leaf water status of plants under water deficit. **Orientation of leaves** in response to sunlight (heliotropism) is important in avoiding incident of excess light energy and hence tolerating drought.

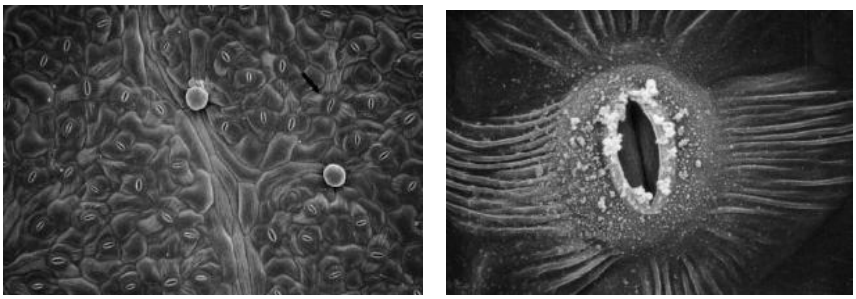
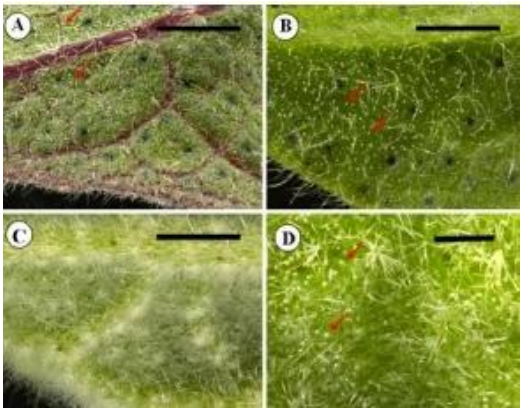


Fig. 1 Stomata on abaxial surface of the cotton leaf (Image Source: Bondada and Oosterhuis, 2000)

Fig.2.Wax in the form of striations in the abaxial surface of cotton leaf (Source: Bondada and Oosterhuis, 2000)



Light micrographs of the abaxial surface of three diploid cotton leaves, including:
A) G. arboreum, B) G. herbaceum and C) G. raimondii D) close up of adaxial surface of G.raimondii leaves

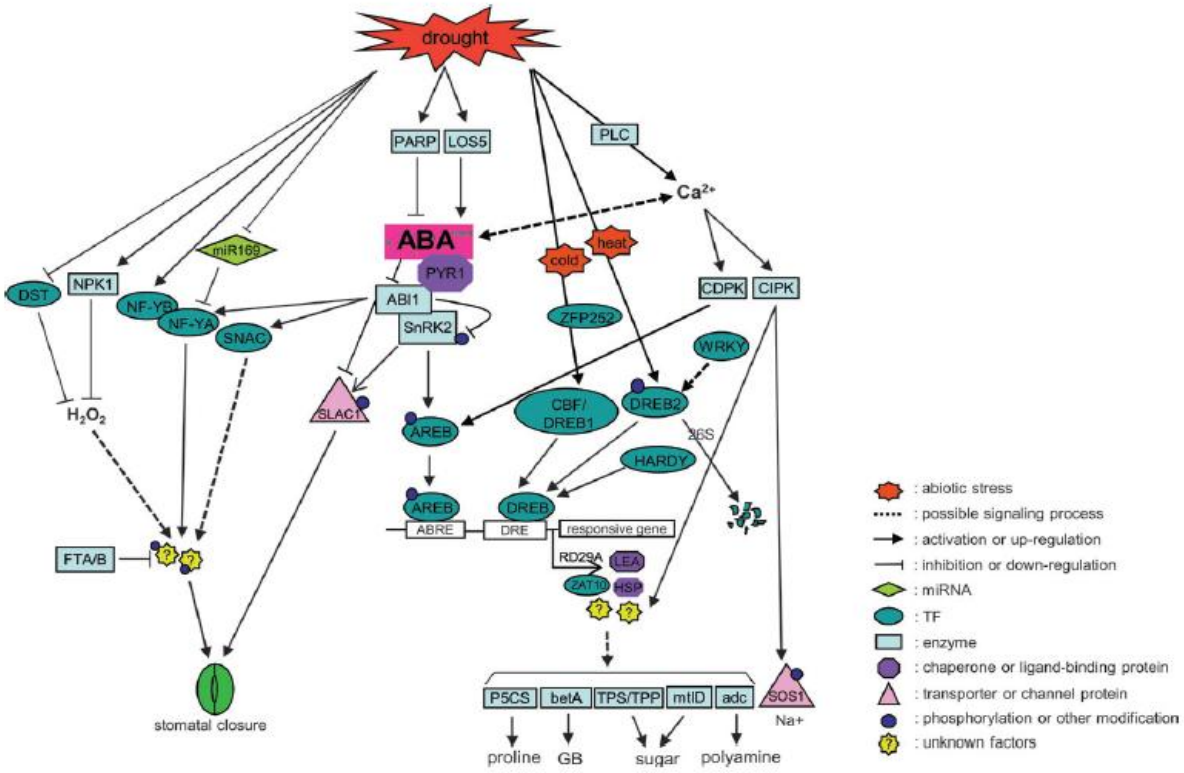
(Image source: Turley and Vaughn,2012)

Metabolic adaptations

Plants senses drought stress by osmotic sensors (receptors) present in the plasma membrane. This is followed by a cascade of signaling events ultimately resulting in transcription of genes imparting drought tolerance. The early transcribed genes are those of transcription factors or regulatory proteins and the late transcribed genes are those coding for functional proteins responsible for the production of compatible osmolytes such as proline, glycine betaine, inositil, pinitol etc., antioxidants such as glutathione, ascorbic acid, tocopherol, and antioxidative enzymes such as super oxide dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR), Peroxidase (POX) etc. and water channel proteins/aquaporins required for drought tolerance.

Drought stress induces the accumulation of ABA as influenced by PARP and LOS5. The plant hormone ABA transmits long distance signals from the root to the leaves where it mediates the partial closure of stomata by Ca-mediated signaling pathway under water deficit conditions. ABA is perceived by the receptors in the plasma membrane and induces downstream transcription factors (TFs) such as NF-YA, SNAC, AREBs and SnRK2 kinase activity. SnRK2 interacts with S type anion channel protein SLAC1 to promote stomatal closure. In response to ABA, an activated AREB/ABF binds to the conserved regulatory cis-element sequence (ACGTGT/GC) called the ABA responsive element (ABRE, Mundy et al., 1990) to trigger gene expression. Drought induces the transcription of TFs that are responsive to dehydration but not to ABA, are called ABA-independent dehydration-responsive TFs (with an exception of DREB 1D/CBF4). These TFs typically bind to a specific and conserved cis-element (A/GCCGAC) in the promoters of target genes to activate their expression. The signature cis-element is termed the dehydration responsive element (DRE). Interestingly, DRE was simultaneously characterized as a C-repeat (CRT) element in the promoters of several cold-responsive genes (Baker et al., 1994). DREB 1A (CBF 3), DREB 1B (CBF 1), DREB1C (CBF 3), DREB 2 pathways are ABA independent pathways whereas DREB 1D (CBF 4) pathway is ABA dependent. Some of the genes has two cis- acting elements ABRE (ABA responsive Elements) and DRE. For example, the rd29A gene has at least two cis-acting elements. (i)The 9-bp direct repeat sequence, TACCGACAT, termed the dehydration-responsive element (DRE), functions in the initial rapid response of rd29A to drought, salt, or low temperature. (ii) The slower ABA response is mediated by another fragment that contains an ABRE. DREBs and AREBs regulates the transcription of downstream genes to synthesize osmoprotectants such as LEA, HSP, Proline, Glycine Betaine(GB), sugars and polyamines.

Other ABA independent transcription factors induced by drought include ZFP, NF-YB and ZAT10. Farnesyl transferase α/β subunit (FTA/B), SNAC, NF-YA5, DST and NPK1 regulates the stomatal movements through H_2O_2 and Ca signaling.



PARP – Poly (ADP-ribose) polymerase ; LOS5 –Low expression of Osmotically responsive gene 5; ABA- Abscissic Acid;ABI1-ABA Insensitive 1; SnRK2- Sucrose non fermenting 1 –Related Kinase 2; SLAC1- Slow Anion Channel 1; miR169- microRNA 169; DST- Drought and Salt Tolerance; NPK1-Nicotiane Protein Kinase1; NF-YB- Nuclear Factor-YB; NF-YA- Nuclear Factor-YA; SNAC- Stress responsive NAC {NAM (No Apical Meristem), ATAF (Arabidopsis Transcription Activation Factor), CUC2(Cup Shaped Cotyledon 2)}; FTA/B- Farnesyl Transferase α/β subunit; AREB-ABA responsive element binding Protein; ZFP 252- Zinc Finger Protein 252; CDPK- Calcium Dependent protein Kinase; CIPK- Calcineurin B- Like Interacting Protein Kinase; DREB- Dehydration Responsive Element Binding protein; CBF- C-Repeat Binding Factor; LEA- Late Embryogenesis Abundant; HSP- Heat Shock Proteins; DRE- Dehydration Response Element; ABRE- ABA Responsive Element; RD- Responsive to Dehydration; ZAT- ERF associated Amphiphilic Repression(EAR) Zinc Finger Protein;P5CS- Pyrroline 5 Carboxylate Synthase, betA-; TPS-Trehalose Phosphate Synthase; TPP – Trehalose Phosphate Phosphatase; mtlD- Mannitol Dehydrogenase; adc- Arginine decarboxylase; SOS1- Salt Overly Sensitive
Image Source: Yang *et. al.,* 2010

Proteins and other osmoprotectants protect the membranes and macromolecules of the cell under drought conditions. Compatible osmolytes/osmoprotectants are a small group of chemically diverse organic compounds that are highly soluble and do not interfere with cellular metabolism even at higher concentrations. Proline, glycine betaine, sugars such as glucose and fructose, polyhydric alcohols such as pinitol and mannitol and polyamines are few examples of osmoprotectants.

LEA proteins are first characterised in cotton and wheat plants . It comprises upto 4% of cellular proteins. It offers desiccation tolerance in seeds, pollen and anhydrobiotic plants. LEA proteins are induced by cold, osmotic stress, exogenous ABA and other stresses. The proteins encoded by these genes are typically hydrophilic, Gly rich with amphipathic α-helices and strongly bind water. Their protective role might be associated with an ability to retain water and to prevent crystallization of important cellular proteins and other molecules during desiccation. They might also contribute to membrane stabilization.

Proline (Pro) is an amino acid accumulated under stress conditions. Under stressed conditions, Pro acts as a mediator of osmotic adjustment, stabilizer of sub cellular structures, a scavenger of free radicals, a buffer in cellular redox potential and a major constituent of cell wall structural proteins (hydroxyl proline) that may provide mechanical support for cells. In higher plants, proline is synthesized via both the glutamic acid (Glu) and ornithine (Orn) pathways. The former is considered to be a major pathway, especially under osmotic stress. In the Glu pathway, proline is synthesized from Glu via 2 intermediates, glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C). Two enzymes catalyze this pathway, P5C synthetase (P5CS) in the first step and P5C reductase (P5CR) in the final step. P5CS is the rate-limiting enzyme in proline biosynthesis in higher plants.

Glycine Betaine (N, N, N, trimethyl glycine) is a quarternary ammonium compound occuring naturally in plants, animals and microorganisms. They are dipolar, but electrically neutral molecules at physiological pH. GB acts as osmoprotectant by stabilizing both the quarternary structure of proteins and highly ordered structure of membranes against adverse effects of stress.

The **polyamines** are organic compounds having two or more primary amino groups - such as putrescine, spermidine, and spermine that are growth factors in both eukaryotic and prokaryotic cells. They are of polycationic nature. As cations, they do bind to DNA, proteins, membrane phospholipids and pectic polysaccharides. They are localised in vacuoles, mitochondria and chloroplasts. Polyamines in particular spermidine prevent senescence of leaves under stress conditions. Spermidine binds to the pectic polysacharides of membranes offering cell membrane stability under stress conditions.

Water channel Proteins or **Aquaporins** are a class of integral membrane proteins more commonly referred to as a class of major intrinsic proteins (MIP) that form pores in the membrane of biological cells. Aquaporins selectively conduct water molecules in and out, while preventing the passage of ions and other solutes. The gating of aquaporins is carried out to stop the flow of water through the pore of the protein as the plant contains low amounts of cellular water due to drought. The gating of an aquaporin is carried out by an interaction between a gating mechanism and the aquaporin which causes three dimensional change in the protein so that it blocks the pore and thus disallows the flow of water through the pore. Under normal conditions the aquaporin is phosphorylated (indicated by P) & the water channel is open. Under drought stress, closure of the plant plasma membrane is triggered by the dephosphorylation of two serine residues: Ser 115 in the cytosolic loop B and Ser 274 in the carboxy-terminal region. Both residues are situated in consensus phosphorylation sites. Greatest transcript concentrations, enhanced translation, and activation of existing proteins each may constitute a mechanism for regulating aquaporin abundance and activity in response to drought stress.

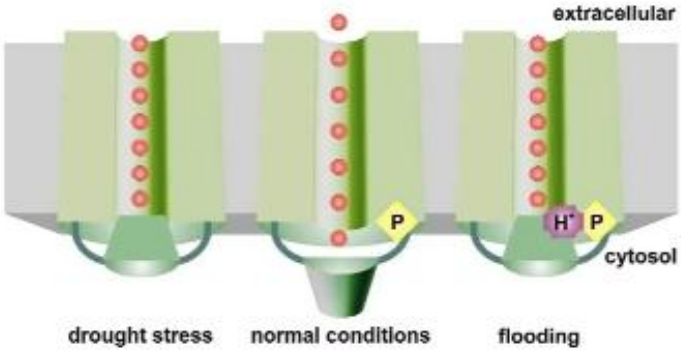


Diagram illustrating the structural mechanism of aquaporin gating in plant plasma membranes
Image Source: Tornoth -Horsefield *et. al.*, 2006.

Conclusion

Plants acquire inherent ability to exhibit drought tolerance. Understanding the mechanisms of drought tolerance helps us in alleviating the stress by agronomical, physiological and biotechnological means to improve the productivity of cotton under water limiting environments.

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Determinants of fibre quality in Cotton: A physiologist's perspective

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Introduction:

Cotton fiber is an important natural textile fiber due to its exceptional length and thickness. Cotton fibre quality improvement is of vital importance to textile industry. These properties arise largely through primary and secondary cell wall synthesis. The cotton fiber of commerce is a cellulosic secondary wall surrounded by a thin cuticulated primary wall. Cotton fiber harvested from *Gossypium* species is the world's most important renewable textile fiber. These single-celled fibers are highly elongated and thickened seed epidermal cells, and their useful properties depend on cell walls deposited during a staged cellular differentiation program lasting about seven weeks. In cotton fiber, a thin primary cell wall is synthesized while the fiber achieves its extraordinary length of, 2.5 to 3.5 cm. Cotton fibre cells are tubular outgrowth of single celled trichomes which arises in near synchrony from the epidermis of the ovule and may elongate at peak rates in excess of 2 mm per day during rapid polar expansion phase of development (Basra and Malik, 1984). The process of modification of epidermal cells to fibre initials starts 4-6 days before anthesis that has shown up by down regulation of biochemical constituents. The final trigger is provided at anthesis where the solutes help in fibre initiation and elongation process accounting for the involvement of multiple components (Gopalkrishnan et al., 2011). Cotton fibre initiates by swelling above the ovule surface near the day of flower opening (or anthesis).

During the transition to secondary wall cellulose synthesis, primary wall remodeling occurs and a thin intermediary cell wall ''winding'' layer is deposited. The transition stage ends when secondary wall thickening begins via the deposition of nearly pure cellulose. At harvest, the cotton fiber is composed of a thick cellulosic secondary wall that is surrounded by a, 200 nm thick cuticulated primary wall on the fiber perimeter (Avici et al., 2013). Despite similarities in these basic cotton fiber characteristics, there are important differences in the fiber produced by the two commercial allotetraploid species, *Gossypium hirsutum* (Gh) and

Gossypium barbadense (Gb). Polyploidization about 1–2 million years ago unified ancestral diploid A and D genomes to form the progenitors of modern allotetraploid Gh and Gb cotton, followed by independent evolution and then domestication of the two species (Avici et al.,2013). Upland cotton, accounts for the majority of current cotton fiber production because of its high yield and wider environmental adaptability. However, Gb cotton is grown in selected environments because its fiber (Egyptian cotton) is longer, stronger, and finer (having less mass per unit length), which makes it preferred for spinning the stronger and silkier yarns that can be woven into luxury cotton clothing. A major goal of modern cotton breeding and biotechnology is to determine the controls of these superior fiber properties in Gb, followed by selective transfer of improved fiber traits to Gh without compromising its high yield and environmental adaptability. Under suitable growing conditions, Gb fiber has a prolonged elongation phase that generates its longer fiber compared to Gh (Raun et al., 2004). Similarly, finer fiber in Gb correlates with less cellulose per unit of fiber length (Chen et al., 2012). The yield of cotton fibre (lint) is usually negatively associated with fibre quality (Meredith, 1996).

Molecules of cell walls in cotton:

Cotton fibers are single-celled outgrowths from individual epidermal cells on the outer integument of the ovules in the developing cotton fruit. Fibers of upland cotton (*G. hirsutum* L.) generally grow up to 30 to 40 mm in length and ~15 μm in thickness at full maturity. Their development consists of four overlapping stages: fiber initiation, cell elongation, secondary wall deposition, and maturation. The thickened secondary walls of mature cotton fibers have long been considered unique in that they were thought to consist of nearly pure cellulose and to be devoid of hemicellulose and phenolics. However, molecular and biochemical evidences have also showed phenylpropanoid synthesis and presence of wall-linked phenolics in white, soft cotton fibers (Fan et al., 2012). The content of lignin-like phenolics in the cotton fibre in a single boll was kinetically increased with the fibre developing during 20 DPA to mature. These results suggested that monolignol biosynthesis and wall-linked lignin-like phenolics involved in the secondary wall thickening of cotton fibers (Fan et al., 2009).

Biologically, cotton fibre is an excellent model system for the study of plant cell elongation and cell wall and cellulose biosynthesis (Kim and Triplett, 2001). Differences in cellular processes are also being discovered between the two species of cotton (*G. hirsutum*

and *G.barbadense*). For example, the duration of plasmodesmatal closing at the base of the fiber was much longer in Gb compared to Gh, which was predicted to allow high turgor to persist longer in Gb fiber as a driver of elongation.Higher vacuolar invertase gene expression levels, vacuolar invertase activity, and hexoses, along with a somewhat faster fiber elongation rate, were observed in one Gb cultivar compared to Gh. Similar observations were made for phosphoenolpyruvate carboxylase, a key malate biosynthetic enzyme, which may promote fiber elongation through effects on turgor, lipid synthesis, or other processes (Avici et al.,2013). Although other studies have implicated differences related to genetic control of cell wall synthesis in Gh and Gb fiber, there was previously little evidence about how the fiber cell wall chemistry of these two commercial cotton species compared. Recently discovered unexpected features of cotton fiber cell walls for example, Gh fibers are joined together during elongation by a special outer layer of the primary wall called the cotton fiber middle lamella (CFML) (Avici et al., 2013).

Cotton fiber has an outer primary wall layer called the cotton fiber middle lamella (CFML). The CFML joins adjacent elongating fibers together through forming a unified cell wall between them (Singh *et al.*, 2009). The CFML facilitates the formation of tissue-like fiber bundles, which become organized into a packet around each seed. This orderly fiber packing, in turn, likely facilitates the extreme elongation of 100,000 fibers within a confined space inside each locule (or carpel) of the cotton boll (or fruit).

From the physiologist's perspective, the fiber quality of a specific cotton variety is a composite of fiber *shape* and *maturity* properties that depend on complex interactions among the genetics and physiology of the plants producing the fibers and the growth environment prevailing during the cotton production season. Fiber properties, particularly length and diameter, are largely dependent on genetics. Fiber maturity properties, which are dependent on deposition of photosynthate in the fiber cell wall, are more sensitive to changes in the growth environment. The effects of the growth environment on the genetic potential of a genotype modulate both shape and maturity properties to varying degrees. Anatomically, a cotton fiber is a seed hair, a single hyper-elongated cell arising from the proto-dermal cells of the outer integument layer of the seed coat. Like all living plant cells, developing cotton fibers respond individually to fluctuations in the macro- and microenvironments. Thus, the fibers on a single seed constitute continua of fiber length, shape, cell-wall thickness, and physical maturity (Bradow et al., 1997a). Environmental variations within the plant canopy, among the

individual plants, and within and among fields ensure that the fiber population in each boll, indeed on each seed, encompasses a broad range of fiber properties and that every bale of cotton contains a highly variable population of fibers.

Fibre Morphogenesis:

Fiber morphogenesis proceeds through several stages that will be described further in this chapter: initiation, elongation, transitional primary wall remodeling, secondary wall synthesis, and maturation. These differentiation processes, which typically last at least 50 days, directly determine cotton fiber quality characteristics. The number of fibers initiated in the outer integument of the ovule is a major factor in fiber yield. Fiber fineness (weight per unit length) and micronaire are determined by the fiber perimeter and the extent of secondary cell wall thickening. Fiber length and length uniformity are both valued in modern spinning mills, and cell elongation is strongly affected by developing and maintaining high turgor pressure within the central vacuole as well as carbon supply in different regions of the seed. Fiber strength is affected by properties of the transitional "winding" cell wall layer as well as secondary wall cellulose (*e.g.*, degree of polymerization and microfibril angle). High fiber tensile properties (including strength and mechanical elongation, or elongation-to-break) help to preserve fiber length during processing, and they are also required to produce strong yarns and fabrics. The potential of cotton fibers to pickup dye molecules and to absorb water are determined by the amount and the degree of crystallinity of the secondary wall cellulose. The final collapse of the fiber into the typical kidney bean shape that facilitates spinning (through improved friction properties) relies on adequate filling, but not over-filling, with secondary wall cellulose. Since cotton fiber is a cell wall composite, many of its important developmental processes relate to plant cell wall deposition (Haigler *et al.*, 2012).

Fibre Development stages in cotton:

Cotton fibre development can be divided into four phases: initiation, elongation, secondary wall thickening, and maturation (Fig1.). These phases will be discussed in more detail below.

Fibre Initiation

Fibre initiation on an ovule starts one to two days before flowering, beginning at the chalazal (round) end of the seed and moving towards the micropylar (pointy) end of the seed.

The time for initiation sometimes takes up to three days from one end to of the seed to the other. On the day of flowering most of the fibre cells on the seed coat have swelled into small balloons, although some fibres can still be initiated four to five days later. This ballooning stage is considered critical to determining fibre perimeter. Following this initial burst of fibre initiation a second set of fibres are initiated on the seed. These develop into the fuzz left on cotton seed following ginning. The majority of fibres that initiate remain as fuzz (up to 80%). The number of fibres initiated can vary between 12,000 and 20,000 per seed and is also variety dependant. There is little understanding of the effects of plant stress on fibre initiation although stress is known to reduce the number of fruit, ovules per fruit and seeds per ovule.

Fibre Elongation

Fibre elongation does not start until flowering despite some fibres having been initiated a few days before this. From flowering fibres elongate for up to 25 days, reaching variable lengths. The elongation period and the rate of fibre elongation in this period determine the final length of fibres (the reason for differences between varieties). Elongation is the onset of a process in which fibre length increases 1000 to 3000 times the diameter of the cell. The large and vigorous demand on the plant during fibre lengthening makes this process very sensitive to stress. The environment therefore plays an important role in whether fibre length reaches the genetic potential (determined by variety). Firstly, to maintain turgor, the cell must have ready access to potassium, solutes and water to regulate osmosis into the vacuole and produce the turgor pressure essential for fibre elongation. Severe water stress or potassium deficiency will reduce turgor pressure and result in shorter fibres. Temperature also plays an important role in regulating the rate of fibre elongation and influencing the duration of fibre elongation. Cool temperatures may reduce the rate of elongation but this effect will often be compensated by having a longer fibre elongation period. High temperatures and stress however, may reduce fibre length as the elongation period is shortened and access to substrates is limited.

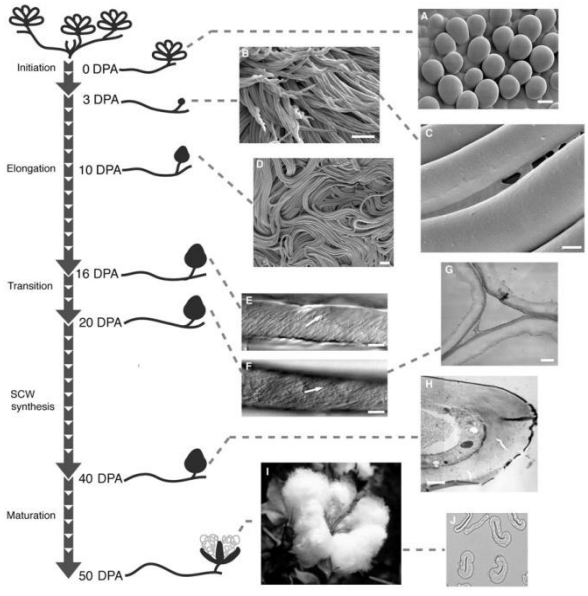
Fibre Thickening

Fibre thickening is sometimes referred to as the secondary wall formation phase. It is the phase where cellulose is laid down in winding sheets on the inner surface of the primary wall of the fibre cell. As a result the cytoplasm is pushed towards the interior of the cell and the vacuole (lumen) is reduced in size. More than 90% of the fibre weight is made up of the secondary wall. Fibre thickening occurs over a period of approximately 40 days but this can

vary depending on cotton species, variety and environment. Hence the degree of deposition of cellulose in the fibre cell is significantly affected by factors that affect photosynthesis. As cotton plants are subjected to fluctuations in the levels of photosynthesis and growth on a daily basis, the production of secondary wall in the fibre forms growth rings like a tree.

Fibre maturity

Fibre maturity is a term that is used to refer to the degree of development or thickening of a fibre (which contributes to the micronaire measurement incorporated in HVI (High Volume Instrument) testing). Soon after boll opening, the cotton fiber dries and collapses into the kidney bean cross-sectional shape that aids spinning into yarn, assuming that the fiber has an optimal maturity ratio. The fiber of many seeds fluffs into the mass typical of the open cotton boll. The thicker the layers of cellulose during fibre thickening the more mature the fibre. Insufficient supplies of carbohydrate for cellulose production will cause fibres to be more immature. Fibre maturity is an important property for both the physical and chemical processing properties of cotton fibres. Immature fibre is more prone to nep formation and do not take up as much dye as mature fibres. A nep is an entanglement of fibres resulting from mechanical processing.



A representation of a mature cotton plant containing bolls and fibre at all stages of fiber development (Haigler et al., 2013)

Crop Management for improved fibre quality:

Fibre length and micronaire are significantly affected by agronomic and climatic variables; however Fibre strength is more influenced by variety choice. Fibre growth and development is affected by most factors which influence plant growth. Since the fibre is primarily cellulose, any influence on photosynthesis and production of carbohydrate will have a similar influence on fibre growth. Cell expansion during growth is strongly driven by turgor, so plant water relations will also affect fibre elongation1 in the period immediately following flowering. Thus in terms of primary (direct) responses, water status strongly influences fibre growth and ultimately final fibre length. Fibre elongation will also be affected by temperature and carbohydrate limitations.

Fibre thickening are also affected by temperature and radiation effects on photosynthesis with large reductions in fibre thickening leading to low fibre micronaire following long periods of low temperatures or cloudy weather. Delayed sowing may expose more of the fibre thickening phase to lower temperatures and reduce micronaire. Potassium deficiency can have a significant impact on fibre length because of the role of potassium in maintenance of cell turgor by osmotic regulation. Other nutrient deficiencies can also reduce fibre length. However where nutrient deficiencies are not the major factor in a production system, nitrogen or potassium fertilizer treatments will not necessarily improve fibre length. Early crop defoliation or leaf removal can cause substantial reductions in fibre micronaire due to the cessation in carbohydrate supply for fibre thickening. Few agronomic or climatic conditions have been shown to consistently affect fibre bundle strength. One of the aims of High density planting system (HDPS) is to compress fruiting and fibre development to a shorter time period and avoid later moisture stress conditions – to at least achieve more uniform crop fibre properties. Cotton's indeterminate growth habit also leads to many secondary (indirect) impacts of climate and management on fibre properties. Any management which delays crop maturity can lead to reduced micronaire due to exposure of a greater proportion of a crop to unfavorable conditions such as cooler or cloudy weather. Early stress with subsequent recovery, or higher nitrogen fertility and different tillage or rotation systems and insect damage causing compensation and later fruit production are examples. Therefore adoption of appropriate and efficient management (both strategic and tactical) for improving yield will also contribute to improved fibre quality.

Canopy Architecture and Fiber Quality

Cotton canopy architecture, particularly with respect to plant height and branch formation, is modified by such environmental factors as temperature (Reddy et al., 1990); growth-regulator application (Reddy et al., 1990); light intensity (Sassenrath-Cole, 1995). Genotype canopy characteristics, such as solar tracking and leaf shape, and macro- and micro-environmental factors interact to modulate canopy light distribution, which, in turn, alters photosynthetic activity within the canopy and the crop. Thus, reduced photosynthetic rates and the modulation of other metabolic factors, in association with lower light intensities, may result in lower micronaire, fiber strength, and yield (Pettigrew, 1996).

Fibre length and short-fibre content

The fibre length generated through primary wall synthesis is essential for spinning yarn. Due to the variability inherent in cotton fiber, there is no *absolute* value for fiber length within a genotype or within a test sample. Even on a single seed, fiber lengths vary significantly because the longer fibers occur at the chalazal (cup shaped, lower) end of the seed and the shorter fibers are found at the micropylar (pointed) end. Coefficients of fiber-length variation, which also vary significantly from sample to sample, are on the order of 40% for upland cotton. Physiologists follow fiber development in terms of fiber chronological maturity from days after floral anthesis. Variations in fiber length attributable to genotype and fiber location on the seed are modulated by factors in the micro- and macro-environment (Bradow et al., 1997a,b). Environmental changes occurring around the time of floral anthesis may limit fiber initiation or retard the onset of fiber elongation. Suboptimal environmental conditions during the fiber elongation phase may decrease the rate of elongation or shorten the elongation period so that the genotypic potential for fiber length is not fully realized (Hearn, 1976). Further, the results of environmental stresses and the corresponding physiological responses to the growth environment may become evident at a stage in fiber development that is offset in time from the occurrence of the stressful conditions.

Fiber Length and Temperature

Maximum cotton fiber lengths were reached when night temperatures were around 19 to 20 °C, depending on the genotype. Early-stage fiber elongation was highly temperature dependent; late fiber elongation was temperature independent. Fiber length (upper-half mean

length) was negatively correlated with the difference between maximum and minimum temperature (Bradow and Davidonis , 2000).

Fiber Length and Water

Cotton water relationships and irrigation traditionally have been studied with respect to yield. Grimes and Yamada (1982) concluded that fiber length was not affected unless the water deficit was great enough to lower the yield to 700 kg per ha. Fiber elongation was inhibited when the midday water potential was –2.5 to –2.8 mPa. Occurrence of moisture deficits during the early flowering period did not alter fiber length. However, when drought occurred later in the flowering period, fiber length was decreased (Hearn, 1976). Severe water deficits during the fiber elongation stage reduce fiber length, due to the direct mechanical and physiological processes of cell expansion (Hearn, 1994). However, water availability and the duration and timing of flowering and boll set can result in complex physiological interactions between water deficits and fiber properties including length. For example, water deficits which occur during the mid- to late flowering periods reduces fiber-length means for bolls containing zero to two small short-fiber motes were lower in the mid-season population of rainfed bolls than in the mid- to late-season irrigated bolls . In other studies, irrigation increased mean fiber length and upper-half mean length. In India, moisture conservation practices (mulching) increased fiber length and yield.. However, under irrigated conditions, conservation tillage surface residues did not affect any fiber property, including length (Bradow and Davidonis , 2000).

Fibre Strength:

Fibre strength is contributed by a thin "winding" cell wall layer and secondary cell wall cellulose, which allows the fibre to dye deeply and absorb water. The inherent breaking strength of individual cotton fibers is considered to be the most important factor in determining the strength of the yarn spun from those fibers Table 1 (Patil and Singh, 1995). All environmental variables were interrelated, and a close general association between fiber strength and environment was interpreted as indicating that fiber strength is more responsive to the growth environment than are fiber length and fineness. Shading, leaf-pruning, and partial fruit removal decreased fiber strength (Pettigrew, 1996). Selective square removal had no effect on fiber strength in bolls at the first, second, or third position on a fruiting branch (Heitholt, 1997). Fiber strength was slightly greater in bolls from the first 4 to 6 wk of

flowering, compared with fibers from bolls produced by flowers opening during the last 2 wk of the flowering period (Bradow and Davidonis , 2000).

Table.1. Characteristics of ultimate fibre cells and fibre quality of cotton spp.

Trait	Cotton
Fibre strength (g tex-1)	30.0
Length :breadth	25.0
Fibre fineness (tex)	0.2

Value represent average over different economically important cotton sp., viz., *G.aroboreum, G. herbaceum, G.barbadance, G.hirsutum* (source: Wakelyn et al., 2007).

Fiber Fineness and Maturity:

Micronaire is the most commonly used instrumental fiber-quality test (Haigler et al., 2012). Micronaire is an indirect measure of the air-permeability of a test specimen of known mass enclosed in a container of fixed dimensions. The fineness factor in micronaire is considered more important in spinning, and fiber maturity is thought to have more effect on dye-uptake success. However, the finer the fiber, the higher the number of reflective surfaces per unit area and, consequently, the higher the luster of the dyed fabric. Immature fibers have thinner walls and are finer than mature fibers of the same genotype. However, lower micronaire fibers stretch, tangle, and break more easily and do not impart the greater yarn strength and uniformity expected of genetically finer, but still mature, fibers.

Action and interaction of phytohormones in fiber elongation

Phytohormones participate in signaling processes to regulate fibre development. Previously, gibberellic acid (GA) and auxin (IAA) were shown to be required for fiber growth on cultured cotton ovules (Gopalkrishnan et al 2011), and recent large scale gene expression data on -3 to 3 DPA ovules (or ovules with fibers) compared to other cotton tissues implicated extensive networks related to the biosynthesis and signaling of IAA, GA, and brassinosteroids (BR) as important for early fiber development. Plant growth regulators like IAA, GA_3, ethylene and ABA play a decisive role in early fibre development (Prakash et al., 2002). The effect of ABA on cotton fiber elongation was examined for three cotton genotypes representing long, medium, and short fibers: *G. hirsutum* Hybrid-4 and Hybrid-8 as well as *G. arboreum* Gujarat Cotton-15 (Dasani and Thaker, 2006).

Source-Sink Manipulation and Fiber Maturity

Variations in fiber maturity were linked with source-sink modulations related to flowering date (Bradow J.M and Davidonis G H. (2000), fruiting site (Pettigrew, 1996), and seed position within the bolls (Bradow J.M and Davidonis G H. (2000). The increases in micronaire after selective square removals were associated with increased fiber wall thickness, but not with increased strength of elongation percent. Early-season square removal did not affect fiber perimeter or wall thickness (measured by arealometer) (Pettigrew et al., 1996). Further, after more than 70 yr of research, single fiber strength data have yet to be correlated with yarn strength. Yarn strength is the processing result of greatest interest to yarn and textile manufacturers (Bradow, 1999a). Changes in fiber-quality requirements and increases in economic competition on the domestic and international levels have resulted in fiber quality becoming a value determinant equal to fiber yield (Bradow J.M and Davidonis G H. (2000). Indeed, it is the quality, not the quantity, of fibers ginned from the cotton seeds that decides the end use and economic value of a cotton crop and, consequently, determines the profit returned to both the producers and processors.

Conclusions:

Physiological studies and textile-processing models suggest that bulk fiber-property averages at the bale, module, or crop level do not describe fiber quality with sufficient precision for use in a vertical integration of cotton production and processing. More importantly, bulk fiber-property means do not adequately and quantitatively describe the variation in the fiber populations or plant metabolic responses to environmental factors during the growing season. Such pooled or averaged descriptors cannot accurately predict how the highly variable fiber populations might perform during processing. Meaningful descriptors of the effects of environment on cotton fiber quality await examinations of the variability, induced and natural, in fiber-quality averages. Only then can the genetic and environmental sources of fiber quality variability can be quantified, predicted, *and* modulated to produce the high-quality cotton lint demanded by today's textile industry and, ultimately, the consumer. Increased understanding of the physiological responses to the environment that interactively determine cotton fiber quality is essential. Only with such knowledge one can real progress be made toward producing high yields of cotton fibers that are white gold, as fine as silk, and as uniform as genotypic responses to the environment will allow. Next generation cotton

germplasm with higher fiber yield and quality, as well as potentially novel fiber characteristics, will be an important part of providing renewable, sustainable, resources to a growing human population.

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Insecticidal toxins from Entomopathogenic (EPN) nematodes

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Entomopathogenic nematodes (EPN) in recent years have been recognized as potential potent bio-agent against wide array of insect pests. The relatively rapid death of the insect host (24-48 h), wide host range, nontoxicity to mammals, amenability to mass production protocols and host-finding ability of these nematodes has generated great interest in their use as component of Integrated Pest Management protocols.

THE KEY TO SUCCESS WITH ENOTMOPATHOGENIC NEMATODES ARE

- Understanding their life cycle and functions
- Matching the correct nematode species with the pest species
- Appropriate time of application
- Application under appropriate conditions
- EPN are living entities. These need careful handling to ensure their survival and infectivity.

There are two genera of Entomopathogenic nematodes viz. *Heterorhabditis* and *Steinernema spp.* The life cycle includes an egg stage, four juvenile stages and adult stages. Third juvenile stage is called infective juvenile and is the only free living stage. This stage can survive in soil and can search, locate and infect insect hosts. Insects infected with Entomopathogenic nematode are typically characterized by flaccid appearance with characteristic change of color to yellow, brown or orange in *Steinernematid* infection while *Heterorhabditis* infected insects appear brownish red to brick red that faintly luminescence in the dark. . Normal life cycle is completed in 3-7 days. Infective juveniles start emerging from infected host in 6-11 days for steinernematids and 12-14 days for heterorhabditids.

HOW EPN KILL INSECT LARVAE

Infective juveniles locate their hosts by means of two strategies. –‘ambushing‘and ‘cruising’.

Ambushing- Nematodes that use this strategy remain stationary and locate host insects by direct contact. An ambusher searches by standing on its tail so that most of its body is in the air, referred to as 'nictation' The nictating nematodes attached to and attacks passing insect hosts. Highly mobile insect pest are controlled effectively by this. Ambusher species include- *Steinernema carpocapsae.*

Cruising : These are highly mobile and locate their hosts by sensing carbon dioxide or other volatiles released by the host These are effective against sedentary and slow moving insect species. Cruiser species are *Heterorhabditis bacteriophora* and *S. glaseri*.

The nematodes belonging to *Steinernema* penetrate insects through natural body openings as mouth, anus and spiracles while nematodes belonging to *Heterorhabditis* can also directly puncture cuticle due to presence of tooth in mouth region. EPN are symbiotically associated with a bacterium species (*Photorhabdus luminescens* and *Xenorhabdus spp*.) which live within nematode's gut. Once inside the body cavity of insects, these bacteria are released. These bacteria multiply quickly. Insect kill is achieved due to toxin produced by both nematode and the bacterium. When host contents have been consumed, the infective juveniles armed with bacteria emerge from empty shell of insect and move into the soil to search for new host. Gene isolated from these bacteria are now attracting attention for developing next generation transgenics.

Isolation and characterization of bacterial symbiont of EPN

Entomopathogenic nematodes are associated with bacterial symbiont (*Xenorhabdus sp*. and *Photorhabdus sp*.) and toxicity of EPN-bacterial system to insects is largely attributed to toxins produced by bacterial symbiont. The bacterial symbiont (nonluminescent variant of *Photorhabdus luminescens is* associated with *Heterorhabditis spp*. while *Xenorhabdus* is symbiont of *Steinernema spp.*

The colony characters on nutrient agar, NBTA and McConkey agar are used for preliminary identification. Primary colonies are generally smaller and more complex. The two forms were distinguished by the following features.

- On McConkey agar, primary colonies appeares purple blue/ red or bright pink. Secondary colonies are light gray.

- On NBTA, primary forms are green with or without red/ brown/ rust colored center. On nutrient agar, colonies range from creamish, yellowish or offwhite acquiring reddish color after 2-3 days.

The bacteria are motile, medium to long rods gram negative anaerobes with peritrichous flagella forming spheroblasts in older cultures.

Extraction of DNA template

Total genomic DNA extracted by growing single bacterial colonies on LB broth overnight at 37^0C. Culture was pelleted at 300rpm for 15 minutes and pellet resuspended in 500 ul of STET (Sucrose 8%, triton X/SDS 5%, Tris HCL 50mM, EDTA 50mM) at pH 8.0 and Treated with RNAse/Lysoenzume mixture (Lysozyme 10mg/ml, RNAse 1mg/ml) in 50mM Tris HCl. Bacterial cells lysed by boiling pellet for 1.45 minutes and DNA extracted by standard phenol choloroform. Quantification of DNA done by spectrophotometer and quality assessed by running an aliquot of DNA extracted on 1.5% agarose.

Amplification of 16sRNA

For molecular characterization, 16s ribosomal RNA sequence of bacterial symbionts amplified using oligonuceotide primers (5'GGA GAG TTA GAT CTT GGC TC3' sense and 5'AAG GAG GTG ATC CAG CCG CA3'. The standard 25ul PCR mixture contained template DNA 1µl (50ng), 5x buffer 5µl, $Mgcl_2$ (25mM) 1.5 µl, dNTP mixture (10 mM) 0.5µl, 10X BSA 0.5 µl, forward and reverse primers (10µm) 1ul and Taq polymerse (5U/µl) 1µl . Negative control contains all components except template DNA. The reaction run on Eppendorf or Biorad Icycler for 35 cycle amplification after initial denaturation at 95^0C for 5 minutes , each cycle included denaturation at 95 ^{0}C for 45 sec, annealing at 61 ^{0}C for 1 minute and extension at 72^0C for 2 minutes .The final extension carried out at 72^0C for 3 minutes. The reaction products separated on 1.5% Agarose gel containing 0.5ug of ethidium bromide per ml and visualised using Syngene Gel documentation system. The sequence amplified around 1550 bp and cloned in *E.coli* JH109 using Promega pGEMT vector for further sequencing. Blue white screening done to identify transformed colonies which are further confirmed by PCR with specific primers. PCR positive colonies grown in LB broth

and plasmids can be isolated using Axy plasmid miniprep kit. The sequences blasted and sequences of bacterial 16s RNA gene can be deposited with NCBI gene bank.

Characteristics of bacterial symbiont *Photorhabdus spp*. Associated with EPN *Heterorhabditis indica*

Test	**Result**	**Test**	**Result**
Gram stain	-	Lipolytic	+
Motility	+	Oxygen utilization	Facultative anaerobe
Flagellation	+ Peririchous	Tyrosinase	-
Oxidase	_	Peroxidase	+
VP Test	+	Lead Acetate	_
MR Test	+	Utilization of acetate	+-
Gelatin Lignifications	+	Utilization of benzoate	-
Utilization of formate	+	Utilization of citrate	+
Utilization of fumarate	+	Utilization of succinate	+
Utilization of malate	+	Utilization of tartarate	-
Utilization of malonate	-	Utilization of gluconate	-
Utilization of oxalate	-	Protease	+
Utilization of Lactate	+	Argininine Dihydrolase	-

Toxin isolation

For isolation of toxins, the bacteria in two phases are cultured on LB broth for 48 hrs. on shaker. Extracellular and intracellular fractions separated by centrifugation and sonication Protein profile of two phases of the bacterium can be resolved on native and SDS PAGE.

Different fractions from the extracellular and intracellular components of both the phases of bacterium separated using columns, centrifugal devices and gel filteration bioassayed against 3rd instar larva of *Helicoverpa armigera* for insecticidal activity. On SDS page protein is denatured so to get active protein need to run Native PAGE and individual

bands cut, eluted in buffer (140 mm NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mMKH_2PO_4, pH 7.3) and analysed for insecticidal activity.

The elutes of bands are applied to artificial diet for oral toxicity to *Helicoverpa armigera* neonates. These are also injected in intrahaemoceolic for toxicity to *H.armigera.* LC_{50} experiments are conducted for 48 h with neonate larvae, and replicated atleast thrice with 12 larvae per treatment. Growth inhibition studies are 72 h in duration and repeated twice with about 12 individuals per treatment. Mortality data from the LC_{50} experiments analyzed by Probit analysis. Individual band with insecticidal activity can be sequenced for amino acid composition and DNA sequence can be deduced form that.

Inseticidal toxin genes can also be cloned based on information available in NCBI data base. Primers are designed based on sequences available in NCBI data base. To get full length amplicons, amplification with long range Taq polymerse need to be carried out and conditions for amplification of each amplicon standardized. Higher $MgCl_2$ Concentration (2mM) may be required.

The amplicons cloned into pGEMT vector systems (Promega) and transformed in chemically competent *E.coli* strain JM109 and plated on LB agar with Ampicillin, X-Gal and IPTG. Positive clones selected as white colonies on overnight incubation, followed by Colony PCR to check for presence of required insert. The plasmids isolated and insertion further confirmed by Restriction Digestion with Eco RI and PCR with M13 primers. Amplicons up to 3kb can be cloned and sequenced through primer walking. The sequences need to be blasted to ascertain correctness of the sequence.

Some of the Insecticidal toxin genes from bacterial symbionts of EPN

Toxin genes from *Photorhabdus spp.*	Toxin genes from *Xenorhabdus spp.*
Tca, Locus (*tcaA*, *tcaB*, and *tcaC)* Tcb locus (*tcbA)* Tcc locus (*tccA* and *tccB , tccC tccC2-tccC5)* Tcd locus (*tcdA1-tcdA4,tcdB1-tcdB2)* *Photorhabdus* virulence cassettes" (PVCs TXP 40 PIR AB Mcf1	Xpta1, XptA2, XptB1, and XptC1

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Plant Variety Protection, DUS testing and Variety Identification

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Introduction

New varieties with improved yield and, quality are the key elements in any Plant Breeding programme. With the technological advancement in agriculture science, newer varieties suitable for different agronomic conditions are being evolved. Plant breeder develops varieties to suit mankind by systematic, scientific planning and pain staking efforts, investing lot or resources, time and talent. Hence there should be a system of encouraging the breeder through rewarding him/her by providing Plant Variety Protection. To promote this, International Union for the Protection of New Varieties of Plants (UPOV), an inter-governmental organization based in Geneva, Switzerland started implementation of the Plant Breeder's Rights. UPOV advocated to its member countries (71) enact a varietal protection system of their own (*sui generis*).

What is Breeders' Rights?

It is an exclusive control provided to plant breeder over the propagating material, be it, seeds or any other propagule developed by him/her. Breeders Rights is extended to production, selling, distribution, marketing, export and import of breeder's material.

Researcher's Rights

Researchers can freely utilize the protected variety for conducting experiments or to develop newer varieties with prior authorization from the breeder.

Grant of the Breeder(s) Right

A variety shall be granted protection if it meets the following technical criteria

- Novel/new
- Distinct

- Uniform
- Stable

Novelty

A variety must not have been sold or marketed in the member country for more than one year prior to the application for protection, or more than four years in a country other than that of the member of the Union.

Distinctness

A variety is distinct if it is clearly distinguishable from any other variety whose existence is known at the time of filing of the application.

Uniformity

A variety is uniform if, it is sufficiently uniform in its relevant characteristics other than the variation that may be expected from its way of propagation (self/often cross/cross pollinated). The variation is expected to be more in cross pollinated species than often cross/self pollinated species and if it is within the acceptable limit the variety is considered uniform.

Stability

A variety is stable if its relevant characteristics remain unchanged after repeated propagation. It ensures that the identity of the variety is kept throughout the period of protection.

Denomination

The breeder must give the variety an acceptable "denomination", which becomes its generic name and must be used by anyone who markets the variety.

Plant Variety Protection in India

India being a signatory to the Trade Related Intellectual Property Rights (TRIPS) Agreement had to develop and implement a *sui generis* system of plant variety Protection which is a slightly modified form than what is advocated by UPOV.

Protection of Plant Variety and Farmers' Rights Act, 2001

The Act was the first of its kind in the legislative history of India and perhaps the world to recognize the rights of the farmers as conservators, breeders and cultivators. In addition

to Breeder's Rights the Act also covered Farmers' Rights. To implement the Act, Government of India, has set up PPV & FR Authority at New Delhi. Unique features of the Act other than Breeders' Rights are the following:

Farmers' Rights

- Farmers are entitled to save, use, sow, re-sow, exchange, share and sell farm produce of a protected variety except sale under a commercial marketing.
- Farmer is also rewarded for conserving genetic resources/land races.
- He/s is also eligible for variety protection if any improvement of existing variety is made through selection
- Farmers need to be informed about the expected performance of a variety at the time of sale of seed/propagating material.
- A farmer or a group of farmers or an organization of farmers can claim compensation according to the Act, if a variety or the propagating material fails to give an expected performance under given conditions, as claimed by the breeder of the variety. Other important features of the Act are:

Compulsory license

The authority can grant compulsory license to any person or organization to produce seeds of a protected variety, if there is any complaint regarding the non-availability or mis-marketing of seeds of protected. Under such circumstances the Authority will take the responsibility of seed production of the protected variety and will ensure that seeds are available to farmers at reasonable price.

Benefit sharing

Breeder has to share the benefits from a variety developed if it is from indigenously derived plant genetic resources. The authority will look into the quantity of benefit sharing to be shared.

National Gene Fund

There is provision for a National Gene Fund which will be credited with fund from:

(a) The benefit sharing from the breeder.

(b) The annual fee paid to the authority by way of royalties.

(c) By the compensation provided to the communities

Variety denominations under PVP

a. Essentially Derived Varieties (EDV)

EDV means a variety which has been essentially derived from existing variety by any of the means such as genetic engineering, mutation, tissue culture derived, back cross derivative or any other Ploidy change etc. EDV conforms to the existing (initial) variety except for the difference in the expression of the essential characteristics.

b. Farmers' Variety

A variety which has been traditionally cultivated and evolved by the farmers in their fields; or is a wild relative or land race or a variety about which the farmers possess the common knowledge.

c. Extant Variety

A variety, which is–

(i) A farmers' variety; or

(ii) A variety about which there is common knowledge; or

(iii) Any other variety which is in the public domain

d. Candidate variety

Any new variety being claimed for protection

e. Reference variety

Any released extant variety with maximum traits similar to the candidate variety

f. Example variety

Variety that best expresses a particular trait. Morphological characters to be recorded during testing for distinctness, uniformity and stability under Plant variety Protection has been listed as National Test Guidelines for 36 crops and is available online. There are 37 characters for

tetraploid cotton (*G. hirsutum* and *G. barbadense*) and 31 characters for diploid cotton (*G. arboreum* and *G. herbaceum*).

