OPTIMIZATION OF *AGROBACTERIUM* MEDIATED COTTON TRANSFORMATION USING SHOOT APICES EXPLANTS AND QUANTITATIVE TRAIT LOCI ANALYSIS OF YIELD AND YIELD COMPONENT TRAITS IN UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.)

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF TABLES vi

LIST OF FIGURES viii

LIST OF ABBREVIATION x

ABSTRACT xi

INTRODUCTION 1
- I.1 References 6

CHAPTER 1 LITERATURE REVIEW 8
- 1.1 Cotton Tissue Culture 8
- 1.2 *Agrobacterium* – Mediated Cotton Transformation 11
  - 1.2.1 The Genus of *Agrobacterium* 11
  - 1.2.2 T-DNA Binary Vector System 12
  - 1.2.3 The Function of *Vir* Genes 13
  - 1.2.4 *Agrobacterium* – Mediated Cotton Transformation 14
- 1.3 Particle Bombardment Method of Cotton Transformation 18
- 1.4 QTL Analysis of Cotton Traits 21
  - 1.4.1 Linkage Maps 21
  - 1.4.2 QTL Analysis of Cotton Traits 23
- 1.5 References 24

CHAPTER 2 OPTIMIZATION OF SHOOT APEX BASED COTTON REGENERATION SYSTEM 32
- 2.1 Introduction 32
- 2.2 Materials and Methods 33
  - 2.2.1 Seed Disinfection Methods 33
  - 2.2.2 Shoot Apex Isolation 34
  - 2.2.3 Shoot Elongation and Rooting Development 34
  - 2.2.4 Plantlets Graft 36
  - 2.2.5 Experimental Design and Statistical Analysis 36
- 2.3 Results and Discussion 37
  - 2.3.1 Seed Surface Disinfection 37
  - 2.3.2 Effect of Explants Age 39
  - 2.3.3 Root Efficiency of Four Cotton Varieties on MS Medium 41
  - 2.3.4 Effect of IAA Shock 42
  - 2.3.5 Plantlet Grafting 44
  - 2.3.6 Conclusions 44
- 2.4 References 45

CHAPTER 3 OPTIMIZATION OF *AGROBACTERIUM* MEDIATED COTTON TRANSFROMAITON SYSTEM USING SHOOT APICES AS EXPLANTS …… 47
3.1 Introduction …… 47
3.2 Materials and Methods …… 49
3.2.1 Preparation of Shoot Apex Explants …… 49
3.2.2 *Agrobacterium* Strain and Plasmid …… 49
3.2.3 Pretreatment of Shoot Apex …… 50
3.2.4 *Agrobacterium* Co-cultivation and Transgenic Plants Regeneration 51
3.2.5 β-Glucuronidase (GUS) Histochemical Analysis …… 52
3.2.6 Kanamycin and Glufosinate Leaf Test …… 53
3.2.7 Polymerase Chain Reaction Analysis …… 53
3.2.8 Southern Blot Analysis …… 54
3.3 Results and Discussion …… 55
3.3.1 Determination of Suitable Kanmycin Concentration in Selection Medium …… 55
3.3.2 Effect of Inclusion of Acetosyringone During Co-cultivation …… 56
3.3.3 Effect of Concentration of *Agrobacterium* and Duration of Co-cultivation …… 57
3.3.4 Production of Putative Transgenic Plants …… 58
3.3.5 Conformation of Transformation Event …… 59
3.3.6 Production of Herbicide Resistant Cotton …… 63
3.3.7 Conformation of Transformation …… 64
3.3.8 Discussions …… 65
3.4 References …… 66

CHAPTER 4 CHROMOSOMAL ASSIGNMENT OF AFLP MARKERS IN COTTON …… 70
4.1 Introduction …… 70
4.2 Materials and Methods …… 72
4.2.1 DNA Isolation …… 73
4.2.2 Amplified Fragment Length Polymorphism Analysis …… 73
4.2.3 Marker Naming …… 77
4.3 Results and Discussion …… 78
4.3.1 AFLP Markers Frequency in Cotton …… 78
4.3.2 Assignment of AFLP Markers to Chromosomes …… 79
4.3.3 Association of Linkage Groups to Chromosomes …… 81
4.3.4 Discussions …… 82
4.4 References…… 83

CHAPTER 5 IDENTIFICATION OF QUANTITATIVE TRAIT *LOCI* FOR YIELD AND YIELD COMPONENT TRAITS IN UPLAND COTTON…… 86
5.1 Introduction …… 86
5.2 Materials and Methods …… 89
5.2.1 Plant Materials …… 89

5.2.2 Phenotypic Traits Measurement …………………………………… 89
5.2.3 Linkage Analysis …………………………………………………… 89
5.2.4 QTL Analysis …………………………………………………………... 90
5.3 Results and Discussion ……………………………………………………..... 90
5.3.1 Summary Statistics and Normality Test of Traits ………………… 90
5.3.2 Traits Correlations ………………………………………………… 91
5.3.3 Path Analysis of Yield Components ………………………………… 91
5.3.4 QTL Analysis of Lint Yield ………………………………………… 95
5.3.5 QTL Analysis of Bolls per plant ……………………………………… 96
5.3.6 QTL Analysis of Number of Fiber per Seed ………………………… 97
5.3.7 QTL Analysis of Average Weight per Fiber ………………………... 98
5.3.8 QTL Analysis of Seed Numbers per Boll ………………………….… 99
5.3.9 Discussions ……………………………………………………….…. 99
5.4 References ……………………………………………………………….. 102

VITA ……………………………………………………………………………….. 106

LIST OF TABLES

1.1 Reported genetic transformations of cotton ………..…..……………………..……... 16

2.1 Mean number of explants elongated on elongation media from 4 varieties at 4 different ages ……………………………………………………………………….. 40

2.2 ANOVA table for investigation of age effect of explants ...…………………..…... 40

3.1 Number of GUS positive cotton apices after treatment with 100 uM acetosyringone …………………………........…….…………………………… 57

3.2 Survival of cotton shoot apices after co-cultivation with *Agrobacterium* LBA 4404 and selection with 50mg/L kanamycin ..…….………………………………….... 59

4.1 Adapters and primers used for pre-amplification and selective amplification of AFLP procedure ….………………………………………………………………… 74

4.2 Protocol components for digestion and ligation of genomic DNA ………………. 75

4.3 Reagents used in the pre-amplification step and selective amplification step ……. 76

4.4 Number of monomorphic and polymorphic (total) and number of AFLP primer combinations between two lines (Pee Dee 2165 and Paymaster 54) of Upland cotton ……………………………………………………...………………….. 78

4.5 AFLP markers and its chromosome locations ……………………………...…... 80

4.6 Results of assignment of linkage groups to chromosomes …………………...……... 82

5.1 Reported linkage maps for tetraploid cotton ………..……………………………..... 88

5.2 Summary statistics and normality test for yield and yield component traits ……….. 91

5.3 Correlation coefficients among traits in an intraspecific cross of $F_{2:3}$ population ….. 93

5.4 Path analysis of yield components to lint yield in a $F_{2:3}$ population of an intraspecific cross of *G. hirsutum* ……………….……..…………………………. 94

5.5 AFLP markers that were associated with putative QTL influencing lint yield by using single point analysis ……………………………………………………… 95

5.6 AFLP markers that were associated with putative QTL influencing lint yield by using interval mapping (IM) and composite interval mapping(CIM) ………………. 96

5.7 AFLP markers that were associated with putative QTL influencing bolls per plant by using single point analysis ………….……………..…………………………. 96

5.8 AFLP markers that were associated with putative QTL influencing bolls per plant by using interval mapping (IM) and composite interval mapping(CIM) ….………. 96

5.9 AFLP markers that were associated with putative QTL influencing number of fiber per seed by using single point analysis ……………..…………………………………… 97

5.10 AFLP markers that were associated with putative QTL influencing number of fiber per seed by using interval mapping (IM) and composite interval mapping(CIM) …. 98

5.11 AFLP markers that were associated with putative QTL influencing average weight per fiber by using single point analysis ………….……………..………………………. 98

5.12 AFLP markers that were associated with putative QTL influencing average weight per fiber by using interval mapping (IM) and composite interval mapping(CIM) … 99

5.13 F and P value in SPA analysis and it's corresponding LOD score ……………….. 102

LIST OF FIGURES

I.1 Cotton yield trends from 1900 to 2002 in the USA ………………………………… 2

I.2 Transgenic cotton adoption in USA. …………………………………………… 4

1.1 Schematic representation of T-DNA transfer from *Agrobacterium* to the plant genome ………..…..……………………………………………………...…..……... 12

1.2 Schematic representation of binary vector system …..….………………….…..…... 13

2.1 Isolation of shoot apex of cotton …..…………………….……………….…..…….. 35

2.2 Grafting procedures of unrooted shoots …….…………………………….....….. 37

2.3 Mean number of germinated and contaminated cotton seed following three different surface disinfection methods ....……………………………………...………… 39

2.4 Isolated shoot apices growing on elongation media after two weeks ….……….….... 41

2.5 Percent of rooting efficiency of shoot apices from four cotton varieties after 3 weeks culture …………………………………………………………………..... 42

2.6 Regeneration of shoot apices …....……………………..…………………………… 43

2.7 Effect of IAA shock on stimulating the rooting of previously unrooted Coker 312 shoot apices ……………………………………………………...…………… 44

3.1 T-DNA region of pTOK233 ……………..……………...………………………. 49

3.2 Construct of the *bar* and *NPTII* genes on binary vector pBIMC-B …………..…. 50

3.3 Schematic representation of shoot apex meristem ………..…………………………. 51

3.4 Survival rate of shoot apices at different concentration of Kanamycin in 3 weeks …. 56

3.5 Effect of concentration of *Agrobcterium* and duration of co-cultivation ………….. 58

3.6 Production of putative transgenic plants ……….……………………………… 60

3.7 Histochemical staining of leaf discs ..………….………………………………….. 61

3.8 Kanamycin leaf spotting test …………...………………………………………….. 61

3.9 PCR analysis of transgenic plants for integration of the *NPTII* gene …………..... 62

3.10 Southern blot analysis of transgenic plants for integration of the *GUS* gene ……… 63

3.11 Herbicide (Liberty) leaf spotting test ……………………………………………… 64

3.12 PCR analysis of transgenic plants for integration of the *bar* gene ………………… 65

3.13 Southern blot analysis of transgenic plants for integration of the *bar* gene ……….. 65

4.1 AFLP gel image for the primer pair combination EcorI+ACA/MseI+CAA ..……. 81

5.1 Frequency distribution for lint yield and yield components …...…………….……. 92

5.2 Path diagram of cotton yield and yield component traits …………………………… 93

5.3 A comparison of QTL positions for Upland cotton lint yield and yield components.. 100

LIST OF ABBREVIATION

2,4-D	2,4-dichlorophenoxy-acetic acid
AFLP	Amplified fragment-length polymorphism
AHAS	Acetohydroxyacid synthase
Bt	*Bacillus thuringiensis*
CAT	Chloramphenicol acetyltransferease
CIM	Composite interval mapping
EPSPS	5-enolpyruvylshikimate-3-phophate synthase
GUS	β-glucuronidase
HPT	Hygromycin phosphotransferase
IAA	Indole acetic acid
IM	Interval mapping
MS	Murashige and Skoog
NOS	Nopaline synthase promoter
NPTII	Neomycin phosphotransferase II
OCS	Octopine synthase
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
Pha	Polyhydroxyalkanoate synthase
QTL	Quantitative trait loci
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
SSR	Simple sequence repeat

ABSTRACT

Cotton (*Gossypium spp*) is an important world crop. Although great improvements have been achieved through traditional breeding methods, cotton breeders are facing many problems, i.e., narrow genetic base, inability to use alien genes and difficulty in breaking gene linkages. Genetic transformations and quantitative trait loci (QTL) analyses are main tools used by breeders to overcome these problems. In this dissertation, an optimized cotton regeneration system from shoot apices was developed. The regeneration rate was increased to 85% by combining rooting induction, Indole acetic acid (IAA) shock and graft techniques. The regeneration system is genotype-independent and the whole process takes 12 to 16 weeks.

Transgenic cotton plants were obtained via *Agrobacterium*-mediated transformation using shoot apices as explants. Transformation rates were 0.67% and 1.01% for LBA 4404 with β-glucuronidase (*GUS*) gene and EHA 105 with *Bar* gene, respectively. Putative transgenic plants were confirmed by leaf GUS assay, kanamycin or herbicide (Liberty) leaf test, polymerase chain reaction (PCR) and southern blot analysis.

Out of 151 polymorphic markers, 53 amplified fragment-length polymorphism (AFLP) markers were assigned to individual chromosomes or chromosome arms by using a set of aneuploid genetic stock.

In the QTL analysis of cotton yield and yield components was conducted on an $F_{2:3}$ population derived from the intraspecific cross. A previously developed linkage map was used based on same population covering 1733.2 cM (37.7%) cotton genome (4700 cM). A total of 47 markers associated with yield and yield component traits were detected. Nine and seven QTL detected by interval mapping (IM) and composite interval

mapping (CIM) methods, respectively, four of which were detected by both methods. For lint yield, two main QTL, explaining 27% of variation, were detected via CIM method. No QTL was detected for bolls per plant by IM method and one QTL explaining 8.56% variation was detected by CIM method. For number of fibers per seed, 23.7 % of variation was explained by two main QTL detected by both IM and CIM methods. For mean weight per fiber, two QTL were detected via CIM. No QTL was detected for seed number per boll via either method.

