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COTTON BIOTECHNOLOGY

K.R. Kranthi
S. Kranthi
A.B. Dongre

Central Institute for Cotton Research
Nagpur

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FOREWORD

Over the past few years, advances in molecular biology and biotechnology have resulted in the development of powerful tools and techniques that have assisted the enormous strides made in crop improvement. A few years ago it would have been ludicrous to talk about bacterial genes being expressed in higher organisms. But today molecular biology has advanced to such an extent that if scientists of any country are not adequately imaginative they would most certainly be left behind only to put their countries to an economic disadvantage in an open market economy. Cotton crop is a bundle of challenges to the researcher. It has been subjected to stringent research programmes well over the past century. Peculiarly the problems are far from over. At times it seems that the conventional ways of research are perhaps saturated not being able to offer anything drastically different and novel in order to cause a perceptible change in the cotton crop production, protection and improvement programmes. Several conventional projects look ordinary repetitive and jaded. Biotechnological advances are exciting not just because the intricacies of the science are just being unravelled but also because the tools are extraordinarily powerful and can generate products that have a tremendous commercial application in the near and distant future. The commercialisation of Bt cotton and herbicide resistant cotton in most parts of the world, is just the beginning of what will be known as the great biotechnological revolution in years to come. Biotechnological products have attracted the attention of everyone concerned with economic progress, sustainable productivity and environmental compatibility. Though the excitement has been present continuously, apprehensions were also not uncommon. The terminator gene was only an example of how easily a concept can be misinterpreted and misunderstood. This bulletin helps in the simplification of some concepts and perhaps can be used even by beginners to get a feel of things to come. The motivation to encourage scientists to write this bulletin has obviously been the great potential that the science holds in helping researchers in solving persistent problems of biotic and abiotic stresses. Indeed biotechnology makes the unthinkable only a thing of the past. I congratulate the authors for making a good effort in trying to balance the technical and general aspects of biotechnology in order to enable the reader get a grasp on what the science can offer the cotton farmer and researcher.

M.S. Kairon

Preface and Acknowledgement

Biotechnology has the potential to create new plants, new genes and new products that are environmentally safe and economically viable. Cotton biotechnology has tremendous commercial implications. It can change the way cotton had been cultivated thus far. In a few years from now cotton cultivation would certainly be more easier and less input dependent and more compatible ecologically as it was never before. All this without putting any technological load on the farmer for things that he has to remember, or strategies that he has to alter, or inputs that he has to add to his existing list. Over the past one decade cotton crop has been the cynosure of most commercial biotech companies for the huge financial benefits it seemingly offers. And not surprisingly transgenic cotton was one of the first genertically modified crops to be commercially released. The genetic resources of cotton are vast. Cataloguing, classification, charcterization and use of germplasm has always been beset with operational difficulties due to the sheer vastness of germplasm resources and paucity of labour.

Molecular breeding offers great potential to the breeder in facilitating a fast forward operation through time and resources.

Often students, research scholars, scientists, administrators and planners have approached some of us to seek information on serval of the topics mentioned in this bulletin. The subject is certainly not simple enough to be explicitly described in the confines of these forty odd pages. Our attempt has been to layout a basic framework to what can be used as foundation for the reader to build upon for a detailed understanding. How successful our endeavour has been will be reflected in how comfortable the reader would have been with the text and the enthusiasm and benefits accrued due to this bulletin.

The only person we would like to thank is Dr. M. S. Kairon, Director, CICR who was solely responsible for this bulletin to happen.

Keshav Kranthi
Sandhya Kranthi
Ashok Dongre

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Introduction

Biotechnological advances have now become integral to all crop improvement strategies designed to cater to the ever-increasing challenges of the burgeoning population. Problems in cotton sustenance and development have always been a challenge to the researcher. Over the past two decades biotechnology has offered a tremendous scope primarily in the form of creating novel transgenic plants to combat biotic and abiotic stresses and molecular markers that can aid in the quicker selection of parents and progeny in conventional breeding programmes aimed at crop improvement. This bulletin makes an attempt to familiarise the reader with some basic aspects as well as the status and prospects of biotechnological research in cotton. Dealt with, in two parts the first concentrates on the development of transgenic cotton while the next focusses on the potential of biotechnological methods to assist breeding.

Development of cotton transgenics

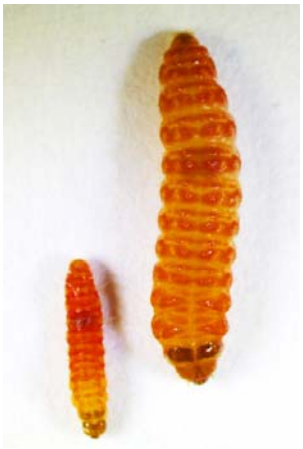
The ability to introduce desirable genes into plants has enabled the genetic manipulation of crop plants to modify them to our advantage and is also an essential component of molecular approaches to plant biology. Genetic transformation has now become a routine technique for several plant species. Introduction of foreign DNA into plants has been achieved using the Ti plasmid of *Agrobacterium tumefacians* or by direct gene transfer methods such as electroporation, PEG (polyethylene glycol) and microprojectile bombardment. The expression of a foreign gene in a multicellular organism could be assayed through the use of selectable markers, which play an important role in confirmation of stable transformation and quantification and visible expression of transformed genes in plant cells. Reporter genes such as chloramphenicol acetyl transferase (cat), neomycin phosphotransferase (nptII), b-glucuronidase (gus), b-galactosidase (lacZ), luciferase (lux) and green fluorescent protein (gfp) have been commonly used in gene expression systems in transformed plants. Confirmation of gene integration is often done using antibiotic markers, enzyme assays of the reporter genes, Southern blotting and labelled probes and PCR of reporter gene or the target gene itself. Expression of the gene is determined by either Northern blotting and use of labelled probes, ELISA (Enzyme linked immunosorbent assay) for the expressing protein and bioassays for the desirable trait.

Cotton crop occupies 32-33 million hectares of world area with a production of 18-19 million tonnes. In India its area currently spans over 9.1 million hectares with an average yield of306 kg/ha of lint and 918 kg/ha of seed cotton. To meet the challenges of 2000 AD with an anticipated population of more than one billion, a total of at least 20 million bales would have to be produced as against the 16-17 million bales of today. This can be achieved by the use of improved crop production practices, generation of novel transgenics coupled with appropriate pest management tactics. Cotton transgenic plants were generated almost a decade ago and are currently under commercial cultivation in the United States of America and Australia since 1996 and China, Argentina, Mexico and South Africa since 1997. Field trials are now underway in Zimbabwe, Columbia, Bolivia, Brazil, EL Salvador, Greece, India, Israel, Paraguay and Thailand. Transgenic crops with in built resistance to insect pests in India could result in at least 25-30% reduction in insecticide use on cotton, resulting in a benefit of about of about Rs 300 crores, apart from the favourable impact on the environment.

Regeneration

- *In vitro* Regeneration of cotton has been a major bottleneck in genetic engineering of cotton. So far generation of transgenic plants, from callus regeneration or somatic embryogenesis have been restricted to a few selected Coker 312 genotypes (Trolinder and Xhixian, 1989).

Helicoverpa armigera



Pectinophora gossypiella

Earias vittella

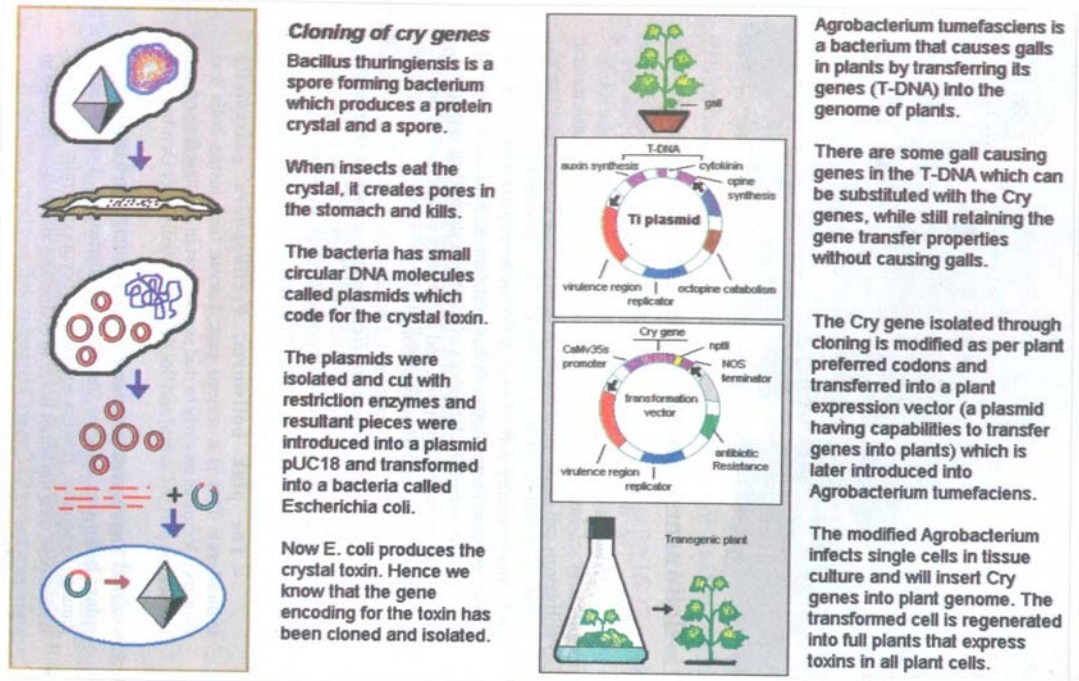
Bollworms –'The notorious three'