Variety Characterization

The trueness of variety is needed to be maintained both for protection and seed distribution. Though morphological characters are available for characterization, they are time and labour consuming. They are few in number and cultivars with high similarity might not be distinguished. Therefore, non-conventional markers especially molecular markers which are more in number and remain stable have been in use for varietal characterization worldwide.

Non-conventional markers

Biochemical markers

Total seed proteins and Isozymes

The electrophoretic analysis of proteins has been applied widely for variety identification, breeding, seed testing, certification etc. The basis for using these markers is that all expressions have a biochemical basis but not all biochemical differences may not be expressed morphologically. Seed protein profiles have been used worldwide for differentiating several crop varieties. These could be total seed proteins or salt soluble proteins. Though these profiles are recommended by UPOV for testing distinctness, these have been advocated to be included as supplementary characters for DUS testing especially of wheat, barley and maize. In cotton, salt soluble protein profiling has been found effective for determination of hybrids and parents.

International Seed Testing Association (ISTA) has adopted a standard reference method of PAGE for identification of varieties of wheat and barley into its International rules, involving separation of gliadins from wheat and hordeins from barley.

Alleles of same enzyme with different size but performing same function are isozymes/allozymes. Some of the major isozymes employed in variety discrimination are esterase, malatedehydrogenase, alcoholdehydrogenase, peroxidase, Superoxide dismutase, Glutamate dehydrogenase etc.

Molecular markers

DNA based molecular markers have been widely employed in various fields such as variety identification, marker assisted selection, genetic diversity analysis, germplasm characterization etc. A wide variety of methods have been developed in the past few years for visualizing DNA sequence polymorphism. DNA fragment patterns is unique to each individual and is considered the final tool for individualization (be it plant, animal or human being). High level of polymorphism and stable inheritance are the major advantages of molecular markers. Various types of molecular markers employed to examine DNA polymorphism are Restricted Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) or Sequence Tagged Microsatellite Markers (STMS), Amplified Fragment Length Polymorphism (AFLP) etc. Being genetically co-dominant, reproducible and amenable to complete automation, SSR markers are the most preferred type of molecular marker. At CICR, cotton germplasm (working collection) has been characterized employing SSR markers. The work on DNA fingerprinting of cotton varieties using SSR markers is in progress.

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Molecular Markers and Construction of Linkage Map

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MOLECULAR MARKER SYSTEMS

Genetic information in the form of DNA is stored in cell at three places, *viz.*, nucleus, chloroplast and mitochondria. The genetic variation arises in this stored information due to the change in the DNA sequence by base substitution, deletion, insertion, duplication and inversion. These variations are manifested as variation in trait expression (at phenotype level) or amino acid sequence variation (at protein level) or nucleotide sequence variation (at DNA level). In general, marker is anything that marks or tags or identify an individual. Any heritable variation following Mendelian inheritance can be called as genetic marker. Genetic markers facilitate mapping and discovery of valuable genes hence, they are widely used in crop improvement programmes. Compared to conventional phenotypic selection, marker assisted selection (MAS) provides more reliability, efficiency and rapidity in plant breeding programmes. Genetic markers are of three types, *viz.*, morphological markers, biochemical markers and DNA based markers.

I. **Morphological markers**: These are related to shape, size, colour and surface of various plant parts.
II. **Biochemical marker**: Such markers are related to variation in protein and amino acid banding patterns.
III. **DNA markers**: Typically, small regions of DNA showing sequence polymorphism in different individuals with in a species, e.g. Restriction Fragment Length Polymorphism- RFLPs; Amplified Fragment Length Polymorphism- AFLP; Random Amplified Polymorphic DNA- RAPD; Cleaved Amplified Polymorphic Sequence-CAPS; Simple Sequence Repeats- SSRs; Single Nucleotide Polymorphism (SNP), etc.

Characteristics of ideal genetic marker:

a. High level of polymorphism
b. Stability, no influence of environment
c. Simplicity of observation

d. Non-epistatic
e. Co-dominancy
f. Genome-wide distribution
g. Low cost
h. Reproducibility
i. Portability between species
j. Mendelian inheritance

Morphological and biochemical markers are have limited applications in crop improvement compared to DNA based markers because they are less in number, not well distributed across the genome, stage or/and tissue specific expression, influenced by external environment, not amenable to complete automation and limited polymorphism. Hence, widely used DNA markers are detailed in length in this lecture notes. There are different classes of DNA markers. Based on their evolution, first generation markers (hybridization based markers like RFLPs); second generation markers (most of PCR based markers like RAPD, AFLP, SSRs) and third generation markers (sequence based markers like SNPs).

Types of DNA markers

i) Restriction Fragment Length Polymorphism (RFLP):

RFLP is hybridization based marker, developed by Botstein *et al.*, (1980). This is the polymorphism which is detected based on the differential hybridization of cloned DNA to DNA fragments in a sample of restriction enzyme digested DNAs. Hence, the polymorphism detected in the fragment length is due to variation in restriction site(s) of the enzyme used and these RFLPs are defined by specific enzyme-probe combinations.

Strength:

- ✓ Robust and reproducible
- ✓ Co-dominant nature and hence can be effectively used to differentiate homozygotes from the heterozygotes
- ✓ Particularly useful in comparative genome mapping

Weakness:

- The assay is tedious and time consuming
- Requires large quantities of high quality DNA
- Not amenable to complete automation
- Associated with health hazards as it used radioactivity

ii) Random Amplified Polymorphic DNA (RAPD):

RAPD markers were given by Williams *et al.,* (1990) and are based on the differential PCR amplification of a sample of DNAs from random oligo-nucleotide sequence. RAPDs are genetically dominant in nature. RAPD uses single random primer usually of 10 nucleotides long, to amplify inverted repeats in the genome and can anneal at multitude of genomic locations.

Strength:

- ✓ Fast and so called Rapid markers
- ✓ Requires less quantities of DNA
- ✓ Needs limited investment in time and training
- ✓ Commercially available
- ✓ No radioactivity involved
- ✓ No need of sequence information

Weakness:

- Lack of reproducibility in markers patterns across lab and experiments
- Dominant in nature

iii) Amplified Fragment Length Polymorphism (AFLP):

AFLP markers were given by Vos *et al.,* (1995) and these polymorphism are generated using a procedure that combines restriction digestion and PCR amplification. The basic procedure is as follows:

- Digestion of genomic DNA with a combination of two restriction enzyme- a rare and a frequent cutter, e.g. *Eco*RI & *MSe*I; *Eco*RI & *PSt*I.
- Ligation of double stranded adapters ot cut ends of DNA fragments

- Pre-selective amplification
- Selective amplification
- Separation of amplified fragments
- Visualization using autoradiography

Strength:

- ✓ Stable amplification and high repeatability
- ✓ Can generate fingerprints of any DNA regardless of their origin, so highly suited for DNA fingerprinting
- ✓ Can act as bridge between genomic and physical maps
- ✓ Hyper-variability

Weakness:

- Time consuming procedure
- Required significant technical skills and financial resources
- Dominant in nature

iv) Simple Sequence Repeats (SSR):

These are also called as microsatellites or Short Tandem Repeats (STRs) or Simple Sequence Length Polymorphisms (SSLPs). These are variable tandem repeats ranging from 1-10 nucleotides, dispersed throughout the genomes of most eukaryotic organisms. These repeats may be di, tri, or tetra-repeats with repeat pattern being perfect, imperfect, compound or complex. If the repeat number is more than 10 nucleotides, they are referred as minisatelites. The conserved regions flanking these repeats are used to design the primers for PCR based amplification. The polymorphism discovered is due to variation in number of repeats in the individuals.

Strength:

- ✓ Abundant and uniformly distributed in the genome
- ✓ Hypervariable (large number of allele per locus)
- ✓ Co-dominant markers
- ✓ Highly reliable and reproducible assay
- ✓ Marker systems of choice in most of crop improvement programmes

Weakness:

- High developmental cost
- Often primers are species specific.
- Needs prior information of the sequence to design primers

v) Single Nucleotide Polymorphisms (SNPs):

The differences which are found at single nucleotide position are referred to as single nucleotide polymorphism or SNPs. This type of polymorphism results due to substitution, deletion or insertions. This is mostly biallelic and co-dominant in nature.

Strength:

- ✓ Abundant
- ✓ Sequence based robust markers
- ✓ Fast and high throughput
- ✓ Genome-wide distribution
- ✓ Possible to discovery of new alleles, hence useful in allele mining
- ✓ Co-dominant

Weakness:

- Most SNPs are biallelic and thus less informative than SSRs
- SNP assay is costly
- Sophisticated equipments and considerable efforts required

Each marker system has its own advantages and limitations. They vary with each other with respect to genomic abundance, locus specificity, polymorphism level, reproducibility, technical requirements and financial investments. Hence, due care has to taken before choosing marker system for different genetic studies.

Applications of molecular markers in crop improvement:

- ❖ Assessment of genetic diversity
- ❖ Differentiation and identification of varieties
- ❖ Construction of genetic maps
- ❖ Mapping and tagging of genes/QTLs

- Marker assisted selection
- Physical mapping
- Map-based gene cloning

CONSTRUCTION OF LINKAGE MAP IN CROP PLANTS

Precise mapping of traits of economic interest is essential for application of molecular marker technology for crop improvement. High resolution genetic mapping requires construction of saturated linkage map which determines the relative distance between the marker(s) and trait of interest. This genetic distance is estimated based on the mean number of recombination events, involving a given chromatid, in that region per meiosis. A linkage map is a genetic map of a species or experimental population that shows the position of its known genes or genetic markers relative to each other in terms of recombination frequency, rather than a specific physical distance along each chromosome. First ever genetic map was developed by Prof. Steve D Tanksley in 1995 in tomato using RFLP markers. Development of appropriate mapping population is first step in construction of linkage map and genetic mapping of traits.

Steps involved in construction of genetic linkage map:

1) *Selection of parents and development of appropriate mapping populations.*

Parents selected should have high genetic divergence both at genotypic level (to be measured by molecular markers) and at the phenotypic level (assessed as diversity for traits of interest). Geographical origin and pedigree or source of the lines selected as parents should be given importance while choosing parents. Different mapping populations can be constructed by planned crosses for mapping both qualitative and quantitative traits. Mapping populations like F_2, $F_{2:3}$, backcross (BC) and near-isogenic lines (NILs) can be utilized for mapping mono/oligogenic traits while immortal populations like recombinant inbred lines (RILs) and doubled haploids (DH) are most preferred for precise mapping of quantitative trait loci (QTL) or polygenic traits. The type and size of mapping populations can exert an influence on the accuracy and economic significance of genetic maps. Larger mapping population is always better especially when the goal is high resolution mapping in specific genomic regions or mapping QTLs of minor effect. Immortal populations of large size (preferably more than 200 individuals), genotyped by co-dominant markers yield more precise and high resolution linkage maps.

2) Identification of informative markers between parents.

Different probe-enzyme combinations (in case of hybridization based markers) and primers (in case of PCR based markers) should be used for assaying the parents selected and polymorphic markers between parents should be identified.

3) Genotype the mapping populations using the informative markers.

The individuals of the mapping population developed should be precisely genotyped using genome-wide distributed markers polymorphic between the parents. The generated molecular profiles should be scored either manually or using software to determines the allelic profiles in the mapping populations.

4) Analysis of segregation pattern of markers in the populations.

A molecular marker is expected to follow Mendelian inheritance, which can be tested using chi square test. The expected ratio for co-dominant markers is 1:2:1 and 3:1 for dominant markers. Wherever there is a problem of segregation distortion (any significant deviation from expected Mendelian ratio), either eliminate those markers from the analysis or increase the log of odds (LOD) score.

5) Establishment of linkage relationship.

Genetic linkage map is developed based on the recombination frequency of the markers segregating in the mapping population. The distance between the markers is determined by the extent of recombination between markers; greater the recombination frequency farther the markers are and lower the frequency closer the markers are. The markers that are genetically linked (less than 50% of recombination frequency) form a linkage group. Possibility of linkage is tested using 2 point or 3 point test, later being more preferred as it will reveal the order of the markers apart from distance between the markers. Linkage between markers is usually calculated using odds ratios (i.e. the ratio of linkage versus no linkage). The ratio is expressed as logarithm of odds ratio (LOD). Higher LOD score, higher is the reliability of the map constructed. Threshold LOD values need to be determined depending upon the situation. Haldane and Kosambi mapping functions are used to convert recombination frequency into centi Morgans (cM). Distance on a linkage map is not directly related to the physical distance of DNA between genetic markers. Linkage map can be constructed using different statistical

packages like, Linkage1, GMendel, MapMaker, MapManager, JoinMap etc. The accuracy of measuring the genetic distance and determining marker order is directly related to the number of individuals in the mapping population. How good or saturated is the map constructed will be indicated by map saturation indices *viz.*,, the number of linkage groups (in any species should be equal to haploid number of chromosomes), no/few gaps and no chromosome end extension.

6) *Defining telomeric and centromeric regions and assigning linkage group to chromosomes.*

Telomeric regions can be identified by techniques like Fluorescent In-Situ Hybridization (FISH) and use of telomeric sequences as probes. Centromeric regions can be mapped using Fluorescent In-Situ Hybridization (FISH) or using cytogenetic stocks, like Telotrisomy and Ditelotrisomy. Once saturated linkage map is constructed, and telomeric and centromeric regions are mapped, we need assign different linkage group constructed to different chromosome which is achieved with the help of known genetic marker or trisomic stocks or translocation stocks. In some crops, special cytogenetic stocks are available such as chromosome additional lines (brassica and wheat) and B-A translocation stocks (maize) which are helpful in assigning linkage groups to chromosomes of the species.

Principles and Practices of Molecular Phylogenetics

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"Nothing in biology makes sense except in the light of evolution"

- Theodosius Dobzhansky

Introduction:

Classification of organisms is very much important because we need to know what's out there and they are related to each other to better understand the underlying biological processes that have generated them. This information is useful for the management and conservation of our biological heritage. After Darwin's book "On the origin of species" that was published in 1859, it was become apparent that classification of biological organisms should be based on evolutionary relationships. The study of evolutionary relatedness among various groups of organisms (eg. Species, populations) is known as phylogenetics (Gr. *Phyle*, tribe + *genesis*, birth). It is also known as systematic or cladistics in which a species is treated as a group of individuals with common lineage over time. The basic idea of phylogenetic analysis is to compare species based on specific characters (features) with the assumption that species with similar characters are genetically related to each other. These relationships are referred as phylogeny and are represented in the form of a phylogenetic tree. They can provide most meaningful insights into biology. This important realization is now apparent to researchers in diverse fields, including ecology, molecular biology and physiology. One example is the value of placing model organisms in the appropriate phylogenetic context to obtain a better understanding of both patterns and processes of evolution (Soltis and Soltis, 2000).

As Baum et al. (2005) pointed out, "phylogenetic trees are the most direct representation of the principle of common ancestry-the very core of evolutionary theory-and thus they must find a more prominent place in the general public's understanding of evolution".The morphological and molecular markers are used in the phylogenetic analysis. DNA and protein sequences are used in the molecular phylogenetic studies. More recently, molecular phylogenetics has become an important tool for genome comparisons. Next

generation sequencing (NGS) technologies are generating huge data sets (Yang and Rannala, 2012).

Evolutionary tree-also known as phylogeny-is a diagrammatic depiction of biological entities that are connected through common descent, such as species or higher level taxonomic groupings. The assumption is that every organism alive today and all those who have ever lived are members of a shared heritage that extends back to the origin of life some 3.8 billion years ago. One might therefore expect it to be possible, at least in principle, to reconstruct the Tree of life, branch by branch and bough by bough, from the current diversity residing at the outermost twigs to a universally shared root. So any reconstructed phylogenetic tree is a hypothesis about relationships and patterns of branching and thus is subject to further testing and revision with the analysis of data derived from organisms alive today or represented in the fossil record (Gregory, 2008).

Types of trees:

In the most general terms, tree diagrams are known as "dendrograms" (after the greek for tree), whereas phylogenies that depict only branching order are known as "cladograms". By contrast, tree known as "phylograms" present branch lengths as being proportional to some measure of divergence between species.

A phylogeny is a tree containing nodes that are connected by branches. Each branch represents the persistence of a genetic lineage through time, and each node represents the birth of a new lineage. If the tree represents the relationship among a group of species, then the nodes represent speciation events.

Phylogenetic trees are not directly observed and are instead inferred from sequence or other data. Closely related sequences occupy neighbouring branches on a phylogenetic tree. Based on phylogenetic study, gene function can be predicted. Phylogeny reconstruction methods are either distance-based or character-based (Yang and Rannala, 2012).

During the course of evolution, a species splits into two or more different species that do not cross among each other. The relationship among different species can be represented in the form of an evolutionary or phylogenetic tree (Fig 1.). In this figure, A, B, C, D, E are the terminal nodes. These are also called Operational Taxonomic Units (OTUs). These can be gene or protein sequences, individuals, populations, species, etc. F, G & H are internal nodes which represent ancestral units. I is the root node i.e. most ancestral node. The pattern of branching known as tree topology. Species A and B share a recent common ancestor that was not shared by the other species and are therefore called "sister taxa". Species E is the most

distantly related of the sample of species and is known as the "outgroup". It is necessary to root a tree and provides stability (Sharma, 2009).

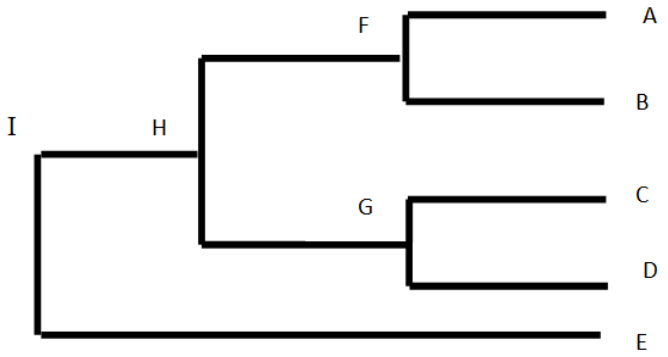

Figure 1. The Anotomy of Phylogenetic tree

A set of all taxa derived from a particular common ancestor is called a clade. A tree that depicts the order of branching of taxa without regard to the distances between them is called a cladogram.

A tree said to be rooted if there is a single ancestral node from which all other nodes descend, and in this case the root node is the parent of two branches. If the tree is rooted, direction is defined by the evolutionary time-scale and we usually associate increasing time with the downward or rightward direction. If there is no root, all we can say is that the leaves correspond to the ultimate descendants of some ancestor, but we do not know whether the internal node adjacent to any leaf is its ancestor, nor do we know the ancestral relationships of the internal nodes.

Phylogenetic analysis establishes the relationships between genes or gene fragments, by inferring the common history of the genes or gene fragments. To achieve this, it is essential that homologous sites be compared with each other (positional homology). For this reason, the homologous sequences under investigation are aligned such that homologous sites form columns in the alignment. Obtaining the correct alignment is easy for closely related species and can even be done manually. The more distantly related the sequences, the less straight forward it is to find the best alignment. Therefore, alignments are usually constructed with specific software packages that implement particular algorithms (Deonier et al. 2005)

Many popular algorithms start by comparing the sequence similarity of all sequence pairs, aligning first the two sequences with the highest similarity. The other sequences, in order of similarity, are added progressively.

Genes originating from a duplication event recent enough to reveal their common ancestry at the nucleotide or amino and level are called paralogous. Comparing such genes by phylogenetic analysis will provide information about the duplication event.

Homologous genes in different species that have started to evolve independently because of the speciation are called orthologous.

When the goal is to understand the evolutionary relationships between organisms, the distinction between a gene tree and a species tree becomes important. A gene tree is a tree drawn from DNA or protein sequences corresponding to a particular gene shared by a set of organisms. A species tree is often produced from set of macroscopic characters but also may be produced from sequence data.

Monophyletic:

A monophyletic grouping is one in which all species share a common ancestor, and all species derived from that common ancestor are included.

Paraphyletic:

A paraphyletic grouping is one inwhich all species share a common ancestor, but not all species derived from that common ancestor are included.

Polyphyletic:

A polyphyletic grouping is one inwhich species that do not share an immediate common ancestor are lumped together, while excluding other members that would link them.

Molecular phylogeny:

Phylogenetic trees are built from molecular data: DNA or Protein sequences. Originally, the purpose of most molecular phylogenetic trees was to estimate the relationships among the species represented by those sequences, but today the purposes have expanded to include understanding the relationship among the sequences themselves without regard to the host species, inferring the functions of genes that have not been studied experimentally (Hall et al. 2009), and elucidating mechanisms that lead to microbial out breaks (Hall and Barlow 2006) among many others. Major assumptions are that molecular sequences used in phylogenetic construction are homologous and each position in a sequence evolved independently. All the process of phylogenetic tree estimation and testing of reliability has been shown as a flowchart as in the figure 2.

Any biological information that can be used to infer the evolutionary relationship among the taxa is known as a phylogenetic information marker. Mitochondrial (cytochrome C oxidase subunit I & II (COX I & II)), chloroplast (trnH-psbA, matK, rpoC, rpoB, rbcL) and nuclear (16S ribosomal RNA) conserved genes are preferred to use for analyzing animal,

plant and microbial species respectively and are called "barcode genes" (Niranjan Reddy B P, 2011). This is followed by polymerase chain reaction amplification of targeted gene/Protein, followed by sequencing and editing of the sequences for further analysis.

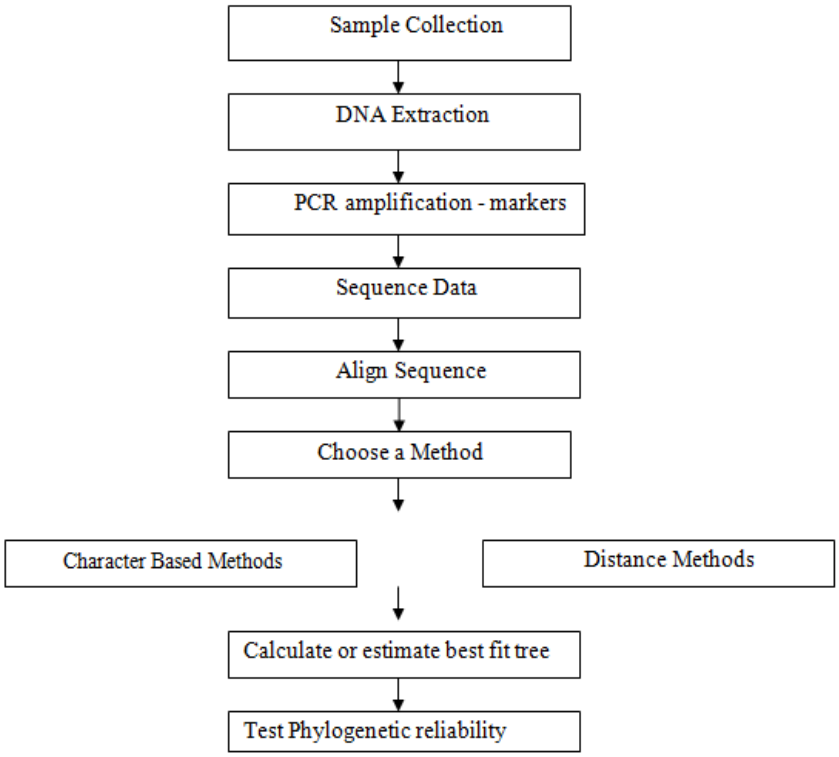

Figure 2. The Flowchart showing all the steps involving Phylogenetic tree Reconstruction

Phylogeny reconstruction methods:

Phylogeny reconstruction methods are either distance-based or character based. The tree with the best score should be identified by comparing all possible trees. In practice, because of the huge number of possible trees, such an exhaustive search is not computationally feasible except for very small data sets. Instead, heuristic tree search algorithms are used. These approaches often generate a starting tree using a fast algorithm and then perform local rearrangements to attempt to improve the tree score. A heuristic tree is not guaranteed to find the best tree under the criterion, but it makes it feasible to analyse large data sets. To describe the data, distance matrix, maximum likelihood and Bayesian inference all make use of a substitution model and are therefore model-based, whereas maximum parsimony does not have an explicit model and its assumptions are implicit (Yang and Rannala, 2012).

Distance matrix methods:

The distance between every pair of sequences is calculated and the resulting distance matrix is used for tree reconstruction. It assumes a Markov chain model of nucleotide substitution. The JC69 model assumes an equal rate of substitution between any two nucleotides, were as the K80 model assumes different rates for transitions and transversions. Both models predict equal frequencies of the four nucleotides. Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Neighbor-Joining method, Least square, Minimum evolution methods are used for phylogenetic analysis based on distance matrix method.

(a) Unweighted Pair Group Method with Arithmetic Mean (UPGMA):

It is a clustering algorithm that works by joining the branches of the tree on the basis of greatest similarity criteria among pairs of sequences and by calculating averages of joined pairs. Because of simplicity, one of the most popular method for tree construction. It first calculates the raw pair-wise distance data from a set of sequences, then constructing a matrix. The algorithm first uses the least distance pairs for grouping and then adds sequences having distance next to it and so on to build a tree from all the sequences in a data set.

(b) Neighbor-Joining Method:

The NJ method is comparatively fast method. It is based on minimum evolution principle. In this method, closest sub-tree are first joined to each other followed by joining of sub-trees far from each other. It is most suitable for the trees of known topology having branch length which stimulates different levels of evolutionary changes.

(C) Minimum Evolution Method:

It seeks to find the shortest tree that is consistent with the path lengths measured. It works by minimizing the squared deviation of observed to tree-based distances. Minimum evolution seems to be the best procedure and it performs nearly identically in simulation studies (Huelsenbeck, 1995). However, the speed of NJ and its ability to produce results very similar to other slower methods ensures that this method continues to be one of the most popular to date.

Character Based Methods:

These methods includes maximum parsimony, maximum likelihood and Bayesian inference methods. These approaches simultaneously compare all sequences in the alignment, considering one character (a site in the alignment) at a time to calculate a score for each tree. The ‘ tree score’ is the minimum number of changes for maximum likelihood and the posterior probability for Bayesian inference.

(a) Maximum Parsimony(MP):

MP is preferred by many phylogeneticists because of its theoretical basis and the diagnosable units it produces (Deonier et al. 2005)It minimizes the number of steps require to generate the observed sequence variation. For any particular site, there are several ways to determine the minimum number of evolutionary events. For the execution of this method on a set of sequneeces, multiple sequence alignment (MSA) is required in which positions of the sequences corresponding to each other are predicted. A phylogenetic tree is constructed for each position in MSA based on the smallest number of evolutionary changes. Each possible tree is evaluated by using this method to give a specific score so that best tree can be selected from the different trees. The tree with minimum number of evolutionary changes is also called the most parsimonious tree. This method is very good for distantly related sequences
(Sharma, 2009).

(b) Maximum Likelihood:

The first algorithm for maximum likelihood analysis of DNA sequence was developed by Felsenstein. The method based on the explicit model of evolution used for phylogenetic analysis. It searches the phylogenetic tree and evolutionary model based on the highest likelihood of producing the observed data set. Two optimization steps are involved in maximum likelihood tree estimation: optimization of branch length to calculate the tree score for each candidate tree and a search in the tree space for the maximum likelihood trees.

Estimating the reliability of the tree:

The most common way to estimate the reliability of a phylogenetic tree is by the bootstrap method. It evaluates the tree topology by constructing phylogenetic trees equal to the given number of pseudo-replicates. The bootstrap percentages, indicate the reliability of the cluster descending from that node; the higher the number, the more reliable is the estimate of the taxa that descend from that node. The tree node having more than 70% bootstrap percentages are generally considered as consistent. This test does not estimate the overall reliability of the tree; instead it estimates the reliability of each node.

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Mapping Populations: Development, Characterization and Utilization

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Genetic mapping (also known as linkage or meiotic mapping) refers to the determination of the relative position and distances between markers along chromosomes based on the mean number of recombination events, involving a given chromatid, in that region per meiosis. Genetic map construction requires that the researcher develop appropriate mapping population, decide the sample size and type of molecular marker(s) for genotyping, genotype the mapping population with sufficient number of markers, and perform linkage analyses using statistical programs (Linkage1, GMendel, MapMaker, MapManager, JoinMap etc.). The construction of detailed genetic map with high level of genome coverage is the first step for localizing genes or quantitative trait loci (QTL) that are associated with economically important traits, marker assisted selection, comparative mapping between different species, a framework for anchoring physical maps, and the basis for positional cloning of genes. Hence, development of appropriate mapping population forms the basic platform for most of the molecular marker assisted plant breeding applications.

Mapping Populations

A population used for gene mapping usually obtained from controlled crosses is commonly called a mapping population. The first and most critical step in producing a mapping population is selecting two genetically divergent parents, which show clear genetic differences for one or more traits of interest at phenotype level. Sufficient variation for the traits of interest at the DNA sequence is equally important to trace the recombination events for the easier identification of polymorphic informative makers.The parents should be genetically divergent enough to exhibit sufficient polymorphism and at the same time they

should not be genetically too distant to cause sterility of the progenies and/or very high levels of segregation distortion during analysis. Consideration must be given to the source of parents used in developing mapping population. Genetic divergence between parents influences marker-trait associations and hence economic significance of map.

Development of mapping populations:

Progenies from the second filial generation (F_2), F_2 derived F_3 (F_2:F_3) populations, backcross (BC) populations, recombinant inbred lines (RILs), doubled haploids (DHs), near isogenic lines (NILs), and immortalized F_2 Population have been used for genetic mapping crop plants. F_2 populations are developed by selfing F_1 hybrids derived by crossing the two parents while BC population is produced by crossing F_1 back into one of the parents (the recipient or recurrent parent)(Fig. 1). $F_{2:3}$ population is generated by selfing the individual F_2 segregants for a single generation. RILs are developed by single-seed selections from individual plants of an F_2 population; such selections continue for 6-8 generations. If backcross selection is repeated at least for six generations, more than 99% of the genome will be derived from recurrent parent. Selfing of selected individuals from BC_7F_1 will produce BC_7F_2 lines that are homozygous for the target gene, which is said to be nearly isogenic with the recipient parent (NILs). A DH population is produced by anther/pollen culture of F_1 plants. Plants will be regenerated using tissue culture techniques after induction of chromosome doubling from pollen grains or haploid embryos resulting from species crosses. Immortalized F_2 populations can be developed by paired crossing of the randomly chosen RILs derived from a cross in all possible combinations excluding reciprocals. The set of RILs used for crossing along with the F_1s produced, provide a true representation of all possible genotype combinations (including the heterozygotes) expected in the F_2 of the cross from which the RILs are derived. The RILs can be maintained by selfing and required quantity of F_1 seed can be produced at will by fresh hybridization. This population therefore provides an opportunity to map heterotic QTLs and interaction effects from multi-location data.

Characterization of Mapping Populations

Precise molecular and phenotypic characterization of mapping population is vital for success of any mapping project. Since the molecular genotype of any individual is independent of environment, it is not influenced by G × E interaction. However, trait

phenotype could be influenced by the environment, particularly in case of quantitative characters. Therefore, it becomes important to precisely estimate the trait value by evaluating the genotypes in multi-locations over years using immortal mapping populations to have a valid marker-trait association.

Utilization of mapping populations:

Currently available literature shows that genetic maps are constructed using different types and sizes of mapping populations, marker systems, statistical procedures and computer packages. Each factor can affect the efficiency of the mapping process because of differences in the genetic distances between markers that can occur by variations in the degree of recombination observed in different crossings. Each mapping population has advantages and disadvantages and the research needs to decide the appropriate population for linkage mapping depending on project objective, time available for developing the population, and whether the molecular markers to be used for genotyping are dominant or co-dominant.

Both F_2 and BC populations are the best populations for preliminary mapping and are the simplest types of mapping populations as they are easy to construct and require only a short time to produce. However, F_2 and BC populations are ephemeral populations because they are highly heterozygous and seed derived from selfing these individuals will not breed true, so cannot be propagated indefinitely through seeds. This limitation can be overcome to a limited extent by cuttings, tissue culture or bulking F_3 plants to provide a constant supply of plant material for DNA isolation. Nevertheless, it is difficult or impossible to measure characters as part of quantitative trait locus (QTL) mapping in several locations or over several years with F_2 or backcross populations. Thus, the effect the G × E interaction on the expression of quantitative traits cannot be precisely estimated. The specific advantage of BC populations is they can be further utilized for marker-assisted backcross breeding. Both F_2 and BC populations are the products of one cycle of meiosis, so are of limited use for fine mapping. The $F_{2:3}$ population can be used for reconstituting the genotype of respective F_2 plants, if needed, by pooling the DNA from plants in the family. Though not an 'immortal' population, it is more suitable for mapping quantitative traits and recessive genes.

RILs, NILs and DHs are permanent populations because they are homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring.

Repeated selfing of F_2 plants leads to RILs that each contains a different combination of linkage blocks from the original parents. The differing linkage blocks in each RIL provide a basis for linkage analysis. However, several generations of breeding are required to generate a set of RILs, so this process can be quite time-consuming. Moreover, some regions of the genome tend to stay heterozygous longer than expected from theory and obligate outcrossing species are much more difficult to map with RILs because of the difficulty in selfing plants. Nevertheless, in cases where it is feasible, seed from RILs is predominantly homogeneous and abundant, so the seed can be sent to any lab interested in adding markers to an existing linkage map previously constructed with the RILs. RILs being obtained after several cycles of meiosis are very useful in identifying tightly linked makers, thus making them ideal for QTL mapping.

Although NILs are frequently generated by plant breeders as they transfer major genes between varieties by backcross breeding, they are suitable populations for molecular tagging and functional genomics but not for linkage mapping. DH populations are completely homozygous inbred populations and are produced quickly than RILs and NILs but the production of DHs is only possible for species with a well established protocol for haploid tissue culture. Anther culture induced variability should be taken care of. So production of DHs demands relatively more technical skills and it accounts recombination only from male side compared to RILs and NILs.

Population size and marker systems

Once an appropriate mapping population has been chosen, the appropriate population size must be determined. The type and size of mapping populations can exert an influence on the accuracy and economic significance of genetic maps. Larger mapping population is always better especially when the goal is high resolution mapping in specific genomic regions or mapping QTLs of minor effect. Immortal populations of large size (preferably more than 200 individuals), genotyped by co-dominant markers yield more precise and high resolution linkage maps.

Different molecular marker systems *viz.,* Restriction fragment length polymorphisms (RFLPs), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs), cleaved amplified polymorphic sequence (CAPS), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeats

(ISSR), diversity array technology (DArT), and single nucleotide polymorphism (SNP) have been used for map construction in several crops. Each marker system has its own advantages and disadvantages. For high throughput screening and high resolution mapping, molecular markers should preferably be highly informative, co-dominant, reproducible, locus specific, cross-transferable, and amenable to complete automation. Although, the scope of EST-derived marker development is limited to species for which sequencing databases already exist, but wherever possible, EST marker should be exploited for mapping as, it is found to be genetically associated with a trait of interest, so offers possibility of direct mapping of trait, candidate gene based and comparative mapping across different species.

Combining Markers and Populations

The genetic segregation ratio at maker locus is jointly determined by the nature of marker (dominant/ co-dominant) and types of mapping populations (Table 1). Therefore, a thorough understanding of the nature of markers and mapping population is crucial for any mapping projects. Markers such as RFLPs, microsatellites and CAPS etc. are codominant in nature, while AFLP, RAPD, ISSR are often scored as dominant markers. Mapping populations such as RILs and DHs equalize marker type because of fixation of parental alleles at marker locus in homozygous condition. These populations result in 1: 1 segregation ratio at marker locus irrespective of genetic nature of markers, while an F_2 population segregates in 1: 2: 1 ratio for a codominant marker and in 3:1 ratio for dominant marker (Fig. 2). Depending upon the segregation pattern, statistical analysis of marker data will vary.

Maximum genetic information is obtained from F_2 population using a co-dominant marker system. Dominant markers supply as much information as co-dominant markers in RIL, NILs and DHs because all loci are homozygous, or nearly so. Information obtained from BC populations using either co-dominant or dominant markers is less than that obtained from F_2 populations because one, rather than two, recombinant gametes are sampled per plant. RILs, NILs and DHs may be powerful tools for QTL detection in some circumstances but provide no information on dominance relationships for any QTL while, F_2 is preferred for detecting QTLs of additive effect.

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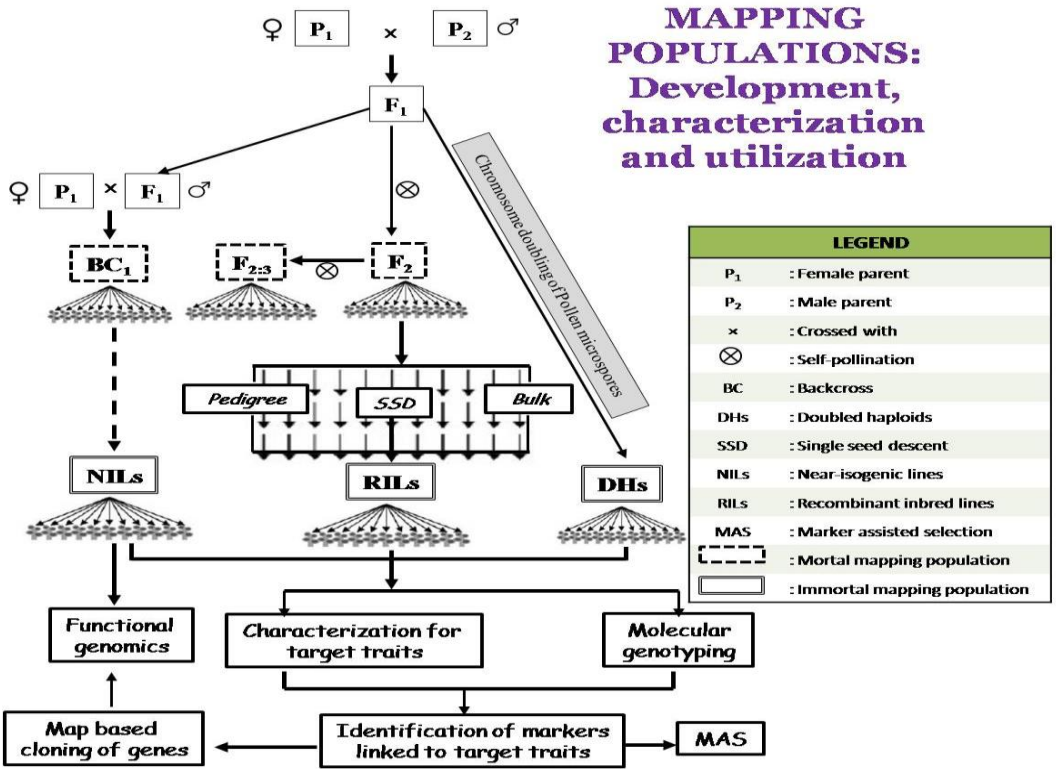

Fig. 1 Development, characterization and utilization of mapping populations

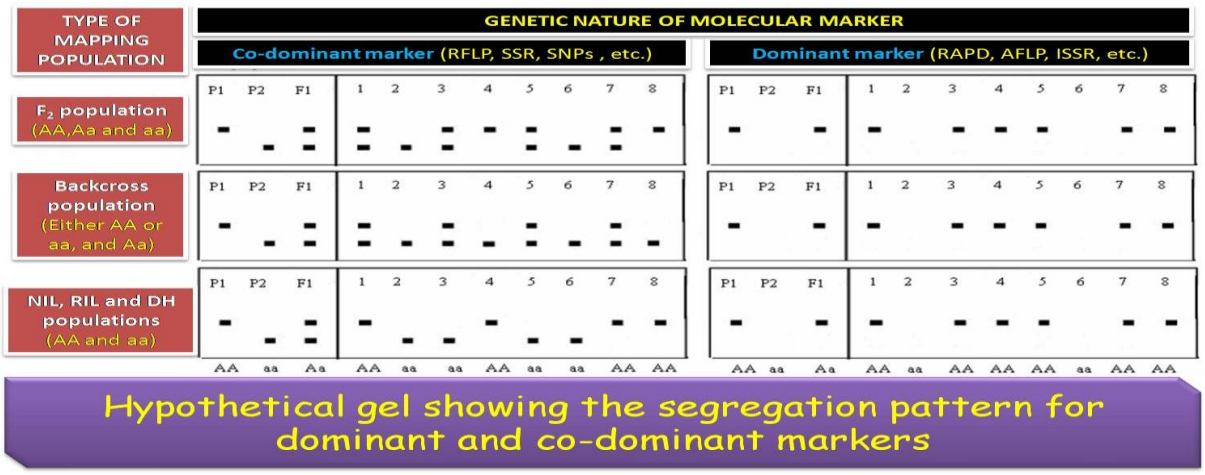

Fig. 2 Characterization of mapping populations' vis-à-vis different marker systems

Nature of Molecular Marker	Genetic Segregation Ratio in Mapping Populations					
	F_2	RILs	DHs	NILs	Backcross Popn.	
					B_1	B_2
Co-dominant	1 : 2 : 1	1 : 1	1 : 1	1 : 1	**1:1**	**1:1**
Dominant	3 : 1	1 : 1	1 : 1	1 : 1	**1:0**	**1:1**

Table 1. Genetic segregation ratio at marker locus in different marker-population combinations

Mapping Quantitative Trait Loci (QTL) in Crop Plants

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The term Quantitative Trait Locus (QTL) was first coined by Gelderman (1975) which defines QTL as "a region of the genome that is associated with an effect on a quantitative trait". Conceptually, a QTL can be a single gene, or it may be a cluster of linked genes that affect the trait. A quantitative trait exhibits continuous variation in its phenotypic expression. The trait expression may be controlled by many genes (a few hopefully of *large effect* and the others of *small effect*). Phenotypic expression of the trait is affected by genetic (*gene* × *gene interaction; epistasis*) and non-genetic factors [(*macro-* and *micro-environment variations* and *measurement errors* (*Noise*)] apart from their interaction with the environment (*gene* × *environment interaction*)**.** A genetic marker that tends to co-segregate with the trait is likely to be close to a QTL controlling that trait. In QTL mapping, we seek an association between marker alleles (genotypes) and trait values (phenotypes). The major purpose of QTL mapping is primarily to estimate the locations, number, magnitude of phenotypic effects, and mode of gene action, of individual genetic loci (QTLs) for its application in marker-assisted breeding. Assessing the relative contribution of a QTL to the total genetic variance for a specific trait (R^2), analysis of QTL x E effects and identification of parental lines contributing the favourable QTL allele are the other important components of QTL analysis. The fundamental challenge in QTL analysis is to disentangle the genetic signal at any individual locus from the noise. With Polymorphic markers, QTL analysis can be done with or without a genetic linkage map. A linkage map needs adequate polymorphic markers and allows mapping of QTL, estimating their number, phenotypic effects, and mode of gene action. Without a linkage map, only QTL-harbouring markers can be identified; QTL location, number, effect and mode of gene action cannot be determined. QTL analysis can be done using a *mapping population* (Linkage analysis) or a *natural/breeding population* (Association analysis). Classical analysis of QTLs employed statistical techniques which were based on means, variances and co-variances provided negligible information on what these genes were, where

they were located, and how they controlled the trait. Major advancements in PCR-based marker systems, applied statistics and quantitative genetics revolutionised the QTL research.

Requirements

1. A suitable mapping population generated from phenotypically contrasting parents
2. A reasonably saturated linkage map based on molecular markers
3. Reliable phenotypic screening of mapping population
4. Powerful statistical packages to analyse genotypic and phenotypic information in combination.

QTL Mapping – Detailed Steps

1. Selection of phenotypically contrasting for target trait (and as many important traits as possible) and genetically divergent parental lines with due consideration of pedigree information (Fig. 1).

2. Identification of a large number of polymorphic markers between parental lines with adequate genomic coverage.

   Different marker systems have different levels of resolution for detecting genomic variation. Select a system that allows experimental detection of heritable genomic variation among individuals of the mapping population. Co-dominant markers provide more power for QTL detection than dominant markers. For eternal RIL and DH, both types of markers are equally informative.

3. Generation of a suitable mapping population.

   Ability to detect QTLs or the information contained in F_2 or F_2-derived populations and RILs are relatively higher than others. $F_{2:3}$ populations are capable to measure the effects of additive and dominance gene actions at specific loci. Recombinant Inbred Lines (RILs) in general and Doubled Haploids (DHs) wherever available are the most preferred mapping populations for high resolution QTL mapping, though additive gene action can only be measured. Though, backcross (BC) population provides limited genetic information, marker-assisted introgression can be integrated with QTL mapping (AB-QTL Method). Mapping population of size 500-1000 is considered ideal for high map resolution and finer marker order. So choice of mapping population and

its size should be, therefore, based upon the objectives of the experiment, the timeframe as well as resources available for undertaking QTL analysis.

4. Partitioning of mapping population into different genotypic classes (based on genotypes at polymorphic marker loci)

   "Genotyping" refers to the process of determining the genotype (the genetic constitution) of an individual by the use of biological assays. For locating QTLs, a dense map is preferable as this allows greater precision of QTL location. QTL mapping, like linkage mapping, relies on the frequency of detectable recombination events, which, beyond a given marker density can only be increased by increasing size of the mapping population. So a possible approach is to initially use an evenly spaced sparse map to detect significant chromosomal regions to which more markers could be subsequently saturated for fine-scale localization of QTLs.

5. Phenotyping of mapping population for target trait

   Phenotyping is as important as genotyping and is an often unrecognized, but the most critical part of QTL analysis. Robust and high resolution QTL mapping demands reliable phenotyping of traits of interest under defined target environmental conditions over locations and years. Phenotypic data that explains the effect of QTLs should come from multi-environment testing. Use appropriate intra-environment experimental designs and biometric tools to obtain accurate and precise estimate of genetic parameters.

6. Apply correlative statistics to determine 'linkage disequilibrium' between a marker and the QTL.

   Assess the probabilities of associations between phenotypic values and genotype scores at each marker locus, across the set of mapping population progenies. QTL Cartographer, MapQTL, PlabQTL, MultiQTL are the most commonly employed QTL mapping softwares. QTL analysis approaches could be classified as follows according to the number of genetic markers used as *unit of analysis* in analysing the data.

   i. ***Single-Marker Analysis:*** Single factor analysis (SFANOVA)
   ii. ***Two-Marker Analysis:*** Simple Interval Mapping (SIM)
   iii. ***Multiple-Marker Analysis:*** Composite Interval Mapping (CIM), Multiple Interval Mapping (MIM)
   iv. ***Non-Parametric Mapping*** employing MapQTL Software

Salient Features of most commonly employed QTL detection methods:

- ***Single point analysis*** analyses one marker at a time for its association with QTL employing linear regression analysis. It is computationally simple, quick and do not require molecular linkage map. But the magnitude of QTL detected is underestimated because of recombination and less likely to detect QTL further from a marker because of cross over effects between the loci. The method cannot determine whether the markers are associated with one or more QTLs.
- ***Simple Interval mapping*** (Lander and Botstein, 1989) localizes QTL into marker intervals and takes full advantage of a linkage map. Presence of a putative QTL is estimated if the log of odds ratio (LOD) exceeds a critical threshold. Formulae available for calculating significance levels are appropriate for interval mapping when the genome size, number of chromosomes, number of marker intervals, and overall false positive rate desired are given. It is most widely used approach. By using tightly linked markers for analysis, it compensates for the recombination between markers and the QTL, thereby increases the probability of statistical detection of the QTL, and provides an unbiased estimate of QTL effect, but when multiple QTLs are segregating in a cross (which is usually the case), SIM fails to take into account genetic variance caused by other QTLs.
- ***Composite interval mapping*** (Zeng, 1994 and Basten *et al.* 2001) employs adjacent markers and/or other QTL as cofactors. It combines interval mapping for a single QTL in a given interval with multiple regression analysis on marker(s) associated with other QTL. It considers a marker interval plus a few other well-chosen single markers in each analysis, so that *n*-1 tests for interval-QTL associations are performed on a chromosome with *n* markers. By using linked markers as cofactors, the test is not affected by QTL outside the region, thereby increasing the precision of QTL mapping.
- ***Advanced Backcross-QTL (AB-QTL) Strategy*** (Tanksley and Nelson, 1996) combines QTL analysis with variety development. It is particularly useful for transfer of valuable QTLs from un-adapted germplasm into elite breeding lines. QTL analysis delayed until the BC_2 or BC_3 generation. QTL-NILs derived from advanced BC populations in one or two additional generations are utilized to validate the QTL activity. QTL-NILs also represent improved commercial inbreds (over the original

parental line) for one or more quantitative traits. So AB-QTL method enables simultaneous QTL mapping and its validation.

- ***Multiple Interval Mapping*** (Zeng *et al.* 1999) *is a* multiple QTL-oriented method that allows estimation of number, positions, effects, and epistatic interactions among significant QTLs simultaneously. ***Multiple-Trait Interval Mapping*** tests and estimates *pleiotropy* and *pleiotropy-vs-linkage*, *QTL x environment* interactions and QTLs affecting multiple traits.

The care and expense invested in generating marker and trait data should be accompanied by *equal care* in biometric analysis of data. A resampling technique to measure the quality of statistical estimates and models called **Bootstrapping** and statistical method to evaluate the predictive capability of a given set of models called as **Cross validation** are utilized to validate the QTL statistical analysis. Once mapped and validated, the QTL mapping information can be utilized for, genotype building/QTL pyramiding, QTL introgression, hybrid breeding, marker-assisted recurrent selection (MARS), and understanding basic mechanisms to resolve complex biological problems.