INTRODUCTION

Cotton, *Gossypium spp*., is an economically important crop that is grown throughout the world. Cotton is grown as a source of fiber, food and feed. Lint, the most economically important product from the cotton plant, provides a source of high quality fiber for the textile industry. Cotton seeds are an important source of oil, and cotton seed meal is a high protein product used as livestock feed. Other products include seed hulls and linters. In the United States, cotton fiber is a major source of export revenue, and over one half of the cotton produced is exported. Cotton has been estimated to contribute US $15-20 billion to the world's agricultural economy with over 180 million people depending on the crop for their livelihood. In 2003, it was grown on more than 15.6 million acres in the United States. In Louisiana, cotton is one of the leading agronomic crops, and it was grown on over 500,000 acres.

The genus *Gossypium* contains about 50 diverse species. Four are cultivated, *G. hirsutum* L. and *G. barbadense* L., which are tetraploid (2n = 4x = 52), and *G. arboretum* L. and *G. herbaceum* L., which are diploid (2n = 2x = 26). The species most widely grown around the world is *G. hirsutum*. Over 95 percent of United States cotton acreage is covered by *G. hirsutum* cultivars followed by *G. barbadense*. *G. hirsutum* is native to Mexico and parts of Central America and *G. barbadense* is native to South America. Cotton was among the first species to which the Mendelian principles of segregation and independent assortment of genes were applied (Balls, 1906). The traditional breeding methods use hybridization, wide-crosses, backcross, mutation…etc. techniques to introduce desirable agronomic traits, such as high yield, good quality and disease resistance, into new breeding lines which may be released after several years of field

testing. Traditional breeding methods have been used with aggressive selection for yield, disease resistance and fiber quality. Significant progress has been made in all breeding objectives. The yield increase contributed by genetic improvement was 7-10 kg/ha/year for the USA (Meredith *et al*., 1984), 23kg/ha/year for Australia (Constable *et al.,* 2001), and 8-10 kg/ha/year for China (Kong *et al.*, 2000).

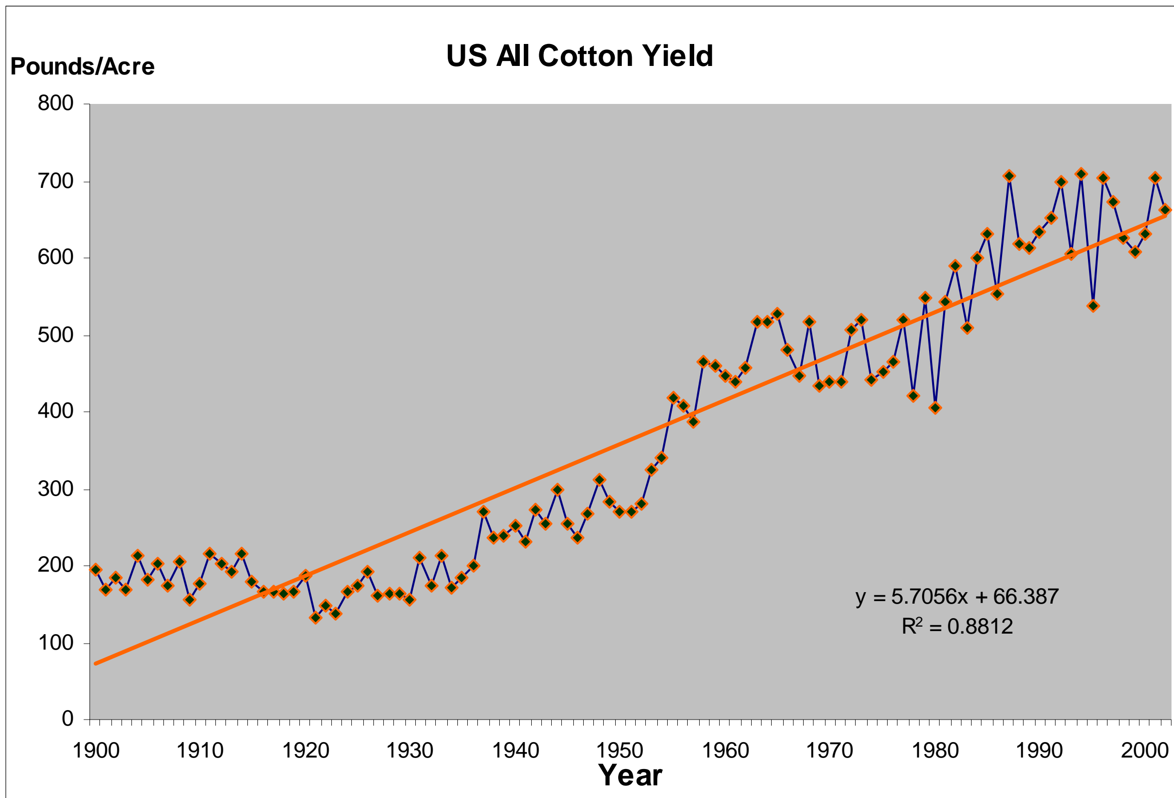

Figure I.1 Cotton yield trends from 1900 to 2002 in the USA. Data source is the USDA National Agricultural Statistics Service.

Despite the steady increase during 1900 to 1990, cotton yield has been erratic over last ten years. Figure I.1 represents cotton yield trend from 1900 to 2002 in theUSA. We can see that cotton yields have been static from 1990 to 2002. This was caused by the limitations of conventional breeding which including:

1) Narrow genetic base of the cultivated species

2) Inability to use sexual crosses for introducing many useful alien genes into the crop
3) The length of time needed for successfully developing crop cultivars
4) The difficulty in breaking gene linkages between useful and useless traits
5) Inefficient selection methods for quantitative traits, such as lint yield

These restrictions have seriously limited new cultivar development. As plant breeders face these challenges, they are increasing funding to two new approaches to overcome these problems. One is the use of genetic transformation to incorporate valuable alien genes into the cotton genome; the other is the use of quantitative trait locus (QTL) analysis to associate molecular markers with interesting traits to facilitate the use of marker assisted selection (MAS) in a breeding program.

With the advent of recombinant DNA technology in the 1970s, the genetic manipulation of plants entered a new age. Genes and traits previously unavailable through traditional breeding became available through DNA recombination and with greater specificity than ever before. This modern genetic technology allows the transfer of genetic material across wide evolutionary lineages and has removed the traditional limits of crossbreeding. Genes from sexually incompatible plants or from animals, bacteria or insects can now be introduced into plants. Modern plant genetic engineering involves the transfer of desired genes into the plant genome, and then regeneration of a whole plant from the transformed tissue. Currently, the most widely used method for transferring genes into plants is *Agrobacterium*-mediated transformation (Chilton *et al*., 1977) and the particle bombardment method (Klein *et al.,* 1987). Others methods, such as polyethylene glycol (PEG)- mediated transformation (Datta *et al*., 1990), and

electroporation (Potrykus *et al*., 1985; Fromm *et al*., 1985) have also been used to transfer genes into plants.

The first transgenic upland cotton, expressing the CryIAc insecticidal protein, was released into commercial production in 1996 on 12 % of the acres in cotton production in the U.S. (Hardee and Herzog, 1997). The overall success of transgenic cotton was soon apparent in the dramatic increase in total acres committed to transgenic cotton within the first few years of production. In less than 5 years, transgenic cotton in the USA accounted for more than 70% of the acreage in the vast majority of cotton –production regions of the Cotton Belt (Figure I.2). There is little doubt that genetic transformation will play a significant role in the future of cotton genetic improvement.

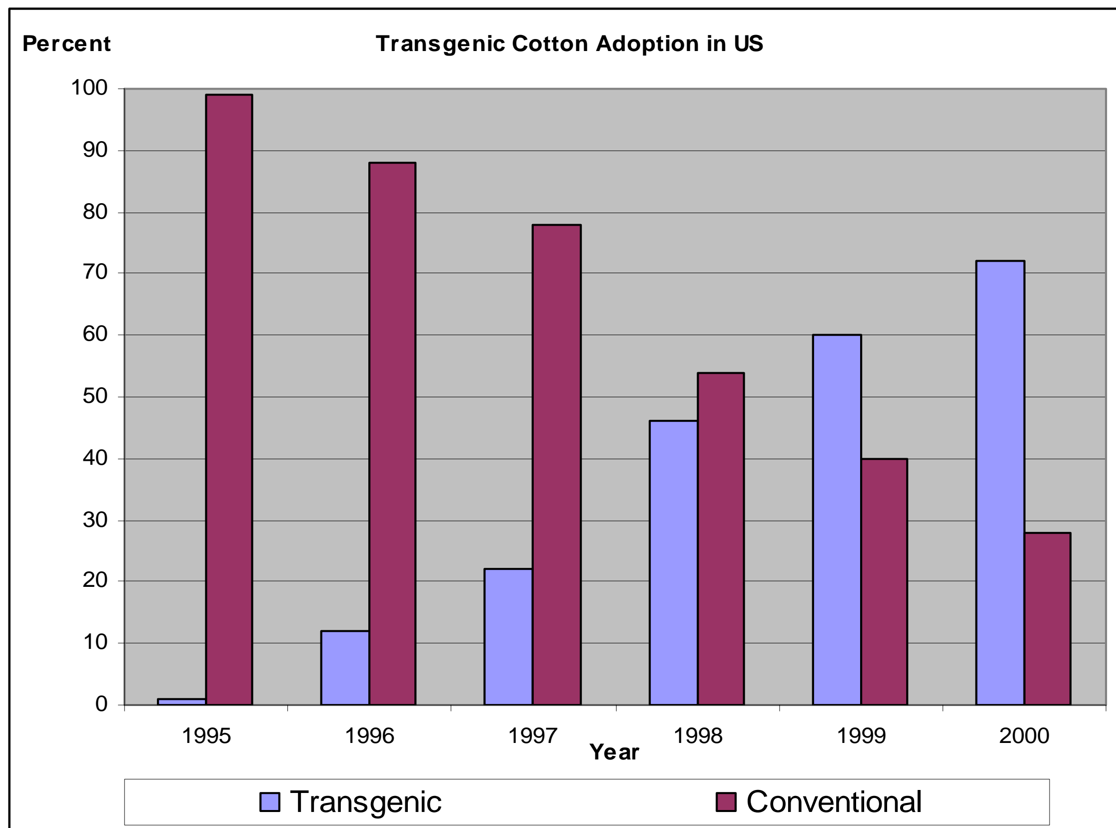

Figure I.2. Transgenic cotton adoption in USA. Data from the USDA National Agricultural Statistics Service.

Another active research field in cotton genetic improvement is QTL analysis. Since many important traits in cotton are controlled by several genes each with small effects, researchers have focused on identifying and controlling those genes for the improvement of cotton yield and fiber quality. Cotton breeders have historically improved quantitative traits by conventional breeding methods based on phenotypic evaluation and selection, which are time and resource consuming and increasingly less effective. With the advent of molecular marker techniques as well as the availability of saturated DNA marker maps, it is now possible to identify and locate genes controlling complex traits like lint yield and its component traits. The first cotton linkage map, reported by Reinish *et al*. (1994), was constructed using 705 restriction fragment length polymorphism (RFLP) markers from an interspecific cross (*G. hirsutum* × *G. barbadense*). After that, several linkage maps were reported based on both interspecific and intraspecific cross. Recently, a more saturated genetic map that was constructed by 3347 markers was reported (Rong *et al.*, 2004). The availability of such saturated molecular maps (Rong *et al.,* 2004; Lacape *et al*., 2003) has made it possible to elucidate the inheritance pattern of QTL. The association of molecular markers with desirable quantitative traits should contribute to the discovery of genetic variability and aid in the selection of desirable parents and progeny through marker-assisted breeding (Paterson *et al.,* 1988).

In this dissertation, the first chapter will provide the literature review on genetic transformation and QTL analysis in cotton research. Chapters 2 and 3 will focus on the development of a regeneration system using shoot apices as explants and the optimization of *Agrobacterium*-mediated cotton transformation. Chapters 4 and 5 will present the

results of the assignment of AFLP markers to chromosomes by using aneuploid genetic stocks and QTL analysis of lint yield and a detailed dissection of yield component traits.

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CHAPTER 1 LITERATURE REVIEW

Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plant. The development and commercial release of transgenic cotton plants relies exclusively on two basic requirements. The first one is a method that can transfer a gene or genes into the cotton genome and govern its expression in the progeny. The two main gene delivery systems for achieving this end are *Agrobacterium* - mediated transformation and particle gun bombardment. The other requirement is the ability to regenerate fertile plants from transformed cells. This is achieved by regenerating plants via somatic embryogenesis or from shoot meristems. The following paragraphs presents reviews of these topics in detail.

1.1 Cotton Tissue Culture

Plant tissue culture or the aseptic culture of cells, tissues and organs, is an important tool in both basic and applied studies. It is founded upon the research of Haberlandt, a German plant physiologist, who in 1902 introduced the concept of totipotency: that all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant. Considerable research work was undertaken in plant tissue culture in the 1950s and 1960s. The focus of research in plant cell culture for many crop species was to be able to put a species into tissue culture, develop callus, and ultimately regenerate a normal plant. For many crops, an efficient tissue culture procedure has been developed, e.g. tobacco, rice and some horticultural crops. In comparison with other crops, successes in cotton tissue culture lag behind those in other crops.