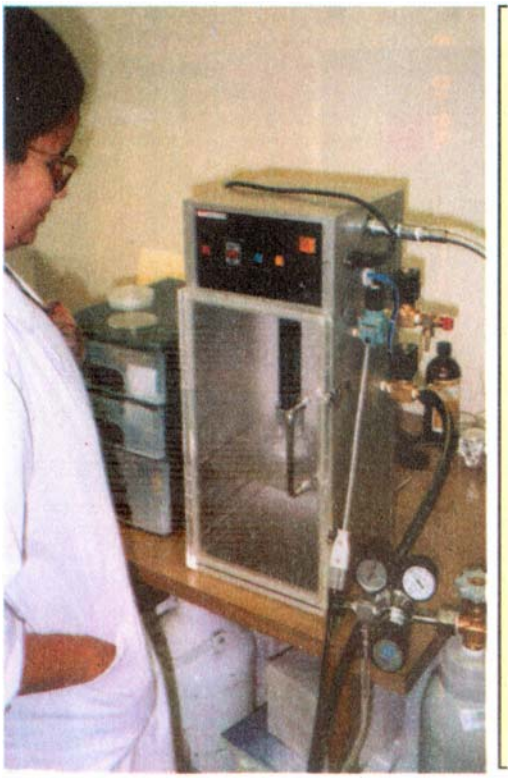
The three cotton bollworms being internal feeders are difficult to control and thrive on fruiting parts of the crop, hence result in heavy economic losses. It is estimated that together the three bollworms can cause yield losses of up to 80% in cotton. Resistance to almost all groups of insecticides has led to persistent insect control problems especially with *H. armigera* thus necessitating the need for viable alternative methods. Insect resistant transgenic cotton is perceived as one of the best tools available to strengthen Integrated pest management programmes, and is certainly considered as one of the most environment friendly options.

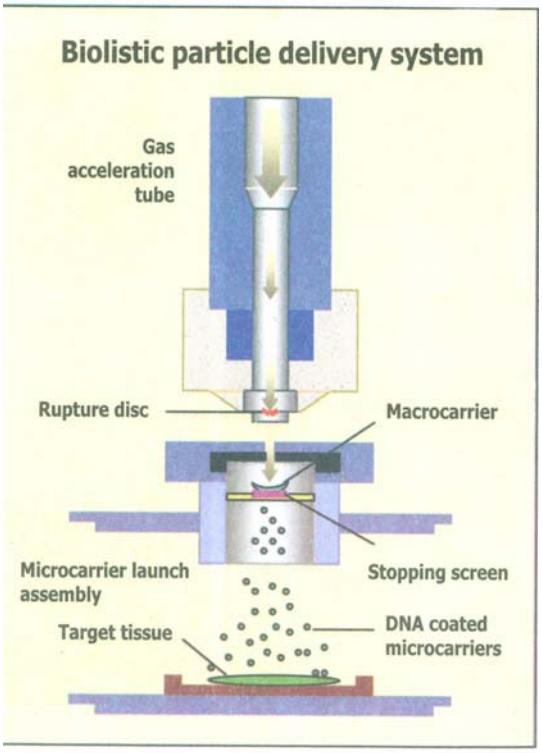
1 **The cotton bollworm, *Helicoverpa armigera* (Hubner):** Larvae feed on squares, flowers and bolls. Typically the larva feeds inside bolls keeping half of its body outside. Each larva can feed on 810 squares and 2- 3 bolls in a single life cycle

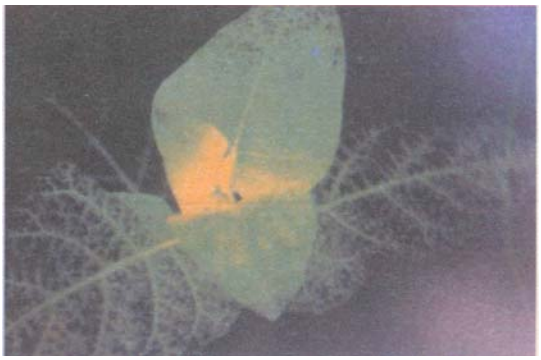
2 **The pink bollworm, *Pectinophora gossypiella* (Saunders):** This is a serious pest. Larvae feed inside bolls and flowers. Occurs as an early to mid season pest in North India and as a late season pest in Central and South India, usually after October.

3 **The spotted bollworm, *Earias vittella* (Fabricius) and the spiny bollworm, *Earias insulana* (Boisduval):** Larvae cause damage to squares, flowers and bolls. It occurs generally as an early season pest, as a top shoot borer in cotton and also causes damage to squares.


Cloning of cry genes
Bacillus thuringiensis is a spore forming bacterium which produces a protein crystal and a spore.
When insects eat the crystal, it creates pores in the stomach and kills.
The bacteria has small circular DNA molecules called plasmids which code for the crystal toxin.
The plasmids were isolated and cut with restriction enzymes and resultant pieces were introduced into a plasmid pUC18 and transformed into a bacteria called Escherichia coli.
Now E. coli produces the crystal toxin. Hence we know that the gene encoding for the toxin has been cloned and isolated.
gall
T-DNA
auxin synthesis
cytokinin
opine synthesis
Ti plasmid
virulence region
replicator
octopine catabolism
Cry gene
CaMv35s promoter
nptII
NOS terminator
transformation vector
antibiotic Resistance
virulence region
replicator
Transgenic plant
Agrobacterium tumefasciens is a bacterium that causes galls in plants by transferring its genes (T-DNA) into the genome of plants.
There are some gall causing genes in the T-DNA which can be substituted with the Cry genes, while still retaining the gene transfer properties without causing galls.
The Cry gene isolated through cloning is modified as per plant preferred codons and transferred into a plant expression vector (a plasmid having capabilities to transfer genes into plants) which is later introduced into Agrobacterium tumefaciens.
The modified Agrobacterium infects single cells in tissue culture and will insert Cry genes into plant genome. The transformed cell is regenerated into full plants that express toxins in all plant cells.




Biolistic particle delivery system
Gas acceleration tube
Rupture disc
Macrocarrier
Microcarrier launch assembly
Stopping screen
Target tissue
DNA coated microcarriers



- However, simultaneously regeneration in other genotypes was also reported from China (Chen *et al.,* 1994), Australia (Cousins *et al.,* 1991), Russia (Kolganova *et aI.,* 1991) and Egypt (Momtaz *et al., 1998).*
- To circumvent the existing problem of genotypic limited regeneration of callus or leaf tissue, other methods such as transformation and regeneration from meristematic tissues have been found useful (Zapata *et al., 1999).*
- Recently, in India, genotypes within variety MCU-5 were regenerated profusely through somatic embryogenesis (Kumar and Pental, 1998).

Transformation

- Though several different methods of plant transformation have been used in various crops, two methods most widely in cotton, involve 1. *Agrobacterium* mediated transfer of DNA and 2. bombardment of cells with DNA coated particles through particle acceleration gene delivery systems.
- *Agrobacterium tumefacians* was successfully used as a vector for cotton transformation (Umbeck *et aI.,* 1987). But this method was limited to only specific cultivars that could be regenerated in tissue culture.
- Later, particle delivery gene transfer systems (Dewang *et aI.,* 1998) were used to transform embryogenic cell suspensions and meristems of elite cotton lines (McCabe and Martinell, 1993) which facilitates the transformation of commercial genotypes thereby eliminating the need for backcrossing with a regenerable genotype.
- Recently, a method to recover genetically transformed cotton plants via *Agrobacterium* - mediated transformation of shoot apex ex-plants, was reported (Zapata *et aI., 1999).*

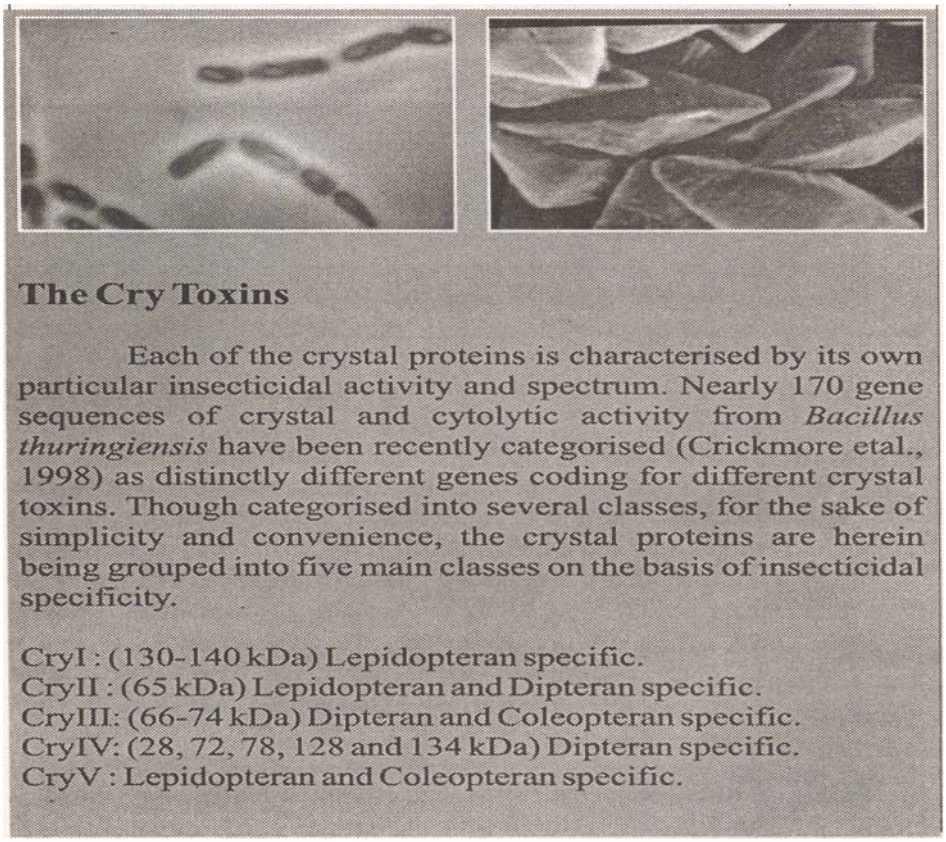
Legends for photographs in opposite page

1. Confirmation of gene integration through PCR for npt II
2. ← Green fluorescent protein expression in transformed leaves
3. Multiplication of transformed plants

Genes used so far

Bollworm resistance

Genes from the soil bacterium *Bacillus thuringiensis* that encode insecticidal crystal proteins and genes that code for inhibitors of insect gut proteases have been used so far for the generation of cotton transgenics.