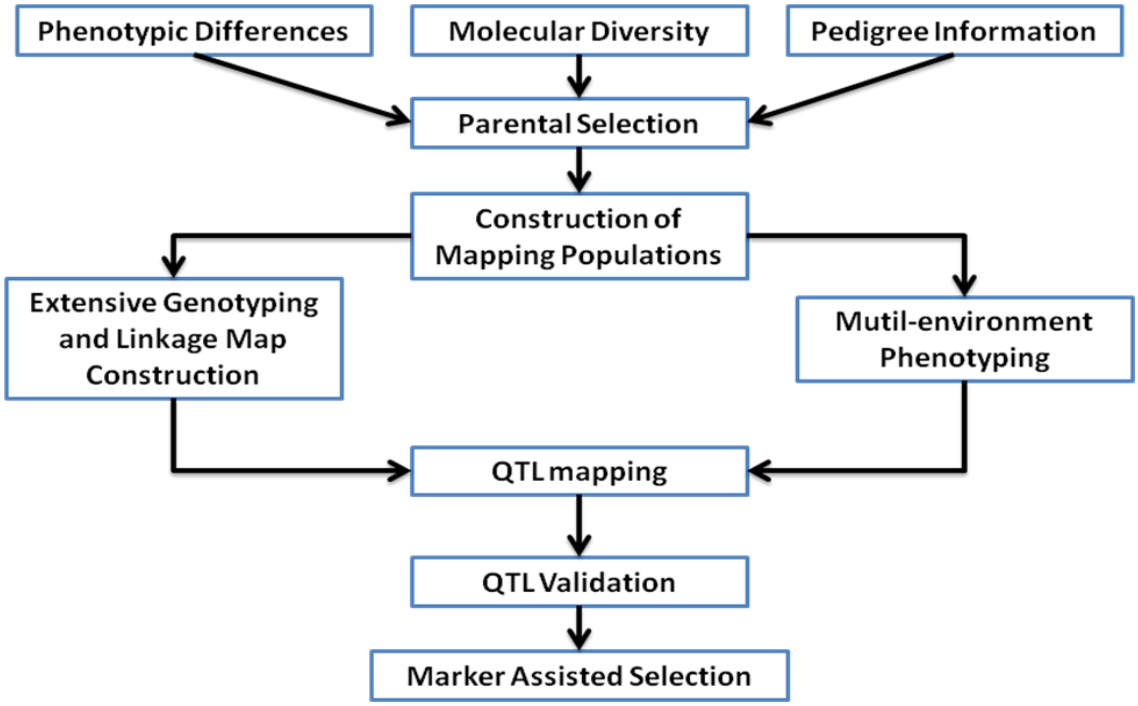

Fig. 1. Frame work of QTL Mapping for Crop Improvement

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Marker Assisted Breeding in Cotton

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Introduction

Genetic improvement of crop plants through conventional plant breeding is primarily based on phenotypic selection of superior individuals among segregating populations. Significant strides have been made in crop improvement through phenotypic selections for economic traits, though considerable difficulties are often encountered during this process, primarily due to genotype-environment interactions. Most of the traits considered in plant genetic improvement programmes are quantitative, i.e. they are controlled by many genes (polygenes), influenced to the large extent by environmental factors and the underlying genes have small effects on the observed phenotype.

In classical genetic improvement programmes, selection is carried out based on observable phenotypes and/or their relatives but without knowing which genes are actually being selected. The advent of molecular markers has revolutionized the scenario and has promise to overcome these limitation.

Molecular Markers

The term marker is most commonly used as genetic marker that indicates morphological, biochemical (isozymes, proteins) and molecular markers (at DNA level). The characteristics of an ideal genetic marker are:

- Abundant
- Highly polymorphic – indication of presence of variability.
- Multiallelic – presence of more than one allele in the population
- Codominant - the heterozygote can be distinguished from each of homozygote.
- Non-epistatic – referred to absence of intra and inter-locus interactions.

- Neutral
- Insensitive to environment

Different types of molecular marker have been developed over the years. Different types of DNA based markers include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), sequenced tagged sites (STS), sequence characterized amplified regions (SCAR), variable number tandem repeats (VNTR), microsatellites or simple sequence repeats (SSR), inter simple sequence repeats (ISSR), amplicon length polymorphism (ALP), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphism (SNP).

Application of Molecular Markers

DNA polymorphism is exploited in plants for differentiation between individuals, accessions and species of plants, pathogens and pests. Their high resolution compared with all other markers makes them a valuable tool for varietal and parental identification for the protection of breeders' rights. DNA markers further add to the repertoire of tools for the determination of the evolutionary relationship between species and families. Molecular markers also allow an understanding of the relationship between chromosomes of the related species.

- Assessment of genetic diversity and DNA fingerprinting
- Establishing phylogenetic relationship of species
- Evolutionary studies
- Genetic linkage maps
- Mapping genes/ QTLs and characterization
- Association studies and
- Marker assisted breeding

Genetic mapping

A genetic map could be thought as road map, reflecting the relative proximity of different landmarks to one another, and molecular markers at defined places along each linear chromosome enabling the geneticist to determine a particular gene of interest (Paterson, 1996). Genetic linkage maps are useful tools for studying genome structure, evolution, identifying introgression and for marker-assisted selection in breeding programme. Modern upland cultivars show significant variation for important traits including fibre production, pest resistance, and

tolerance to environmental adversities such as heat, cold and drought (El-Zik and Thaxon, 1989). In addition, the wild *Gossypium* germplasm also harbors many valuable traits including disease and insect resistance, stress tolerance and fibre quality attributes. However, the transfer of genes from wild species is time consuming and not always successful. DNA markers and genetic maps that assess the introgression of alien genes into cultivated cotton will greatly accelerate breeding efforts (Jiang *et al.*, 2000 and Waghmare *et al.*, 2005). Identification of molecular markers closely associated with important agronomic trait play a critical and increasing role in the development of superior cultivars that combine the favorable traits.

One of the first RFLP based linkage maps of cotton published by Reinisch *et al*. (1994). Efforts to saturate this map continued using the same population (Reinisch *et al*., 1994) and a detailed cotton map consisting of 2584 loci spaced at 1.72 cM in 26 linkage groups has subsequently been published (Rong *et al*. 2004). Coalescence of the map into 26 linkage groups, equal to the gametic chromosome number, has enhanced and extended the association of linkage groups with easily distinguishable chromosomes of the subgenomes. Rong *et al*. (2004) has also made a diploid D genome map consisting of 763 loci spaced at 1.96 cM and recombinational length of 1493.3 cM in 13 linkage groups. Comparison of both diploid (D) and tetraploid (AtDt) map revealed synteny of markers and no major structural changes were observed between Dt and D chromosomes. Till recently, more than 30 linkage maps have been published in cotton, largely based on interspecific crosses (involving *G. hirsutum* and *G. barbadense*) employing various marker types.

Interspecific tetraploid genetic linkage maps were valuable for finding new DNA markers though conventional breeding programmes rarely use interspecific crosses for varietal development. It is widely recognized that increasing marker density of cotton genetic maps is essential for marker assisted selection and genomic studies. Efforts were made to inter-link existing maps based on subsets of shared DNA markers to develop consensus (Rong *et al*. 2004) or integrated maps (Rong *et al*. 2007, Yu *et al*. 2010, Reddy *et al*. 2011, Blenda *et al.* 2012) with as many as 8254 loci (Blenda et al 2012). Recently, a very high density whole genome marker map (WGMM) of cotton was constructed by using 18597 DNA markers corresponding to 48958 loci that were aligned to both a consensus genetic map and a reference genome sequence (Wang *et al*. 2013). The WGMM is a versatile genetic map to be used for marker assisted breeding, fine mapping, cloning of genes/QTLs, developing new markers, genome wide association and genome evolution studies.

Mapping genes/ QTLs in cotton

Molecular markers hold promise for mapping several economic traits in cotton which have been introgressed from exotic germplasm, such as *Verticillium* wilt resistance, bacterial blight resistance, restoration of cytoplasmic male sterility (Weaver and Weaver, 1977), improved fibre quality (Culp et al., 1979). Association of DNA markers with these genes and others of agronomic importance would provide cotton breeders new opportunities for marker assisted selection (MAS). Of the several molecular linkage maps developed in cotton only few have largely been used to identify QTLs for agronomic and fibre quality traits. An RFLP map of Reinisch *et al*. (2004) and Rong *et al*.(2004, 2007) have widely been used to link QTLs, traits related to plant growth, development, and morphology (Jinag *et al*. 2000b; Wright *et al*. 1999), reproductive biology (Lan *et al*. 1999); fiber yield and quality (Jiang *et al*. 1998; Paterson *et al*. 2003), disease resistance (Wright *et al*. 1998) and the preservation of productivity and quality under drought stress (Saranga *et al*., 2001; Saranga *et al.*, 2004).

Mei *et al*. (2004) detected seven QTLs for six fibre related traits, five of them were distributed among A genome chromosomes. More than 100 QTLs associated with agronomic and fibre traits were also mapped in an intraspecific population of upland cotton (Shappley *et al*., 1998). Several cotton genes have been tagged with DNA markers that include leaf shapes (Yu *et al*., 1997), plant trichomes, photoperiodism (Yu and Kohel, 1999), fertility restorer (Lan *et al*., 1992; Guo *et al*., 1998), stomatal conductance (Ulloa *et al*., 2000) and fibre traits (Zhang *et al*., 2003).

Zhang *et al.* (2005) identified four QTLs affecting lint per cent, two QTLs for 2.5% span length, three QTLs for fibre length uniformity, three QTLs for fibre strength, two QTLs for fibre elongation and two QTLs for micronaire have been detected. Shen *et al*. (2005) reported 39 QTLs (17 significant QTLs and 22 suggestive QTLs) affecting fibre traits were found in the three populations. Park *et al*. (2005) suggested that chromosomes 2, 3, 15 and 18 may harbour genes for traits related to fibre quality. Further investigation by Frelichowski *et al*. (2006) revealed that apart from the above mentioned chromosomes, loci on chromosome 12 may also affect variation in fibre quality traits. Abdurakhmonov *et al*. (2007) reported QTLs associated with lint percentage located on chromosomes 12, 18, 23 and 26. He *et al.* (2007) identified 4 QTLs for lint index, 8 for seed index, 11 for lint yield, 4 for seed cotton yield, 9 for number of seed per boll, 3 for fiber strength, 5 for fiber length, and 8 for micronaire value. A study on intra-

hirsutum RIL population by Shen *et al.* (2007) using SSR markers revealed 25 major QTLS, 4 with large effects on fiber quality and 7 with large effects on yield components.

Meta-analysis of QTLs

Lacape et al (2010) carried out meta-analysis of fibre quality QTLs across the environments using the RIL population that displayed a large variability for all major fiber traits. A meta-analysis of more than a thousand putative QTLs was conducted with MetaQTL software to integrate QTL data from the RIL and 3 backcross populations. The QTL clustering was possible for 30 trait x chromosome combinations (5 traits on 19 different chromosomes) where an effective co-localization of unidirectional QTLs from at least 5 different data sets was observed. Most consistent meta-clusters were identified for fiber color on chromosomes c6, c8 and c25, fineness on c15, and fiber length on c3. Meta-analysis provided a reliable means of integrating phenotypic and genetic mapping data across multiple populations and environments for complex fiber traits. The consistent chromosomal regions contributing to fiber quality traits constitute good candidates for the further dissection of the genetic and genomic factors underlying important fiber characteristics, and for marker-assisted selection.

Marker-assisted breeding

Marker-assisted selection (MAS) involves selection of plants carrying genomic regions that are involved in the expression of traits of interest through molecular markers. With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits both governed by major genes as well as quantitative trait loci (QTLs).

For example in cotton, the use of molecular markers enables cotton breeders to identify gene underlying a specific phenotype with the distinct regions of the genome in which the gene resides, e.g., the phenotypic expression for fiber quality in domesticated species. The genetic advances in fiber quality can be made indicative of the existence of genes that contribute to fiber quality in germplasm that does not express the phenotype. Molecular markers could provide the opportunity to use precision in identifying the phenotype of these traits. Molecular markers will allow direct selection for genotypes, thereby providing a more efficient means of selection for fiber properties. The genetic manipulation of cotton fiber properties using molecular strategies relies on the identification and isolation of genes that control fiber development and/or directly affect a particular structural property of fibers. Molecular markers

facilitate to identify and isolate the genes relating to fiber characters by map-based cloning. Once markers for trait of interest are established, these should allow prediction of fiber characters, yield or resistance of individual offspring derived from a cross. Besides the exploitation of genomic polymorphism for germplasm utilization and protection of varieties, interest in molecular markers should focus on following major issues.

- The introgression of single resistant genes for plant pathogens from wild species or cultivated donor lines into otherwise superior cultivars.
- Gene pyramiding of major and/or minor resistance genes into cultivars for generating multiple and durable (horizontal) resistances against several pathotypes of the same pathogen.
- The improvement of the agronomic value of cotton by breeding for quantitatively inherited traits, such as yield and drought tolerance.

In MAS, an empirical association of marker genotypes with trait phenotypes is used for selection. Based on available phenotypic and or molecular information, the parents hosting the different target genes are crossed, after which selection consists of screening- among the different recombinants produced in one or more generations – the one(s) that is closest to the target gene based on marker analysis. Finally, phenotypic evaluation is carried out in order to evaluate the agronomic value of the resulting progenies.

Marker assisted backcrossing for a single target gene:

Backcross breeding is used for introgression of a target gene from a donor line into the genomic background of a recipient line. The target locus is selected in each backcross generation (foreground selection) and simultaneously selected for increasing genomic content of the recipient genotype (background selection). The target gene/ locus can be known gene or a QTL. When the target gene is a QTL, the foreground selection poses additional problems as the exact location of the target is often not known, but rather estimated based on appropriate statistical methods. Hence, the number and chromosomal position of the markers identifying the target gene must take account of the uncertainty of the true target location. The carrier chromosome (carrying the target gene) deserves special consideration in backcross programme than the non-carrier chromosomes due to selection for the target gene in each generation so as to reduce the linkage drag. Clearly, selecting more number of flanking markers close to the target, facilitate

identification of double recombinant types or single recombinant with tightly linked flanking marker in the background selection should help to reduce the linkage drag.

At each generation of backcrossing selection is carried out in three steps-

1. Foreground selection: Selection of all individuals that are heterozygous at the target locus.
2. Minimization of linkage drag: Selection of individuals those are homozygous for the recipient allele at the markers flanking the target locus.
3. Background selection: Selection of the individual that is most homozygous for the most markers on the non-carrier chromosomes.

Marker assisted backcrossing for multiple target genes:

Several genes of interest for one or different characters may also be manipulated at the time. These may include genes or favorable alleles of target QTL in the same breeding population. Use of molecular markers can make such breeding scheme more efficient.

1. ***Marker based population screening***:

When several favorable genes are present in the parental genotypes involved in the development of population (F2, F3, RILs or DH), the population is screened based on molecular markers for individual homozygous at the target loci. Van Berloo and Stam (1998) considered at set of identified QTL, each identified by two flanking markers and studied selection in RIL population based on flanking markers to produce best recombinant. In this case, if all the favorable genes cannot be fixed in a single selection step, it is necessary to cross again among the selected individuals with set of homozygous favorable loci. This strategy is practically feasible with the small number of target loci. With the increase in number of loci, the population size necessary to fix the target genes increases exponentially.

2. ***Marker based recurrent selection***:

If MAS is to be applied for more loci, recurrent selection- a breeding scheme involving several generations of selection and random mating of selected individuals, may be employed. Hospital (2000) studied selection on marker pairs flanking 50 QTL identified in F2 population. In this scheme, a set of individuals are selected that are complementary for their genotypes at flanking markers, such that each target is carried by at least two selected individuals. Selection of 3-5 individuals among a total of 200 for 10 generations increases the

frequency of favorable alleles at the 50 QTL up to 100% when the markers are located exactly on QTL and 92% when the marker QTL distance is 5 cM. In this strategy, marker based selection is limited by recombination taking place between the markers and the QTL.

3. ***Marker based gene pyramiding*:**

When the target genes are present in the multiple parents, it is possible to perform a marker assisted gene pyramiding involving several initial crosses between the parents. For example, four genes (G1-G4) are present in four different genotypes (L1-L4) can be combined in two steps, i.e. two lines homozygous for two target genes may be first combined in single cross (G1/G2: L1xL2; G3/G4: L3xL4) and then individuals with two homozygous loci (G1G1G2G2 and G3G3G4G4) from single crosses may be combined again to select for homozygous individuals with all four homozygous loci.

4. ***Marker assisted backcrossing for several target genes***:

All economic traits are governed by polygenes. For introgression of complex traits, polygenes- several QTL of small or medium effect accounts for the variability. In such cases, several targets are required to be controlled with the genetic background in marker assisted backcross breeding. The number of individuals for genotyping increases exponentially with the number of target loci. Hospital and Charcosset (1997) concluded that in general it is futile to plan to manipulate more than three to four QTL simultaneously in a marker assisted backcross programme. If the targets are known loci identified directly by markers then the maximum number of targets could be slightly higher, but should not exceed five to six.

Selection combining marker and phenotype information

In cases, when the target genes do not account for all the variability of the selected trait(s), the gain expected from the accumulated effects of the target genes might not warrant performing selection based solely on marker information. In such cases, it may be desirable to employ selection strategy based on both the variability accounted for by the target genes and phenotypic expression of the trait. Chakraborty et al (2002) considered the optimization of MAS for identified QTL and a possible polygenic background controlling the genetic variation of the trait not explained by QTL. Such analyses are restricted to one or two identified QTL of sufficiently large effects.

Conclusion

Molecular markers are valuable tools in genetic/ breeding investigations. They offer method of tracing genetic sources of useful variability. Molecular markers facilitates characterization of gene pool, cultivar identification, DNA fingerprint, genome variability, genetic diversity and relatedness, gene mapping, phylogenetic relationships, molecular dissection of complex traits i.e. yield and quality attributes; genome organization, evolution and genome divergence are being studies. Application of marker assisted selection (MAS) would facilitate breakage of linkage and early detection of targeted genes especially for those that would help to break yield plateau and increase productivity and quality. An identification of markers tightly linked to important gene(s)/QTLs would facilitate development of appropriate selection strategies for improvement of specific trait(s).

Marker assisted selection for disease resistance – Bacterial blight of Cotton

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Bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* is one of the most important diseases of cotton which causes approximately 10 to 35% of yield loss. It occurs in all cotton-growing areas throughout the world and one of the most devastating diseases of cotton causing yield losses from 5 – 35%. Yield losses of 10 to 30% are not unusual in Africa or Asia (Thaxton and El-Zik, 2001). The disease prevails all over the India irrespective of regions. The cloudy weather specifically high humidity coupled with continuous rainfall favours the disease spread and severe yield loss. In Australia, resistance to bacterial blight is a mandate for all commercial cotton varieties (Xiao *et al*. 2010). In India, bacterial blight of cotton occurs in almost all cotton growing regions every year and causing yield losses up to 30 per cent (Meshram and Raj, 1992). Bacterial blight symptoms include dark green,water-soaked lesions on leaves, stems, and bolls, followed by premature leaf senescence and reduced lint yields. Boll opening is accelerated, leading to higher proportions of immature cotton fibres, and the lint may be stained and unmarketable (El-Zik and Thaxton 1994).

Several races of Xam have been reported from India and worldwide by different workers. So far, about 19 Xam races have been identified in the USA (Verma, 1986). Of these races, race 18 is the most virulent and present in almost all production areas in the world (Brown, 2001). Races 10 and 18 are widely distributed in most of the cotton growing areas in India and around 80.00 per cent isolates belonged to these races. The race 18 of the pathogen is most virulent which can overcome 5 major bacterial blight resistant genes viz. B2, B7, Bin, BN and B4 and different polygene complexes of cotton differentials is being utilized for identification of resistant sources (Meshram *et al*. 2002). Screening for bacterial blight resistance through artificial inoculation using different races of pathogen in field and pot culture has been performed by many workers and several cultivars and germplasm lines were classified as immune, resistant and susceptible. Resistance to *Xam* was assessed for 3657 lines of upland cotton in field and 15 lines expressed disease free reaction for race 10 and 18 in both field and

pot culture conditions through artificial inoculations (Meshram *et al*. 2002). Race specific RFLP marker has been developed to identify the race 18 of Xam (Chakrabarty *et al*. unpublished).

The deployment of resistant varieties is the most effective and economical means to control the disease and minimize the yield loss (Xiao *et al*. 2010). The breeding for disease resistance is the highly significant method to overcome the problem and the long term solution for disease resistance. Resistance to bacterial blight has been studied extensively (Hillocks, 1992). To date Marker assisted selection is the most successful technique in disease resistance breeding. Conventional screening for disease resistance is the cumbersome process which involves lot of labour and required controlled environment conditions which is very difficult to create and costly. Alternatively, MAS is the cheap and easy method to follow and less time consuming. Marker assisted selection for resistant individuals can be performed at any development stage of the plant including seeds, and even has a low cost and fast turnaround of information compared to the phenotype-based selection (Xiao *et al*. 2010).

Molecular markers provide a novel avenue for the genotype based resistance selection (Xiao *et al.* 2010). For major gene traits such as many disease resistances, gene validation is fairly straight forward (Dwivedi *et al*. 2007). Genotyping the elite lines for the markers flanking the resistance gene can identify the putative resistant lines based on their haplotypes at the resistant gene region (Xiao *et al*. 2010). Resistance germplasm that are elite and locally adapted are the most desirable as the resistant parents and identification of such resistance germplasm is very important in the breeding for the resistance (Xiao *et al*. 2010).

There are at least 22 reported resistance genes in cotton that confer differing degrees of resistance to various *Xcm* races carrying different avirulence genes in a typical gene-for-gene manner. Of these 22 reported resistance genes, B_{12} confers a high level of resistance to all *Xcm* races presently found in the US and also other races found in Africa (Wallace and El-Zik, 1990). Wright et al. (1998) mapped B2, B3 and b6 genes with restriction fragment length polymorphism (RFLP). They also placed B12 gene on chromosome 14, but the closest linked RFLP marker was 11.4 cM. Later Rungis et al. (2002) tried to tag B12 gene with amplified fragment length polymorphism (AFLP) and SSR markers but failed to identify any closer markers (Xiao *et al.*2010).In breeding of resistance that is controlled by a single gene like the B12 gene for bacterial blight resistance, selection of resistant individuals is most desirable in early generations like F2 or BC1F1 if possible. Selection at such an early stage when population is still segregating is only possible by the application of markers tightly linked to the resistant gene (Xiao *et al.* 2010). A SNP haplotype that flanks the resistant gene greatly facilitates the selection of the resistance genotypes (Xiao *et al*. 2010).

The markers SSR/SNP have been reported as linked to bacterial blight resistance and may be utilized in resistance breeding programs. Three SSR markers, CIR 246, BNL 3545 and BNL 3644 on chromosome 14, were found closely linked to B_{12} gene (Xiao *et al*. 2010) which is a major gene contributing maximum resistance to almost all the races of *Xam*. Based on Monsanto SSR/single nucleotide polymorphism (SNP) consensus map, SNP markers closely linked to CIR 246 were used to screen the resistant and susceptible genotype for polymorphism (Xiao *et al*. 2010). The four SNP markers spanning 3.4 cM were found to flank the resistance gene B_{12} on chromosome 14 and the linkage between B12 and 4-SNP marker haplotype can be used for marker assisted selection for breeding programs (Xiao *et al*. 2010). The genetic makers for trait of interest can be identified through construction of linkage maps and QTL analysis. Instead of linkage mapping, short cut methods like Bulk segregant analysis (used for markers located in specific chromosomal regions) and selective genotyping (selecting individuals from a population that represent the phenotypic extremes or tails of the trait being analysed (both require mapping populations) can be used for gene tagging.

The validation of the available and reported markers available in public domain and identify the tightly linked markers and utilize them in breeding programs through marker assisted selection will be one of the best possible option to incorporate resistance trait to desirable cultivars which are susceptible to the disease.

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Biosafety Regulation in India

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Introduction

Biotechnology has been recognized as an important tool for improving agricultural production in a suitable and environmentally friendly manner. Genetically modified crop (popularly called as GM crop) is one of the agricultural technologies where the adoption rate is sky-scraping compared to other recently developed technologies. Dr. Norman Borlaug (2000) said that **"Transgenic plants have been hailed as a powerful way to increase agricultural productivity and to help alleviate hunger in underdeveloped nations"**, but also have raised concern over possible health, biosafety and environmental risks, pointing to the need for risk assessment and management (Haslberger, 2006). The inserted gene sequence, known as the *transgene*, may come from an unrelated plant, or from a completely different species, for instance, bacterial Bt gene in cotton plant (Bt cotton). One of the purposes of inserting a combination of genes in a plant is to make it as useful and productive such as higher yield, improved quality, pest or disease resistance, and tolerance to heat, cold and drought.

The first transgenic Bt cotton experience in India has been very positive and encouraging. However, for harnessing full potential of biotechnology (particularly GMOs), it is essential to understand and follow certain foremost regulatory measures and guidelines lay down by regulatory authorities of India. Thus, biosafety concerns on GMO at primary level (contained use) are imperative and essential for every laboratory involved in the GM crop development. Contained use is where control measures are used to limit contact between GMOs, humans and the environment so as to provide a high level of safety. In practice, this involves work in laboratories, animal houses, plant growth facilities (including growth rooms in buildings and suitable glasshouses) and large-scale production facilities on industrial sites.

The potential risks associated with GMOs have made it essential for governments and civil societies to address the issue of 'biosafety' in all major sectors of biotechnology: medical

/ pharmaceutical, agricultural, industrial and environmental. In the context of GM-crops, the concept of 'biosafety' is a broad one, covering three areas: the health safety of humans and livestock, the safety of the environment (i.e. ecology and biodiversity) and socio-economic safety (i.e. the economic and social impact on farmers, consumers and different social classes, as well as on trade and economy in general). The biosafety guidelines and regulations are made very cumbersome, stringent and time consuming in India. Although some literal compliance would be too difficult to achieve, one has to comply with all the stipulated guidelines, since prevention is better than cure. Further, concerted efforts are needed to educate and make people aware of the technology, which has already gained access. This lecture notes outlines the regulatory framework of India, various technical terms used on GM crop, the order of biosafety experiments to be carried out especially on GM crops before it released into the environment, biosafety experiments etc are dealt in details.

The Convention of Biological Diversity (CBD):

CBD is an international legally binding treaty, which was opened for signature at the Earth Summit (United Nations Conference on Environment and Development) in Rio de Janeiro, Brazil in 5th June 1992.

The objectives of the CBD are as follows:

1. the conservation of biological diversity,
2. the sustainable use of its components and
3. the fair and equitable sharing of the benefits arising out of the utilization of genetic resources".

When developing the Convention, the negotiators recognized that biotechnology can make a contribution towards achieving the objectives of the Convention, if developed and used with adequate safety measures for the environment and human health. The Contracting Parties agreed to consider the need to develop appropriate procedures to address the safe transfer, handling and use of any Living Modified Organism (LMO) resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity (Article 19.3 of the CBD). The development of biosafety protocols was the outcome of this process.

Cartagena Protocol on Biosafety:

The Cartagena Protocol on Biosafety is an international agreement on biosafety, as a supplement to the Convention on Biological Diversity. The Cartagena Protocol derives its name from a city in Colombia where the Biosafety Protocol was originally scheduled to be concluded and adopted in February 1999. The Protocol was finalized and adopted a year later on 29 January 2000 in Montreal, Canada by more than 130 countries. The full name of the Biosafety Protocol is "the Cartagena Protocol on Biosafety to the Convention on Biological Diversity" The protocol entered into force on September 11, 2003. As on date 99 countries have ratified the protocol. India has ratified the Cartagena Protocol on Biosafety on 23rd January 2003.

Regulatory Frame work in India:

Government of India has evolved regulatory mechanisms for their development and use of GMOs. A national biosafety framework to regulate production and release of genetically modified organism was established. The Indian Government had issued Rules and Regulation for handling GMOs and hazardous organisms through a Gazette Notification No. G.S.R. 1037 (E) dated December 5, 1989 from the Ministry of Environment and Forests, Government of India. The Rules cover the areas of research as well as large-scale applications of all kinds of GMOs and its products, which are controlled commodities for handling and use in the country under the Environment (Protection) Act (EPA). The rules also cover the application of hazardous microorganisms which may not be genetically modified. The Department of India (DBT) under Ministry of Science and Technology (MoST) along with Ministry of Environment and Forestry (MoEF) are implementing agency for biosafety regulation in India. They formed several committees to advise, facilitate & regulate modern biotechnology work at different stages to achieve the objectives of protecting environment including human and animal health from the possible adverse effects of GMOs and its products.

Recombinant DNA Advisory Committee (RDAC)

This committee constituted by the Department of Biotechnology. Takes note of developments in biotechnology at national and international levels. The RDAC recommendations include, from time to time, the technologies/processes suitable for implementation for upholding the safety regulations in research and applications of GMOs and its products. This Committee prepared the "Recombinant DNA Biosafety Guidelines in

1990", which was adopted by the Government for conducting research and handling of GMOs in India.

Institutional Biosafety Committee (IBSC)

It is necessary that each institution intending to carry out research activities involving genetic manipulation of microorganisms, plants or animals should constitute the IBSC. All the IBSCs, need to have one nominee from the DBT (Government agent). The IBSC is the nodal point for interaction within the institution for implementation of the guidelines.

Review Committee on Genetic Manipulation (RCGM)

The RCGM functions as a body under the Department of Biotechnology and has the functions like, to bring out manuals of guidelines are specifying producers for regulatory process on GMOs in research, use and applications including industry with a view to ensure environmental safety and to review all ongoing r-DNA projects involving high risk category and controlled field experiments.

Genetic Engineering Approval Committee (GEAC)

This committee is apex body functions under Ministry of Environment and Forestry. Functions are to adopt producers for restriction or prohibition, production, sale, import & use of GMOs both for research and applications under Environment (Protection) Act, 1986. This committee approves large-scale production and release of GMOs and products thereof into the environment. GEAC is authorized agencies or persons to have powers to take punitive actions under the under Environment (Protection) Act, 1986.

State Biotechnology Coordination Committee (SBCC)

This is state level committee, which is headed by the Chief Secretary of the State. They have powers to inspect, investigate and to take punitive action in case of violations of statutory provisions through the State Pollution Control Board or the Directorate of Health etc. To review periodically the safety and control measures in various institutions handling GMOs. This committee act as nodal agency at State level to assess the damage, if any, due to release of GMOs and to take on site control measures.

District Level Committee (DLC)

This Committee, constituted at the district level, is considered to be smallest authoritative unit to monitor the safety regulations in installations engaged in the use of GMOs in research and applications. The DLC is headed by the District Collector who can induct representatives from State agencies to enable smooth functioning and inspection of the installations with a view to ensure the implementation of safety guidelines while handling GMOs, under the Indian EPA.

Development and establishment of GM crop:

Past 3 decades our ability to alter life-forms has been revolutionized by modern biotechnology. We have learned to manipulate and transfer strands of DNA and entire genes, which contain the biochemical instructions governing economically important traits. Although more than 30 crop species have been genetically engineered, the technology for most species is inefficient and hampered by cultivar restrictions for *in vitro* regeneration. Plant tissue culture plays a pivotal role in the development of transgenic crop along with other molecular biological techniques. Some plants are amenable for somatic embryogenesis and some are not, thus one should have first a complete reproducible protocol for successful induction and regeneration of somatic embryos. Secondly, the selected gene is normally equipped with proper regulatory sequences to ensure production of the respective protein in certain organs and/ or under certain conditions. However, the transformation event is random, as a result the site of insertion in the genome, the transgene activity does not always meet our expectations and the site of insertion cannot be predicted and influenced in advance. Only it can be analyzed after transformation to exclude lines with any unwanted changes in essential or potentially harmful genes. Similarly, only lines with suitable transgene activity are selected for further application. Low transformation frequencies and ambiguities of the integration events, such as gene copy numbers and position effects, influence the levels of gene expression and regulation. Further much remains to be learned in terms of basic methods and procedures needed for efficient manipulation of plants *in vitro.*

Worth bearing in mind is that insertional effects are related to the specific transformation event. It would be extremely rare for a significant improvement in ecological fitness or substantial compositional changes to result (Petersen *et al*. 2005). Transgene activity is influenced not only by the number and sites of transgene insertion, but it differs also among genetically identical cells or plants (with transgene inserted in the same genome location) due to epigenetic changes randomly occurring in the inserted DNA. The introduced

gene can be silenced as late as one or more years after transformation. However, the expression and stability of a transgene, it has been a general practice in the laboratory level to discard T0 transgenic lines with multiple copies of T-DNA. Such practice limits the number of useful lines into further development. They demonstrated that clean single copied transgenic plants could be obtained from two T1 populations derived from T0 plants with multiple copies of T-DNA and vector backbone sequence. This validates the alternative approach to proceed with multiple copied T0 plants with desirable phenotypes and obtain clean single copy segregants in the next generation. Thus one should select properly the perfect transgenic event which fulfils the requirement of targeted traits and trounce the perceived problem in crop improvement.

After completion of molecular characterization the transgenic plants and events should be subjected to contained (controlled like Green house condition) trial after obtaining permission through Institute Biosafety Committee (IBSC) and informed to Review Committee on Genetic Manipulation (RCGM) New Delhi. Primarily the type of information requested by the regulatory authority would be related to the details of plant species, biological document, category of genetic manipulation, detailed molecular characterization of the inserted DNA, as well as host genomic flanking sequences, e.g. the nature and source of vector used, including maps of all functional elements, PCR primer positions and sequences, and a table of descriptions and functions, sources and construction, insertion site(s) and copy numbers of all inserted DNA (transgenes, regulatory sequences, vector backbone) etc.

Further, the information on gene constructs, map of vector molecules, border sequences the insert or T-DNA, regulatory sequences of transgene, marker gene such as promoter, terminator, plant selection marker gene, gene of interest, introns if any introduced etc. Further, molecular confirmation of transgene integration and its expression data should be documented and submitted for the support of events. (This was briefed in GM crop development topic).

Transgenic Event

Crop breeding programs are devised to accumulate alleles from within the gene pool and from cross-compatible wild relatives. But with advent of genetic engineering through recombinant DNA technology and spatial and temporal targeted expression of genes facilitated the transfer of precise gene sequences and the transfer of genes across gene pools.

Asexual techniques of this gene transfer helped us to engineer new transgenic event with useful gene isolated from any living organism. The point of foreign gene integration and successful expression in the new genetic background made the transgene is unique event and the introgressed position in the chromosome of host cell remains unchanged during segregation.

What is an event?

Event refers to the unique DNA recombination incident that took place in one plant cell transformed with foreign DNA, which was then used to generate entire transgenic plants. Every cell that successfully incorporates the gene of interest represents a unique "event". Each event will have different point of transgene integration and its positional effects in the host genome.

Event selection

Marker genes are used to identify transformed cells, and each resulting transgenic plant is the result of one event. The derived transgenic line is identified by an abbreviation. Although many different transgenic plants are made with the same gene construct, only a handful merits further use. Different events can have much different consequences. This depends on the number of times the gene construct was added to the cell's genome and may also have something to do with the placement of the new genes. The events that result in optimal transgene expression and traits are considered "elite" events. Every plant line derived from a transgenic event is considered a GMO. Its commercial use and release into the environment require authorisation according to biosafety regulations. Events can be introduced to other cultivars by breeding. This is why certain events are available in many different genotypes.

Contained trials should be conducted to screen and identify the best event. A number of events are generated during transformation. Forwarding all the transgenic events is difficult and laborious and requires large investments. Thus, one should select the best event satisfying all the set parameter during contained event selection trial and forwarded to BRL trials and biosafety experiments. An application should be submitted through IBSC to obtain permission from RCGM, New Delhi to conduct the event selection trial. Currently, India’s apex biotech regulatory body – the GEAC approved for commercial cultivation of a number of events of Bt

cotton so far namely MON531 event (*cry*1Ac gene), MON 15985 (*cry*1Ac and *cry*2Ab genes), GFM event (*cry*1Ab-*cry*1A), Event-1 (*cry*1Ac) and event 9124 (c*ry*1Ac).

Event-specific flanking sequence identification

The characterization of foreign gene (transgene) point of integration in the host genome and sequencing of flanking region of transgene is called event specific flanking sequence identification. The flanking sequence of an exogenous integrant in the transgenic plant should be characterized. In general, the 3′-integration junction between host plant DNA and integrated DNA of transgenic must be isolated using thermal asymmetric interlaced (TAIL)-PCR. The event-specific primers and TaqMan probe have to be designed based upon the isolated 3′-integration junction sequence, and qualitative and quantitative PCR systems may be established employing these designed primers and probe. Event-specific qualitative and quantitative PCR detection system based on the 3′-integration junction is reliable, sensitive and accurate.

Homozygosity test

Homozygous describes a genotype consisting of two identical alleles at a given locus. An organism is homozygous for a particular gene when identical alleles of the gene are present on both homologous chromosomes. Often one can perform a test to confirm the homozygosity of the gene of interest. An individual that is homozygous dominant for a particular trait carries two copies of the allele that codes for the dominant trait. When a transgenic line contains a T-DNA insertion at a single locus, the initial transformant plant T_0, is hemizygous at that locus. For example, R (transgene for resistance) present in hemizygous state in the T_0 generation, selfing of any individual will give progeny segregating 3:1 for the transgene. (The null allele is represented by "0").

To identify individuals homozygous for a transgene first, grow 10 - 20 plants until several true leaves are available, but well before flowering. Extract DNA from each individual, and test for presence of the transgene by PCR. Use primers specific for the transgene, not the vector. Discard null segregants, which do not give a band, and cover the positive plants flowers with bag during flowering to prevent cross-pollination. Collect seed from each plant and keep in separate envelopes. For 5 positive parents, grow at least 15 (to test for homozygosity) plants until several true leaves are available. Extract DNA from each

individual, and test for the presence of the transgene by PCR. (You may save some work by freezing leaf tissue for 5 sets of progeny, but only testing one individual at a time, until you get a true homozygote). If any line tests positive in all individuals, that line must be homozygous. If some individuals test negative, it may be because of bad DNA preps. It is sometimes worth re-doing the DNA preps and doing PCR on the new DNA. All seed from that parent for which no null segregants were seen should be homozygous, and it should be possible to propagate this line indefinitely with no loss of the transgene due to segregation (http://home.cc.umanitoba.ca).

Genome Position Effect:

One of the big challenges facing genetic engineers today is the regulation of transgene expression, with the position of integration of a transgene within a genome influencing its expression. This is known as the genome position effect. The insertion of multiple random copies of a transgene in the genome can effectively abolish its expression and the insertion of a transgene in or close to another gene can result in the production of an undesirable phenotype. Therefore, to ensure long term stable expression of a transgene post-transformation, the insertion of a single copy of a gene into a location in the genome where expression of the transgene is not adversely affected by the surrounding genomic sequences is desirable. One way of isolating the transgene from the potential deleterious effects of the surrounding plant genomic DNA is to include nuclear matrix attachment regions (MARs) as part of the chimeric binary construct. Pleiotropy is recognised as frequently being associated with the introduction of novel genes by hybridisation. This is likely to apply to the introduction of transgenes. The successful Bt and herbicide resistant genes act peripherally to host pathways thus pleiotropy is minimised. Where host metabolic pathways are altered by the transgene, pleiotropic effects might be predicted or transgenic modification may be restricted by compensation of the host metabolism due to attempts to maintain homeostasis.

Phenotype of the GM crop

The plant phenotype may be affected by the insertion or expression of transgene. These effects of should be examined and recorded. Some unexpected phenotype changes were observed in certain GM potato and tobacco lines, as well as in other plant species. These changes were traced to genetic or epigenetic effects connected with the site of foreign gene insertion or with “somaclonal and proclonal variations“ that are based on natural instability of plant genomes combined with an increase of mutability by the transformation/regeneration

process (Fischer *et al.* 2008). Plants are generally very flexible, having the possibility to meet certain targets through multiple routes (and usually also some detours). Thus, for instance, complete inactivation or over-activation of many (essential) plant genes has often no visual effect on the phenotype. On the other hand detailed analysis of transgenic potato plants exhibiting strong spontaneous tuberization proved that their phenotype was not related to inserted DNA designed to randomly activate genes at the site of insertion. The inserted DNA was localized in the non-coding region, which indicates that the phenotypic change was caused by mutation associated with either the transformation process or with somaclonal variation – transgenic plants are regenerated from differentiated somatic cells which can already harbour mutations or whose genetic information can be modified during the regeneration process (Fischer *et al.* 2008).

Target trait expression

In genetics gene expression is the most fundamental level at which genotype gives rise to the phenotype. In the development of transgenic program no matter what gene insertion method is used, a series of events must occur to allow a whole genetically modified plant to be recovered from a single genetically modified cell. The genes put into plants using genetic engineering can come from any organism.Thus, cell must incorporate the new DNA into its own chromosomes without rearrangement and the inserted gene must continue to work properly ("gene expression") in the regenerated plant. Most genes used in the genetic engineering of plants have come from bacteria. However, we learnt more about the genetic makeup of plants and genes are modified accordingly to express in the plant genome.

GM technology of targeted genetic modifications is not absolutely precise in respect of introduction of unintended changes to the genome of the target plant. However, the frequency of these changes is still much lower than that introduced by chemical or physical mutagenesis used in classical breeding. Considering that plant genomes are still very dynamic and full of mobile genetic elements, which can occasionally change their positions and insert into other places in the genome, current genotypes of crop plants have a “safe history of use” in naturally occurring processes of genetic modification. If natural or human-induced mutation of these plants had not resulted in a “dangerous crop”, we cannot expect that possibility to arise as a result of transgenic technology itself. Thus introduction of properly selected safe transgenes, together with reasonable testing of the final GM plant and products is a very efficient and reasonable way of targeted and quick improvements of plant features.

The general discussion should not always be directed towards the technology itself, but should instead address the proper selection of genes and applications for this technology.

Growth and development parameters

The selected transgenic lines should be subjected to growth and development parameters studies. There are number of parameters apart from DUS characters (Distinctness, Uniformity and Stability) are generally recorded as plant phenotypic and morphological characters such as plant growth habit (determinate /indeterminate), plant type (open/compact), pigmentation (on stem, leaf, petiole etc), leaf shape and appearance, flower and petal colour, pollen colour, time of flowering (50%) and other quality parameters or economically important traits (yield) should be documented and submitted to regulators or for registration under Protection of Plant Varieties and Farmers' Rights Act (PPV&FR).

Yield parameters

The ultimate goal of GM crop is to increase the yield and concurrently overcome the biotic and abiotic stress. The yield data should be recorded along with non-GM crop and percentage increase over may be calculated. Pest-resistant genetically modified crops can contribute to increased yields and agricultural growth in those situations, as the case of Bt cotton in India demonstrates. In India on average, conservative estimates for small farmers indicate that yield increased by 31%, insecticide application decreased by 39%, and profitability increased by 88% equivalent to US$250 per hectare (www.isaaa.org).

Biosafety experiments:

What is biosafety?

Biosafety is a term used to describe efforts to reduce and eliminate the potential risks resulting from biotechnology and its products. With specific reference to genetically modified organisms (GMOs), biosafety aims at "ensuring that the development and use of GMOs, and products derived from them, do not negatively affect plant, animal and human health, agricultural systems, or the environment". For the purposes of the Biosafety Protocol, this is based on the precautionary approach, whereby the lack of full scientific certainty should not be used as an excuse to postpone action when there is a threat of serious or irreversible damage. While developed countries that are at the center of the global biotechnology industry

have established domestic biosafety regimes, many developing countries are only now starting to establish their own national systems (http://www.cbd.int/biosafety/).

Contained and Confined trial

Contained and confined trials are refers to research experiment to test the GM crops. Contained conditions experiments are carried out with genetically modified organisms within contained facilities, such as a laboratory, a greenhouse, a net house, and areas used for the storage and handling of experimental GE organisms. Under contained conditions there is a physical barrier or barriers that contain material under research and development so there is virtually no direct contact of viable GE organisms with the environment. Activities carried out within such contained facilities are generally performed subject to specific biosafety guidelines and under specified levels of containment as detailed in Guidelines for Research in Transgenic Plants, 1998, wherein three different categories of containment levels have been defined for genetic engineering experiments on plants. These guidelines are primarily based on guidelines on containment issued by OECD (igmoris.nic.in).

Confined field trials are field experiments carried out to evaluate the performance of genetically modified (GM) plants. They are an essential step for technology assessment and development. Confined field trials are carried out under stringent terms and conditions that confine the experimental material. They are similar to field experiments done for conventional breeding, but plant material and genes are confined to a limited area. However "confinement" of a field trial also refers to reproductive isolation, but depending on circumstances, may also include some degree of physical isolation. The purpose of confined field experiment is to test GM plants under real field conditions and to test the value of the trait in local environment. The trials are also used to breed biotech traits into local varieties and to enable selection of the best lines and eventual scaling-up of production material, prior to regulatory approval for commercialisation. Confined field trials also serve to generate safety data needed for subsequent risk assessment and approval.

A single confined field trial may be comprised of one or more events of a single plant species that are subject to the same terms and conditions of confinement which include, but are not limited to, reproductive isolation, site monitoring, and post-harvest land use restrictions. It is understood that the experimental plant/species/varieties/hybrids grown in confined trials are those that have yet to receive regulatory approval for environmental release

from GEAC. This confinement is also to be understood in terms of confinement of a particular GE plant in a particular region, state, village or a research farm of the applicant and is not accessible by other parts of the country in environmental terms.

Approval of confined field trials refers to three important considerations. Firstly, confined field trials are typically carried out on a small scale, usually to a maximum of one hectare (ha). There may be exceptions to this *e.g.*, the cultivation of larger areas so that sufficient plant material may be harvested for livestock feeding trials. Secondly, a confined trial is an experimental activity conducted to collect data on potential biosafety impacts. The collection of such field trial data is a prerequisite for safety assessment of the GE crop under evaluation. Additionally, field trials are carried out to produce sufficient plant material so that the developer can undertake research to address the information and data requirements for livestock feed and human food safety assessments. Finally, the trial is conducted under conditions known to mitigate pollen- or seed-mediated dissemination of the experimental plant, persistence of the GE plant or its progeny in the environment, and introduction of the GE plant or plant products into the human food or livestock feed pathways (igmoris.nic.in*).*

Environmental study

The transgenic crops have been subjected to biosafety regulations, so that any potential risks may be adequately assessed. Once the GM crop is tested under contained condition, the next step is to go for biosafety experiments with confined conditions after obtaining permission from RCGM. The biosafety experiments can be grouped broadly into three categories: 1. Environmental safety studies, 2. Toxicity and allergenicity studies and 3. Livestock feeding study. Under the environment safety study pollen flow or gene flow, soil microflora and fauna and effect on non-target organism experiments should be completed. All biosafety experiments targeted to test the safety measures of genetically modified organism and to find out any adverse effect on environment, human health and biological diversity.

Pollen flow study

The possibility that genes introduced by genetic engineering may “escape” (be transferred via pollen) to wild or weedy related species growing nearby is often cited as one of the major risks of GMOs. Gene flow between crops and the wild species, from which they were derived however, is a well documented natural phenomenon. Over the course of evolution, familiar crop species – wheat, potatoes, corn, canola, and numerous others – were

modified from their original form because of hybridization with related species or weedy or cultivated strains growing nearby. Through this long established mechanism for gene transfer, any gene in a cultivated crop or plant, irrespective of how it got there, can be transferred to its wild or semi-domesticated relatives. Thus experiments should be conducted to find out the outcross with non-GM crop and wild relatives.

Different experimental lay outs for cross pollination studies (e.g. Bt cotton)

- Central pollen source surrounded by border receptors
- Source on the borders and the receptor at the centre
- Combination of above with receptors on the corners and larger source plot in the centre

All experiments present clear evidence that there is reduction in Pollen Gene Flow (PGF) rate as one goes inside the receptor plot. Highest % cross pollination always occurred in plants near the pollen source, drastically reducing as distance increases, apparently in an exponential way.

The consequence of gene flow will depend on the crop, the transgene, the trait encoded the particular environment and risk management practices. Case by case study is required for a complete risk analysis. Combination of factors such as differences in experimental design, environmental conditions and changing pollinator populations affect the results. Crops of little or no external trade: more amenable to engineering and in extensively traded crops, transgenic forms might not be useful.

Soil micro flora and fauna

An imperative aspect of the biosafety assessment of genetically modified plant is to study its impact on soil ecosystem including changes in plant associated microflora. The report of the FAO on environmental effects of GM crops recommended that environmental effects of *Bt* crops or any transgene proteins should be assessed on a case-by-case basis, including their potential impact on local soil microflora and biodiversity. Microbes are in close contact with all three soil phases (Solid, water and air); they can sensitively and rapidly probe responses to soil perturbations. With respect to the impact of GM plants on the soil ecosystem, the key consideration is whether the effect(s) actually matter(s) in terms of the overall functioning of the ecosystem. The soil macro fauna community studies are tested with

GM and non-GM field condition. The earthworm biomass and decomposer communities and degradation speed of plant residues should be studied with new event of transgenics. The study of the macro fauna community, including both the litter layer and the superficial soil layers, allows identifying the plant species/management combinations which favor the increase of the diversity of the invertebrates.

Laboratory studies (Venkateswerlu and Stotzky 1992) have shown that insecticidal Cry proteins from *B. thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* are readily adsorbed at equilibrium and bound to clay minerals and humic acids, and that their insecticidal activity is maintained or enhanced in the soil-toxin complexes. Based on those laboratory findings, these researchers hypothesized that incorporation of Bt proteins into soil from repeated large-scale use of transgenic crop plants could exceed the rate of natural degradation and inactivation, thereby leading to an accumulation of the protein in the soil that could reach biologically active levels. However, this hypothesis has been evaluated under field conditions case-by-case of all transgene before it release into the environment. Till date no studies have reported that the levels of Bt protein in soils of agricultural field where transgenic Bt crops have been repeatedly planted and residues of the crops incorporated into the soil. Head *et al*. (2002) used both insect bioassays and enzyme-linked immunosorbent assays (ELISA) to evaluate levels of Cry1Ac protein in soils where transgenic Bt cotton producing Cry1Ac protein had been continuously grown, and subsequently incorporated into soil by postharvest tillage, for 3 to 6 yr.

Non-target organism

There are some concerns regarding the safety of new proteins expressed in transgenic plants. Even low-level expression of a new transgene potentially may have an unintended, deleterious effect on other organisms including birds, insects, browsing animals, and soil organisms in the local environment. This is particularly the case when the protein has no prior history of being found in plants, or is not found at the levels expected in the GMO. Proteins intended to control specifically targeted pests may be harmful to non-target species. In terms of plant-produced insecticides, the only insecticidal compounds that currently are commercialized are the toxin proteins naturally produced by *Bacillus thuringiensis* (Bt) These proteins are highly specific in their toxic effects. One group of these proteins affects only certain species of caterpillars whereas others affect only a restricted set of beetles. None of these proteins has been shown to have a significant disruptive effect on predators of pest species or beneficial insects. The toxicity issue (and any potential risk issue) can sometimes

be inflated to alarming proportions. Studies conducted during the multi-location field trials with Bt revealed that the Bt cotton hybrids do not have any toxic effects on the non -target species, such as sucking pests (aphids, jassids, white fly and mites). The population of secondary lepidopteron pests, namely tobacco caterpillar remained negligible during the study period in both Bt and non Bt cotton plants. The beneficial insects (lady beetle, spiders) remained active in both Bt and non Bt varieties.

Toxicity and Allergenicity

The toxicity and allergenicity testing of transgenic crops must be tested before forwarding to BRL trials.

i) Acute oral toxicity test should be undertaken with purified protein and sub-chronic 90 day toxicity should be undertaken with whole plant material, along with daily intake food/feed.
ii) Allergenicity testing should comprise a battery of tests including amino acid sequence homology using bioinformatics tools from the allergen databases, pepsin digestibility and protein thermal stability. These tests should be mandatory and the use of other tests *viz*. serum testing (specific/targeted) and use of animal models should be recommended on a case by case basis, based on the source of the gene/protein and the results of the above three mandatory tests.