Cotton somatic embryogenesis was first observed by Price and Smith (1979) in *Gossypium koltzchianum,* but no plantlet regeneration was reported. Davidonis and Hamilton (1983) first described plant regeneration from two-year old callus of *Gossypium hirsutum* L. CV Coker 310 via somatic embryogenesis. The procedure, however, involved a lengthy culture period, was not successful with other cultivars, and was difficult to repeat. Other researchers (Rangan *et al.*, 1984; Shoemaker *et al.*, 1986; Gawel *et al.*, 1986) also reported the successful initiation of somatic embryos and regeneration of cotton plants. A common feature of those reports is that the procedure is restricted to only a few genotypes. In their research, they found that only slow-growing, gray, opaque calli were embryogenic, while pale yellow, or light to dark green and fast-growing calli was not embryogenic. The critical examination of callus cultures under a stereomicroscope was important in successfully establishing cotton cultures that could regenerate.

In vitro cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies (Shoemaker *et al.*, 1986; Chen *et al.*, 1987; Trolinder and Goodin, 1987; Kolganova *et al.*, 1992; Zhang, 1994a; Zhang *et al.*, 1996, 1999). Regenerated plants have been obtained from explants such as hypocotyls, cotyledon, root (Zhang, 1994a) and anther (Zhang *et al.*, 1996), and from various cotton species (Zhang, 1994b). In 1987, Trolinder and Goodin reported cotton regeneration from suspension cultures. Eight cotton cultivars were screened for their ability to form embryogenic callus from hypocotyl sections and Coker 312 was described as having a high embryogenic response. A system that is simple, easy to manipulate, and can provide large numbers of somatic embryos for study in a short time was described. A limitation,

however, was that among the 78 flowering plants obtained, only 15.4% set seed. Finer (1988) reported establishing a high-frequency embryogenic suspension culture of Coker 310. High numbers of somatic embryos were formed and normal, fertile plants were regenerated. Suspension culture of cotton remained limited to a few Coker cultivars, and cotton plants developed from cell culture methods demonstrated a disturbing level of cytogenetic abnormalities (Li *et al.*, 1989; Stelly *et al.*, 1989).

Another approach to develop a cell culture system for cotton that was genotype-independent was first reported by Renfroe and Smith (1986). This system used the isolated shoot meristem from seedlings of *G. hirsutum* L. cv. Paymaster 145. Isolated shoots could be cultured into rooted plants. Gould *et al.* (1991) extended this approach by using two *G. barbadense* cultivars and 19 *G. hirsutum* cultivars and was successful in establishing cotton regeneration methods that were independent of genotype; however, rooting efficiency was low. Since this method did not involve a callus intermediate stage, it was genotype-independent and saved a considerable amount of time. Nasir *et al.* (1997), Morre *et al.* (1998) and Zapata *et al.* (1999) also reported the regeneration of cotton plants from shoot meristems. This method has also been successfully used in cotton transformation when combined with particle bombardment (McCabe and Martinell, 1993).

Although the efficiency of regeneration via somatic embryogenesis has been improved significantly in recent years, some difficulties still remain. Only a limited number of cultivars can be induced to produce somatic embryos and regenerative plants, and the most responsive lines are Coker varieties, which are no longer under cultivation (Feng *et al.*, 1998). This genotype-dependent response restricts the application of cotton

biotechnology in cotton breeding and production. Therefore, before plant tissue culture techniques are widely applied to cotton improvement programs, plant regeneration must be possible for a broad range of genotypes. The focus of improving the rooting rate in shoot apex culture was undertaken and the results are presented in chapter 2.

1.2 *Agrobacterium* -Mediated Cotton Transformation

1.2.1 The Genus of *Agrobacterium*

The genus *Agrobacterium* has been divided into a number of species based on its disease symptomology and host range. *A. radiobacter* is an 'avirulent' species, *A. tumefaciens* causes crown gall disease, *A.rhizogenes* causes hairy root disease and a new species, *A. vitis*, which causes galls on grape and a few other plant species (Otten *et al.*, 1984). The host range of *Agrobacterium* is extensive. As a genus, *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species and gymnosperms. In addition, *Agrobacterium* can transform fungi, including yeast, ascomycetes and basidiomycetes (Stanton, 2003).

The most widely used specie in plant transformation is *A. tumefaciens*. *A. tumefaciens* is a naturally occurring soilborne pathogenic bacterium that causes crown gall disease. The crown gall disease has been shown to be due to the transfer of a specific fragment, the T-DNA (transfer DNA), from a large tumor-inducing (Ti) plasmid within the bacterium to the plant cell (Zaenen *et al.* 1974). After transfer, the T-DNA becomes integrated into the plant genome and its subsequent expression leads to the crown gall phenotype (Chilton *et al.*, 1977). There are two bacterial genetic elements required for T-DNA transfer to plants. The first element is the T-DNA border sequences that consist of 25 bp direct repeats flanking and defining the T-DNA. The borders are the only

sequences required in *cis* for T-DNA transfer (Zambryski *et al.*, 1983). The second element consists of the virulence (*vir*) genes encoded by the Ti plasmid in a region outside of the T-DNA. The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome (Godelieve Gheysen *et al.*, 1998). Figure 1.3 shows the mechanism of T-DNA transfer to a plant's genome.

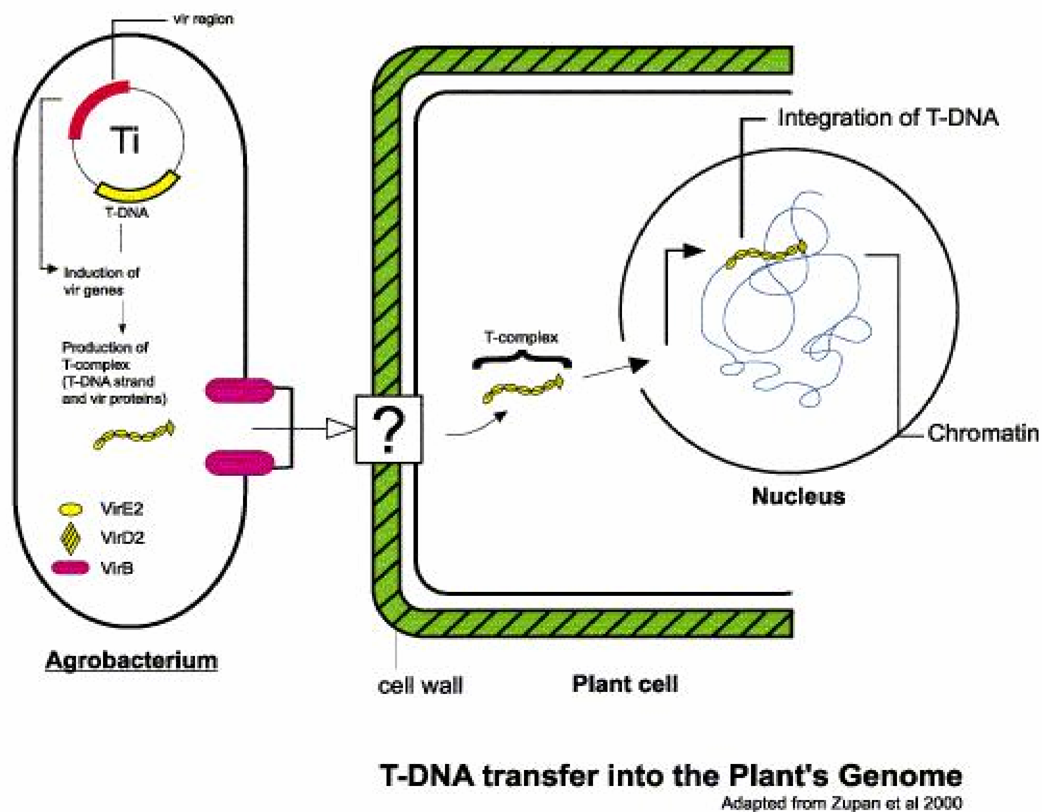

Figure 1.1 Schematic representation of T-DNA transfer from *Agrobacterium* to the plant genome (picture from http://www.cambiaip.org/Whitepapers/Transgenic/AMT/Scientific_aspects/agri_page4.htm)

1.2.2 T-DNA Binary Vector System

Scientists have taken advantage of this naturally occurring transfer mechanism, and have designed DNA vectors from the tumor-inducing plasmid DNA to transfer desired genes into the plant. The development of DNA vectors using *A. tumefaciens* is

based on the fact that besides the border repeats, none of the T-DNA sequences is required for transfer and integration. This means that the T-DNA genes can be replaced by any other DNA of interest, which will be transferred into the plant genome. Also the length of the T-DNA is not critical. Small (a few kb or less) as well as large T-DNAs (150kb)(Hamilton *et al.*, 1996) will be transferred by the *A. tumefaciens* into plant cell. It has also been found that T-DNA and *vir* genes do not have to be in the same plasmid for transfer of T-DNA (Hoekema *et al.*, 1984). This achievement has allowed development of a binary vector system to transfer foreign DNA into plants. Two plasmids are used in the binary method, i.e., the Ti plasmid containing the *vir* genes with oncogenes eliminated, a so called 'disarmed' plasmid or '*vir* helper', and a genetically engineered T-DNA plasmid containing the desired genes (An *et al.*, 1986). The plasmids in T-DNA binary vectors are smaller than plasmids in *Agrobacterium* and easier to manipulate in both *E. coli* and *Agrobacterium*. This has allowed researchers without specialized training in microbial genetics to easily manipulate *Agrobacterium* to create transgenic plants.

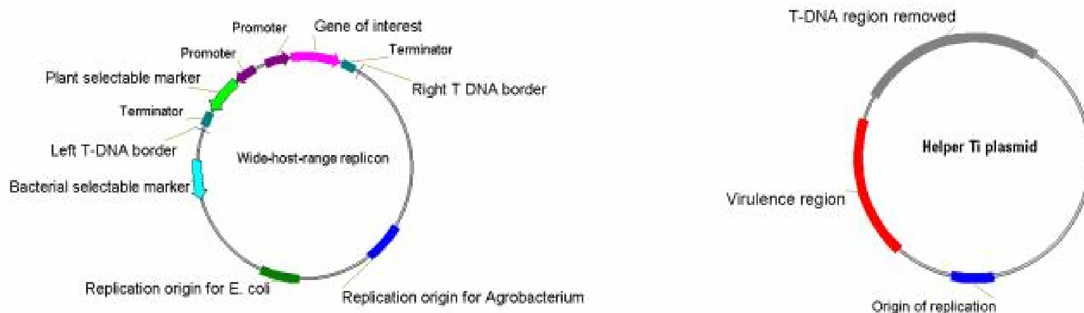

Figure 1.2 Schematic representation of binary vector system. (picture from http://www.cambiaip.org/Whitepapers/Transgenic/AMT/Scientific_aspects/agri_pge6.htm)

1.2.3 The Function of *Vir* Genes

The processing and transfer of T-DNA from *Agrobacterium* to plant cells is regulated by the activity of the vir genes. At least 24 *vir* genes in nine operons (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G ,*vir*H and *vir*J) have been identified. The *VirG*, a cytoplasmic response regulator, specifically reacts to the presence of exudates of wounded plant cell and promotes transcriptional activation of the *vir* gene. (Winans, 1991). It was shown that by increasing the copies of v*ir*G genes that it is possible to increase the transient transformation of rice and soybean from two to sevenfold (Ke *et al*., 2001). Also, presence of acetosyringone can help *Agrobacterium* to transfer T-DNA to recalcitrant plant species (Ashby *et al.*, 1987). With the induction of plant phenolic exudates, *vir*A and *vir*G expressed and induced expression of other *vir* genes. Expression of *vir* genes leads to the production of a single-stranded T-DNA copy, termed the T-strand, which is then transported into the host cell. The *Vir*D and *Vir*E, alone with T-strand form the T-complex, is transferred to plant cells by *Vir*B and other genes. A detailed review of all the *vir* genes and their function can be found in Tzvi Tzfira and Vitally Ctovsky's paper (2000). Based on the findings of the key role of *vir* gene expression in T-DNA transfer, vectors have been made to provide constitutive expression of *vir* genes to enhance transformation efficiency (Hansen *et al.*, 1994; Ishida *et al.*, 1996).

