# Expression profiling of fiber traits in Genus *Gossypium*


*By*

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**2006**

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**A dissertation submitted for partial fulfillment of the degree of MASTER OF PHILOSOPHY in BIOTECHNOLOGY**

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# CERTIFICATE

This dissertation submitted by Mr. Muhammad Nawaz is accepted in its present form by National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, the department of biological sciences, Quaid-i-Azam University Islamabad, Pakistan as satisfying the thesis requirements for the degree of Master of Philosophy in Biotechnology.

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Dated 1-3-2006

**Copy No. 210**

*Dedicated*

*To*

*My Dear*

*Madni*

# Table of Contents

# ACKNOWLEDGEMENT

*In the name of Allah, the merciful, the beneficent*

In the name of Allah, the most gracious and the merciful, the creator, the most supreme whose mercy enabled me to accomplish this task and bestowed me with success.

I acknowledge the kind help of all the concerned persons without which I would never be able to complete this manuscript. First of all, I would like to pay attributes to my worthy supervisor Dr.Yusuf Zafar T.I. Deputy Chief Scientist, Director, NIBGE, Faisalabad, Pakistan for his guidance and encouragement; he provided me all the time.

I feel great pleasure to express my sincere thanks to my Co-supervisor Dr.Mehboob-ur-Rahman, SS, Group Leader Plant Genomics & Mol Breeding Lab. Plant Biotechnology Division, NIBGE Faisalabad whose insightful feedback and personal interest were always with me. His hardworking attitude and personal interest always kept me inspiring and ever ready for work.

I would like to express my sincere thanks for Dr.Rob. W. Briddon who helped in conducting dot blot experiments and using different software for sequence homology with known gene sequences of different organisms. I am also thankful to Mr.Imran Amin S.S, NIBGE and Ms Javaria Qazi for their guidance in dealing with radioactivity business. I am also thankful to Dr Aftab Bashir, PS, NIBGE for providing me sequencing facility.

.

My acknowledgement will remain incomplete if I do not acknowledge all my lab fellows Mr. Muhammad asif (S.S) NIBGE, Ms. Nabila Tabbasum, Mr.Ihsan-ul-Allah, Mr. M.Younas, Mr. Ali Manan Mohsin, Mr. Muhammad Zaman, Mr. Kashif, Ms. Huma Mumtaz and Ms.Tayabba Shaheen for their friendly behavior and inspiring cooperation.

Last but not the least; I cannot forget the sincere love, moral support, countless prayers and endless efforts of my mother, father and brothers for my success.

# List of Tables

# List of Figures

# Abbreviations

| | |
|---|---|
| AFLP | Amplified fragment length polymorphism |
| AGP | Arabinogalactans protein |
| ATP | Adenosine tri phosphate |
| BC | before Christ |
| bp | base pair |
| LDCs | Least developed countries |
| cDNA | Complementary deoxyribonucleic acid |
| dd | double distilled |
| DAP | days after pollination |
| DEPC | Diethyl pyrocarbonate |
| DPA | day post anthesis |
| DNA | deoxyribonucleic acid |
| *E-coli* | *Escherechia coli* |
| EDTA | Ethylene diamine tetra acetic acid |
| GDP | Grass domestic product |
| GhTUB | *Gossypium hirsutum* tubulin |
| Kb | Kilo base pair |
| LB | Lauria Bertani |
| LTP | Lipid transfer protein |
| Min | minute |
| M-MuLv | moloney murine leukemia virus |
| mRNA | messenger RNA |
| MT | microtubules |
| PCR | Polymerase chain reaction |
| PCW | Primary cell wall |
| PRP | Proline rich protein |
| RAD | Representational difference analysis |
| RNA | ribonucleic acid |
| SCW | Secondary cell wall |
| TSR | Template suppression reagent |
| U | Unit |
| UV | Ultra violet |
| w/v | weight/ volume |

# Abstract

In recent years, cotton fiber growth and development have been studied using genomic approaches to formulate strategies for fiber modification, the ultimate aim of which is to get good quality fiber. In the present study, we made an attempt for identification and expression profiling of fiber related genes in Genus *Gossypium* using different functional genomic approaches. We selected *G. arboreum* as a model to avoid redundancy regarding polyploidy effects. Gene specific primers were designed after searching the available data. Four different genes with partial sequences were identified using reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR products were cloned in pTZ57/RT vector and sequenced. The identified genes encode ubiquitin extension protein, proline rich protein and myb related proteins. The nucleotide and amino acid alignments revealed that ubiquitin extension genes are highly conserved having more than 90% homology with different species of plant and animal kingdom. Nucleotide tree alignment analysis also exhibited a highly conserved nature of ubiquitin sequences with rice, maize and *Arabidopsis thaliana*. RT-PCR and RNA dot blot results showed that the ubiquitin gene is constitutively expressed while; the expression of the gene coding proline rich protein was up regulated during fiber development. It was also investigated that the myb gene expressed constitutively in the developing fiber. This study will act as a milestone for identification and characterization of fiber specific genes, which will pave the way for understanding the genetics of fiber in the complex cotton genome.

# Chapter 1

## INTRODUCTION AND REVIEW OF LITERATURE

The word cotton has been derived from Arabic origin. The word 'qutun' or 'kutun', which means any fine textile, represents one of the oldest known fibers. It is the earliest fabric remainders found in excavations of ancient civilizations. The word 'cotton' refers to four species in the genus *Gossypium* (*Malvaceae*) i.e. *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L., that were domesticated independently as source of textile fibre (Brubaker *et al.*, 1999a). Globally, the *Gossypium* genus comprises about 50 species, of which 45 are diploid and 5 are allotetraploid (Brubaker *et al.*, 1999a; Percival *et al.*, 1999; Ulloa *et al.*, 2005). The place of origin of the genus is not known, however the primary centers of diversity for the genus are West Central and Southern Mexico (18 species), Northeast Africa and Arabia (14 species) and Australia (17 species). DNA sequence data from the existing *Gossypium* species suggests that the genus arose about 10- 20 million years ago (Wendell and Albert, 1992; Seelanan *et al.*, 1997; Wilkins and Arpat, 2005).

Plant trichomes found on vegetative and reproductive organs throughout the plant kingdom exhibit considerable diversity in terms of size and morphology, distribution, and origin (Werker, 2000). A number of biologically important roles have been ascribed to trichomes, including protection against biotic and abiotic factors, water absorption, secretion, alluring mechanisms, and seed dispersal. While the majority of plant trichomes are multicellular, cotton (*Gossypium* spp.) produces unicellular seed trichomes commonly called ''fibers'', which are of considerable economic importance.

Cotton, a prized agricultural commodity for more than 8000 years, is the world's leading natural fiber and a foundation of economy for most of the countries round the globe. A multi-billion dollar industry, the production, marketing, consumption and trade of cotton-based products further stimulate the economy, making cotton the number one value added crop. However, yield and fiber quality have declined over the last decade (Meredith, 2000) – a downward trend that has been attributed in part to erosion in genetic diversity of cotton varieties and due to increased vulnerability to environmental stresses (Meredith, 2000).

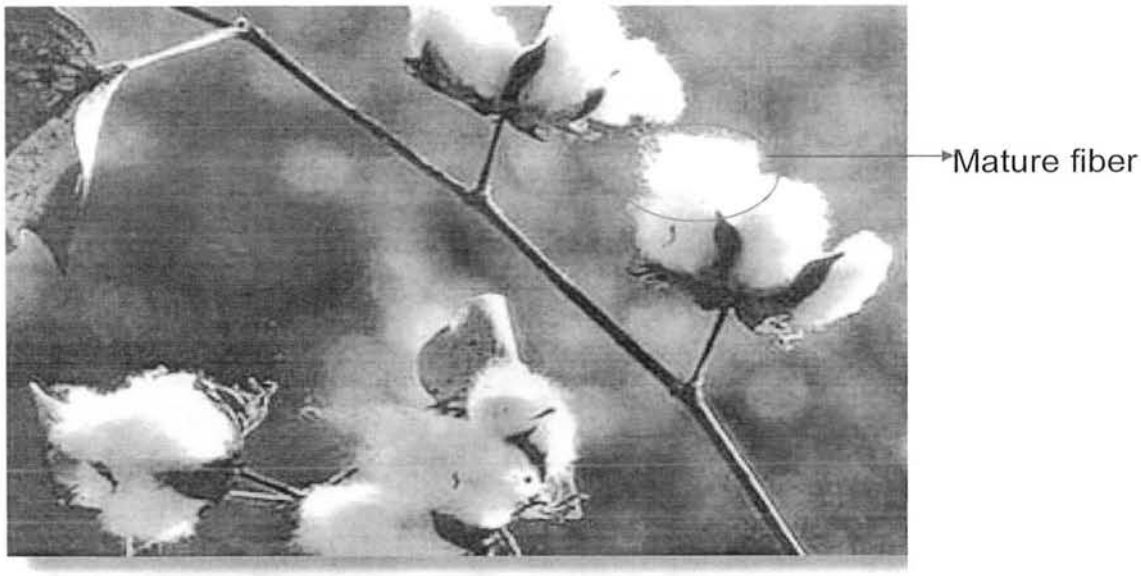



**Figure-1.1**

A monopodial branch of *Gossypium hirsutum* plant having opened bolls with mature fibers from NIBGE cotton field, which is ready for picking.

Cotton is the largest revenue-earning crop in the world. It is the most important economic wheel in developing as well as in industrialized countries. Cotton is grown in more than 70 countries but the top ten countries having an 85% share of world production are, Australia, Brazil, China, Greece, India, Pakistan, Syria, Turkey, USA and Uzbekistan. Pakistan is in the list of five largest cotton-producing countries i.e. China, India, Pakistan, USA and Uzbekistan. These countries share 70% of the total crop production. Agriculture is the backbone of Pakistan's economy. Although Pakistan is one of the large cotton producing countries there is much potential to increase both the quality and quantity. Cotton produced in Pakistan provides raw material to 503 textile mills, 1135 ginning factories and 5000 oil expellers located in this country (Anonymous, 2004).

Two strategies are being followed to reverse the trend of declining quality: introgression of traits from exotic germplasm using traditional breeding methods, and the use of genomic tools and resources to facilitate breeding using molecular approaches. Conventional cotton breeding methods improve fiber quality through the time consuming process of selection based on measuring the fiber traits in advanced generations of the selected lines (Green and Gulp, 1990) but genetic improvement has been hampered by the association of poor fiber

properties with high yields in cotton (Kloth, 1998; Kohel, 1999). Stable introgression of fiber quality is difficult to achieve by incorporating genes of *G. barbadense* into *G. hirsutum* (McKenzie, 1970). Another barrier to fiber quality improvement through conventional breeding in cotton is the length of time required for ultimate selection. The development of an alternative approach to introduce fiber quality genes of *G. barbadense* into *G. hirsutum* (the most cultivated species) is necessary to avoid these difficulties. Genetic engineering is a highly desirable approach for meeting these objectives. Genetic transformation enables the transfer of individual genes from *G. barbadense* into *G. hirsutum*. A logical prelude to cotton improvement strategies through a molecular approach is the establishment of programs that identify and characterize fiber-associated genes in cotton. Identification and characterization of the cotton fiber transcriptome (Arpat *et al.*, 2004) has been a tremendous boon to the understanding of the molecular mechanism of fiber development. It will significantly contribute to the development of functionally anchored genetic maps as a step toward marker-assisted selection and the implementation of strategies for manipulating the transcriptome for the genetic improvement of agronomic traits. This study was therefore undertaken to clone fiber development specific genes from *Gossypium arboreum.* The availability of the reported genes and identification of new genes will help to understand the fiber development pathway and this information may be used for improvement of cotton fiber traits in future studies.

## 1.1- Cotton plant

Cotton represents one of the most important industrial crops in the world and is the most common natural fiber used in the textile industry. Cotton is an annual, biennial or perennial plant but when it is cultivated in the field as a crop, it is generally treated as an annual herbaceous to short shrub or small tree if left to grow on its own in nature, the cotton plant can attain height of 3.5 meters. However when it is grown on a farm, it can grow only to about 1.2 meters. There are many different varieties of cotton; the most common type grown in Pakistan is American Upland cotton (*Gossypium hirsutum*). This is a leafy green shrub that produces cream and pink flowers that turn into the fruit or cotton bolls. The Plant body consists of a primary axis; erect and branched with a vegetative lower zone having

monopodial branches and a fruiting upper zone with sympodial branches. The leaves of the cotton plant alternate, cordate, petiolate, three to nine lobed and palmately veined, with varying size, texture, shape and hairiness. The large, flashy, creamy yellow, pink or purple

**Figure-1.2** A complete cotton (*G. hirsutum*) plant (vegetative plant body) showing stem, branches, flowers and developing bolls.

flowers are extra axillary, terminal, solitary, and borne on sympodial branches. The calyx (sepals) consists of a very short cup-shaped structure at the base of the corolla. The five petals of the corolla are either free or slightly united at the base of the convoluted bud (Sundararaj, 1980). There are colored varieties of cotton including green, brown and blue shades.

### 1.2-Origin of cultivated cotton

History of cotton is as old as human civilization. Cotton cultivation on commercial bases began in Queens land and New South Wales in the 1860s when the American Civil War caused shortages in world cotton supplies. Later on cultivation was attempted in the Northern Territory (1882) and the Kimberley's, Western Australia (1947), although in these northern regions, the prevalence and impact of insect pests limited the commercial viability of continued plantings. One of the most remarkable stories in the annals of crop domestication is the origin of cultivated cotton. Perhaps the most striking aspect of this history is that it is global in scope, involving ancient human cultures in both the Old and New Worlds and a convergent or parallel plant domestication process from divergent and geographically isolated wild ancestors. Indeed, cotton is unique among crop plants in that four separate species were independently domesticated (Brubaker et al., 1999a; Brubaker and Wendel, 1994; Percy and Wendel, 1990; Wendel and albert, 1992; Wendel *et al.*, 1989) for the specialized single-celled trichomes or fibers that occur on the epidermis of the seeds.

### 1.3-Domestication history and cultivated species of Gossypium

The history of domestication of the four species can be summarized as follows.

#### 1.3.1-*Gossypium herbaceum* (African-West Asian Cotton)

Where it grows as a perennial shrub it is native to sub-Saharan Africa and Arabia in semi-desert and savanna. It was probably domesticated in Ethiopia or southern Arabia and its cultivation spread to Persia, Afghanistan, Turkey, North Africa, Spain, Ukraine, Turkistan

and China (first cultivation in China was in about 600 AD). Domestication included selecting for cultivars that grew as annuals.

### 1.3.2-*Gossypium arboreum* (Pakistani-Indian Cotton)

Native to Northwest India and Pakistan and as far back as 2000 BC it was being used by the Harappan civilization of the Indus Valley in the production of cotton textiles. Some cultivars are tall perennial shrubs, others short annuals. One of the perennial cultivars was introduced to East Africa and 2000 years ago was being grown by the Meroe people of Nubia who are considered to be the first cotton weavers in Africa. This variety of cotton was spread to other parts of Africa including Kano in Nigeria, which from the 9th century onwards became a cotton-manufacturing center.

### 1.3.3-*Gossypium barbadense* (South American Cotton)

Probably once widespread along Pacific and Atlantic coasts of South America, wild populations of this species are now only known from coastal Ecuador. The oldest cotton textiles recorded from South America are from archaeological excavations in the northern Chilean desert and date to 3600 BC. The first clear sign of domestication of this cotton species comes from an archaeological site on the Peruvian coast where cotton bolls dating to 2500 BC were found that show characteristics intermediate between wild and modern domestic forms. By 1000 BC Peruvian cotton bolls were no different to modern cultivars of *G. barbadense*. Cotton growing became widespread in South America and spread to the West Indies where Columbus encountered it. Cotton became a commercial slave plantation crop in the West Indies so that by the 1650's Barbados had become the first British West Indian colony to export cotton. In about 1670, planting of *G. barbadense* began in the British North American colonies when cotton planters were brought in from Barbados.

### 1.3.4-*Gossypium hirsutum* (Mexican Cotton)

Wild populations of *G. hirsutum* are found in coastal vegetation of Central and southern North America and were also encountered on islands of the West Indies and islands in the Pacific. Cotton remains dating to 3500 BC have been found in the Tehuacan Caves in Mexico and by 200 BC there is evidence of cotton string and fabric. Spanish explorers in the

1500's found cotton under cultivation throughout the Mexican and Central American lowlands. It was being grown and manufactured into textiles not only by the great Maya and Aztec civilizations but also by smaller tribes. Trade was often in the form of mantas (i.e. strips of textile as they come off the loom) and these were in beautiful colours and patterns. The last Aztec emperor, Moctezuma, demanded cotton mantas as tribute from 34 of 38 provinces under his control. Cotton cultivation and utilization had also spread into southern North America. This required the selection of annual forms capable of growing in long summer days outside the tropics. Cottonseeds in archaeological deposits in Arizona dating to 100 AD suggest that cotton was under cultivation in this region at that time. With the arrival of the Spaniards in the Americas, the annual forms of Mexican cotton were spread to other parts of the world and during the past 200 years, commercial cottons have been derived mainly from Mexican Cotton (Sauer, 1993). No one knows exactly how old cotton is? Scientists searching caves in Mexico found bits of cotton bolls and pieces of cotton cloth that proved to be at least 7,000 years old. They also found that the cotton itself was much like that grown in America today .In the Indus River Valley in Pakistan, cotton was being grown, spun and woven into cloth 3,000 years BC. At about the same time, natives of Egypt's Nile valley were making and wearing cotton clothing. Arab merchants brought cotton cloth to Europe about 800 A.D. When Columbus discovered America in 1492, he found cotton growing in the Bahama Islands. By 1500, cotton was known generally throughout the world. Cottonseed is believed to have been planted in Florida in 1556 and in Virginia in 1607. By 1616, colonists were growing cotton along the James River in Virginia. Cotton goods were brought into Europe by the army of Alexander the Great around 300BC and cotton cloth was only affordable by rich families. Cultivation of cotton and processing cotton fiber to make coarse clothe for their own use in Southern American colonies was started in early 17th century. A revolutionary development of cotton industry occurred in 18th century when Britain acquired colonies suitable for growing of cotton and also improvements in the textile machinery made it possible to spin stronger yarn. Southern American states played an essential role in the 19th century when they became the biggest single supplier of cotton to the flourishing Britain textile mills. It was the end of 19th century when United States was growing more than half of the world cotton. Since then other countries have adopted new

strategies to increase their production while Europe is involved in making progress in manufacturing of textiles goods and machinery.

### 1.4-Why Cotton is important

#### 1.4.1-*Cotton a global crop*

Cotton is an important agricultural commodity providing income to millions of farmers in both industrial and developing countries. Cotton serves as an engine for growth of economy because it is used both as fiber as well as food. According to International Cotton Advisory Committee cotton is the largest revenue-earning crop produced in the world, providing cash income of over 250 million people worldwide. China, United States of America, India, Pakistan, and the Central Asian Republics are leading producer of cotton, share 77 percent of global cotton output and 73 Percent of the global cotton cultivated area. The current market share of cotton is 56 percent of all fibers.

#### 1.4.2-*Pakistan economy and cotton*

The wheel of Pakistan economy moves around the agriculture sector. Agriculture accounts for around 25% of GDP. In the agriculture sector cotton provides the raw material to the textile industry and textile exports contribute more than 60% to the total exports of Pakistan. This means that quality and quantity of the cotton crop has a direct effect on economy of Pakistan. In 1960 the seed cotton yield in Pakistan was 835 kg/ha while in 2003 it was 1818 kg/ha indicating a healthy increase, which is vital for the Pakistan economy. Pakistan is the 5$^{th}$ largest producer and the 3$^{rd}$ largest exporter of raw cotton and 60% of foreign exchange earning is on the behalf of cotton (Rahman *et al.*, 2002) and plays a pivotal role in uplifting the economy of Pakistan.

Cotton is the first hand source of livelihood of 1.3 million farmers in Pakistan who cultivate cotton over 3 million hectares mainly in the provinces of Punjab and Sind, which is 15% of total cultivatable area of the country. Agriculture credit companies, commission agents, 1.3 million farmers, more than 20 pesticide companies, 114 seed companies, ginners, government seed corporation, government seed certification department, irrigation

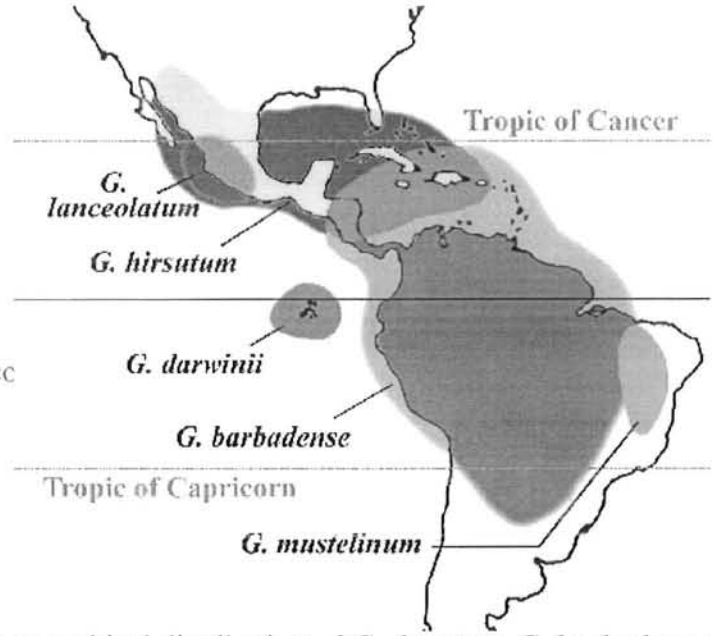
department, cotton research institutes have their individual roles in production of good quality cotton in large quantities (Banuri, 1998).

### 1.5-Geographical distribution

Although the cotton plant is native to tropical countries, cotton production is not limited to the tropics. Indeed, the emergence of new varieties, as well as advances in cultivation techniques led to the expansion of its culture within an area straddling from approximately 47 degrees North latitude (Ukraine) to 32 degrees South (Australia). Although cotton is widely planted in both hemispheres, it remains a sun-loving plant highly vulnerable to freezing temperatures. Cotton is crucially important to several developing countries. Out of the 85 cotton-producing countries in 2005, 80 were developing countries, 28 of which were indexed by the United Nations among the least developed countries (LDCs).