Acute Oral Safety Limit Study in Rats or Mice

Acute toxicology studies are often conducted via oral exposure because that is the most likely route of exposure to the transgenic protein, and mice are used because less test substance is required. Oral exposure is usually accomplished by gastric gavage, wherein a tube is inserted through the oral cavity and the esophagus of the test animal and the test substance is injected directly into the stomach. An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects. However, some studies using other routes of exposure, such as intraperitoneal or intravenous administration, have been conducted. Depending on the results from the Tier I assessment, additional toxicology studies and hypothesis-based testing could be considered on a case-by-case basis.

In general, proteins exhibit toxicity wield their effect at low dosages and in a short time frame, acute toxicity tests have been considered adequate for evaluating potential toxicity (EPA, 2000, NRC, 2000). If toxicity testing of a protein is considered necessary then acute exposure studies in laboratory animals should be sufficient, since, if toxic proteins are known to act via acute mechanisms. Therefore, when a protein demonstrates no acute oral toxicity in high-dose testing using a standard laboratory mammalian test species, this supports the determination that the protein will be nontoxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposure (Sjoblad *et al*. 1992). In the evaluation of the toxic characteristics of a protein, the determination of oral toxicity is routinely carried out by acute testing. Such studies provide information on the possible health hazards likely to arise from dietary exposure to a novel protein. This method comprises the basic single dose toxicity study that is commonly used for proteins for which low toxicity is expected due to prior knowledge of the source and previous exposure. The duration of post exposure observation is 14 days. Lack of mortality, moribundity or evident toxicity is generally interpreted as a lack of oral toxicity associated with the test substance (DBT, 2008).

Subchronic Feeding Study in Rodents

Foods are complex mixtures of compounds characterized by wide variations in composition and nutritional value. Due to their bulk and effect on satiety, they can usually be fed to animals only at low multiples of the amounts that might be present in the human diet. The risks associated with whole foods and detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can be extremely difficult. However, there may be circumstances where chronic or subchronic whole food feeding studies might contribute to an assessment of potential toxicity. Ninety days animal feeding studies are recommended with transgenic crops in rodents to evaluate the safety of whole food. The 90-day whole food feeding study is not intended to assess the potential toxicity of the protein expression product(s) of the inserted gene(s) as this is accomplished via the 14-day acute oral toxicity study in rodents.

The rationale of subchronic assessment and evaluation of potential toxicity associated with a whole food derived from a GE plant. The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time

covering post-weaning maturation and growth well into adulthood. The study will provide information on the major toxic effects, including possible target organs, and the possibility of cumulative effects. These data may also be useful in assessing whether there have been any unintended effects as a result of the genetic modification process. The need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. This study should allow for the assessment of potential to cause neurotoxic, immunological or reproductive organ effects, which may warrant further in-depth investigation (*www*.igmoris.nic.in*)*.

Protein Thermal Stability

Proteins are charged biomolecules that can fold into compact structures that are very sensitive to solution conditions. As an extreme case, thermal denaturation can lead to irreversible loss of structure and function of the molecule. All newly expressed proteins in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of the source of the introduced protein, significant similarity between the amino acid sequence of the protein and that of known allergens and its structural properties, including its susceptibility to enzymatic degradation and heat stability. Resistance to heat denaturation has been observed in several food allergens; thus a correlation exists between heat stability and allergenic potential. Therefore, the retention of biological activity after incubation under high temperature conditions may indicate that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic (igmoris.nic.in*)*.

Protein thermal stability study should be conducted to measure the thermolability of recombinant protein when exposed to heat. Purified protein samples are dissolved in a buffer and incubated at a range of temperatures from 25°C to 95°C for up to 30 minutes. This is followed rapid cooling of the sample and assays of the biological activity of the protein are performed. Proteins which show less than 10% of the non-treated activity after 30 minutes incubation are considered heat labile at that temperature, although there needs to be consideration of the relevance of the particular temperature to human exposure, for example, whether the food is processed or cooked before consumption (www.igmoris.nic.in).

Pepsin Digestibility Assay

Proteins introduced into GM crops are compared to endogenous and safe dietary proteins. One safety assessment method is to evaluate the potential digestibility of introduced proteins *in vitro* using simulated gastric and intestinal fluids, because safe dietary proteins must be digestible to provide a dietary source of amino acids. Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential (Astwood *et al*., 1996). The resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis has to be conducted to determine the likelihood of the newly expressed protein being allergenic. A consistent and well-validated pepsin degradation protocol may enhance the utility of this method and is strongly recommended. However, it is recognized that other enzyme susceptibility protocols also exist and these may be used with adequate justification.

Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in many laboratories (Thomas et al., 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but inactive at pH 3.5 and irreversibly denatured at pH 7.0. The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. The original assay recommends performing the digestion at pH 1.2, however, the FAO/WHO (2001) recommends using two pH conditions. The assay is performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and sodium dodecyl sulfate (SDS-) polyacrylamide gel electrophoresis (PAGE) loading buffer, then heating to more than 70°C for 3 to 5 minutes. The timed digestion samples are separated by SDS-PAGE and stained with coomassie or colloidal blue to evaluate the extent of digestion. A review of the digestibility assay5 indicates that most of the non-allergenic food proteins that have been tested are digested by approximately 30 seconds, while major food allergens are stable, or produce pepsin-stable fragments that are detectable for from eight to 60 minutes (www.igmoris.nic.in).

Livestock Feeding Study

To feed the growing population, modern methods of biotechnology must be used to produce crops that supply feed for livestock and food for humans. We must be sure that these

and future products produced using modern techniques of biotechnology are safe for both livestock and humans if they are to be eaten now and in the future. Beever and Kemp (2000) concluded that there is no significant risk associated with the consumption of Bt DNA or the resulting proteins from GM crops that are registered in any of the countries. Feeding of Bt corn grain and corn silage (Folmer *et al*., 2001) based diets also did not have any adverse effect on milk production performance of dairy cows. Singh *et al*., (2003) concluded that feeding of transgenic (Cry I Ac) and non-transgenic cottonseed based rations to the lactating Murrah buffaloes did not have any adverse effects on their feed intake and health status as assessed from haemato-biochemical constituents. Based on the safety analyses required for each crop, consumption of milk, meat and eggs produced from animals fed GM crops should be considered to be as safe as traditional practices.

Animal feeding studies should be conducted with rats, broiler chickens, catfish, goat and dairy cows as part of a safety assessment program GM crops. These studies are designed to compare the feeding value of genetically engineered plants with non-GM crop. The aim of livestock feeding trials in the safety assessment of a food derived from a recombinant DNA plant is primarily to evaluate the nutritional parameters (*e.g.*, wholesomeness and nutrient bioavailability) of the food and/or feed under relevant circumstances. Compositional analysis is the basis of the nutritional assessment of a food derived from a GM plant. Once the compositional equivalence between the GE food and its conventional counterpart has been established, the results of numerous published livestock feeding trials with GE varieties of maize, soybean, canola, cotton, or sugarbeet, also confirmed no significant differences in digestibility of nutrients, animal health or animal performance (Flachowsky *et al*., 2005). Therefore, once compositional and phenotypic equivalence has been established, nutritional equivalence may be assumed, and livestock feeding trials are conducted only to confirm the same.

The following test protocols are set by regulatory authorities to test the novel proteins expressed in recombinant DNA plants and foods derived from these plants. The GM crop product should be mixed with animal food and feed with animal system like milking cows or buffalos, goat, broiler chicken, fish and rats and assess the safety level and test data generated must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under Rules, 1989, of the Environmental Protection Act, 1986.

Broiler chicken

To assess comparative nutrient utilization and carcass quality in broiler chickens feed diets containing transgene protein, for instance, solvent extracted cottonseed meal (CSM) processed from Bt and parental non-Bt line cottonseeds. These experiments can be carried out with this institute by submitting a small project with fund to meet the expenditure especially consumables and manpower. Ideally, the animal species should be one which: (1) can be used in relatively large numbers to increase the statistical power of the study; (2) can be obtained as a nearly genetically uniform population in order to negate any effects of genetic background; and (3) is sensitive to the effect of small changes in nutritional quality on its growth and performance. One example of an animal species that meets these requirements is the broiler chicken, which has emerged as a useful animal model for assessing the nutritional value of foods and feeds derived from GE cotton, maize or soybean crops. Under typical conditions, a broiler chicken has a daily consumption of *ca*. 60 g maize kernel/kg body weight, compared to 45 g/kg body weight for a growing pig and *ca*. 0.2 g/kg body weight for an adult human. Hence, the broiler chicken model offers the advantage of significantly higher exposure over nearly the complete life span of the animal. Fast growing species such as the broiler chick increase their body weight approximately 45-fold during the approximately 40 days they take to reach market weight. Because of this rapid weight gain, broilers are particularly sensitive to any change in nutrient supply or the presence of toxic elements in their feed (OECD, 2003). Broilers have advantages over many other species used in commercial production, as they tend to provide a genetically homogeneous population and can be used in relatively large numbers to increase the statistical power of the experiment.

This study conducted to determine the nutritional equivalency of cottonseed meals derived from Bt transgenic or any GM crops, related non-transgenic isoline cotton and two sources of commercial cotton following 42 days of dietary exposure to broiler chickens. Individual animals, or all the animals in an experimental unit (*e.g.*, a pen if it is a poultry feeding study), should be weighed at several pre-determined times throughout the course of the study, including at the study termination. Measurements of feed consumption should be made on the same schedule as collecting body weight data.

Goat

GM crop seeds / leaf should be tested on ruminants (goat) for their any toxic effects. A 90 days feeding study on goats should be conducted. The methods, species of animals and the

route of administration described in this protocol are based up on the standard OECD guidelines No. 408 (1993). This procedure deals with handling, maintaining and other procedures to be followed while dealing with feeding studies with goats. The availability of standard genetically defined goats and dietary and husbandry conditions also make goats ideal in the Indian context and safety data on this ruminant model will be appropriate. In order to maintain even distribution, the goats will be provided a number, based on random selection. The test material will be administered in the diet. This route of administration was selected because it represents the most likely route of exposure of goat species in their natural habitat. The GM plant materials such as grain, forage or meal can be used to test the effect on the ruminants compared to non-GM seeds.

A group of 12 goats (6 males and 6 females) normally assigned to each (test and control) group. Each group is fed for 90 days and observed. The animals will undergo an acclimation for a period of not less than 15 days prior to the actual studies. The test material normally crushed and mixed with the feed. Feed consisting of wheat bran, gram, salt, minerals, cotton seeds or any GM crop test seeds and grass would form the daily diet of the goats. Analysis would be initiated during this period itself *viz.*, feed consumption, weight gain etc. This would facilitate accurate statistical analysis. All the test and control animals observed daily for morbidity, mortality and clinical signs. The general health of all the animals monitored daily and relevant records maintained. Any adverse observation would be documented. Animals found moribund or dead during the study period will be necropsied to the extent necessary to determine the probable cause. Animals were assessed for gross pathology and histopathology. Hematological observations such as total RBC and WBC count, differential leucocytic count, haemoglobin concentration, clotting time, ESR immunoglobulin profile etc and clinical biochemistry such as total serum protein, glucose, blood urea, nitrogen, histamine, alkaline phosphatise etc would be studied. All the animals are sacrificed on day 91 and internal organs viz., adrenals, heart, liver, gonads (testes and ovaries), brain, kidneys, spleen would be compared.

Milking cow

The biosafety experiments of GM crops on milking cows or buffalos should be conducted. The main aim of the experiment is to test the effect of GM crops feed intake, change in body weight of cows during experimental period, production and composition of milk in dairy cows, milk production performance etc assessed. Generally, all animals used in

a study should be healthy, free of parasites, and have a similar genetic history and 28-days experiment are conducted with milking cows. First the non-GM crop seeds are feed with all the test animals and brought to adaptation then divided into two groups, one group feed with GM crop plant product (*e.g.*, grain, forage, meal etc) another group with Non-GM crop feed. Body weight of cows should be recorded before staring the experimental feeding as well as at the end of trial period and assess the increase or decrease of body weight of each cow. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health.

Measurements of body weight and feed consumption are taken periodically and data should be collected. The chemical composition of GM and non-GM seeds should be identical or quite close to the specifications given by Bureau of Indian Standards (BIS) for Type I cattle feed. Daily milk yield of cows during adaptation period as well as during experimental period need to be recorded and compared. Milk production efficiency in terms of milk yield/ kg dry matter intake in Non GM and GM seeds must be recorded. The composition of milk (*e.g.*, fat, protein, and lactose) samples drawn periodically in both groups are analysed and compared. Transgene protein in the milk, somatic cell counts in milk as well in blood plasma of cows fed with GM crop should be tested. The significance and likely impacts of any abnormal findings should be reported. Where there are statistically significant differences in parameters between test and control groups, these should be discussed in terms of their biological significance and impact on animal performance and health.

Insect bioassay

The eventual target of insect resistant transgenic GM crop is to control the target pest. Insect bioassays studies are the direct method to assess the efficacy of GM crop on the target insect system. For instance, insect infestation on transgenic Bt and non-Bt cotton varieties. The laboratory experiments need to be conducted with fully opened young leaves, squares etc., with neonate larvae (Perlak *et al.,* 1990). Most of the studies conducted with released Bt cotton event in India showed that transgenic Bt cotton was highly toxic to *Helicoverpa armigera* causing 100% neonate larval mortality. They also demonstrated that the Cry 1Ac protein found to be excellent delta endotoxin which gives complete protection against the major pest of cotton *H. armigera* across the season (Olsen and Daly, 2000).

LOD/LOQ

To comply with international labeling regulations for genetically modified (GM) crops and food, and to enable proper identification of GM organisms (GMOs), effective methodologies and reliable approaches are needed. The spurious and unapproved GM planting has contributed to crop failures and commercial losses. To ensure effective and genuine GM cultivation concurrently evaluates the structural and functional stability of the transgene insert. A multiple polymerase chain reaction (PCR) approach is used for detection, identification, and gene stability confirmation. Limits of Detection (LOD) are the concentration of the least amount of analyte that can be reliably detected. For DNA-based detection methods, the LOD is dependent on genome size and the number of transgene inserts per genome, The LOD test is affected by the sampling size, type of matrix present, extraction efficiency, and sensitivity of the test. To determine whether a matrix alters either the LOD, a comparison can be made between an analyte extracted from a feed sample with a known content of GM plant and a standard obtained separately from the sample. The lowest concentration or amount of analyte that can be identified, measured, and reported. For instance one GM crop seed DNA in 10,000 non- GM crop DNA, is 0.1% LOD, which can be detected as the lowest quantity of the target that can be quantified with a probability of 95% (Berdal and Holst-Jensen, 2001). The limit of detection cannot be common to all crop species since the LOD test is dependent on genome size and the number of transgene inserts per genome. Genome size is fixed for each species and determines the number of genomes present in a fixed amount of DNA sample being used per PCR test. Further the LOD test is affected by the sampling size, type of matrix present, extraction efficiency, and sensitivity of the test. All these factors are influenced by the biological characteristics of each crop.

Amino acid Sequence homology

Amino acid sequence similarity using bioinformatics and genetic databases can help to identify structural and functional relationships between the introduced protein and potential allergens or toxins. Some cases few amino acid or peptide sequences act as allergens and some peptide are combining with other biochemical molecules and be active as allergens and all these can be studied by using a structural consensus approach. Sequence searches can also establish the degree of relatedness of the introduced proteins to proteins already present in food and feed with a long history of safe use and consumption.

Biosafety Research Level – I (BRL-I) Trial

The revised guidelines for biosafety experiments and application form can be retrieved from the website www.dbtbiosafety.nic.in and or www.igmoris.nic.in. Currently, the field trials are categorized into two types: Biosafety Research Level I (BRL-I) and Biosafety Research Level II (BRL-II) trials. RCGM, functioning in the DBT, is the regulatory authority for BRL-I trials, can be conducted maximum of 1 acre (0.4 ha) per trial site location and a cumulative total of 20 acres (8.1 ha) for all locations for each plant species/construct combination (e.g., one or more events originating from transformation of a plant species with the same genetic construct), per Applicant, per crop season.

Trial site Map

RCGM or GEAC approved BRL I or II Trial site map should be prepared with details and submitted along with application. In the event this latter requirement is not met, RCGM/GEAC reserves the right to cancel the authorization and require termination of the confined field trial. The provision of draft maps at the time of application is recommended as this will facilitate the assessment of conduct of trials. The field trial site should be labelled and a map of the trial site will be prepared by the Trial In-Charge and appended to the Record of Planting. Maps of confined field trials must be noted clearly with precise information. Maps must provide details on the layout of the site and distances between the field trial site and surrounding features like names of the land owners/farmers, any specific marks/features etc Maps should also depict the legal or descriptive land location, compass direction, date of sowing, physical landmark, border or surrounding crops, isolation distance etc. Maps must provide sufficient detail to allow regulatory officials/monitoring agencies to locate each field trial site during the planting season and any required period of post-harvest land use restriction. The dimensions of the trial site and distances to physical landmarks must be accurately reported. For more detailed information log on to www.igmoris.nic.in

Trial in-charge

The experiment on BRL trials at different centre are conducted by the technical person assigned by the GM crop developer as responsible for management of the field trial, ensuring compliance with the terms and conditions of a confined field trial authorization and providing information as on required by Regulatory Authorities. The details of conduct person especially name, designation, organization, fax and telephone or mobile number email id

should be submitted to the regulators. The Trial In-Charge must, at a minimum, be an agriculture graduate.

Biosafety Research Level – II (BRL-II) Trial

GEAC, functioning in the MoEF, is the Regulatory Authority for Biosafety Research Level II trials, the trials are conducted to limited in size and not more than 2.5 acres (1 ha) per trial site location and to no more than eight locations within India for each plant species/construct combination (e.g., one or more events originating from transformation of a plant species with the same genetic construct), per Applicant, per crop season. Biosafety Research Level II trials: The application form must be completed using the proforma provided in the website (www.dbtindia.nic.in and www.igmoris.nic.in) and submitted by regular mail. For environment release of new event of GM crop one should carry out the following trials as per the sequence specified by the Biosafety authority. After event selection trial, one should complete confined field trials at the level of Biosafety Research Level I for a crop season and followed by second crop season of confined field trials at the level of BRL-I or BRL-II and finally a third crop season of confined field trials at the level of BRL-II and the it will be approved for released into the environment.

Records and Reporting:

Compliance Records

As per the RCGM and GEAC guidelines records of all confined field trials, including pre- and post-harvest site monitoring, activities related to trial site compliance, cleaning of equipment, transportation, disposition and storage of all surplus and harvested seed and plant material, shall be maintained by the GM crop developer (RCGM/GEAC permitted party) and shall be made available to regulatory authorities or the designated monitoring agencies upon request. Mandatory recording formats are referenced in the RCGM/GEAC Standard Operating Procedures (SOPs) for Confined Field Trials of Genetically Engineered Cotton: Transport, Storage, Management, Harvest or Termination and Post Harvest Management and can be downloaded from http://igmoris.nic.in/.

Field Trial Report

After completion of confined field trial the Permitted Party should submit a comprehensive field trial report to RCGM/GEAC within 3 months. The field trial report must

summarize the completed trial, including methods, observations recorded and analysis of any effects of the trial plants on other plants, non-target organisms, or the environment. These data would be critically reviewed by regulatory authorities and informed accordingly for further advancement of GM crop (www.dbtbiosafety.nic.in) or environment release if completes all the biosafety requirements.

Mandatory Information Submissions by Applicant

Planting Information Submission:

The anticipated date of sowing and harvesting must be submitted to RCGM/GEAC, however the exact date can also informed later within a week time after sowing and labeled the trial site. A Record of Planting shall be submitted and must reference the confined trial permit number, document the amount of material planted, the planting date, the transportation of plant material to the trial site, the cleaning of any equipment used during planting, and the disposition of any surplus plant material remaining after planting. If it was not provided with the application, this notification must also include a detailed map of the trial site (www. www.dbtbiosafety.nic.in).

Harvest Information Submission:

After completion of each confined field trial site a record of harvest (or termination) shall be prepared by the permitted party and shall document the date and method of harvest, the amount of harvested material, the disposition of any harvested materials, the cleaning of any equipment used during harvest, and the method of destruction of any residual plant material on the trial site. This record must be verified and signed by a member of the Monitoring Agency or any nominee of RCGM / GEAC / SBCC / DLC / SAU authorized by RCGM / GEAC during the conduct of a field trial site inspection during harvest, or within 15 days of the completion of harvest (www.dbtbiosafety.nic.in).

Accidental Release Information:

The applicant should inform to RCGM / GEAC, New Delhi immediately if any mishap or upon discovery by telephone but positively within 24 hours in writing (submission to be received by RCGM/GEAC within 24 hours by facsimile, e-mail or other means) of any incident involving an accidental or unauthorized escape like spillage, theft, encroachment by

unauthorized persons, vandalization etc. of regulated GE plant material during transportation, storage within a contained facility, or during any other activity associated with the conduct of a confined field trial. For the purposes of these Guidelines, any breach of the authorized terms and conditions of reproductive isolation shall be considered an accidental release and subject to risk assessment and management if any at the cost of the applicant (www. www.dbtbiosafety.nic.in).

Cost of Biosafety experiments

Biosafety experiments on GM crops are an endeavor to prevent any accident that could be harmful to people, animals, plants and environment. India is one of the first Asian countries to invest in agricultural biotechnology research and to set up a biosafety system to regulate the approval of genetically modified crops. Despite the Government of India's acknowledged interest in encouraging growth in the biotechnology sector and the increasing number of research initiatives in the public and private domains, the approval of new applications of transgenic crops has been rather slow. The country has only approved one GM crop, Bt (*Bacillus thuringiensis*) cotton, which was planted on 10.5 million hectares in 2012. There are several other GM crops and traits in the biosafety regulatory pipeline including herbicide resistant cotton and Bt Brinjal.

Most countries have or are developing a system to regulate transgenic crops in response to concerns of environmentalists and consumers about the crops' safety and pressures from the biotechnology companies and government scientists who want regulations that assure people of their products' safety and value. Huge cost is involved for commercial release of an event (of transgenic crop) after completion of all the above mentioned biosafety experiments. Approximately, rupees 100 to 150 lakhs are required to release an event in India. However the regulatory authorities suggested that the biosafety issues are concerned with safety of environment and human and not concerned with cost, so the norms are followed for all the transgenic events to make sure that the GM is safe to human, health and environmental.

Summary and Conclusions

The prerequisite for successful transformation is regeneration protocol from single somatic cell. Secondly, the gene of interest (economically important), gene construct (border, promoter, selector gene) should be effective during transformation process. A number event would be generated during transformation and all the event should be subjected to first event

selection trial. However the best only one event would be forwarded to further Biosafety Research trails. Environmental safety studies, toxicity and allergenicity studies and food/feed studies must be completed for environmental release of transgenics. In India so far 6 Bt cotton events were released and all the transgenic crop is a thoroughly researched biotech product that has undergone all the tests pertaining to bio-safety and agronomic traits as prescribed by the concerned regulatory authorities. During these eight years in India, no untoward incident has occurred with regard to bio-safety or pest resistance. The proper application of GM strategies provides means for improving the quality of life and protecting the environment. The major benefits from Bt-cotton include effective control of bollworms leading to significant yield increase, drastic reduction in chemical sprays and substantial increase in net profit to farmers. Efforts are being made to sustain these social, economical and environmental benefits. Criticism based only speculation has no credibility. It should be backed by scientific evidence. If anyone has any genuine issue or better suggestions for safety assessment, these should be addressed to the concerned regulatory authorities for necessary action. One should follow the biosafety rules and regulation, respect nation and worship Motherland.

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Risk assessment of growing transgenic cotton on soil biological attributes

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Biological indicators of soil quality

Soil health has been defined as the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality and promote plant and animal health (Doran et al. 1996). Sustainability of agricultural systems has become an important issue all over the world. Many issues of sustainability are related to soil quality and its change with time (Karlen *et al.* 1997). There is a surge of interest in soil quality assessments through measurement of various physical, chemical and biological attributes that are sensitive to soil management. Assessing land use-induced changes in soil properties is essential for addressing the issue of agro-ecosystem transformation and sustainable land productivity. Soil quality cannot be measured directly, so we evaluate indicators. Soil biological parameters have been suggested as important indicators of soil quality (Dick, 1994). It has been proposed that the microbiological and biochemical status of a soil can be used as an early and sensitive indicator of soil ecological stress or restoration processes in both natural and agro-ecosystems (Bolton et al. 1985; Ruf et al. 2003). Soil quality indicators are useful to policy makers to monitor the long-term effects of farm management practices on soil quality; assess the economic impact of alternative management practices designed to improve soil quality by including not only environmental values but also taking into account economic and social factors. Some of the key indicators of soil biological quality are presented below:

***Soil respiration*:** Soil respiration is a useful index of the overall biological activity in soil and is a critical determinant of ecosystem carbon storage. It reflects the intensity of the soil organic matter decomposition and mineralization and the incidence of the microorganisms in soil, and it is often used for the biomass determination.

***Fluorescein diacetate hydrolysis*:** The fluorescein diacetate (FDA) hydrolysis assay measures the enzyme activity of microbial populations and can provide an estimate of overall microbial activity in an environmental sample. The assay is considered non-specific because it is sensitive to the activity of several enzyme classes including lipases, esterases, and proteases.

***Soil microbial biomass*:** Soil microbial biomass is a living pool containing 1-5% of the soil organic matter. Microbial biomass determinations indicate changes in the soil organic matter before they can be detected by measuring total soil carbon making possible its use as an indicator of early changes in soil organic matter content. Microbial biomass consists of both dormant and metabolically active organisms and has been considered as an integrative indicator of microbial significance of soils.

***Soil urease*:** Urease plays an important role in the efficient use of urea fertilizer in soils and the changes in urease activity can be used as an indirect indicator of the variation in the pool of potentially available N in a soil. While, low urease activity might cause added urea to be lost by leaching; on the other hand, a higher activity might result in excessive hydrolysis of added urea and subsequently ammonia can be lost by volatilization.

***Soil dehydrogenase*:** The dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils. This enzyme is considered to exist as an integral part of intact cells but does not accumulate extracellularly in the soil. Dehydrogenase enzyme is often used as a measure of any disruption caused by pesticides, trace elements or management practices to the soil.

***Phosphatases*:** Phosphatases are a broad group of enzymes that are capable of catalysing hydrolysis of esters and anhydrides of phosphoric acid. In soil ecosystems, these enzymes are believed to play critical roles in P cycles.

***ß glucosidases*:** This enzyme plays an important role in soils because it is involved in catalysing the hydrolysis and biodegradation of various ß-glucosides present in plant debris decomposing in the ecosystem. Its final product is glucose, an important C energy source of life to microbes in the soil.

***Arylsuphatases*:** They are responsible for the hydrolysis of sulphate esters in the soil and are secreted by bacteria into the external environment as a response to sulphur limitation.

***Soil microorganisms*:** Microorganisms have double role in relation to soil fertility. On one hand, the microbes are the agents that mineralise and liberate plant nutrients from the organic material. On the other hand, the microorganisms can also be viewed as a collective observer of the soil environment. Since the microbes are in close contact with all three soil phases (Solid, water and air), they can sensitively and rapidly probe responses to soil perturbations.

Mechanisms by which transgenic plants affect soil microorganisms

There are a variety of considerations as to how transgenic plants may affect soil microorganisms that include both direct and indirect effects of the plant that has been modified. Direct effects will depend on the spectrum of activity of the transgene proteins (Oger et al. 1997) and the quantities of the protein that accumulate in the environment. In contrast, indirect effects are mediated by changes in plant protein and root exudates composition that arise as a result of modifying the metabolic pathways in the plant tissues. The direct effects are of the most concern since the introduction of transgene proteins for pest and disease resistance can involve the production of chemical substances that are potentially toxic to non-target soil organisms, including mycorrhizal fungi and soil microfauna that are involved in organic matter decomposition. Indirect effects caused by changes in root exudates are also possible consequences of transgenic plants, but are much more difficult to evaluate since so many factors may affect root exudates composition and microbial community structures in soil.

Effects of growing transgenic cotton on soil biological properties

Development of insect resistant genetically modified crops has been postulated as a promising method to minimize the use of toxic chemical pesticides in agriculture with sustained or improved crop yield (Romeis et al. 2006). On the other hand, it is also reported that growing of transgenic crops may induce adverse effect on soil ecology (Wolfenbarger and Phifer, 2000; O'Callaghan et al. 2005; Griffiths et al. 2006) and the soil biochemical properties (Fang et al. 2005). However, till date the use of genetically modified crops has remained nascent largely owing to the lack of systematic information about ecological consequences associated with their release in the natural ecosystems (Manda et al. 2006). This included the potential impact of transgenic crops on soil microbiology (Saxena and Stotzky, 2001; Gupta and Watson, 2004).

Transgenic cotton, *Gossypium hirsutum*, expressing the insecticidal *Cry1Ac* gene, from the soil bacterium *Bacillus thuringiensis* (*Bt*), was introduced commercially in India in 2002 (Morse et al. 2005), and presently *Bt* Cotton occupies more than 90% of cultivated area in India, which covers an area of approximately 11 million ha representing about one quarter of the global area of 33 million ha under cotton (Singh and Ahlawat, 2011). *Bt* cotton offers resistance to an important pest, the American bollworm (*Helicoverpa amigera*), that has developed resistance to all the commonly used insecticides in the country (Kranthi and Kranthi, 2004). *Bt* cottons not only give higher yields, but also higher net income over traditional cottons due to reduced plant protection costs (Venugopalan et al. 2009).

While there are various benefits of *Bt* cotton, in recent past, there has been wide concern about growing transgenic cotton as *Bt* toxin produced in leaves, stems and roots of *Bt* cotton plants is introduced in soil which might affect general soil health. Several workers have studied the effects of transgene products and transgenic cotton on the soil biological properties (Table 1 to 4). Any undue disturbances in soil nutrient status, enzymatic activities, and microbial diversity are of significance and concern since these factors are essential for maintenance of ecological stability and productivity of the environment.

Table 1. Persistence of Cry proteins in soil: Studies from Bt cotton

Protein	Location	Experimental variable	Persistence of protein in soil	References
Cry 1Ab Cry1Ac Cry3Aa	Lab	Soil amended with biomass of *Bt* cotton	No persistence of proteins in soil; proteins degraded in soil with a half-life of 20 days	Ream et al. (1994)
Cry 1Ab Cry1Ac	Lab	Soil amended with purified protein or biomass of *Bt* cotton	Purified proteins and Cry proteins from cotton tissue decreased rapidly, with a half-life of approximately 4 and 7 days, respectively, by ELISA	Palm et al. (1996)
Cry 1Ab Cry1Ac	Lab	Soil amended with purified protein or biomass of *Bt* cotton	Purified protein was detected up to 28 days and the protein from *Bt* cotton was detected up to 56 days	Donegan et al. (1995)
Cry 2A	Lab Field	Soil amended with biomass of *Bt* cotton *Bt* cotton cultivation	Half-life of bioactivity was estimated at 15.5 days by insect assay Half-life of bioactivity was estimated at 31.7 days by insect assay	Sims and Ream (1997)
Cry 1Ac	Field	*Bt* cotton cultivation	No detectable level of protein in soil for 3-6 consecutive years	Head et al. (2002)

Table 2. Effects of Cry proteins from *Bacillus thuringiensis* on soil-dwelling invertebrates: Studies from Bt cotton

Organism	Species	Location	Experimental variable	Protein	Effect	References
Earthworm	*Eisenia fetida*	Lab	Transgenic cotton	Cry1Ac	No adverse effect on earthworms	Valasubramanian (2001)
Collembola	*Folsomia candida*	Field	Cultivation of Bt and non-Bt cotton	Cry1Ab Cry1Ac	No effects on numbers	USEPA (2001)
Collembola Mites	*Folsomia Candida* *Oppia nitens*	Lab	Fed leaves of Bt and non-Bt cotton	Cry1Ab Cry1Ac	No significant effects on oviposition, numbers of eggs, and body length	Yu et al. (1997)
Micro, meso and macro fauna	Nematodes, Collembola and ants	Field	Cultivation of Bt and non-Bt cotton	Cry1Ac	micro, meso and macro fauna were more in Bt cotton rhizosphere as compared to non Bt cotton rhizosphere	Usha et al. (2011)

Table 3. Effects of Cry proteins from *Bacillus thuringiensis* on the microbial diversity: Studies from Bt cotton

Microorganisms	Experimental variable	Protein	Effect	References
Culturable bacteria and fungi	Soil with Bt and non-Bt cotton	Cry1Ac	A significant, but transient, increase in numbers in soil with Bt cotton	Donegan et al. (1995)
Composition of soil microbiota	Rhizosphere soils of Bt cotton versus herbicide-tolerance (Roundup Readys) cotton	Cry1Ac	Significantly different microflora in soil with Bt cotton residues than in soil with herbicide-tolerant cotton	Gupta and Watson (2004)
Composition of soil microbiota	Rhizosphere soils of Bt and non-Bt cotton	Cry1Ac	More extensive fungal colonization, higher ratios of fungi to bacteria, and different types of fungal spores in soil with Bt cotton than non-Bt cotton	Gupta and Watson (2004), Gupta et al. (2002)
Culturable functional bacteria (potassium and inorganic phosphate dissolving bacteria, and nitrogen-fixing bacteria)	Rhizosphere soils of Bt and non-Bt cotton	Cry1Ac	Higher culturable functional bacteria in soil of non-Bt cotton than in soil of Bt cotton in early and middle growth stages; no significant differences in numbers after the growing season	Rui et al. (2005)
Microbial population	Rhizosphere soils of Bt and non-Bt cotton	Cry1Ac	No adverse effect on soil microbial populations	Valasubramanian (2001)
Functional diversity of microbial communities	Soil with Bt and non-Bt cotton	Cry1Ac	No adverse effects of Bt cotton on soil ecosystem	Shen et al. (2006)
Diversity of plant-associated methylobacteria	Phyllosphere, rhizoplane, stem internal tissues of Bt-cotton & non-Bt cotton	Cry1Ac	No adverse effects of Bt cotton on diversity of plant-associated methylobacteria	Balachandar et al. (2008)
Culturable functional bacteria (nitrogen-fixing, organic, P dissolving, inorganic P Dissolving)	Multiple-year (0–5 years) cultivation of Bt and non-Bt cotton	Cry1A & CpTI	No adverse effects of Bt cotton on functional bacterial populations in rhizosphere soil	Hu et al. (2009)
Culturable and non-culturable microbial diversities	Soil with Bt and non-Bt cotton	Cry1Ac	Bt cotton did not adversely affect the diversity of the microbial communities	Kapur et al. (2010)
Culturable microbial population and diversity	Soil with Bt and non-Bt cotton	Cry1Ac	Higher microbial population and diversity in soil grown with Bt cotton than the non-Bt cotton	Velmourougane and Sahu (2013)

Table 4. Effects of *Bt* cotton on microbe-mediated process and functions in soil

Process/Function	Experimental variable	Protein	Effect	References
Ureases, alkaline Phosphatases, dehydrogenases, phenol oxidases	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	No differences in the activities of enzymes between soil with *Bt* and near-isogenic non-*Bt* cotton	Shen et al. (2006)
Ureases, acid phosphomonoesterases, invertases, cellulases and arylsulfatases	Soil amended with *Bt* and non-*Bt* cotton biomass	Cry1Ac	The addition of biomass of Bt cotton to soil stimulated the activities of all enzymes, except that of arylsulfatase, which was inhibited	Sun et al. (2007)
Dehydrogenase activity	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	Significant reduction in dehydrogenase activity in the rhizosphere of Bt-cotton over non-Bt isoline.	Sarkar et al. (2008)
CO_2 evolution	Soil amended with biomass of various *Bt* and non-*Bt* plants	Cry1Ab Cry1Ac Cry3A	Lower evolution of CO_2 from soils amended with biomass of *B*t cotton, than with biomass of near-isogenic non-*B*t counterparts	Flores et al. (2005)
Soil respiration	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	Significant reduction in soil respiration in the rhizosphere of Bt-cotton over non-Bt isoline.	Sarkar et al. (2008)
N mineralization and Olsen-P	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	Total mineral-N was reduced in Bt cotton, whereas Olsen-P was increased	Sarkar et al. (2008)
Root biomass	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	Root biomass were not different but root volume was significantly higher in Bt than non-Bt isoline	Sarkar et al. (2008)
Microbial biomass C, N and P, organic carbon, microbial quotient, Potential N mineralization, nitrification, nitrate reductase, and acid and alkaline phosphatase activities, root volume	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	There were some positive or no negative effects of *Bt*-cotton on the studied indicators.	Sarkar et al. (2009)
Urease activity, nitrate reductase, acid and alkaline phosphatase	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	No significant difference in alkaline phosphatase, nitrate reductase and urease activity between Bt and non- Bt cotton rhizosphere	Usha et al. (2011)
Dehydrogenase activity and $KMnO_4$-N content	Soil with *Bt* and non-*Bt* cotton	Cry1Ac	Positive correlations between *Bt* cotton cultivation and $KMnO_4$-N content and dehydrogenase activity in soil	Singh and Ahlawat (2013)
Soil respiration, fluorescein diacetate activity, urease, dehydrogenase, microbial biomass carbon	Soil with Bt and non-Bt cotton	Cry1Ac	Higher biological activities in soil grown with Bt cotton than the non-Bt cotton	Velmourougane and Sahu (2013)

Conclusion:

In general, Cry proteins released in root exudates and from plant residues of Bt cotton appear to have no consistent, significant, and long-term effects on the soil biological properties. Some differences in total numbers and community structure of microorganisms in soil between Bt and non-Bt crops have been indicated. However, many of these observations were not statistically significant, were transient, were not related to the inserted transgene, or were the result of altered plant characteristics. Although Cry proteins bind rapidly on clays and humic substances, there is little evidence for the accumulation of the proteins in soils in the field, even after years of continuous cultivation of Bt cotton. However, a few studies have reported significant differences in microbial community structure in soils with Bt and non-Bt crops using various classical and molecular techniques.

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PART –II

TRAINING MANUAL

Preparation of Stock Solutions

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Solution: Solution is a homogenous mixture of two or more substances in which the molecules or ions of one substance are dispersed throughout the medium of other substance. The substance, which is dispersed, is called solute and the substance in which solute particles are dispersed is called solvent. In other words solution is a homogenous mixture of a solute in a solvent

The concentration of a solution can be expressed as

- Per cent solution
- Molar solution
- Molal solution
- Normal solution
- Parts per million (ppm)
- Parts per billion (ppb)

Per cent solution:

A per cent solution is one, which contains unit weight or volume of salt or liquid in 100 ml of water. Per cent solution can be prepared in two ways:

Weight/volume basis (1 g/100 ml=1%)
Volume /volume basis (1 ml in 99 ml = 1%)

Molar solution:

A molar solution can be prepared by dissolving any substance corresponding to one molecular weight (1 mole) in water (solvent) so as to obtain a final volume of exactly one litre 20^0 C. This is termed as volume molar or molar solution (M).

Molal solution:

Molal solution can be prepared by dissolving gram molecular weight of any substance in 1000 g of water (solvent). This is termed as weight molar or molal solution (m). The final volume of this solution, unlike molar solution, will naturally be a little more than one litre. This increase in volume is known as the solution volume of the solute.

Normal solution:

A normal solution is one, which contains a gram equivalent weight of a substance in one litre of solution. This can be obtained by dissolving gram equivalent weight of any substance in water so as to make the final volume to 1litre. This is termed as normal solution or equivalent solution.

Equivalent weight = Molecular weight/ Valency

Parts per million solutions (ppm):

When one part of a substance is dissolved in one million part of the solvent i.e. if 1 mg of a substance is dissolved in 10^6 mg of water, the resulting solution will be 1 ppm. In other words mg substance per litre of solvent water is the simplest expression of ppm solution. This system is based on the fact that a litre of water weigh 1000 g or (10^6 mg) at 4^0C.

Parts per million billion (ppb):

When one part of a substance is dissolved in one billion part of the solvent i.e one part per 1,000,000,000 parts, one part in 10^9, 1/1,000,000,000 * 100% = 0.0000001% (or 1% = 10,000,000 ppb). In other words 1µg substance per litre of solvent water is the simplest expression of ppb solution.

Preparation of solutions:

Examples

1. NaCl (5%) and Ethyl alcohol (20%)

Weight 5 g of NaCl and dissolve in 100 ml of distilled water. This gives 5% solution of NaCl w/v basis. A 20% aqeous solution of ethyl alcohol (v/v basis) is prepared by mixing 20ml of

ethyl alcohol with 80 ml of water. Solution of liquids is designated as per cent solution (v/v) basis.

2. 1M NaCl

Weigh 58.44 g of NaCl and dissolve in a little volume of distilled water and then make up the final volume to exactly one litre. This gives 1M solution of NaCl. Solution of different molarity viz., 0.01M, 0.02 M, 0.04 M and 0.08M from 1M stock solution can be prepared by using the following formula:

$V_1 \times C_1 = V_2 \times C_2$

Where

V_1	Volume of the stock solution
C_1	Concentration of stock solution
V_2	Volume of the desired solution
C_2	Concentration of the desired solution

3. 1 Molal (m) NaCl

Weigh 58.44 g of NaCl and dissolve in 1000 g of distilled water. This will give 1 molal solution of NaCl.

4. 500 ppm NaCl

Weigh 500 mg of NaCl and dissolve in a little volume of distilled water and makeup the final volume to exactly 1 litre.

Different concentrations of ppm solution viz., 10, 50 100 and 200 ppm from 500 ppm stock solution can be prepared by using the same formula $V_1C_1 = V_2C_2$ mentioned for the dilution of molar solution.

1N H_2SO_4

Determine the equivalent weight of the acid (98/2 = 49 for sulphuric acid). Now calculate 1N solution of H_2SO_4.

Specific gravity = 1.84 g/ ml; Purity – 98%

Normality = Equivalent weight x 100 / Specific gravity x % of purity

1N= 49 x 100 / 1.84 x 98 =27.17

27.17 ml of given H_2SO_4 in 972.83 ml of water gives 1N H_2SO_4.

The final solutions of desired normality can now be prepared by simple dilutions as per the following equation:

$V_1N_1 = V_2N_2$

Where

V_1	Volume of the stock solution
N_1	Normality of stock solution
V_2	Volume of the desired solution
N_2	Normality of the desired solution

Simple Dilution:

A simple dilution is one in which a unit volume of a desired liquid is mixed with appropriate volume of a solvent liquid to achieve the desired concentration. The dilution factor is the total number of unit volumes in which the material will be dissolved. The diluted material should be thoroughly mixed to achieve the true dilution. A 1:10 dilution denotes combining 1 unit volume of the material to be diluted + 9 unit volumes of the solvent medium (1+9 =10 = dilution factor)

Example 1. To dilute a Folin reagent 1:10

Mix 1 unit reagent of folin reagent with 9 volumes of water

Example 2. In some cases, solutions are expressed in terms of "X", an indicator of relative solute concentration. A 2X solution of sodium phosphate buffer can be prepared from 20X stock solution by mixing two volumes of 20 X stock with 18 volumes of water.

Primer Dilution:

The dry oligos/primers should be dissolved in TE buffer (10 mM Tris, pH 8.0, 1mM EDTA) rather than water.

1. Preparation of 100 µM stock

   µl TE needed = 10X nanomoles of oligo (as given in tube/spec sheet)

   For e.g. 500 µl TE + 50 nmoles oligo- 100 µM stock

2. Preparation of working concentration

   Dilute the stock 1: 10 in water – 10 µM oligo

   0.4µl of 10µM oligo in 20 µl of reaction volume = 0.2 µM

Exercise:

1. 1M of Tris Buffer (Mol.wt.121.4)
2. 0.5 M EDTA (Mol.wt. 372.24)
3. 1% PVP
4. 2N HCl (Mol.wt= 36.46)
5. 150 ppm kanamycin

Plant Genomic DNA isolation

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Isolation of plant DNA:

Nucleic acids (DNA and RNA) are vital micro-molecules in all living cells. DNA contains the basic genetic information of an organism. In prokaryotic cells, genetic activity occurs throughout the cell while in eukaryotic cells it lies in the discrete particles within the cells. Most of the DNA of eukaryotes exists in the nuclei and remaining DNA in the partially self-duplicating mitochondrial and chloroplast particles. The nuclear DNA combines with histone proteins in an orderly manner to form chromatin. Extraction of DNA is done by number of methods. The efficiency and recovery of extraction depends on the sample material, ionic conditions of the extraction medium and type of lysing agent used, etc.

Extraction of DNA is accomplished by the rupturing of cell wall and nuclear membrane followed by deproteinization and precipitation of nucleic acid using ethanol. A number of methods are available for the extraction of plant DNA depending upon the starting tissue, homogenization conditions, etc. in the present investigation we are following the isolation method developed by Paterson *et al.*,(1994) .

Protocol used for isolation of DNA from Cotton

Materials

- Sample material (Leaf or Seed from cotton)
- Extraction buffer (pH 7.8)

➢ 100mM Tris-HCl -1.576 g (Used to maintain a stable pH). 10mM EDTA Na_2 - 0.372 g (EDTA binds and chelates divalent cations such as calcium and magnesium thus helps to protect DNA from DNases. . Na^+ an ionic bond with the negatively charged phosphates on the DNA, neutralizing the negative charges and allowing the DNA molecules to come together)

500mM NaCl - 2.992 g

- Distilled Water - 100 ml

- Suspension Buffer (pH-8)
  - 50mM Tris-HCl - 0.788 g
  - 10mM EDTA Na_2 – 0.372g
  - Distilled Water - 100 ml
- 7.5M Ammonium acetate -57.81g in 100ml (precipitates the protein)
- 20% SDS (Sodium Dodecyl Sulphate solubilizes the membrane fragments)
- 20% PVP (Poly Vinyl Pyrrolidine forms hydrogen bonds with the phenolic compounds)
- 80% Ethanol(precipitates the nucleic acid)
- TE Buffer (pH-7.5)
  - 10mM Tris-HCl - 0.157 g
  - 1mM EDTA Na_2 - 0.37 g
  - Distilled Water - 100 ml

Method:

- Weigh 0.1 g of plant tissue, quickly freeze in liquid nitrogen (it freeze the tissue to become fragile to be a fine powder which increase the surface area of extraction, and the very low temperature prevent DNase activation) and grind to fine powder in a pestle mortar.
- Add 2 ml of extraction buffer in small aliquots and grind thoroughly.
- Add 1 ml of suspension buffer and make the suspension.
- Transfer the homogenate to a centrifuge tube and add 250 µl of 20% SDS and 20% PVP. Mix the contents properly.
- Keep the tube in water bath at 65˚C for 30min with inversion at every 10min. Take out the tube and add 3ml of 7.5M ammonium acetate and keep in ice for 1hr.
- Centrifuge the contents at 15000rpm at 4˚C for 15min.
- To the aqueous layer add $6/10^{th}$ volume of isopropyl alcohol and keep at -20˚C overnight for complete precipitation of DNA.
- Pellet the DNA by spinning at 15000rpm for 15min. dissolve the pellet in 25µl of TE buffer add 1.5µl RNase for 15 min at 37˚C.
- Pellet the DNA by centrifugation at 1500rpm for 15min. if DNA is not pure, go for phenol:chloroform and isoamyl alcohol(25:24:1) mixture. Proteins are efficiently denatured

by phenol, RNase activity is not completely inhibited. Therefore, a small amount of isoamyl alcohol is added to further ensure the deactivation of RNase activity. Mix properly by inverting the tube 4 to 5 times.

- Centrifuge and pull out the aqueous layer in a fresh tube leaving the interphase.
- Add equal volume of ice-cold ethanol and pellet the DNA by centrifugation at 15000rpm for 15min (repeat this step at least 2 times).
- Drain off the ethanol and invert the tubes on tissue paper towel for 1min. allow the pellet to air dry, take care that not to dry the pellet to much.
- Dissolve the pellet in suitable volume (30µl) or TE buffer.

Quantification of DNA

The isolated DNA was quantified in spectrophotometer at 260 nm. Concentration of 50µg/ml shows absorbance of 1.0 at 260nm. The 260/280 ratio should be 1.8-2.0 for good quality DNA.

Introduction to Cloning vectors and Plasmid DNA Isolation

J. Amudha

Senior Scientist (Biotechnology)
Central Institute for Cotton Research, Nagpur

Cloning vectors are DNA molecules that are used to "transport" cloned sequences between biological hosts.

CLONING VECTORS SHARE FOUR COMMON PROPERTIES:

1. Ability to promote autonomous replication.
2. Contain a genetic marker (usually dominant) for selection.
3. Unique restriction sites to facilitate cloning of insert DNA.
4. Minimum amount of nonessential DNA to optimize cloning.

TYPES OF CLONING VECTORS

Different types of cloning vectors are used for different types of cloning experiments. The vector is chosen according to the size and type of DNA to be cloned.

Plasmid: Plasmid vectors are used to clone DNA ranging in size from several base pairs to several thousands of base pairs (100bp -10kb).

ColE1 based, pUC vehicles, Commercially available ones, eg pGEM3, pBlueScript .

Phagemids (virus): Phage lambda is a bacteriophage or phage, i.e. bacterial virus that uses *E. coli* as host. Its structure is that of a typical phage: head, tail, tail fibres. Lambda viral genome: 48.5 kb linear DNA with a 12 base ssDNA "sticky end" at both ends; these ends are complementary in sequence and can hybridize to each other (this is the cos site: cohesive ends). Lambda tail fibres adsorb to a cell surface receptor, the tail contracts, and the DNA is injected. The DNA circularizes at the cos site, and lambda begins its life cycle in the *E. coli* host.

Cosmid: Clone large inserts of DNA of size ~ 45 kb.Cosmids are Plasmids with one or two Lambda Cos sites. Presence of the Cos site permits *in vitro* packaging of cosmid DNA into Lambda particles. Thus, have some advantages of Lambda as Cloning Vehicle.Strong

selection for cloning of large inserts.Infection process rather than transformation for entry of chimeric DNA into *E. coli* host. Maintain Cosmids as phage particles in solution.But Cosmids are Plasmids thus do not form plaques but rather cloning proceeds via *E. coli* colony formation.

Yeast Artificial Chromosome (YAC): YAC are special linear DNA vectors that resemble normal yeast chromosome. YAC contain telomers that stabilize chromosome ends, centromere, that ensures chromosome partitioning between two daughter cells and a selective marker gene. This cloning vehicle propogate in eukaryotic cell hosts as eukaryotic Chromosomes. Clone very large inserts of DNA: 100 kb - 10 Mb.YAC cloning vehicles are plasmids. Final chimeric DNA is a linear DNA molecule with telomeric ends.

Expression vectors: This vector allows a cloned segment of DNA to be translated into protein inside a bacterial or eukaryotic cell. Vectors contain the following features.*(a) in vivo* promoter(b) Selection marker (c) Multiple cloning site (d) Protein purification tag. It produces large amounts of a specific protein. This vector permits studies of the structure and function of proteins.