1.2.4 *Agrobacterium*-Mediated Cotton Transformation

Agrobacterium-mediated transformation is the most widely used method to transfer genes into plants. Transformation is typically done on a small excised portion of a plant known as an explant. The small piece of transformed plant tissue is then

regenerated into a mature plant through tissue culture techniques. The first reported plant transformation by A*grobacterium* was in 1983 (Fraley *et al.*, 1983). Since then, major advances have been made to increase the number of plant species that can be transformed and regenerated using *Agrobacterium*. In cotton, the first report of a genetically engineered plant was in 1987 (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987). In the report by Umbeck *et al.* (1987), hypocotyl explants of *G.hirsutum* cv. Coker 312 were transformed by *Agrobacterium tumefaciens* strain LBA4404 with neomycin phosphotransferase II (*NPT* II) and chloramphenicol acetyltransferease (*CAT*) genes regulated by the nopaline synthase promoter (*NOS*). Molecular analysis confirmed that the genes were in the primary plants, but progeny evaluation was not reported. A comprehensive list of successful transformations using the *Agrobacterium* method is listed in Table 1.1. These early cotton transformation experiments were not thoroughly characterized and were difficult to repeat in other laboratories. Umbeck *et al.* (1989) first reported progeny analysis of transgenic cotton containing foreign genes. Segregation ratios of 3:1 (selfed) and 1:1 (backcrossed) were reported. These ratios were expected for a single gene trait. Perlak *et al.* (1990) were the first to insert an agronomically important gene into cotton, cv. Coker 312 by using *Agrobacterium* strain A208. The gene was the cryIA (b) gene from *Bacillus thuringiensis*(Bt) for insect resistance regulated by the CaMV 35S promoter. Insect feeding bioassays and immunological (Western) analysis confirmed the expression of the *Bt* protein in the primary transgenic plant. The progeny expressed the *Bt* gene as a single dominant Mendelian trait and the phenotype appeared normal. In 1992, field tests showed good protection from cotton bollworm and *Pectinophora zea*, the pink bollworm. Transgenic cotton resistant to the herbicide 2,4-D

Table 1.1 Reported genetic transformations of cotton

Transgenic trait	Introduced gene	Method of transformation	explant	Reference:
Selectable markers	NPTII and OCS	*Agrobacterium*	Cotyledon	Firoozabady *et al.*, 1987
	NPTII and CAT	*Agrobacterium*	Hypocotyl	Umbeck *et al.*, 1987
	HPT	Particle bombardment	Embryogenic suspension culture	Finer and McMullen, 1990
	GUS	Particle bombardment	Zygotic embryo meristem	McCabe and Martinell, 1993 Chlan *et al.*, 1995
	NPTII	*Agrobacterium*	Cotyledon and hypocotyl	Cousins *et al.*, 1991;Rejasekaran *et al.*, 1996
	NPTII	*Agrobacterium*	Shoot tips	Zapata *et al.*, 1999
	NPTII and GUS	Particle bombardment	Embryogenic suspension culture	Rajasekaran *et al.*, 1996, 2000
Insect resistance	CrylAc		Hypocotyl	Perlak *et al.*, 1990
	Protgeinase inhibitors	*Agrobacterium*	Cotyledon	Thomas *et al.*, 1995
	Bromoxynil tolerance	*Agrobacterium*	hypocotyl	Fillati *et al.*, 1989
Herbicide tolerance	2,4-D mono-oxygenase for 2,4-D resistance	*Agrobacterium*	hypocotyl	Bayley *et al.*, 1992;Lyon *et al.*, 1993
	CP4 (CP4 EPSPS)for glyphosate tolerance	*Agrobacterium*	Hypocotyl	Nida *et al.*, 1996
	Mutant AHAS for sulfonylurea tolerance	*Agrobacterium*	Hypocotyl	Rajasekaran *et al.*, 1996

Table 1.1 *continued*

Transgenic trait	Introduced gene	Method of transformation	explant	Reference:
Herbicide tolerance	Mutant AHAS for sulfonylurea tolerance	Particle bombardment,	Embryogenic suspension culture	Rajasekaran *et al.*, 1996
	Bialaphos resistance	Particle bombardment	Zygotic embryo meristem	Keller *et al.*, 1997
Stress tolerance	Mn superoxide dismutase	*Agrobacterium*	Hypocotyl	Payton *et al.*, 1997
Fiber genes	E6 antisense RNA	Particle bombardment	Zygotic embryo meristem	John, 1996
	E-6 promoter +pha	Particle bombardment	Zygotic embryo meristem	John and Keller, 1996
	FbL 2A promoter + pha	Particle bombardment	Zygotic embryo meristem	Reinhardt *et al.*, 1996

Note: NPT II – Neomycin phosphotransferase II; CAT – Chloramphenicol acetyltransferase;
OCS – Octopine synthase; GUS – β-glucuronidase;
HPT – Hygromycin phosphotransferase; EPSPS – 5-enolpyruvylshikimate-3-phophate synthase;
AHAS – Acetohydroxyacid synthase; Pha – Polyhydroxyalkanoate synthase;

was reported by Bayley *et al.* (1992). Transgenic primary plants and progeny were tested by spraying with 2,4-D and recording damage at 3 weeks. Molecular analysis was done using PCR analysis. Progeny were also assayed for 2,4-D monooxygenase activity and a 3:1 segregation pattern of inheritance was confirmed. Although cotton has been transformed via *Agrobacterium* and plants have been subsequently regenerated, commercially important cultivars have proven very difficult to regenerate due to the inability to generate embryogenic cells. To circumvent the problem of genotype-dependent regeneration of cotton, shoot apices were used as explants in the reports by Zapata *et al.* (1999). The seedling shoot apex was transformed using *Agrobacterium tumefaciens* LBA4404 to transfer the nptII and GUS genes driven by a CaMV 35S promoter. Transformation was confirmed by the Kanamycin resistant phenotype in progeny and by Southern hybridization analysis of the progeny. Unfortunately, the transformation efficiency was low (only 0.8%) and further research is needed to improve the transformation rate.

1.3 Particle Bombardment Method of Cotton Transformation

Biolistic transformation was initially welcomed as an alternative method for generating transgenic plant species but is not yet amenable to *Agrobacterium*-mediated transformation methods. Particle bombardment utilizes high velocity metal particles to deliver biologically active DNA into plant cells. The technology was first reported by Klein *et al.* (1987). In their experiments, transient expression of exogenous RNA or DNA was demonstrated in the bombarded epidermal cells of onion (*Allium cepa*). The concept of particle bombardment (also known as biolistics, microprojectile bombardment, gene gun, etc.) has been described in detail by Sanford (1990). Following these experiments, the technique was shown to be a versatile and effective way for the creation of transgenic

organisms including microorganisms, mammalian cells and a large number of plant species.

The first transgenic cotton plants created using the particle gun method was reported by Finer and McMullen (1990). Embryogenic suspension cultures of *G. hirsutum* L. cv. Coker 310 were transformed using particle bombardment. Southern hybridization confirmed the presence of the transgene in embryonic tissue and in regenerated plants. Three years later, McCabe and Martinell (1993) reported a successful transformation of cotton by using excised embryo axes as explants through bombardment methods. Since embryonic axes can regenerate into plants without a callus intermediate, this was considered a genotype-independent transformation method. Chlan *et al.* (1995), Keller *et al.* (1997) and Rajasekaran *et al.*, (1996, 2000) also reported the successful transfer of a foreign gene into cotton by bombardment methods.

There are two main types of explants used in particle bombardment methods. One is the embryo meristem (shoot apex) and the other is embryogenic cell suspension cultures. The advantage of using the embryo meristem as an explant is that it allows genotype-independent transformation and the relatively rapid recovery of transgenic progeny (Christou, 1996; John 1997). The disadvantage of using embryonic meristems is that the preparation of shoot tip-meristems is an extremely tedious, labor – intensive task, which involves the surgical removal of leaf primordia to expose the meristem, followed by the careful excision of meristem explants from imbibed seeds. Also, the stable transformation rate is very low (0.001 to 0.01 %). The advantages of using embyrogenic suspension cultures are: 1) it is easy to produce a large amount usable cells in a short time; 2) the regeneration rate is high; and 3) when combined with multiple bombardments, the transformation rate is high (4%). The disadvantage of using embyrogenic suspension

culture is that suspension cultures are genotype-dependent, only a few varieties can be regenerated into plants; and also the recovery of fertile transgenic plants with normal morphology is largely dependent on the use of embryogenic suspension cell cultures less than 3 months old.

In cotton, *Agrobacterium tumefaciens*-mediated transformation (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987; Bayley *et al.*, 1992) and particle bombardment methods (Finer and McMullen, 1990; McCabe and Martinell, 1993) have been successfully used to obtain transgenic plants. Nevertheless, genetic transformation of cotton remains far from being a routine process; improvement of transformation efficiency is necessary before the technique becomes common in cotton improvement. The particle bombardment method provides a means to introduce foreign genes into any elite cotton variety, however, the transformation efficiency is low (1 transgenic plant per 1,000 bombarded explants) (McCabe and Martinell, 1993), and germline transformants are even rarer. This method is also more expensive than *Agrobacterium*- mediated transformation and is not available in many laboratories. While the transformation efficiency and the technical requirement for *Agrobacterium*-mediated transformation is attractive, the method suffers from the need for plant regeneration via somatic embryogenesis, which has been successfully applied to only a few cotton cultivars (e.g., the Coker lines). Nearly 100 cotton cultivars are under cultivation in the United States and they are, in general, not as amenable to tissue culture techniques as the Coker lines (Trolinder and Chen, 1989; Firoozabady and Deboer, 1993; Koonce *et al.*, 1996). Therefore, an elite regenerable line of the upland cultivar Coker 312 currently serves as the industry standard for *Agrobacterium*-mediated transformation of cotton. The transfer of transgenes into commercial cultivars is accomplished via selection for an active transgene in a

conventional backcross program. This strategy requires 10-14 months to obtain mature transgenic plants of Coker 312 and an additional 3-4 years to backcross the value-added traits into more productive agronomic cultivars. Moreover, plants regenerated from an embryogenic callus phase are sometimes sterile and / or show signs of somaclonal variation, which affect both the phenotype and genotype of the plant (Stelly *et al.* 1989; Firoozabady and Deboer, 1993). Recently, several researchers have regenerated plants from shoot tip meristems (Zapata *et al.*, 1999). In this method, shoot tips regenerated directly without a callus phase. This method has the advantage of being genotype-independent; almost all cultivars can be regenerated from shoot tips. The use of shoot tips as explants in an *Agrobacterium*-mediated transformation system is a good way to overcome the obstacles in traditional *Agrobacterium*-mediated transformation. An optimized *Agrobacterium*- mediated cotton transformation system by using the shoot apex as explant is presented in chapter 3.

1.4. QTL Analysis of Cotton Traits

1.4.1 Linkage Maps

Construction of a genetic linkage map is based on the observed recombination between marker loci in an experimental cross. Segregating families, e.g. F_2 or BC_1 progenies, F_3 families, or recombinant inbred lines are commonly used. In cotton, most reported linkage maps were based on the use of F_2 plant populations. Genetic map distances are calculated based on recombination fractions between loci. The Haldane or Kosambi mapping functions are commonly used for converting the recombination fractions to map units or centiMorgans (cM). The Haldane mapping function takes into account the occurrence of multiple crossovers, while the Kosambi function accounts also

for interference (Ott, 1985). Computer programs performing full multipoint linkage analysis include Mapmaker/Exp (Lander *et al*., 1987) and Joinmap (Stam, 1993).

The first linkage map of tetraploid cotton was report by Reinish *et al.* (1994). A total of 705 RFLP markers was sorted into 41 linkage groups, covering 4675 cM of the cotton genome. Currently, 14 of 26 chromosomes have been associated with linkage groups by using a series of monosomic interspecific substitution stocks developed previously (Stelly, 1993). An updated linkage map was reported by Rong *et al.* (2004) by using the same mapping population. The linkage map was composed of 2584 loci in 26 linkage groups, covering 4444.5 cM of the cotton genome (1.72 cM interval). This was an 1879-locus increase compared with the previous report.

A new mapping population based on an interspecific cross of *G. hirsutum* (TM1) and *G. barbadense* (3-79) was developed by the USDA-ARS, Crop Germplasm Research Unit in Texas. Both TM1 and 3-79 are considered as genetic standards of their species. A linkage map based on this population was reported (Yu *et al*., 1998; Reddy *et al*., 1997). Several different types of markers (RFLPs, RAPDs, SSRs, AFLPs and morphological markers) were assembled into 50 linkage groups, which covered nearly 5000 cM of the cotton genome. Of cotton's 26 chromosomes, 18 were identified with the linkage groups by using aneuploid cotton stocks. Another interspecific mapping population (*G. hirsutum* × *G. barbadense*) using different parents was developed at CIRAD/ Montpellier (France). The updated linkage map based on this population consists of 888 loci, including 465 AFLPs, 229 SSRs, 192 RFLPs, and two morphological markers, ordered in 37 linkage groups, and covering 4400 cM of the cotton genome (Lacape *et al*., 2003).

The first linkage map based on an intraspecific cross (*G. hirsutum* × *G. hirsutum*) was reported by Shappley *et al.* (1998). 120 RFLP markers were assembled

into 31 linkage groups, covering 865 cM or about 18.6 % of the cotton genome. Another intraspecific linkage map was reported by Ulloa and Meredith (2000). Hence, 81 RFLP loci were assigned to 17 linkage groups with a total map distance of about 700 cM of the cotton genome. Akash (2003) reported an intraspecific map, which was constructed into 28 linkage groups using 143 AFLP markers. The 28 linkage groups covered a genetic distance of 1773.2 cM, about 39% of the cotton genome.

There are several difficulties in genetic mapping of intraspecific cross populations, The main difficulty is that all the mapping populations used were tentative (such as F_2) rather than from permanent populations (such as DH or RIL) and were not available for continuous and cooperative research. Another problem is the low number of molecular markers available for mapping due to insufficient genetic polymorphism within *G. hirsutum*. The linkage groups constructed to date from intraspecific cross populations only cover 19 % to 39 % of the cotton genome. A third complicating factor is the allotetraploid nature of cotton, despite its functional behavior as a diploid. Clearly, a more saturated linkage map is needed to do QTL analysis of specific traits. Further research on finding more polymorphic markers and developing a saturated map is underway.