1. Currently, about 20 insecticidal **crystal (Cry) proteins produced by *Bacillus thuringiensis*** are recognised for their potential in plant protection. Twelve of these, the Cry 1 and Cry II toxins are toxic towards various pest species of lepidoptera. When ingested by insects, the Bt

crystal toxin, binds to specific receptors in the epithelial cells of the larval midgut, disrupts the potassium pump and induces pore formation in the plasma membrane of columnar cells. This leads to an osmotic imbalance and subsequently causes death of the insect. The expression of Cry toxins in cotton plants was enhanced from an initial 0.00 1 % of total soluble leaf protein to 0.1 % by modification of codons of the *cry 1 Ac* gene to make it more compatible with the plant preferred codons (Perlak *et al.*, 1991). Attention is now being focussed currently in obtaining tissue specific expression in boll-rind, squares, flowers, pollen and other plant parts. The commercially released transgenic Bt cottons have a single *cry 1 Ac* gene (Bollgard) derived from *Bacillus thuringiensis* (Bt).

2. **Protease inhibitor genes** from legumes and also insects themselves, which have been used to generate insect pest resistant transgenic cotton, govern expression of proteins that inhibit midgut proteases in lepidopteran larvae. Cotton transgenic plants resistant to *H. armigera* have been developed using the cowpea trypsin inhibitor gene in China (Wei Wang *et al., 1998).*

Both genes, 'Bt toxins' and 'protease inhibitors' used thus far, are extremely specific in their target range and have been conclusively demonstrated to be safe to the environment.

Herbicide resistance

1. The **enzyme nitrilase from a bacterium *Klebsiella* sp** removes the nitrile atom from the herbicide, bromoxynil and detoxifies it (Stewart, 1995) thus conferring resistance.
2. A **monooxygenase gene from a bacterium *Alcaligenes eutrophus*** which detoxifies 2,4-D was engineered into cotton to confer 2,4-D resistance (Lyon *et al., 1993).*
3. Mutated genes for **acetolactate synthase an enzyme** involved in synthesis of branched chain amino acids have been used for resistance against sulphonyl urea and imidazolinone class of herbicides (Yadav *et al., 1986).*
4. Over-expression and/or expression of a **mutated form of an EPSP (5- enol pyruvyl shikimate-3 phosphate) synthase,** a key catalyst in the production of aromatic amino acids for glyphosate resistance has been achieved by the use of a strong constitutive promoter.

Genes under active consideration

Insect resistance

1. **Cholesterol oxidase from a *Steptomycete* fungus** was found to be active on the boll weevil, *Anthonomas grandis* and *H. virescens* (Purcell *et al., 1993).*

2. **'Iso-pentenyl-transferase *(ipt)*'** is a micro-organism derived enzyme that affects feeding of the tobacco homworm, *Manduca sexta* and the potato aphid, *Myzus persicae.* The over expression of this enzyme hinders the cytokinin-biosynthetic pathway.

3. **Lectin genes** have been under active consideration since these produce proteins that act on the digestive system by binding to a sugar moiety causing blood cells to agglutinate.

4. **Spider and scorpion venom genes** that code for a variety of insecticidal peptides have been subject to intense scrutiny due to a potential negative public reaction. *Helicoverpa armigera*

stunt virus is a small RNA virus contains three genes and attacks the midgut cell of *Heliothis* species and causes cessation in feeding. Among the advantages of expressing the virus in transgenic plants is that the plant needs to make only minor amounts of the virus which later amplifies itself in the target insect (Hanzlik *et al.*, 1994).

5. **a- amylases,** which inhibit digestive enzymes in insects, have also been used to modify bean plants for protection against bruchid beetles (Ishimoto et al., 1996) and are under consideration for efficacy against lepidopteran insects.

6. **Insect neuropeptides.** Fifteen neuropeptide hormones have been identified and characterised. Most of these are very small peptides ranging from 5-50 aminoacids, yet can have a devastating effect when over produced or blocked in insects. Because of the small gene size it is easier to use these genes for genetic engineering to over express such neurohormones in plants.

7. **A novel gene from Photorhabdus luminiscens** which is highly virulent against certain insects and genes from straw itch mite (Perlak, 1998) are a few others that are actively being pursued.

Disease resistance

1. **Chitinases, glucanases and glucose oxidases** which act on the cell wall of invading fungi, make the pathogen more susceptible to natural plant defenses.
2. Use of **viral coat protein genes or replicase genes** is another approach for generating disease resistant transgenics.
3. **Magainin I and II, from frogs and antibacterial cecropins from silkmoth** and other insects are under investigation for disease resistance.
4. Recently, an **antisense DNA of CLCuV DNA-A borne ACI gene along with the antisense DNA of the AC2 and AC3 gene** was used for the vector construction and transgenic cotton resistant to the CLCuV (Asad et al, 1998).

Resistance to environmental stress

Till date, environmental stress resistance has not yet been engineered into any plant because of the complexity of plant's response to stress, which is governed by many genes. However a few attempts are being listed here.

1. Expression of **superoxide dismutase gene** in cotton resulted in resistance to chilling stress. Superoxide dismutase confers resistance to chilling stress by virtue of being a free radicle scavenger, in removing reactive oxygen radicals generated during stress which would otherwise result in metabolic damage to cells.

2. **Overproduction of proline** with the bacterial osmogene under the regulation of a constitutive promoter could lead to drought resistance.

3. Successes have been achieved only recently to obtain transgenic crop plants resistant to stress, using genes such as the **antifreeze protein genelala-3** which leads to improved freeze

tolerance and thermo-tolerance using heat shock protein expression.

4. Metabolic engineering of crop plants has also been done leading to enhanced biosynthesis of **'glycine betaines'** which are known to confer tolerance to salt and cold (Scott *et al., 1999).*

Improved fibre quality

1. The production of **polyhydroxy butyrate** (biopolymers which are polyester like compounds produced by certain bacteria) by transgenic cotton fibres has already been demonstrated in the US.
2. Blue cotton through gene manipulation by synthesis of **pigments such as indigo** in fibres.
3. Fibre specific expression of **bioremediation enzymes, melanins** and a number of enzymes and peptides to enhance fibre quality and strength, superior dye binding, absorbency and thermal properties are the other parameters being dealt with through genetic engineering.

Field performance

The commercially cultivated Bt transgenic cotton crops express Cry l Ac toxin. The Cry l Ac toxins are not at all toxic to insect pests belonging to insect orders other than lepidoptera, importantly, the sucking pest complex, which includes jassids, aphids and whiteflies. Hence, insecticides would still have to be used on Bt transgenic cotton. Moreover, Cry lAc is more effective on the pink and spotted bollworms as compared to the American bollworm, *Helicoverpa armigera* and only marginally toxic to the tobacco caterpillar *Spodoptera litura.*

Bt transgenic cotton crops expressing Cry1Ac were found to cause 100% mortality in susceptible *H virescens* in the U.S (Mahaffey *et ai.,* 1995) and far less than 90% mortality of *H. armigera* and *Helicoverpa punctigera* (Wallengren) in Australia (Forrester and Pyke, 1997). In general, *Helicoverpa* species appear to be innately tolerant to the Bt toxins when compared to the *Heliothis* species.

Bt cotton was first cultivated commercially in 1996 in the US and Australia. The area now under Bt cotton has expanded to about 8 lakh hectares in the US, followed by 0.8, 0.5, 0.4, 0.12 and 0.08 lakh hectares in Australia, China, Mexico, South Africa and Argentina respectively (ICAC, recorder, 1999).

Large scale cultivation of Bt cotton has resulted in the significant reduction of insecticide use to the tune of 40 to 60% less than the intensity on the corresponding non-transgenic varieties.

Several studies have evaluated the economic benefits accrued due to the cultivation of Bt transgenic cotton versus the corresponding non-transgenic cultivar.

However, some problems were also encountered in the US and Australia. The com ear worm *Helicoverpa zea,* which is comparatively less susceptible to Cry1Ac, was found to survive on about 1 % of the total area under Bt cotton in the US (Jocelyn, 1996).