**Table1.1** Cotton-growing countries by geographical area, 2005

| | Developed Countries | LDCs | Other | Total |
|---|---|---|---|---|
| Africa | 1 | 21 | 15 | 37 |
| North and Central America | - | 2 | 14 | 16 |
| South America | - | - | 7 | 7 |
| Asia | 1 | 5 | 16 | 22 |
| Europe | 2 | - | - | 2 |
| Oceania | 1 | - | - | 1 |
| Total | 5 | 28 | 52 | 85 |




(http://www.perunaturtex.cc

**Figure-1.3** Map showing geographical distribution of *G. darwinii*, *G. barbadense and G. mustelinum* in Southern America and that of *G. lanceolatum* and *G. hirsutum* in Central America.

**1.6-Uses of cotton**

Cotton is a miracle of nature...It's a fiber, feed and food crop. The fiber of a thousand faces and almost as many uses, cotton is noted for its versatility, appearance, performance and– above all its natural comfort.

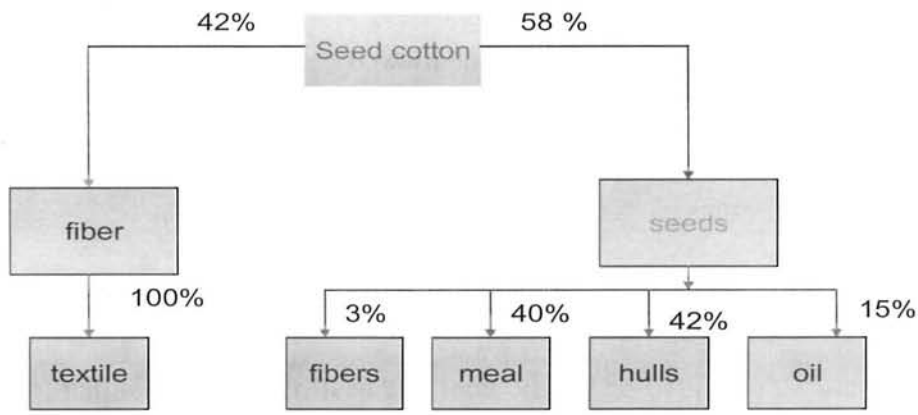



**Figure-1.4** Seed cotton produces only 42% fiber but 58% of cotton seeds which gives only 3% fiber in the form of linters.

### 1.6.1-*Major uses of cotton fiber*

From all types of apparel...to sheets and towels...tarpaulins and tents...cotton in today's fast-moving world is still nature's wonder fiber, providing thousands of useful products and supporting millions of jobs as it moves year after year from field to fabric. The major end uses for cotton fiber include wearing apparel, home furnishings, and other industrial uses (such as medical supplies).

The cotton fiber is made primarily into yarns and threads for use in the textile and apparel sectors (wearing apparel would account for approximately 60% of cotton consumption). Cotton is also used to make home furnishings, such as draperies (eventually the third major end use) or professional garments (about 5% of cotton fiber demand). Besides traditional uses and as a result of different finishing processes that have been applied to the cotton fiber, cotton is made into specialty materials suitable for a great variety of uses.

Cotton fabrics with specialty applications include, for example, fire-proof (flame resistant) apparel, which is suitable for professional uses and provides effective protection against potential risks associated with high temperature and particularly flashover. Flame resistant cotton fabrics are treated with chemicals. Without chemical treatment, cotton would burn up releasing very strong heat, just like the major part of synthetic fibers, which melt when they are exposed to high temperatures.

Cotton also finds special applications in medical and hygienic uses. Most notably, the fiber is used to manufacture hydrophile cotton (cotton wool), compress, gauze bandages, tampons or sanitary towels, and cotton swabs. In this field, the most suitable cotton variety is the species *Gossypium herbaceum* with short-staple thick fibers. (Anonymous, 2004)

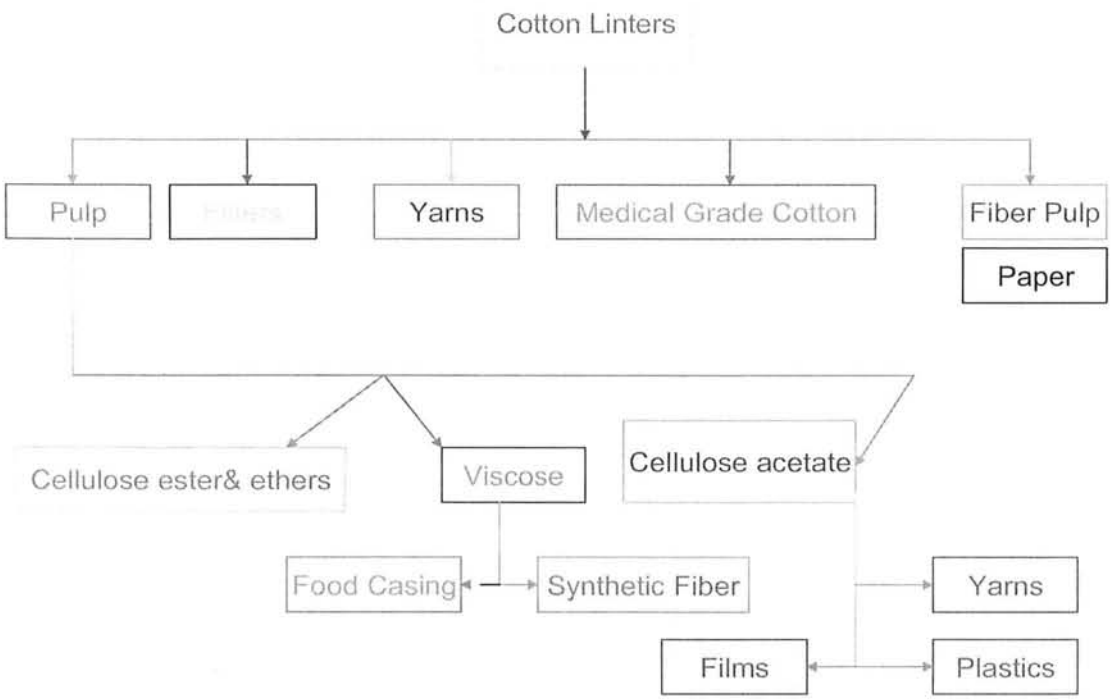



**Figure-1.5** A variety of final products is obtained from cotton fibers.

### 1.6.2-*Major uses of cottonseed*

Cottonseed oil is mechanically extracted from the cottonseed by means of screw or press. Cottonseed oil ranked fifth in production and consumption volume among vegetable oils over the period 1961-2003, accounting for approximately 8% of the world's vegetable oil consumption (close to the ratio of groundnut oil). In many countries of francophone Africa (notably, Mali, Chad, Burkina Faso, Togo, Ivory Coast, and Cameroon), cottonseed oil (used as oil or margarine) provides the main source of fat and oil supply and has several food applications. Cottonseed oil was one of the world's first vegetable oils, is cholesterol-free and high in poly-unsaturated fats. It also contains high levels of antioxidants (Vitamin E) that contribute to its long shelf life. (anonymous, 2004)

The whole seed including the hull (shell) is used as stock feed. The stalk of the cotton plant is processed for the development of ethanol for petrol and diesel blends and is also used as

mulch to improve soil organic matter. Cottonseed oil is also further refined for use in soaps and cosmetics.

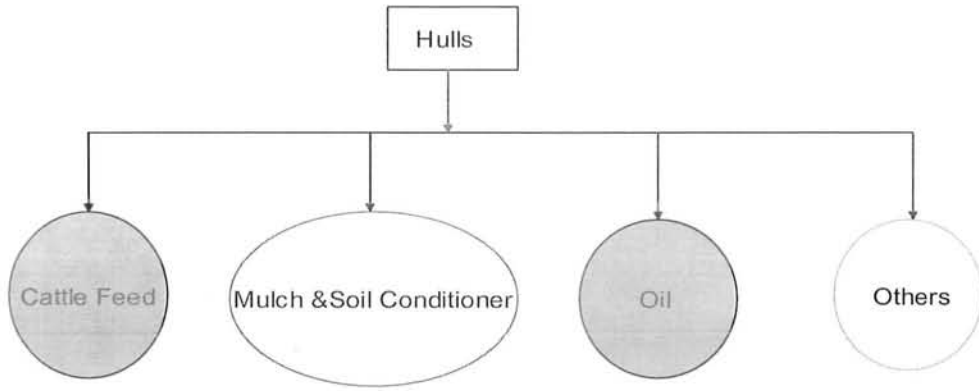



**Figure- 1.6** Major use of Cottonseed is in the production of oil and cattle feed.

### 1.6.3-Some figures about the cottonseed oil market

*Cottonseed oil production*

The five largest producers of cottonseed oil over the period 1995-2003 (four are developing countries and countries in transition) accounted for a combined 70% of global output. Their relative shares were:

- China: 27%,
- United States: 12%
- Former Soviet Union: 10% (of which: Turkmenistan: 10% and Uzbekistan: 72%, --- -- Tajikistan: 8%, Kazakhstan: 6%),
- India: 11%,
- Pakistan: 9 %

The foundation on which Pakistan's economy stands is the agriculture sector with approximately 25% share of GDP. Within the agriculture sector cotton crop is the vital raw material for the textile industry. As textile exports comprise more than 60% of Pakistan's total exports, thus the success or failure of cotton crop has a direct concern regarding textile exports. Cotton production is the inherent comparative advantage of the textile sector of Pakistan.

### 1.7-Genome of *Gossypium species*

The genus *Gossypium,* a member of the family *Malvaceae*, consisting of 50 species, four of which (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L.) were domesticated autonomously as sources of textile fiber (Brubaker *et al.*, 1999a; Ulloa *et al.*, 2005; Fryxell, 1984). The New World tetraploid species are allotretraploids, which means that their chromosomal makeup represents the genomes of two distinct diploid species (Munro, 1987) whose phylogenetic relationships have been explored using multiple molecular data sets (Seelanan *et al.*, 1997; Small *et al.*, 1998; Cronn *et al.*, 2002b). New World, diploid *Gossypium* species comprise a morphological and cytogenetic (D genome) assemblage (Cronn *et al.*, 2002; Endrizzi *et al.*, 1985; Wendel, 1995; Wendel and Cronn 2003) that taxonomically is recognized as subgenus Houzingenia (Fryxell, 1969; 1979; 1992).

The genus Gossypium contains eight genomic groups, which are represented as A (*G. herbacium*), B, C (*G. sturtianum)*, D (*G. ramondii*), E, F, and G (*G. australe)*, K (*G. anapoides)* (Endrizzi *et al.*, 1984; Edwards and Mirza, 1979; Endrizzi *et al.*, 1985; Stewart, 1995). Each genome can be distinguished by a group of morphologically parallel species and the chance of forming hybrids with species of other genomic groups. Most of the genus is diploid (n = 13, 2n=2x=26), but five species from the western hemisphere are classic genomic allopolyploids ('AD-genome'; n = 26, 2n=4x=52), (*G. hirsutum* and *G. barbadense*) formed as a result of hybridization between two diploid species ('A-genome' (*G. arborium*, *G. herbacium*) and 'D-genome' *G. ramondii*), possibly in the mid-Pleistocene (Wendel *et al.,* 1989; Seelanan *et al.*, 1997; Cronn *et al.*, 2002). How and when the original crosses occurred is a matter of intense investigation

### 1.8-Polyploidy in cotton

Organisms are said to be polyploid if they have more than two haploid (n) sets of chromosomes, so their chromosome number is some multiple of n greater than the 2n content of diploid cells, which is because of chromosomal replication without nuclear division. For example, triploid (3n) and tetraploid cell (4n) cells show polyploidy.

One generalization that has emerged from the recent massive effort in genome sequencing and mapping in a diversity of organisms is that genome doubling through polyploidy is a prominent process in plant evolution and has played a major role in the evolution of eukaryotic nuclear genomes (Hughes *et al.*, 2000; Makalowski, 2001; Smith *et al.*, 1999; Wolfe, 2001; Wolfe and Shields, 1997). Polyploidization has been especially active and ongoing in higher plants, with up to 70% of all angiosperms having experienced a relatively recent episode of genome doubling (Leitch and Bennett, 1997; Masterson, 1994; Soltis and Soltis, 1999; Soltis *et al.*, 1992).

Although there are various types of polyploidy (Grant, 1981) the most common is allopolyploidy, whereby two differentiated genomes, usually from different species, become reunited in a common nucleus as a consequence of a hybridization event. In the simplest case, allopolyploids have one complete diploid set of chromosomes derived from each parental species, and thus contain a doubled complement of genes (homoeologues). Examples of such polyploids abound and include many of the world's most important agricultural commodities (Hilu, 1993) including cotton, wheat, watermelons and marigolds.

*Gossypium hirsutum* and *Gossypium barbadense* are thus classic allopolyploids, resulting from the merger of two formerly isolated diploid genomes. This history may have promoted morphological, ecological, and physiological adaptation, mediated by natural selection on a greatly enhanced level of variation resulting from an instantaneously doubled complement of genes (Fryxell, 1979; Grant, 1981; Ohno, 1970; Otto and Whitton, 2000; Soltis and Soltis, 2000). For the same reasons, genome doubling may have offered novel opportunities for agronomic improvement through human selection (Jiang *et al.*, 2000; Wright *et al.*, 1998). The cotton genus presents its own evolutionary mysteries associated with domestication, some of which have begun to be clarified. Phylogenetic analysis by *Cronn* et *al.* (Cronn *et al.*, 2002) has clearly outlined a sequence-based phylogeny for members of *Gossypium* as well as its tribe, *Gossypieae*. These studies have led to a good understanding of the relationships between the diploid genome groups, which contain two domesticated species (A

genome) and how a single merger of an A genome and D genome species resulted in the formation of a polyploidy clade, which also contains two domesticated species (Wendel and Cronn, 2003) .

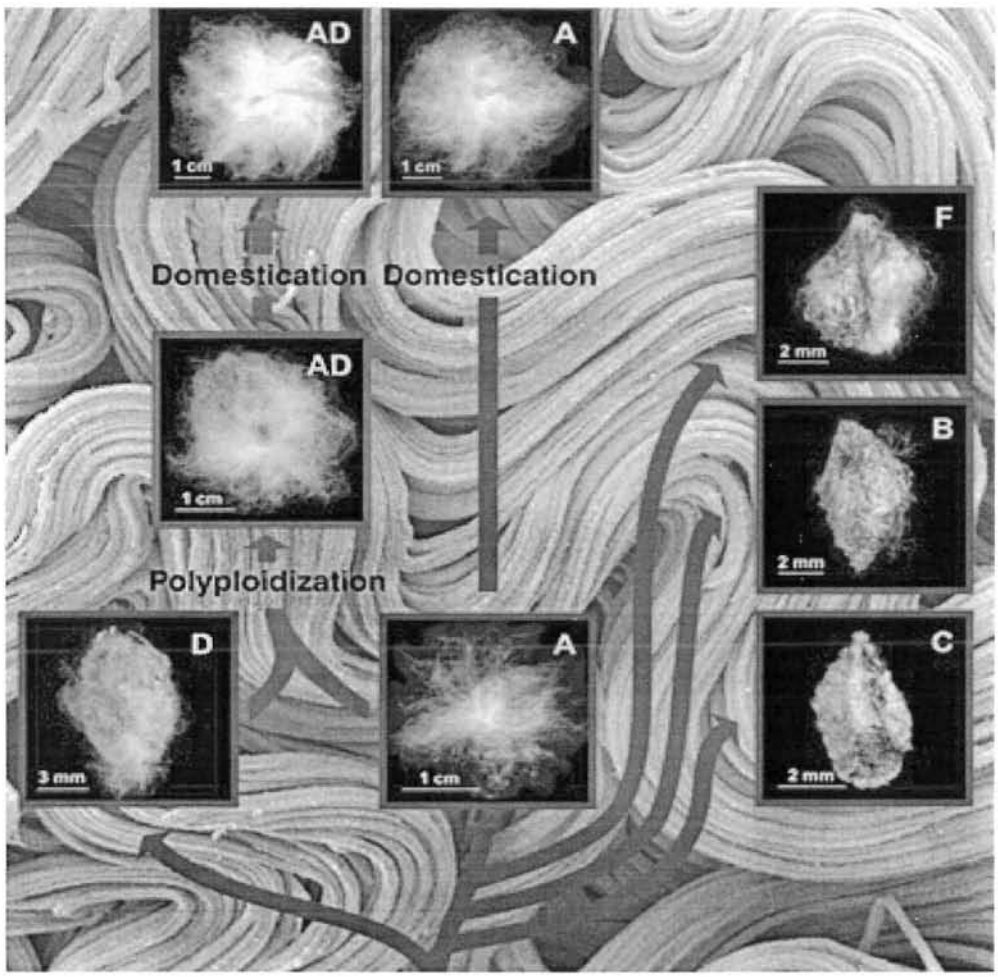



**Figure-1.7** The merger of cotton genome A and D to produce AD alltetraploid cotton and its domestication.

These classic cytogenesis studies demonstrated that the American tetraploid species are true allopolyploids and that they contain two resident genomes, an A genome from Africa or Asia, and a D-genome similar to those found in the American diploids. Additional and conclusive support (Endrizzi *et al.,* 1985) for the hypothesis of an allopolyploid origin of the

American tetraploid emerged in subsequent decades from diverse sources of evidence, including genetic studies of duplicate factors controlling morphology (Stephens, 1944, 1951) meiotic pairing behavior in synthetic polyploids (Gerstel and Phillips, 1958; Phillips, 1964) phytochemical analysis isozyme markers (Saha and Zipf, 1998) comparative genetic mapping (Brubaker *et al.*, 1999 b; Reinisch *et al.*, 1994) and comparative analysis of DNA sequences(Cronn *et al.*, 1999; Small *et al.*, 1998; Small and Wendel, 2000) These latter studies provide particularly compelling proof that the allotetraploid (AD Polyploidy genome) species formed from hybridization between A- and D-genome ancestors: most nuclear genes are duplicated in the AD-genome cottons, and when both copies are isolated and sequenced, they correspond phylogenetically and phenetically to those of the antecedent A- and D-genome diploids.

### 1.8.1-*Polyploidy and fiber*

A final consequence of polyploidy is one of paramount agronomic importance, as it concerns fiber. Four separate species of *Gossypium* have been independently domesticated for their seed hairs. The characteristic that attracted the attention of the earliest domesticators, the seed lint itself, however, evolved only once in the progenitor of all four cotton species, which highlights the fact that the ancestral condition of *Gossypium* species is to have seeds with epidermal seed trichomes that typically are short and tightly adherent to the seed. While mature seeds from wild species exhibit great diversity in fiber length, color, and other properties; it has recently been shown that the earliest developmental stages are similar among all species (Applequist *et al.*, 2001). Indeed, there is little difference in fiber density or developmental profile for the first several days following anthesis.

To identify developmental differences that might account for variation in fiber length and to place these differences in a phylogenetic context, (Applequist *et al.*, 2001) conducted scanning electron microscopy of ovules at and near the time of flowering, and generated growth curves for cultivated and wild diploid and allopolyploid species. Trichome initiation was found to be similar in all species, with few notable differences in fiber density or early growth. Developmental profiles of the fibers of most wild species are similar, with fiber elongation terminating at about two weeks post-anthesis. In contrast, growth is extended to 3 weeks in the A- and F-genome diploids. When this observation is considered in light of the

phylogeny of the genus, it becomes clear that this prolonged elongation period represents a key evolutionary event in the origin of long fiber, and that it happened in the common ancestor of these two groups of diploid cottons prior to domestication in Africa. This observation has a fascinating implication; namely, that the domestication of the New World cottons that presently dominates cotton agriculture worldwide was first precipitated by a developmental switch that occurred millions of years ago in a different hemisphere. In light of the foregoing, several recent studies have suggested that allopolyploidization provided novel opportunities for agronomic improvement. (Jiang *et al.*, 1998; 2000; Wright *et al.*, 1998)

## 1.9-Cotton fiber

Plant trichomes found on vegetative and reproductive organs throughout the plant kingdom exhibit considerable diversity in terms of size and morphology, distribution, and origin (Werker, 2000). A number of biologically important roles have been ascribed to trichomes, including protection against biotic and abiotic factors, water absorption, secretion, alluring mechanisms, and seed dispersal. While the majority of plant trichomes are multicellular, cotton (*Gossypium spp.*) produces unicellular seed trichomes commonly called ''fibers'', which are of considerable economic importance.

The cotton fiber is remarkable and unique in the plant kingdom. It is a modified plant hair or trichome that differentiates from an epidermal cell of the seed coat. It is a single living cell that can grow extremely fast to achieve an average length of 25-30 mm. There are about 10,000 to 20,000 fibers per seed. Fiber number per seed and weight per seed are now being exploited by cotton breeders as a component /building block for higher crop yields since there is significant variability for this trait.

## 1.10-Fiber properties

The physical properties of the fiber give it unique and most significant characteristics to judge the quality of cotton. For the assessment of economic value, as well as for determining the end use of cotton physical properties such as fiber length, strength, fineness and micronaire play a vital role.

Better fiber spins into better yarn, which enables cotton to compete effectively with synthetic fiber. Therefore, the thriving future of the cotton industry depends on high-quality fiber. Three of the most important fiber traits for textile operations are fiber length, strength and micronaire.

### 1.10.1-*Fiber length*

The importance of fiber length to textile processing is significant. Longer fibers produce stronger yarns by allowing fibers to twist around each other more times for more valuable end products. Due to the variability inherent in cotton fiber, there is no *absolute* value for fiber length within a genotype or within a test sample (Behery, 1993). 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. 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 a.,* 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.

### 1.10.2-*Fiber strength*

Fiber strength is directly related to yarn strength. Fiber strength is governed largely by genetics, with some loss attributed to severe weathering between boll opening and harvest, extreme plant stress during fiber development or harsh ginning practices.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 (Munro, 1987; Patil and Singh, 1995; Moore, 1996). Recent developments in high speed yarn spinning technology, specifically open end rotor spinning systems, have shifted the fiber quality requirements of

the textile industry toward higher-strength fibers that can compensate for the decrease in yarn strength associated with open-end rotor spinning techniques (Patil and Singh, 1995). Compared with conventional ring spinning, open-end rotor-spun yarn production capacity is five times greater and, consequently, more economical. Rotor-spun yarn is more even than the ring-spun, but is 15 to 20% weaker than ring-spun yarn of the same thickness. Thus, mills using open-end rotor and friction spinning have given improved fiber strength (together with fiber fineness) highest priority. Length and length uniformity, followed by fiber strength and fineness, remain the most important fiber properties in determining ring-spun yarn strength (Patil and Singh, 1995; Moore, 1996).