1.4.2 QTL Analysis of Cotton Traits

In cotton, several QTL studies have been conducted using both intra- and inter-specific crosses. Among other agronomic traits, fiber quality and lint yield are the most frequently reported traits in cotton QTL analysis. Jiang *et al.* (2000) identified 14 QTL affecting fiber related traits: there QTL (explaining 31 % of phenotypic variance) were detected for fiber strength, one QTL (explaining 15 % of phenotypic variance) was detected for fiber length, and one QTL (explaining 13 % of phenotypic variance) was

detected for fiber thickness. For yield components, two QTL (explaining 59% of phenotypic variance) were detected for bolls per plant and two QTL (explaining 15 % of phenotypic variance) for mass of seed cotton. Those results were based on a F_2 population of an interspecific cross (*G. hirsutum* × *G. barbadense*). Based on an interspecific cross of TM1 and 3-79, Kohel *et al.* (2001) detected 13 QTL that were responsible for fiber quality. Those QTL explained the phenotypic variances ranging from 30 to 60%. The results indicated that the majority of QTL for fiber quality were recessive, making marker-assisted selection more desirable in cotton breeding programs. Shappley *et al.* (1998b), Ulloa and Cantrell (1998) and Zhang *et al.* (2003) reported QTL analyses based upon an intraspecific cross. Akash (2003) reported QTL analysis of cotton yield and fiber quality traits based on a $F_{2:3}$ population derived from a cross of Paymaster 54 and Pee Dee 2156. In this research, 5 QTL were detected for yield and 9 QTL were detected for fiber quality. These QTL collectively explained 4 % to 69% of the total phenotypic variation.

In chapter 4, the assignment of AFLP markers to chromosome is presented and the results used to associate linkage groups created in previous research to chromosomes (Akash, 2003). Chapter 5 presents the results of QTL analysis of cotton lint yield and a detailed dissection of yield component traits.

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CHAPTER 2 OPTIMIZATION OF SHOOT APEX BASED COTTON REGENERATION SYSTEM

2.1 Introduction

Cotton (*Gossypium hirsutum L.*) is an important crop in the USA. Genetic transformation plays an important role in modern cotton breeding and has had a significant impact on production. To take advantage of this promising technology, a reliable and genotype-independent regeneration system is essential. Although cotton plants can be regenerated from callus by somatic embryogenesis (Trolinder and Goodin, 1987), and the efficiency of regeneration via somatic embryogenesis has improved significantly in recent years (Trolinder *et al*., 1989; Rajasekaran *et al*., 1996 and Zhang *et al*., 2001), some difficulties still remain. Only a limited number of cultivars can be induced to produce somatic embryos and regenerative plants, and the most responsive lines are Coker varieties, which are no longer under cultivation, however. (Feng *et al*., 1998). Aside from the genotype limitation, many of the plants regenerated from callus as somatic embryos are abnormal (Cousins *et al*., 1991; Trolinder and Goodin, 1987 ; Rajasekaran *et al*., 1996). This troublesome and time-consuming procedure restricts the application of cotton biotechnology in cotton breeding and production. Another approach to regenerating cotton was first reported by Renfroe and Smith (1986). This system used the isolated shoot meristem from seedlings of *G. hirsutum* L. cv. Paymaster 145 to obtain regenerated plants. Gould *et al.* (1991) extended this approach by using two G. *barbadense* cultivars and 19 *G. hirsutum* cultivars in his research, which showed that regeneration from shoot tips was genotype-independent. Saeed *et al*., (1997), Morre *et al*., (1998) and Zapata *et al*., (1999) also reported the regeneration of cotton plants from shoot meristems. However, rooting efficiencies were low in these reports (from 38% to

58%). The objective of this research is to improve rooting efficiency in shoot apex based cotton regeneration system. Three factors that could affect the rooting efficiency of shoot apices were investigated in this research: 1) Effect of seed sterilization method, 2) Effect of shoot apex age, and 3) Effect of concentration of IAA shock. In the end, an improved regeneration protocol with rooting efficiency up to 85% was developed. The protocol uses cotton shoot apices as explants and combines basic rooting, IAA shock and grafting steps to increase rooting efficiency up to 85%.

2.2 Materials and Methods

2.2.1 Seed Disinfection Methods

Cotton variety Coker-312 was used in this study. Cotton seeds were disinfected via three methods:

Method 1: Cotton seeds were treated with 70% ethanol for 2 minutes prior to a 20 minute exposure to 10% Clorox ® (5.25% sodium hypochlorite (NaOcCl))solution with two drops of Tween 20 per 100 ml, and rinsed three times with sterile double-distilled water. The seeds were then placed on seed germination medium.

Method 2: Cotton seeds were treated with a 50% Clorox® (5.25% NaOcCl) solution with two drops of Tween 20 per 100 ml on a rotary shaker at 50 rpm for 20 minutes and rinsed at least three times with sterile double-distilled water. The seeds were then placed on seed germination medium.

Method 3: Cotton seeds were treated with 20% hydrogen peroxide for 2 hours and rinsed three times with double-distilled water. The seeds were then placed overnight on a rotor shaker at 100 rpm. After removing the seed coat, the seeds were then placed on seed germination medium.

After surface disinfection, 50 seeds from each treatment were placed on seed germination medium. This was replicated three times. The seed germination medium contained 4.3g Murashige and Skoog (MS) salts (Sigma, Product No. M2909) (Murashige and Skoog, 1962) per liter, plus 3% sucrose and 0.8% agar (Sigma, USA). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 ºC for 20 min. from four to six seeds were placed in each Petri dish (100 X 20 mm) (figure 2.3 A) The seeds were incubated in the dark at 25 ºC for 5 days. Up removal from incubation, the number of elongated shoots as counted. Contamination was determined by visual inspection for fungal and / or bacterial growth.

2.2.2 Shoot Apex Isolation

Shoot apices were isolated from 3 to 11- days old seedlings with the aid of a dissecting microscope. The seedling apex was exposed by pushing down on one cotyledon until it broke away, exposing the seedling shoot apex. The apex was removed just below the attachment of the largest unexpanded leaf. Additional tissue was removed to expose the base of the shoot apex (Figure 2.1 A - B). The unexpanded primordial leaves were left in place to supply hormones and other growth factors. The isolated shoot apex was then placed on shoot elongation and rooting medium.

2.2.3 Shoot Elongation and Rooting Development

The isolated shoot apices from four different cotton varieties: Coker 312, LA98405052, LA 95402069 and LA 96110067) were placed on MS medium+0.1mg/L Kinetin (Gould *et al.*, 1991) for two weeks to induce shoot elongation. The number of elongated shoots was recorded for each variety and then the shoots were transferred to MS medium for rooting. After three weeks, the number of rooted shoots was recorded.

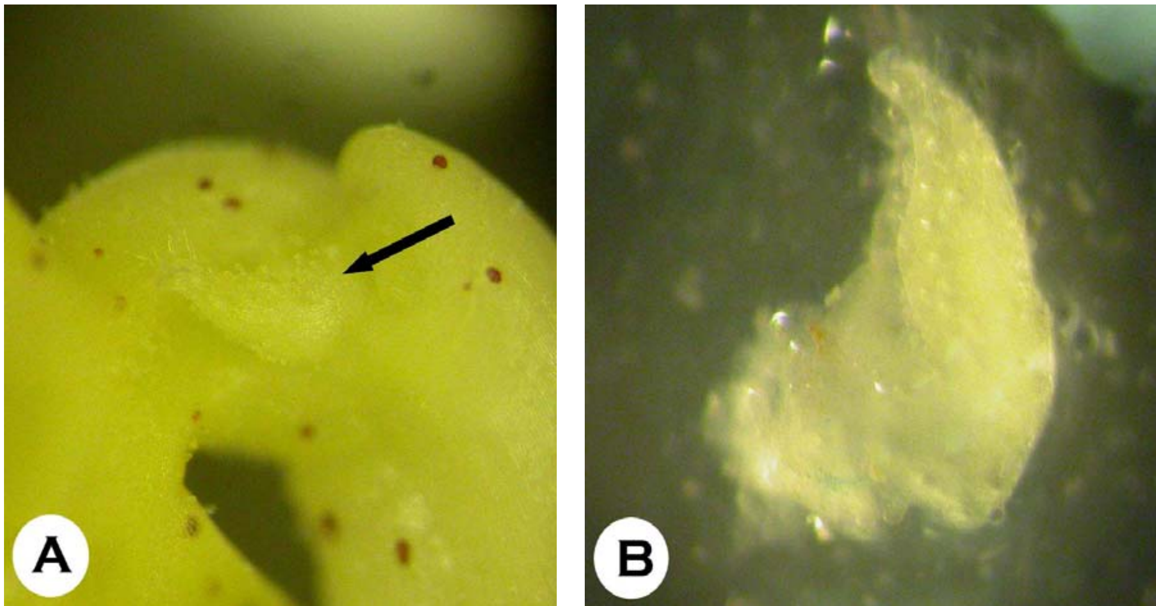

Figure 2.1 Isolation of shoot apex of cotton. A: Cotton shoot apex with one cotyledon broken away. B: Isolated cotton shoot apex

The rooted shoots were then transferred to Magenta boxes containing MS medium and incubated in a culture chamber (27 °C) for four weeks and then transferred to the greenhouse. The shoots without root development were subjected to an IAA shock at different concentration (from 0.1 to 2.0 mg/ml) for one minute. The treated shoots were then transferred to fresh MS medium for another three weeks. The number of rooted plants was recorded and the rooted plants were transferred to Magenta boxes containing MS medium and incubated in a culture chamber for four weeks before being transferred to the greenhouse. The remaining shoots without root development were then grafted to a germinated seeding of the same variety. By definition in this dissertation, The MS medium contained 4.3g/L MS salts (Sigma, Lot. 129H2365), and 1 ml/L MS vitamins (Sigma, Lot. 122K2314). The pH of all medium was adjusted to 5.8 before autoclaving, and all medium were solidified with 8.0g/L agar (Sigma). The medium were dispensed (25 ml) into 100 X 20 mm Petri dishes. Ten shoot apices were placed in a Petri dish. All

cultures were maintained at 27±2 ºC at a constant light intensity of 985 umol m^{-2} s^{-1} under a 16 hour photoperiod in the culture chamber. The light source consisted of cool white fluorescent lamps.

2.2.4 Plantlets Graft

Elongated shoots that did not develop roots on the MS medium after IAA shock were grafted onto the seedling stocks of the same variety. These seedlings stocks were the healthy normal plantlets with two to four true leaves grown from seed in plant pots. The scions were cultured shoots without root development. The first step was to cut the bottom of the scion into a wedge with a scalpel blade (figure 2.2B), then the upper part of the seedling stocks was cut under the first true leaf; and a slit (about 1.0 cm) on the stem was cut vertically (figure 2.2 A). The decapitated end of the root stocks and matching cut ends of the scions were treated with 0.1 mg/L IAA + 0.2mg/L GA. for 2 minutes. Then the treated scion was inserted into the slit and the cambiums were lined up. Final step was to bind the grafted parts together with Parafilm™ (Figure 2.2 C). The grafted plant was then covered by a 1000 ml flask and kept in a humid chamber for a week. Next step was to remove the flask and keep the plants in the humid chamber for another week before being transferred to the greenhouse. It was important to keep proper humidity in the chambers. The graft is successful if the scion does not wilt or rot after grafting for a week (Figure 2.2 D).

2.2.5 Experimental Design and Statistical Analysis

All experiments were conducted as a randomized complete block design (RCBD) with three or four replications. The data were analyzed via Proc Mixed in SAS 9.0 (SAS Institute, Cary, NC).