In Australia, where the principal pests are bollworms *(H.armigera* and *H. punctigera),* which are also moderately tolerant to Cry lAc, the efficacy of Bt cotton lasted only for approximately half the season. The transgenic plants were found to express Bt proteins only till the 95th day after which fluctuations were observed (Daly and Fitt, 1998). The reduction in expression was primarily due to down regulation and post-transcriptional changes of the unstable RNA. It was also reported that cotton tannins which increase with growth phase, act as antagonists with Bt toxins.

In addition, cotton pest spectrum in the US was found to alter after the introduction of Bt cotton. Unsprayed Bt cotton sustained 4 times more attack of tarnished bugs, 2.4 times more with boll weevil, 2.8 times more with stink bugs and *Spodoptera.* Due to these changes in pest complex, farmers had to spray 3-5 times on bollgard as compared to 6-8 times on non-Bt cottons (Bacheler and Mott, 1997).

Herbicide resistant transgenic cotton crops have been under commercial cultivation in the US since 1997. 'BXN cotton', resistant to bromoxynil and 'Roundup Ready cotton' resistant to glyphosate, were planted in an area of about 13 lakh hectares. Recently stacked gene varieties 'Bt + Roundup Ready' and 'Bt + BXN' cotton expressing combined resistance to herbicides and bollworms were released for commercial cultivation in 1998 (ICAC recorder 1999).

Despite the progressive growth in its area of adoption, some problems have also been observed with herbicide tolerant transgenic cotton. Abnormal boll shedding was reported from 'Roundup ready' transgenic cotton in some parts of US. However, this was found to be due to the use of glyphosate on cotton plants after the fourth leaf stage (ICAC recorder 1999)

Bt cotton-the Indian context: socio-economic issues

Areas with maximum pesticide use per hectare, necessitated mostly due to bollworms, are likely targeted market niches for the bollworm resistant transgenic cottons. Growing Bt cotton transgenics and herbicide tolerant transgenics on marginal land and rainfed areas is not recommended by the companies as these would not show any impact in the absence of strong insect pressure and broad leaved weeds.

Research on transgenic cotton in government funded research labs in India is almost in the final stages. Press reports suggest that Bt transgenic cotton is expected to be introduced soon for commercial cultivation in India by Monsanto-Mahyco biotech, India. Such seeds may also be priced at a premium and it remains to be seen how enthusiastic the response of Indian farmers would be to the expensive input. Transgenic releases from government organisations would definitely enforce a competitive pricing to restrict the monopoly of the private companies.

Transgenic crops with resistance to a specific herbicide encourages the use of only the particular herbicide for weedcontrol and the chemical company holding the patent on the herbicide might be tempted to overprice its product because of lack of competition. In this case, the technology might prove to be beneficial to the resource rich farmers. Clearly, here the targeted market is primarily a high input farmer. The monopoly of transgenics due to patenting rights held by the biotech companies may also lead to high pricing of the seeds.

A significant socioeconomic issue that arises from the introduction of transgenics into the Indian farming system is that the high priced seeds may benefit the prosperous and large farmers thus providing a negative externality on small and marginal farmers. On the other hand it is also argued that the developments from the application of biotechnology would be beneficial to low input farming practices wherein the cost of chemical inputs can be minimised.

The terminator genes

In the developed countries such as US and Australia, the technology fee for transgenic B t cotton is currently charged at US $ 80-112. In addition farmers were required to sign agreements with the commercial companies to prohibit the storing of seeds for the following year and also the transfer of seeds to other growers.

To combat the potential threat of illegal use and transfer of seed the USDA and Delta and Pineland company developed a technology called 'Technology Protection System (TPS)' which produces infertile seeds. The technology is a transgenic system comprised of a complex array of genes and promoters that, in the normal state, are inactive. Seeds with the TPS system are treated with tetracycline that acts as an inducer that triggers the production of a 'ribosomal inactivating protein (RIP)' thus causing infertility in the seed of the next generation without affecting its current germination.

The TPS technology was met with a strong public reaction in almost all developing countries, due to fears of a possible spread of the 'infertility causing terminator gene' in other cultivars of the same or related species. It was also perceived as exploitation of poor farmers, particularly in small farming communities (Rakshit, 1998).

Resistance development to Bt transgenics

The development of bollworm resistance to Bt toxins is an accepted inevitability. The concern was elevated when it became known that Bt cotton crops were overwhelmed by the bollworm in about 8000 hectares out of the 8 lakh hectares planted in 1996 in the US. However, the increase in insect numbers on transgenics was reportedly due to the unusual hot weather and the nearby com crop which served as a breeding ground for the pest.

In India, Bt sprays have not been commonly used and almost all *Helicoverpa* populations tested so far has indicated susceptibility to Bt toxins (Kranthi, unpublished). This might provide a good opportunity to exploit the full potential of the first few generations of the Bt transgenic cottons before eventually the pest develops resistance.

Laboratory selection programmes have generated resistance upto 10,000-fold in the cotton bollworm *Heliothis virescens* (Gould *et aI.*, 1995). However, It would still be a matter of speculation if the rate of resistance development through laboratory selection could be related to what might happen under field conditions wherein a continuous gene-flow occurs in the form of dispersing and immigrant susceptible individual moths. Additionally, in farming systems such as in India where several crops are grown adjacent to each other, in vast mosaic patterns, the selection pressure may also be diluted due to the non-transgenic crops serving as hosts to *H.*

armigera thereby acting as reservoirs of susceptible refuges.

Interestingly, resistance to Cry lAc in field populations of any of the lepidopteran insect pests, is yet to be detected in any part of the world, despite the fact that Bt transgenic cotton was being cultivated on a large scale in the U.S, China and Australia over the past three years. This may have been made possible due to the implementation of Bt resistance management programmes right through inception of field use of the transgenic technology.

Resistant Management strategies

Some of the universally proposed strategies include; use of multiple toxins, rotation of toxin genes, crop rotation, seed mixes, gene pyramiding, high or ultra high dosages, and spatial and temporal refugia.

However, the resistance management strategies required for India will have to be specifically designed keeping the Indian farming situation and the characteristically different cotton pest profile, in mind. *H.armigera* is not a leaf feeder hence, it would be useful to regulate high toxin expressions confined to fruiting parts, throughout the fruiting phase. Cry lAc is only marginally toxic to the tobacco caterpillar *Spodoptera litura* (Kranthi et al., unpublished data). It is important to prevent the re-emergence of the Cry lAc tolerant tobacco caterpillar, *Spodoptera litura* (Fab.) as a major pest, especially in the wake of reduced pyrethroid usage on Bt cotton transgenics. One of the strategies towards this end would be to use toxins such as Cry 1F which is toxic to S. *litura* (data not shown here) in conjunction with Cry lAc which was demonstrated as a synergistic combination against *H. armigera.*

Additionally, in order to effectively reduce the total insecticide use on cotton, it would be a good idea to transform cotton genotypes that are resistant to sucking pests, with Cry toxins, so that the plants would resist a wider range of pest complex.

Bt cotton transgenics, which are the products of intense scientific research involving high costs and efforts, indeed represent the state of-art in pest management technology. Apart from the likelihood of reduction in insecticide use on transgenic cotton by at least 50-90%, it is also expected to ensure favourable ecological, economic and sociological returns, in contrast to the harmful effects due to the use of conventional insecticides. It is in the best interests of the farming benefits of such a technology must be conserved and extended for the longest possible time. Since development of resistance is an evolutionary eventuality, it is imperative that studies must be initiated to understand the basic nature of the phenomenon to enable combat the problem more effectively.

MOLECULAR MARKER ASSISTED COTTON BREEDING

An important prerequisite for Crop improvement is the selection of genotypes possessing desirable traits. Such selection is usually made, based on the phenotypic characteristics that are associated with specific desirable features. Usually the conventional screening and selection procedures against biotic and abiotic stresses are time consuming, laborious and generally less accurate due to environmental and pleiotropic effects. Over the past few years it has been conclusively demonstrated that small pieces of DNA called 'molecular

markers' which are heritable and associated with economically important traits could be used by plant breeders as selection tools which is known as 'Marker assisted selection' . The value of molecular markers in selection depends on their repeatability, map position and linkage with desirable traits. Reliability is often dependent on the tightness of linkage (< 10 centiMorgan) between the marker and the economic trait. For example resistance breeding relies heavily on the availability of strong sources of resistance with definite closely linked markers associated with the trait. In addition, screening for a resistant source involves time and effort. The use of molecular markers simplifies and hastens the selection of desirable parents and thus the transfer of desirable genes among varieties and to introgress novel genes from related wild species.

Traditionally breeders have been using one or more of several approaches for screening and selection, which include the use of morphological traits, biochemical markers or by scoring host plant response to the insect pest. So far about 80 morphological traits in *Gossypium hirsutum* have been used, but most of these have major effects on other important quantitative traits and have very limited usefulness in selection. In many cases the heterozygous condition is not identifiable. Since identification of morphological attributes involves considerable time and effort for selection, apart from association with undesirable traits, it would be ideal to identify molecular markers that co-segregate with resistance and hence can be used for screening and selection. Moreover, molecular markers are new genetic tools with potential to enhance selection efficiency, and are advantageous since these have no effect in themselves on the phenotype. The number of availability of molecular markers is enormous and also have the advantage that the heterozygote of these can be identified. Most morphological markers require mature plants or sometimes progenies, while molecular markers can be used to evaluate seedlings or any plant part. Hence this enables rapid screening of germplasm material and saves on time and effort. Additionally co-dominance is much more common in molecular markers. Molecular markers are inherited as single factor loci while morphological markers are often conditioned by two genes. One of the most commonly used biochemical markers - 'isozymes' has thus far not been identified for insect resistance in cotton. However, the paucity of isozyme loci and the fact that they are subject to post translational modifications often restricts their utility. Currently one of the most viable options for the identification of markers seems to be through RFLPs (Restriction fragment length polymorphism) and PCR (Polymerase chain reaction) based strategies including RAPDs (Randomly amplified polymorphic DNA) and AFLP (Amplified fragment length polymorphism). Marker assisted selection for desirable traits could accelerate the introgression of the desirable gene in current cotton cultivars and also desirable plants in a backcross population could be easily selected without screening through biotic or abiotic pressures, by detection of the presence of any coupling markers. This bulletin lists out various methods and techniques used in the identification and isolation of molecular markers with specific reference to cotton.