### 1.10.3-*Micronaire*

Micronaire is determined both by the diameter of the fiber (biological fineness) and the amount of secondary wall development prior to boll opening (gravimetric fineness). It is an indirect measure of both fiber maturity and fiber fineness.

Micronaire is the most commonly used instrumental fiber-quality test (Lord and Heap, 1988; Moore, 1996). Micronaire is measure of the air-permeability of a test specimen of known mass enclosed in a container of fixed dimensions. Initially, air-permeability of the sample was thought to depend on fiber linear density, and the empirically derived curvilinear micronaire scale was set in gravimetric fineness units of fiber weight per inch (Ramey, 1982; Lord and Heap, 1988). However, basic fluid-flow theory states that air permeability is inversely dependent on the square of the fiber surface area, and linear density units were subsequently dropped from the micronaire scale. Now micronaire (also, *mike* or *mic.*) is treated as a dimensionless fiber property quantified against an empirically derived scale and standardized for each annual crop. 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 (Ramey, 1982). 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. The complex interactions among fiber fineness, fiber maturity, fiber spinning properties, and fiber

dye-uptake characteristics are difficult to interpret or predict and can cause confusion and frustration for breeders and physiologists who engage in research designed to improve fiber quality (Cooper *et al*, 1996; Palmer *et al*, 1996).

At the moment people are interested in cotton with long staple length. Strong fiber with long staple length, strength and fineness survives against the harsh practices of ginning, opening, cleaning, carding, combing and drafting. While the fiber with short staple length is lower yarn strength, reduce spinning efficiency, limit the use of yarn and increase imperfections in the yarn. The industry needs stronger yarns. Since fiber strength translates directly into the strength of rotor yarns, it must posses a higher average level of strength and, most importantly, a lower variability of strength to cope with ever-increasing processing speeds in spinning, weaving and knitting (Benedict *et al* 1999; Deussen, 1992).
Identifying and modifying the genes that control the development of fiber, promises many potential benefits like how these unique cells develop and may ultimately provide the basis for genetic engineering of the most wanted fiber properties in indigenous cotton varieties.

**1.11-Fiber development**

Cotton is still the most marvelous fiber through out the world although it has been used for thousands of years. Fiber is a biological composite of cellulose, small quantities of hemicelluloses, pectin and proteins that provide excellent wearability, comfort and aesthetics. Cotton fiber or seed hair consists of single cell of 30 40 mm in length and 15 μm in thickness. Approximately one hundred million individual seed hairs are present in one pound of cotton and each hair is made of a single cell that is 3000 times longer than its width. Approximately 30% of ovular epidermal cells are differentiated into commercially important lint fibers (Ramsey and Berlin, 1976). Each ovary contains about 25 to 30 ovules, a single ovary contains about one half million synchronously elongating cells, and attains a length of 2.5-3.0 cm after 16-20 days post anthesis (Basra and Malik, 1984; Tiwari and Wilkins, 1995). The rate at which fiber elongates and the final length it attains are more than other plant cells (Cosgrove, 1997). Developing cotton fibers offer both a unique developmental model, as well as an excellent single-celled model for studying many basic biological processes in plants. Indeed, developing cotton fiber has been instrumental in providing a

novel approach towards the mechanisms of cellulose biosynthesis during secondary cell wall modification (Kurek *et a*l., 2002; Peng *et al.*, 2002). Growth of fiber occurs by deposition of materials through entire length of fiber and by tip growth (Seagull, 1990). During cell expansion the movement of solute is the result of developmentally reversible gating of plasmodesmata, the most vital factor in the elongation of fiber cell (Ruan *et al.*, 2001). Reversible gating of plasmodesmata, the expression of cell wall loosening gene and plasma membrane transporters for sucrose and K all are coordinated. Coincident with the transient closure of plasmodesmata, sucrose and K transporter express maximally in fibers at 10 DPA. At the same time fiber osmotic and turgor potential are elevated driving the rapid phase of elongation. Elongation of cotton fiber is terminated by the increased wall rigidity and loss of higher turgor due to the down regulation of the transporter genes and re-opening of plasmodesmata (Cassman *et al.*, 1990).

Cotton fiber is composed of concentric layers. The cuticle layer on the fiber is separable from the fiber and consists of wax and pectin materials. Primary wall composed of celluloses, hemicelluloses, pectin, waxes and other plant materials (4%) while secondary wall is purely cellulosic (96%) and contains no lignin like other plant cells (Arthur, 1990; Ryser, 1985).

Fiber growth usually consists of four distinct but overlapping developmental stages: **Initiation** (Fiber initiation and differentiation), **Elongation** (Cell elongation (primary cell wall synthesis), **Secondary cell wall thickening:** Cell wall deposition (secondary cell wall synthesis), **Maturation** (changes in mineral content and protein level) (Turley and Ferguson, 1996; Orford and Timmis, 1997; John and Keller; 1995; Basra and Malik, 1984; Jasdanwala *et al.*, 1977).

Fiber cell elongation begins about 1 day postanthesis (dPA) (Stewart, 1975) and continues until 22-26 dPA (Schubert *et al.*, 1973; Meinert and Delmer, 1977). Along with the elongation stage, deposition of the secondary cell wall overlaps between 16-18 dPA. (Schubert *et al.*, 1973; Meinert and Delmer, 1977). The cells continue depositing layers of SCW up to 40-45 dPA. Finally as the carpel dehisces; fiber cells dehydrate and become mature fiber. At maturity cotton fiber is 89 percent cellulose (Arthur, 1990; Basra and Malik, 1984). The fiber cell with in a boll instigates growth at about the same time and continue its development in a nearly synchronous manner (Stewart, 1995). Each of developmental phases overlaps the subsequent phase and duration of each phase is temperature and genotype

dependent. The fiber cells attain the length during elongation phase, which is 1000-3000 times of their diameter (diameter 20 μm) (DeLanghe, 1986). The probable thickness of primary wall is 100 to 200 molecules (0.1 to 0.2 μm), and is made up of cellulose (30 percent), other neutral acid polysaccharides, waxes, pectic compounds, and proteins (Arthur, 1990; Ryser, 1985). The secondary wall (8 to 10 μm) is made up of cellulose that is deposited during the third developmental stage (Arthur, 1990; Basra and Malik, 1984).

Each of the developmental phases overlaps the succeeding stage. The Duration of each phase is dependent on temperature and genotype.

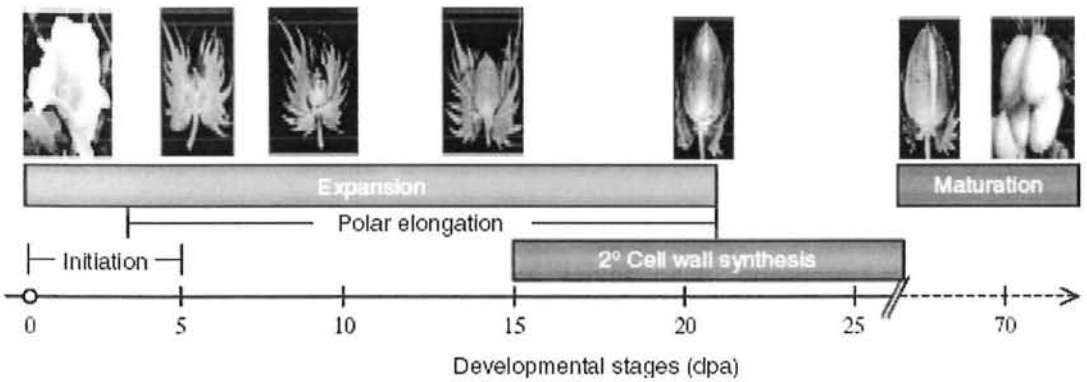



**Figure-1.8** Different development stages of cotton fiber, which are over lapped with each other.

### 1.11.1-Transcriptome of a developing fiber

Cotton fibers, or seed trichomes are unique and the longest cell types still known in the plant kingdom. Cotton fibers i.e. single cells that are differentiated from trichome primordia present at the epidermis (protoderm) of the ovule. Fiber initials are seen as balloon like outgrowths on the surface of ovule at anthesis, that signals entry into the phase of rapid cell expansion, in which approximately 30% of the primordia go through the process of morphogenesis, producing approximately 20000 fibers/ovule (Berlin, 1986). Fiber elongation process continues for approximately 21 days, with overstated growth rates (in excess of 2 mm /day) reaching the peak point levels at approximately 10–12 dpa. Dropping off in growth rate, transcriptional activity (Kosmidou- Dimitripoulou 1986) and protein complexity (Graves and Stewart, 1988) indicates the execution of elongation phase. At this stage, cross-linking of cellulose micro fibrils and non-cellulosic matrices, most likely ''fix'' the structure

primary cell wall (PCW) (Wilkins and Jernstedt, 1999) resulting in the first significant increase in fiber strength (Hsieh, 1999). Cotton fibers enter a ''transition'' phase at approximately 15 dpa, which signals the developmental switch from PCW to secondary cell wall (SCW) synthesis. The fiber SCW is deposited in extensive amounts and as a result imparts the fiber with its distinctive characteristics suitable for spinning into threads and yarns. The fiber SCW consists of >94% cellulose. Although it has been speculated that programmed cell death plays a role in fiber maturity, practically nothing is known about the latter stages of fiber development as molecular studies are hindered by the inability to isolate RNA from fibers much past 25 dpa when cellulose is being deposited. The type of growth mechanism (diffuse versus tip) associated with the overstated growth rate of rapidly elongating cotton fibers has been hotly debated for decades (Seagull, 1990; Wilkins and Jernstedt, 1999). Diffused growth is supported by structural and physiological data (Tiwari and Wilkins, 1995). The microtubule (MT) cytoskeleton is re-orientated from longitudinal to transverse helical arrays in fiber primordial that are perpendicular to the axis of elongation, and hence, direct polar elongation. This role of the MT cytoskeleton is supported by the observation that polar elongation is aborted by MT inhibitors, whereas disruption of the actin cytoskeleton only serves to retard growth. A number of secretory vesicles having newly synthesized cell wall material can be observed in the process of fusing with the plasma membrane and depositing contents into the extra cellular matrix. There is marked asymmetric distribution of vesicles along the length of the fiber, telling that polar elongation occurs via a biased diffuse growth entailing preferential deposition of cell wall materials in the region of the tip. However, the fiber is highly pointed, and vesicles distributed along the entire length of the cell occur in support of lateral expansion of the fiber as well, in which the diameter of the fiber is determined at the base of the cell by 3–4 dpa. Polar elongation of developing cotton fibers via diffuse growth is therefore controlled at the cellular level by the transverse orientation of MTs, the sites of cell wall loosening, and cell wall deposition in the extra cellular matrix, and polar vesicular trafficking (Cosgrove, 2001; Tiwari and Wilkins, 1995). In any case, the bias in distribution of secretory vesicles toward the tip of the fiber would certainly account for the final few millimeters in length attained, as the last residue of cell wall material is deposited in the region of the tip in the final days of fiber elongation. Interestingly, no genes known to be specific to tip-growing cell types (e.g. pollen and root

hairs) have been identified in developing cotton fibers, supporting a diffuse-growth mechanism during rapid polar elongation. Accumulating evidence therefore does not support contentions that fibers supposedly switch from a diffuse- to a tip-growing mechanism. As all plant cells experience cell expansion to some degree during growth and development, so cotton fibers which are elongating very swiftly offer a matchless single-celled model to study the molecular and cellular mechanisms that regulate the rate and duration of cell expansion, and hence, manage cell size and shape, and in the case of cotton, key agronomic traits as well.

The transcriptome of developing cotton fiber represents approximately 18 000 genes in the genome of cultivated diploid species (Wilkins *et al.*, 2005). The cotton fiber transcriptome in allotetraploid species is similarly estimated at approximately 36 000 genes and to contain homoeologous loci from both the $A_T$ and $D_T$ genomes. Genetically highly complex cotton fiber transcriptome in both diploid and tetraploid species accounts for a significant proportion (45–50%) of all the genes in the cotton genome (Arpat *et al.*, 2004; Wilkins and Arpat, 2005; Wilkins *et al.*, 2005). Approximately 1.5 million years (myr) of evolutionary history followed the polyploidization event and polyploidy has not been accompanied by rapid genome change, as the genome organization and gene sequences of orthologous loci from the A and D genomes of diploid and tetraploid cotton species are highly conserved (Senchina *et al.*, 2003; Rong *et al.*, 2004). Moreover, spatial and temporal expression patterns have been evolutionarily conserved (Cedroni *et al.*, 2003). So this suggests that fiber gene function is highly conserved in the genomes of wild and cultivated species, as well as diploid and tetraploid species, despite millions of years of evolutionary history. Quantitative differences in gene expression are responsible for phenotypic variation in fiber properties as opposed to differences in the genotype at the DNA level. Almost 80% of the cotton fiber transcriptome has been identified (Arpat *et al.*, 2004). This study was made to target the genes involved in rapidly elongating cotton fibers for a number of compelling reasons (1) The rate and duration of fiber elongation governs important agronomic properties, such as yield and fiber length, (2) The exaggerated growth of elongating fibers, underpinned by high levels of metabolic activity, was therefore expected to be especially generich from a gene discovery perspective, and (3) Isolation of fibers free of contamination from surrounding complex tissues permit an

unmistakable look at the transcriptome of a single cell within a biologically relevant framework. The judicious selection of a cultivated diploid species *(G. arboreum* Ravi.) as a model for fiber development was because the rate of gene discovery is expected to be enhanced at least two-fold simply by avoiding the redundancy due to polyploidy. The fact that a progenitor of *G. arboreum* served as the maternal parent during polyploidization approximately 1.5 myr (Galau and Wilkins, 1989) tilted selection in favor of this diploid species as a model.

In the developing fibers the high level of metabolic activity during turgor-driven cell expansion and elongation (Smart *et al.*, 1998) is reflected in the genetic complexity of the fiber transcriptome. Almost two-thirds of annotated genes assigned to functional categories based on gene ontology fall into three major groups (energy/carbohydrate metabolism, cellular structure/organization/biogenesis, and protein metabolism) out of approximately 14,000 unique gene sequences identified in elongating cotton fibers, a profile that is certainly consistent with metabolically active cell types (Arpat *et al.*, 2004). Cytoskeleton and cell wall-related genes are among the most copious gene transcripts during PCW synthesis in elongating fibers, while the genes involved in metabolism reflect the vast majority of reasonably expressed genes. This indicates that the transcriptional activity and protein complexity are significantly lower in other developmental stages (Ryser, 1999). It also suggests that the genetic complexity of elongating fibers accounts for a major portion of the cotton fiber transcriptome.

Compared with the *Arabidopsis* trichome, little is known about the molecular control of the cotton fiber development. So far, a number of genes differentially expressed during different stages of fiber development have been identified, but their roles in cotton development are not yet clear. Several cloned genes are fiber-specific, for example, H6 (John and Keller, 1995), Rac13 (Delmer *et al.*, 1995), FbL2A (Rinehart *et al.*, 1996), FS5 (Orford and Timmis, 1997), FS6 (Orford and Timmis, 1997) and GhEXP1 (Orford and Timmis, 1998). Other genes are preferentially expressed in fiber with additional expression in other tissues. For example, further expression was detected in ovary, flower and leaves for E6 (John and Crow, 1992), in leaves for B6 (John, 1995) and LTP6 (Ma *et al.*, 1997) and in root, flower and seed

for CelA1 (Pear *et a.,* 1996), respectively. In addition, their expression patterns are usually developmentally regulated. Based on mRNA transcript accumulation, they could be divided into three groups. The first group includes those expressed in the stages of fiber elongation, such as E6 (John and Crow, 1992) FS6 (Orford and Timmis, 1997), GhEXP1 (Orford and Timmis, 1998), GH3 (Ma, 1997) and GhCAP (Song and Allen, 1997) which peak their transcription during the elongation stage or a little earlier. The second group of the genes has the highest expression during the thickening stage of the cellulose cell wall, for example, H6 (John and Keller, 1995) and Fbl2A (Rinehart *et al.*, 1996) but with little expression in the early stages of fiber development. The third group has a constant expression during the complete developmental stages of fiber, for example, actins (Shimizu *et al.*, 1997) and PPase (Smart *et al.*, 998).

Previous studies about molecular genetics of cotton were mainly focused on partial cDNA libraries obtained by RT-PCR and cDNA arrays/filter arrays of developing cotton fibers (Cosgrove, 1986; Cosgrove *et al.*, 1998; Cosgrove, 2000; Cosgrove, 1995; Cosgrove, 2001; Cosgrove, 1997) or BAC libraries obtained from genomic DNA (DeLanghe, 1986). These libraries have been used to establish the expressed sequence tags (EST's) to link the relationship of pre-reported genes in partial cDNA/BAC libraries. Now the emphasis is on isolation and characterization of differentially expressed genes in fast elongating cotton fiber through suppression subtractive hybridization, fluorescent differential display, cDNA filter array, cDNA representational difference analysis (RDA) and microarray. (Ji *et al.*, 2003; John and Crow, 1992; John and Keller, 1995; Zhao *et al.*, 2001; Feng *et a*l., 2004; Li *et al.,* 2002).

Zhao *et al,* (2001) isolated ten new cDNAs from developing cotton fiber cells, which show high sequences homology to previously reported cDNAs. Five cDNA encode bisphosphate nucleotidase, alpha-tubulin, beta-galactosidase, annexin, and reversibly glycosylated polypeptide while the functions of five other cDNAs were not elucidated. Dot blot analysis of these cDNAs showed that these preferentially accumulated in fiber cells during early phase of cotton fiber development except one hypothetical protein, which accumulated at high level during late phase of cotton fiber development.

Li *et al,* (2002) reported 14 genes and identified ten after RT-PCR analysis that were highly transcribed in cotton fiber. Among them an RD22-like protein (GhRDL),a putative acyltransferase (GhACY), a Fiddlehead homolog(GHFDH),a serine carboxypeptidase-like protein(GHSCP),two tubulin components (GHTUA6 and GHTUB1) and the already reported protein E6 .While three further genes actin (GhACT), a putative cellulose synthase catalytic subunit(GhCesA-5), and a putative 24-sterol-C-methyltransferase (GhSMT) were actively transcribed in elongating cotton fibers, but their transcripts were also present in other tissues. Ji *et al,* (2002) isolated Gh-BTubL from fast elongating cotton fiber having high sequence identity with known plant and yeast beta-tubulin. Over expression of this GhBTub in fission yeast induced longitudinal growth of the host cells with no apparent effect on other aspects of the host cells. This shows that beta tubulin plays an important role in cotton fiber elongation. Thus elucidation of the control mechanisms for expression of tubulin-like proteins may help to improve fiber quality and productivity. Feng *et al*, (2004) reported five gene families; which are proline-rich proteins (PRPs), arabinogalactan proteins (AGPs), expansins, tubulins and lipid transfer proteins (LTPs). Expression profiling of these proteins showed that most of these gene families were expressed during 0-20 dPA (the early cotton fiber developmental stage) while many transcripts accumulated 50 times more after 10 dPA as compared to 0 dPA. Proline rich proteins (PRP) and arabinogalactin proteins (AGPs) are two important cell wall structural proteins. PRPs are widely distributed in plants and are encoded by gene families. PRPs members showed to be regulated both temporarily and spatially during plant development. AGPs are family of proteoglycans that have been implicated in various aspects of cellular activities.

# Chapter 2
## MATERIALS AND METHODS

### 2.1-Plant growth

The allotetraploid cotton *Gossypium hirsutum* .L (Fh-901), *Gossypium barbadense* and *Gossypium arboreum* were used in this study. Seeds were delinted in 10% $H_2SO4$, washed with tap water and air-dried. The seeds were sown in pots that were kept in environmentally controlled growth chamber (Conviron, Canada) and monitored daily. Plants were grown in three replicates. The growth parameters maintained in growth Chamber are given below (Table 2. 1).

**Table2.1**- Growth Parameters

| Time 24 hr/day | Temperature C | Relative Humidity % | Fluorescence Light | Incandescence Light |
|---|---|---|---|---|
| 0:00 | 25 | 65 | 0 | 0 |
| 6:00 | 25 | 65 | 2 | 2 |
| 10:00 | 30 | 65 | 2 | 2 |
| 14:00 | 35 | 65 | 2 | 2 |
| 21:00 | 30 | 65 | 0 | 0 |
| 23:59 | 25 | 65 | 0 | 0 |

### 2.2-RNA extraction from cotton fibers

The seed germinated 4-10 days after sowing. When the plants attained a height of about 10 to 12 inches, approximately two months after sowing, they were transplanted into the field. On the commencement of flowering each flower was tagged. The first day of flower opening was taken as 0 DPA (day post anthesis). Cotton plants typically bloom for about 6 weeks. The tagging of flowers was continued up to the third weak of flowering.

The development and growth of cotton 'boll' begins immediately after fertilization although the most rapid phase of growth and development ranges between 5-16 days after anthesis (Oosterhuis and Jernstedt, 1999). The developing cotton bolls are spherical to ovoid in shape and pale green in colour. At approximately 25 days post anthesis the developing cotton boll attains maximum size and reaches full maturity 20 days later. Mature bolls split open to give seeds and fibers. Fresh sample of ovules at different developmental stages (0DPA, 3DPA, 5DPA and 10 DPA) were collected from the field, wrapped in aluminum foil and preserved in liquid nitrogen. This protects RNA transcripts from degradation and also to make the boll and fibers easier to process; making the boll cover easily removable and the fibers easier to homogenize. Cotton fibers were separated from seed using forceps. Fibers were ground into fine powder and total RNA was extracted by using Quiagen mini plant RNA isolation kit, (Appendix1a). Care was taken during processing to ensure that all plastic/glassware were clean and RNAase-free (Appendix 1b)

Before RNA isolation the working area was cleaned with bleach, ethanol and RNase Away solution (Invitrogen) to avoid the RNAse activity (Appendix 2 a). The pestle and mortar, spatula, forceps, eppendorf tubes and tips to be used in the extraction process were autoclaved. The eppendorf tubes and tips were treated with 0.01% DEPC (diethyl pyrocarbonate), autoclaved and baked in oven at 80oC overnight before use in RNA extraction. Gloves were worn all the times during RNA handling and manipulations. The integrity of the isolated total RNA was checked by electrophoresis on 1% non-denaturing agarose gels (Appendix1b). The concentration of the RNA isolated was measured spectrophotometrically and the RNA was stored at -70°C after making suitable aliquots. (Fig. 3.1)

## 2.3-Synthesis of the first strand cDNA suitable for PCR profiling of cotton fiber specific genes

The messenger RNA at five different cotton fiber developmental stages was reverse transcribed to cDNA (Appendix2a) using 'Revert aid H minus First Strand cDNA Synthesis kit' (Fermentas, USA). This cDNA was used as template in PCR profiling of fiber genes at

different developmental stages of cotton fiber development using gene specific primers (Table 2.2). A Master Cycler Gradient **5331** (Eppendorf, Germany) was used for PCR amplification. PCR products were analyzed by electrophoresis on 1.2% TBE agarose gels visualized by ethidium bromide staining and illumination with UV light.