Introduction

Procedures for a large-scale isolation and purification of plasmid DNA are elaborate, time consuming and require ultra centrifugation. However, there shall be occasions when one has to analyze a large number of samples for the presence of plasmid and also does not need either to isolate large number quantity of plasmid DNA or of high purity. There are several procedures that describe the isolation of small amounts of partially purified plasmid DNA from large number of bacterial clones in a relatively short period of time. One such 'miniscreen' procedure is described below. This method does not require a large input of cells and can be carried out in small Eppendorf tubes.

Principle:

The isolation procedure is based upon the release of soluble high MW DNA from disrupted cell wall and membranes, dissociation of nucleoprotein complexes by denaturation and proteolysis and separation of DNA from other macromolecules.

Materials:

1		**Solution A (pH 8.0)**
	25mM Tris-HCl	30.3mg
	50mM Glucose	90mg
	Lysozyme	100mg (Freshly added before use)
	Water	10ml
		Solution B
2	0.2M NaOH	0.8g
	1.0% SDS	1.0g
	Water	100ml
		Solution C
3	3M Sodium acetate (pH4.8)	24.6g
	Water	100ml
4		**Solution D**
	50mM Tris-HCl (pH.8.0)	0.60g
	100mM Sodium acetate	8.20g
	Water	100ml

Procedure:

Transfer 1ml of an overnight cell culture (*E. coli* carrying plasmid pBR328 or any other plasmid) into an Eppendorf tube. Sediment the cells by centrifuging briefly in the microfuge. Drain off excess liquid.

1. Resuspend the cells in 100µl of solution A. Incubate on ice for 30min.
2. Add 200µl of freshly prepared solution B. Vortex briefly and keep on ice for 5min. The cells will lyse immediately and the solution will become viscous.
3. Add 150µl of solution C. Vortex briefly and keep on ice for 60min (15-30 min). The bulk of chromosomal DNA and cell material will precipitate into white viscous clump.
4. Remove this by centrifuging in the microfuge for 5min.
5. Transfer 500µl of the cleared lysate to a clean eppendorf tube. Add 1ml of ethanol, mix thoroughly, and cool in a freezer (-70^{o} C) to precipitate DNA. Sediment the DNA by spinning for 5min.
6. Discard the supernatant from the DNA pellet and dissolve the precipitate in 100µl of

solution of D. Add 200µl of ethanol mix and store in the freezer for 10min. Pellet the DNA by centrifuging for 2 min.

7. Repeat step 6 twice more. Remove residual ethanol from final DNA pellet by drying under vacuum. Dissolve the pellet in a suitable volume of TE buffer for further analysis.
8. Test the plasmid DNA preparation by agarose gel electrophoresis.

Checking Quality and Quantity of isolated DNA:

Checking DNA yield with Spectrophotometer

Procedure:

- Take 8.0µl of DNA with 632µl of sterile distilled water (total 640µl) and read at 260nm and 280nm in a Spectrophotometer.
- Use 260nm reading for calculation.
- In shortcut rout multiply the 260nm readings with 4 for miniprep (2 for macroprep*).

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Agarose Gel Electrophoresis

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Preparation of Agarose Gel

For checking the quality of DNA, 0.8% agarose was used. For electrophoresis of PCR amplified DNA products 1.5% agarose gel was used.

Materials:

10X Tris-Borate EDTA (TBE) buffer (pH 8.0)

Tris buffer -108.0 g
Boric acid - 55.0 g
0.5M EDTA (pH 8.0)- 2.925 g
Distilled water-1000 ml

The solution was prepared and autoclaved

Gel Loading Dye

Sucrose - 66.7 mg
Bromophenol blue - 4.2 mg
Distilled water - 1.0 ml

- Gel electrophoresis unit and power pack (Bangalore Genei Pvt. Ltd.).

Method

- The ends of the gel casting plate were sealed with cello-tape and kept on perfectly horizontal leveled platform.
- Agarose was melted in 1X TBE buffer and allowed to cool lukewarm temperature and then ethidium bromide (10 mg/ml) was added at final concentration of 1 µl per 25 ml of agarose gel.

- The agarose was poured into the gel casting plate with the comb fixed on one end of plate and the gel was allowed to solidify.
- After solidification of the gel, the comb was carefully removed from the gel plate without disturbing the wells.
- The gel cast was now placed on the electrophoresis unit and submerged with 1X TBE buffer.
- The DNA samples were mixed with the gel loading dye and carefully loaded into the wells. A suitable DNA ladder was also loaded.
- The cathode and anode were connected to the power pack and the current was adjusted to 80V.
- The negatively charged DNA molecules moved towards the anode and they separated according to their molecular weight. The power was turned off when the tracking dye reached at about 2 cm from the anode end.

Viewing the Gel

After disconnecting the power supply, the gel was transferred on UV-transilluminator and viewed. DNA in the gel appeared pinkish-orange owing to fluorescence of bound ethidium bromide (intercalating chemical). The gel was photographed and was used for further interpretation of band patterns.

Standardization of Polymerase Chain Reaction (PCR) condition

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Components of the PCR Reaction Mixture:

- TemplateDNA
- Primers
- Mg^{2+} Concentration
- dNTPs
- Enzyme (Thermostabile DNA Polymerases)

➢ **Template DNA:** Optimal amounts of template DNA in the 50 µl reaction volume are in the 0.01-10 ng range for both plasmid and phage DNA, and in the 0.1-1 µg range for genomic DNA. Higher amounts of template increase the risk of generation of nonspecific PCR products. Lower amounts of template reduce the accuracy of the amplification.

Note: Trace amounts of certain agents used for DNA purification, such as phenol, EDTA and proteinase K, can inhibit thermostable DNA polymerases. Ethanol precipitation and repeated washes of the DNA pellet with 70% ethanol normally remove trace contaminants from DNA sample

➢ **Primers:** The recommended concentration range of primers is 0.1-1 µM. Too high primer concentrations increase the probability of mispriming and thereby appearance of nonspecific PCR products. Therefore start optimization from standard concentrations and increase if necessary

➢ **Mg^{2+} Concentration:** Mg^{2+} in general stabilizes primer-template complexes. PCR buffers for *Taq* DNA Polymerase are supplemented with Mg^{2+}. Due to the binding of Mg^{2+} to dNTPs, primers and DNA templates, Mg^{2+} concentration needs to be optimized for maximal PCR yield. The recommended concentration range is 1-4 mM.

Recommended Mg^{2+} concentration:

Taq DNA Polymerase is supplied with two buffers: *Taq* buffer with KCl and *Taq* buffer with $(NH_4)_2SO_4$. Therefore for standard PCR with *Taq*DNA Polymerase and 0.2 mM dNTPs the recommended $MgCl_2$ concentrations are in general lower 1.5±0.25 mM when using *Taq* buffer with KCl compared to 2.0±0.5 mM when using *Taq* buffer with $(NH_4)_2SO_4$. Due to antagonistic effects of NH_4^+ and Mg^{2+}, *Taq* buffer with $(NH_4)_2SO_4$ offers higher primer specificity in a broad range of magnesium concentrations at variety of annealing temperatures. Volumes of 25 mM $MgCl_2$ or 25 mM $MgSO_4$ solutions required to reach a specific concentration of magnesium ions in the 50 µl reaction volume

If the Mg^{2+} concentration is too low, the yield of PCR product could be reduced. On the contrary, non-specific PCR products may appear and the PCR fidelity may be reduced if the Mg^{2+} concentration is too high. If DNA samples contain EDTA or other metal chelators, the Mg^{2+} ion concentration in the PCR mixture should be increased accordingly (1 molecule of EDTA binds 1 molecule of Mg^{2+}(1))

Final concentration, mM	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$ or $MgSO_4$, µl	2	2.5	3	3.5	4	5	6	8

➢ **dNTPs:** The recommended concentration of each dNTP is 0.2 mM. In certain PCR applications higher dNTP concentrations are required. Due to the binding of Mg^{2+} to dNTPs, Mg^{2+}concentration needs to be adjusted accordingly. It is essential to have equal concentrations of all four nucleotides (dATP, dCTP, dGTP and dTTP). If the nucleotide concentrations are not balanced, the PCR error rate may dramatically increase. The concentrations of all four dNTPs are perfectly balanced to provide fidelity and to increase the yield of PCR products. To achieve 0.2 mM concentration of each dNTP in the PCR mixture, use the following volumes of dNTP Mixes

Volume of PCR mixture	**dNTP Mix, 10 mM each**
50 µl	1 µl
25 µl	0.5 µl
20 µl	0.4 µl

➢ **Thermo stabile DNA Polymerases : *Taq* DNA Polymerase.** *Taq* DNA polymerase is the most commonly used enzyme for PCR. It is suitable for most amplifcation reactions that do not require high fidelity enzyme or PCR products longer than 3 kb. Normally, 1-1.5 u of *Taq* DNA Polymerase are recommended for a 50 µl volume of a PCR mixture. Nonspecific PCR products may appear at higher concentrations of the polymerase. However, it may be necessary to increase the amount of *Taq* DNA Polymerase to 2-3 u, if the PCR mixture contains inhibitors, for instance, due to contamination of the template DNA. *Taq* DNA polymerase, if PCR is assembled at room temperature, exhibits low but noticeable activity during the reaction set-up. As a result, non-specific priming events, such as mispriming or formation of primer dimers, which occur at ambient temperatures, will lead to generation of nonspecific amplification products during PCR. Therefore, PCR reaction set-up should always be performed on ice

PCR Cycling Parameters:

Amplification parameters greatly depend on the template, primers and parameters of the thermal cycler used.

Initial DNA Denaturation: It is essential to completely denature the template DNA at the beginning of PCR to ensure efficient utilization of the template during the first amplification cycle. If GC content of the template is 50% or less, an initial 1-3 min denaturation at 95°C is sufficient. For GC-rich templates, this step has to be prolonged 5-10 minute.

Denaturation: Normally 0.5-2 min DNA denaturation at 94-95°C per cycle is sufficient. For GC-rich DNA templates, this step could be prolonged.

Primer Annealing: Annealing temperature should be 5°C lower than the lowest primer-template melting temperature (Tm). Annealing for 0.5-2 min is normally sufficient. If nonspecific PCR products appear the annealing temperature should be optimized stepwise in 1-2°C increments.

Extension: The rate of DNA synthesis by *Taq* DNA Polymerase and *Pfu* DNA Polymerase is highest at 70-75°C. As a general rule, the extension step with *Taq* DNA Polymerase is 1 min at 72°C for PCR products up to 1 kb.

Number of Cycles: The number of cycles may vary depending on the amount of template DNA in the PCR mixture and the expected yield of PCR product. If less than 10 copies of the template are present in the reaction, about 40 cycles are required. With higher template amounts 25-35 cycles are sufficient

Final Extension: After the last cycle, it is recommended to incubate the PCR mixture at 72°C for additional 5-15 min to fill-in the protruding ends of reaction products. If PCR product is to be cloned into TA vectors (to ensure the highest efficiency of 3'-dA tailing of PCR product)

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www. Fermentas.com

Optimisation of Polymerase Chain Reaction condition

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Materials:

- Eppendorf Tubes: 1.5ml, 0.5 ml, 0.2ml PCR tubes (autoclaved)
- 8-strip PCR tubes with lids (autoclaved)
- Tips 200 µl and 10 µl
- 96 well stands.

Chemicals:

- 10X Taq Buffer
- 25 mM $MgCl_2$
- 10mM dNTPs
- 10µM Forward Primer
- 10µM Reverse Primers
- Taq.DNA Polymerase
- PCR grade Water

Apparatus:

- Thermocycler
- Mini centrifuge

Procedure:

1. Prepare the Master mix in an eppendorff tube as follows:

   (for one sample reaction mixture)

PCR Mix Composition:

PCR Stock solutions	Working concentrations	For 1 reaction	For 12 reactions
PCR grade Water	--	16 µl	
10X PCR Buffer	1X	2 µl	
25mM MgCl2	1.5 mM	1.4 µl	
10mM dNTPS	0.2 mM	0.4 µl	
10µM Primer Forward	0.1 µM	0.2 µl	
10µM Primer Reverse	0.1 µM	0.2 µl	
Taq Polymerase enzyme	1 unit per reaction	0.2 µl	
DNA Template	50-100 ng	1.0 µl	
Total reaction volume		20 µl	

Calculation of working solutions:

A. for buffer: Provided Taq Buffer is 10X concentrated , make it to 1X in the final PCR reaction volume, using the following formula:

C1V1=C2V2 where C1-concentration of stock solution that you have

V1- volume of stock required

C2-concentration of working solution that you want

V2- volume of working solution required.

For calculation of buffer

10X x X µl = 1X x20 µl

.

X µl = 1X x 20 µl / 10X = 2 µl

B. For calculation of $MgCl_2$ (stock concentration =25 mM; required concentration=1.5mM)

C1V1=C2V2

25mM x Xµl = 1.5 mM x 20 µl

X µl =_____

2. Prepare an appropriate amount of master mix and distribute in each tube.

3. Place the tubes thermocycler and set gradient of temperatures from __ to ____.

After the completion of all the cycles, visualise on _____ %agarose gel .

STEPS INVOLVED IN PCR:

Step	Temperature	Time	
Initial denaturation	94^0C	5 min.	
Denaturation	94^0C	45 secs	**35 Cycles**
Annealing	_____	45 secs	
Extension	72^0C	2 min.	
Final extension	72^0C	2 min.	
On-Hold	15^0C		

Initially primer annealing temperature is tested across a gradient of _____ ^{o}C. The temperature in each block is as follows:

The instrument is programmed such that the steps would be repeated for 35 cycles to get the amplified DNA. Samples are loaded into _____% agarose gel and the amplified bands visualized.

Primer Designing

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Primer: Single stranded DNA oligonucleotide containing complementary sequence for target gene of interest. It provides 3'OH group, needed for DNA polymerase to add nucleotide and It always be in 5' to 3' direction

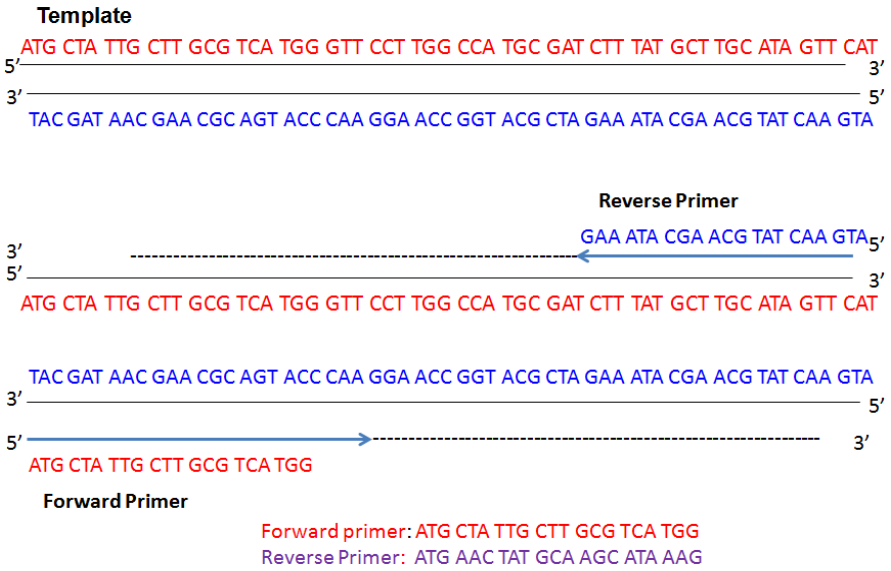
Basic consideration for Primer Designing:

- PCR primers should be generally 15-30 nucleotides long
- Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer
- Prefer one or two G or C at the 3'-end of the primer, but avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of nonspecific priming
- Avoid primer self-complementarities, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization
- Differences in melting temperatures (Tm) of the two primers should not exceed 5°C for conventional PCR

Estimation of Primer Melting Temperature(Tm) :

The approximate melting temperature (Tm) of primer sequence can be calculated using the following equation: Tm = 4 (G + C) + 2 (A + T), where G, C, A, T – number of respective nucleotides in the primer.

Manual Primer designing:



Exercise: **Design forward and Reverse primers to amplify the Gene X with 979bp**

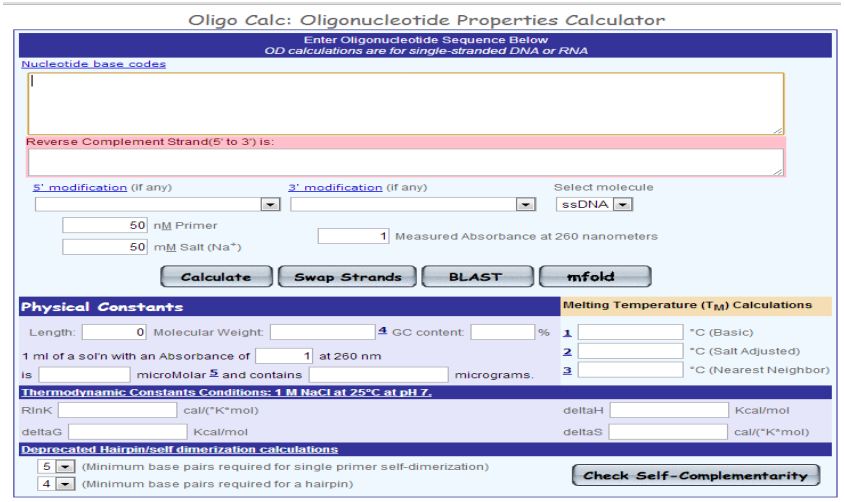
CAAGTTTCTCAAATGCCTTCTTCATCACCCCTTTCTTCCAATAAGGATGAAATGCG
TCCCAAAGCCGATTTCAGCCTAGCATTTGGGGAGATTTCTTCCTCAATTGTCCCGA
CAAGAATATTGATGCTGAAACTCAAAAACGCCACCAACAATTGAAAGAAGAAGT
GAGGAAGATGATTGTGGCACCAATGGCTAATTCAACCCAAAAGTTAGCCTTCATT
GATTCAGTCCAAAGACTGGGTGTGAGTTACCATTTCACCAAGGAGATCGAAGATG
AACTAGAGAATATCTACCATAACAACAATGATGCCGAGAACGACCTCTACACCA
CATCCCTTCGATTCCGACTACTCCGAGAGCATGGATTCAATGTTTCATGCGACGTA
TTCAACAAGTTTAAAGACGAGCAAGGGAATTTCATTGTAACACCAGAAACCTTTA
AATGGGCAGCCAATGACCCTAAGATAATTCAAGCTTCCACAATTATTTGTAGGTT
TATGGATGATGTTGCTGAACACAAGTTCAAACATAGGAGAGAAGACGATTGCTC
AGCAATTGAGTGTTACATGGAAGAATATGGTGTAACAGCACAAGAGGCATATGA
TGTATTCAACAAGCATGTTGAGAGTGCTTGGAAGGATGTGAATCAAGAGTTTCTG
AAACCAACAGAAATGCCAACAGAAGTTTTGAATCGTAGCTTAAACCTTGCAAGG
GTGATGGATGTACTCTACAGAGAAGGTGATGGCTACACATATGTTGGAAAAGCG
GCTAAGGGTGGAATCACTTCATTACTCATTGAACCAATTGCACTTTGAAATCGTA
TTAAATTTTCCTCTTCTGTTCCTTAAGGAATAGTTATTAAGTTATAATTAATAATG
TTTTATAATATATATATTATAAAAGAAATTCTAAATCAACTGTCTTTGTATTCATT
TCCTTGTATTGATCTAATAAAGTTCTTTTCAAGCTTTCATTTGTG

Answer: Forward primer: **CAAGTTTCTCAAATGCCTTCTTC**

Reverse Primer: **CACAAATGAAAGCTTGAAAAGAAC**

Validation of manually designed primer:

Online validation manually designed primer for the desirable parameters such as GC content, hairpin structure and primer self complementarily using **Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/OligoCalc.html)**



Online Tools for Primer designing:

1. Integrated DNA Technologies (IDT) (https://eu.idtdna.com/site): Primer and probe design and selection
2. Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) : Utility for locating oligonucleotide primers for PCR amplification of DNA sequences

Integrated DNA Technologies:

Online primer designing stepwise:

1. Log on to web site : (https://eu.idtdna.com/site)
2. Go to Scitools , scroll and click on Primer Quest
3. Enter sequence manually or Download sequence using genebank or Accession ID
4. Click on design PCR 2 primers
5. List of forward and reverse primers with amplicon size, position of prime will be displayed
6. Select the best one and synthesize

Isolation of *Bacillus thuringiensis* strains from environmental samples

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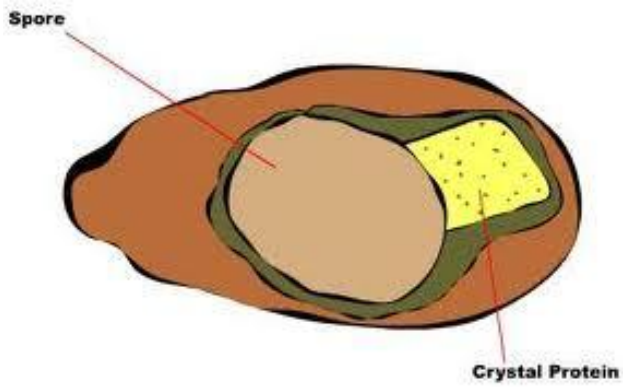
Control of insect pests in agriculture and of insect vectors of important human diseases is mainly achieved using chemical insecticides. However, the use of these chemical pesticides has led to several problems, including environmental pollution and increase in human health effects, such as cancer and several immune system disorders (Devine and Furlong, 2007). The most successful insect pathogen used for insect control is the bacterium *Bacillus thuringiensis* (Berliner) (Bt), which presently is 2% of the total insecticidal market. *B. thuringiensis* is a member of the genus *Bacillus* and like the other members of the taxon has the ability to form endospores that are resistant to inactivation by heat, desiccation and organic solvents (Crickmore et al., 1998). The bacterium is a gram-positive and facultative anaerobe. The shape of the cells of the organism is rod. The width of the rod varies 3-5 µm in size when grown in standard liquid media. Because of its insecticidal activity, it has been used for nearly fifty years to control certain insect species among the orders Lepidoptera, Coloeptera, and Diptera.

Morphological Properties of *Bacillus thuringiensis* and Cry toxins classifications

Colony morphology can help to distinguish *B. thuringiensis* colonies from other *Bacillus* species. The organism forms white, rough colonies, which spread out and can expand over the plate very quickly. *B. thuringiensis* strains have un-swollen and ellipsoidal spores that lie in the sub-terminal position. The presence of parasporal crystals that are adjacent to the spore in the mother cell is the best criteria to distinguish *B. thuringiensis* from other closely related *Bacillus* species. The morphology, size, and number of parasporal inclusions may vary among *B. thuringiensis* strains. However, four distinct crystal morphologies are apparent: the typical bipyramidal crystal, related to Cry 1 proteins (Aronson

et al., 1976); cuboidal inclusions related to Cry 2 proteins and usually associated with bipyramidal crystals (Ohba and Aizawi, 1986); amorphous and composite crystals releated to Cry 4 and Cyt proteins (Federici et al., 1990); and flat, square crystals, related to Cry 3 proteins (Hernstadt et al., 1986; Lopez-Meza and Ibarra, 1996). Spherical and irregular pointed crystal morphologies can also be observed in *B. thuringiensis* strains.

Cry toxins are classified by their primary amino acid sequence and more than 500 different cry gene sequences have been classified into 67 groups (Cry1-Cry67) (Crickmore et al., 2010). These cry gene sequences have been divided in to at least four phylogentically non-related protein families that may have different modes of action: the family of three domain Cry toxins (3D), the family of mosquitocidal Cry toxins (Mtx), the family of the binary-like (Bin) and the Cyt family of toxins (Bravo et al., 2005). Also, some Bt strains produce additional insecticidal toxins named VIP. VIP toxins, in contrast to Cry, are produced during the vegetative growth phase. At least three VIP toxins have been characterized, VIP1/VIP2, a binary toxin, and VIP3 (Estruch et al., 1996).



Mode of Action of cry toxins

The crystal proteins of *B. thuringiensis* show host specificity. For this reason, each type of Cry protein can be toxic to one or more specific insect species. Because of this specific toxicity, they do not affect many beneficial insects, plants and animals including humans. The specificity of these insecticidal crystal proteins (ICPs) derives from their mode of action (Adang, 1991). The parasporal crystals of *B. thuringiensis* contain the ICPs in the form of protoxins. After ingestion of parasporal crystals by the susceptible insect, the crystals are dissolved in alkaline conditions (pH 10-12) in the insect mid-gut, generating 130 to 135

kDa protein chains called protoxin. These proteins are then processed to the actual toxic fragments of 60-65 kDa by the gut proteases (Gill et al., 1992; Höfte and Whiteley, 1989). Finally, these activated toxins bind to specific receptors present in the larval mid-gut epithelia. The activated toxin binding to the specific receptors on the cell membrane creates ion channels or pores. The pore formation causes osmotic shock. As a result of this process, the cell membrane lyses, paralysis occurs and consequently, the insect stops feeding and dies from starvation (Knowless, 1994).

Isolation Methods to Establish *Bacillus thuringiensis* Strain

Isolation of *B. thuringiensis* from soil and other natural environments is greatly facilitated by the use of selective techniques. There are many selective enrichment methods described in the literature (Travers et al., 1987; Johnson and Bishop, 1996). The sodium acetate selection method has been used routinely for the isolation of *B. thuringiensis* from environmental samples (Martin and Travers, 1989). The germination of spores in crystal forming bacilli, including both *B. thuringiensis* and *B.sphaericus*, is inhibited by sodium acetate concentrations of approximately 0.25M. Soil is inoculated into a nutrient medium containing the sodium acetate. After a period of growth, the vegetative cells are eliminated by heat treatment and remaining spores are isolated on a nutrient medium without acetate. The survivors from this treatment range from 20-96% *B. thuringiensis* and *B. sphaericus*.

Protocol for isolation of Bt strains from environmental samples (Travers et al., 1987**)**

1g of sample (soil, plant material, dead insects etc.)

↓

20 mL of LB medium + 0.25 M sodium acetate

↓

Mixture incubated in a rotary shaker
at 250 rpm for 4 h at 30 °C

↓

After incubation, 1.5 mL of the mixture was
placed into microfuge tubes and heated at 80 °C for 5 min

↓

Samples plated on LB agar medium and
incubated overnight at 30 °C.

↓

White, rough colonies were selected from LBA plates and cultured on T3
agar medium overnight at 30 °C

↓

Endospore staining (Coomassie blue) and observation under
Phase contrast microscope for crystal morphology

T3 Media (g/L): 3 g Tryptone, 2 g Tryptose, 1.5 g Yeast extract, Sodium phosphate buffer (pH 6.8) 0.05 M, 0.005 g $MnCl_2$, 15 g Agar

LB agar media (g/L): 10 g Tryptone, 5 g Yeast extract, 5 g Sodium Chloride, 18 g Agar, pH 7.2

Coomassie brilliant blue stain (0.25%): 0.25 g Coomassie brilliant blue in 100 ml (50% ethanol (50 ml) + 7% Acetic acid (7 ml) + 43 ml water)

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Gene cloning: Methods

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Cloning is widely used techniques in array of biological experiments and practical applications ranging from genetic fingerprinting to large scale protein production. Cloning of any DNA fragment essentially involves following steps

- **Isolate** gene of interest to be cloned (using PCR based method) and choose a suitable plasmid vector for amplification and manipulation of foreign DNA
- **Cut** for Directional cloning : restriction endonuclease digestion of vector and insert (foreign) DNA or T/A cloning or Blunt end cloning
- **Ligate:** covalently join (paste) vector and insert DNA using DNA ligase
- **Transform:** introduce recombinant molecule into prokaryotic or eukaryotic cell
- **Select:** identify cells that received a plasmid
- **Screen:** identify cells containing recombinant DNA of interest (clones)

Subcloning: is a technique used to move a particular gene of interest from a parent vector to a destination vector in order to further study its functionality.

There are three ways: 1.T-A Cloning,2. Directional Cloning

- Blunt Ended Cloning
- **TA cloning** is a subcloning technique that doesn't use restriction enzymes and is easier and quicker than traditional subcloning. PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together.

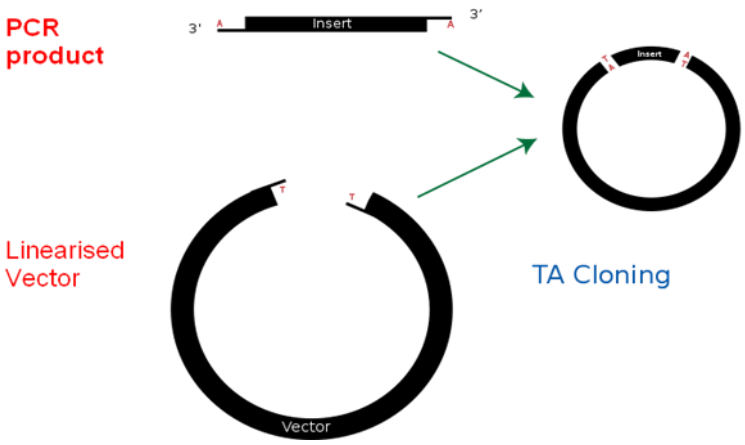
- **Directional Cloning**, the DNA fragment is digested with single or two Restriction Enzymes producing Cohesive/Sticky ends. The digested fragment is then ligated into a vector digested with the same set of enzymes and then introduced into a suitable host.
- **Blunt Ended Cloning**: the DNA fragment is digested with Restriction Enzymes producing Blunt ends. The digested fragment is then ligated into a vector digested with the same enzyme and then introduced into a suitable host.

T/A cloning:

PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs. The technique relies on the ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together.

Steps in T/A cloning:

1. PCR amplification of target gene of interest
2. Agarose gel electrophoresis and
3. Gel elution of the PCR product
4. Ligation of PCR product (Insert : Vector) in different molar ratio



1. Bacterial Transformation of ligated product
2. Selection based on blue white colony and selection marker
3. Screening for confirmation through Colony PCR/Plasmid PCR and Restriction digestion

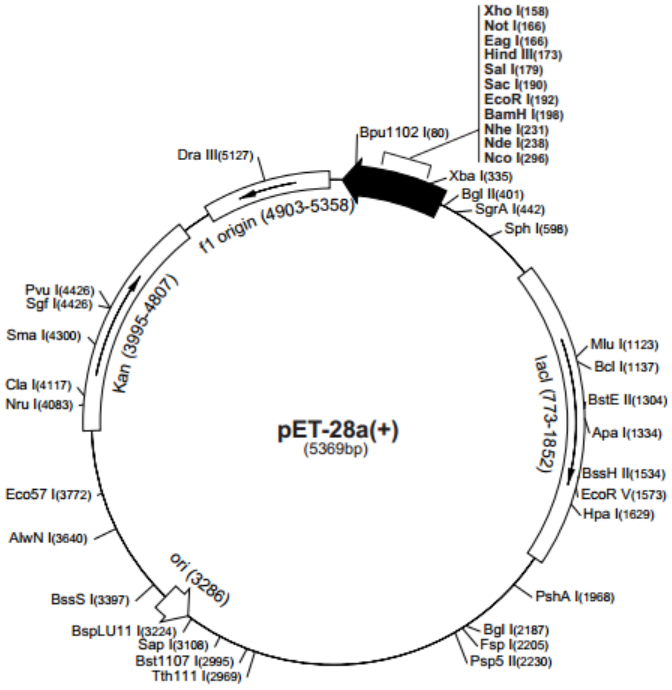
Directional cloning:

Directional Cloning, the DNA fragment is digested with single or two Restriction Enzymes producing Cohesive/Sticky ends. The digested fragment is then ligated into a vector digested with the same set of enzymes and then introduced into a suitable host.

Steps in directional cloning:

1. Isolation and sequencing of the target gene of interest (GOI)
2. Analyze and select of restriction sites present in vector for directional cloning

   Example: Directional cloning Gene of interest in between BamHI and EcoRI in pET-28a for protein expression



3. **Restriction Analysis of target gene nucleotide sequence using online tools such as NEB cutter (http://tools.neb.com/NEBcutter2/) and engineering them into primers:**

Paste Plain or fasta format nucleotide sequence of gene of interest

>Gene of interest
CAAGTTTCTCAAATGCCTTCTTCATCACCCCTTTCTTCCAATAAGGATGAAATGCG
TCCCAAAGCCGATTTCAGCCTAGCATTTGGGGAGATTTCTTCCTCAATTGTCCCGA
CAAGAATATTGATGCTGAAACTCAAAAACGCCACCAACAATTGAAAGAAGAAGT
GAGGAAGATGATTGTGGCACCAATGGCTAATTCAACCCAAAAGTTAGCCTTCATT
GATTCAGTCCAAAGACTGGGTGTGAGTTACCATTTCACCAAGGAGATCGAAGAT
GAACTAGAGAATATCTACCATAACAACAATGATGCCGAGAACGACCTCTACACC
ACATCCCTTCGATTCCGACTACTCCGAGAGCATGGATTCAATGTTTCATGCGACG
TATTCAACAAGTTTAAAGACGAGCAAGGGAATTTCATTGTAACACCAGAAACCTT
TAAATGGGCAGCCAATGACCCTAAGATAATTCAAGCTTCCACAATTATTTGTAGG
TTTATGGATGATGTTGCTGAACACAAGTTCAAACATAGGAGAGAAGACGATTGCT
CAGCAATTGAGTGTTACATGGAAGAATATGGTGTAACAGCACAAGAGGCATATG
ATGTATTCAACAAGCATGTTGAGAGTGCTTGGAAGGATGTGAATCAAGAGTTTCT
GAAACCAACAGAAATGCCAACAGAAGTTTTGAATCGTAGCTTAAACCTTGCAAG
GGTGATGGATGTACTCTACAGAGAAGGTGATGGCTACACATATGTTGGAAAAGC
GGCTAAGGGTGGAATCACTTCATTACTCATTGAACCAATTGCACTTTGAAATCGT
ATTAAATTTTCCTCTTCTGTTCCTTAAGGAATAGTTATTAAGTTATAATTAATAAT
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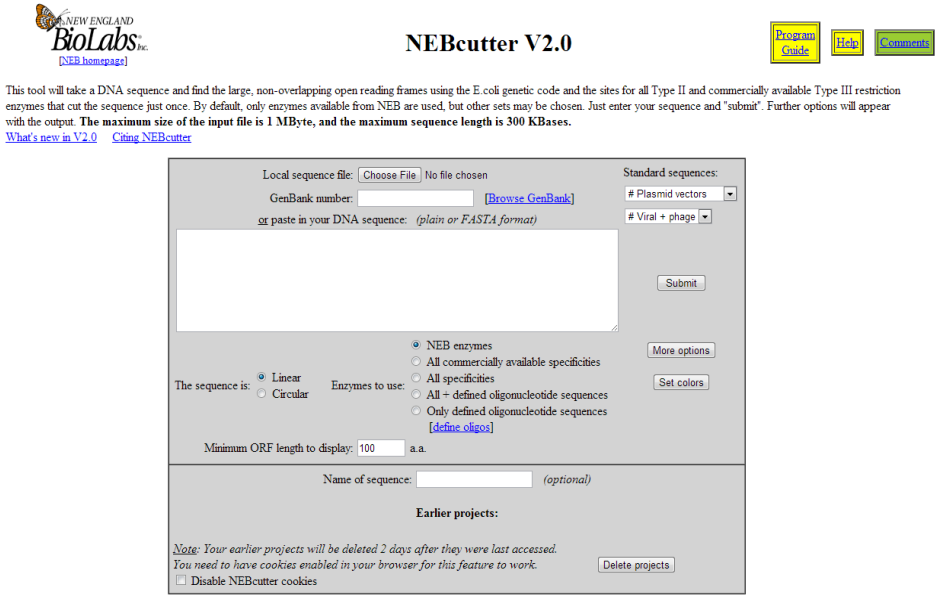
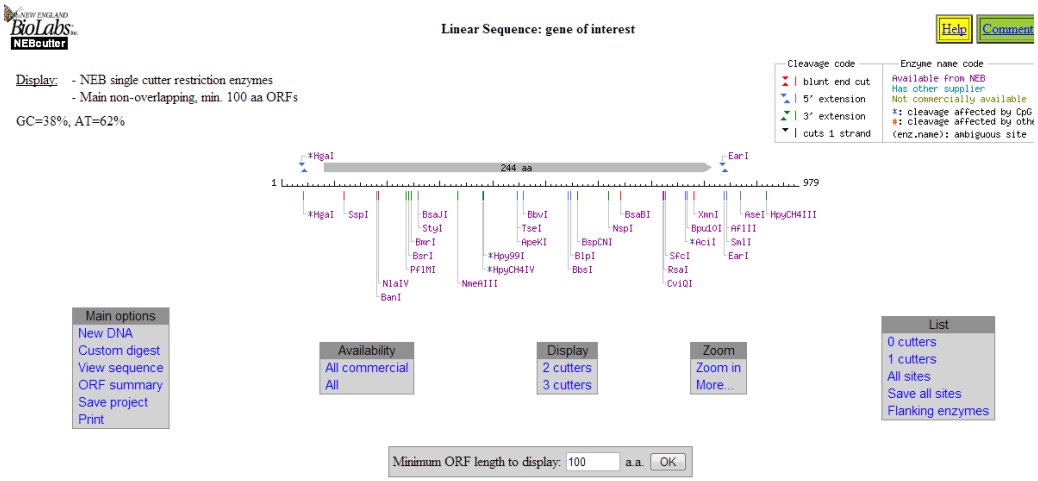


- Submit for restriction analysis



• Analyze and find out the whether the selected restriction sites on the vector is available for cloning**(** Means- The nucleotide sequence of the gene of interest should not contain restriction site for the selected enzymes (In this case BamHI and EcoRI), those enzymes are called as **zero cutter for sequence i.e., Enzymes that don't cut)**

Enzymes that don't cut

gene of interest

Number of cuts = 0 OK Save as text file

#	Enzyme	Specificity
1	AatII	G_ACGT^C
2	Acc65I	G^GTAC_C
3	AccI	GT^MK_AC
4	AclI	AA^CG_TT
5	AcuI	CTGAAG(N)$_{14}$_NN^
6	AfeI	AGC^GCT
7	AflIII	A^CRYG_T
8	AgeI	A^CCGG_T
9	AhdI	GACNN_N^NNGTC
10	AleI	CACNN^NNGTG
11	AlwI	GGATCNNNN^N_
12	AlwNI	CAG_NNN^CTG
13	ApaI	G_GGCC^C
14	ApaLI	G^TGCA_C
15	AscI	GG^CGCG_CC
16	AsiSI	GCG_AT^CGC
17	AvaI	C^YCGR_G
18	AvaII	G^GWC_C
19	AvrII	C^CTAG_G
20	BaeGI	G_KGCM^C
21	BaeI	_(N)$_5$^(N)$_{10}$ACNNNNGTAYC(N)$_7$_(N)$_5$^
22	BamHI	G^GATC_C
23	BanII	G_RGCY^C

If the selected enzymes are in zero cutter, then add the recognition nucleotide sequence of the enzymes to the 5' end of the forward (BamHI) and Reverse Primer(EcoRI)

CAAGTTTCTCAAATGCCTTCTTCATCACCCCTTTCTTCCAATAAGGATGAAATGCG
TCCCAAAGCCGATTTCAGCCTAGCATTTGGGGAGATTTCTTCCTCAATTGTCCCGA
CAAGAATATTGATGCTGAAACTCAAAAACGCCACCAACAATTGAAAGAAGAAGT
GAGGAAGATGATTGTGGCACCAATGGCTAATTCAACCCAAAAGTTAGCCTTCATT

GATTCAGTCCAAAGACTGGGTGTGAGTTACCATTTCACCAAGGAGATCGAAGAT
GAACTAGAGAATATCTACCATAACAACAATGATGCCGAGAACGACCTCTACACC
ACATCCCTTCGATTCCGACTACTCCGAGAGCATGGATTCAATGTTTCATGCGACG
TATTCAACAAGTTTAAAGACGAGCAAGGGAATTTCATTGTAACACCAGAAACCTT
TAAATGGGCAGCCAATGACCCTAAGATAATTCAAGCTTCCACAATTATTTGTAGG
TTTATGGATGATGTTGCTGAACACAAGTTCAAACATAGGAGAGAAGACGATTGCT
CAGCAATTGAGTGTTACATGGAAGAATATGGTGTAACAGCACAAGAGGCATATG
ATGTATTCAACAAGCATGTTGAGAGTGCTTGGAAGGATGTGAATCAAGAGTTTCT
GAAACCAACAGAAATGCCAACAGAAGTTTTGAATCGTAGCTTAAACCTTGCAAG
GGTGATGGATGTACTCTACAGAGAAGGTGATGGCTACACATATGTTGGAAAAGC
GGCTAAGGGTGGAATCACTTCATTACTCATTGAACCAATTGCACTTTGAAATCGT
ATTAAATTTTCCTCTTCTGTTCCTTAAGGAATAGTTATTAAGTTATAATTAATAAT
GTTTTATAATATATATATTATAAAAGAAATTCTAAATCAACTGTCTTTGTATTCAT
TTCCTTGTATTGATCTAATAAAGTTCTTTTCAAGCTTTCATTTGTG

BamHI

Forward primer: *GGATCC* **CAAGTTTCTCAAATGCCTTCTTC**

Reverse Primer: *GAATTC* **CACAAATGAAAGCTTGAAAAGAAC**

EcoRI

4. Perform standard PCR to amplify gene of interest, then perform either Protocol A or B (Protocol B is giving better results in our case)

Protocol A	**Protocol B**
• Gel elution or PCR clean up • Digest PCR product with restriction enzyme(s) to create digested ends and digest the vector with same restriction enzymes • Ligate PCR product (insert) into your linearized vector	• T/A cloning, identify positive colony and isolate Plasmid • Digest Plasmid with restriction enzyme(s) to release insert and to create digestive end and digest the vector with same restriction enzymes • Gel elution of Insert and PCR cleanup of vector • Ligate insert into your linearized vector

5. Bacterial transformation of the ligated product

6. Selection and screening for positive colonies

Restriction digestion

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Restriction enzymes are Nucleases which can cleave the sugar-phosphate backbone of DNA, since they cut within the molecule, are commonly called restriction endonucleases. They specifically cleave the nucleic acids at specific nucleotide sequence called Restriction sites. Restriction enzymes form the defense system in bacterial cells through restriction and modification against invasion by foreign DNA.

There are three types of Restriction Enzymes: **Type I, Type II and Type III**

Type II Restriction Enzymes are commonly used one's in molecular biology and recombinant DNA techniques. Unlike Type I and III, these enzymes cleave specifically at the recognition sequence site by hydrolyzing phosphodiester bonds in both strands. One of the salient feature of commonly used restriction endonuclease is that the recognition sequences are mostly palindromes - they shows the same forward (5' to 3' on the top strand) and backward (5' to 3' on the bottom strand) sequences. Mg2+ acts as co-factors for all restriction enzymes. The recognition sequences usually vary between 4 and 8 nucleotides, differ with restriction enzymes and produce over hangs (5' or 3' Sticky ends) or blunt ends.

Examples of Restriction enzymes and their recognition sequence for Sticky end and Blunt end

Cleavage type	Enzyme	Recognition sequence
Sticky end	**BamHI**	GGATCC CCTAGG
	EcoRI	GAATTC CTTAAG
Blunt end	**RsaI**	GTAC CATG
	SmaI	CCCGGG GGGCCC

Temperature and time of incubation for the restriction digestion will vary with amount of template and enzyme efficiency. There are many commercial companies are involved in manufacture and supply of restriction enzymes. The standardized manufactures protocol can be used with modification for your experimental condition.

Ligation, transformation and Screening of transformants

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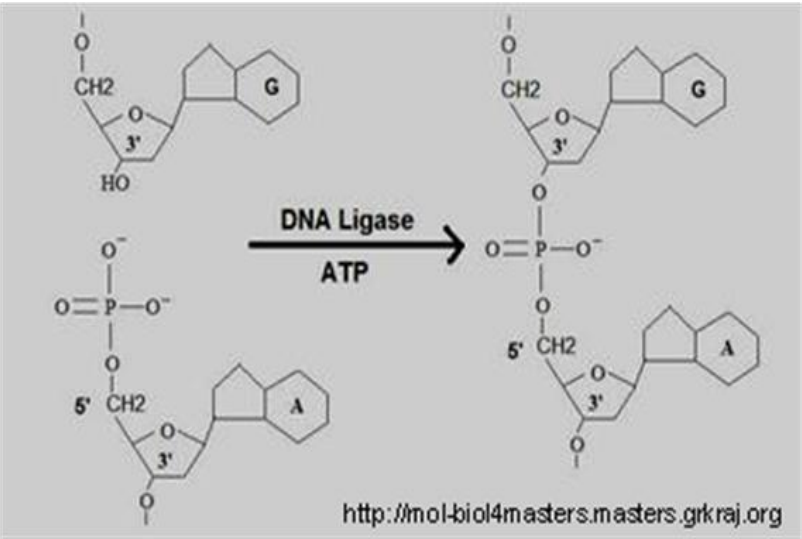
Ligation :

The isolated gene of interest and vector was suitably prepared based on method of choice(T/A cloning or blunt end cloning or directional cloning) before subject them for a process called ligation. Ligation is often termed as joining reaction where both gene/fragment of interest and vector (plasmid) will be covelantly linked by the action of DNA ligase. DNA ligase catalyses covalent bond formation between 3'OH and 5'PO4 on DNA.



DNA ligase: Phosphodiester bond formation

Calculation for optimization of vector: insert ratio in ligation reaction:

$$Conc.Of\ insert = \frac{[(conc.Vect.) \times (Size\ of\ insert) \times (ratio\ of\ insert)]}{size\ of\ vector \times ratio\ of\ vector}$$

Ligation reaction mixture:

Vector	= 1 µl	
Insert	= µl	
Ligase buffer (2X)		= 5 µl
Ligase	= 1 µl	
H_20		= µl
	= 10 µl	

Incubate the ligation reaction at 4 ^{0}C/16 ^{0}C for overnight.

Stop the reaction and use it for transformation

Bacterial Transformation

One of the most common procedures used in laboratories to genetically modify bacterial cells is known as **Transformation**. A commonly used bacterium for transformations is *E.coli*. Bacterial cells that are actively growing are most amenable to bacterial transformation. However, they must undergo a series of treatments before they are competent for transformation. There are several ways to make cells competent for transformation, but the simplest methods require calcium chloride, ice, and heat.

Role of Calcium Chloride in making cells competent:

A cell is surrounded by a **cell membrane** composed of phospholipid molecules. The outside of the bacterial membrane is negatively charged and the phosphate group in DNA molecules gives DNA a negative charge. Since , the DNA and the cell membrane have the same charge, it is difficult to push the DNA into cell. The calcium chloride treatment of bacterial cells results in creation of layer of calcium ions on the negatively charged cell membrane. During the transformation, calcium ions also bind the DNA and thus resulted close proximity of the positively charged ions to these negative charges effectively

neutralizes the charge on both the cell membrane and the DNA molecules. This enables the DNA plasmid to move toward the cell membrane without being repelled.

Heat Shock treatment:

During the transformation protocol, the cells and DNA are incubated with the calcium chloride solution; the entire solution is chilled on ice. During the procedure, a brief heat shock is given at 42 ^{o}C. Hypothesis is that, the heat shock treatment effectively opens pores in the cell membrane through which a plasmid molecule could travel.

Optimal Growth and Recovery: After heat shock, nutrient broth is added and *E. coli* cells are given time to recover.

Selection and Screening of transformants:

Selection of transformed cells with plasmid is usually achieved with selection marker such as Ampicillin, Kanamycin and other antibiotic markers. Only bacteria that have been transformed with the plasmid will grow under the antibiotic conditions because they express a protein that confers resistance to antibiotic such as kanamycin or ampicillin and others present only on transformed plasmid.

Blue White screening of transformants:

Modern bacterial cloning vectors available in the market use the **Blue-White Screening** to distinguish colonies of transformed one from non transformed. The method is based on the principle of **α-complementation of the β-galactosidase gene**. This phenomenon of α-complementation was first demonstrated by F.Jacob and J.Monod.

β-galactosidase is active only when it contains both N terminal α-peptide and C-terminal ω-peptide. In this method of screening, the host E. coli strain carries the lacZ deletion mutant- which contains the **ω-peptide**, while the plasmids used carry the lacZ α sequence which encodes the N terminal residues of β-galactosidase, the **α-peptide**. Neither is functional by individually. However, when a plasmid containing the lacZ α sequence is transformed into a lacZΔM15 cells, the two peptides are expressed together and form a functional β-galactosidase enzyme. . The process is called as α-complementation. This enzyme in presence of substrate X-gal **results in blue color**

The **Blue/White Screening Method** works by disrupting this α-complementation process. The plasmid within the lacZα sequence carries a **Multiple Cloning Site (MCS).** This MCS within the lacZα sequence can be cut by restriction enzymes so that the foreign DNA may be inserted within the lacZα gene, thereby disrupting the gene and thus production of α-peptide. Consequently, in cells containing the plasmid with an insert, no functional β-galactosidase may be formed. Hence the positive bacterial transformants with plasmid containing gene of interest wiil be **white in colour**.

The *lacZ* promoter is **induced** (turned on) by lactose or a nonhydrolyzable analog, **IPTG** (isopropyl thiogalactoside) and Lactose analog X-gal (**5-bromo-4-chloro-3-indolyl β -D-galactopyranoside)** is used as substrate in.

Colony PCR : PCR confirmation of the selected positive colonies using gene specific primers and colony as template.

PROTOCOL FOR RESTRICTION DIGESTION*

(*Adopted from New England Biolabs Protocol)

High Fidelity (HF) Restriction Enzymes have 100% activity in specified Buffer; single-buffer simplicity means more straightforward and streamlined sample processing. HF enzymes also exhibit dramatically reduced star activity. HF enzymes can therefore cut substrate DNA in minimum time with the flexibility to digest overnight without degradation to DNA. ngineered with performance in mind, HF restriction enzymes are fully active under a broader range of conditions, minimizing off-target products, while offering flexibility in experimental design.

Unit Definition: One unit is defined as the amount of enzyme required to digest 1 µg of λ DNA in 1 hour at 37°C in a total reaction volume of 50 µl.

Storage Temperature: -20°C

There are several key factors to consider when setting up a restriction endonuclease digest. Using the proper amounts of DNA, enzyme and buffer components in the correct reaction volume will allow you to achieve optimal digestion. By definition, 1 unit of restriction enzyme will completely digest 1 µg of substrate DNA in a 50 µl reaction in 60 minutes. This enzyme: DNA: reaction volume ratio can be used as a guide when designing reactions. However, most researchers follow the "typical" reaction conditions listed, where a 5–10 fold over digestion is recommended to overcome variability in DNA source, quantity and purity. NEB offers the following tips to help you to achieve maximal success in your restriction endonuclease reactions.