Potential for insect resistance markers

Host plant resistance is one of the key components in IPM programmes worldover. Breeding for insect resistance in cotton is of utmost importance, especially in developing countries like India, since it makes pest management easier for resource poor farmers and helps in reducing the need for insecticide use. Successful and economical cotton production programmes depend to a greater extent on the choice of cultivar, which also happens to be the first most important consideration in any pest management decision. Breeding for insect

resistance relies heavily on screening segregating progeny populations derived from crosses between resistant and susceptible sources by artificial insect releases or by cultivation in hot spots. The methods have been useful so far but are cumbersome, uncertain due to escapes and time consuming. With the availability of molecular markers breeders would be able to screen a large number of progeny reliably in a short time using markers on small quantities of DNA isolated from individual seedlings of the progeny. In India crop losses due to bollworms, jassids and whiteflies are considerable, commonly destroying more than half the yield. Recently the problem has been compounded with leaf curl virus being transmitted by the whitefly thus resulting in crop failures in most northern parts of the country. Breeding for insect resistance in cotton has been slower than expected due to problems mostly related to reliable screening techniques to identify sources for resistance. Some of the common constraints include unavailability of regular supply of insects for screening, lesser facilities for large scale cultivation under controlled conditions and screening being dependent on the age and stage of the crop. Moreover, screening and evaluation for resistance are done in the field under unsprayed conditions, which is often characterized by operational difficulties such as absence of adequate natural infestations and problems with assessment of exclusive varietal reaction to specific pests. For example, most exotic germplasm fail to survive an initial jassid attack and hence become inaccessible to screening for sources of resistance to other pests. Under the current predicament wherein the time taken to develop a resistant variety can be inordinately long owing to the cumbersome screening procedures, the availability of molecular markers can greatly hasten the screening and selection process. So far, a number of biochemical and morphological mechanisms in *Gossypium spp.* have been found to mediate resistance against bollworms, jassids and whiteflies. Morphological characters such as hairiness of leaves, toughness of leaf veins, thickness of leaf lamina, length of hair and angle of insertion were reported to be associated with jassid resistance. The pubescent genes H1 and H2 were used to provide jassid resistance to cultivars in India. However, extreme pubescence has an adverse effect on agronomic traits. Cultivars resistant to jassids were also characterized by high levels of non-reducing sugars, tannins, free gossypol, silica, total phenols and epicuticular waxes. *Gossypium arboreum* types as compared to *Gossypium hirsutum* are known to be more resistant to *Helicoverpa* and whiteflies. Morphological attributes such as red leaf, glabrousness, okra leaf and frego bract were reported to confer a high degree of tolerance to *Helicoverpa* and whiteflies. Among other important attributes contributing to resistance against *Helicoverpa* are nectariless, thick boll rind wall etc. Biochemical factors such as high levels of gossypol, heliocides H1 and H2, hemigossypolone, catechin, quercetin and isoquercetin were found to mediate tolerance to *Heliothis spp.* Conversely, total sugars, tannins, flavonols, phenols and gossypol were negatively correlated with population density of whitefly. Such a contrast in the relative importance of diverse attributes mediating resistance against different pests also underlines the importance of the neccessity to identify genotypes with multiple pest resistance, with the aid of molecular markers. Cultivars such as Abhadita, Kanchana, LK 861 and Supriya which are tolerant to *Helicoverpa* and whiteflies have been developed. Genotypes DHY 286, Mahalaxmi, MCU5, Krishna, Sujatha etc. have been identified for resistance against jassids. Such genotypes can be used as one the parents to be crossed with a susceptible counterpart and progeny screening can be initiated to isolate molecular markers linked with resistance.

Potential for disease resistance markers

Cotton crop suffers from several diseases. Some of the important ones are Bacterial blight, *Xanthomonas campestris* pv. *Malvacearum;* Fusarium wilt, *Fusarium oxysporum* F. sp. *Vasinfectum;* Root rot, *Rhizoctonia bataticola* (Taub); Verticillium wilt, *Verticillium dahliae* (Kleb.); Anthracnose, *Colletotrichum gossypii* (South.); Grey mildew, *Ramularea areola* (Atk.); Foliar spots, *Alternaria macrospora* (Zimm.); *Helminthosporium spiciferum* (Bain.) and *Myrothecium roridum* (Tode Ex. Fr.). Verticillium wilt is localized to Tamilnadu and leaf curl virus to north India. Since several traits morphological such as few, small and sunken hairy stomata have been associated with disease resistance, the same have also been often used in screening and selection programmes. Other traits such as red pigmentation for resistance to *fusarium,* and biochemical factors such as high terpenoid and tannin content have been associated with resistance to *Rhizoctonia* root rot and *Verticillium* wilt respectively. Disease resistant genotypes/sources have been identified in both cultivated varieties and wild species. For example MCU-5 VT tolerant to *Verticillium* wilt was isolated from the commercial variety MCU-5. Resistance to some races of *Xanthomonas campestris pv. Malvacearum* and a few other species of bacteria and fungal pathogens has been found among A genome cottons. Wild species such as *Gossypium anomalum, G. armourianum, and G. davidsonii* were found to exhibit resistance to *Xanthomonas; G. sturtuanum, G. harknessii and G. thurberi* to Fusarium wilt and *G. harknessii, G. thurberi, G. sturtianum,* and *G. raimondii to Verticillium* wilt. Almost all the varieties of *G. arboreum* that have so far been released for cultivation have been found to be resistant to Fusarium wilt. Resistance breeding has resulted in the release varieties such as SRT-1, Bikaneri Narma, MCU-8, MCU-10, Suvin, Khandwa-2, Sujay, Cot-13 and Sharda resistant to bacterial bligh. With access to immune and susceptible germplasm lines, it is now possible to initiate a breeding programme to obtain segregation progeny population to identify markers closely linked with the resistant traits.

Potential for drought and salinity resistance markers

In India about 70 per cent of the total cotton area is under rainfed conditions and thus is under constant pressure of abiotic stress such as soil moisture deficit otherwise referred to as drought. Drought resistance in cotton has been associated with morphological characters such as few, small and sunken stomata with capability to close rapidly; thick cuticle, hairy, waxy leaf surface and a deep root system. Biochemical attributes such as high praline content and physiological characters such as high leaf turgidity and osmotic concentration of cell sap are associated with drought resistance. Both the diploid cotton species G. arboretum and G. herbaceum have been found to tolerate severe drought conditions. Several genotypes such as Indore-2, SRT-1 TCH 1002, LH 900 have been identified as drought resistant/tolerant and MESR 17, DHY 286 and Lakshmi as salinity tolerant. Search for markers can be very useful for such polygenic traits as drought and salinity.

Potential for heterosis and quality character markers

Utilization of heterosis in cotton began with the convincing demonstration of heterotic vigour in Hybrid-4 in India. Since the introduction of the first hybrid as much as 42% of the cotton produced in India is from hybrid cotton. Breeders in India and USA have been making attempts to use F2 hybrids. One of the problems faced by breeders is the selection of

good combiners and attempts are being made to determine if genetic distance of some markers could be used as tool to select good combiners to produce good F2 hybrids. So far except a direct estimation of yield and boll number few other parameters have been considered as markers. The use of molecular markers in other crops has been investigated for the verification of pedigree records, assigning inbreds to heterotic groups and quantifying relatedness in the parental stocks used in hybrid programmes. Several researchers have also attempted to characterize and isolate markers for male sterility and fertility restoration so as to hasten the screening process in the development of male sterile lines. Additionally attempts are being made to understand the basis of heterosis and thereby to be able to predict heterosis and performance of hybrids. Breeding for fibre qualities such as colour, strength, length, fineness and maturity has resulted in the release of varieties with superior fibre quality. Cotton varieties, MCU-5, MCU-9, MCU-10, Suvin, K-9, K10, Saraswati, AKA 8401 are good examples of quality cotton. Since recurrent selection method has also been used to iprove fibre quality, it would be easier to initiate work with the existing progeny material that was used in the screening process.

Basic techniques involved

DNA isolation:

Initially cells are disrupted using gentle methods such as enzymatic degradation of cell wall and detergent lysis of cell membranes. Deproteinisation is done by partitioning the extract with phenol chloroform and precipitation of DNA from aqueous phase with isopropanol or ethanol usually in the presence of 0.2 M salt. The enzyme ribonuclease (Rnase) is used to digest and remove and interfering RNA from the preparation. Purity of DNA preparation is determined by estimating absorbance at A_{260}/A_{280} such that the ratio is close to 1.8 to indicate pure DNA. An A_{260} of 1.0 is equivalent to a concentration of 50µg/ml for single stranded DNA.