### 2.4-Primer design

Information about the sequences of cotton fiber specific genes as well as the genes involved in trichome initiation in different plant species was retrieved from the already available data. Twenty primer pairs were designed at the conserved regions of trichome development related genes from different species along with cotton by using the tools from www.expasy.ch and www.justbio.com

### 2.5-Criteria for primer designing

1. The length of the primers was kept close to 18 nucleotides.
2. The melting temperature (2*(A+T) +4*(C+G)) of forward and reverse primer was estimated using OligoCalc (www.justbio.com) and was kept similar by increasing or decreasing the nucleotides of one of the primers in a given pair.
3. Care was taken to ensure that primers did not have complementarity to each other in order to avoid primer dimer formation.
4. The average GC content of each primer was kept close to 50%.

**Table 2.2- Primer sequences**

| NO | NAME | SEQUENCE | FUNCTION |
|---|---|---|---|
| 1 | **SNP 10** | F-CATCATTGGGCTGGACATTG<br>R-GTACACATCGGCATAGGTAG | Proline rich cell wall protein |
| 2 | **SNP 19** | F- CTCCAGTTCTCAACGAATCC<br>R-AGGTACAATCCAGTTGGAGC | profilin |
| 3 | **SNP 23** | F-CGTCAAGATGCAGATCTTCG<br>R-CTTCTTCCTCTTCTTGGCAC | Proloine rivh protein |
| 4 | **SNP 24** | F-CCAATGTGATGAAGCTCC<br>R-GGTGTACCTGAACCATTG | Ubiquitin extension protein |
| 5 | **SNP 76** | F-CTGAGGAAGCTGCTATTGC<br>R-CCAGTCATCCAGTACCATC | Translation elongationfactor1-gamma |
| 6 | **SNP 5** | F-GATTCTACTGCTGGGATACC<br>R-GATTAGGATCACCTGCAAGC | Translation elongationfactor1-beta |
| 7 | **SNP 7** | F-TCCGAGTCGTATCAGAAG<br>R-CAGTTTCCTGATGAGGAG | Transcription factor |
| 8 | **Y23** | F-GGCTTCTTGCCTTCTTCACC<br>R-ATTCGGCACGAGAAAAGCC | Myb like protein |

## 2.6- Polymerase chain reaction

***The PCR reagents for profiling and cloning of fiber specific genes***

| PCR Reagents | Concentration | Volume |
|---|---|---|
| Template cDNA | 1:30 dilution | 5µl |
| dNTPs (10 mM) | 10 mM | 1µl |
| Buffer (10x) | 10x | 5µl |
| $MgCl_2$ (25 mM) | 25 mM | 4µl |
| Forward primer (1µg/µl) | 50 ng/µl | 1µl |
| Reverse primer(1µg/µl) | 50 ng/µl | 1µl |
| Taq Polymerase (5 U/ ul) | 2.5 U | 1µl |
| Double distilled $H_2O$ | | 32.00µl |
| Total Volume | | 50µl |

### 2.6.1-*PCR profile for the amplification of fiber specific genes*

| | |
|---|---|
| Initial denaturation temperature | 94 0C |
| Time | 4 min |
| Cycle | 1 |
| Denaturation temperature | 94 0C |
| Time | 1min |
| Annealing temperature | Variable |
| Time | 1 min |
| Extension temperature | 72 0C |
| Time | 1 min |
| Cycles | 40 |
| Extension Temp | 72 0C |
| Time | 10 min |

Annealing temperature was variable and depends upon the primer length and its GC content. The annealing temperatures for each primer pair for the amplification of genes are given in (table 2.2). All the procedures in the preparation of reaction mixture were carried out on ice. The amplified products were analyzed by electrophoresis on 1.2% agarose gel (Appendix 1c) along with standard 100 base pair DNA ladder (GIBCO) (Appendix 2b)

### 2.7-Agarose-gel electrophoresis

Amplified DNA fragments as a result of PCR were separated by electrophoresis on 1.2% (w/v) agarose gel in 0.5XTBE(Appendix3a) buffer containing ethidium bromide (0.5µg/ml). The gel was examined under UV light. Fragment sizes were estimated by comparison with 100 bp DNA ladder (Fermentas). Fermentas 6 X DNA loading dye was used (Appendix3a).

### 2.8-Elution of DNA fragments from agarose gel

Amplified PCR products were eluted from the agarose gel using DNA elution kit (Qiagen) (Appendix7) for cloning into the pTZ 57/RT-cloning vector. (Appendix 3b)

### 2.9-Ligation

Eluted PCR products were ligated into Fermantas pTZ 57 /RT TA cloning vector (Appendix 3b).

1. Ligation Reaction

| | |
|---|---|
| Sterile water | 4µl |
| 10x Ligation Reaction | 1µl |
| Vector (25ng/ul) | 1µl |
| Fresh PCR product (10ng) | 1µl |
| T4 DNA Ligase | 1µl |

2. Incubate ligation reaction at 14°C for at least 4 hours (preferably overnight)
3. Centrifuge the ligation reactions briefly and place on ice.

## 2.10-Transformation

The ligated mixture was transformed into chemically competent *E. coli* DH-5 α (Appendix 4a) by the heat shock method (Appendix 4b).

## 2.11-Plasmid isolation from *E. coli*

For screening of cloned fiber specific genes on large scale the plasmids were isolated as given below.

### 2.11.1-*Plasmid isolation for restriction digestion analysis of clones*

1. Following protocol was used for the isolation of plasmid DNA from *E. coli.*
2. A single *E. coli* colony was cultured in 5 ml liquid LB (Appendix4c) medium containing 100μg/ml Ampicillin and grown overnight in a controlled temperature shaker set at 37°C.
3. The *E. coli* culture was centrifuged in 1.5 ml eppendorf tube at 14000 rpm for 5 minutes.
4. The supernatant was discarded; step 2 was repeated to collect the cells from 3 ml culture.
5. 100 μl of solution I (Suspension buffer, Appendix5) was added to eppendorf tube and the pellet was suspended by vortexing.
6. 150μl of solution II (Lysis solution, Appendix5) was added to eppendorf tube and mixed well by inverting gently.
7. 200μl of solution III (Neutralization solution, Appendix5) was added to eppendorf tube mixed by inverting 3-4 times and centrifuged at 14000 rpm for 10 minutes.
8. The supernatant was taken in fresh eppendorf tube and two volumes of 100% ethanol were added.
9. Eppendorf was kept at -20°C for 30 minutes and centrifuged at 14000 rpm for 10-15 minutes.
10. The supernatant was discarded and the pellet was washed with 100μl of 70% ethanol.
11. After centrifugation for 2 minutes, supernatant was discarded and pellet was air dried.

12. 30 µl of distilled water was added to the pellet to dissolve DNA and stored at 20°C for further use.

But for DNA sequencing purpose the plasmids were isolated using QIAprep Spins Miniprep Kit (QIAGEN) and protocol is as under.

1. 1-5 ml overnight cultures of E.*coli* in LB (Luria-Bertani) medium were centrifuged at 500x g for 10 minutes.
2. The pellet was resuspended in 250 µl of Buffer P1 and transferred to a microfuge tube.
3. 250 µl of buffer p2 were added and inverted the tube 4-6 times to mix.
4. 350 µl of Buffer N3 were added and inverted the tube immediately but gently 4-6 times.
5. Centrifuged it for 10 minutes @ 10,000 x g (or 13,000 rpm).
6. Applied the supernatants to the QIAprep column by decanting of pipetting.
7. Centrifuged 30-60 seconds. Discarded the flow-through.
8. Washed QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuged 30-60 seconds and discarded the flow-through.
9. Washed QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging 30-60 seconds.
10. The flow-through was discarded, and centrifuged for an additional 1 minute to remove residual wash buffer.
11. Placed QIAprep column in a clean 1.5-ml microfuge tube.
12. To elute DNA, 50 µl of sterile Milli-Q $H_2O$ were added to the center of each QIAprep column, let stand for 1 minute, and centrifuged for 1 minute.

**2.12- Screening of the transformants**

The isolated plasmids were digested with the restriction enzyme *EcoRI* (Fermentas). The restriction digestion reactions were performed as described below:

| | |
|---|---|
| Plasmid DNA | 2µl |
| *Eco*R1 enzyme | 1µl |
| 10X Buffer | 2µl |
| Water | 15µl |
| Total | 20µl |

The reaction mixture was incubated at 37°C for 1 hour. The digested products were run on 1% agarose gels along with 1Kb and 100bp DNA ladder (Appendix 2b). The clones having the correct size inserts were selected for further analysis. (Fig. 3.2)

### 2.13-DNA sequencing

Sanger's dideoxy chain termination method (Sanger *et al.*, 1997) using the fluorescent-labeled dye terminator was used for sequencing the cloned genes. The PCR-based version of the said method was used as described below.

#### 2.13.1-*Sequencing reaction:*

ABI Prism 310 Cycle Sequencer was used for the sequencing of cloned fragments.
The protocol, which was used for sequencing PCR, is as under.

1. Remove Big Dye Ready Reaction Mix (Manufacturer) from -20C freezer and place in the icebox.
2. Thaw the reagent on ice. Once thawed, open the tube to mix all the reagents and place back on ice.
3. In a 0.2 ml PCR tube reaction mixture was prepared using barrier tips and wearing gloves:

| | |
|---|---|
| Plasmid (1µ g/ l) | 2µ l |
| Big dye mix | 6µ l |
| Primer (10ng/µ l) | 1µ l |
| Double distilled deionised $H_2O$ | 11µ l |
| Total | 20 µl |

4. Mix the reaction mixture well.

5. Run PCR reaction:

| | | |
|---|---|---|
| Hot start 96°C | | |
| 1) Denaturing Temp. | 96°C | 10 seconds |
| 2) Annealing Temp. | 50°C | 5 seconds |
| 3) Extension Temp. | 60°C | 4 minutes |
| 4) Repeat step | 1-3 for | 24 times |
| 5) Hold | 4°C | 10 minutes |

Samples can be frozen at this point for several days.

The PCR products were purified and precipitated using any of the two methods given bellow. The salt precipitation is simple and is the method of choice.

**2.13.2-*Salt precipitation***

To each sequencing reaction tube add:

| | |
|---|---|
| Double distilled deionized water | 90 µl |
| Glycogen if available | 1 µl |
| 3M Na Acetate | 3 µl |
| Mix all and add Absolute Ethanol | 250 µl |

1. Mix and leave at room temperature for 10 minutes.
2. Spin 14000 rpm for 20-30 minutes.
3. Aspirate the liquid with out disturbing the tiny reddish pellet
4. Wash pellet with 100 µl of 70% ethanol.
5. Spin 14000 rpm for 10-15 minutes.
6. Completely pipette out all the liquid.
7. Leave tube open at room temperature to completely dry the DNA pellet

**2.13.3-*Sample loading preparation***

1. Ensure that samples are completely dry; Sequence will not work if there is any trace of liquid in sample.
2. Add 20 µl of TSR (Template Suppression Reagent) to sample.
3. Mix thoroughly on a vortex mixer.

4. Denature the sample for 2 minutes at 95°C
5. Chill samples on ice and vortex to mix.
6. Spin at 13,000 rpm for 30 seconds to collect volume at the bottom of tube.
7. Transfer entire volume of sample to sample tube. Make sure you do not introduce any air bubbles.
8. Cap sample tube with grey rubber gasket and make sure it is evenly sealed.
9. Place sample on ice or in the refrigerator until you load it into the ABI Prism 310 Instrument.
10. Load into machine within a few hours after step 8.

### 2.14-Sequence manipulation and analysis

Sequence information was stored, assembled, and analyzed using Laser gene version (DNASTAR, Madison, USA). Sequence alignments and phylogenetic analyses were generated using CLUSTAL X (Thompson et al., 1997) running on an IBM compatible personal computer. Phylogenetic dendrograms were viewed and manipulated using Tree view (Page, 1996).

### 2.15-cDNA AFLP

Amplified restriction fragment polymorphism (AFLP) is a technique for fingerprinting of genomic DNA (vos *et al.*, 1995, Zabeau, 1992). This technique is used as a tool to identify a specific template or to assess the relatedness between the samples. As in this (cDNA AFLP) technique cDNA is used as template in place of genomic DNA, hence the name cDNA AFLP. The working principle of this technique is based on selective amplification of a subset of template restriction fragments using PCR.

cDNA AFLP was performed with cDNA of different developmental stages of ovule of *G. arboreum* according to manufacturer instructions (GIBCO BRL-AFLP ANALYSER SYSTEM). The number of bands was reduced by using C and G composition of selective nucleotides and amplifying the cDNA with random decamer primers before restriction ligation reaction (appendix-8)

#### 2.15.1-*Restriction endonuclease digestion*

The restriction fragments for amplification were generated by two restriction endonucleases: *Eco*RI and *Mse*I.these enzymes generate small template fragments, which amplify well.

### 2.15.2-*Ligation of adapters*

Following the heat inactivation of the restriction endonuleases, template fragments were ligated to *Eco*RI and *Mse*I adapters to generate the template for final amplification.

### 2.15.3-*Final amplification reaction*

This step was done in two consecutive reactions; first one is the preamplification in which template is amplified with AFLP primers each having one selective nucleotide. The products of this pre amplification were diluted in 1:35 ratio and were used for final selective amplification by using different AFLP primer combinations.

AFLP Primer combinations, which were used, are as under.

**Table 2.4** Combinations of different cDNA-AFLP primers, which were used in modified cDNA-AFLP to amplify different fragments from cDNA of different developmental stages of cotton fiber.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| E-AAC | M-CTG | M-CAC | M-CAA | M-CAT | M-CTC | M-CAG | M-CTT | M-CTA |
| E-ACC | M-CTG | M-CAC | M-CAA | M-CAT | M-CTC | M-CAG | M-CTT | M-CTA |
| E-AGG | M-CTG | M-CAC | M-CAA | M-CAT | M-CTC | M-CAG | M-CTT | M-CTA |
| E-ACG | M-CTG | M-CAC | M-CAA | M-CAT | M-CTC | M-CAG | M-CTT | M-CTA |

### 2.15.4-*Separation of amplified fragments on agarose gel*

Products from selective amplification were separated on 1.5% agarose gels (appendix-1c). Differential fragments were observed showing polymorphic amplified PCR products with respect to leaf and fiber tissues. (Fig.3.11)

### 2.16-RNA dot blot

RNA dot blot was performed to confirm that the cloned, partial genes show expression in different developmental stages of fiber and to assess whether their expression varies in different tissues and at different stages (Fig. 3.12), (Fig. 3.13) and (Fig. 3.14).

10 µg total RNA were dissolved in 10mM NaOH and 1mM EDTA and applied to HYBOND (Amersham Biosciences) membrane. Subsequently the membrane was neutralized by immersing in 2 x SSC for 5 minutes. A piece of filter paper was dampened in 2 x SSC solution (Appendix 6) and the HYBOND membrane with RNA blots was put on it. The membrane was exposed to UV radiation in a Stratalinker (Stratagene, USA) to bind nucleic acids. The membrane was then air dried and stored in a Ziploc pouch until hybridization.

### 2.16.1-*Probe labeling*

Cloned DNA fragments were radioactively labeled with α-32P dCTP, using a Hexa Label™ DNA Labeling kit (Fermantas).

| | |
|---|---|
| Plasmid | 10µl |
| 5x Reaction Buffer | 10µl |
| Mix C | 3µl |
| Ultra pure $H_2O$ | 23µl |

All the reagents were put in an eppendorf tube and were heated to 95 C for 10 minutes to denature the template and then quenched on ice for 5 minutes. One µl of Klenow Fragment was added along with 3µl of radioactive dCTP. The reaction mixture was incubated at 37 C for one hour and was then stored at-20 C until use.

### 2.16.2-*Prehybridization*

Prehybridization was done to block nucleic acid binding sites on the membrane. Prehybridization solution was made as follows. The membrane was prehybridised at 65°C in a hybridization oven (Hybaid, USA).

| | |
|---|---|
| 20x SSC | 30ml |
| 50x Denhard solution | 10ml |
| 10% SDS | 10ml |
| Denatured Salmon Sperm DNA | 20µl |
| Deionized $H_2O$ | 49.80ml |

### 2.16.3-*Probing*

After prehybridization 25µl of already prepared radio labeled probe was added in the hybridization bottle containing prehybridization solution and blot for a minimum of 2 hours. It was incubated overnight at 65°C in a hybridization oven.

### 2.16.4-*Washing*

After over night incubation at 65°C, the blots were washed with washing solution to remove unbound probe. Varying the amount of salt in the washing solution controlled the stringency of the washing was controlled. The blots were washed until no further radioactive label was removed by an intermediate stringency wash.

| | |
|---|---|
| 20xSSC | 10ml (2xSSC) 5ml (1xSSC) |
| 10%SDS | 1ml |
| Distilled water | 79ml |
| Total Volume | 100ml |

### 2.16.5-*Autoradiography*

After successive washings the blots were exposed to x-ray film for twenty four hours. The film was then developed to viewalise the results.

### 2.17-RFLP-Hybridization

It is highly reliable technique to study the polymorphism among the species or with in the same species. This technique was applied to check the presence or absence of identified fiber genes in different cotton genomes.

RFLP-Hybridization was done using genomic DNA of four different Gossypium species with different genomes.

### 2.17.1-*Restriction of DNA*

Genomic DNA from four different Gossypium species having different genomes was digested with two restriction enzymes i.e. *Eco*RI and *Bam*HI.

| | |
|---|---|
| DNA | 10µg |
| Restriction enzyme | 10µl |
| Buffer | 20µl |
| Distilled$H_2O$ | variable |
| Total volume | 100µl |

### 2.17.2-*Gel electrophoresis*

After the restriction digestion of genomic DNA from different cotton genomes, phenol chloroform precipitation was done. Then it was loaded into 1% w/v agarose gel along with 1kb DNA ladder (Fermantas).

### 2.17.3-*Membrane transfer*

After gel electrophoresis the restricted DNA bands were transferred to HYBOND membrane by using standard southern blotting apparatus. After over night blotting the membrane was

washed in 2xSSC solution for 5 minutes and then the DNA bands were fixed by UV cross-linking.

### 2.17.4-*Prehybridization*

The blot was prehybridized to block the non-specific binding of probe. Prehybridization solution (Appendix6) was made as under.

| | |
|---|---|
| 20x SSC | 35ml |
| 50x Denhard solution | 15ml |
| 10% SDS | 10ml |
| Salmon Sperm DNA | 20µl |
| Deionized $H_2O$ | 39.80ml |

### 2.17.5-*Hybridization*

After prehybridization 25µl of already prepared radio labeled probe was added in the hybridization bottle containing prehybridization solution and blot. It was incubated overnight at 65°C.

### 2.17.6-*Washing*

After over night incubation at 65°C, the blot was washed with washing solution to remove the unhybridized or poorly hybridized probe.

| | |
|---|---|
| 20xSSC | 30ml |
| 10%SDS | 1ml |
| Distilled water | 69ml |
| Total Volume | 100ml |

### 2.17.7-*Autoradiography*

After successive washings the blot was exposed to x-ray film for twenty four hours. Then it was developed to viewalise the results.

# Chapter 3
## RESULTS

The purpose of the current study was to develop an understanding towards molecular approach to dissect the complex process of fiber development. In the current study of cotton fiber development the available information in the data bank along with unpublished information was used to design primers to amplify fiber development related genes. Forward and reverse primers were designed to amplify Ubiquitin extension protein, proline rich proteins, translation elongation factors and transcription factors. The genes encoding these proteins were amplified from the reversed transcribed total RNA from *G.arboreum*.