A "Typical" Restriction Digest

Restriction Enzyme	10 units is sufficient, generally µl is used	
DNA	1 µg	
10X NEBuffer	5 µl (1X)	
Total Reaction Volume	50 µl	
Incubation Time	1 hour*	
Incubation Temperature	Enzyme dependent	

Enzyme

- Keep on ice when not in the freezer
- Should be the last component added to reaction
- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.
- In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1 hour digest.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents or excessive salts. Extra wash steps during purification are recommended.

Buffer

- Use at a 1X concentration

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt) as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol) can be problematic in smaller reaction volumes. The following guidelines can be used for techniques that require smaller reaction volumes.

	Restriction Enzyme*	DNA	10X NEBuffer
10 µl rnx**	1 unit	0.1 µg	1 µl
25 µl rnx	5 units	0.5 µg	2.5 µl
50 µl rnx	10 units	1 µg	5 µl

* Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed.
** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation.

Incubation Time

- Incubation time is typically 1 hour
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours: usually done for genomic DNA.

Stopping a Reaction

If no further manipulation of DNA is required:

- Terminate with a stop solution (10 µl per 50 µl rxn) [50% glycerol, 50 mM EDTA (pH 8.0), and 0.05% bromophenol blue]

When further manipulation of DNA is required:

- Heat inactivation can be used
- Remove enzyme by using a spin column or phenol/chloroform extraction

Storage

- Storage at -20°C is recommended for most restriction enzymes. For a few enzymes, storage at-70°C is recommended for periods longer than 30 days. Please refer to the enzyme's technical data sheet or catalog entry for storage information.
- 10X NEBuffers should also be stored at -20°C

Stability

- All enzymes are assayed for activity every 4 months. The expiration date is found on the label.
- Exposure to temperatures above -20°C should be minimized whenever possible

Control Reactions

If you are having difficulty cleaving your DNA substrate, the following control reactions are recommended:

- Control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) with restriction enzyme to test enzyme viability

If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.

Ligation Reaction:

Calculation for optimization of vector: insert ratio in ligation reaction:

$$Conc.Of\ insert = \frac{[(conc.Vect.) \times (Size\ of\ insert) \times (ratio\ of\ insert)]}{size\ of\ vector \times ratio\ of\ vector}$$

Ligation reaction mixture:

Vector	= 1 µl
Insert	= µl
Ligase buffer (2X)	= 5 µl
Ligase	= 1 µl
H_20	= µl
	= 10 µl

Incubate the ligation reaction at 4 ^{0}C/16 ^{0}C for overnight.

Stop the reaction and use it for transformation

TRANSFORMATION PROTOCOL

A. For chemically Competent Bacterial Cells (Adopted from Hannahan Method : Molecular cloning Sambrook & Russel)

Transformation Protocol from Overnight Bacterial Culture (for 2 transformations)

• The day before the transformation seed overnight culture by inoculating 2 ml* of C-medium with a single bacterial colony. Make sure to use freshly streaked bacterial colonies (not older than 10 days**). Incubate the culture overnight at 37°C in a shaker.

• The day of transformation pre-warm culture tubes containing the required amount of C-medium (1.5 ml for each 2 transformations) at 37°C for at least 20 min. Pre-warm LB agar plates, supplemented with appropriate antibiotic in a 37°C incubator for at least 20 min before plating .

Step Procedure:

1. Add 150 µl of the overnight bacterial culture to 1.5 ml of pre-warmed C-medium. Suspend the cells by gently mixing and incubate the tubes at 37°C for 2 hours in a shaker.

2. Pellet bacterial cells by 1 min centrifugation, discard the supernatant.

3. Resuspend cells in 600 µl of TSB solution. Incubate on ice for 15 min.

4 . Centrifuge for 1 min in a microcentrifuge, discard the supernatant.

5. Resuspend pelleted cells in 120 µl of TSB solution. Incubate 10 min on ice.

6. Add 4.2 µl of DnD solution to each vial and incubate on ice for 5 min.

7. Add an additional 4.2 µl of DnD solution and aliquot 60 µl of cells into fresh 1.5ml tubes.

8. Add 5 µl of ligation mixture to the tubes and incubate on ice for 2min.

9. Heat shock the cells, without shaking, at 42 ^{o}C, for 45 sec and immediately transfer on ice.

10. Add 500 µl of pre warmed C medium to each vial and incubate at 37^{o}C, 200 rpm for 45-60 min.

11. Collect cells by centrifugation and add 100 µl fresh C medium to the vials , resuspend the cells and plate the cells on suitable antibiotic .

12. Incubate overnight.

Recipes

TSB buffer (Hannahan Method : Molecular cloning Sambrook & Russel)

1M MES buffer(2-(*N*-morpholino)ethanesulfonic acid) pH 6.3 10 ml/ L

$MnCl_2.4H_2O$ 45 mM 8.91 g/L

$CaCl_2.2H_2O$ 10mM 1.49 g/L

KCl 100mM 7.46 g/L

Hexamino Cobalt Choride 3mM 0.8 g/L

Distilled water upto 1L

Filter sterilize and store at -20 ^{O}C in 40 ml aliquots.

DnD solution

DTT (Dithiothreitol) 1.53 g

DMSO (Dimethyl sulphoxide) 9ml

100 µl 1M Potassium acetate

Distilled water up to 10 ml.

TRANSFORMATION PROTOCOL:

(B: Adopted from Thermo fisher- transform Aid Bacterial Transformation Kit)

Transformation Protocol from Bacterial Colonies (for 2 transformations)

• Seed a LB plate with a single bacterial colony using the streak plate method and incubate the plate overnight at 37°C. Use freshly streaked bacterial colonies (not older than 10 days*), for preparation of competent cells.

• Before the transformation pre-warm culture tubes containing the required amount of C-medium (1.5 ml for each 2 transformations) at 37°C for at least 20 min. Pre-warm LB antibiotic agar plates in a 37°C incubator for at least 20 min before plating.

• Prepare T-solution: thaw T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µl of T-solution (A) and 250 µl of T-solution (B) in a separate tube and keep on ice.

Step Procedure:

1. Transfer a portion of freshly streaked bacterial culture (4 x 4 mm size) to 1.5 ml of pre-warmed C-medium using an inoculating loop. Suspend the cells by gently mixing and incubate the tubes at 37°C for 2 hours in a shaker.

2. Pellet bacterial cells by 1 min centrifugation, discard the supernatant.

3 .Resuspend cells in 300 µl of T-solution. Incubate on ice for 5 min.

4 .Centrifuge for 1 min in a microcentrifuge, discard the supernatant.

5. Resuspend pelleted cells in 120 µl of T-solution. Incubate 5 min on ice.

6 .Add up to 5 µl of ligation mixture (containing 10-100 ng vector DNA) or 1 µl of

supercoiled DNA (10-100 pg) into new microcentrifuge tubes. Chill on ice for 2 min.

7. Add 50 µl of the prepared cells to each tube containing DNA, mix and incubate on ice for 5 min.

8. Plate immediately on pre-warmed LB antibiotic agar plates. Incubate overnight at 37°C.

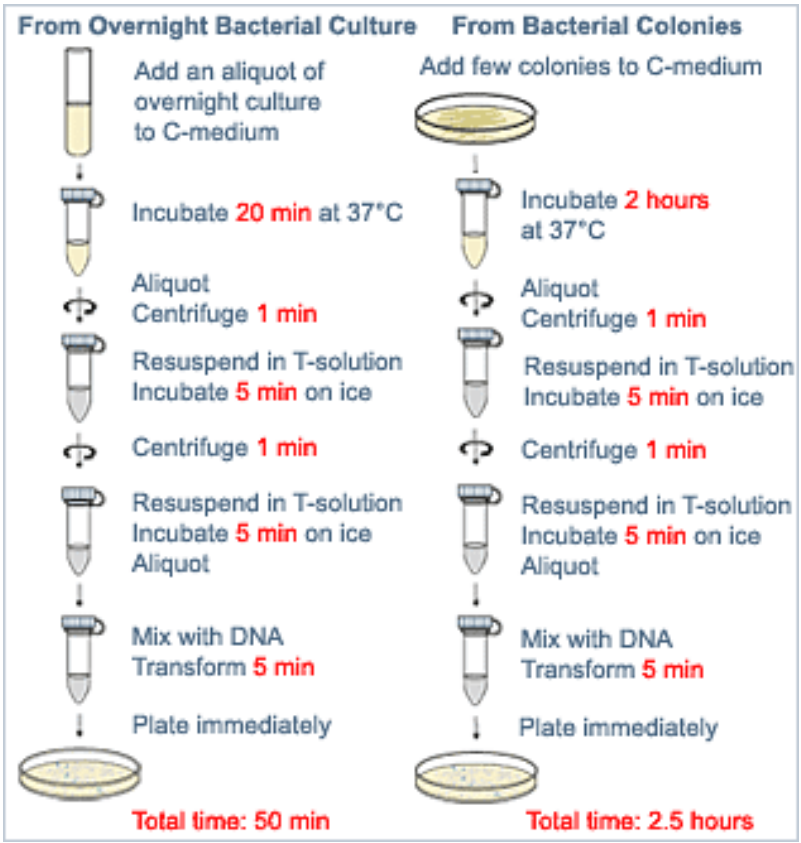
IMPORTANT NOTES

• All procedures are performed on ice. All short centrifugations can be carried out at room temperature in a regular table-top mini centrifuge (10000-12000 x g). Make sure the cells are not left in the centrifuge at ambient temperature for more than 5 min as this will significantly decrease the transformation efficiency.

• Competent cells prepared with TransformAid bacterial transformation kit are suitable for direct use only. Freezing down and storage at -70°C is not recommended.

• The TransformAid kit works with ampicillin, tetracycline and chloramphenicol antibiotic resistance systems. Kanamycin systems show reduced transformation efficiencies of 105 cfu/µg DNA, which are still acceptable in routine cloning experiments.

• Do not use more than 5 µl (containing 10-100 ng of vector DNA) of ligation reaction mixture per 50 µl of competent cells.



Recipes:

Ampicillin Stock Solution (50 mg/ml)

Dissolve 2.5 g of ampicillin sodium salt in 50 ml of deionized water. Filter sterilize and store in aliquots at -20°C.

X-Gal Stock Solution (20 mg/ml)

Dissolve 200 mg of X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (#R0401) in 10 ml dimethylformamide (DMF) or dimethylsulfoxide (DMSO). Store at -20°C in a dark. Alternatively, use X-Gal Solution, ready-to-use (#R0941). Spread 40 µl on plate surface.

IPTG Stock Solution (100 mM)

Dissolve 1.2 g of IPTG (isopropyl-b-D-thiogalactopyranoside) (#R0391) in 50 ml of deionized water. Filter-sterilize, aliquot and store at -20°C. Alternatively, use IPTG Solution, ready-to-use (#R1171). Spread 40 µl on plate surface.

LB antibiotic Plates

Prepare LB agar medium (1 liter), weigh out:
Casein Hydrolysate 1.0 g,
Bacto Yeast extract 0.5 g
NaCl 0. 5 g.

- Dissolve in 80 ml of water, adjust pH to 7.0 with NaOH and adjust the volume with water to 100 ml. Add 1.5 g of Agar and autoclave.
- Before pouring LB ampicillin agar plates, allow the medium to cool to 55°C. Then, add 0.2 ml of ampicillin stock solution (50 mg/ml) to a final concentration of 100 µg/ml*. Mix gently and pour plates. Allow the LB ampicillin agar medium to solidify.
- For blue/white selection, spread 40 µl of each X-Gal stock solution (20 mg/ml) and IPTG stock solution (100 mM) on the surface of the plate. Alternatively, for batch use, add 1 µl of each stock per 1 ml of LB agar (cooled to 55°C).
- For fast and easy preparation of LB medium and LB agar plates supplemented with ampicillin, IPTG and X-Gal, use pre-mixed and pre-sterilized microwaveable media Thermo

Scientific FastMedia LB Liquid Amp (#M0011), FastMedia™ LB Agar Amp (#M0021), or FastMedia LB Agar Amp IPTG/X-Gal (#M0031).

- When using other antibiotics, the following final concentrations are recommended: kanamycin, 30 µg/ml; tetracycline, 12 µg/ml; chloramphenicol, 20 µg/ml.

Colony PCR : PCR confirmation of the selected positive colonies using gene specific primers and colony as template.

Requirements:

- dNTP
- MgCl2
- Buffer
- Primer
- Taq.DNA Polymerase
- PCR grade Water
- PCR tubes
- Pippetts
- Thermo cycler

Procedure:

1. On LB-ampicillin agr plate supplemented with X-gal and IPTG, make a grid
2. Take 5µl of PCR grade water in sterile conditions.
3. Pick up a white colony from the master plate containing blue and white colonies using a sterile tip.
4. Spot or streak the colony on the square marked and the dissolve it into PCR tube containing 5µl water. Repeat the process for required number of colonies.
5. Close the PCR tubes and incubate them at 94^{o}C for 5 min.
6. Immediately transfer tubes on ice.
7. Prepare the master mix as follows:

PCR reaction mixture	For 1 reaction
PCR grade Water	10.15 µl
10X PCR Buffer	2 µl
25mM MgCl2	1.2 µl
10mM dNTPS	0.4 µl
10µM Primer Forward & Reverse	0.5 µl each
Taq Polymerase enzyme	0.25 µl
Total reaction volume	15 µl

8. Add 15 µl of the reaction master mix to the dissolved and boiled colonies.
9. Place the tubes thermocycler and set the required temperature.

Step	Temperature	Time	
Initial denaturation	94^0C	5 min.	
Denaturation	94^0C	45 secs	**35 Cycles**
Annealing	55^0C	45 secs	
Extension	72^0C	2 min.	
Final extension	72^0C	2 min.	
On-Hold	10^oC		

Expression analysis of target gene at transcript level using RT-PCR

Total RNA isolation

(Adopted from Spectrum™ Plant Total RNA Kit as per manufacturers instruction)

Requirements:

1. Lysis solution
2. Binding solution
3. Wash solution 1and 2(concentrate)
4. Elution columns
5. Filtration and binding columns.
6. Liquid nitrogen
7. Mortar and pestle.

Procedure:

1. Grind 100mg plant sample (Leaf, flower and ovule tissue of cotton) to fine powder. Take 1ml lysis solution and 10µl β-mercaptoethanol.
2. Mix the plant sample with the lysis solution and vortex the solution for 30 sec.
3. Incubate at 56ºC for 3-5 mins and centrifuge at 12000g for 3 mins at 4ºC.
4. Collect the supernatant into blue ring retainer column centrifuge at 12000g for 3mins at 4ºC.
5. Discard the flow through and add 500µl solution.
6. Again centrifuge at 12000g for 1 min at 4ºC.
7. Discard the flow through and add 500µl wash solution centrifuge at 12000g for 1 min at 4ºC and repeat the step.
8. Discard the flow through and dry spin for 1 min at 12000g at 4ºC.
9. Transfer the column to 2 ml collection tube and add 30µl elution buffer.
10. Centrifuge at 12000g for 1 min at 4ºC.
11. Take aliquot and run on the gel.

Quantification of Total RNA

Requirements

- RNA elution buffer
- Molecular Biology grade Water
- Pipettes
- Tips
- Kimwipe tissue
- UV-Visible spectrophotometer/ Nonovalue calculator

Procedure:

1. Switch on the instrument, i.e. Nanovalue calculator and set it on DNA quantification setting. Alternatively set wavelength of 260 nm on UV-Visible spectrophotometer.

2. Pour a little excess of MB grade water on the marked surface. Wipe clean with a Kimwipe tissue.
3. Place 1µl of RNA elution buffer on the marked spot and set Blank value with it.
4. Mix the DNA sample well with gentle tapping. Place 1µl of Sample on the marked spot and read at 260 nm. Again read the same sample at 280 nm .
5. Wipe the marked spot clean with Kimwipe tissue.

First strand cDNA synthesis (Reverse Transcription) and PCR:

Protocol for first strand cDNA synthesis from total RNA was adopted from SuperScript® III First-Strand Synthesis from Invitrogen

Requirements:

- OligodT Primers (50 µM)
- 10X RT buffer
- mM MgCl2
- 0.1 M DTT
- 10 mM dNTP mix
- Reverse Transcriptase enzyme (RT) (200 U/µL)
- E. coli RNase H (2 U/µL)
- DEPC-treated water

Procedure:

For cDNA synthesis, SuperScript® III First-Strand Synthesis System from Invitrogen was used. The procedure is as follows:

The following procedure is designed to convert 1 pg–5 µg of total RNA or 1 pg–500 ng of poly(A)+ RNA into first-strand cDNA:

1. Mix and briefly centrifuge each component before use.
2. Combine the following in a 0.2- or 0.5-mL tube:

Component	Amount
up to 5 µg total RNA	n µL
Primer* *50 µM oligo(dT)20	1 µL
10 mM dNTP mix	1 µL
DEPC-treated water	Upto to 10 µL

3. Incubate the tube at 65°C for 5 min, then place on ice for at least 1 min.

4. Prepare the following cDNA Synthesis Mix, adding following components in specified order, as follows:

Component	1 Rxn
10X RT buffer	2 µL
25 mM MgCl2	4 µl
0.1 M DTT	2 µL
RNaseOUT™ (40 U/µL)	1 µL
SuperScript® III RT (200 U/µL)	1 µL

1. Add 10 µL of cDNA Synthesis Mix to each RNA/primer mixture mix gently, and collect by brief centrifugation.

2. Incubate as follows :

- Oligo(dT)20 or GSP primed: 50 min at 50°C
- Random hexamer primed: 10 min at 25°C, followed by
- 50 min at 50°C

3. Terminate the reactions at 85°C for 5 min. Chill on ice.

4. Collect the reactions by brief centrifugation. Add 1 µL of RNase H to each tube and incubate the tubes for 20 min at 37°C.

5. cDNA synthesis reaction can be stored at −30°C to −10°C or used for PCR immediately .

The PCR conditions are as follows:

PCR reaction mixture	For 1 reaction
PCR grade Water	14.15 µl
10X PCR Buffer	2 µl
25mM MgCl2	1.2 µl
10mM dNTPS	0.4 µl
10µM Primer Forward & Reverse	0.5 µl each
Taq Polymerase enzyme	0.25 µl
cDNA Template	1.0 µl
Total reaction volume	20 µl

TISSUE CULTURE TECHNIQUES

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Introduction

Plant tissue culture is the technique of growing plant cells, tissue and organs in an artificial nutrient medium under aseptic conditions. Plant cells are having totipotent; i.e. a single cell has the capacity and ability to develop into whole plant (animal cells lacks this capacity). Tissues from any part of the plant like shoot apex, bud, leaf, mesophyll cells, epidermis, cambium, anthers, pollen, fruit etc., can be used for *in vitro* culture called as explant. After inoculating the explant in a suitable medium under aseptic condition, the explant under goes formation of an amorphous mass of cells or a mass of disorganized, mostly undifferentiated cells known as callus (pl. calli). Callus can be induced to re-differentiate on appropriate medium to develop embryoids, which directly develop into the plantlets, eventually giving rise to a whole viable plant. Plant regeneration can also be achieved by organogenesis i.e. directly from meristamatic tissues or embryonic axes.

Tissue culture has many potential applications for crop improvement viz.,

1. Micropropogation of elite lines/ single superior plant.
2. Induction of somaclonal variation to develop new strains for crop improvement.
3. Tissue culture is an important tool for successful gene transfer and development of transgenic plants.
4. Fusion of protoplasts to obtain interspecific and inter-generic hybrids (which are difficult to obtain by conventional sexual hybridization).
5. Anther culture for development of elite plants to reduce breeding cycle.
6. Embryo rescue technique for interspecific hybridization (when conventional breeding fails due to post zygotic incompatibility).
7. Cloning of virus free plants. (Cloning means an organism obtained from a single cell through mitotic divisions. It is even applied to DNA multiplication i.e. cloning of genes in bacteria).

8. Development of synthetic seeds.
9. Conservation of genetic resources.

Stock preparation

Stock of MS medium (macro and micro ingredients)

A Media composition varies for different purpose such as callus induction, somatic embryogenesis, axillary bud proliferation, organogenesis, anther culture, embryo rescue etc. Tissue culture medium generally contains inorganic salts, growth hormones, carbon source, vitamins and gelling agent. Other components added for specific purposes include organic nitrogen compounds, hexitols, amino acids and plant extracts. Preparation of stock would help us to make precise concentration of ingredients. Secondly, the required medium can be prepared faster by mixing of stock solution without wasting much time on weighing each and every chemical.

Composition of Macro and Micro inorganic salts for MS medium and stock preparation (Murashige and Skoog, 1962)

Stock No.	Ingredient	Quantity / litre (1X) in mg	Stock/100ml In gm
	Macro-ingredients		
M1	Ammonium nitrate (NH_4NO_3)	1650	16.50 (10X)
M2	Potassium nitrate (KNO_3)	1900	19.00 (10X)
M3	Calcium chloride ($CaCl_2.2H_2O$)	440	4.40 (10X)
M4	Magnesium sulphate ($MgSO_4.7H_2O$)	370	3.70 (10X)
M5	Potassium dihydrogen phosphate ($KH_2 PO_4$)	170	1.70 (10X)
	Micro-ingredients		
M6	i. Manganese sulphate ($MnSO_4.4H_2O$)	22.3	1.115 (50X)
	ii. Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	0.430 (50X)
	iii. Boric acid (H_3BO_3)	6.3	0.315 (50X)
	iv. Potassium iodide (KI)	0.83	0.0415 (50X)
	v. Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25	0.0125 (50X)
*M7	i. EDTA disodium salt	37.3	1.865 (50X)
	ii. Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	1.390 (50X)
M8	i. Copper sulphate ($CuSO_4.5H_2O$)	0.025	0.00125 (50X)
	ii. Cobaltous sulphate ($CoSO_4.6H_2O$)	0.025	0.00125 (50X)

*** Preparation of iron stock (M7)**

Weigh the required quantity of Ferrous sulphate and EDTA disodium salt and dissolve in 50 ml warm distilled water separately and then mix ferrous sulphate into Na_2 EDTA.

Stock of Growth hormones:

Growth hormones are not nutrients, but they have influence on plant growth and development. There are four classes of growth hormones are commonly used in tissue culture media i.e. auxins, cytokinins, gibberellins and abscisic acid. In general, auxins promote cell enlargement, root initiation and adventious bud formation, whereas cytokinins promote cell division, somatic embryogenesis and shoot initiation. The type of growth hormones and concentration used will vary according to the cell culture purpose.

Auxins:

1. Indole acetic acid (IAA): Dissolve 100mg of IAA in 1ml of 1 N NaOH and make up the volume to 100ml to give a concentration of 1mg/ml.
2. Indole butyric acid (IBA): Dissolve 100mg of IBA in 1ml of 1 N NaOH and make up the volume to 100ml to give a concentration of 1mg/ml.
3. 2,4 Dichlorophenoxy acetic acid (2,4-D): Dissolve 100mg of 2,4-D in 1ml 1M NaOH, if not dissolve in little alcohol and make up the volume to 100ml to give a concentration of 1mg/ml.
4. Gibberellic acid (GA_3): Dissolve 100mg of GA_3 in 1ml 1M NaOH and make up the volume to 100ml to give a concentration of 1mg/ml
5. Naphthalene acetic acid (NAA): Dissolve 100mg of NAA in 1ml 1N NaOH, if not dissolve in little alcohol and make up the volume to 100ml to give a concentration of 1mg/ml.

Cytokinins:

1. Benzyl aminopurine (BAP or BA): Dissolve 100mg of BAP in 1ml 1N NaOH and make up the volume to 100ml to give a concentration of 1mg/ml.
2. Kinetin: Dissolve 100mg of Kinetin in 1ml 1M NaOH and make up the volume to 100ml to give a concentration of 1mg/ml.
3. 2- Isopentenyl adenine (2-ip): Dissolve 100mg of 2-ip in 1ml 1N NaOH and make up the volume to 100ml to give a concentration of 1mg/ml.

Note: prepare all the stocks in sterile bottle and with sterile distilled water.

Organic supplements:

Myo-inositol	100 mg/l
Nicotinic acid	0.05mg/l
Pyridoxine HCl	0.05mg/l
Thiamine HCl	0.05mg/l
Glycine	0.20mg/l

Activated Charcoal

It frequently added to media to adsorb toxic phenols. It usually added at 0.6g/L. Sometimes it is added to rooting media to adsorb root-inhibiting agents.

Casein hydrolysate

It is an undefined protein mixture that is occasionally used in media as a non-specific source of organic nitrogen. Casein, a phosphoprotein in milk, is hydrolyzed (treated with water) to form this weak acid.

Antioxidants

The antioxidants such as citric acid, ascorbic acid, pyrogallol, phloroglucinol and L-cysteine are used in tissue culture to reduce excessive browning of the explants. Adsorbents like PVP and activated charcoal are also used for checking excessive browning.

Natural Complexes

The natural complexes such as coconut milk, yeast extract, malt extract, tomato juice, potato extract etc., are used in tissue culture for various purposes. Addition of these complexes in the medium make the medium undefined, since variation in growth promoting or inhibiting compounds in these complexes.

Preparation of MS Agar medium (1-Lit.)

1. Pour 600ml of distilled water into a flask (2-litre capacity).
2. Add 10 ml stock of macro (M1-M5) and 2 ml of micro (M6-M8) + 1ml vitamin + auxin and cytokinins as per the requirement. It varies with the application.
3. Weigh and add 30g sucrose and 100mg-meso inositol.

4. Place the flask on the hot plate / stirrer, slide a magnetic stir bar and turn on the stirrer.
5. Turn off the stirrer after dissolving the sucrose completely and add distilled water to the flask up to 950ml.
6. Adjust the pH of the medium to 5.6 - 5.8 with 0.1N NaOH or 0.1N HCl and make up the volume to 1000ml.
7. Weigh and add 8g of agar powder.
8. Turn on the hot plate/ stirrer heat. Continue to heat and stir until the medium boils (but do not allow to boil over)
9. Turn off the hot plate and make up the volume to one lit.
10. Disperse the medium at 10-15ml per test tube/ petri plates or conical flasks.
11. Cap the container and sterilize it in an autoclave at 15lb/sq.inch pressure (121º C) for 15 min.
12. After sterilization prepare the slants. After solidification label the test tubes and store the medium in a cool and clean place.

Preparation of MS Liquid medium

For liquid medium preparation prepare 1 litre medium as described above without agar powder. Liquid medium is faster to make than agar medium, as there is no need of dissolving agar before dispensing to the test tube. This medium is used for protoplast culture.

Initiation and Maintenance of Callus and call suspension culture

Callus and suspension cultures are widely employed for the regeneration of somaclonal variation, selection of various kinds of cell lines, production of somatic embryos in-vitro production of secondary metabolites and in cell genetic manipulations. Although callus can be induced from any explant under appropriate conditions, young tissues in the meristematic zone give best response. Nutritional and hormonal requirement are earliest to elicit desired response.

Plant material: In-vitro germinated cotton seedlings.

Equipments/ reagents/glassware:

- Erlenmeyer flask (250 ml) containing 100 ml 70% ethanol
- Erlenmeyer flask (250 ml) containing 100 ml of 0.1% mercuric chloride ($HgC1_2$) made in distilled water
- Two scalpels
- Two large forceps
- Beakers
- Spirit Lamp and matchbox
- Glass marking pen, petri dishes.

Culture media:

(a) Solid medium for initiation and maintenance of callus culture.
(b) Liquid medium for initiation of suspension culture.
(c) Half MS medium for seed germination.

Experimental procedure :

- ✓ Wash the seed material with tap water 2-3 times
- ✓ Wash the seeds with detergent by continuous shaking.
- ✓ Transfer the seeds in 250 ml glass beaker and add 0.2% mercuric chloride solution keep the seeds for 7 min.
- ✓ Wash 3-4 times with autoclaved Double distilled water.
- ✓ Flame the individual seeds after dipping one by one in rectified spirit.
- ✓ Inoculate the individual seed in culture tube containing 1/2 MS medium.
- ✓ Allow the seeds to germinate under dark for 6-8 days.
- ✓ Take out the individual seedling from culture tube and harvest the hypocotyls piece of about 0.5 -1.0 cm length and cultured in MS medium containing growth hormone.
- ✓ Transfer the cultures in culture room unit 16:8 hrs. Photoperiod and temperature of 25 + 2°C and illuminated by cool fluorescent light.
- ✓ Observe the culture for callus induction.

Initiation and maintenance of suspension culture

Transfer small pieces (about 2-4g) of callus into 40 ml of liquid medium. Place the culture flasks on a gyratory shaker of about 100-120 rpm. If the callus in friable, item in 2.3 weeks, the cells of callus disperse and grow to form cell suspensions.

They are now ready for subculture.

-Take 10 ml a aliquots of the suspension and pour them into culture flasks containing 30 ml fresh medium of same composition.

- The flasks arc incubated on a gyratory shaker in a culture room. At the end of every 2-4 weeks when the growth of suspension cultures becomes stationary the process of sub-culture is repeated.

Observation: Callus culture

After a week of inoculation cell proliferate at the cut end of the explants result into a dumb-bell shape structure. Continued cell division will covered the entire explants with unorganized callus growth in 2-3 week:. At the end of 4-6 weeks the callus mass is divided into 2-4 equal segments & transfer into fresh culture medium of the same composition. After several such sub-culture callus growths become uniform and the stock callus cannot be used to measure the growth and to carry out various morphogenic physiological and genetic Experiments, by altering the level of nutrients in the medium and by supplementing it with plant growth hormones in various concentration and combinations. Very useful information can be generated on growth and nutritional aspects of the tissue as well as about their effects on morphogenesis and other physiological and biochemical parameters.

Suspension culture:

During initial passages, cells in suspension culture remains as large aggregates but with subsequent sub-culture, they become friable. After 6-8 sub-cultures cell suspension can be filtered though appropriate pore size nylon mesh to remove large clamp.

Shoot tip and Meristem culture

In-vitro multiple shoot induction is an alternate means of asexual propagation of the plant species, aims of reproducing plant of desirable quality uniformly and in quality. It can proceed via enhanced axillary branching or through formation of adventitious shoot/somatic embryos on the explant. The procedure is simple and includes initiation of culture; shoot multiplication rooting and transplantation in the field.

Plant material:

In-vitro germinated seeding field grown plant material can also be taken.

Equipment/glassware: Laminar airflow chamber, scalpel, sterilized, petri dishes, plastic pots, polythene bags, forceps etc.

Chemicals/Reagents: Ethanol, 0.1% mercuric chloride, 0.2% Bavistin, teepol, media, sterile distilled water soilrite.

Protocol: Shoot tip culture

-Isolate shoot tip along with meristem from in-vitro germinated seedlings.

-Remove both the cotyledonary leaf.

-Inoculate shoot tips placing vertically into the medium (MS medium) supplemented by growth regulator.

-Incubate the cultures at 25+2° C and 16:8 hr. photoperiod for one month with 3-4 sub-cultures weekly in the same medium.

Shoot generation:

After one month shoot buds appeared on the surface of the explants.

-Transfer the multiple shoot bud stock into the fresh medium.

-After 20-25 days, buds will elongate into well define shoots.

-Excise the individual shoot and transfer into root induction medium (MS) and keep the cultures at 25+2° C and 16:8 hrs. Photoperiod.

-Transfer remaining portion of multiple shoot stock into fresh medium and keep at 25+2°C & 16 hrs. Photoperiod.

Field transfer:

-Remove the plantlet from culture tube.

-Wash carefully so as to remove the agar.

-Dip the roots in 0.2% Bavistin for 2 min.

-Transfer in plastic pot containing soil and water lightly.

-Cover with perforated polythene bags to maintain high humidity around. Keep in net house.

-Remove the polythene bags after 2 weeks

-After 4-6 weeks, remove the plants along with a soil and transfer to soil.

Test for determining cell viabi1ity

A cell or an organism is said, to be viable if it is able to perform various metabolic functions such as respiration, synthesis of new compounds etc. Hence viability tests attempt to assess one or more of the above vital function. Germplasm conservation requires that the cells or tissues should not only remain alive but also be capable of multiplication (mitoses). But assessing capability for cell division and growth is time consuming and technically demanding. Since viability is a prerequisite for growth, viability test provide a quick assessment and set the upper limit *of* growth potential. Therefore for a reliable result viability test should be compared. The principles, procedures, advantages, and limitations of various tests used for determining viability are given below.

Tetrazolium test :

Principle: Reduction of triphenyl tetrazolium chloride (TCC) to formazan (a colored compound) by succinate in respiratory chain in mitochondria.

This list can be used for visual assessment of viability; alternatively, formazan can be extracted from cells with 95% ethanol and absorbance recorded at 485 mm (using a spectrophotometer) for quantitative assessment of viability.

Protocol:

1. Prepare 1% stock solution of TTC in 0.05 M phosphate buffer (pH 7.5)
2. Take an aliquot of suspension culture and add an equal volume of stock solution of TTC.
3. Mix and incubate at 22° C for 18-22 hrs. in the dark.
4. Determine the proportion of cells with red colour and express as percentage.

Application: Widely used in viability of seeds, embryos cells etc. and in general shows good correlation with germination or cell division. However, it is not a rapid method and photo reduction of TTC can lend to erroneous results. Further, colored compounds in tissue/cell many interfere with the test.

Fluorescein diacetate (FDA) test (Rotman and Papermaster, 1966):

Principle: Hydrolysis of FDA by esterases to yield fluorescein leading to fluorochromasia.

$$\text{FDA} \xrightarrow{\text{Esterase}} \text{Fluorescein}$$

FDA, a non-polar compound enters into cells where it is hydrolyzed by esterases yielding fluorescein, a polar compound. Non-polar FDA passes through plasmalemma at a faster rate than the outward movement of fluorescein, the latter accumulates inside the cell which can be visualized under a UV microscopic.

Protocol:

*Prepare a stock solution of FDA in acetone (5 mg/ml) and store in a freezer.

*Dilute stock solution and use within 30 minutes

For cells and protoplasts: Dilute in culture medium to a final concentration of 0.01% FDA (Widholm, 1972)

For Pollen: Dilute in 0.5 M sucrose solution to a final concentration of approximately 10^{-6} M FDA (Heslop-Harrisoon & Heslop Horrisoon, 1970)

* Observe under UV microscope with appropriate filter sets (Excita filter Be-12 and Barria filter No.4) giving transmissions of 330-500 nm and 460 nm respt.)

* Viable cells will fluoresce green while dead cells show no florescence (which can be verified by viewing under visible light)

* Determine proportion of viable cells to dead cells in at least 10 different fields and express as percentage.

This is a rapid test and gives reliable information not only of the enzymatic activity within the cell but also the functional integrity of the plasma membrane. If there is membrane damage, fluorescein will leak out and no fluorochromasia will be observed. Further, it is a non-destructive test and the stained cells can be cultured to obtained plants (Nadel, 1989). Need for a UV microscope and unstable nature of FDA are the drawbacks of this method.

Phenosafranine Staining (Widholm, 1972)

This is a negative test because phenosafranine selectively stains dead cells. The exact principle of this staining reaction is not known, perhaps plasmalemma acts as & barrier to the entry of stain into the cell.

Protocol:

-Prepare a stock solution of phenosafranine (0.1% W/V) in culture medium.

-Mix 1 drop of stain with drop of tobacco cell suspension on a slide and lower a cover slip over the cells.

-Observe under a light microscope (Dead cells with stain red)

-Determine viability percentage.

-This is, a simple and rapid test, staining occurs soon after the addition of dye and distinct differences between viable and dead cells arc seen even after 30 minutes.

The solution is stable and only ordinary microscope is sufficient. But dead cells devoid of cell contents will not take stain and hence may be miscounted as viable.

Evan' s blue staining:

This is also a negative test like phenosafranine and only dead cells take up the stain.
The principle of dye exclusion may be similar to the previous test.

Protocol :

- Prepare stock solution of Evan's blue (0.025% W/v) in culture medium.
- Mix 1 drop of stain with 1 drop of suspension culture on a slide and place a cover glass.
- Incubate for 5-10 minutes and observe under a light microscope (Dead cells will stain blue)
- Determine viability percentage

This is also simple and less demanding test.

Protoplast Streaming or Cyclosis (Kamiya, 1959):

This is another method of measuring viability of cells. This is usually best-observed using phase contrast microscopy. However, cyclosis cannot be readily visualized in many cells. This method is cumbersome and quantification is difficult especially in cell clumps. Several other methods such as measurement of oxygen uptake, use of bio-luminescence etc. are also used in some cases but they are technically demanding and have only a narrow application.

Appendix:

SI Units of Mass (Weight)

Weight	**Symbol**	**Multiple of gram**	**Lower Equivalent**
Mega gram	M	10^{6}	1000 kilogram
Kilogram	Kg	10^{3}	1000 gram
Gram	g	1	1000 milligram
Milligram	mg	10^{-3}	1000 microgram
Microgram	µg	10^{-6}	1000 nanogram
Nanogram	ng	10^{-9}	1000 picogram
Picogram	pg	10^{-12}	1000 femtogam
Femtogam	fg	10^{-15}	1000 Attogram

Plant Transformation and Tissue Culture

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Transgenic Cotton also called as Genetically Modified Cotton, contains a gene (DNA fragment) of desire trait. The genetic material is isolated from soil bacterium *Bacillus thuringinesis* and with the help of *Agrobacterium thuringinesis*, it is transferred into the plant cell through genetic engineering. The transformed plant cells are then selected on kanamycin containing medium and thereafter they are regenerated into whole plants by Tissue Culture methods such as organogenesis or somatic embryogenesis.

The genetically modified cotton, popularly known as "Bt-Cotton" was first commercialized by Monsanto (USA) and later by various multinational companies such as Syngeuta, Bayer, DOW, CAAS, JK seeds and Meta helix. So far in India more than 1500 Bt hybrids belonging to different events have been released for commercial cultivation. Because of Bt cotton cultivation the insecticides usage has declined to 25 % compare to 46 % used before release of technology. The Bt technology has tremendous potential and helped to boost cotton production and productivity.

Approved events of Cotton in India:

1. *Cry* I Ac (Mon 531) - Mahyco Monsanto biotech limited.
2. *Cry* 2 Ab (Mon 15985) - Mahyco Monsanto biotech limited.
3. *Cry* I Ac (Event 1) - J.K Agri Genetics seeds limited sourced by IIT Kharagpur.
4. *Cry* I Ab and Cry IAc (GFM event) - Nath seeds.
5. *Cry* I Ac - Dharwad event.
6. Cry Iac(9124) - Metahelix

Steps for Plant Transformation

Agrobacterium mediated transformation is an important technique of gene transfer for the development of transgenic cotton. Through this technique we can incorporate any gene including Bt gene in cotton plants. The following protocol has standardized at CICR, Nagpur for incorporation of different genes and steps involve are given below

Delinted seeds of any cotton variety

↓

After ginning the seeds are treated with dil. Sulphuric acid to remove fuzz. After washing 3-4 times the seeds are treated with lime water and again washed with water several times and dried in sunlight.

The Delinted seeds are first rinsed in tap water and then treated with Bavistine for 15-20 min. After thoroughly washing with DDW, seeds are inoculated in half MS medium in conical flask.

Surface sterilization

↓

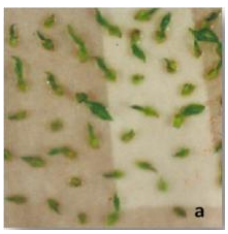
In vitro seed germination (3 to 7 days seedlings)

↓

Excise Shoot tips / embryo axis

↓

Infection of above explants by overnight grown *Agrobacterium* culture for 3 min



Shoot tips are isolated by removing two cotyledonary leaves from the seedlings. A sharp oblique cut is made just below the cotyledonary node. The shoot tips are collected in Petri dish and infected with overnight grown culture of *Agrobacterium*

↓

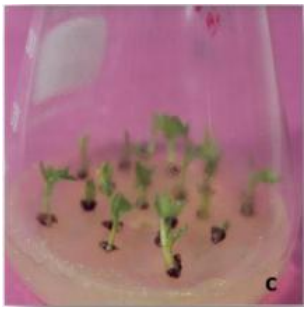
Decant *Agrobacterium* suspension, blot dry and transfer them on MS plane medium, with 30 gm/L glucose, inositol (100 mg/L) and thiamine (10 mg/L) 4 days for co-cultivation.

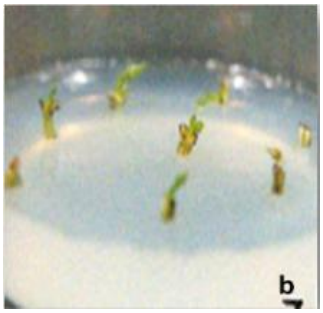
↓

Remove explants form the above medium and culture on MS medium containing carbenicillin / cephotaxime (500 / 250mg/L) individually or in combination depending on the intensity of *Agrobacterium* growth for one week. Depending on the Agrobacterium contamination, one more passage of sub culturing can be given on the same media.

↓

Remove the explants and culture them on MS medium containing kanamycin (50 mg/L) and carbenicillin (500 mg/L) 10-15 days for selection.





↓

The transformed explants can grow on medium containing kanamycin. Isolate growing explants and culture them for regeneration in MS medium supplemented with glucose (3 mg/L) + inositol (100 mg/L) + thiamine (10 mg/L) and kinetin (0.1 mg/L) + kanamycin (50 mg/L)

The shoot tip explants are either regenerated directly into shoots or multiple shoots are induced. The well differentiated shoots are isolated and rooted.

↓

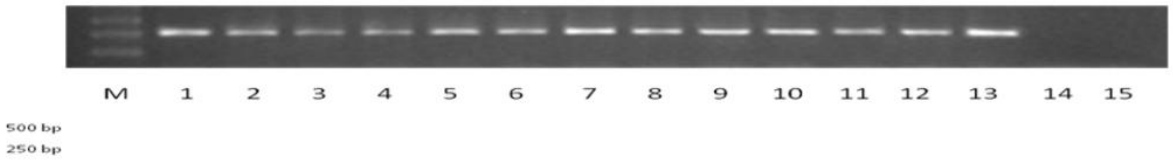
The elongated shoot are transferred on MS medium containing NAA (0.1 mg/L) for rooting

↓

The rooted plantlets are hardened and established in the pot containing soil (To plant)

↓

T0 plants are characterized by PCR for the presence of gene introduced through *Agrobacterium*



M, 1 Kb Ladder, 1-12, +ve transgenic plants, 13, Bunny Bt +ve sample, 14, Non-transgenic RG8 sample, 15-PCR –ve.

↓

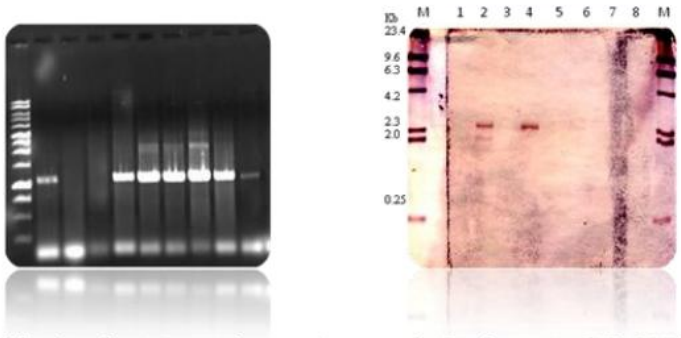
The PCR positive T0 Plants are selfed and seeds collected on boll basis individually.

↓

Seeds of individual selfed boll are sown as boll to row progeny

↓

Progenies of individual boll are characterized for gene integration and expression by **PCR** and **southern blot** and **ELISA** and homozygous plants are identified



PCR amplification of cry1Ac gene fragment in transgenic *G. arboreum* cv. RG8. M,1 kb ladder; 1, positive control; 2, negative control; 3, non-Bt; 4–9, Bt-RG8—lines 12, 16, 18, 37, 59, and 82. **b** Southern hybridization of genomic DNA of transgenic *G. arboreum* cv. RG8 cotton using fragment of cry1Ac gene as DNA probe. M, λ-HindIII ladder; 2, transformed plant, 4, CryIAc positive, 6, Non-transgenic –ve.

↓

Homozygous plants are selfed and forwarded to next generation

↓

RCGM / GEAC Trials (Point of integration, Biosafty analysis and insect bioassay)

↓

Commercial release

↓

Cultivation by Farmer

Molecular characterization of transgenics

Dr. J. Amudha

Senior Scientist (Biotechnology)

A. Transgene integration analysis

1. PCR amplification of transgene

Polymerase chain reaction is an ingenious new tool for molecular biology. PCR is very sensitive method that a single DNA molecule has been amplified and single copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels. Polymerase is an enzyme that polymerizes any DNA material *in vitro*, if it is supplemented with other minimal requirements such as dNTPs (deoxy nucleotide triposphates) and the primers.

The cocktail is incubated at different temperatures e.g. 94°C, 45°C and 72°C which are responsible for the denaturation of the DNA, annealing temperature of primers with the DNA and polymerization of nucleotides on template DNA strand respectively. The PCR protocol differs with different situations.

PCR Principle: The core of the gradient PCR (Biometra®) is the high performance thermo block high power Peltier elements temper a sample block made of gold plated solid silver. This achieves high heating and cooling rates. Also there is a facility of setting a temperature gradient to carry out the priming reaction at different temperatures; here a range of temperature is entered in to the instrument e.g. 55°C-65°C, accordingly the instruments sets 12 different temperatures in 12 different wells in a row of sample block.

Materials:

- DNA sample
- 25mM dNTPs
- 25mM MgCl2
- 10 X PCR buffer (Taq buffer B)
- Primer

- Sterile distilled water
- Taq DNA polymerase

PCR protocol

Method: A master mix (reaction mixture) was first prepared in one eppendorff tube as follows, For one sample (reaction mixture):

Sr. No.	Reagents	Quantity
1	Sterile distilled water	17.1µl
2	Taq buffer B	2.5µl
3	MgCl2	1.6µl
4	DNTPs	0.5µl
5	Primer (forward)	1µl
6	Primer (reverse)	1µl
7	Taq polymerase	0.3µl
8	DNA template	1µl
	Total	**25µl**

- The master mixture was mixed properly and 19µl of it was distributed to each tube in which then 1µl of template DNA was added.
- The tubes was placed in the PCR machine, and the gradient was set having temperature range 55°C-65°C with following PCR program:

Step 1: Temp-94°C, Time-5min

Step 2: Temp-94°C, Time-45sec

Step 3: Temp- 56 °C Time-45sec

Step 4: Temp-72°C Time-60sec

Step 5: **35 cycles**

Step 6: Temp-72°C, Time-5min

Step 7: Temp-4°C Time- variable

- After completion of all the cycles, the tubes was taken out and preserved at 4°C.
- The amplified samples were resolved on 1.5% agarose gel and observed the banding pattern under UV-light.

2. Southern Blotting Technique

1. Isolate the DNA from the transgenic plant.
2. Digest the DNA with an appropriate restriction enzyme.
3. Run the digest on an agarose gel.

Further experiment on the DNA fragments in agarose gel is difficult as the gel is very difficult to handle. This has been made possible by transferring the DNA fragments to nitrocellulose. This blotting technique was first described by Southern in 1975 and is called `Southern Blotting'.

By this technique the separated DNA fragments are transferred and immobilized on the nitrocellulose. It can then be hybridized using a radioactive/nonradioactive labeled probe to identify the appropriate gene among the fragments. The procedure for blotting is described below.

PRINCIPLE

The DNA molecules in the agarose gel are transferred to nitrocellulose membrane either by capillary action (capillary blotting) or by electrophoresis (electroblotting). The smaller fragments are more readily transferred than the longer ones. The DNA molecules are immobilized to nitrocellulose when heated at 80 under vacuum after transfer.

Southern blotting was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s.

Materials:

1. Nitrocellulose filters
2. Whatman 3 filter paper
3. **Depurination solution**

Conc. HCI 2mI

Distilled Water 100mI

4. **Denaturing solution**

NaOH (0.5 M) - 20g

NaCl (1.5M) 87g

Water to 1L

5. **Neutralizing solution (pH 7)**

Tris-HCI (0.5M) – 78.8g

NaCl(3.0M) 174g

Water to 1 L

6. **Saline sodium citrate (SSC) pH 7 20X**

Sodium citrate (0.3M) 88.2g

Sodium chloride (3M) 175.3g

Water to 1L

Prepare 10-fold concentrated SSC and store.

BLOTTING ON MEMBRANE

1. Transfer the agarose gel, after electrophoresis, to a suitable large dish. A thin sheet of plastic slightly larger than the gel is used for this transfer.
2. Immerse the gel for 15min in depurination solution. Decant the solution and repeat the process.

Note: **Depurination**: A depurination step is optional. Fragments greater than 15 kb are hard to transfer to the blotting membrane. Depurination with HCl (about 0.2M HCl for 15 minutes) takes the purines out, cutting the DNA into smaller fragments. Be aware, however, that the procedure may also be hampered by fragments that are too small.

3. Decant the depurination solution and immerse the gel for 15min in denaturing solution.
4. Replace with fresh solution and continue soaking for another 15 min.

5. Repeat the procedure twice with neutralizing solution.

Note: Separate double-stranded DNA into single-Stranded DNA. Only ssDNA can transfer.

6. In a suitable glass tray place a glass plate (somewhat larger than the gel) on the platform at a height of about 2cm.

7. Place on top three large pieces of Whatman 3MM paper to serve as a wick. Add 1.0 x SSC to the tray and wet the wicks completely.

8. Over the wick, place 10 sheets cut exactly the same size of the gel to be blotted. Wet these sheets also and press out any air bubbles between the sheets.

9. Place the pre-treated gel on top of this stack between two plastic spacers across the wick.

10. Cut the nitrocellulose filters to the same width but slightly longer than the size of the gel, gently float it on 2X SSC, thoroughly wet and immerse down.

11. Wet the surface of the gel with 2X SSC. Place the wet nitrocellulose on the gel starting at the middle and extending along both the ends, finally the ends lying over the spacers. Express out any bubbles between the gels and filter carefully using forceps.

12. Place five sheets of Whatman 3MM paper (gel size) above the gel and then a wad of tissue papers. Place a glass or plastic plate over the top and add a weight on it (a brick or a flask with one litre of water).

13. Allow the transfer to proceed overnight or even longer. The buffer moves upward by capillary action via the gel and filter. During this process the DNA fragments are transferred out of the gel and retained on the nitrocellulose because of its greater affinity, thus making a replica of gel pattern.

14. Dismantle the assembly and recover the filter. Soak in 2X SSC for 15min. Observe under UV transillumination. The ethidium bromide will transfer with the DNA so it should be possible to visualize some pattern.

15. Dry the filter by placing between two layers of 3MM paper and placing in a vacuum oven (3h at 80°C). The DNA will be immobilized by this drying.