2.3 Results and Discussion

2.3.1 Seed Surface Disinfection

Cotton seeds from the field are highly contaminated as they contain large

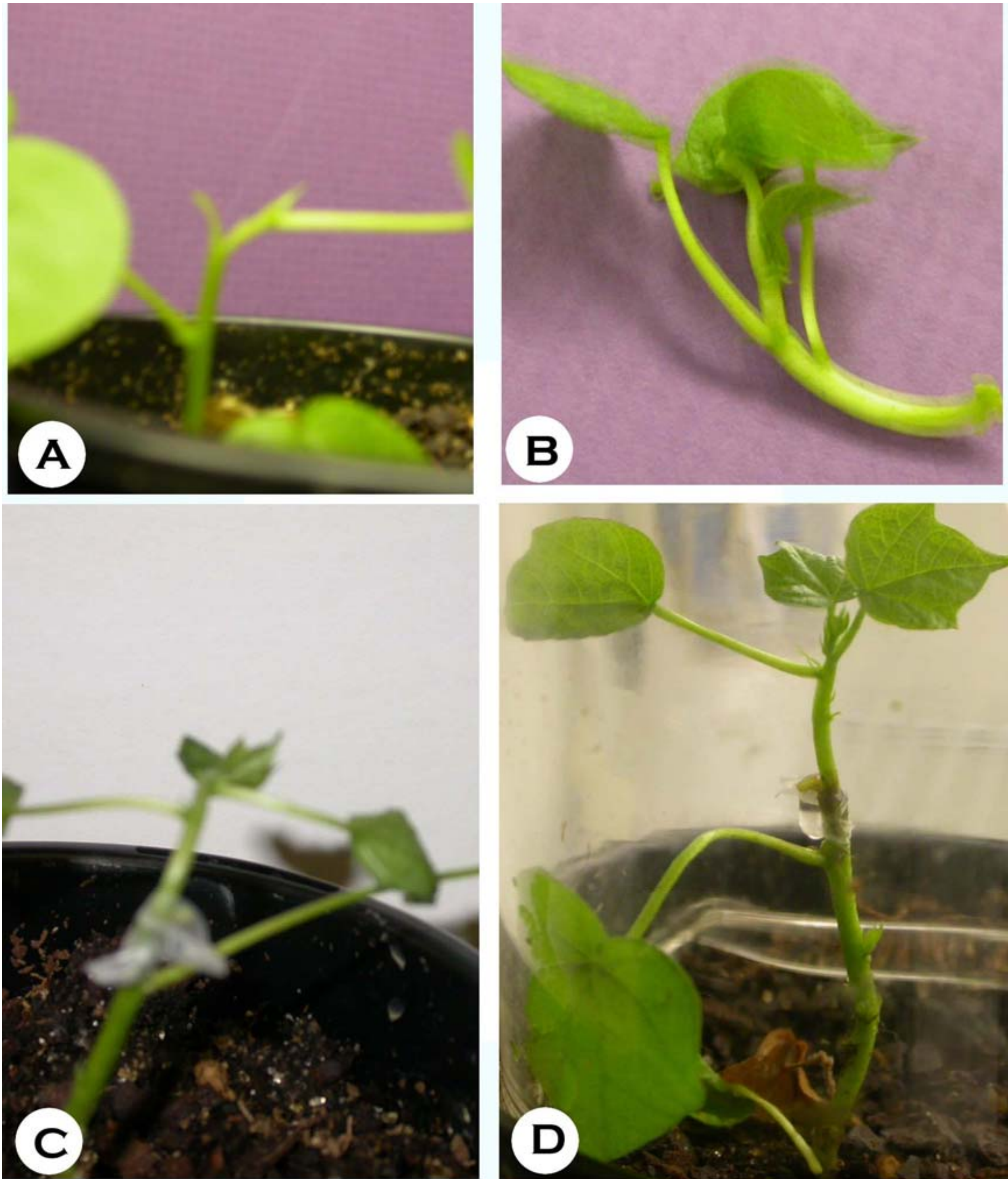

Figure 2.2 Grafting procedures of unrooted shoots. A: treated seedling stock with 2 true leafs (cut a 1 cm crack on the stem). B: treated scion with sharpened bottom (from unrooted shoots). C: grafted stock with scion banded by parafilm. D: grafted plant after one week in the culture chamber.

numbers of small hairs that can hold spores of fungi and bacteria. Delinting with H_2SO_4 is a highly effective way to remove the hairs and reduce the risk of contamination in the cultures. For any tissue culture study, the surface of explants must be fully sterilized. In previous research, different sterilization methods were used to sterilize delinted cotton seeds surface (Gould *et al*., 1991; Chen *et al*., 1987; Zhang, 1994). To obtain the best explants for isolating the shoot apex, three seed sterilization methods were compared in this research. Fifty seeds of the variety Coker 312 were sterilized by the three methods (Method 1: 70% ethanol for 2 minutes +10% Clorox ®(5.25% NaOcCl) for 20 minutes; Method 2: 50% Clorox® (5.25% NaOcCl)for 20 minutes and Method 3: 20% hydrogen peroxide for 2 hours) with three replications. The disinfected seeds were then cultured on MS medium for 5 days. The number of visually contaminated seeds and the number of germinated seeds (shoot elongation) were recorded after 5 days. The results show that method 3 gave the best surface disinfection (number of contaminated seed is zero) (Figure 2.3). Methods 1 and 2 did not give perfect sterilization. Use of only 50% Clorox® gives the least sterilization. Combining Clorox® and ethanol gave the better results, but this was still not as efficient as hydrogen peroxide. From the germination results, all seeds sterilized by hydrogen peroxide germinated in 5 days (Figure 2.3); seeds sterilized by both Clorox® methods had a lower germination rate (85% and 49%, respectively). The reason for those results may be that the residual of Clorox, specifically, chlorine, suppressed the germination of cotton seeds, while the residual of hydrogen peroxide is water and CO2, which did not affect the germination of cotton seeds.

2.3.2 Effect of Explants Age

Using sterilization method 3 (20% hydrogen peroxide for 2 hours), cotton seeds germinated in 5 days and hypocotyls enlarged up to 5-10 cm in one week with expanded cotyledons covering an area of 2 cm^2. Shoot apex growth started after 3 days of seed culture. The age of explants used for isolating shoot apices was examined in the next experiment. Thirty of 5, 7, 9 and 11 day-old seedlings of each of the four varieties were used to isolate shoot apices. The isolated apices were placed on MS medium+0.1mg/L Kinetin (Gould *et al.*, 1991) to induce shoot elongation for two weeks. The number of elongated shoots was recorded for each variety and the results are presented in table 2.1.

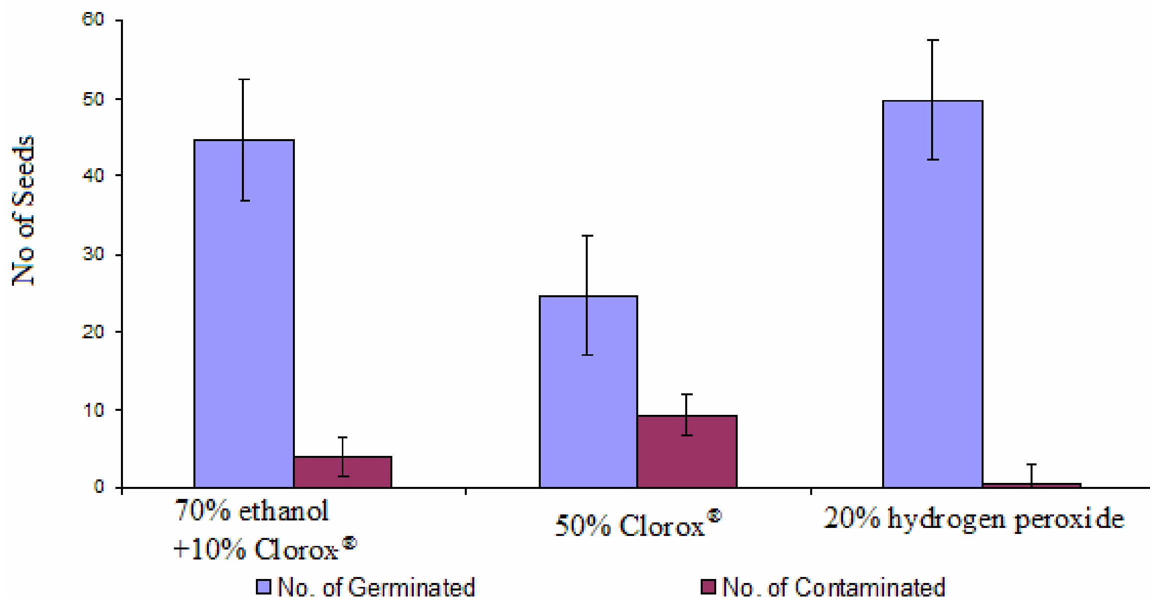

Figure 2.3 Mean number of germinated and contaminated cotton seed following three different surface disinfection methods. Vertical bar represent the standard error of three treatments.

The age of explants has a significant effect on shoot tip elongation (Table 2.2). On average, 42.5 % of shoot tips from 5 day-old explants had elongated; 85.5% of shoot tips from 7 day-old had elongated; 94.7% of shoot tips from 9 day-old explants had elongated and 99.2% of shoot tips from 11 day-old explants have elongated. The elongation rates

between 9 days of age and 11 days of age were not significantly different. The elongation rates of the four varieties were not significantly different from each other (p=0.1573) (Table 2.2), which indicates that the elongation of shoot tips on elongation medium was not genotype-dependent.

Table 2.1 Mean number of explants elongated on elongation medium from 4 cotton varieties at 4 different ages

Cotton Variety	Age of Explants				
	5 days	7 days	9 days	11 days	Mean
Coker 312	11.0±2.0[++]	25.33±2.08	28.67±0.57	30±0.0	23.75[a]
LA 98405052	13.33±3.06	26.7±0.57	28.0±1.0	29.33±0.57	24.33[a]
LA 95402069	12.0±2.0	24.33±1.52	28.33±1.15	29.66±0.57	23.58[a]
LA 96110067	14.67±3.21	26.67±2.08	28.67±0.57	30±0.0	25.00[a]
Mean	12.75[c+]	25.75[b]	28.41[a]	29.75[a]	

Note: + different letter label significant at p=0.05 level using LSD method.
++ Mean ± Std.

Table 2.2 ANOVA table for investigation of age effect of explants

Source	DF	Mean Square	F Value	Pr>F
Variety	3	4.944	1.85	0.1573
Age	3	728.333	273.12	<0.0001
Variety*Age	9	2.388	0.90	0.5400
Error	32	2.667		

The isolated shoot tips began to grow in one week. The elongation rate was also affected by the size of isolated tips. It was observed that if the starting size of the apex was less than 1mm, the tips would not grow at all. This may be because there was too

much leaf tissue removed and / or the tips themselves were damaged. Shoot tips sizes between 1.0 to 1.5 mm had a greater chance of surviving under experimental conditions as shown in Figure 2.4. It was also observed that some tips with small size grew into callus; this may be because the kinetin was used in the medium to promote cell division and aid in growth. No multi shoot formation was observed in this experiment. It may be because of apical dominance.

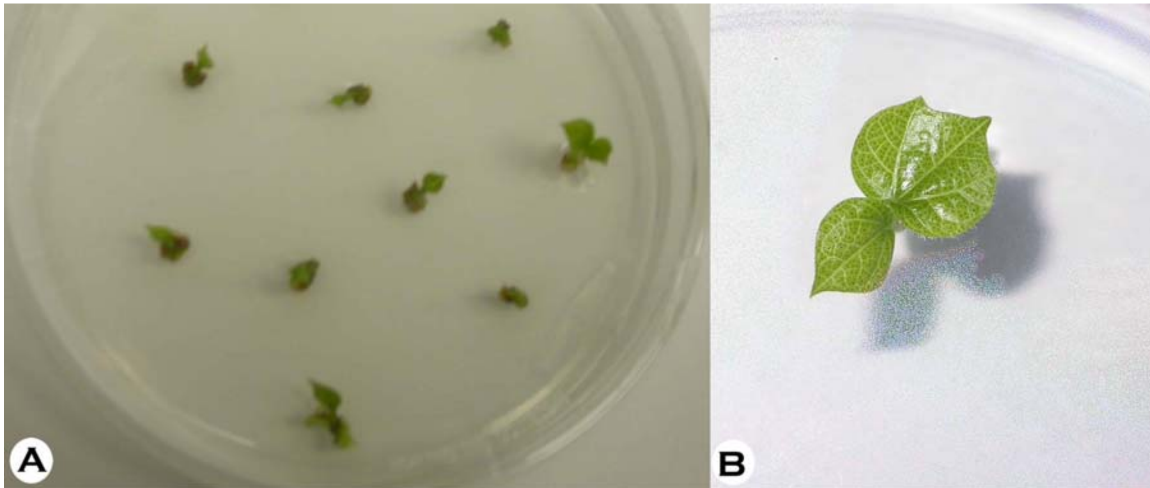

Figure 2.4 Isolated shoot apices growing on elongation medium after two weeks. A: shoot tip growing on petri dish. B: close up of elongated shoot tip.

2.3.3 Root Efficiency of Four Cotton Varieties on MS Medium

Thirty elongated shoot tips of each variety were transferred to MS medium without hormones to induce rooting for 3 weeks. The experiment was repeated three times. The number of rooted shoot tips was recorded. The results are shown in Figure 2.6. From the results we can see that the rooting efficiency of the four varieties were from 36% to 47%. Coker 312 had the highest rooting efficiency (47%), and LA 95402069 had the least rooting efficiency (36%). The difference of rooting efficiency was not significantly different in the four varieties (P=0.08). This result indicated that rooting efficiency is genotype independent.

The rooted plantlets were transferred to Magenta boxes with MS medium to hasten development of roots. After two weeks culture, the plantlets were transferred into pots containing autoclaved soil and cultured in the chamber under high humid for one week. Plantlets were watered every two day, and then the plantlets were transferred to the greenhouse (Figure 2.5). The plants appeared normal.