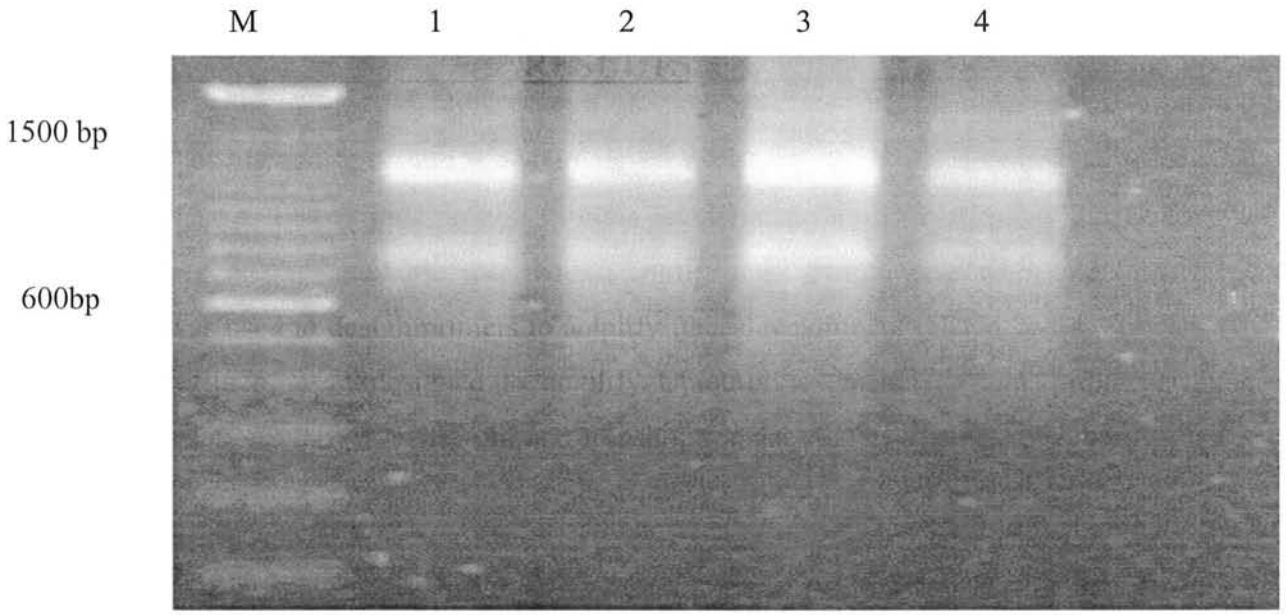
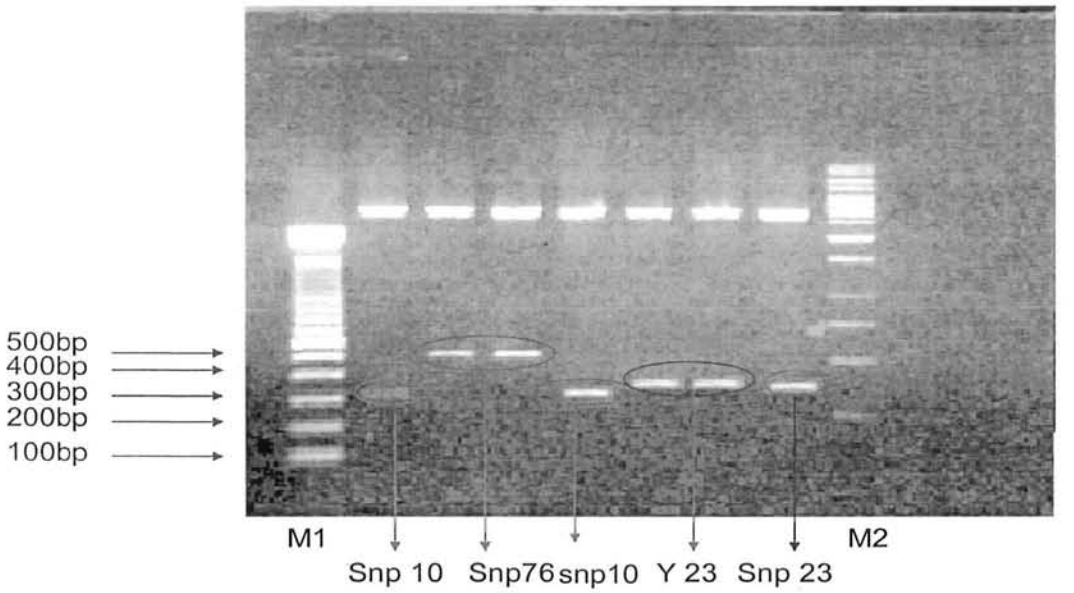



**Fig. 3.1** - RNA extraction from ovule and leaf of *G. arboreum.* Lanes 1-3 contained RN from *G. arboreum* ovules at 0, 5 and 10 dpa respectively. Lane 4 contained RNA from leaf of *G. arboreum*.100 bp ladder (Fermentas) was run in lane M

The PCR amplification products were cloned in to pTZ57 R/T cloning vector. Clones having the inserts of interest were analyzed by restriction enzyme digestion. Clones with right size were selected for further analysis.




M1:100bp ladder (Gibco BRL) M2:1kb ladder (Fermentas)

**Figure 3.2-** Restriction enzyme digestion analysis of clones; snp 10, snp 23, snp76, y23 are different primers which were used in PCR to amplify these fragments from cDNAs of *G. arboreum.* In lane M1 100 bp DNA ladder (GIBCO) and lane M2 1kb DNA ladder (Fermentas) were run.

Transcription of these genes encoding different proteins was studied at different days of post anthesis, so that their up regulation and down regulation may be seen. Thus the expression profiling of the cloned genes was observed. The expression profiling was done by PCR using gene specific primers as well as by RNA dot blots.

### 3.1-Ubiquitin-extension protein- (Clone no 4)

A partial gene sequence approximately 300 bp; encoding Ubiquitin extension protein was amplified with forward primer 5' CGTCAAGATGCAGATCTTCG 3' and reverse primer 5' CTTCTTCCTCTTCTTGGCAC 3' from the cDNA template of 0dpa, 5dpa and 10dpa developing ovule of *G.arboreum*. The PCR products were run on 1.2 % (W/V) agarose gel

with 100 bp DNA ladder. These transcripts began to express at 0dpa stage and went on increasing to the 10dpa.

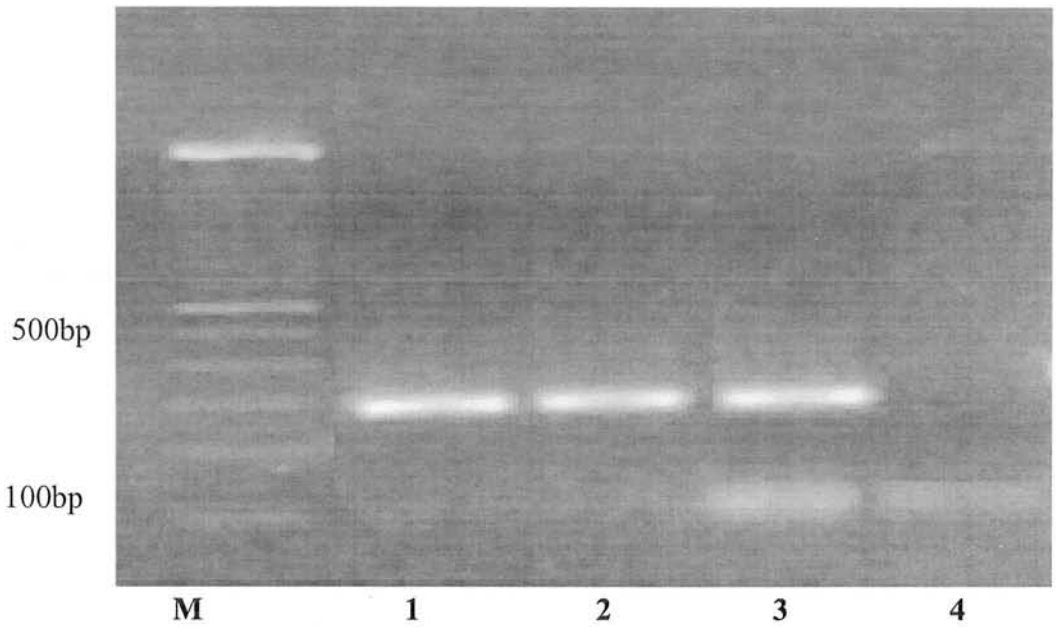



**Figure.3.3**- Ethidium bromide stained agarose gel of PCR amplifications of cDNAs with primer snp23. The reactions in lanes 1 to 3 contained cDNAs derived from *G. arboreum* ovules at 0, 5 and 10 dPA respectively. Sample in lane 4 derived from a PCR reaction containing cDNA from a *G. arboreum* leaf. A molecular weight size marker was run in lane M (100 bp GIBCO). The sizes of relevant marker bands are shown on the left.

The PCR products were cloned in to pTZ57 R/T vector. After restriction enzyme digestion analysis plasmid DNA was isolated and purified with QIAprep spin mini Kit (QIAGEN). Sequencing pcr was done with M13 reverse and forward primers. The sequence was blast searched which showed homology with other reported sequences .The partial gene sequence of Ubiquitin extension protein was translated into amino acid sequence using translated blast (NCBI). This was found to be homologues with other species as well.

### 3.1.1-*Partial nucleotide sequence of clone 4 encoding Ubiquitin extension protein*

After the completion of sequencing reaction, the trace files were checked by using the programme Seq Man (DNA Star). Edit Seq Man (DNA Star) was used to edit the sequence. After removing the vector sequence following sequence was obtained.

CGTCAAGATGCAGATCTTCGTCAAAACCCTAACGGGGAAGACCATAACCTTG
AGGTTGAATCCTCCGATACAATCGACAACGTCAAGGCCAAGATCCAAGACAA
GGAGGGCATCCCACCCGACCAACAGCGCCTTATCTTCGCCGGGAAGCAGCTC
GAAGACGGCCGCACCTTAGCCGACTACAACATCCAGAAGGAATCCACTCTCC
ACCTCGTCCATCCGTCTCAGGGGTGGTGCCAAGAAGAGGAAGAAG

### 3.1.2-*Amino acid sequence of clone 4 encoding Ubiquitin extension protein*

The cleaned DNA sequence of clone 4 was put in to open reading frame finder (ORF finder) in NCBI (National Center For Biotechnology Information).This sequence was also put into translated blast at NCBI and finally following amino acid sequence was obtained.

M Q I F V K T L T G K T I T LE V E S S D T I D N V K A K IQ D K
E G I P P D Q Q R L I FA G K Q L E D G R T L A D Y NI Q K E S T
L H L V H P S Q G W C Q E E E E

### 3.1.3 - *Amino acid sequence alignment of clone 4 encoding Ubiquitin extension protein*

Amino acid sequence of clone 4 was put in to protein blast at NCBI. Homologous sequences from different species were selected and were aligned by using MegAlign (DNA Star) programme.

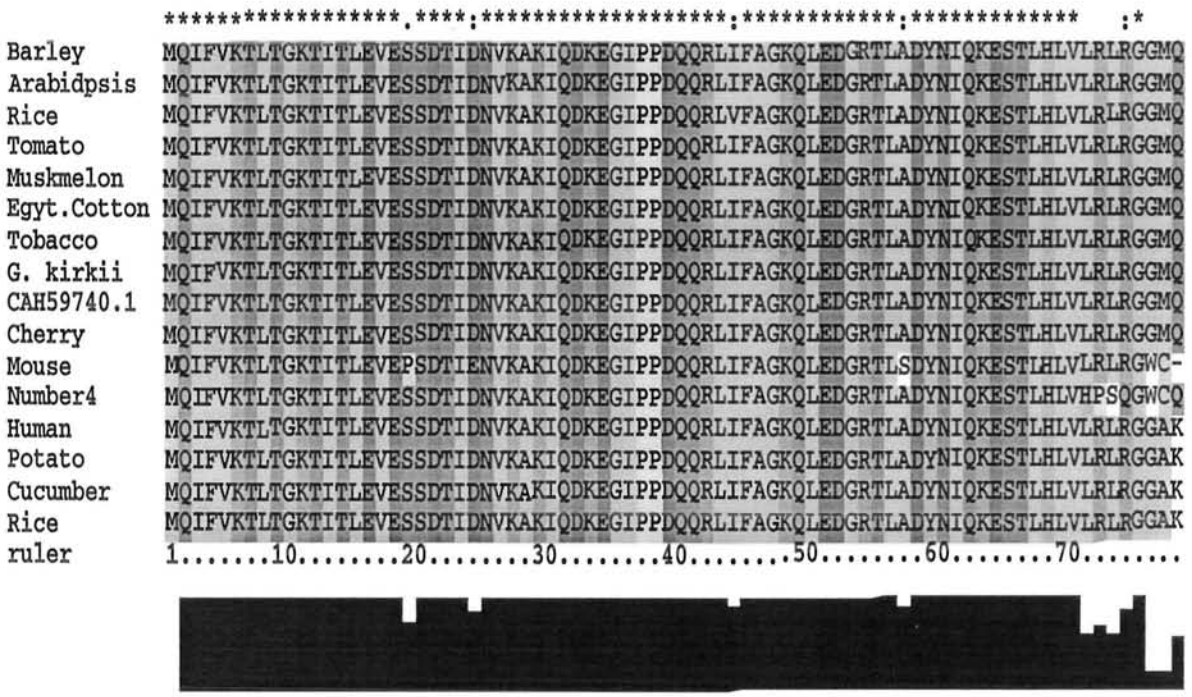



**Fig. 3.3**- Amino acid Sequence alignment of clone 4 (Ubiquitin Extension Protein) showing homology with amino acid sequence of same protein in different species.

### 3.1.4- *Nucleotide sequence alignment tree of clone 4 encoding Ubiquitin extension protein*

The cleaned nucleotide sequence of clone 4 was put in to nucleotide- nucleotide exact blast at NCBI. Homologous sequences from different species were selected and an alignment tree was constructed by using programme Tree view.

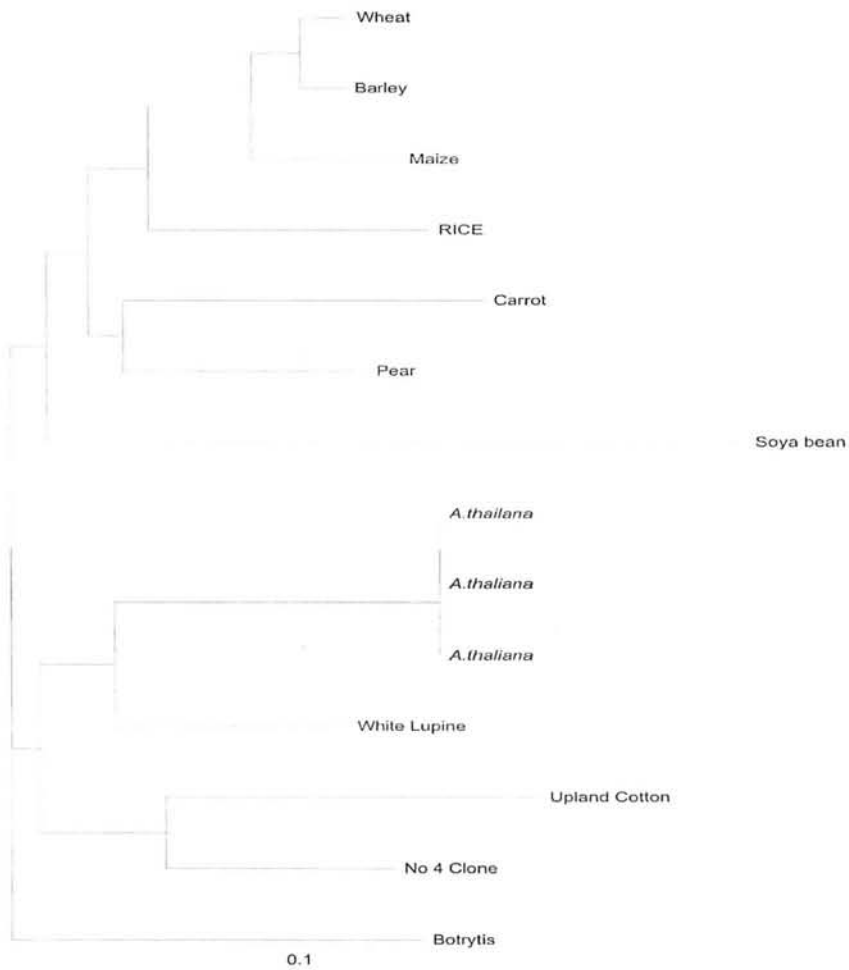



**Figure 3.5**-Nucleotide sequence alignment tree of clone 4 encoding Ubiquitin extension protein showing phylogenatic relation for the same gene in different species.

### 3.2- Proline rich proteins- (Clone no 7)

A partial gene sequence approximately 300 bp; encoding Ubiquitin extension protein was amplified with forward primer 5' CATCATTGGGCTGGACATTG 3' and reverse primer 5' GTACACATCGGCATAGGTAG 3' from the cDNA template of 0dpa, 5dpa and 10dpa developing ovule of *G.arboreum*. The PCR products were run on 1.2 % (W/V) agarose gel with 100 bp DNA ladder. These transcripts began to express at 0dpa stage and went on increasing to the 10dpa.

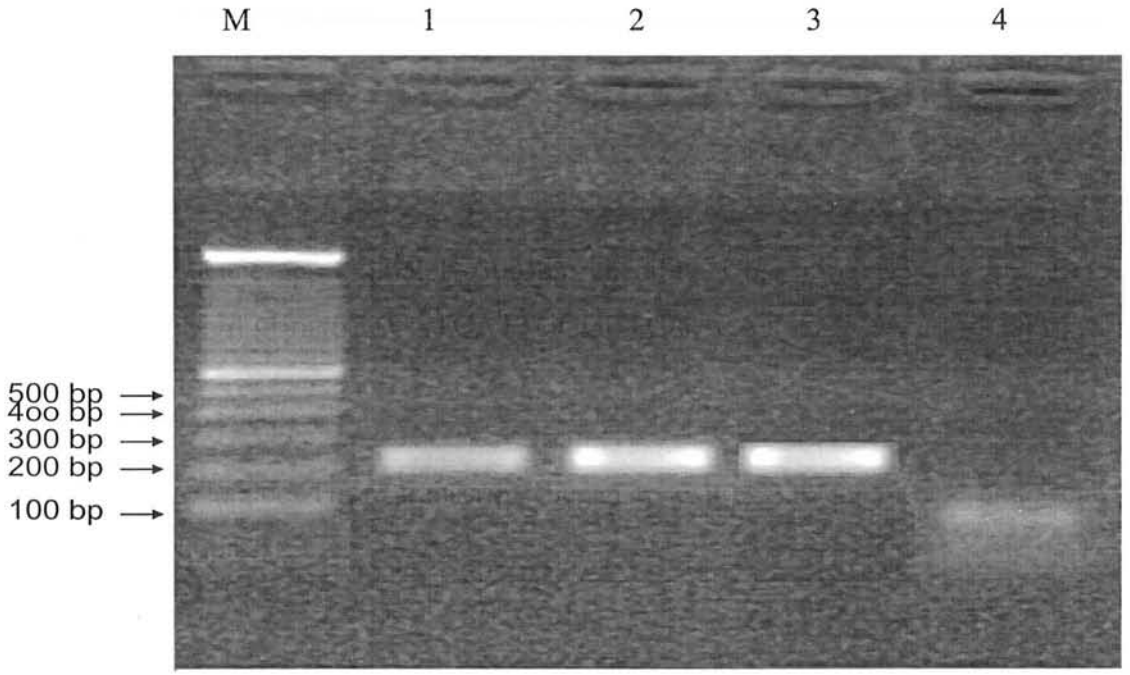



**Figure.3.6**- Ethidium bromide stained agarose gel of PCR amplifications of cDNAs with primers snp 10. The reactions in lanes 1 to 3 contained cDNAs derived from *G. arboreum* ovules at 0, 5 and 10 dPA respectively. Lane 4 contains sample for negative control reaction. A molecular weight size marker was run in lane M (100 bp GIBCO). The sizes of relevant marker bands are shown on the left.

### 3.2.1- *Partial nucleotide sequence of clone 7 encoding proline rich protein*

After the completion of DNA sequencing reaction, the trace files were checked by using the programme Seq Man (DNA Star). Edit Seq Man (DNA Star) was used to edit the sequence. After removing the vector sequence following sequence was obtained regarding clone 7, which is partial nucleotide sequence for proline rich protein gene.

CAAAGACACTTGCTGCCCTGTACTACAAGGGCTGTTGGATTTAGACGCCGCATTT
GTCTCTGTACTACGATCAAGGCCAAACTTTTGAATATCAATATTATAATCCTCAT
AGCTCTTCAGGTCCTCATCGACTGTGGCAAAACTCCACCTCCAGGGTCCAATGTC
CAGCCCAATGATG

### 3.2.2-*Nucleotide sequence alignment of clone 7 encoding proline rich protein*

DNA sequence of clone 7 was put in to blast at NCBI. Homologous sequences from different species were selected and were aligned by using Megalign (DNA Star) programme.

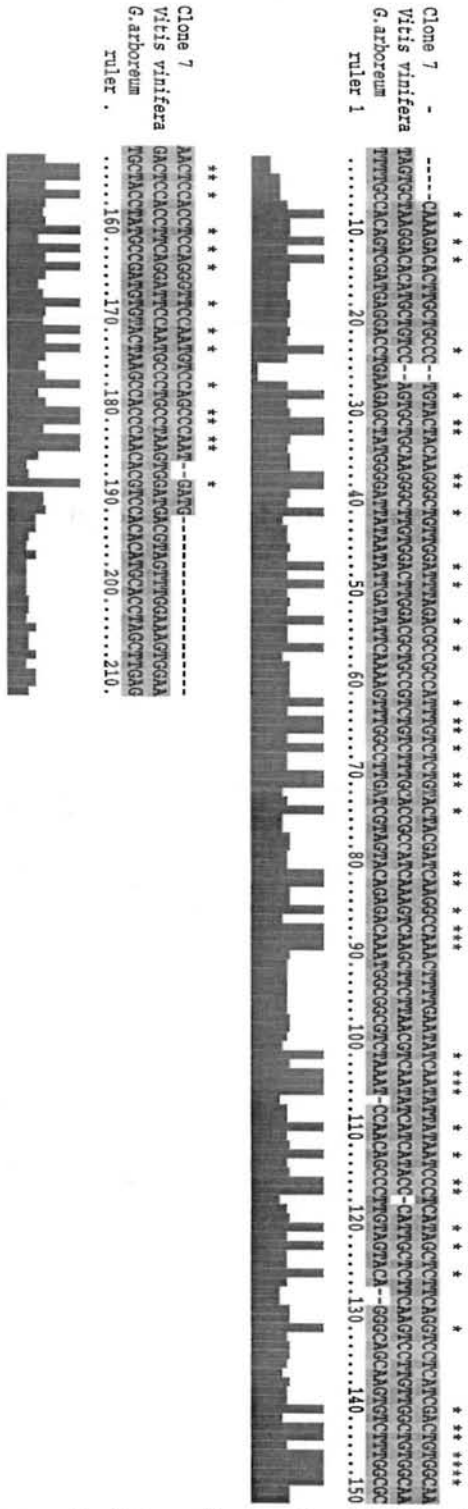



**Figure 3.7** Nucleotide sequence alignment of clone 7 encoding proline rich protein with other species.

### 3.3 –MYB protein (Clone no 5)

A partial gene sequence approximately 300 bp; encoding Ubiquitin extension protein was amplified with forward primer 5' GGCTTCTTGCCTTCTTCACC 3' and reverse primer 5' ATTCGGCACGAGAAAAGCC 3' from the cDNA template of 0dpa, 5dpa and 10dpa developing ovule of G.arboreum. The PCR products were run on 1.2 % (W/V) agarose gel with 100 bp DNA ladder. These transcripts began to express at 0dpa stage and went on increasing to the 10dpa.