Probe Preparation: Dig DNA Labelling and detection

Labelling principle

DIG-labeled DNA probes are generated according to the method of random primed labeling which is based on the hybridization of random oligonucleotides to the denatured DNA template. The complementary DNA strand is synthesized by Klenow enzyme which uses the [3`OH] termini of the random oligonucleotides as primers and a mixture of deoxyribonucleotides containing DIG-11-dUTP, alkali-labile for elongation. DIG dUTP is incorporated every 20-25 nucleotides into the newly synthesized DNA. This density of haptens in the DNA yields the highest sensitivity in the detection reaction.

Test principle

The DIG DNA Labeling and Detection Kit uses digoxigenin (DIG), a steroid hapten, to label DNA probes for hybridization and subsequent color detection by enzyme immunoassay.

- Denature the DNA by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath.

Note: Complete denaturation is essential for efficient labeling.

- Add the following to the freshly denatured probe or control DNA.

Reagent

1. Hexanucleotide Mix, 10 × (vial 5)
2. dNTP Labeling Mix (vial 6)
3. Klenow enzyme labeling grade (vial 7)

- Incubate for 1 h to 20 h (overnight) at +37° C.
- Stop the reaction by adding 2 l 0.2 M EDTA (pH 8.0) and/or by heating to +65°C for 10 min.

Note: The length of the DIG-labeled fragments range from 200 to 1,000 bp.

Hybridization

- Pre-heat an appropriate volume of **DIG Easy Hyb buffer** (20 ml/100 cm^2 filter) to hybridization temperature.
- Prehybridize filter for 30 min with gentle agitation in an appropriate container.

Note: Membranes should move freely, especially if you use several membranes in the same

prehybridization solution.

- Denature **DIG-labelled DNA probe** (about 25 ng/ml) by boiling for 5 min and rapidly cooling in ice/water.

Note: As DIG-11-dUTP is alkali-labile, DNA probes cannot be denatured by alkali treatment (NaOH).

- Add denatured DIG-labelled DNA probe to pre-heated DIG Easy Hyb buffer (3.5 ml/100 cm membrane) and mix well but avoid foaming bubbles may lead to background).
- Pour off prehybridization solution and add probe/hybridization mixture to membrane.
- Incubate at least 6 hours to O/N with gentle agitation at hybridization temperature.

Stringency washes

- Wash 2 × 5 min in ample 2 × SSC, 0.1% SDS at +15 to +25° C under constant agitation.
- Wash 2 × 15 min in 0.5 × SSC, 0.1% SDS (prewarmed to wash temperature) at +65 to +68° C under constant agitation.

Immunological detection

- After hybridization and stringency washes, rinse membrane briefly (1-5) min in **Washing buffer**.
- Incubate for 30 min in 100 ml **Blocking solution**. Incubate for 30 min in 20 ml **Antibody solution.** Wash 2 × 15 min in 100 ml **Washing buffer**.
- Equilibrate 2-5 min in 20 ml **Detection buffer**.
- Incubate membrane in 10 ml freshly prepared **Color substrate solution** in a appropriate container in the dark. **Do not shake** during color development.
- ***Note:*** The color precipitate starts to form within a few minutes and the reaction is usually complete after 16 h. The membrane can be exposed to light for short time periods to monitor color development. Stop the reaction, when desired spot or band intensities are achieved, by washing the membrane for 5 min with 50 ml of sterile double dist. water or with **TE-buffer**.
- Results can be documented by photocopying the wet filter or by photography.

B. Gene expression study by Real Time-PCR

Real time PCR

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for the detection of products in real-time PCR are:

(1) non-specific fluorescent dyes that intercalate with any double-stranded DNA,

(2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

Frequently, real-time PCR is combined with reverse transcription to quantify messenger RNA (mRNA) and non-coding RNA in cells or tissues. qPCR is the abbreviation used for real-time PCR. Real-time reverse-transcription PCR is often denoted as: qRT-PCR. The acronym "RT-PCR" commonly denotes reverse transcription polymerase chain reaction and not real-time PCR, but not all authors adhere to this convention.

Basics of Real Time PCR

PCR can be broken in to four major phases the linear ground phase, early exponential phase, log linear phase (also known as exponential phase),and plateau phase (Tichopad et al.,2003). During the linear ground phase (usually the first 3-15 cycles), PCR is a just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence has calculated at this time. At early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. During the exponential phase, the

amount of PCR products approximately doubles in each cycle. As the reaction proceeds however, reaction components becomes limiting. At this point, the reaction slows and enters the plateau phase.

Relative Quantification:

Relative quantification describes a real time PCR experiment in which the gene of interest is one sample (i.e .treated) is compared to same gene in another sample (i.e untreated). The results are expressed as fold up or fold down regulation of the treated in relation to the untreated. A normalize gene (such as β – actin) is used as a control for experimental validity in this type of quantification. Relative quantification or comparative quantification measures the relative change in mRNA expression levels .It determines the changes in steady state m RNA levels of gene across multiples sample and expresses it relative to the levels of other RNA.

Procedure:

Primer designing:-Sequence of gene *DREB 1A*: Forward primer: GCGA TATTTGAGATGCCGAGTT Reverse primer: CGCCGTCGACTTCATGATTA

The primer for gene *DREB1 A* was prepared by using softwares from the MxPro software by selecting QPCR Internet Links from the Tools menu.

Note: - When using a software program to design primers and probes, it is important to set the concentration of monovalent ions (Na+/K+) and divalent ions (Mg2+) to those that are used in your reaction for accurate melting temperature prediction. (The buffer conditions will generally be in the range of 50–100 mM monovalent cation and 1.5–5.5 mM Mg2+.)

Reference dye: -

Reference dye has been used to correct for sample to sample signal variation that is not due to the chemistry itself (e.g., aliquotting errors or deficiencies in the signal uniformity due to the instrument optical system). ROX was used as reference dye.

Procedure: -

RNA sample preparation: -

Purified RNA was used for RT-PCR. All the cells contain ribonucleases it was difficult to isolate RNA. This difficulty was overcome by proper handling of instruments and

sample in RNA isolation protocol. This was done by Rnases free reagents and plastic wares. For preparing Rnase free 0.1% DEPC and Rnases away solution were used.

Measuring RNA quality: -

Absorbance

RNA purity was measured using nanodrop by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm (A260/A280).

Gel Electrophoresis

Gel electrophoresis technique was used to check the quality of isolated RNA. By this technique discrete intact ribosomal bands were visualized and determined the degree of degradation of RNA. Ribosomal RNA bands were seen at the ratio of 28s to 18s 2:1.

Prepare the following reaction mixture by carefully pippeting into the bottom of microfuge tubes-

Reaction mixture (µl)	Master mix
Reaction buffer	10µl
Forward primer	0.5µl
Reverse primer	0.5µl
water	7µl
cDNA	2µl
Total Volume (µl)	20µl

- The software actually measures for each well the cycle number at which the fluorescence crosses an arbitrary line called the threshold. This crossing point is known as the Ct value. More dilute samples will cross at later Ct values.
- The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level).
- Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).

Cry1Ac-ELISA kit (Bt-Quant)

Cry1Aa, Cry1Ab and Cry1Ac -toxin detection and quantification Immunoassay kit.

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Introduction:

Cry1Ac (Crystal 1Ac), Cry1Ab or Cry1Aa proteins are being expressed in transgenic crop plants developed specifically for insect pest management. It is essential to monitor the levels of toxin expression both in the initial and final stages of development of transgenic plants. This has necessitated the development of some reliable tools for the detection of these proteins in transgenics.The ELISA kit developed by CICR facilitates a precise quantification of all three toxins, expressed in transgenic plants. The kit is simple, cost effective and very reliable. It takes about 2.5 hrs for completion of one set of ELISA assay. Each ELISA plate can be used for 96 samples (including four wells for standards and two for blank). Depending on the capabilities of a laboratory hundreds of samples can be processed in a single day.

Additionally the ELISA kit can also be used for the quantification of Bt toxins in Bt *(Bacillus thuringiensis*) insecticide formulations. Calibration standards are included with the kit.

Test Principle:

Antigen (Cry toxin) is detected through the use of sandwich ELISA. AntiCry1Ac antibodies are coated on ELISA plates, which capture the antigen (cry toxin) present in unknown or standard samples. HRP-conjugated antibodies are then used to bind to the captured antigen and HRP enzyme-assay is carried out in the plates by adding TMB/H_20_2 (HRP substrate) to quantify the amount of antigen present in the sample. Absorbance at 450 nm is read in an ELISA reader. The minimum limit of detection is 1.0 ng (1×10^{-9} gm) of Cry1Ac or Cry1Ab or Cry1Aa, per gram of leaf material.

Cross-reactivity:

The kit is based on anti-Cry1Ac antiserum, but also detects other Cry1A toxins efficiently.

Principle of ELISA:

Enzyme-Linked Immuno Sorbent Assay, also called **ELISA**, **Enzyme ImmunoAssay** or **EIA**, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Older ELISAs utilize chromogenic substrates, though newer assays employ fluorogenic substrates enabling much higher sensitivity.

The method which is exploited in the development of our Bt Quant Cry1Ac Kit is a type of ELISA which has been described below.

SANDWITCH ELISA:

A less-common variant of this technique, called "sandwich" ELISA, is used to detect sample antigen. The steps are as follows:

1. Prepare a surface to which a known quantity of capture antibody is bound.
2. Block any non specific binding sites on the surface.
3. Apply the antigen-containing sample to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. Apply primary antibodies that bind specifically to the antigen.

6. Apply enzyme-linked secondary antibodies which are specific to the primary antibodies.
7. Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
8. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
9. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

The above method includes the use of a secondary antibody conjugated to an enzyme. The major advantage of a sandwich ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present. Without the first layer of "capture" antibody, any proteins in the sample (including serum proteins) may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized.

Material and Methods:

Glassware's and equipments:

All the glassware used in the development of the kit are of Borosil or schott Duran company. Plastic wares such as disposable microfuge tubes, Micro tips, Tip boxes were purchased from M/s Tarson, Axygen and Hi Media. Micropipettes, T-10, T-20, T-200 and T-1000 were of Accupipette and Eppendorf Company.

Following equipments and consumables are used.

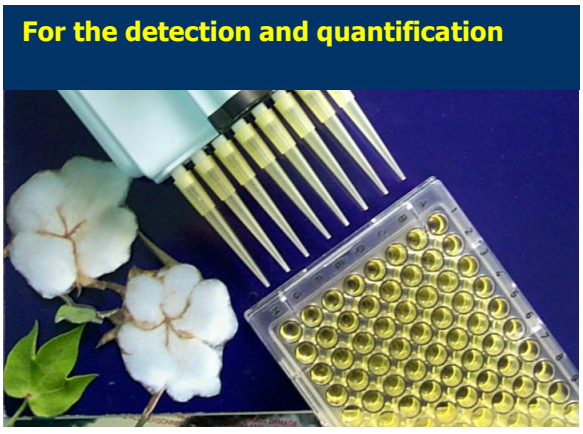
1. For crushing the seeds:
a: Plier (for removal of the seed coat)
b: Micropipettes (T-20,T-200 and T-1000) (Eppendorfs)
c: Plastic Pestles (for crushing the seed mechanically)
d: 96 well Plastic Block (deep well plates) (for crushing large no. of seeds at one time)
e: Express Grinder (for grinding of Seeeds-Innonative Bioscience Company)
f: Plastic mats (for covering the Blocks while being crushed, Axygen Company)
2. Deep freezer (-20^{0}C, Vest frost)

3. Centrifuge (REMI, Sigma, Eppendorf)
4. Refrigerator (LG, Samsung)
5. Microfuge (Sigma)
6. Plastic mats (for covering the Blocks while being crushed, Axygen Company)
7. ELISA Washer (For washing the Plates, Bio-Tek instruments)
8. ELISA Reader (for measuring the absorbance of the plate, Bio-Tek instruments)

Materials provided:

1. 96 well ELISA plate coated with antibody (Store refrigerated).
2. **Extraction buffer** (Store refrigerated).
3. **Substrate** (Store refrigerated).
4. **PBST** (10x) Dilute it before use. Add 90 ml distilled water to 10ml of the 10x PBST to prepare a working solution of PBST.
5. **IgG-conjugate** (Store refrigerated).
6. **Stop Solution (**ready to use)

A 96-well microtiter plate being used for ELISA.



Procedure:

- Remove the seed coat and crush the embryo. Seeds can also be crushed directly. If the assay is used for leaves, Punch out 2 leaf discs using a lid of eppendorf vial.

- Grind it into a fine solution with 0.5 ml sample extraction buffer
- Centrifuge the sample at 10,000 rpm (optional)
- Pipette out 50 µl of antibody-conjugate directly into each well.
- Add 50 µl (micro liter) of the sample into each well of the ELISA plate
- Pipette out buffer only in one or two wells of the ELISA plate to maintain blanks.
- Pipette out each Bt and NBt seeds in two wells and also at least three standards of required proteins of known concentrations (for reference). That means the first lane of eight wells should be for Bt, NBt, standards and Blank. From the next lane onwards, samples can be loaded
- Incubate at room temperature for 50-60 minutes.
- Wash the plate with wash buffer (PBST) repeatedly 3 times and empty wells.
- Add 100 µl substrate to each well. Incubate for 20 minutes (not more). Blue color develops in positive samples.
- Add 50 µl stop solution to each of the wells. Positive samples turn yellow
- Read absorbance at 450 nm.

Calculations for ELISA to quantify Cry1Ac

Example:

Seed weight (without seed coat) = 68 mg
Buffer quantity = 500µl
Crush the sample thoroughly
50µl was pipetted into each well

Standards:

1 ppm (1µg Cry1Ac per 1000 µl)
0.2 ppm (200 ng Cry1Ac per 1000 µl)
0.04 ppm (40 ng Cry1Ac per 1000 µl)
0.008 ppm (8 ng Cry1Ac per 1000 µl)

Standards		Cry1Ac quantity	
	1 ml (1000 µl)	**100 µl**	**50 µl**
1 ppm	1000 ng	100 ng	50 ng
0.2 ppm	200 ng	20 ng	10 ng
0.04 ppm	40 ng	4 ng	2 ng
0.008 ppm	8 ng	0.8 ng	0.4 ng

50µl was pipetted into each well; hence each well contains the following amount of Cry1Ac

Results:

	Standards				
	Cry1Ac ng/well	O.D			
	50	1.6			
	10	0.44			
	2	0.22			
	.4	0.17			
	0	0.11			
Steps			1	2	3
Samples	**Seed weight in mg**	**O.D**	**ng/well**	**ng/seed**	**ng/gm**
1	68	0.67	18.1	180.7	2657
2	72	0.12	-0.8	-8.3	0
3	54	0.98	28.7	287.2	5319
4	48	1.12	33.5	335.3	6986
5	77	0.88	25.3	252.9	3284
6	82	0.76	21.2	211.6	2581
7	59	0.65	17.4	173.8	2946
8	49	0.11	-1.2	-11.8	0
9	55	0.74	20.5	204.7	3723
10	62	0.82	23.2	232.2	3746

To construct the standard curve, use INSERT-CHART-XY (SCATTER)-trend line-options to obtain regression equation. (Please see EXCEL spread sheet)

Regression equation with standards:

y=bx + a

y=0.0291x +0.1442

y represents O.D (optical density or absorbance)

x represents amount of Cry1Ac

b represents slope

a represents constants

Step 1. Use formula to derive x = (O.D-a) to get ng/well

= (O.D._b)/a

Step 2. Multiply the value of ng/well with 10 (because $1/10^{th}$ of the sample was pipetted into each well)

Step 3. Calculate ng/well using the following formula

= (ng/seed x 1000)/weight of seed

Example-1st Sample

X=ng/well = (0.67-0.1442)/0.0291=18.17

Total qty of Cry1Ac (ng) in seed = 18.17x10=180.7

The amt of Cry1Ac in ng/gm = (180.7x1000)/68

= 2657.16

Cry1Ac in µg/gm **(ppm)** **=2.657**

Notes:

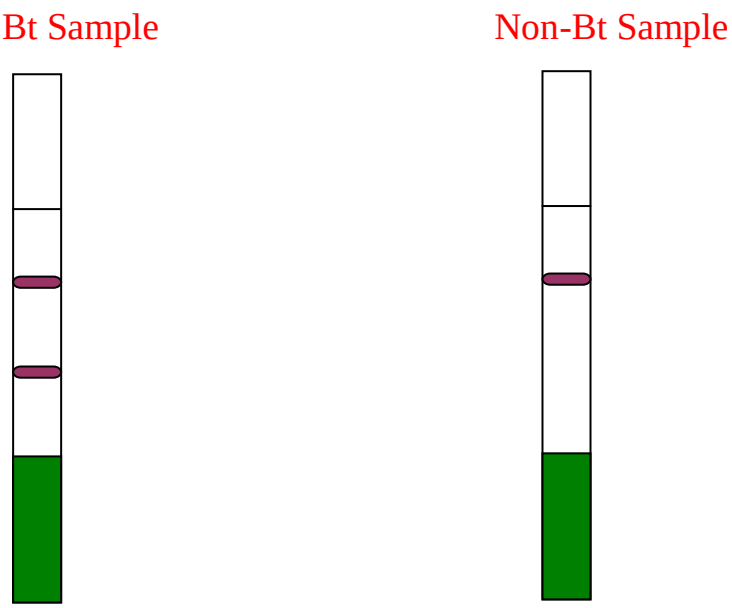
1 g (gram) =1000mg (milligram)
1 mg (milligram) =1000µg
1 µg (microgram)=1000ng(nanogram)
1 L (litre) =1000ml (milliliter)
1 ml (millilitre) =1000µl (microlitre)
1 µl (microlitre) =1000 nl (nanolitre)
Ppm=parts per million (1000, 000)
1mg in 1 litre=1ppm
1 mg in 1 kg=1 ppm
1 µl in 1 litre=1 ppm

Applications:

1. Quantification of Cry1A toxin expression in plants or plant callus.
2. Quantification of Bt toxins in Bt-spray formulations (based on *Bt*K-HD1, *Btt* and other strains wherein Cry1A is the prominent bio-active toxins)

Bt Express Dip Stick Kit

Bt express Dip-Stick Kit is a kit developed by CICR for the qualitative analysis of Cry1Ac in various parts of cotton Plant. **Cry 1 Ac Bt express** is a dip-stick format and can be used by even a layman, for instantaneous detection of Bt toxin in either seeds or plant tissue. It takes about 10 minutes for the test to be completed. The test can be used in fields and does not require any additional facilities for use. The kit is rapid, reliable and ready to use.



Position of Bands on strips in respective samples after the test.

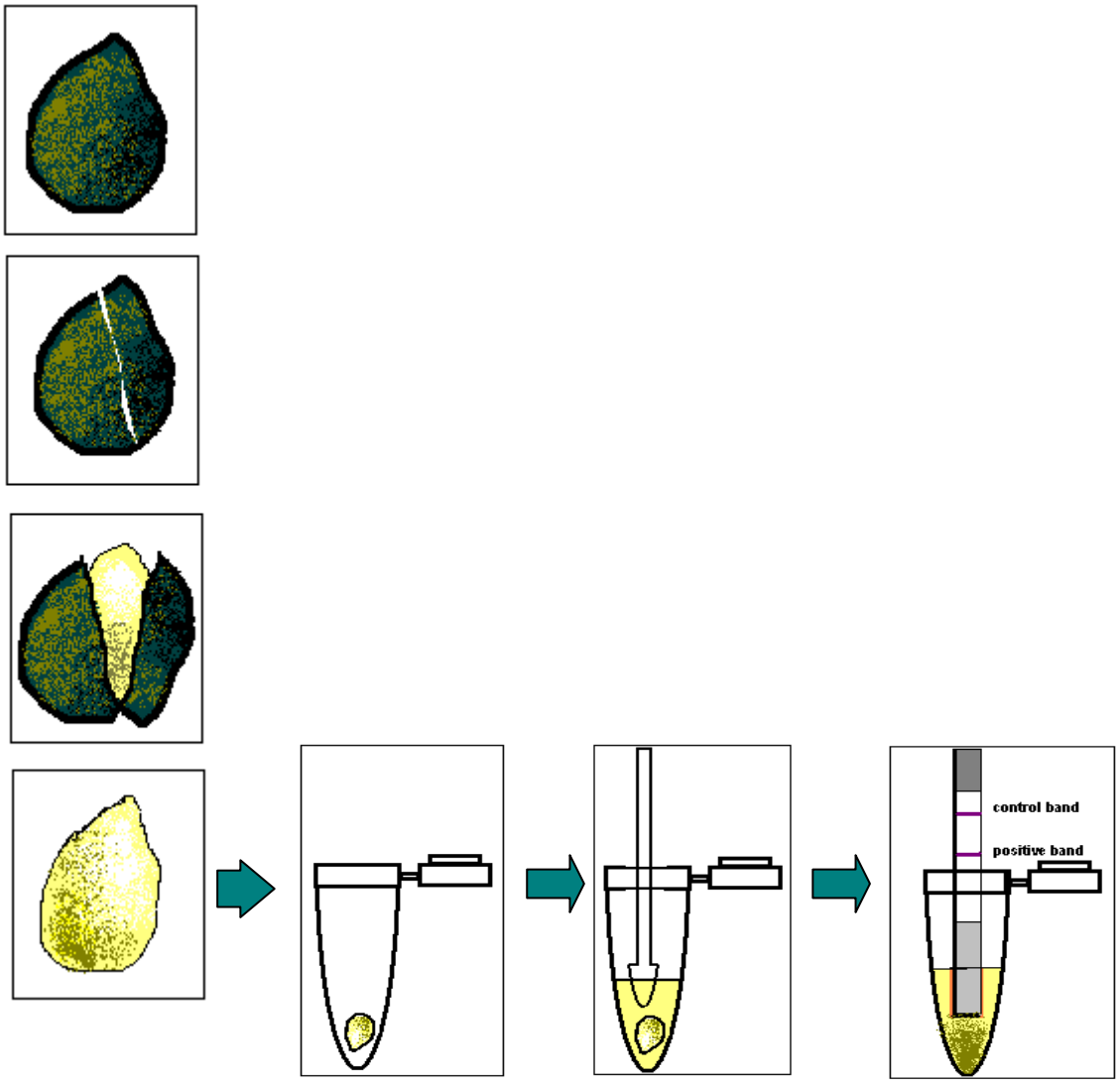
Bt Express

INSTRUCTIONS FOR USE:

1. Pick up a cotton seed and break it open or a piece of leaf tissue (One disc of about 1.0 cm diameter).
2. Take the white colored internal embryo matter and transfer it into the vials provided
3. Add 0.5 ml (marked on the vial) of sample extraction buffer provided.
4. Crush the embryo/leaf disc using the pestle provided with the kit.
5. Dip the 'Cry1Ac Bt instant-check' strip into the vial, to take care that only the end marked as sample is dipped into the sample. Wait until the sample solution (purple colored) travels till the top end of the strip and the filter pad at top-end is almost completely wet. This should take about 15-20 minutes.
6. If one band develops halfway through the strip along with another at the top (two bands), it indicates presence of Cry1Ac in the sample). It is a positive Bt-cotton sample.
7. If only one band develops at the top end of the strip it indicates that the sample is negative for Cry1Ac and is a non-Bt sample.

PRECAUTIONS:

1. Clean the pestle thoroughly before re-use. Any minor contamination of the sample with a positive sample can lead to false positives.
2. Keep the strips in airtight pack in refrigerated condition to ensure sustained activity.
3. If unused the strips may lose activity on prolonged storage of 6 months.
4. Do not keep strips outside the seal pack. In a humid environment the strips absorb moisture on prolonged exposure and have a slow flow rate.
5. Leaves of Bt-plants older than 90 days have less of Cry1Ac, hence the sample line intensity is usually light. Care must be taken in interpreting results when the strips are used on very old plant tissue samples.
6. Sometimes, if the sample extract is colored or concentrated, there may be a light grey-colored band in the sample line region. This should not be considered positive.



Contents of the Kit:

The Kit comprises of following items:

1. 25 micro centrifuge tubes.
2. 2 plastic pestles.
3. 1 bottle containing 25 dip sticks.
4. 1 15 ml buffer bottle.

Cost of the kit: Each kit costs Rs.1000/-.The kit is meant for twenty five tests. That means each test costs Rs.40/-.

GUS DETECTION KIT FOR DETECTION OF Cry2Ab GENE

It is a colorimetric test for the detection of GUS glucuronidase, which is a marker for the presence of Cry2Ab in seed samples of BG II (Mon 15985 event). It involves the use of specific reagents called Substrate and developer. The results are available at the end of 20 min. It is a qualitative test requiring minimal skill. The seed under test is cut longitudinally into two halves and 50 µl substrate is pipetted on to the cut end portion and is incubated in dark for 20 mins. After that, 50µl developer is added and again incubated in dark for 20 min. Development of blue green color indicates positive results for the GUS marker of Cry2 Ab. This test has been validated at NBPGR, New Delhi. The cost of kit is Rs. 100/- for 90 samples.

Cry1B AND Cry1C QUANT KITS

Similar to the method described above, Cry1B and Cry1C quant kits have also been developed successfully.

The production of antibodies, purification of antibodies, desalting the purified IgGs, production of enzyme labeled proteins and finally the quant kits (Elisa plates) were prepared with above described methods.

STANDARDIZATION OF PROTOCOL:

Various parameters such as serum dilution (while plate coating), conjugate dilutions (while setting up the reaction) and concentration of antigens (protein of interest) have been taken into account to reach the optimum conditions.

CRY1C PLATE DEVELOPMENT:

The Cry1C plates were tested with:

1. The cry1C seed powder (transgenic seeds of cry1C in which cry1C is present at a concentration of 12µg/gm of seed powder.)
2. A commercial formulation of Cry1C, "Xentari" which contains 3% Cry1C.
3. Highly purified (HPLC purified) Cry1C toxin.

It was observed that the plate detected the toxin at even a concentration of 0.004 ppm (seed powder), 1.11ppm (Xentari) and 0.01 ppm (HPLC purified toxin)

CRY1B PLATE DEVELOPMENT:

Cry1B plates were tested with highly purified (HPLC purified) toxin.It was observed that the plates detected the toxin at a concentration of 0.06 ppm. Also the cross-reactivity of both Cry1C and Cry1B plates is also checked. In another experiment with cry1B plates, the cross-reactivity of the plates have been tested using both cabbage and cauliflower NBt leaves with TBS leaf extraction buffer and artificial insect gut fluid buffer. The plate showed the expected negative results.

Application of software (MEGA) for phylogenetic inference- Practical approach

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A phylogenetic tree is an estimate of the relationships among taxa (or sequences) and their hypothetical common ancestors (Nei and Kumar 2000; Felsenstein 2004; Hall 2011.) today most phylogenetic trees are built from molecular data: DNA or protein sequences. Building a phylogenetic tree requires four distinct steps: (Step1) identify and acquire a set of homologous DNA or Protein sequences, (Step 2) align those sequences, (Step 3) estimate a tree from the aligned sequences, and (Step 4) Present that tree in such a way as to clearly convey the relevant information to others. MEGA5 (Molecular Evolutionary Genetic Analysis) is an integrated program that carries out all four steps in a single environment, with a single user interface eliminating the need for interconverting file formats. MEGA5 is, thus, particularly well suited for those who are less familiar with estimating phylogenetic trees (Hall 2013).

The MEGA5 has to be downloaded from http;//www.megasoftware.net and installed on a desktop.

Step 1. Open MEGA5 (Tamura et al. 2011) software by clicking on the icon of Mega on the desktop.

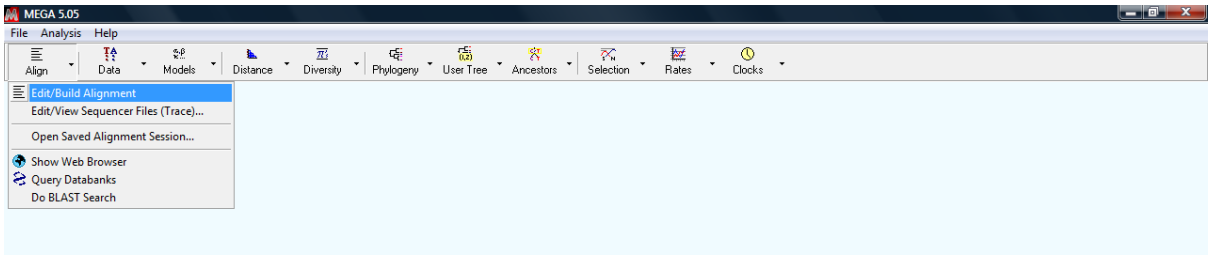


Figure 1: Mega Window and Edit/build Alignment Options

Step 2. The Mega 5 window will appear on the screen as in figure 1. Select Edit/Build Alignment from the Align menu.

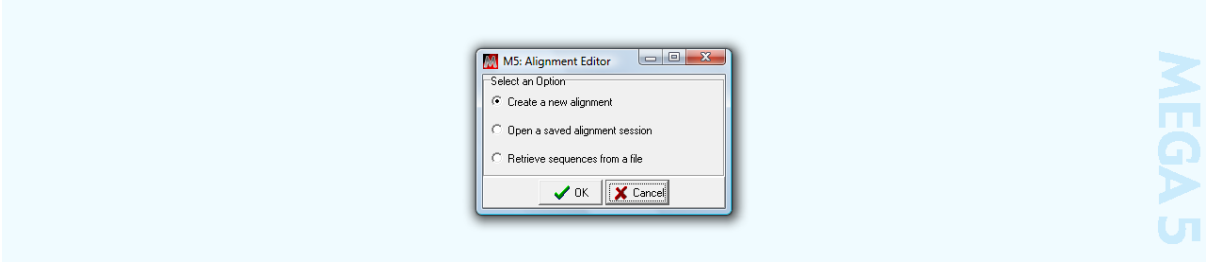


Figure 2: Alignment Editor with select options

Step 3. An alignment dialog box will open to select an option. Select "Create a new alignment" from the list and click on **OK** (figure 2.)

Step 4. As we select "Create a new alignment" option, a new dialog box will open asking for type of input sequence (figure 3). Choose DNA or Protein based on the input sequences.

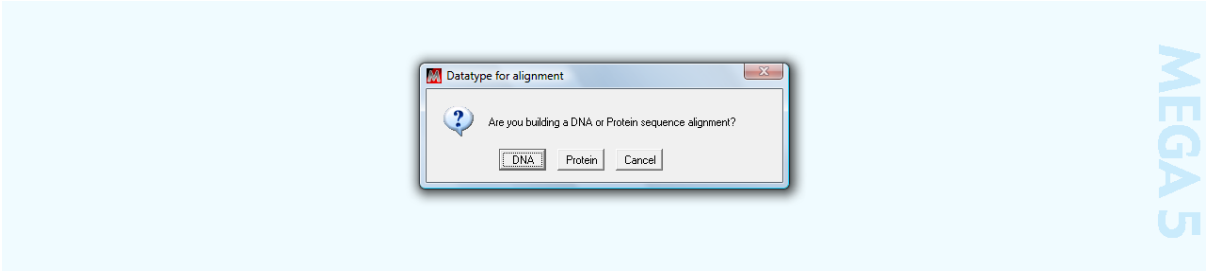


Figure 3: Input sequence type selection

Step 5: As we click on DNA option to select DNA sequence type, the alignment explorer window will appear as shown in figure 3. Select **Data** then go to >> **OPEN and** click on >> **Retrieve sequences from file** to load the input sequences.

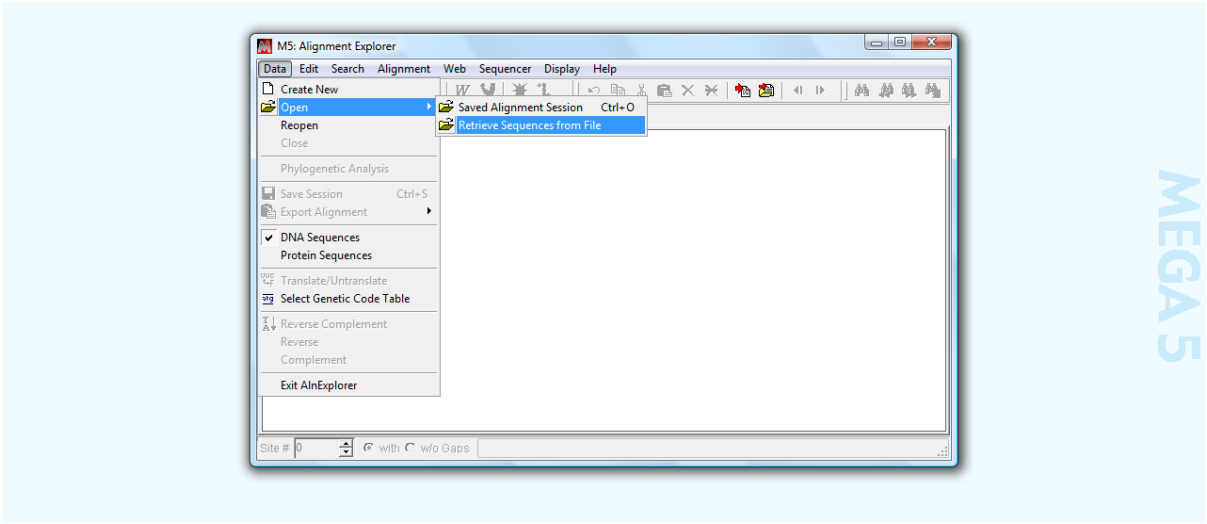


Figure 4: Alignment Explorer window and loading input sequences

Step 6. As we click on **Retrieve Sequence from File,** a file browsing dialog box will open to select the input FASTA file. Select the file and click on OPEN as shown in figure 5.

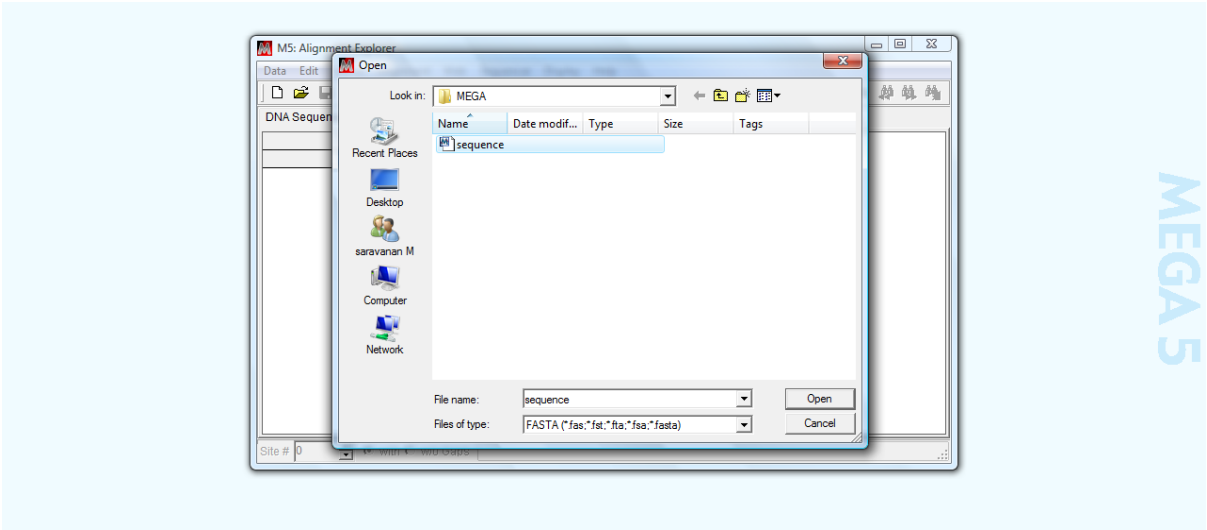


Figure 5: File browsing window

Step 7. Sequences will open in Alignment Explorer as given in Figure 6. Two alignment methods are provided: ClustalW (Thompson et al. 1994) and MUSCLE (Edgar 2004a, 2004b). Click one of those buttons or choose Clustal or Muscle from Alignment menu. Select

"Align by ClustalW" from the Alignment menu. A dialog box will appear from which select all sequences.

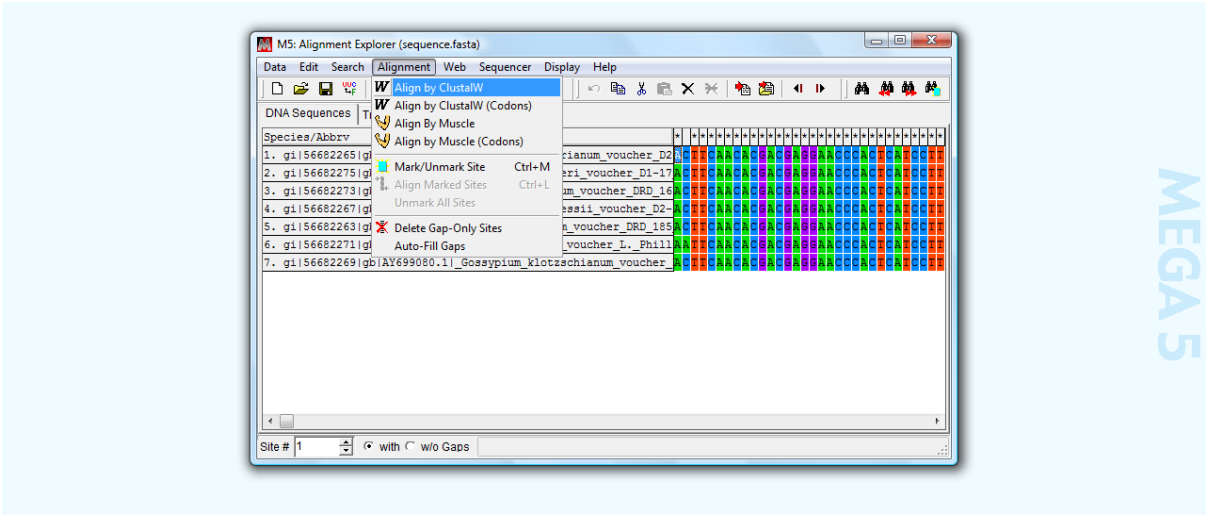
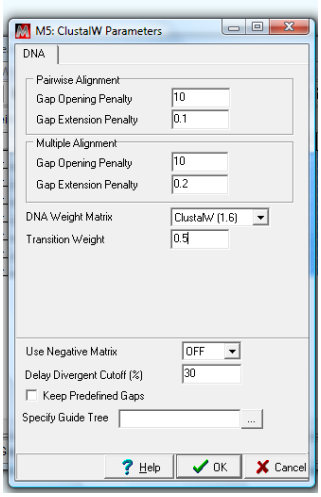


Figure 6: Alignment Explorer

Step 8. The "ClustalW Parameters" dialog box will open to select the alignment parameters.



Click on OK after selection of the parameters (Figure 7).

Step 9. After alignment, save the alignment session for further uses and save the alignment in MEGA format for further analysis using MEGA software. (Figure 7,8 and 9). MEGA software asks for the title of the data for saving in MEGA Format.

Figure 7: ClustalW Parameters Dialog Box

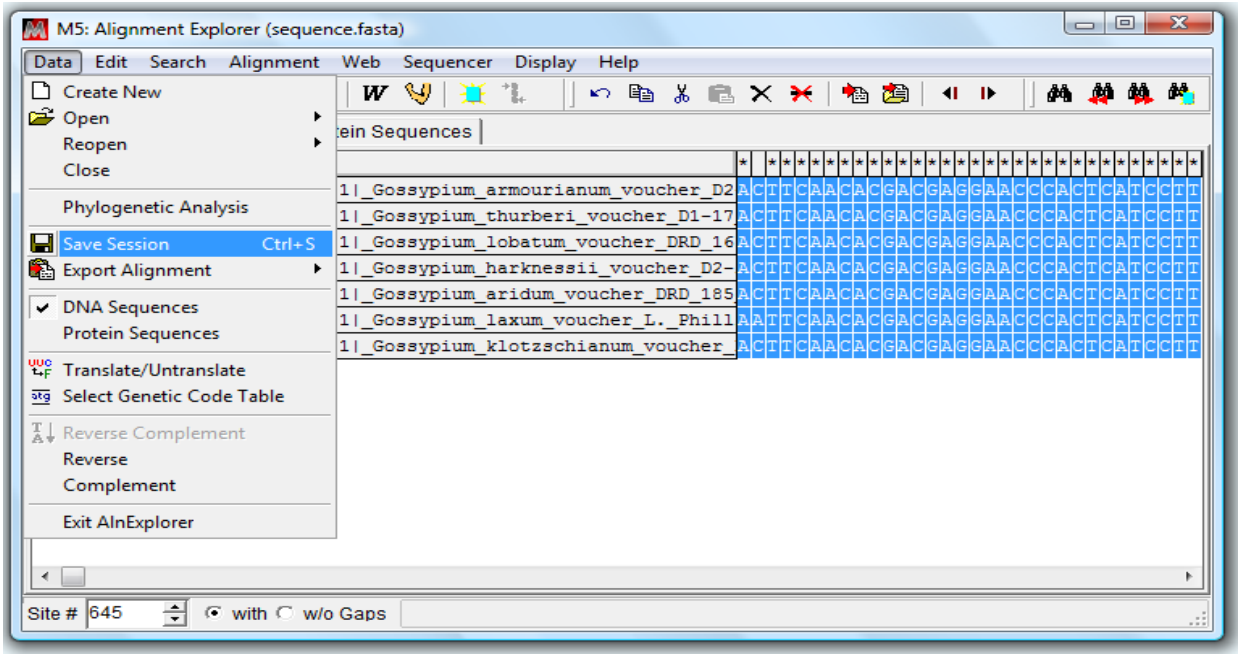


Figure 8: Saving alignment session

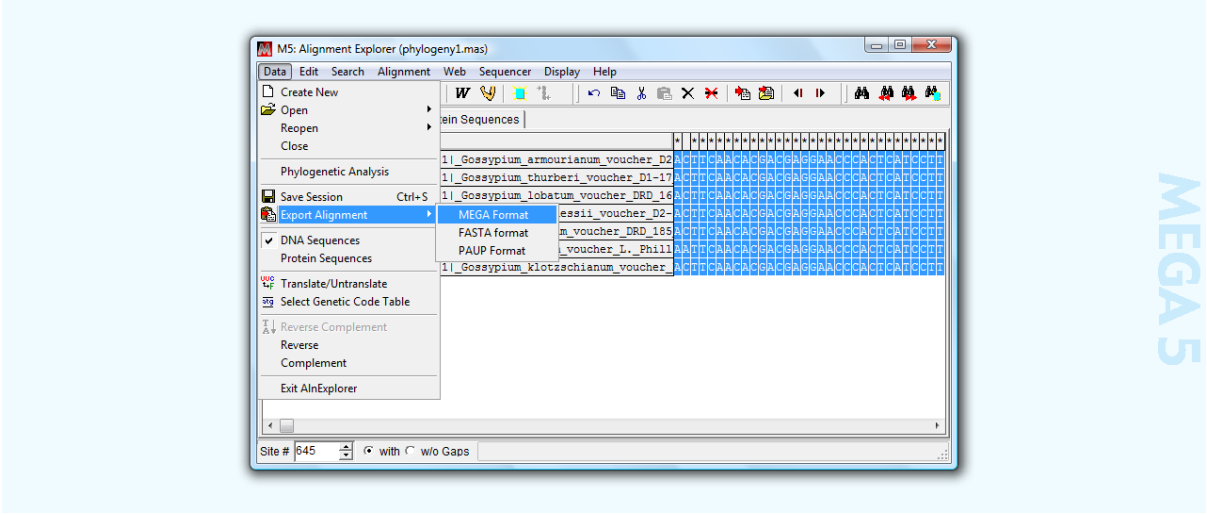


Figure 9: Saving alignment in MEGA format

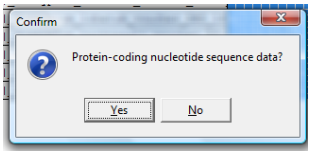


Figure 10: Confirmation of the data opening in MEGA software

Step 10. After saving the alignment session and alignment file, close the alignment explorer window. As we close the alignment explorer window, a dialog box will open to confirm the opening of the data file in MEGA software for further analysis (figure 10). Click on **YES** to open the data. Data will be opened in MEGA as in Figure 11.

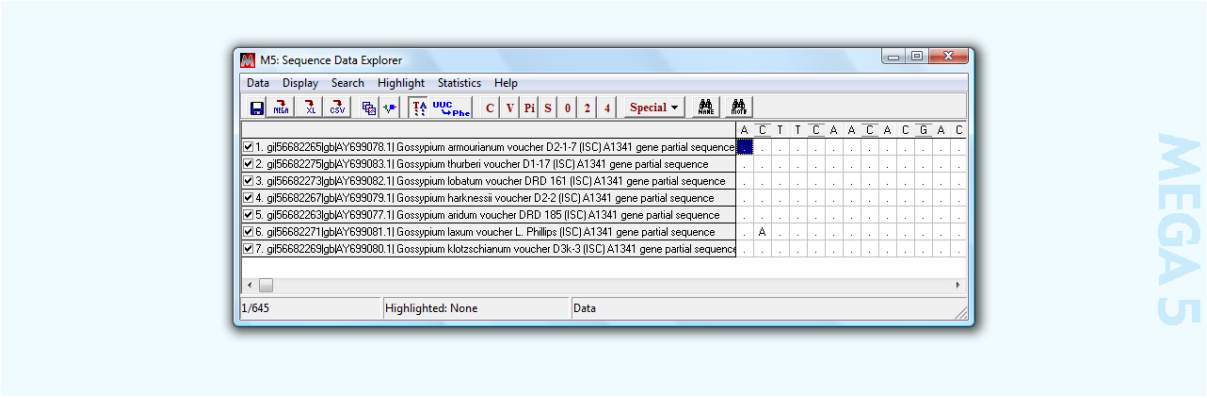


Figure 11: Data opened in MEGA software

Step 11. To perform phylogenetic analysis of the data, select any of the options from the PHYLOGENY menu as per requirement. Here we selected **PHYLOGENY** >>**Bootstrap test of Phylogeny** >> **Neighbour joining tree** for phylogenetic tree construction (Figure 12).

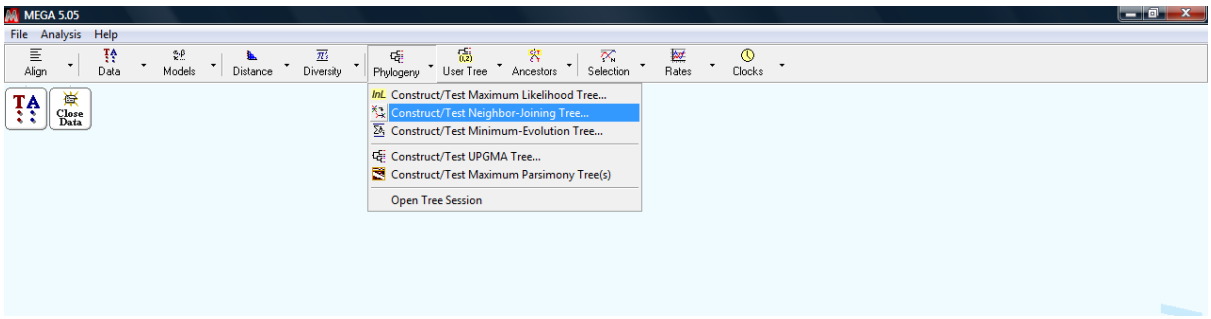


Figure 12: Selection of methods for phylogenetic analysis

Step 12. Select the parameters from analysis preferences window and click on COMPUTE (Figure 13).

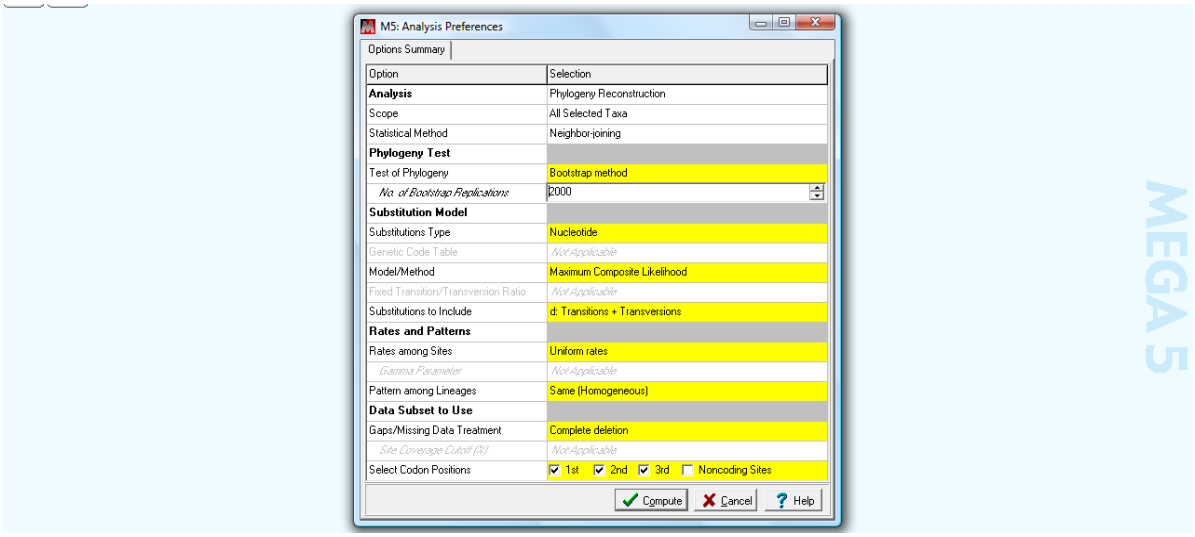


Figure 13: Parameter selection for phylogenetic analysis

Step 13. After computing the phylogeny, MEGA generates the phylogenetic tree with bootstrap values given on the nodes of the branches. The names of individuals are given on the termini of the branches. Save the current tree session (Figure 14).

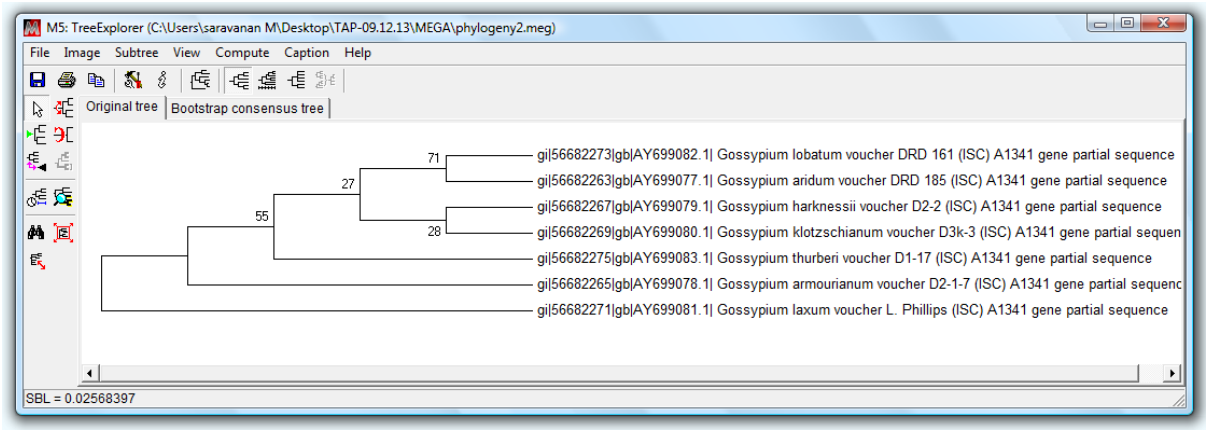


Figure 14: Phylogenetic tree

Although you can print the tree for your own purposes,to publish it you must save it in a graphics file format that is acceptable to the journal. The portable document format (PDF) is almost universally acceptable. Choose **save as PDF file** from the **image menu.**

Reference:

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Hall BG. 2011. Phylogenetic trees made easy: a how –to manual. 4th ed. Sunderlands (MA): Sinauer Associates.