Restriction enzymes:

Restriction enzymes cut DNA at specific sites. The endonucleases which are the most commonly used class of restriction enzymes, cut at an internal position in a DNA. There are several different restriction enzymes isolated from bacteria based on which they are named. Each enzyme recognizes a specific sequence of 4,5 or 6 bases in the DNA. The expected frequency of recognition sites within the DNA can be calculated as 4^n, where n is the length of the recognition sequence. An appropriate amount of the restriction enzyme is added to the target DNA and incubated in a buffer solution at 37°C. The number of fragments generated due to digestion of DNA by the restriction enzyme depends on the frequency of occurrence of the recognition sequence.

Polymerases

Polymerase enzymes assist in the synthesis of copies of nucleic acid molecules by joining together nucleotides whose bases are complimentary to the template strand in a 5' to 3' direction.

DNA ligases

DNA ligase repairs broken phosphodiester bonds and is used to seal discontinuities in the sugar-phosphate chains that arise when recombinant DNA is made by joining DNA molecules from different sources.

DNA sequencing

Sequencing is done either by Maxam-Gilbert or Sanger Coulson's methods. In the Maxam-Gilbert method the DNA fragment is cloned in a plasmid vector. The DNA is radiolabelled with ^{32}p at 5' ends of each strand, and denatured, separated and purified to give a population of labelled strands for the sequencing reactions. Later the bases of the DNA strand are chemically modified such that only one modification is introduced into each copy of the DNA molecule. The modified bases are then removed from their sugar groups and the strands cleaved at these positions using a chemical called piperidine. The process generates a set of fragments which terminate at different bases and differ in length by one nucleotide. In the Sanger - Coulson's method a copy of the DNA to be sequenced is made by the Klenow fragment of DNA polymerase. The template for this reaction is a single stranded DNA, and a primer is used to provide the 3' terminus for DNA polymerase to synthesise a copy from the template. The production of nested fragments is achieved by the incorporation of a modified dNTP in each reaction. The dNTPs lack a hydroxy 1 group at the 3' position of deoxyribose, which is necessary for the chain elongation to proceed. Such modified dNTPs are known as dideoxynucleoside triphosphates (ddNTPs). Four ddNTPs (A, G, T and C) are included in a series of four reactions each of which contains the four normal dNTPs. Each reaction produces a series of fragments terminating at a specific nucleotide, and the four reactions together provide a set of nested fragments. The DNA chain is labelled by using a radiolabelled or fluorescent labelled dNTP.

Labelling of nucleic acids

Nucleic acids are labelled using either radioactive or non radioactive molecules to generate probes. There are three methods of labelling nucleic acids. 1. End labelling, 2. Nick translation and 3. Primer extension labelling. End labelling is done through the transfer of radiolabelled terminal phosphate group of ATP onto 5' -hydroxy termini of nucleic acid molecules using the enzyme polynucleotide kinase. In 'Nick translation' a nick is created in the DNA using Dnase 1 and a strand replacement reaction is initiated with incorporation of new dNTPs, one of which would be radiolabelled, into the DNA chain using DNA polymerase 1 to create a radiolabelled DNA molecule. In 'Primer extension labelling' the DNA is heated and random oligonucleotide primes are annealed to the single stranded DNA. The KIenow fragment of DNA polymerase is then used to synthesise a copy of the template, primed from the 3' -hydroxyl group of the oligonucleotide through the addition of dNTPs one of which would be radiolabelled. The radiolabelled DNA is separated from the non radiolabelled DNA molecules by gel filteration.

Gel electrophoresis

DNA or DNA fragments can be separated based on their molecular weight dependent migration towards anode through agarose or polyacrylamide gels. The migration of DNA fragments within the gels is proportionate to their molecular weight due to the negatively charged phosphate groups and a uniform charge density of the basic phosphodiester backbone.

Southern blotting and autoradiography

Fragments of DNA are separated on electrophoresis and then transferred to a nitrocellulose or nylon membrane by blotting technique such as capillary blotting, electro blotting or vacuum blotting. In a typical nucleic acid blotting procedure, the gel is placed on a filter paper wick and a nitrocellulose or nylon filter placed on top on which a stack of paper tissues are placed under weight. Transfer buffer is drawn through the gel by capillary action and the nucleic acid fragments are transferred from the gel onto the membrane. The filter can then be hybridised with a radiolabelled probe, washed and exposed to an X-ray film to prepare an autoradiogram. Detection of hybridised bands is also accomplished by colorimetric methods such as biotin labelling or digoxygenin labelling among several other methods.

Polymerase chain reaction (PCR)

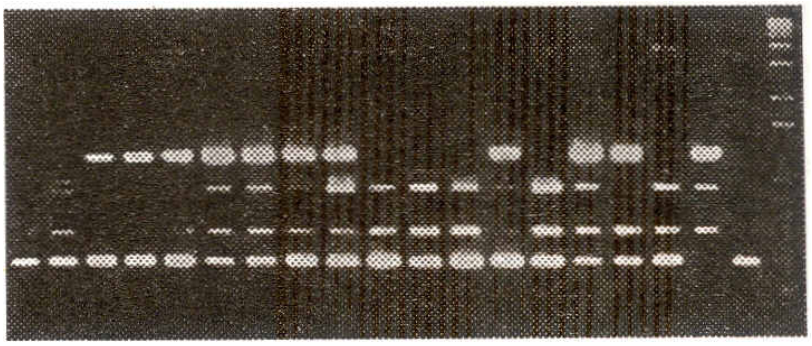
The PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment. PCR has been used extensively in plant breeding to identify polymorphism and thereby for screening and selection of desirable genotypes. The future potential is enormous and must be fully utilized in detection of resistant genotypes in plant populations for reliable diagnosis. Differences in DNA sequences between resistant and susceptible plants, are exploited to synthesize primers, which can be used to selectively amplify the polymorphic DNA to assist in screening programmes.

Genome mapping

Plant genome mapping can assist primarily in identification and tagging of desirable genes, determining gene function, regulation and expression. Through linkage analysis, it is possible to estimate genetic distances between polymorphic traits in the form of isozymes or differential display of DNA fragments or nucleotide sequences. Polymorphism in individuals is a result of base pair changes in DNA sequences due to inversions, translocations, deletions or transpositions. Thus there is an enormous amount of variation present in natural populations. Such polymorphic patterns in DNA of individual organisms are characterised based on the specific differences in sequence of the DNA through restriction mapping or polymerase chain reaction (PCR) DNA amplification methods as listed below:

Isoenzymes:

Isoenzymes are proteins which catalyse the same reaction as that of the same enzyme class but have a different molecular structure as apparent through differential electrophoretic mobility. Isozymes were the first molecularrruukers to be used in plant genetics to map polygenes and were successfully used for genomic characterisation. However the use of isoenzymes is limited due to the paucity of adequate number of isoenzymes to map an entire genome.



Atypical RAPD Mapping gel

RFLP (Restriction Fragment Length Polymorphism):

A typical RFLP analysis consists of five steps. 1. Digestion of DNA samples with restriction enzymes, 2. Separation of the digested fragments on agarose gel, 3. Transfer of the separated fragments to nitrocellulose ornylon filters through Southern blotting, 4. Hybridisation with sequence specific probes and 5. Visualisation with either radioactive or non radioactive methods. The key to RFLP analysis is obtaining appropriate probes which are prepared from gene libraries. Probe selection for linkage map construction depends on identifying homozygous and heterozygous DNA segments that segregate in a predictable fashion in the progeny of a particular cross. It is very critical to select appropriate genetically divergent parent plants if the RFLP programme has to fruitful. Generally it would be very advantageous to construct maps based on crosses of wild relatives with cultivars and screen segregating progeny for desirable traits. After selecting parents DNA is isolated from individual plants of each of the accessions" digested with restriction enzymes, screened for polymorphisms with random probes. For RFLP probes, all loci behave as if they were genetically co-dominant, which is another way of interpretation that both alleles at a locus are visualised. Of the several methods available, polymorphism identified through RFLP is most reliable. Libraries constructed out of cDNAs can facilitate in the development cDNA markers with known gene function, with which the position of specific genes can be marked on chromosomes. Single copy sequences with little repetition can be obtained from cDNA libraries. The DNA probes used in RFLP analysis are not necessarily homologous with known genes.

RAPD (Randomly Amplified Polymorphic DNA):

The assay is based on polymorphism of DNA and the amplification of random DNA sequences using single primers of arbitrary sequence. Polymorphisms produced using this

technique are called RAPD markers. The method is also effective to construct genetic linkage maps because the amplified DNA fragments originating from segregating populations are inherited as dominant or recessive genes following classical Mendelian patterns. RAPDs are usually dominant markers with polymorphisms between individuals identified based on the presence or the absence of a particular RAPD band.

AFLP (Amplified Fragment Length Polymorphism):

The strategy is designed to selectively amplify restriction fragments generated by specific restriction enzymes using oligonucleotide adapters of a few nucleotide bases. Analysis of DNA thus generated on denaturing polyacrylamide gels produces at least 50 or more bands per individual sample. The intensity of the AFLP bands can differentiate between homozygous and heterozygous genotypes.

SCAR (Sequence Characterised Amplified Regions):

The method relies on PCR through designing of longer (20-24 oligonucleotides) primers based on the end sequences of genomic DNA clones obtained from RAPD or RFLP markers. The presence or absence of the single unique SCAR primer amplified DNA band characterises polymorphism. The SCAR markers are nearly identical to sequence-tagged sites in application and have the advantage of being inherited in a co-dominant manner, unlike RAPD markers.