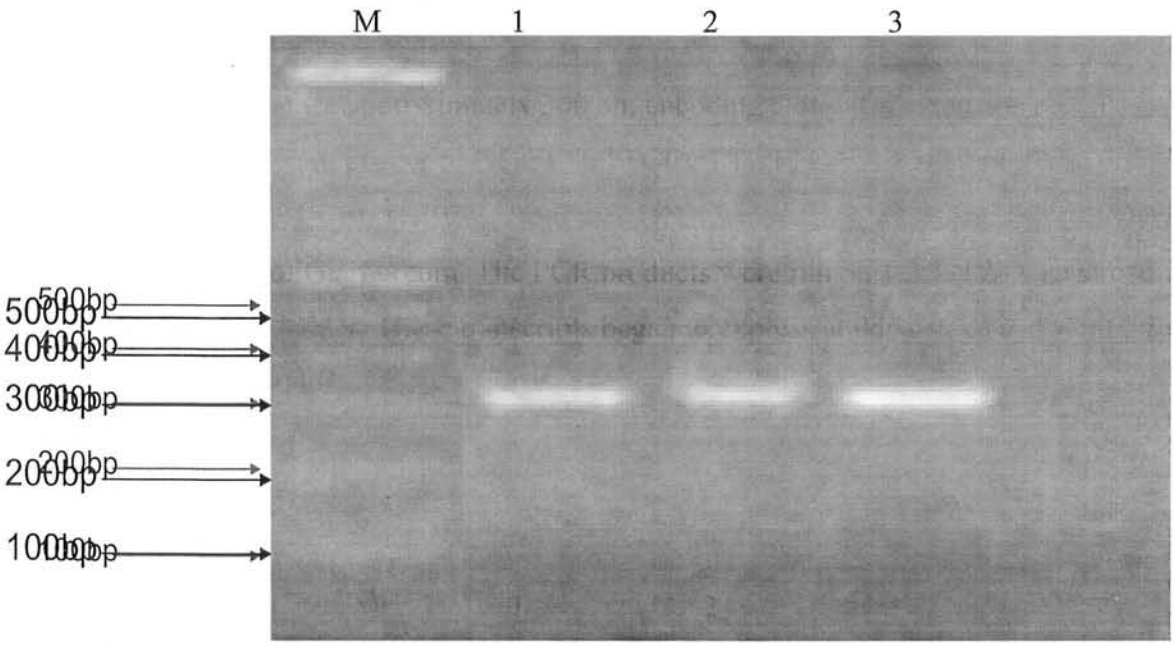



**Figure.3.8**- Ethidium bromide stained agarose gel of PCR amplifications of cDNAs with primers Y23. The reactions in lanes 1 to 3 contained cDNAs derived from *G. arboreum* ovules at 0, 5 and 10 dPA respectively. A molecular weight size marker was run in lane M (100 bp GIBCO). The sizes of relevant marker bands are shown on the left.

### 3.3.1- *Percentage homology of predicted amino acid sequence of clone 5 with selected myb sequences from data*

Nucleotide sequence of clone 5 was put into translated blast (NCBI) and predicted amino acid sequence was used to find % homology among different species.

**Table 3.1-** ***Homology %age of predicted amino acid sequence of clone 5 with selected myb sequences from data***

| *Arabidopsis* (np_195443) | *Arabidopsis* (aac83628) | Clone no 5 | Tobacco (bac53938) | *Arabidopsis* (np_191132) | Sequence (accession no.) |
|---|---|---|---|---|---|
| 100 | 98.5 | 73.5 | 44.7 | 29.1 | *Arabidopsis* (np_195443) |
| | 100 | 72.3 | 38.4 | 23.1 | *Arabidopsis* (aac83628) |
| | | 100 | 67.5 | 45.8 | Clone no. 5 |
| | | | 100 | 31.0 | Tobacco (bac53938) |
| | | | | 100 | *Arabidopsis* (np_191132) |

### 3.3.2-*Partial nucleotide sequence of clone 5 encoding myb like protein*

After the completion of DNA sequencing reaction, the trace files were checked by using the programme Seq Man (DNA Star). Edit Seq Man (DNA Star) was used to edit the sequence. After removing the vector sequence following sequence was obtained regarding clone 5, which is partial nucleotide sequence for myb protein gene.

CTCCGCAAGTTGAGCATCGGGCCTTCACCCCCGAGGAAGACGAGACCATCAC
AGAGCTCATGCCCGATTTGGTAACAAGTGGGCCACAATAGCCCGACTCCTCA
CGGTCGTACGGACAACGCCATTAAAAACCACTGGAACTCAACGCTAAAACGA
AGTGCTTGTCGGTTGGGGAAGAGAGTAATTTCATTATACATGGTGGGTATGC
GGTAATTTGGGAGGGGAAAGGAAGCAACAACCGTTGAAT

### 3.3.3- *sequence alignment of predicted amino acid of clone 5 with selected myb sequences.*

Amino acid sequence of clone 5 was put in to protein blast at NCBI. Homologous sequences from different species were selected and were aligned by using Megalign (DNA Star).

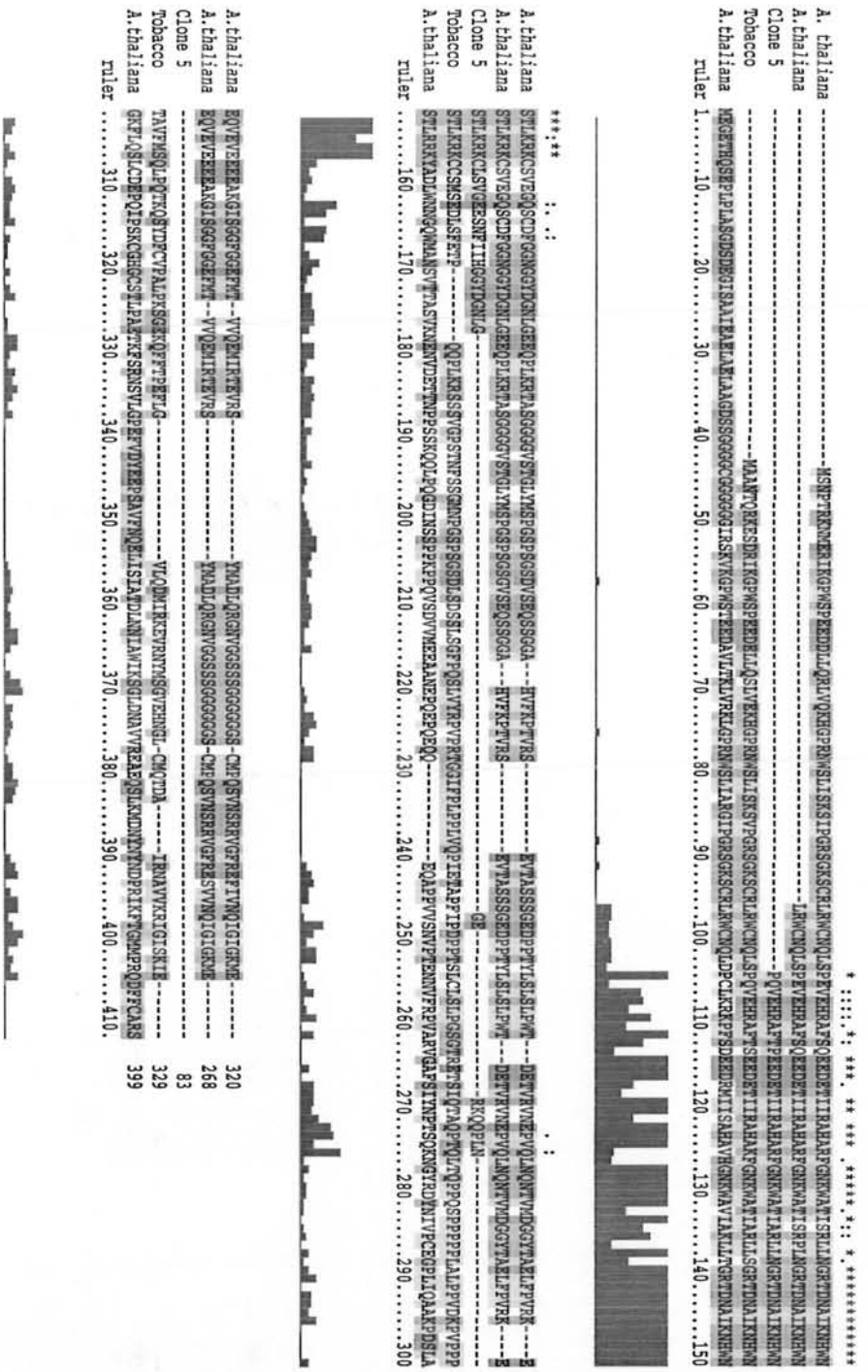


**Figure3.9** Amino acid Sequence alignment of clone 5 (myb like protein) showing homology with amino acid sequence of same protein in different species.

### 3.3.4- *Amino acid sequence alignment tree of clone 5 encoding myb like protein*

The cleaned nucleotide sequence of clone 5 was put in to translated blast at NCBI. Homologous amino acid sequences from different species were selected and an alignment tree was constructed by using programme Tree view.

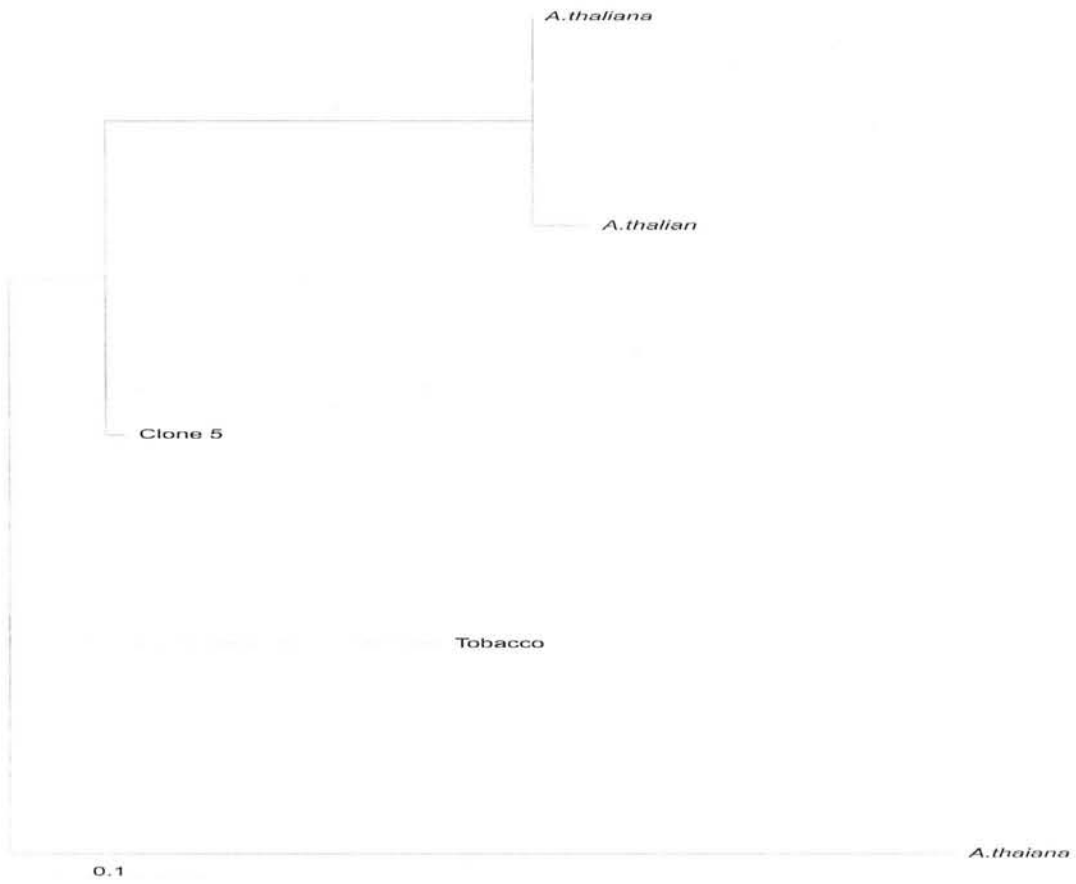



**Figure 3.10**

Amino acid sequence alignment tree of clone 5 encoding myb like protein showing phylogenatic relationship for myb like protein in different species.

### 3.5-cDNA-AFLP

To study the polymorphic patterns of amplification with respect to fiber tissues and leaf cDNA-AFLP was performed with little change in the standard operating procedure. Before restriction of cDNA with enzymes, a PCR reaction was performed with random decamer primers and then the product was restricted, ligated with adaptors and used for final PCR amplification with different combination of primers (Table 2.4). Sixty four cDNA-AFLP primers combinations were used in this study. Eight polymorphic amplification products in different primer combinations were observed with respect to fiber development stages.

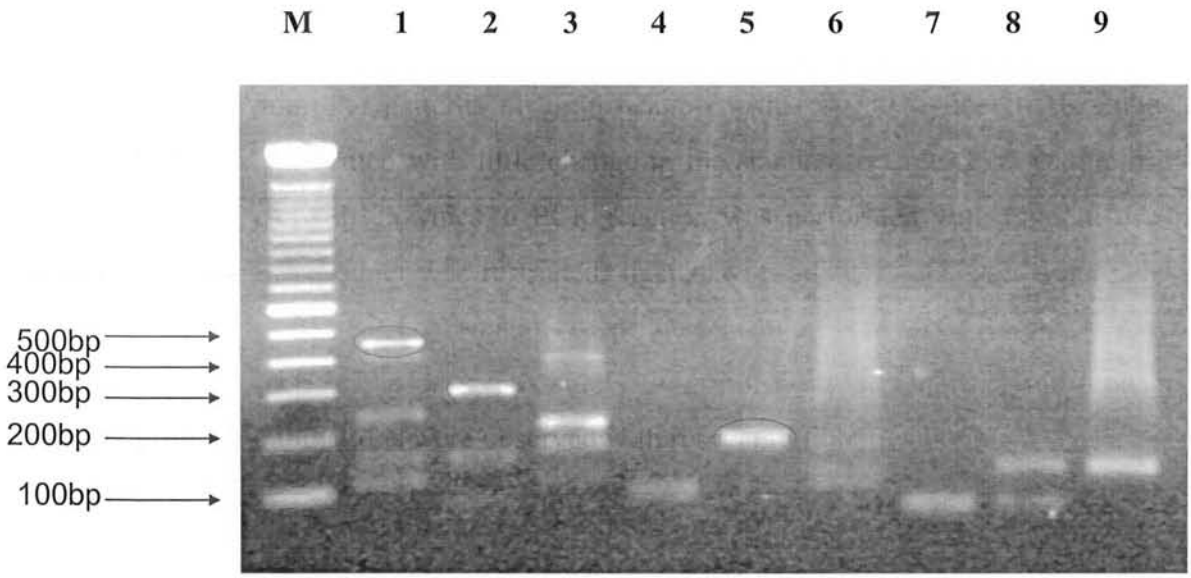



**Figure 3.11-** cDNA-AFLP PCR. Lanes 1-2 represent 0 and 5 dpa, lane 3 represents leaf cDNAs amplification with primer pair E-ACC, M-CTA; Lanes 4 -5 represent 0 and 5 dpa, lane 6 represents leaf cDNAs amplification with primer pair E-ACC, M-CTT; Lanes 7-8 represent 0 and 5 dpa, lane 9 represents leaf cDNAs amplification with primer pair E-ACC, M-CTC from *G.arboreum*.100bp DNA ladder (GIBCO-BRL) was run in lane M.

### 3.6-RNA dot blot

This method allows the rapid analysis of numerous small samples for the sequence of interest and is less time consuming than the gel electrophoresis methods. Dots of RNA are made onto a filter using a manifold and the filter is then hybridized with a labeled probe.

RNA dot blot was performed to check the presence of expressing partial genes sequences in the mRNA transcripts. By using this technique the expression profiling of the partial sequences of different genes was also done.

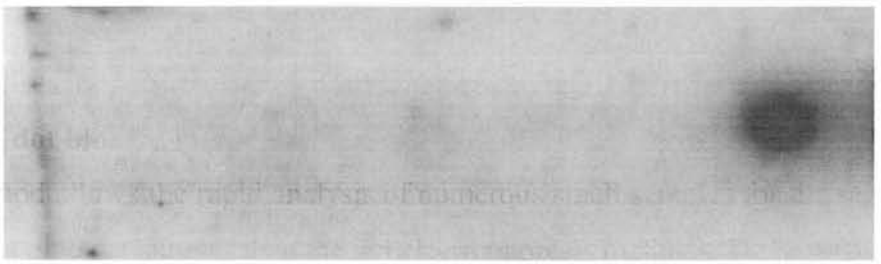



**Figure 3.10-** RNA dot blot of clone 4 (ubiquitin extension protein) with 0dpa (1) 5dpa (2), 10dpa (3), leaf (4) mRNA transcripts of *G. arboreum.* (5) is positive control.

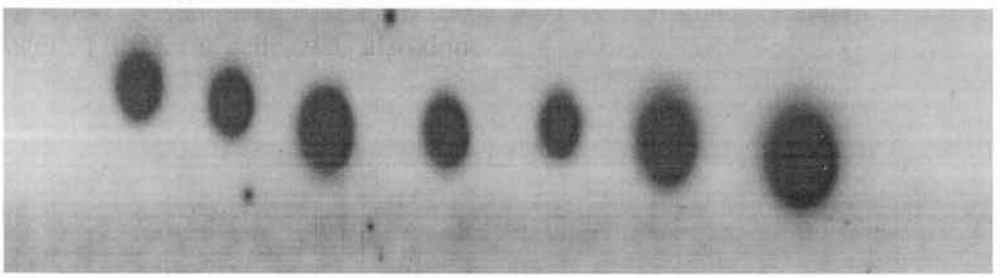



**Figure 3.11-** RNA dot blot of clone 7 (proline rich protein). 1,2 and 3, are 0,5 and 10 dpa mRNA transcripts of *G.arboreum* while 4,5 and 6 are 0,5 and 10 dpa mRNA transcripts of *G. hirsutum* and 7 is positive control.

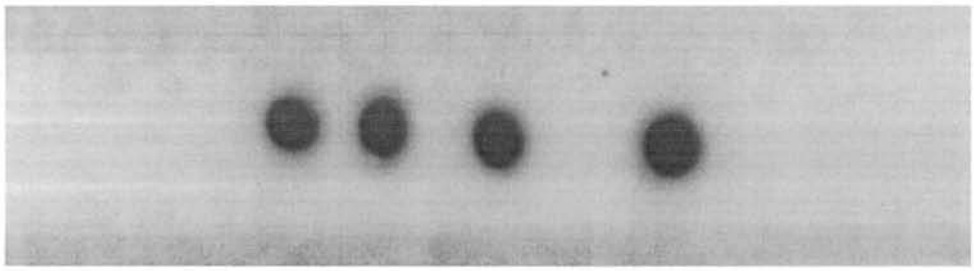



**Figure 3.12-** RNA dot blot of clone 5 (Myb protein). 1,2 and 3 are 0, 5 and 10-dpa mRNA transcript of *G. arboreum.* 4 is positive control.

# Chapter 4

## DISCUSSION

The complexity of cotton fiber development suggests that large numbers of plant genes are involved, especially during initiation, elongation and maturation (Smart *et al.*, 1998, Ruan and Chourey, 1998). The cloning of partial cDNA sequences encoding Ubiquitin extension protein, translation elongation factor, myb protein and proline rich protein was done in this study. The expression profiling of these partial cDNAs encoding different protein has also been studied by PCR amplification using gene specific primers along with RNA dot blots. These studies indicate that these genes are expressed and differentially regulated in the developing cotton fibers. Three species of Genus *Gossypium* were used in this study i.e *Gossypium arboreum*, *Gossypium hirsutum* and *Gossypium barbadense*.

Mainly the work was focused on *G. arboreum* because it is diploid (2n =26) with simple genome as compared with allotetraploid species. The process of gene discovery whose purpose at the end of the day is to get the high quality fiber to meet the needs of ever wanted textile industry can be speed up by just avoiding the redundancy due to polyploidy. The basic understanding of the fiber development process will lay the foundations for genetic engineering of the most desireable characters in the cotton fiber. This will provide the information that which genes are involved in the regulation of developing fiber cells and what different metabolic pathways are contributing towards fiber elongation. The elongation of plant cells occurs by diffuse and tips growth mechanism (Martin *et al.*, 2001).

A cotton fiber cell normally elongates as a single ovule epidermal cell. Regulation of cell division during the early stages of fiber development may trigger specific epidermal cells to elongate into what are called fibers. Usually fibers initiate at or just before anthesis and soon enter a rapid elongation period that lasts until 25 days post anthesis (dpa). Fiber cells display the most rapid elongating rate around 10 dpa. During secondary wall deposition stage fibers undergo mainly cellulose biosynthesis, which results in very high cellulose content (about 90%) in these cells. No single crucial fiber factor has been discovered, which suggests that

regulation of fiber development may require a number of genes (Ji *et al.*, 2003). Generally, for plant growth and development cell expansion is very necessary and particularly, in the cotton developing fiber, it plays a very vital role. Experimental evidences show that cell enlargement is the result of relaxation of the cell wall, which is produced by flow of water into the central vacuole of an expanding cell (Cosgrove, 2001). This important process is controlled by two different mechanisms namely tip growth and diffuse growth. In the tip growth mechanism, post-Golgi vesicles are targeted and fused to a defined region of the plasma membrane, resulting in extension of both the cell membrane as well as the cell wall in the same direction. Morphogenesis of pollen tubes and root hairs is known to depend on tip growth mechanism (Jones *et al.*, 2002). Most of the cell types in plants expand using diffuse growth mechanism, in which cell expansion is driven by turgor pressure and it occurs diffusely throughout the entire cell surface.

Proline-rich proteins (PRPs) and arabinogalactan proteins (AGPs) are two important cell wall structural proteins (Cassab, 1998; Minorsky, 2002). PRPs are widely distributed in plants and are encoded by gene families. PRP members showed to be regulated both temporally and spatially during plant development (Bernhardt and Tierney, 2000).

During growth and elongation, plant cells undergo many cellular changes such as modification of cell wall composition and organization. Structural proteins including PRPs and AGPs are known to play crucial roles in restructuring the cell wall (Varner and lin 1989). PRPs may function by regulating actin polymerization and promoting membrane protrusions (Holt and Koffer, 2001).