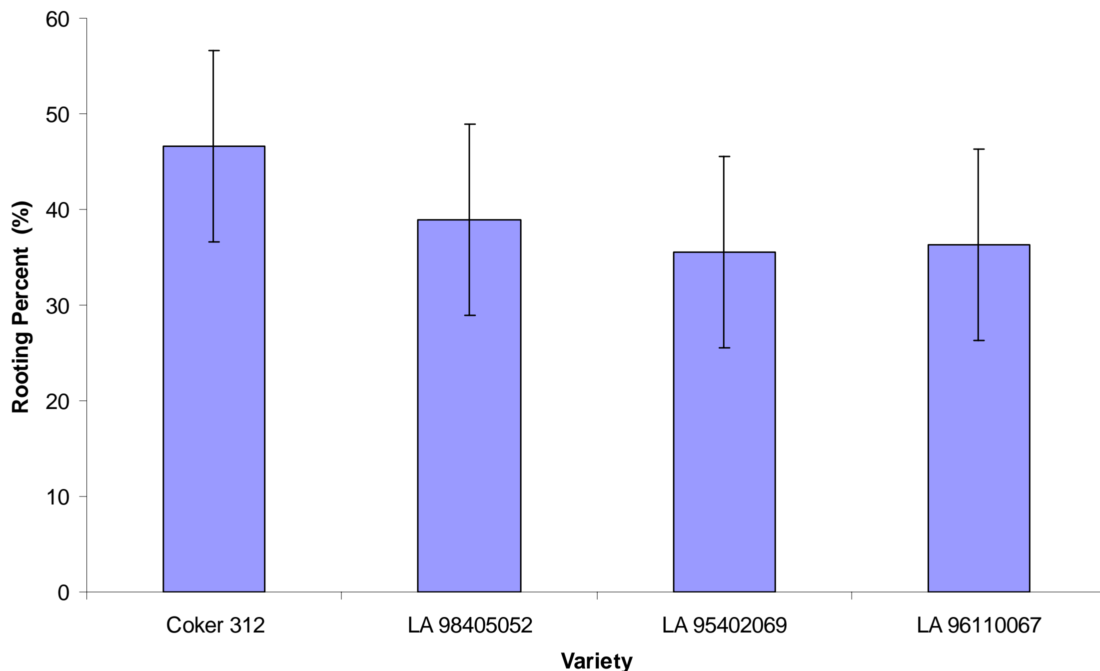

Figure 2.5 Percent of rooting efficiency of shoot apices from four cotton varieties after 3 weeks culture. Vertical bar represents the standard error of 4 varieties.

2.3.4 Effect of IAA Shock

Twenty unrooted shoot tips of Coker 312 from previous experiments were subjected to an IAA shock. The shoot tips were put in an IAA solution (concentration 0.1, 0.5, 1.0, 1.5, 2.0 mg/ml) for 1 minute and then transferred to fresh MS medium without hormones after rinsing three times with water. The number of rooted plants was recorded after three weeks culture. The rooting efficiency was significantly different in different concentrations of IAA ($p=0.027$) (Figure 2.7). The effect of different IAA shock

concentrations varied from 6.7% to 25%. The highest efficiency (25%) was observed for a 1.5 mg/ml IAA and the lowest efficiency (6.7%) was observed for 0.1mg/ml IAA. So the concentration of 1.5 mg/ml IAA was choose in the regeneration system.

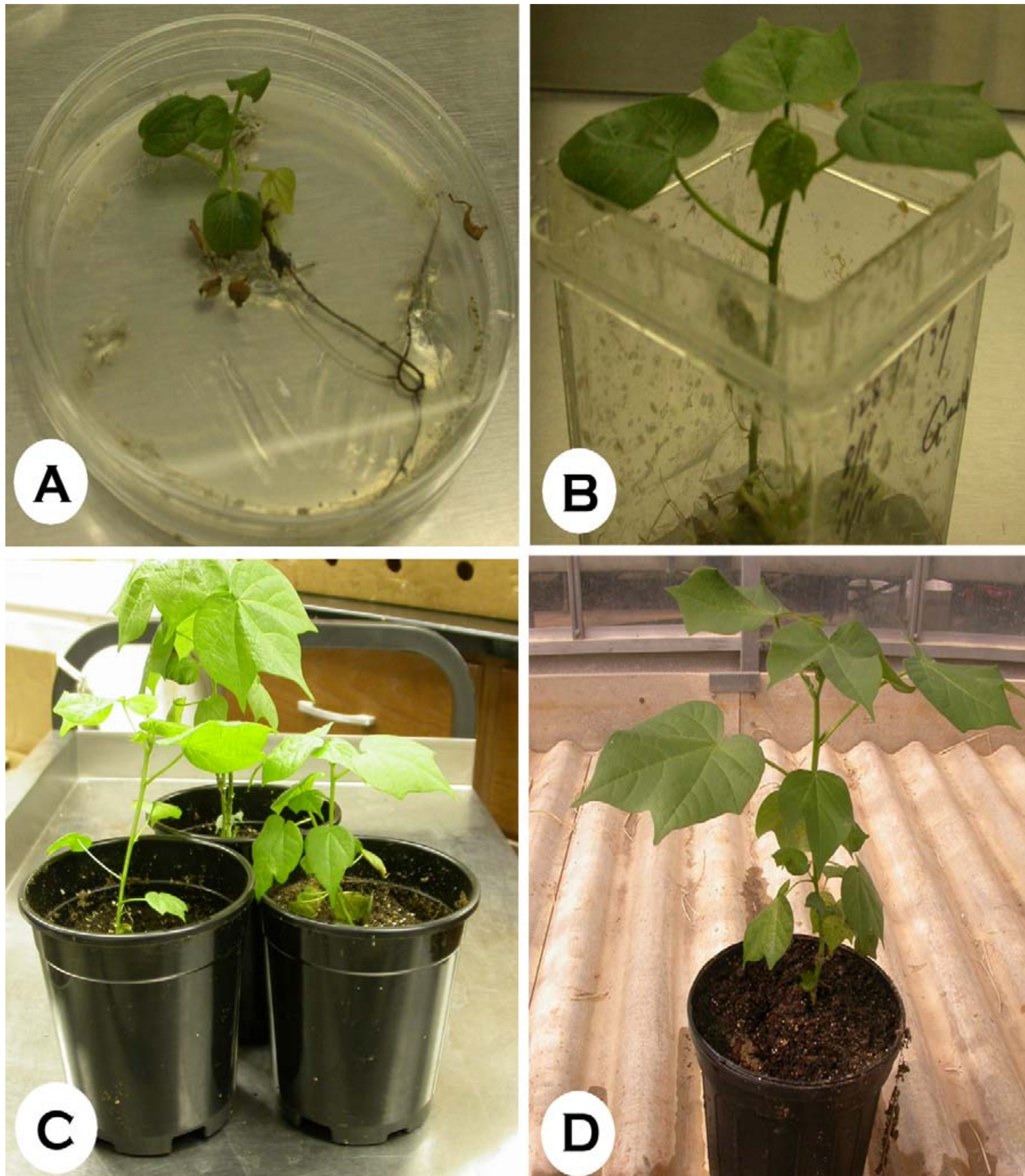

Figure 2.6 Regeneration of shoot apices. A: Rooted shoot tips on MS medium. B: Small plantlet in Magenta box. C: Regenerated plants. D: Regenerated plant in green house.

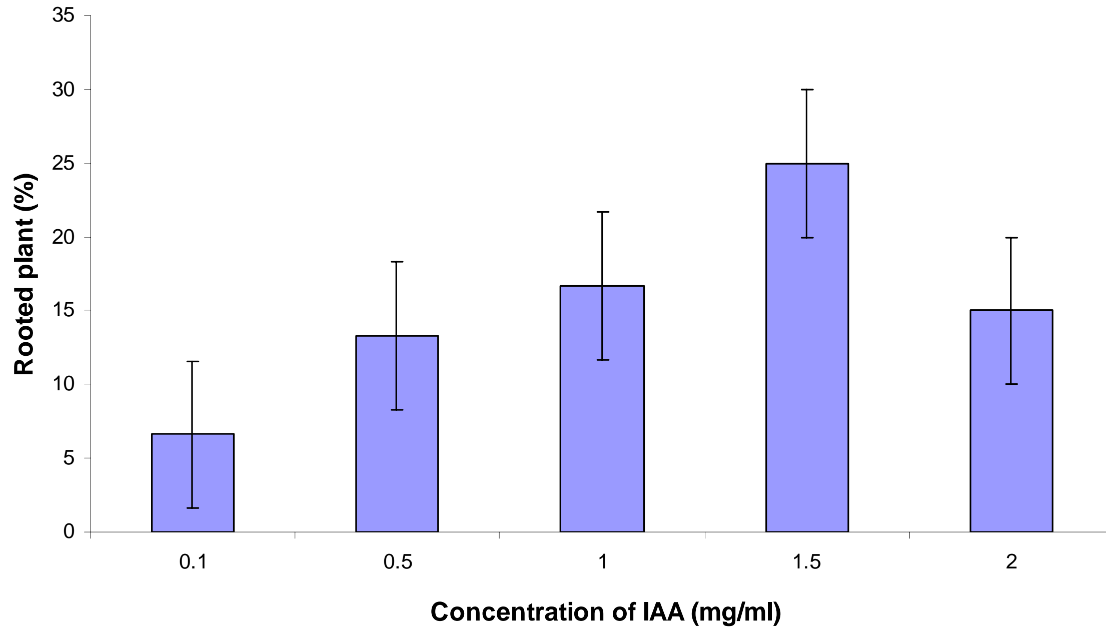

Figure 2.7 Effect of IAA shock on stimulating the rooting of previously unrooted Coker 312 shoot apices. Vertical bar represents the standard error of the 5 treatments of IAA

2.3.5 Plantlet Grafting

Grafting is a very useful technique and is commonly used in horticultural crops. The unrooted shoot tips (> 2cm) after IAA shock treatment were grafted to normal plants as previous described method. Eight out of 10 grafted plants survived. In the grafting procedure, it was important to keep the plant humid, also pretreatment of the scion and stock with 0.1mg/L IAA + 0.2 mg/L GA improved the survival rate.

2.3.6 Conclusions

To fully take advantage of gene transfer techniques, it is important to develop a reliable and efficient regeneration system for cotton. In recent years, there has been a focus in the development of regeneration systems through shoot apices. Regeneration from the shoot apex was direct and simple. Theoretically, each excised apex should develop into a rooted plant; however, the yield of shoots *in vitro* from isolated apices

depends on the incidence of contamination and rooting efficiency (Gould *et al*., 1991). In recent years, protocols involving proliferation of cotton shoots (Agrawal *et al*., 1997; Hemphill *et al.*, 1998) have been published. The rooting efficiency ranged from 38 % to 58 % in their reports. In this experiment, sterilizing seed surface with 20% hydrogen peroxide greatly lowered the chance of contamination. Remove of the seed coat may also explain the lower contamination rates of this method. By combining IAA shock and grafting technique, the rooting efficiency was increased up to 85%. The regeneration was carried out without a callus phase. Cotton plants rooted in an MS medium without hormones for a period of 3 to 6 weeks, and they could be transferred directly to soil without further steps. Two weeks later they could be transferred to the greenhouse and all plants were fertile and grown to set seed. Efforts have been made to couple this regeneration procedure with *Agrobacterium* mediated transformation for rapid introduction of value-added traits directly into high-fiber-yielding cotton germplasm. The results are presented in Chapter III.

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CHAPTER 3 OPTIMIZATION OF *AGROBACTERIUM* MEDIATED COTTON TRANSFORMATION SYSTEM USING SHOOT APICES AS EXPLANTS

3.1 Introduction

Cotton (*Gossypium spp.*) is the world's leading fiber crop and an important source of oil as well. Although significant progress has been made in cotton breeding programs, traditional breeding techniques have several limitations, such as access to a limited gene pool, crossing barriers, inefficient selection and being time consuming. Recent advances in transgenic technology now make it possible to deliver and express various genes in many agriculturally important species, including cotton (*Gossypium hirsutum*). The rapid development of cotton transformation technology not only provides a valuable method for introducing useful genes into cotton to improve important agronomic traits, but also helps in the study of gene function and regulation. Although transformation rates have been significantly improved since the first report of success in the transformation of cotton (Firoozabady *et al.* 1987; Umbeck *et al.* 1987)), increasing its efficiency is still needed.

Transformation efficiency is influenced by several factors, including *Agrobacterium* strain, addition of phenolic compounds (e.g., acetosyringone) in the co-cultivation medium, wounding treatment of the target tissue (Godwin *et al*., 1991, Norelli *et al*., 1996) and appropriate selection of transformed cells or tissue from majority of untransformed tissue. In the published protocols of *Agrobacterium*- mediated transformation of cotton, hypocotyls, cotyledons and embryogenic suspension culture cells have been used as explants (Firoozabady *et al*., 1987; Umbeck *et al*., 1987; Rajasekaran *et al*., 1996). The limitations of these explant types are their low

regeneration rate and their genotype-dependence limiting application to a select group of cultivated varieties. With the development of a shoot apex-based cotton regeneration system, it has been possible to improve transformation rates. To date, the meristem-based transformation method has been used successfully in *Agrobacterium*-mediated transformation of petunia (Ulian *et al*., 1988), pea (Hussey *et al*. 1989), sunflower (Bidney *et al*., 1992), corn (Gould *et al*., 1991), banana (May *et al*., 1995), tobacco (Zimmerman and Scorza 1996), and rice (Park *et al*., 1996). This chapter will present the optimization of shoot apex based *Agrobacterium*-mediated cotton transformation.