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Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol.* 28: 2731-2739.

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DNA FINGERPRINTING IN CROP PLANTs

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DNA identification analysis, identity testing, profiling, fingerprinting all refer to the characterization of one or more relatively rare features of an individual's genome or hereditary make- up (Kirby, 1990). An unambiguous characteristic pattern of cultivars obtained using DNA markers is called as a DNA fingerprint. Alec Jeffery et al. (1985) developed the technique of DNA fingerprinting in human beings for the first time while working at University of Leicester, England. The usefulness of DNA fingerprinting technique for cultivar identification was demonstrated first by Dallas (1988) in rice. The principle behind DNA fingerprinting is "every individual is unique" and the very basic objective of DNA fingerprinting is to identify molecular loci that are polymorphic enough to produce 'band profiles' which are 'genotype specific'. Besides helping in varietal identification, DNA fingerprinting has numerous other applications in genetics such as:

- ✓ Study of genetic diversity within a taxa;
- ✓ Study of evolutionary and genetic relationships;
- ✓ Tagging of economically useful traits;
- ✓ Assessment of genetic purity of inbred lines and varieties;
- ✓ Selection of parents in a breeding programme;
- ✓ Paternity analysis in perennials and tree crops;
- ✓ Identification of hybrids and maternal seedlings in plants with polyembryony e.g. Citrus, Mango etc.;
- ✓ Monitoring genetic stability of germplasm conserved in form of seeds or tissues;
- ✓ Detection of somaclonal variants;
- ✓ Screening of duplicate accessions in gene banks and
- ✓ Crime solving in forensics.

Procedure:

1. Procurement of genuinely pure seeds of genotypes to be fingerprinted
2. Extraction of genomic DNA from genotypes and its quantification
3. Selection of markers from published literature and DNA databases
4. Polymorphism survey using selected subset of diverse lines
5. Genotyping of all the genotypes using identified, polymorphic markers
6. Scoring of molecular profiles
7. Data analysis and results interpretation

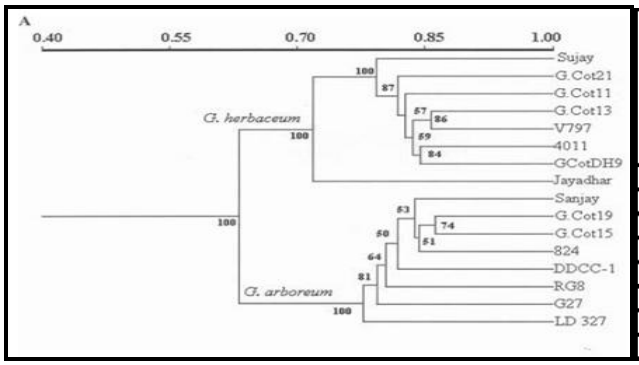

Fig. 1. Dendrogram

MARKERS/LINES	bnlg1429-190	bnlg1420-180	bnlg1429-200	bnlg1429-175	bnlg1429-210	bnlg1429-220	bnlg1866-185	bnlg1866-200	bnlg1866-190	bnlg1866-225
1	■								■	
2	■									
3			■				■			
4	■									
5		■								■
6	■						■			
7	■						■			
8									■	

Fig. 2. Barcode DNA fingerprint

Though, same molecular data can be used for DNA fingerprinting and molecular diversity analysis, the basic objective differs. In diversity analysis, aim is to assess the diversity or similarity between two or more individuals (Fig. 1) and it is desirable that markers should represent all genomic regions of the species. While in DNA fingerprinting, basic question to be answered will be how two individual can be identified or distinguished and genome-wide distribution of markers is not mandatory. After genotyping the individuals, score the molecular profiles and study the number of alleles present and their distribution across genotypes. Identify the rare alleles (alleles with frequency of less than 0.20), null alleles (no allele detected, even after repeated amplification) and unique alleles (allele specific to particular genotype). Polymorphism Information Content (PIC) of marker indicates ability of the marker to detect polymorphism among individuals. Highly polymorphic markers (markers with high PIC) can be used to construct robust DNA fingerprint of genotypes (Fig. 1) which will clearly indicate which allele is present each individual genotype for each marker included in the study. The robustness of the barcode DNA fingerprint generated is decided by statistical parameter called 'Probability

of Identical Match' which estimate the probability of DNA fingerprints of two genotypes being identical by chance. The probability of identical match can be calculated by employing the formula X^f, where X is the average similarity index and f is the average number of amplicons per genotype. Lower the X^f value, higher will be the reliability of DNA fingerprint developed. Reliable DNA fingerprint developed can serve as ready reference for cultivar identification, differentiation and protection.

Marker Assisted Breeding

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MAS refer to the use of DNA markers that are tightly-linked to target loci as a substitute for or to assist phenotypic screening. By determining the allele of a DNA marker, plants that possess particular genes or quantitative trait loci (QTLs) may be identified based on their genotype rather than their phenotype.

Advantages of MAS

- Time saving from the substitution of complex field trials with molecular tests
- Elimination of unreliable phenotypic evaluation associated with field trials due to environmental effects
- Selection of genotypes at seedling stage
- Gene pyramiding or combining multiple genes simultaneously
- Avoid the transfer of undesirable or deleterious genes (linkage drag)
- Selecting for traits with low heritability
- Testing for specific traits where phenotypic evaluation is not feasible
- Selections can be made on the genotype rather than the phenotype, which may increase the speed and efficiency of selection
- Useful if conventional screening methods are laborious, costly, or environmentally dependent
- Selections for disease and insect resistance can be made in the absence of the pathogen or pest
- Greatest potential advantage over phenotypic selection for traits with low penetrance or low heritability
- May reduce population sizes needed for phenotypic selection
- May permit selection of individual plants
- May speed up the breeding process
- May be effective for early generation testing
- Selections at the seedling stage can be a great advantage in crops with a long generation time
- Reduce number of generations in a backcrossing program by selecting for recovery of the recurrent parent genome as well as genes of interest from the donor parent

Types of markers used in MAS

- Although there are exceptions, SSRs, ESTs, and SNPs have generally been the markers of choice for MAS, because results are highly repeatable and they are amenable to high throughput technologies such as microarrays.
- Co-dominant markers are needed for MAB

The utility of a marker depends on

- How tightly it is linked to genes controlling important traits
- The relative importance of those genes in determining the phenotype
- The consistency of linkage disequilibrium between the marker and QTL
- The frequency of the QTL (MAS will be more beneficial when the QTL is in low frequency)

Limitations of MAS

- Globally, greatest successes with MAS have so far been achieved with simply inherited traits, while examples of success with polygenic traits are limited
- Lack of precision in mapping QTL
- QTL identified from mapping populations often cannot be extrapolated to breeding populations
- QTL x environment interactions are common
- the equipment and consumables required to establish and maintain a marker lab is considerable
- There is a large initial cost in the development of markers which is seldom reported. For marker assisted backcrossing, the initial cost of using markers would be more expensive compared to conventional breeding in the short term however time savings could lead to an accelerated variety release which could translate into greater profits in the medium to long term

Marker assisted backcrossing

- Select for both the gene of interest and for a high frequency of markers from the recurrent parent across the genome
- Reduces the number of backcross generations required to recover the desired phenotype

- 90% of recurrent parent genotype can be recovered in two generations when sufficient number of markers (e.g., 1 per 10 cM) and adequate numbers of progeny are utilized
- Reduce linkage drag around the favorable QTL

Marker Assisted Breeding – Flow diagram

Screening and selection of resistant/tolerant and susceptible parental genotypes for biotic or abiotic stress

⇩

Development of mapping populations by crossing

⇩

Identification of polymorphism and Linkage analysis of markers

⇩

Construction of linkage maps and QTL analysis (QTL confirmation and Validation)

(or)

Instead of linkage mapping –short cut methods can be used for gene tagging 1. Bulked segregant analysis (used for markers located in specific chromosomal regions) 2. Selective genotyping (selecting individuals from a population that represent the phenotypic extremes or tails of the trait being analysed (both require mapping populations)

⇩

Identification of genetic makers for trait of interest through Linkage map or Bulk segregant analysis

⇩

Validation of markers (reliability of markers) – effectiveness in determining the target phenotype in independent of populations and different genetic backgrounds

⇩

Transfer of gene/QTL to agronomically superior genotypes by using foreground and background selection

Case study: Bacterial blight of Cotton

Causal organism: *Xanthomonas axonopodis pv. Malvacearum*

Isolation and identification of race 18 - *Xanthomonas axonopodis* pv. *malvacearum* from infected plants

⇩

Screening of genotypes against *Xam* race 18 under artificial inoculation in glasshouse conditions

⇩

Grouping of genotypes as resistance and susceptible based on disease Reactions

⇩

Testing of reliability of publicly available and reported markers to the genotype groups

⇩

Identification and confirmation of tightly linked markers to disease resistance gene

⇩

Validation of markers using different genotypes

⇩

Utilization of resistance markers in breeding programs

References

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2. Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005)
3. www.cropandsoil.oregonstate.edu/sites/default/.../css650/.../M13_MAS_GS

FISH (Fluorescent *In Situ* Hybridization) and Genomic in situ hybridization (GISH) Technique

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Molecular cytogenetic techniques, especially Fluorescence In situ Hybridization (FISH) and Genomic In situ Hybridization (GISH), are excellent tools to study the structure and function of genomes, chromosome landmarks, distribution of recombination across the chromosomes, polyploidy, aneuploidy, alien gene introgression and genome evolution and physical mapping of genes. FISH was developed in the early 1980s that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH allows direct mapping of DNA sequences on chromosomes and mapping of small low-copy of DNA sequences in various species. In situ hybridizations require reliable and efficient methods of chromosome preparation with well preserved and dispersed chromosomes and little or no cell wall debris.

Essential requirements for FISH in cotton:

1. Mitotic or Meiotic chromosome preparation
2. Fluorescence in situ hybridization using DNA probes and labelling
3. Banding of chromosome using fluorescent dyes eg Geimsa stain, DAPI etc
4. Fluorescence microscope to observe the hybridization using cell sense dimension imaging software

Preparation of Mitotic Metaphase Chromosome Compatible for Fluorescence in situ Hybridization in Cotton.

Cotton is dicot and hence only main tap root available for mitotic chromosomal studies.

Procedure:

1. The desired seeds (diploid or tetraploid species of Gossypium) are kept in dark at 30°C in a incubator for 2-3 days for germination.

2. 2 - 4 cm long root tips are removed and placed in the micro centrifuge tubes. Place 1-2 roots in each tube and sprinkle double distilled water to avoid drying of these root tips. The root tips are then placed in 1.5 ml micro centrifuge tubes.

3. These 1.5 mL tubes are then placed in a gas pressure chamber (160 psi) and expose them to N_2O for 1 hour and 45 minutes. Standardise gas pressure and N2O for different species and samples etc.

4. Cells in root tips are then fixed by filling each tube with 90% ice cold acetic acid and incubating on ice for 15 minutes.

5. Root tips are then transferred to fresh tubes containing ice cold 70% ethanol. Gently mix the solution. Ethanol is removed and then replaced with fresh ice –cold 70% ethanol. Repeat this 3 times to remove the acetic acid residue.

6. Add ice cold 1x citric buffer. Mix the samples gently and remove citric buffer constantly and replace fresh buffer for 10 minutes.

7. Root tips are cleaned on filter paper. A 1 - 2 mm long section of the milky white region is removed and transferred to a standard 0.7mL Eppendorf tube containing 20µL of ice-cold enzyme solution (1% pectolyase Y-23, 4% cellulase in 1x citric buffer)

8. The sample tubes are then placed in a Hybridization Oven for incubation at 37°C for standardized time. Immediately following digestion tubes. The digested root tips are then washed with ice-cold 1x TE buffer, followed by 2-3 washes in ice cold 100% ethanol using a standard glass pipette.

9. After thoroughly removing the ethanol, 30µL of freshly prepared 75% acetic acid – 25% methanol was added. The remains of the root tips, sufficiently weakened by the enzymatic degradation, are then ground gently using a rounded dissecting needle.

10. Approximately 8 µL of the cell suspension is dropped onto the marked spot on the slide. The slides are then allowed to dry by placing them inside a humidity chamber for 3 - 5 minutes. After drying, slides are viewed under a phase contrast microscope in order to ensure selection of slides with good chromosomal spread for hybridization. Chosen slides are then placed on a thin piece of cardboard and cross-linked on a SpectroLinker XL-1000 UV Crosslinker.

11. Slides are then stored overnight at -20°C in a standard microscope slide box.

Fluorescence in situ hybridization.

1. The DNA insert in clone PTA71 is used as a probe. Clone PTA71, the 18S-28S rDNA, is a 9-kb *Eco*RI fragment from common wheat.
2. One microgram of plasmid DNA is labeled with fluorescein-12-dUTP using nick translation.
3. Denaturation: Five µL of denatured salmon sperm DNA (140ng/µL in 2x SSC) is dropped onto the center of each cell spread and covered with a cover slip. Root-tip DNA (on slide) and probe DNA (in 200µL thin-wall PCR tubes) are denatured by floating in the boiling water baths for five minutes at 100°C.
4. After denaturation, probes are plunged directly into ice. Denatured slides are placed on a thin metal sheet that had been placed upon the ice. The tube containing the probe is spun briefly to collect the total volume. The plastic cover slip is removed and 5 µL of the probe solution (40ng of labeled probe/ µL) is placed at the center of the slide. After addition of the probe, the slides were incubated in a humid storage container lined with Kimwipes soaked with 2x saline-sodium citrate (SSC) and placed in the Hybridization Oven at 37°C overnight.
5. Wash slides in 2x SSC at room temperature for five minutes in a Coplin jar. They were then washed at 42°C in 2x SSC for 20 minutes in an incubating shaker. After removing the slides from the bath, the bottoms and sides are briefly dried to remove excess SSC without allowing the tops to dry. One drop of ice-cold propidium iodide (PI) pre-mixed with Vectashield mounting medium is added to each slide in order to stain the chromosomes. Slides are covered with cover slips prior to visualization. Cells were examined under a Dark Fluorescence microscope connected to a light source. Images were captured using a camera and processed using cell sense dimension imaging software

C-banding.

Chromosome preparations from root tips treated with N_2O for two hours are used for Giemsa staining in cotton. Chromosome preparations that were cross-linked are stored in -80°C and dehydrated in ethanol for five minutes. C-banding was done using a 10% solution

of Giemsa stain and visualized with a photomicroscope and photographed using a DP71 digital camera and images were captured.

Preparation of Meiotic Pachytene Chromosomes for FISH in cotton:

1. Plant material : Diploid or Tetraploid cotton species
2. DNA probe and labelling

   Use amplified Arabidopsis – type telomeric repeats and lable them with biotin by nick translation. The genomic DNA of diploid or tetraploid species is used as DNA probe and labelled with digoxigenin-11-dUTP by nick translation

3. Preparation of Pachytene chromosomes

   - Young flower buds about 5-6 mm long are selected
   - Appropriate meiotic stage is determined
   - Anthers from a bud are sqashed in 45 % acetic acid on a slide under a phase contrast microscope
   - Isolation of nuclei and preparation of DNA fibres
   - Fix PMCs in prophase I directly in Cornoy's solution (3 : 1 Alcohol : Acetic acid) for 30 min.
   - Wash twice in deionized water in a Petri Dish
   - Remove anther from buds soaked in 30mmolL^{-1} citrate into 1mL of 30mmol L^{-1} citrate buffer (pH 4.5) for 10 min
   - Transfer buffer PMCs into 1.5mL micro centrifuge tube
   - Digest in 50µL of enzyme mixture containing 4% (w/v) cellulose, 0.5 % pectolyase Y-23 and cytohelicase at 37°C for 4 hrs.
   - Digested PMCs are collected

- Remove supernatant & PMCs are resuspended in a solution in acetic acid and enzyme mixture.
- Collect PMCs again and transfer on slides and incubated at 50°C for 1 min. to clear cytoplasm. Add Conroy's solution around PMCs on the slide and mix with PMC suspension so that the chromosomes spread on the slide.
- Dry for 20 minutes at room temperature.
- Use these slides directly for in situ hybridization or store at -20°C for several months.
- Isolation of nuclei and p[reparation of DNA fibres
- Fluorescence in situ hybridization and detection using the stain DAPI (6-diamido, 2-2 phenylin dole)
- Hybridization signals are observed under a Fluorescence microscope.
- Images are captured by a CCD and FISH images are further processed using appropriate Adobe Photoshop software.

Fluorescent signal strength depends on factors such as:

a. probe labelling efficiency,

b. type of probe,

c. Type of dye.

Genomic in situ hybridization (GISH)

Genomic In Situ Hybridization (GISH) as a Tool to Identify Chromosomes of Parental Species in Interspecific Hybrids

Genomic in situ hybridization (GISH) can detect alien chromosomes or segments in the interspecific or intergeneric hybrids, translocation breakpoints, chromosome pairing activity, and the genome composition of polyploidy plants. This technique has been applied in many crops including rye, wheat, barley, beet, rice, potato, tomato, brassica, and cotton.

Method:

- Genomic DNA of wild species are used as a probe after being sheared in boiling water for 10 min and labelled with digoxingenin-11-dUTP using the nick translation method.
- Genomic DNA is used as blocking DNA after shearing, with ratios of blocking DNA to probe DNA ranging from 35:1 to 120:1.
- Different washing stringencies are used for different wild species.
- Labelled probes are detected
- Chromosomes are counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) or any other fluorescent dye.
- Slides were analyzed under a fluorescence Imaging microscope.
- Images are captured by a charge coupled device (CCD) camera and processed using Axiovision 3.1 or any other available software and Adobe Photoshop.

Other methods

Comparative Genomic Hybridization (CGH)

This can be described as a method that uses FISH in a parallel manner with the comparison of the hybridization strength to recall any major disruptions in the duplication process of the DNA sequences in the genome of the nucleus.

Virtual Karyotype

Virtual karyotyping is another cost-effective, available alternative to FISH panels using

thousands to millions of probes on a single array to detect copy number changes, genome-wide, at unprecedented resolution. This type of analysis can only detect gains and losses of chromosomal material and will not detect balanced rearrangements, such as translocations and inversions.

Spectral Karyotype

Spectral karyotyping is an image of colored chromosomes. Spectral karyotyping involves FISH using multiple forms of many types of probes with the result to see each chromosome labeled through its metaphase stage. This type of karyotyping is used specifically when seeking out chromosome arrangements.

Uses of FISH and GISH:

- Fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity.
- Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes.
- To measure the length and copy numbers of repetitive sequences of genome
- To determine physical distance between genes
- Physically map BAC or YAC contigs with high resolution
- To analyze gap size in physical map.
- Often used for finding specific features in DNA for use in species identification.
- Also used to detect and localize specific RNA targets (mRNA, lncRNA and miRNA) in cells.
- It can help define the spatial-temporal patterns of gene expression within cells and tissues.

DARwin

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1 Introduction

DARwin is a versatile free to use software package developed by CIRAD for diversity and phylogenetic analyses. This can be downloaded from http://darwin.cirad.fr/Home.php. This software can handle different data types like DNA sequences, dominant and codominant molecular marker data and data on qualitative and quantitative variables. In this package, several algorithms are implemented to perform dissimilarity and factorial analyses and construction of dendrogram. There are options to assess the reliability of constructed tree or to compare several trees on the same data set. This software produces good quality graphics which are easy to read and include in publications. DARwin has been developed with Microsoft Windows Visual Basic 6.0 SP6. It can be operated in any Microsoft Windows operating systems.

2 Data format

DARwin uses its own format for data file but options are available to import data from other phylogenetic software such as PHYLIP, NEXUS, Mega2. The software requires two types input files. One of the files is called '.VAR' that contains data on variables being analyzed. The other file is called '.DON' that contains descriptions of the units being analyzed (e.g. name of the genotype and other known information related to the units being analyzed). Data can be entered in MS Excel and can be saved as 'Text (tab delimited)' format.

2.1 components of .VAR file

The data is arranged in the standard row-column format with units in the row and variable in the columns. Three types of data can be stored in .VAR file: single data, allelic data and sequence data. 'Single data' type includes any numeric data. 'Allelic data type' is meant for co dominant markers like SSR, RFLP, SNP where different alleles for a locus can

be scored. The 'Sequence' data type accepts the DNA sequences of individual samples. Irrespective of type, the data is organized in the following structure:

1st line – header

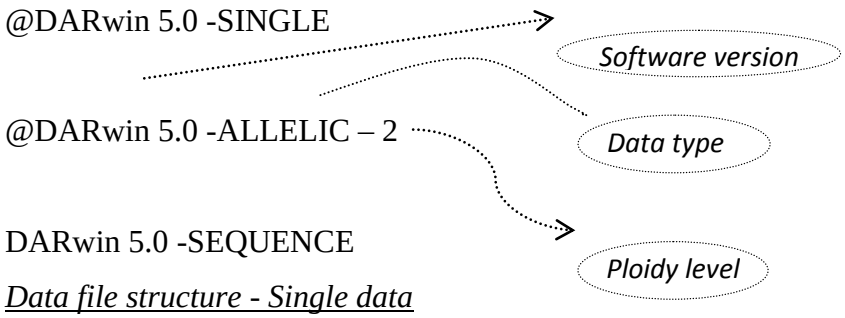
2nd line – number of units followed by number of variables

3rd line – name of the variable in each column

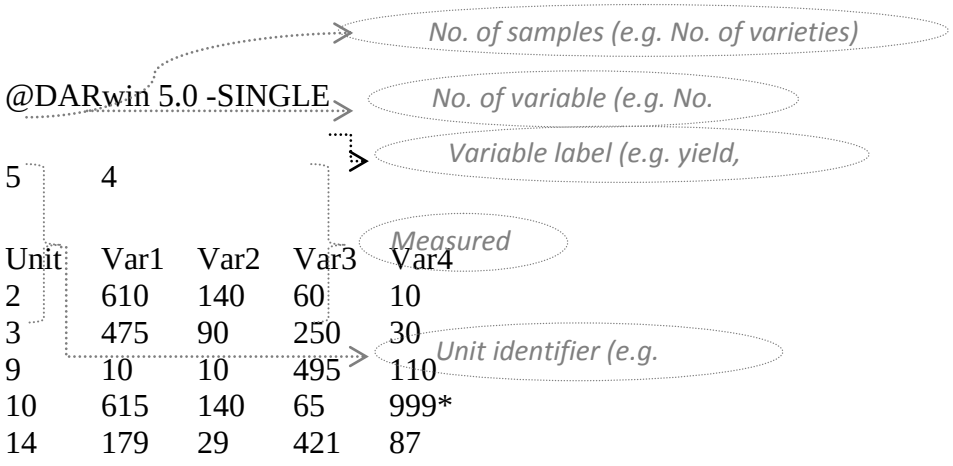
4th line onwards: the first field is the numerical identifier for the unit followed by the values for each variable in the corresponding columns

Header

The version of the software and the type of data are given as header. Any one of the following three lines should be given as header depending on the data type (with the exact syntax):

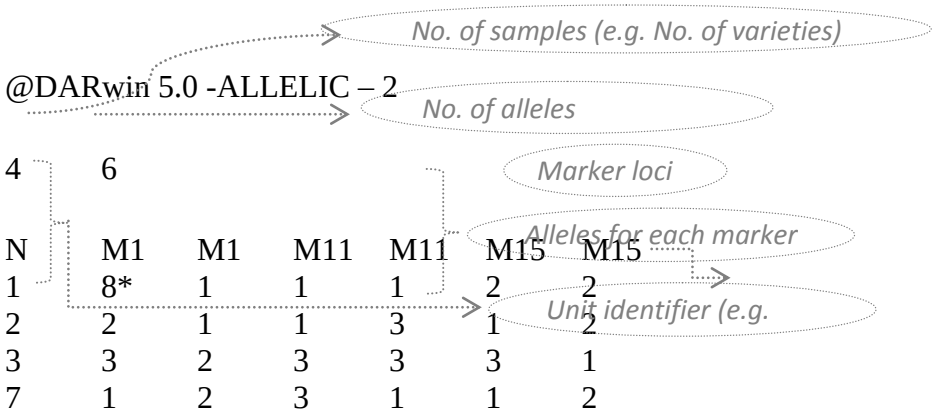


Data file structure - Single data



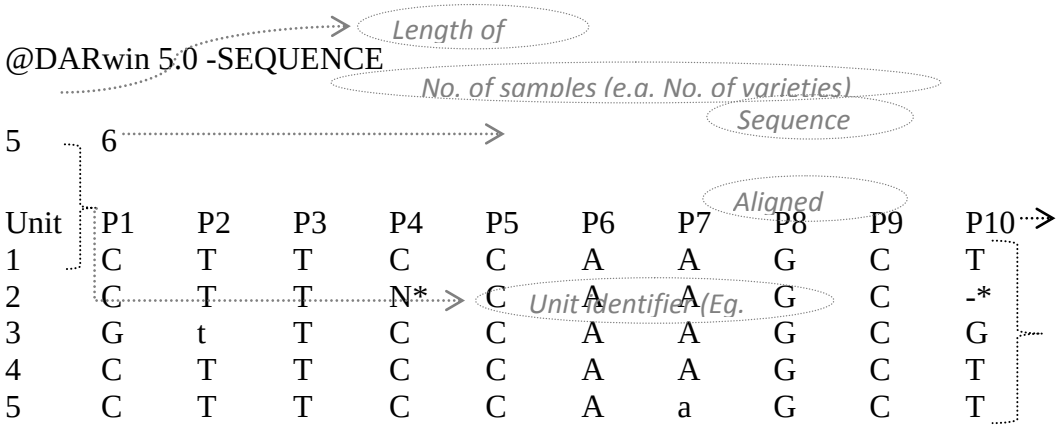
* Missing data (can be coded with any integer)

Data file structure - Allelic data



* Missing data (can be coded using any integer)

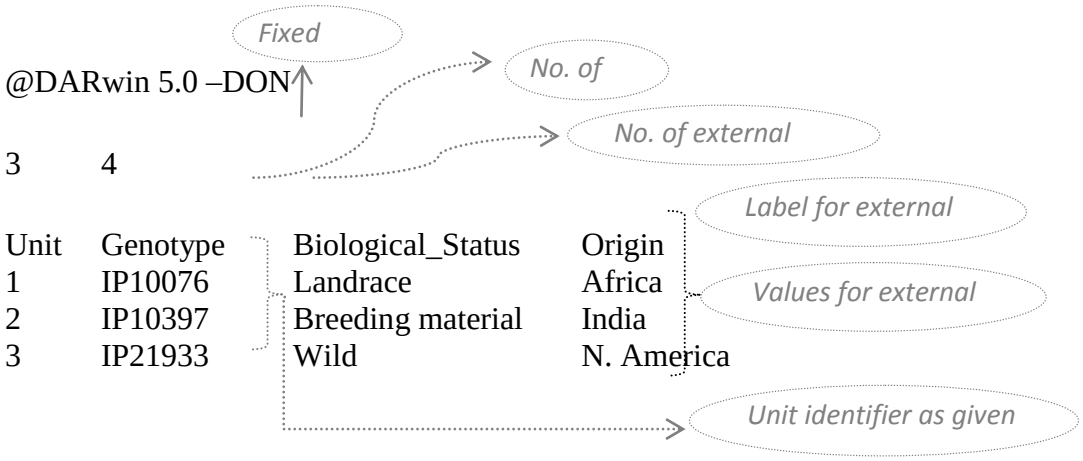
Data file structure - Sequence data



* Missing data (hyphen or characters other than A, T(U), G, C)

2.2 Components of .DON file

The unit identifier given in .VAR file can be supplemented with further descriptions such as variety name, botanical classification, geographical origin, reaction to pest etc. This can be used to illustrate and interpret graphical displays. The details pertaining to the units can be stored in the .DON file giving correspondence to the unit identifier given in .VAR file. Such a way, DARwin uses double level of identification. The first one is referred to as 'internal identifier', the numerical code used in .VAR to identify each unit. The second level is information stored in the .DON file giving correspondence between the internal identifier and a set of other identifiers, i.e. external identifiers. The internal identifier is always used by default but user can always invoke a .DON file and select a more convenient identifier. The following is an example of .DON file

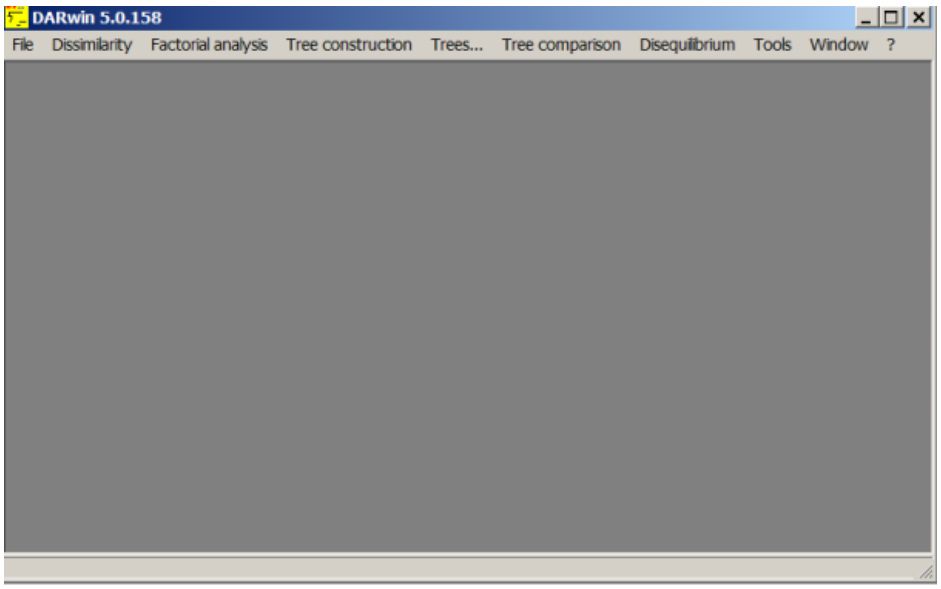


3 Running DARwin

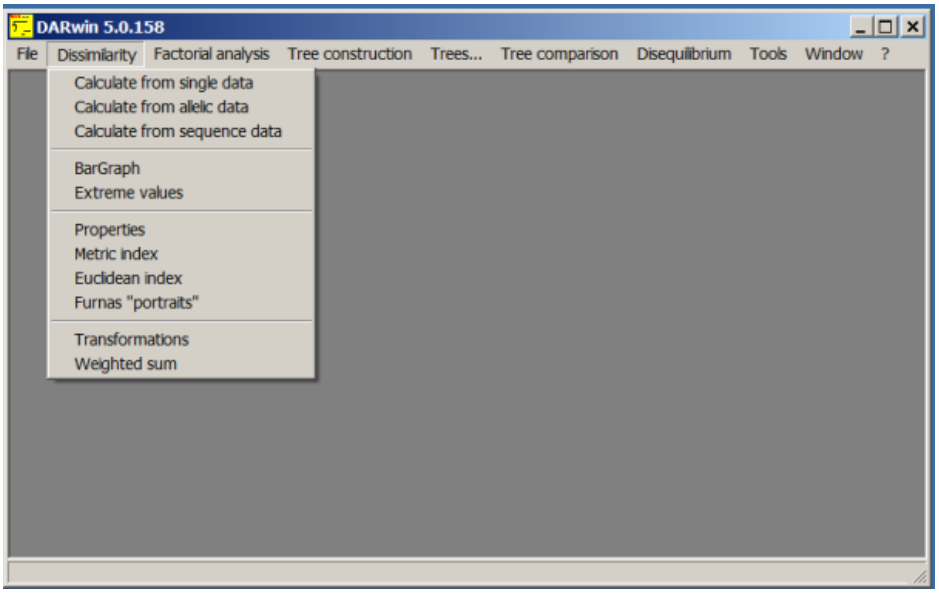
DARwin can perform dissimilarity estimation based on different parameters relevant to different data types such as single, allelic and sequence data and construct the tree using hierarchical or neighbour joining clustering method. It can also perform Factorial analysis.

A sample work-flow in analyzing the example data set for studying the genetic dissimilarity and construct a dendrogram is provided below. The example data set contains data on nine castor accessions genotyped for 40 SSR markers. The files are named as 'castor.var' and 'castor.don'. The file 'castor.var' contains the allele length of each marker across nine samples. The file 'castor.don' contains the genotype names.

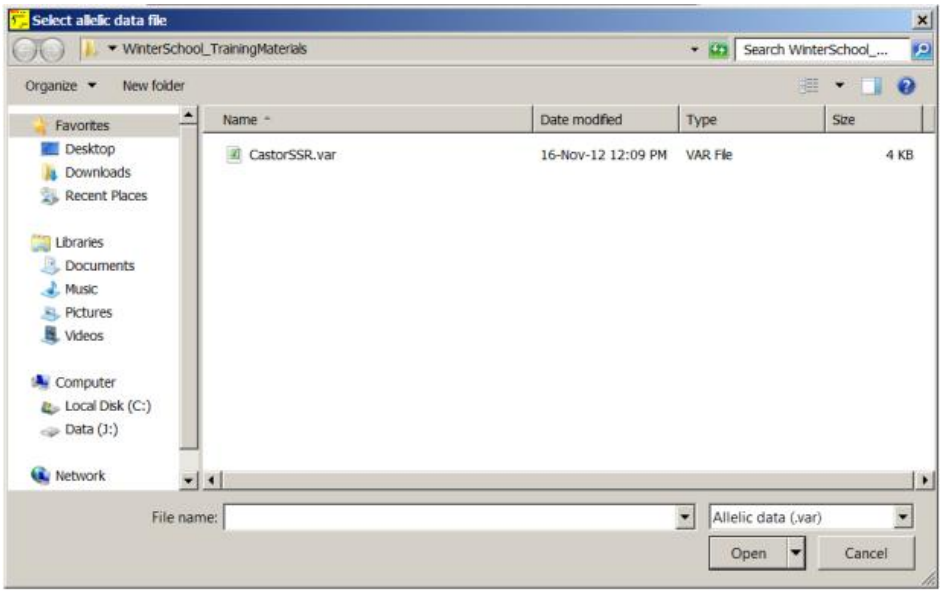
1. Double click the DARwin executable file or the shortcut in the Desktop or Programme menu. In the opening window, you can see different tabs like File, Dissimilarity, Factorial analysis, Tree construction etc.



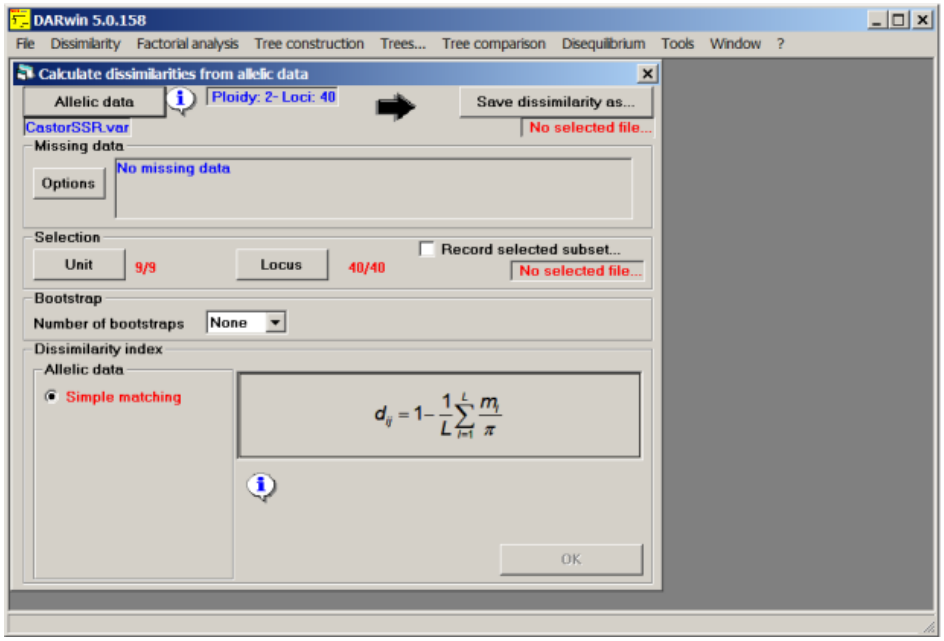
2. Click Dissimilarity



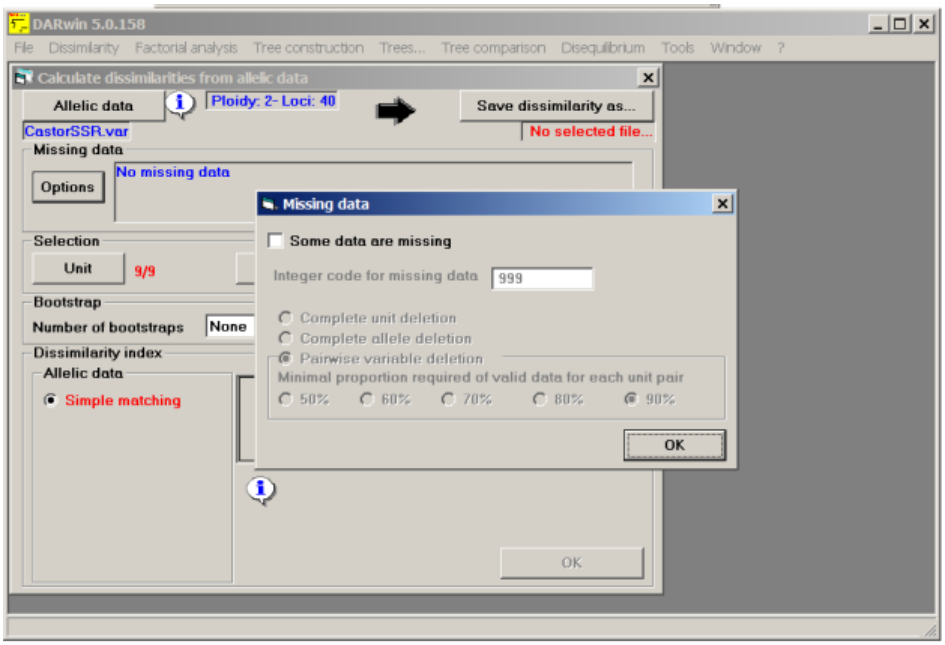
3. Select ‘Calculate from allelic data’. A popup window, as below is seen:



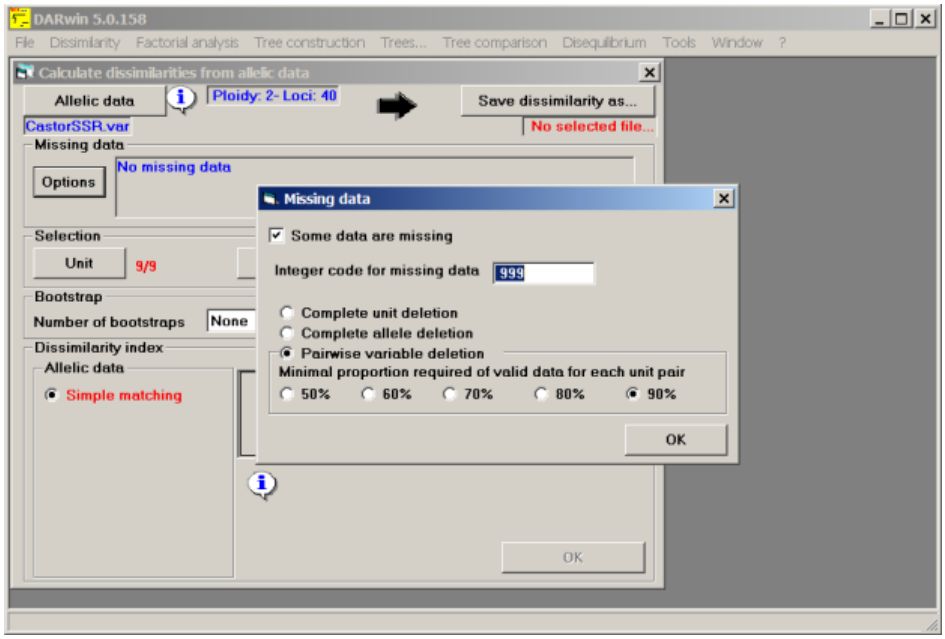
4. Browse the data file ‘castor.var’ in the computer from where it is stored (C:\Desktop\WinterSchool_TrainingMaterials) and click open. If the file is in right format, the following window will appear



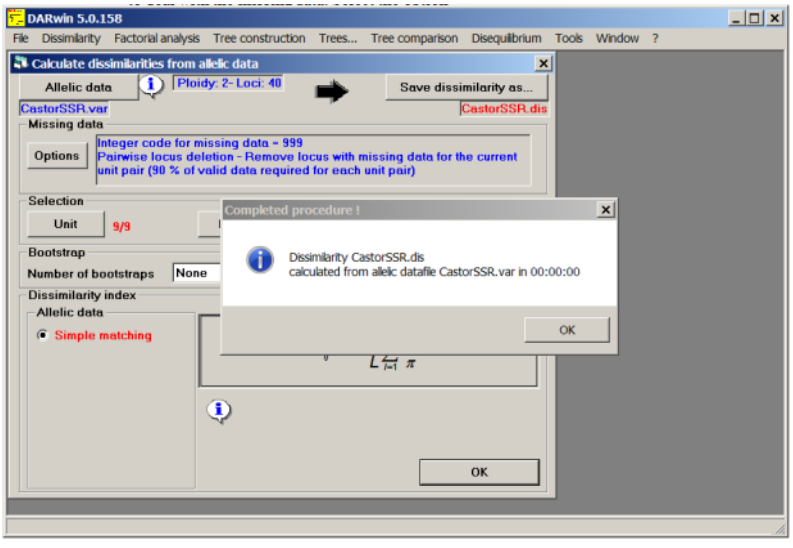
5. Check if the ploidy level, number of units and number of loci displayed are correct. In our example, ploidy level is 2, number of loci is 40 and the number of unit is 9. Click ‘Options’ button under ‘Missing data’. The following window will appear



6. The example data set contains missing data and coded as ‘999’. Hence, check the box against ‘Some data are missing’ and enter the integer code as ‘999’. There are three ways to deal with the missing data. Select one of the options

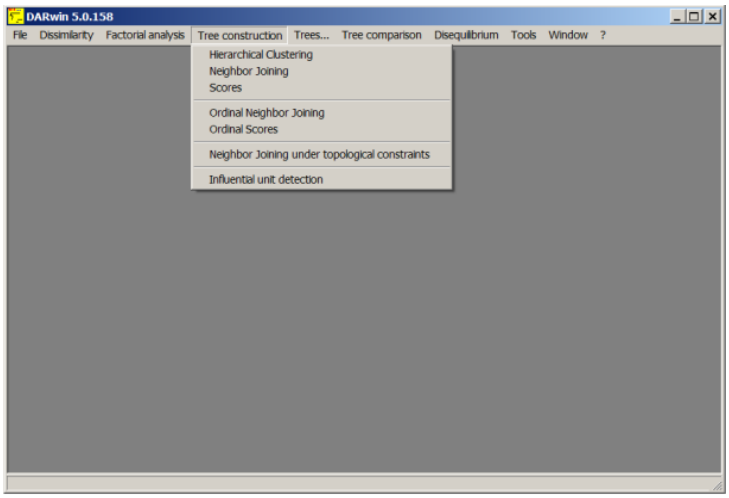


7. Click OK. The following window will appear. In that click 'Save dissimilarity as' button and give a file name and location where the file should be saved. By default, it will give the data file name with .dis extension (casto.dis) and store in the directory where the data file exists (C:\Desktop\WinterSchool_TrainingMaterials)

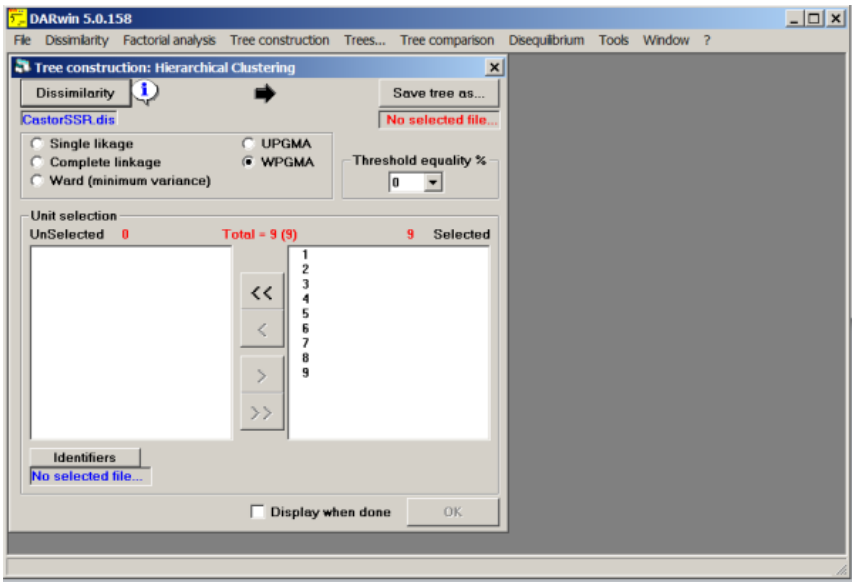


8. The dissimilarity matrix will be saved as a text file (castor.dis) in the desired location (C:\Desktop\WinterSchool_TrainingMaterials), which can be opened in any text editor or in MS Excel.

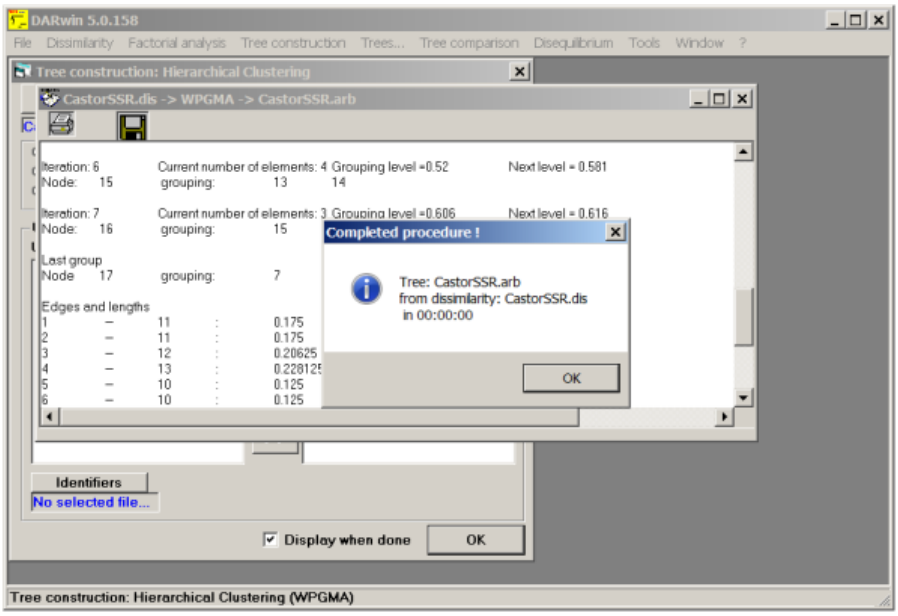
9. To construct the dendrogram, click ‘Tree construction’ option in the menu bar



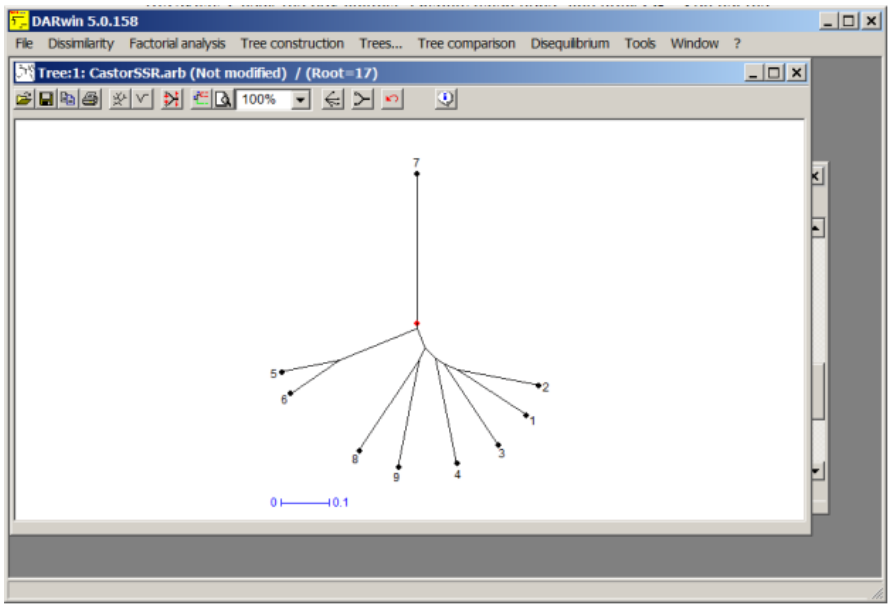
10. You have several options to construct the tree. Choose ‘Hierarchical Clustering’. A popup will open to choose the file. Select the dissimilarity file (‘castor.dis’) created previously. The following window will appear



11. You have few algorithms for clustering. ‘WPGMA’ is the default option. Click ‘Save tree as..’ button and give a file name and location where the file should be saved. By default, it will give the data file name with .arb extension (castor.arb) and store in the directory where the data file exists (C:\Desktop\WinterSchool_TrainingMaterials). Check the box against ‘Display when done’ and click OK. You get the following window



12. Click OK. You will get the tree displayed as below. Mouse over the different buttons available on the menu bar which will give option for changing the display pattern, identifier and illustrations



3 Conclusion

DARwin is sophisticated and user-friendly software. The graphics produced by the software is unique and helps to understand and display the results effectively. A PDF of the manual for operating this software is available at http://darwin.cirad.fr/Home.php. The manual provide simple to follow operational guidelines as well as details of algorithms implemented in the software. The users are encouraged to refer the manual to explore the richness of the software.

Cotton Technical Assistance Programme For Africa

Strengthening the Value Chain

Fellowship Programme on Cotton Biotechnology, 21 October – 20 December 2013, CICR (Nagpur)

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Agrisearch with a human touch