In the present study partial cDNA sequence of a gene encoding proline rich protein was cloned from the developing ovule. PRPs play a structural role in cell wall synthesis so as the fiber development proceeds its expression goes on increasing.

Ubiqitin is a highly conserved protein that appears to be present in all eukaryotes. Several biological roles for ubiquitin have been proposed; the best characterized is as a covalently bound recognition signal for proteolysis. Ubiquitin becomes covalently ligated to target proteins via a three- enzyme pathway, resulting in an iso-peptide linkage between the ubiquitin C-terminal carboxyl group and lysyl t amino groups of target proteins. These ubiquitin-protein conjugates are recognized by a protease that degrades the target protein, releasing ubiquitin intact (Hershko, 1988).

Like ubiquitin polyprotein genes, ubiquitin extension protein genes from higher plants, animals, and fungi have a remarkably conserved gene structure. A single ubiquitin cod- ing region is present in the ubiquitin extension protein genes from Arabidopsis, humans, and fungi, in contrast to the tandem repeats found in ubiquitin polyprotein genes ( Callis and Vierstra, 1989). Extension proteins are constituents of ribosomes and accumulate as free proteins, suggesting that their role in ribosome function is highly conserved and that their association with ubiquitin is transitory. The steady state mRNA level for the extension protein genes appears to be regulated by cell growth and division, because mRNA levels are higher in meristematic than in more mature tissues.

In the present study partial cDNA sequence of Ubiquitin extension protein was cloned and its expression suggests that it is more expressed in the dividing cells. As the dividing cells need moer energy so high activity of riosomes is required. More mRNA transcripts are present in dividing cells suggesting that it is highly expressed in these cells.

MYB genes comprise a large family of transcription regulators in eukaryotes and are involved in a variety of biological functions. In plants, MYB genes are well documented (Romero et al, 1998) and over 100 members have been identified in Arabidopsis (Arabidopsis genome initiative, 2000). In contrast to animals, most plant MYB genes belong to the R2R3-MYB subfamily. Plant MYB genes have been shown to be involved in the regulation of many aspects of plant development, hormone signaling and metabolism. AtMYBGL1 and AtWER are two typical MYB genes critical to trichome initiation in shoot and non-hair fate in root (Lee and Schiefelbein, 1999).

PCR amplification with specific primers at different developmental stages of fiber is found to be good enough for gene identification and expression profiling. RNA dot blots confirmed the results as well. At the end it is concluded that fiber development is highly complex process involving thousands of genes regulated temporally or differentially.

Working on RNA is the most difficult task in molecular biology work due to its high vulnerability to RNAses, which are very difficult to inactivate. In this study we optimized the most suitable working practices. It's not only the clean bench and materials practices but also the speed of carrying out the RNA isolation and a cDNA synthesis reaction is very important.

cDNA-AFLP protocol was modified by PCR amplification of cDNA with random primers before the ligation of adaptors. This practice was done to decrease the number of bands in final selective amplification. By this modified protocol we were able to see the final amplification of fragments on common 1.5% agarose gel thus avoiding the use of polyacrylamide gel electrophoresis. This modified protocol is time saving as well economical because there is no need of silver staining of gel.

# Chapter 5

## REFERENCES


Anonymous, (2004). Economic survey of Pakistan. Ministry of Food, Agriculture and Livestock. Govt. of Pakistan. Finance Division, Economic Wing. Islamabad.

Applequist, W.L., Cronn, R.C. and Wendel, J.F. (2001) Comparative development of fiber in wild and cultivated cotton. *Evol. Dev.* 3: 3–17.

Arpat, A.B., Waugh, M., Sullivan, J.P., Gonzales, M., Frisch, D., Main, D., Wood, T., Leslie, A., Wing, R.A. and Wilkins, T.A. (2004) Functional genomics of cell elongation in developing cotton fibers. *Plant Mol. Biol.* 54: 911–929.

Arthur, J. C. (1990). Polymers, Fibers and Textiles, A Compendium, (J.I. Kroschwitz, (editor) Wiley, New York, pp 118-141.

Banuri, T. (1998) Pakistan: Environmental Impact of Cotton Production and Trade.

Basra, A.S.and Malik, C.P. (1984) Development of the cotton fiber. *Int. Rev. Cytol.* 89: 65-113.

Behery, H.M. (1993) Short-fiber content and uniformity index in cotton. p.40. International Cotton Advisory Committee review article on cotton production research No. 4, CAB Int., Wallingford, UK.

Benedict, C.R., Kohel, R.J. and Lewis, H.L. (1999). Cotton fiber quality. In: (Smith W. C., (editor) Cotton: origin, history, technology, and production. John Wiley and Sons, Inc, pp: 269-288.

Berlin, J.D. (1986) The outer epidermis of the cottonseed. In: Mauney JR, Stewart J McD (eds) Cotton Physiology. The Cotton Foundation, Memphis, pp 375–414.

Bernhardt, C. and Tierney, M.L. (2000) Expression of AtPRP3, a proline-rich structural cell wall protein from Arabidopsis, is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physio.* 122:705-714.

Bradow, J.M., Bauer, P.J., Hinojosa, O. and Sassenrath- Cole, G.F. (1997a) Quantitation of cotton fibre-quality variations arising from boll and plant growth environments. *Eur. J. Agron.* 6:191–204.

Bradow, J.M., Bauer, P.J., Sassenrath-Cole, G.F. and Johnson, R.M. (1997b) Modulations of fiber properties by growth environment that persist as variation of fiber and yarn quality. p. 1351–1360. *In* Proc. Beltwide Cotton Conf., New Orleans, LA. 7–10 Jan.1997. Natl. Cotton Counc. Am., Memphis, TN.

Brubaker, C. L., and Wendel, J. F. (1994) Reevaluating the origin of domesticated cotton (Gossypium hirsutum; Malvaceae) using nuclear restriction fragment length polymorphisms (RFLPs). *Am. J. Bot.* 81: 1309–1326.

Brubaker, C.L., Bourland, F.M., Wendel, J.E. (1999a) The origin and domestication of cotton. In CW Smith, JT Cothren, eds Cotton: Origin, History, Technology, and Production. John Wiley and Sons, Inc., New York, pp 3-31.

Brubaker, C. L., Paterson, A. H., and Wendel, J. F. (1999b) Comparative genetic mapping of allotetraploid cotton and its diploid progenitors. *Genome* 42: 184–203.

Callis J. and Vierstra R.D. (1989) Ubiquitin and ubiquitin genes in higher plants. Oxf Surv *Plant Mol.Cell Biol.* 6: 1-30

Cassab, G. I. (1998) Plant cell wall proteins. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* 49: 281-309

Cassman, K.G., Kerby, T.A., Roberts, B.A., Bryant, D.C., and Higashi, S.L. (1990) Potassium nutrition effects on lint yield and fiber quality of *Acala* cotton. Crop Sci. 30: 672–677.

Cedroni, M.L., Cronn, R.C., Adams, K.L., Wilkins,T.A. and Wendel, J.F. (2003) Evolution and expression of MYB genes in diploid and polyploid cotton. *Plant Mol. Biol.* 51: 313–325.

Cooper, H.B., Jr., J. Pellow, McRae, K. and Anderson, D. (1996). Evaluation of the processing efficiency of two Acala cottons using bale lots. p. 1687–1689. *In* Proc. Beltwide Cotton Conf., Nashville, TN. 9–12 Jan. 1996. Natl. Cotton Counc. Am., Memphis, TN.

Cosgrove, D.J. (1995) Molecular cloning and sequence analysis of expansins, a highly conserved, multigene family of proteins that mediate cell wall extension in plants. Proc Natl Acad Sci USA, 92: 9245-9249.

Cosgrove, D.J. (2001) Wall structure and wall loosening: a look backwards and forwards. *Plant Physiol.* 125: 131–134.

Cosgrove, D. J. (1986) Biophysical control of plant cell growth. *Annu. Rev. Plant Physiol.* 37: 377–405.

Cosgrove, D. J., Durachko, D. M. and Li, L.-C. (1998) Expansins may have cryptic endoglucanase activity and can synergize the breakdown of cellulose by fungal cellulases. *Annu. Meeting Am. Soc. Plant Physiol. Abstr.* 171.

Cosgrove, D.J. (1997) Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement. *Plant Cell* 9: 1031–1041.

Cosgrove, D.J. (2000) Loosening of plant cell walls by expansins. *Nature* 407: 321-326.

Cotton Facts, ICAC (2003).

Cronn, R., Small, R. L., Haselkorn, T.and Wendel, J. F. (2002) Rapid diversification of the cotton genus (Gossypium: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. *Amer. J. Bot.* 89: 707–725.

Cronn, R., Small, R. L., and Wendel, J. F. (1999) Duplicated genes evolve independently following polyploid formation in cotton. Proc. *Natl. Acad. Sci.* USA 96, 14406–14411.

DeLanghe, E.A.L., (1986) Lint development *In* JR Mauney, JM Stewart, eds, Cotton Physiology. The Cotton Foundation, Memphis TN, pp 325-351.

Delmer, D.P., Pear, J.R., Andrawis, A. and Stalker, D.M. (1995) Genes encoding small GTP-binding proteins analogous mammalian rac are preferentially expressed in developing cotton fibers, *Mol. Gen. Genet.* 248: 43– 51.

Deussen,H.,(1992) Improved cotton fiber properties:the textile industry;a key to success in global competition. In:Proc Cotton Fiber Cellulose:Structure,Functionand Utilization Conference.Memphis,National Cotton Council of America,Tennessee, pp 43-64.

Edwards, G. A. and Mirza, M. A. (1979). Genomes of the Australian wild species of cotton II: The designation of a new G genome for *Gossypium bickii*. *Can. J. Gen. Cytol.* 21: 367-372.

Endrizzi, J.E., Turcotte, E.L., Kohel, R. (1985) Genetics, cytology and evolution of *Gossypium*. *Advances in Genetics* 23: 271-375.

Endrizzi, J. E., Turcotte, E. L., and Kohel, R. J. (1984) Qualitative Genetics, Cytology, and Cytogenetics. pp. 82-129. In Kohel, R. J. and Lewis, C. F., Editors. Cotton. American Society of Agronomy,Crop Science Society of America, and Soil Science Society of America. Madison, Wisconsin. 605 pp.

Feng, J. X., Ji, S. J., Shi, Y. H., Xu, Y., Wei, G. and Zhu, Y. X. (2004) Analysis of five differentially expressed gene families in fast elongating cotton fiber. *Acta. Biochim. Biophys.* 36: 51-6.

Fryxell, P. A. (1969) A classification of Gossypium L. (Malvaceae). *Taxon* 18: 585–591.

Fryxell, P. A. (1979) Genus Gossypium Linnaeus. In: Fryxell P. A. (ed.) The natural history of the cotton tribe. Texas A&M University Press, College Station, Texas, pp. 37–71.

Fryxell P. A. (1992) A revised taxonomic interpretation of Gossypium L. (Malvaceae). *Rheedea* 2:108–165.

Fryxell, P. A. (1984). Taxonomy and Germplasm Resources. pp. 27-57. In Kohel, R. J. and Lewis, C. F., Editors. Cotton. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. Madison, Wisconsin. 605 pp.

Fryxell, P.A. (1979). The Natural History of the Cotton Tribe (Malvaceae, Tribe Gossypieae). Texas A&M University Press. College Station and London. 245 pp.

Galau, G.A. and Wilkins, T.A. (1989) Alloplasmic male sterility in AD allotetraploid Gossypium hirsutum upon replacement of its resident A cytoplasm with that of D species G. Harknesii. *Thero Appl Genet* 78: 23–30.

Gerstel, D. U., and Phillips, L. L. (1958) Segregation of synthetic amphiploids in Gossypium and Nicotiana. Cold Spring Harbor Sympp. *Quant. Biol.* 23, 225–237.

Grant, V. (1981) In "Plant Speciation". Columbia University Press, New York.

Graves, D.A. and Stewart, J.Mc.D. (1988) Analysis of the protein constituency of developing cotton fibers. *J. Exp. Bot. 39*: 59–69.

Green, C.C. and Gulp T.W. (1990) Simultaneous improvement of yield, fiber quality, and strength in Upland cotton. Crop Sci. 30:66-69.

Hearn, A.B. (1976) Response of cotton to nitrogen and water in a tropical environment. III. Fibre quality. *J. Agric. Sci.* (Camb.) 84:257–269.

Hershko, A. (1988). Ubiquitin-mediated protein degradation. J. Biol. Chem. (1988). 263:15237–15240

Hilu, K. W. (1993) Polyploidy and the evolution of domesticated plants. *Am. J. Bot.* 80: 1494–1499.

Holt, M.R. and Koffer, (2001) A Cell motility: Proline-rich proteins promote protrusions. *Trends Cell Biol.* 11: 38-46.

Hsieh, Y.L. (1999) Structural development of cotton fibers and linkages to fiber quality. In: Basra AS (ed) Cotton Fibers. Hawthorne Press Inc, New York, pp 137–144.

Hughes, A. L., A, G. J., Garbayo, J. M., and Roberts, R. M. (2000) Adaptive diversification within a large family of recently duplicated, placentally expressed genes. Proc. *Natl. Acad. Sci.* USA 97: 3319–3323.

Jasdanwala, R. T., Singh, Y. D., Chinoy, J. J. (1977) Auxin metabolism in developing Cotton hairs.*J.Exp.Bot.*28: 1111-1116.

Ji, S.J., Lu, Y.C., Feng, J. X., Wei, G., Li. J., Shi, Y. H. and Fu, Q. (2003) Isolation and analyses of genes preferentially expressed during early cotton fiber development by subtractive PCR and cDNA array. *Nucleic Acids Res.* 31: 2534-2543.

Jiang, C. -X., Chee, P. W., Draye, X., Morrell, P. L., Smith, C. W., and Paterson, A. H. (2000) Multilocus interactions restrict gene introgression in interspecific populations of polyploidy Gossypium (cotton). *Evolution* 54:798–814.

Jiang, C., Wright, R., El-Zik, K., and Paterson, A. H. (1998) Polyploid formation created unique avenues for response to selection in Gossypium (cotton). Proc. *Natl. Acad. Sci.* USA 95: 4419–4424.

John, M.E., (1995) Characterization of a cotton (Gossipium hirsutum L.) fiber mRNA (Fb-B6). *Plant Physiol.* 107: 1477– 1478.

John, E.M. and Keller, G. (1995) Characterization of m RNA for a Proline-Rich Protein of cotton fiber. *plant physiol.*108: 669-676.

John, E.M., and Crow, J.L. (1992) Gene expression in cotton (*Gossypium hirsutum* L.) fiber:cloning of mRNAs.Proc.*Natal.Sci.*USA.89:5769-5773.

Jones, M.A., Shen, J.J., Fu, Y., Li, H., Yang, Z. and Grierson, C. S. (2002) The *Arabidopsis* Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. *Plant Cell*, 14: 763–776.

Kloth, R.H. (1998) Analysis of commonality for traits of cotton fiber. *J. Cotton Sci.* 2:17-22.

Kohel, R.J. (1999) Cotton germplasm resources and the potential for improved fiber production and quality. p.167-182. *in* A.S. Basra (ed.) Cotton Fibers. The Haworth Press, inc., Binghamton, NY.

Kosmidou-Dimitripoulou, K. (1986) Hormonal Influences on Fiber Development. In: Mauney JR, Stewart JMcD (eds) Cotton Physiology. Cotton Foundation, Memphis, pp 361–373

Kurek, I. Yu, J., Jacob-Wilk, D., Doblin, M., and Delmer, D. (2002) Dimerization of cotton fiber cellulose synthase catalytic subunit occurs via oxidation of the zinc-bing domains. Proc. Natl. Acad. Sci. USA 99: 11109-11114.

Lee, M..M., Schiefelbein, J. (1999) WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning, *Cell* 99: 473–483.

Leitch, I. J., and Bennett, M. D. (1997) Polyploidy in angiosperms. *Trends Plant Sci.* 2: 470–476.

Li, X. B., Cai, L., Cheng, N. H. and Liu, J. W. (2002) Molecular characterization of the cotton GhTUB1 gene that is preferentially expressed in fiber. *Plant Physiol.* 130: 666- 674.

Lord, E., and S.A. Heap. (1988) The origin and assessment of cotton fibre maturity. Int. Inst. for Cotton. Manchester, UK.

Ma, D.P., Liu, H.C., Tan, H., Creech, R.G., Jenkins, J.N. and Chang, Y.F. (1997) Cloning and characterization of a cotton lipid transfer protein gene specifically expressed in fiber cells. *Biochim. Biophys. Acta.* 1344: 111 – 114.

Makalowski, W. (2001) Are we polyploids? A brief history of one hypothesis. *Genome Res.* 11: 667–670.

Masterson, J. (1994) Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science* 264: 421–424.

Martin, C., Bhatt, K. and Baumann, K. (2001) Shaping in plant cells. *Curr Opin Plant Biol.* 4: 540-549.

Mckenzie, W. H., (1970) Fertility relationships among interspecific hybrid progenies of *Gossypium*. Crop Sci. 10: 571-574.

Meinert, M.C., and Delmer, D.P. (1977) Changes in biochemical composition of the cell wall of the cotton fiber during development. *Plant Physiol.* 59: 1088-1097.

Meredith, Jr. W.R. (2000) Continued progress for breeding for yield in the USA? In: U. Kechagia (Ed.), Proceedings of the World Cotton Research Conference II, Athens, Greece, pp. 97–101.

Minorsky, P.V. (2002) The wall becomes surmountable. *Plant. Physiol.* 128: 345-353.

Moore, J.F. (1996) Cotton Classification and Quality. p. 51–57.*In* E.H. Glade Jr., L.A. Meyer, and H. Stults (ed.) The cotton industry in the United States. USDA-ERS Agric.Econ. Rep. 739. U.S. Gov. Print. Office, Washington,DC.

Munro, J. M. (1987) Cotton 2nd ed. New York: Longman Scientific and Technical, Harlow, Essex, UK.

Ohno, S. (1970) In "Evolution by Gene Duplication". Springer, New York.

Oosterhuis, D. M. and Jernstedt, J. (1999) Morphology and anatomy of the cotton plant. InWC Smith, JT Cothren, eds Cotton: Origin, History, Technology and Production. John Wiley and Sons, Inc., New York, pp. 175-206.

Orford, S.J., and Timmis, J.N. (1998) Specific expression of an expansin gene during elongation of cotton fibers. *Biochem. Biophys.Acta.* 1398: 342–346.

Orford, S.J. and Timmis, J.N. (1997) Abundant mRNAs specific to the developing cotton fibre. *Theor. Appl. Genet.* 94: 909–918.

Otto, S. P. and Whitton, J. (2000) Polyploid incidence and evolution. *Annu. Rev. Genet.*

Page, R. D. M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358.34: 401–437.

Palmer, J.C., Cooper Jr., H.B., Pellow, JW. and McC rae, K.E. (1996) E1 Dorado, a new high qualityAcala cotton for San Joaquin Valley. p. 45-46. In Proc. Beltwide Cotton conf., Nashville, TN. 9-12 jan. 1996. Natl.Cotton Counc. Am.,Memphis,TN.

Patil, N.B. and M. Singh. (1995) Development of medium staple high-strength cotton suitable for rotor spinning systems, p. 264–267. *In* G.A. Constable and N.W. Forrester (ed.) Challenging the future. Proc. World Cotton Conf. I, Brisbane, Australia, 14–17 Feb. 1994. CSIRO, Melbourne, Australia.

Pear, J.R., Kawagoe, Y., Schreckengost, W.E., Delmer, D.P., Stalker, D.M. (1996) Higher plants contain homologs of the bacterial CelA genes encoding the catalytic subunit of cellulose synthase, Proc. Natl. Acad. Sci. U. S. A. 93: 12637–12642.

Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002) Sitosterol-β-glucosidase as primer for cellulose synthesis in plants. *Science:* 147-150

Percival, A.E., Wendel, J.F., Stewart, J.M. (1999) Taxonomy and germplasm resources. In: Smith CW, Cothren JT (eds) Cotton: origin, history, technology, and production. Wiley, New York, pp33–63.

Percy, R. G., and Wendel, J. F. (1990) Allozyme evidence for the origin and diversification of *Gossypium barbadense* L. *Theor. Appl. Genet.* 79: 529–542.

Phillips, L. L. (1964) Segregation in new allopolyploids of *Gossypium*. V. Multivalent formation in New World of Asiatic and New World of wild American hexaploids. *Am. J. Bot.* 51,

Rahman, M., Hussain, D. and Zafar, Y. (2002). Estimation of genetic divergence among elite cotton cultivars-genotypes by DNA fingerprinting technology. *Crop Sci.* 42: 2137-2144.

Ramey, H. H. (1982) The meaning and assessment of cotton fibre fineness. Int. Inst. For Cotton, Manchester, UK.

Ramsey, J. C. and Berlin, J. D. (1976) Ultra structure of early stages of cotton fiber differentiation. *Bot.Gaz.* (Chicago)137:11-19.

Reinisch, A. J., Dong, J., Brubaker, C. L., Stelly, D. M., Wendel, J. F., and Paterson, A. H. (1994) A detailed RFLP map of cotton, *Gossypium hirsutum* and *G. barbadense*: Chromosome organization and evolution in a disomic polyploid genome. *Genetics* 138: 829–847.

Rinehart, J.A., Petersen, M.W. and John, M.E. (1996) Tissue-specific and developmental regulation of cotton gene FbL2A. Demonstration of promoter activity in transgenic plants. *Plant Physiol.* 112:1331–1341.

Romero, A. Fuertes, M.J. Benito, J.M. Malpica, A. Leyva, J. Paz- Ares, (1998) More than 80R2R3-MYB regulatory genes in the genome of Arabidopsis thaliana. *Plant J.* 14: 273– 284.

Rong, J., Abbey, C., Bowers, J.E., Brubaker, C.L., Chang, C., Chee, P-W., Delmonte, T.A., Ding, X., Garza, J.J., Marler, B.S., Park, C-H., Pierce, G.J., Rainey, K.M., Rastogi, V.K., Schultze, S.R., Trolinder, N.L., Wendel, J.F., Wilkins, T.A., Williams-Coplin, D., Wing, R.A., Wright, R.J., Zhao, X., Zhu, L. and Paterson, A.H. (2004) A 3347-locus genetic recombination map of sequence tagged sites reveals features of genome organization, transmission and evolution of cotton (Gossypium). *Genetics* 166: 389–417.