ASAP (Allele Specific Associated Primers):

Allele specific associated primers are used to generate a single DNA fragment from a DNA template extracted under alkaline conditions. Ethidium bromide is then used as a fluorescent indicator of the presence of the amplified double stranded DNA, which characterises the presence of the appropriate allele. Since ethidium bromide does not bind to free nucleotides in the PCR mixture polymorphism is clearly exhibited in the form of the fluorescing double stranded amplified DNA band.

Microsatellites, SSRs (Single Sequence Repeats) or STRs (Short Tandem Repeats):

The SSRs represent an additional level of genomic information that can be used to characterise polymorphism. Microsatellites are regions of small repeated di or tri nucleotide DNA sequences within the genome which do not code for genes and hence are not associated with genes themselves. However, since the sequence repeats can often recombine and expand more frequently, these regions are highly variable and thus could be useful in measuring similarities between closely related species. The method involves the construction of a random genomic library and screening using a repeat element as a probe (e.g. $(AT)_n$ in plants). The deoxynucleotide sequence of the DNA from these clones is determined in order to identify the sequences of nucleotides flanking the repeat element. PCR primers based on the flanking sequences are then synthesised and used to detect polymorphism based on the PCR amplified DNA bands. Several strategies such as **SSR-anchored PCR and SPARs (Single primer amplification reaction)** have been used through microsatellite based methods. The SSR-anchored PCR employs single primers of dinucleotide simple sequence repeats for amplification

of markers. The primer is anchored at 3' or 5' termini with 2-4 nucleotides. Multiple bands containing inter-SSR regions are amplified and visualised on polyacrylamide gels. The bands are generally dominant markers. The SPARs (Single primer amplification reaction) primers which are based on microsatellites or simple sequence repeats (SSR) are used to amplify inter SSR DNA sequences to identify polymorphic patterns between individual samples. An unlimited number of primers can be synthesised from tetranuc1eotide repeats to be used to detect polymorphism. Most SPAR markers are dominant in nature.

SSCP (Single Stranded Conformation Polymorphism):

If suitable primers are designed for economically important traits, SSCP could be used to discriminate progenies using relatively short DNA fragments. The major advantage of SSCP is that it can identify heterozygosity of DNA fragments in nucleic acids of similar molecular weight and can even detect changes in a few nuc1eotides.

CAP (Cleaved Amplified Polymorphic Sequences):

Specific primers are designed based on sequence information obtained from genomic or cDNA clones. The DNA fragments thus amplified are digested using restriction enzymes to observe the polymorphism present between individual samples. The CAP markers are co-dominant.

Mapping populations

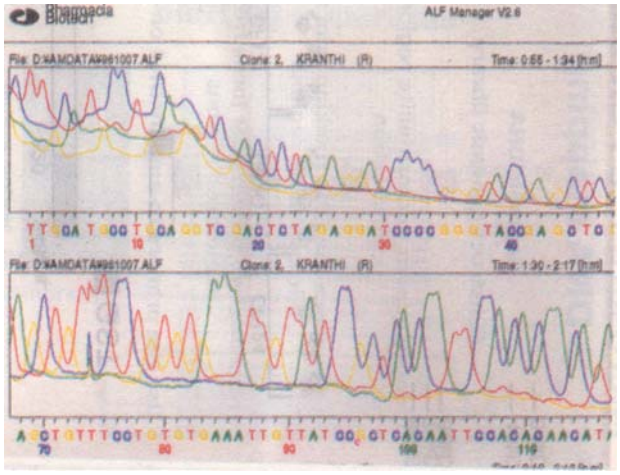
The mapping population is generally derived from either a segregating progeny population or recombinant inbred lines or doubled haploids. Maximum genetic information is obtained usually from a completely classified F2 population using a co-dominant marker system or from a dominant marker system from progeny tests i.e., F_3 or F $_2$BC used to identify heterozygous F_2 individuals. Dominant markers supply as much information as co-dominant markers in recombinant inbred lines, doubled haploids, or back cross populations in coupling phase. Backcross populations can be used to map dominant markers if all loci in the recurrent parent are homozygous and the donor and recurrent parent have contrasting polymorphic marker alleles. Near isogenic lines (NILs) developed through extensive backcrossing can be efficiently used to identify markers, especially employing PCR strategies. This sometimes may cause linkage drag problems i.e., genes incorporated into lines by backcrossing are flanked by DNA segments introduced from the donor parent. Alternatively bulk segregant analysis can be used wherein two bulked DNA samples are drawn from a segregating population originating from a single cross. These bulks contain individuals that are identical for a particular trait (resistant or susceptible) or genomic region but arbitrary at all unlinked regions. The bulks are screened for polymorphisms and these differences compared against a randomized genetic background of unlinked loci. Once the chromosome regions are identified for important quantitative traits, molecular markers can be a tremendous aid in selecting parents and can also be useful in 'finger printing' cultivars and detecting variability within and among cultivars and genetic populations.

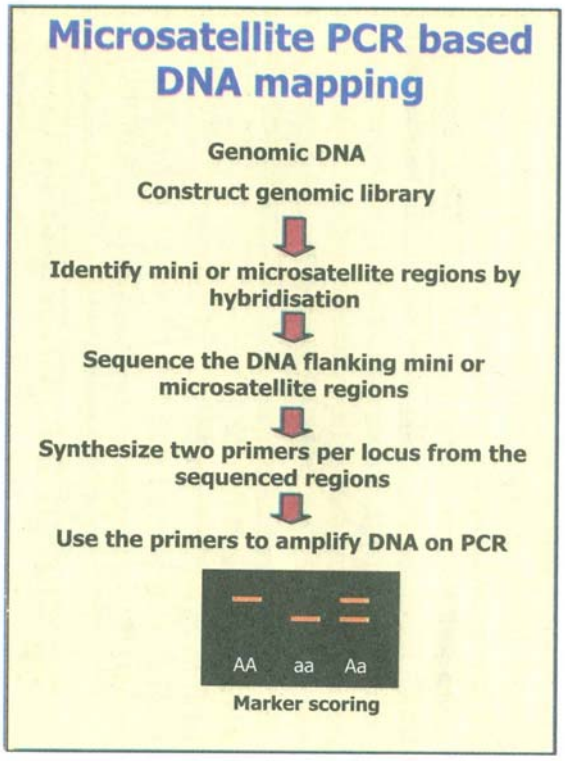
Analysis of QTL (Quantitative Trait Loci) Linkage analysis and physical mapping

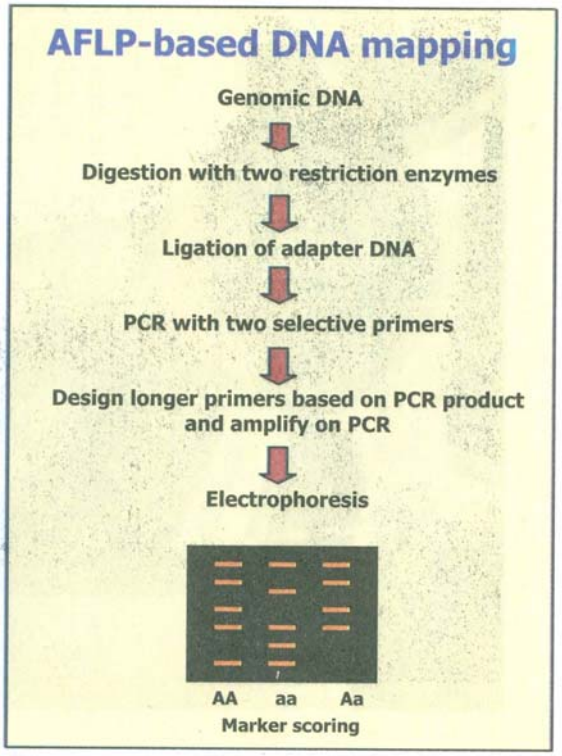
Polygenic controlled characters such as yield, boll weight, boll number, plant height, duration etc. can be identified using any of the DNA markers as has been done in several plants so far. Linkage analysis is derived from scoring a mapping population sequentially with probes from a library used to construct the linkage map. Statistical tests such as chi-square analysis can be used to test the randomness of segregation and thus estimate linkage. Initially there would be more linkage groups than the number of chromosomes themselves, but with increase in marker numbers the linkage groups converge with the chromosome numbers. Map distances are measured using an algorithm such as maximum likelihood. Recombination data can be converted to map distances using any of the commonly used mapping functions. Linkage patterns among molecular markers also can be determined. When large number of markers are available the information can be used to define linkage maps with programmes such as MAPMAKER. For the purposes of physical mapping, initially large genomic DNA fragments of upto 40 to 600 Kb are cloned in YAC (yeast artificial chromosome), BAC (bacterial artificial chromosome) or cosmid vectors. Then genes corresponding to the desirable trait are isolated and characterised before being able to create a physical map.

DNA fingerprinting

Genetic biodiversity can be ideally classified and categorised in a near perfect manner through the generation of DNA fingerprints which are heritable and represent the concerned individuals. The development of genetic fingerprinting has had a major positive influence in areas of basic research and paternity testing. The technique is based on the fact that the plant/animal genome contains polymorphic loci known as hypervariable regions made up of variable number of tandem repeats (VNTRs) of short core sequences. The VNTRs can be detected with probes that hybridise the regions. Initially genomic DNA is digested with a restriction enzyme that cuts outside the repeated regions and specific probes are used to hybridise the fragments on Southern blots to give a genetic fingerprint which is unique to the individual from which the DNA was prepared.