Ruan,Y.L., and Chourey, P.S. (1998) A fiberless seed mutation in cotton is associated with

lack of fiber cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. *Plant Physiol.* 118: 399–406.

Ruan, Y.L., Llewellyn, D.J., and Furbank, R.T. (2001) The control of single-celled cotton fiber elongation by developmentally reversible gating of plasmodesmata and coordinated expression of sucrose and K transporters and expansin. *Plant Cell* 13: 47–63.

Ryser, U. (1985) Cell wall Biosynthesis in differentiating cotton fiber.*European Journal of Cell Biology*.39:236-256.

Ryser, U. (1999) Cotton fiber initiation and histodifferentiation. In: Basra AS (ed) Cotton Fibers. Haworth Press, New York, pp 1–45.

Sauer, J. D. (1993). Historical geography of crop plants-a select roster. CRC Press, Boca Ratony, Florida.

Saha, S., and Zipf, A. (1998) Genetic diversity and phylogenetic relationships in cotton based on isozyme markers. *J. Crop Prod.* 1: 79–93.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74**:** 5463-7.

Schubert, A.M., Benedict, C. R., Berlin, J. D. and Kohel, R. J. (1973) Cotton fiber development kinetics of cell elongation and secondary wall thickening. *Crop Sci.*13: 704-709.

Seagull, R.W. (1990) Tip growth and transition to secondary cell wall synthesis in developing cotton hairs. In: Heath IB (ed) Tip Growth in Plant and Fungal Cells. Academic Press, San Diego, pp 261–284.

Seelanan, T., Schnabel, A., Wendel, J. F. (1997) Congruence and consensus in the cotton tribe.*Stematic Botany* 22:259-290.

Senchina, D.S., Alvarez, I., Cronn, R.C., Liu, B., Rong, J., Noyes, R.D., Paterson, A.H., Wing, R.A., wilkins, T.A. and Wendel, J.F. (2003) Rate variation among nuclear genes and the age of polyploidy in Gossypium. *Mol. Biol. Evol.* 20: 633–643.

Shimizu, Y., Aotsuka, S., Hasegawa, O., Kawada, T., Sakuno, T., Sakai, F. and Hayashi, T. (1997). Changes in levels of mRNAs for cell wall-related enzymes in growing cotton fiber cells. *Plant Cell Physiol.* 38: 375 378.

Small, R. L., and Wendel, J. F. (2000) Copy number lability and evolutionary dynamics of the Adh gene family in diploid and tetraploid cotton (Gossypium). *Genetics* 155: 1913–1926.

Small, R. L., Ryburn, J. A., Cronn, R. C., Seelanan, T., and Wendel, J. F. (1998) The tortoise and the hare: Choosing between noncoding plastome and nuclear Adh sequences for phylogeny reconstruction in a recently diverged plant group. *Am. J. Bot.* 85: 1301–1315.

Smart, L.B., Vojdani, F., Maeshima, M. and Wilkins, T.A. (1998) Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibers are differentially regulated. *Plant Physil.* 116: 1539–1549.

Smith, N. G. C., Knight, R., and Hurst, L. D. (1999) Vertebrate genome evolution: A slow shuffle or a big bang? *BioEssays* 21: 697–703.

Soltis, D. E., and Soltis, P. S. (1999) Polyploidy: Origins of species and genome evolution. *Trends Ecol. Evol.* 9: 348–352.

Soltis, P. S., and Soltis, D. E. (2000) The role of genetic and genomic attributes in the success of polyploids. Proc. Natl Acad. Sci. USA 97: 7051–7057.

Soltis, P. S., Doyle, J. J., and Soltis, D. E. (1992) Molecular data and polyploid evolution in plants. In "Molecular Systematics of Plants" (P. S. Soltis, D. E. Soltis and J. J. Doyle, Eds.), pp. 177–201. Chapman & Hall, New York.

Song, P., Allen, R.D. (1997) Identification of a cotton fiber-specific acyl carrier protein cDNA by differential display. *Biochim. Biophys. Acta.*1351: 305–312.

Stephens, S. G. (1944) Phenogenetic evidence for the amphidiploid origin of New World cottons. *Nature* 153: 53–54.

Stephens, S. G. (1951) Evolution of the gene—"Homologous" genetic loci in Gossypium. Cold Spring Harbor Sympp. *Quant. Biol.*16: 131–140.

Stewart, J. Mc.D. (1995) Potential for crop improvement with exotic germplasm and genetic engineering, CSIRO, Melbourne. pp. 313-327.

Sundararaj, D. D. (1980) Cotton. Botany of field crops. India: The MacMillan Company.

Taylor, J. S., and Brinkmann, J. S. (2001) 2R or not 2R? *Trends Genet.*17: 488–489.

Tiwari, S.C., Wilkins, T.A. (1995) Cotton (Gossypium hirsutum) seed trichomes expand via a diffuse growing mechanism. *Can. J. Bot.* 73: 746–757.

The Arabidopsis Genome Initiative, (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana, *Nature* 408: 796–815.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The Clustal_X windows interface; flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876-4882.

Turley, R.B., Ferguson, D.L. (1996) Changes of ovules proteins during early fiber development in a normal and a fibreless line of cotton (*Gossypium hirustum*).J.*Plant Physiol.*149: 695-702.

Ulloa, M., Mc Stewart, J., Garcia, C.E., Godoy, A.S., Gaytan, M.A. and Acosta, N.S. (2005) Cotton genetic resources in the western states of Mexico: in situ conservation status and germplasm collection for ex situ preservation. *Genet Resour Crop Evol.* In press.

Varner, J.E and Lin, L.S. (1989) Plant cell wall architecture. *Cell,* 56: 231-C239.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, Pot J., Peleman, J., Kulper, M. and Zabeau, M., (1995), AFLP: a new technique for DNA fingerprinting. *Nuceic Acids Research* 23: 21 4407-4414

Wendel, J. F., Cronn, R. (2003) Polyploidy and the evolutionary history of cotton. *Adv. Agron.* 78: 139–186.

Wendel, J. F. and Albert, V.A. (1992) Phylogenetics of the cotton genus (*Gossypium*): Character-State Weighted Parsimony Analysis of chloroplast DNA restriction site data and its systematic and biogeographic implications. *Systematic Botany* 17: 115-143.

Wendel, J. F. (1995) cotton.In;N.Simmonds and J.Smartt(eds), Evolution of crop Plants.Longman,London pp358-366.

Wendel, J. F., Olson, P. D., Stewart, J. Mc. D. (1989). Genetic diversityn ,intrigression and independent domestication of Old World cultivated cotton.*Amer. J.Bot.*76: 795-806.

Werker, E. (2000) Tri home diversity and development. *Adv. Bot. Res.* 31: 1–35.

Wilkins, T.A., Arpat, A.B. (2005) The cotton fiber transcriptome. *Plant Physiol.* In press.

Wilkins, T.A., Arpat, A.B., Sickler, B.A. (2005) Cotton fiber genomics: developmental mechanisms. *Pflanzenschutz Nachrichten* 58:119–139.

Wilkins, T.A., Jernstedt, J.A., (1999) Molecular genetics of developing cotton fibers. In: Basra AS (ed) Cotton Fibers. Haworth Press, New York, pp 231–267.

Wolfe, K. H. (2001) Yesterday's polyploids and the mystery of polyploidization. *Nat. Rev. Genet.* 2: 333–341.

Wolfe, K. H. and Shields, D. C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 387: 708–713.

Wright, R. J., Thaxton, P. M., El-Zik, K. M., and Paterson, A. H. (1998) D-sub genome bias of Xcm resistance genes in tetraploid Gossypium (cotton) suggests that polyploid formation has created novel avenues for evolution. *Genetics* 149: 1987–1996.

Zabeau, M. and P. Vos, (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application No.0534858 A 1. European Patent Office, Paris.

Zhao, G. R., Liu, J. Y. and Du, X. M. (2001). Molecular cloning and characterization of cotton cDNAs expressed in developing fiber cells. *Biosci. Biotechnol. Biochem.* 65: 2789 2793.

# Chapter 6
## Appendices

**Appendix 1a**

**Cautions during the experiment**

1) If there is any chance of spilling something that is probably DNA contaminated, do a spot clean with a bit of bleach on a tissue towel.

2) Do not let the cleaning solution touch with samples. Cleaning solution can destroy DNA/RNA – even the one kept around. When in doubts change the gloves.

3) Use the sterile inside technique to retrieve tubes and tips from their containers. The out sides of these containers will not be super clean. These procedures are meant to ensure that inside of the containers, where the important stuff is, stay uncontaminated.

a) To get a micro centrifuge tube out of beaker, take the tinfoil top off, and shake several tubes into the inside of the tin foil cover. Pick out the required tubes and shake the rest back into the beaker. This will keep sample from smearing contaminants all over the inside of the beaker.

b) Do not handle pipette tips with hands. Open the box, and use only the tip of the pipettor to pick up the tip. Do not close the box every time.

c) When weighing out reagents, do not stick a spatula into the bottle unless it has already been cleaned with RNAse AWAY. Always use a fresh piece of weighing paper or weighing boat. There is a chance that one can contaminate precious sample with RNase with a dirty reagent or dirty pipette during work.

## Appendix 1b

### Laboratory bench clean up protocol

Before performing RNA experiment the lab bench was cleaned as given below to remove any traces of RNAses:

1) Put on a pair of gloves.
2) Remove pipettes, pipettors, beakers, tubes, and boxes of pipette tips, from the bench surface.
3) Wipe the entire surface down with the bleach solution, followed by a wipe down with 70% ethanol. Allow to air dry. Now bench surface is free of all DNA.
4) Wet a few kitchen towels with a little diluted bleach solution. Wipe down the bottom of beakers and tip boxes, place them on bench and through out the tissue towels.
5) Wet a tissue towel with some RNAse away solution. Carefully wipe down completely all the pipettors. Clean away the solution with a fresh tissue and change gloves after cleaning.
6) If there is something that cannot be cleaned this way, such as some powdered reagents, then use a short wave UV-light for 15-30 minutes.

## Appendix 1C

### Agarose gel electrophoresis

Fermentas 6X DNA loading dye was used to load the DNA samples on agarose gel. DNA Fragments were separated by electrophoresis on 1.5% (w/v) agarose gels in 1X TBE buffer containing ethidium bromide (0.5μg/ml). The gels were examined under UV light. The DNA fragment sizes were estimated by comparison with 1Kb DNA ladder (Fermentas).

## Appendix 2a

### First strand cDNA synthesis

When RNA is reverse transcribed using reverse transcriptase, it forms complementary

DNA (cDNA), which is double stranded and much stable as compared to single stranded RNA. For the synthesis of cDNA, RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) was used.

The kit contained RevertAid H Minus M-MuLV reverse transcriptase (RevertAid H Minus M-MuLV RT), genetically engineered version of the Moloney Murine Leukemia Virus reverse transcriptase. The enzyme lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids. Therefore, degradation of RNA does not occur during first strand cDNA synthesis, resulting in higher yield of full-length cDNA from long templates (up to 13Kb).

The first strand cDNA was synthesized by Oligo(dT) primer: Oligo(dT)18: It binds at the 3 end of poly(A) mRNA. In this case mRNA with 3 poly (A) tails are templates for cDNA. The cDNA can be used as a template in polymerase chain reaction (PCR) and also for second strand synthesis.

**Protocol**

RevertAid H Minus First Strand cDNA Synthesis Kit protocol is given below.

1. Prepare the following reaction mixture in a tube on ice.

Template RNA:

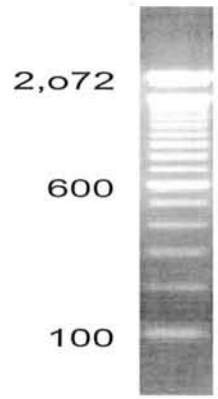
| | |
|---|---|
| total RNA | 10 ng-5μ g. |
| Primer: | |
| Oligo(dT) primer (0.5 g/μ l) | 1μ |
| deionized water, nuclease free to bring the final volume to | 12μ l |

Mix gently and spin down for 3-5 seconds in a micro centrifuge.

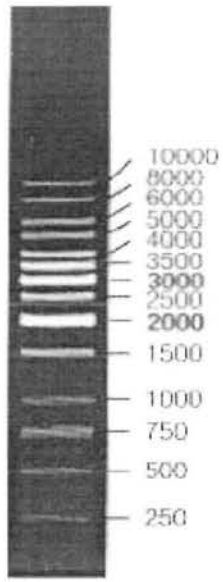
2. Incubate the mixture at 70°C for 5 min, chill on ice and collect drops by brief centrifugation.

**Appendix 2b**

100 bp DNA ladder (GIBCO-BRL)



**1 Kb DNA Ladder**
**Fermentas Cat # SM0311/2/3**




**Appendix3a**

**6X Gel loading dye**

| | | |
|---|---|---|
| 1) | Bromophenol blue | 0.25% (w/v) |
| 2) | Xylene cyanol FF. | 0.25% (w/v) |
| 3) | Glycerol | 30.0% (v/v) |

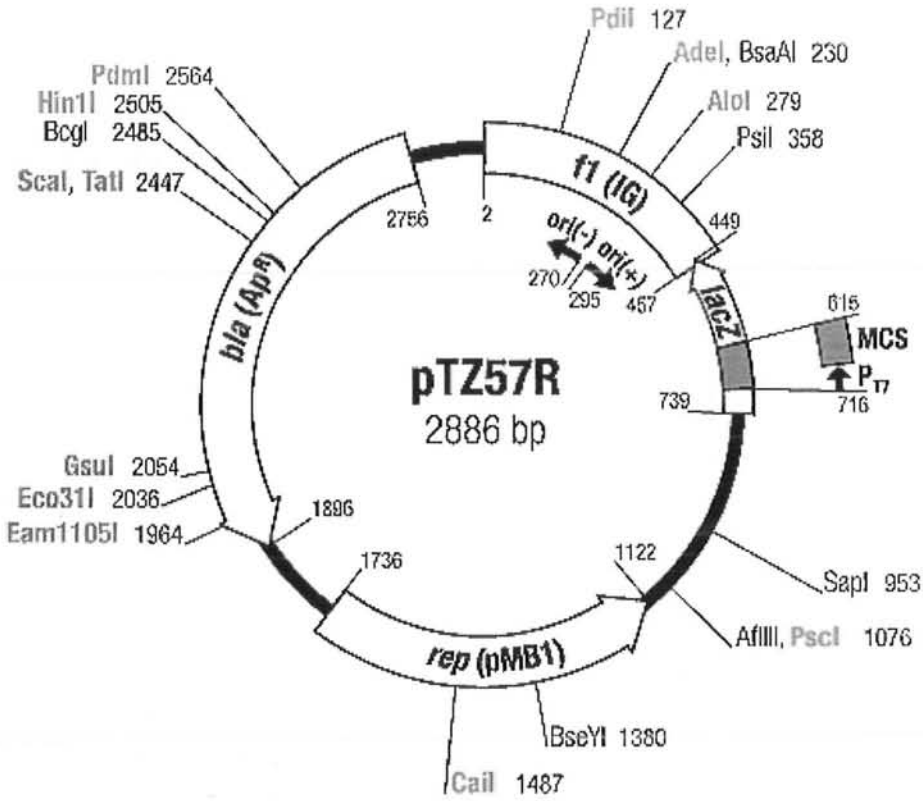
Dissolve in distilled water.

**5x TBE Buffer**

| | | |
|---|---|---|
| 1. | Tris base | 54g |
| 2. | Boric acid | 27.5g |
| 3. | EDTA | 4.65g |
| 4. | Total volume | 1L |
| 5. | Ph. 8.3 | |

**Appendix 3b**

**pTZ57R Cloning Vector**
(Fermentas)




**Appendix4a**

**Preparation of chemically competent cells of *E.coli* (DHα-5)**

Competent cells are prepared for transformation of the ligation reactions. Te competent cells are kept on ice during manipulations after those are taken out of freezer at -70°C. When subjected to heat shock treatment (42°C) for 1-2 minutes, the pores in bacterial cell membrane become open and take up the foreign DNA in their surroundings. These pores are later closed when cells are kept on ice immediately after heat shock.

**Protocol**

1. A single colony from a freshly grown plate of *E. coli* is picked and transferred into 50 ml LB (Appendix 9) medium in a 100ml flask. Incubate the flask at 37°C overnight with vigorous shaking (150-200 rpm).
2. 0.5 ml of the overnight culture is taken and added to 50 ml of fresh LB (Appendix 9) medium and shaken vigorously at 37°C until an OD of 0.5-0.6 ($10_{10}$ cells/ml) is obtained.
3. Culture is kept on ice for 30 minutes and equally divided into two pre-cooled 50 ml sterile Falcon tubes.
4. The cells are pelleted by centrifugation at 5000 rpm at 4°C for 10 minutes and resuspended in 25 ml of ice cold 0.1M $CaCl_2$ and kept on ice for 1 hour.
5. The cells are again pelleted by centrifugation at 5000 rpm for 5 minutes and resuspended finally in 0.1M $CaCl_2$ ($1/10_{th}$ volume of original culture volume, i.e. 2.5 ml). Finally, 250 l of sterile cold 100% glycerol is added to obtain a final concentration of 10% v/v.
6. The cells are stored in aliquots of 200 l at 70°C.

**Appendix 4b**

**Transformation of competent cells of *E.*coli (DHα-5) by heat shock method**

1- 1-2µ l of ligation mixture was added in 200µ l competent cells aliquot and kept on ice for half an hour.
2- Heat shock was given on dry bath at 42°C for 1 minute.
3- Cells were kept on ice for 2 minutes.
4- 1 ml LB was added in the mixture.
5- Cells were kept at 37°C shaker for 1 hour.
6- 100µl of transformation mixture was spread on the LB agar plates containing Ampicillin (100 g/ml), 40µl of X-gal (40mg/ml) and IPTG (100mM) (Appendix 5)

**Appendix 4C**

**LB (Luria-Bertani) liquid medium**

Tryptone 1.0 %
Yeast extract 0.5 %

NaCl 0.5 %

Dissolve in 100 ml double distilled water adjust pH 6.2 - 7.2 and autoclave.

## Appendix 5

### Solution I (Suspension buffer)

Tris (pH 7.4-7.6) 50 mM

EDTA 1 mM

RNase 100 g/ml

### Solution II (Denaturation soln.)

NaOH 0.2 N

SDS 1 %

### Solution III (Neutralization soln.)

Sodium acetate 3 M

Glacial acetic acid 11.5ml/100ml

(pH 4.8-5.0)

### IPTG (isopropyle-thio- -D-galactoside) stock solution (0.1 M)

Make a stock solution of 100 mM concentration in distilled water. Spread 40µl on LB agar plate.

### X- Gal stock solution

The concentration of X-Gal (5-bromo-4-chloro-3-indolyl- -D-galactopyranoside) used was 40 mg/ml in N, N dimethyl formamide. Spread 40µl on LB agar plate.

### Ethidium bromide.

10 mg/ml Stock Solution.
Add 5µl/100 ml of 1x TAE buffer. The concentration of ethidium bromide will be 0.5µg/ml.

### Ampicillin

100 mg/ml stock solution

100 µg/ml working concentration.

**Appendix 6**

**Prehybridization Solution:**

**20x ssc solution**

| | |
|---|---|
| NaCl | 175.3 g |
| Sodium citrate | 88.2 g |
| Final volume | 1 L |

Ph 7.00

**50x Denhard solution**

| | |
|---|---|
| Ficol | 5g |
| PVP | 5g |
| BSA | 5g |
| Total volume | 500ml |

**Appendix 7**

**Qiagen gel extraction method for purification of DNA bands from agarose gel**

The DNA fragments are visible under UV light when separated by electrophoresis in agarose gel in the presence of ethidium bromide. The purpose of elution is to extract DNA fragments (70bp-10Kb) from agarose gel in sterile pure $H_2O$ or TE buffer for using the cleaned DNA fragments for cloning or other enzymatic treatments as given below:

Fluorescent and radioactive sequencing.

Restriction.

Labeling.

Hybridization.

Ligation and transformation.

Amplification.

In vitro transcription.

Microinjection.

The gel elution of DNA fragments in this study was used to clone the purified fragments into pTZ57 RT cloning vector. (Fermentas)

**Protocol**

Perform all centrifugations at room temperature. Equilibrate a water bath or heating block to 50°C. Verify that ethanol has been added to wash buffer.

1) **Gel slice excision**

Cut the area of gel containing the DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.

2) **Gel slice weighing**

Weigh the gel slice. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg is approximately 100 µl). For example, add 300 l of Buffer QG to each 100 mg of gel. For > 2% agarose gel add 6 volumes of Buffer QG.

**Gel solubilization**

Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation. IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

3) **Column loading**

Place a QIAquick spin column in a provided 2-ml collection tube. Pipette the mixture from step 3 into the spin column. Centrifuge the mixture in a microcentrifuge at 14,000 rpm for 1 min. Discard the flow through.

Note: Load no more than 400 mg agarose per column.

4) **Optional column wash**

Place the spin column back into the 2 ml collection tube. Add 500 l of Buffer QG to QIAquick column and centrifuge for 1 min at 14,000 rpm for 1 minute. Discard the flow through. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing.

**5) Column wash**

Place the spin column back into the 2 ml wash tube. Add 750 l of Buffer PE (containing ethanol) to the spin column and incubate for 2-5 min at room temperature. Centrifuge at > 12,000 rpm for 1 minute. Discard the flow through. Centrifuge again for 1 minute to remove residual wash buffer.

**6) DNA elution**

Place the spin column into a 1.5 ml micro centrifuge tube. Add 20 l of Buffer EB (10mM Tris.HCl, pH 8.5) or water to the center of the QIAquick membrane of spin column. Incubate for 1 min at room temperature, then centrifuge at 14,000 rpm for 1 minute.

**Appendix 8**

**Random decamer primers used in cDNAs amplification**

| Primer Name | Primer Sequence |
|---|---|
| OPA1 | CAGGCCCTTC |
| OPA10 | GTGATCGCAG |
| OPA13 | CAGCACCCAC |
| OPJ4 | CCGAACACGG |
| OPJ5 | CTCCATGGGG |
| OPJ10 | AAGCCCGAGG |
| OPJ11 | ACTCCTGCGA |
| OPJ13 | CCACACTACC |
| OPJ14 | CACCCGGATG |