Microsatellite PCR based DNA mapping
Genomic DNA
Construct genomic library
Identify mini or microsatellite regions by hybridisation
Sequence the DNA flanking mini or microsatellite regions
Synthesize two primers per locus from the sequenced regions
Use the primers to amplify DNA on PCR
AA
aa
Aa
Marker scoring


AFLP-based DNA mapping
Genomic DNA
Digestion with two restriction enzymes
Ligation of adapter DNA
PCR with two selective primers
Design longer primers based on PCR product and amplify on PCR
Electrophoresis
AA
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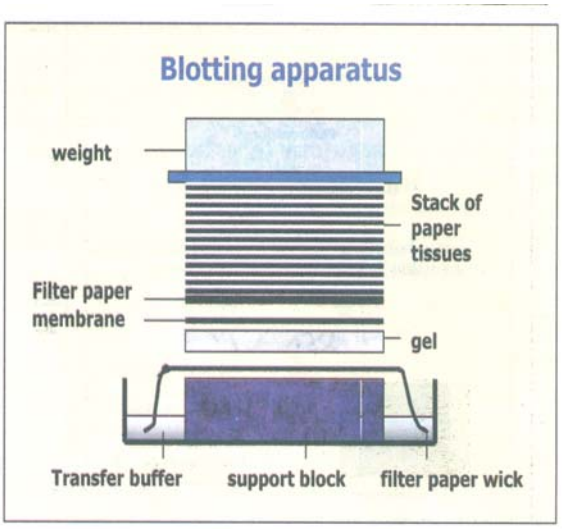
Aa
Marker scoring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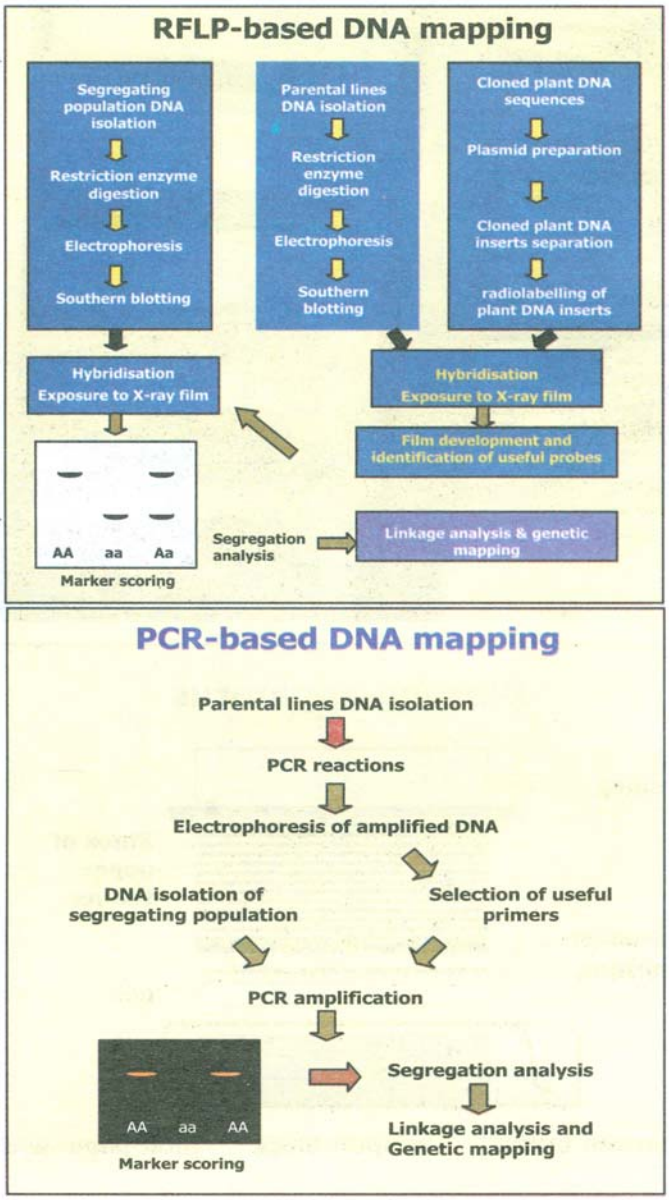
Stack of paper tissues
Filter paper membrane
gel
Transfer buffer
support block
filter paper wick



Cotton genome mapping

The genus *Gossypium* comprises of a total of 49 species which includes four domesticated species comprising of the new world allopolyploids G. *hirsutum* and G. *barbadense* (2n = 52) and the old world diploids G. *arboreum* and G. *hirsutum* (2n = 26). Cotton genome has a total recombinational length of about 5200 cM (centi Morgan, cM = 400 kb) across the 2200-3000 Mb of DNA. The cotton genetic map constructed through crosses of G. *hirsutum* x G. *barbadense* comprised of 705 loci and 41 linkage groups spanning 4675 cM (Reiniscq. et al, 1994).

The levels and patterns of RFLP variation across gene pools in cotton were examined at Texas through the use of 1376 DNA probes and four restriction enzymes to understand the variation among and within tetraploid species. A total of 462 loci were scored as co-dominant alleles. The levels of DNA polymorphism among the tetraploid species were found to be high. It was seen that the average G .*Barbadense* accession was comprised of 8.9% G .*hirsutum* alleles and that Pima cultivars (7.3%) had fewer G .*hirsutum* alleles than sea island (9.0%) or Egyptian cotton (9.6%). Mapping is ideally done through interspecific crosses where polymorphism is high. Biochemical and molecular markers have also been used in mapping the cotton genome. However, only 24 out of the 59 biochemical markers identified were found to be polymorphic. Only a few traits such as phosphoglucomutase 7 *(pgm7),* heat shock proteins and a-amylase were mapped to linkage groups and localised on chromosomes 10 and 12 (Saha and Stelly, 1994). Fluorescence *insitu* hybridisation (FISH) is a molecular cytogenetic tool which was used to reveal a homeologous nuclear organising region (NOR) on chromosomes 26 and 16 (Stelly et al, 1992). So far only thirteen linkage groups could be associated with specific chromosomes. Following is a brief list.

Homeologus chromosome pairs	Linkage group	Character, Loci	Reference
1,15	VII, II	Leaf shape L_1^L, L_2^0 Virescent leaf colouration v_5,v_6 Leaf palisade, lp_p lp_2	Endrizzi and Stein, 1975 Kohel,1973 Kohel,1964
7,16	I,. III,	Anthocyanin pigmentation, R_1 and R_2; Cluster fruiting loci, cl_1,cl_2; Yellow green loci, yg_1, yg_2,: Lint colour loci, Ic_1, D_w	Rhyne and Carter, 1991 Silow, 1946 Rhyne, 1960 Kohel,1985
6.25	IV	Tomentum loci T_1, T_2	Endrizzi and Ramsay, 1979
12,26	V,XIII, IX	Withering bracts bw_1, bw_2; Nectariless ne_1, ne_2; Naked seed N_1, n_2; Lethal Le_1, Le_2; Glandless gl_2, gl_3	Rhyne 1965 Meyer and Meyer, 1961 Ware et al, 1947 Lee, 1981 Barrow and Davis, 1974
A,18	XII, XVI	Open bud trait ob_1, ob_2 Flower colour Y_1, Y_2	Kohel,1973 Stephen, 1954
4	VI	Fregobract fg Stigma-style St_1; Mosaic leaf ml_1	Konel, 1967 McMichael, 1965 Lewis, 1958
5	VIII	Blight resistance B_4	Knight, 1948
20	XVI	Yellow veins yv	Kohel,1983
D	XIV	Virescent v_8, Dwarf red Rd_{33}	Kohel, 1974 McMichael, 1942

In order to make the best use of markers in cotton breeding programmes it is necessary to isolate a large number of markers to select informative markers for all regions of the chromosome or to detect more rapidly evolving regions of the chromosome. In addition the identification of SSR (simple-sequence repeats) based markers can be used to identify cotton cultivars through generation of fingerprints as well as for marker assisted breeding. A significant number of simple sequence repeat loci have been mapped on the cotton genome comprising of the most abundant poly (A) followed by (AT)n, (GA)n and (CA)n. Genetic mapping of 13 SSRs identified 20 polymorphic loci on 12 different linkage groups and all the 16 SSRs that were tested amplified DNA fragments in both A and D genome diploid progenitors of the cultivated AD genome (paterson and Smith, 1999). With the existing markers it is possible to detect the introgression events such as transfer of genomic regions from G. hirsutum to G. barbadense and traits such as verticillium wilt and bacterial blight resistance; nectariless leaves; restoration of cytoplasmic male sterility and improved fibre quality.

Isolation of genes through chromosome walking

A large number of DNA markers that ideally flank or are close to the target gene (ca. 1 cM) are usually required to be able to initiate chromosome walking in order to track down the gene of interest. To enrich the availability of markers, techniques such as RAPD and AFLP are being used all over the world. On the other hand chromosome walking is also facilitated through the development of large-insert cotton DNA libraries. It is estimated that even if the duplicated sites for many loci are inferred, it may be necessary to isolate at least 3000 DNA markers to reach an average 1 cM density. In addition to this the physical genome in cotton will require approximately 60 to 80 thousand YACs (yeast artificial chromosomes) or BACs (bacterial artificial chromosomes) of an average size of 150 kb for 5x coverage (paterson and Smith, 1999).

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