The use of herbicides to reduce loss in crop yield due to weeds has become an integral part of modern agriculture. There is continuous search for new herbicides that are highly effective and environmentally safe. A new class of herbicides that fulfils these needs acts by inhibiting specific amino acid biosynthesis pathways in plants. However, most of these herbicides do not distinguish between weeds and crops. Modifying plants to make them resistant to such broad-spectrum herbicides would allow their selective use for crop protection. Several herbicide resistance genes have been cloned and transferred into crops, such as the *bar* gene (Thompson *et al.*, 1987), the PAT (phosphinothricin-N-acetyl-transferase) gene (Wohlleben *et al*.,1988) and the ALS (Acetolactate synthase) gene (Sathasivan *et al*., 1990), This chapter describes the development of an *Agrobacterium*-mediated cotton transformation protocol using shoot apex as explants. Factors that affect transformation rate, such as the *Agrobacterium* strain and concentration, co-culture time and selective antibiotics, were tested with the aid of a vector expressing the GUS gene. By using a well-developed transformation system, a herbicide resistant gene (*bar* gene) was transferred into cotton.

3.2 Materials and Methods

3.2.1 Preparation of Shoot Apex Explant

Cotton variety Coker 312 was used in the transformation experiments. Seeds of Coker 312 were treated with 20% hydrogen peroxide for 2 hours and rinsed three times with double-distilled water. The seeds were then placed on a rotor shaker at 100 rpm overnight. After removing the seed coat, seeds were germinated in MS basal medium (Murashige and Skoog 1962) for 9 days in petri dishes at 28 ºC in a dark incubator. The shoot apices were dissected from seedlings as described in Materials and Methods in Chapter II. Shoot apices were cultured on MS medium with 0.1mg/L Kinetin (Gould *et al.*, 1991a) for 3 days.

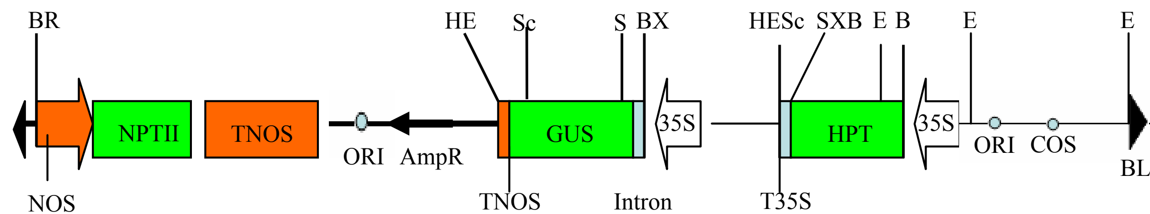

Figure 3.1 T-DNA region of pTOK233. Abbreviations: BR, right border; BL, left border; NPTII, neomycin phosphotransferase; GUS, β-glucuronidase; NOS, nopaline synthase promoter; HPT, hygromycin phosphotransferase, TNOS, 3' signal of nopaline synthase; T35S, 3' signal of 35S RNA; ORI, origin of replication; AmpR, ampicillin-resistance gene active in *E. coli*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S. *Sal*I; Sc, *Sac*I; X, *Xba*I. This vector was kindly provided by Dr. James Oard.

3.2.2 *Agrobacterium* Strain and Plasmid

Agrobacterium strain LBA4404 harboring a 'super-binary' vector pTOK233 (Hiei *et al.*, 1994) was used to develop the optimized transformation protocol. This strain has been successfully used in transformation of rice (Hiei *et al.*, 1994, Jiang *et al.*, 1999). The T-DNA of pTOK233 (Figure 3.1) contains a hygromycin-resistance gene (*HPT*), a kanamycin-resistance gene (*NPTII*), and a *GUS* gene which has an intron in the N-

terminal region of the coding sequence and which is fused to the CaMV35S promoter (Odell *et al*., 1985). The intron –gus gene expresses GUS activity in plant cells, but not in cells of *A. tumefaciens* (Ohta *et al*., 1990).

Agrobacterium strain EHA 105 harboring both NPT II and *bar* genes was used to transfer a herbicide resistance trait into cotton (Figure 3.2). The *bar* gene was originally cloned from the bacterium *Streptomycin hygroscopius*. It encodes for phosphinothricin acetyltransferase (*PAT*) (Thompson *et al.* 1987) that detoxifies phosphinothricin or glufosinate, the active ingredient of the herbicides Liberty and Basta (DeBlock *et al.* 1987). Therefore, plants expressing the *bar* gene are tolerant to herbicides Liberty and Basta.

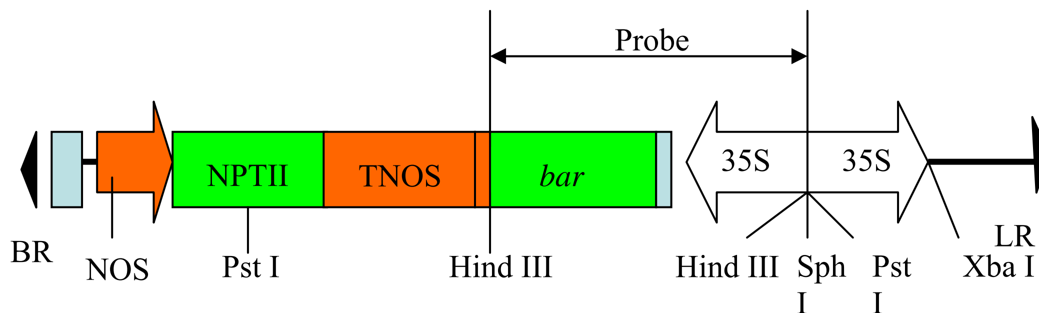

Figure 3.2 Construct of the *bar* and *NPTII* genes on binary vector pBIMC-B. Probe indicated was used in southern hybridization. 35S: 35 S promoter; NOS: NOS promoter. This binary vector was kindly provided by Dr. Yao Shaomian.

3.2.3 Pretreatment of Shoot Apex

The shoot apical meristem (SAM) is a population of cells located at the tip of the shoot axis. The shoot apex is divided into three layers (Figure 3.3). Layer 1 (L1) is a single layer of cells that generally only undergoes anticlinal divisions, and gives rise to the epidermis. Layer 2 (L2) is also a single layer, and gives rise to ground tissue, while the innermost layer (L3) forms the body of new tissues, including vasculature and germline tissue. Only transformation events that occur in the L3 layer will result in germline transformation. Transformation that occurs in the L1 and L2 layers will result in

chimeric phenotypes. To obtain germline transformation, the shoot apices were wounded in the middle tip by using a scalpel to expose layer III cell before co-culturing with *Agrobacterium* .

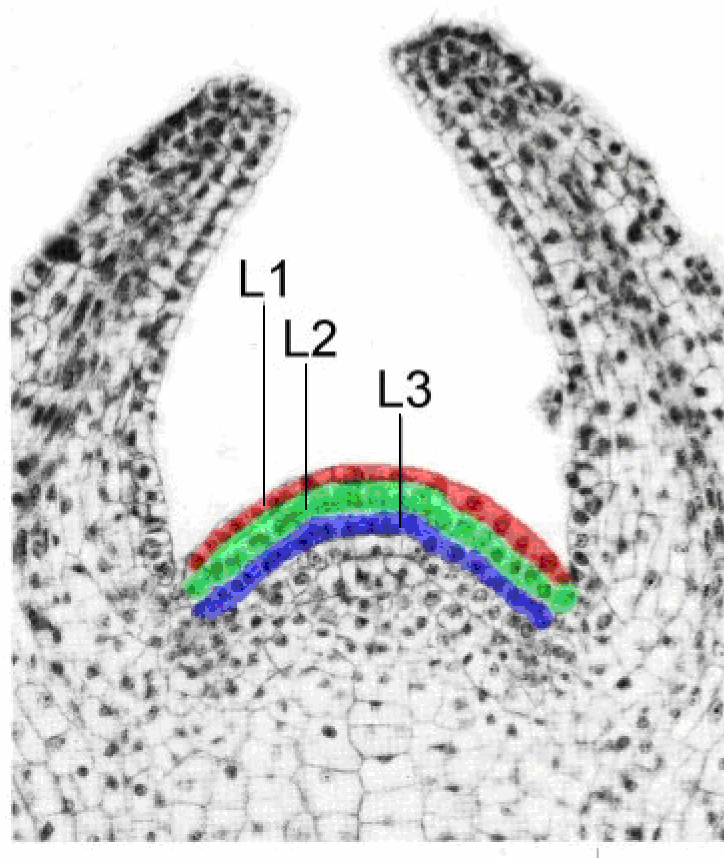

Figure 3.3: Schematic representation of shoot apex meristem (from http://www.dev-biologie.de/arabidopsis/meristem/meristem.htm)

3.2.4 *Agrobacterium* Co-cultivation and Transgenic Plants Regeneration

The *Agrobacterium* strains were cultured in LB medium (contains 10g/L Bacto Tryptone, Bacto, 5g/L Yeast extract and 10g/L NaCl). Twenty ml of LB medium plus antibiotics (50mg/L kanamycin and 50 mg/L hygromycin for strain LBA 4404 or kanamycin 50mg/L for strain EHA 105) was inoculated with *Agrobacterium* and incubated in a 100ml Erlenmeyer flask overnight (about 17 hours) on a shaker set for 180 to 220 rpm at 28°C. Then 2ml of the overnight culture was withdrawn and used to

inoculate 50ml of LB medium without antibiotics. Acetosyringone was added to the culture at a final concentration of 100 μM. After incubation for 3 to 4 hours at 28°C with shaking, those cultures were diluted with additional LB medium (containing 100 μM acetosyringone) to a concentration (OD_{600} 0.6) for transformation. Equal numbers of shoot apices were randomly distributed to two independent treatments, one with *Agrobacterium* co-cultivation and one without *Agrobacterium* co-cultivation. Shoot apices were inoculated by placing one drop of *Agrobacterium* solution onto each shoot apex in co-culture medium (MS + 100 μM acetosyringone) and incubating at 28 ºC under dark conditions for approximately 1 to 4 days. After co-cultivation, explants were washed three times with sterile distilled water. Cleaned apices were blotted dry using a sterile paper towel and cultured on the selection medium consisting of MS with 400 mg/L timentin and 50 ml/L kanamycin. Shoot apices not inoculated with *Agrobacterium* were plated on the selection medium as a negative control. Timentin was included in the selection medium to suppress the *Agrobacterium* growth. The Petri dishes were incubated at a temperature of 28 ºC under an 18 hours photoperiod and sub-cultured every 3 weeks. The process was repeated until controls, not co-cultivated with *Agrobacterium*, were totally dead. After this period the surviving shoot apices were transferred to an MS medium without kanamycin to root the plants. Rooted plants were then transferred to soil and grown to maturity in a greenhouse.

3.2.5 β-Glucuronidase (GUS) Histochemical Analysis

The histochemical assay for GUS gene expression was performed by established methods (Jefferson, 1987; Kosugi *et al*., 1990). Following co-cultivation, apices were harvested for GUS staining. The apices were incubated overnight in a solution containing 25 mg/l X-gluc, 10 mM EDTA, 100 mM NaH_2PO_4, 0.1% Triton X-100 and

50% methanol, pH 8.0) at 37 ºC. The number of apices that stained with blue spots was recorded. Young leaves of putative transgenic plants were also collected for GUS staining to confirm the transformation event.

3.2.6 Kanamycin and Glufosinate Leaf Test

In the putative transgenic plants, expression of the transgene (*NPT II*) or *bar* gene was analyzed by first establishing the lowest concentration of Kanamycin or glufosinate that would kill untransformed plants. Leaves of control plants were painted with a cotton swab when they had two totally opened true leaves using 0, 0.1, 1, 2, or 3% (W/V) of kanamycin or 0, 0.1, 0.2, 0.3, 0.4, and 0.5 ml/L Liberty. The lowest level (2%) of kanamycin that caused damage to the controls was used to evaluate for resistance to kanamycin in the greenhouse. The lowest level (0.3 ml/L) of Liberty was used to evaluate for resistance to glufosinate. Plants were evaluated for resistance 7 days after leaf application of kanamycin or Liberty.

3.2.7 Polymerase Chain Reaction Analysis

DNA was isolated from young leaves of putative transgenic plants using the DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA). The DNA samples were tested for the presence of the T-DNA region using a pair of *nptII* specific primers (upstream 5'-AGAACTCGTCAAGAAGGCGA-3' and downstream 5'-CTGAATGAACTGCAGGA CGA-3') to amplify the 700 bp *nptII* fragments. Regenerated plants transformed by EHA101 were screened for the presence of the *bar* gene by PCR using the *bar* gene specific primers (upstream 5'- CATCGTCAACCACTACATCGAG-3' and downstream 5'- CAGCTGCCAGAAACCCACGTCA-3').

The PCR reaction mixture was prepared as described by Altaf *et al.* (1997). The 25 uL amplification mixture contained 2.5 uL 10X PCR II buffer (50mM Tris (PH 8.3);

500 mM KCl);1.5 mM MgCl2; 1.0 mM dNTP mix (Pharmacia Biotech); 0.2 uM primer; 0.5 unit of AmpliTaq DNA polymerase (Promega); and 20 ng of genomic DNA as template.

DNA was amplified in a Perkin Elmer Geneamp PCR System 9600, programmed for a first denaturation step of 2 minutes at 94 ºC followed by 45 cycles of 94 ºC for 1 minute, 35 ºC for 1 minute, and 72 ºC for 2 minutes. After the completion of 45 cycles, a final extension at 72 ºC was carried out for 5 minutes. The completed reactions were then held at 4 ºC until electrophoresis was done.

PCR products were separated by loading 12 uL of each sample and 2 uL of loading buffer type II on a 1.2 % agarose gel prepared with 1.0X TBE buffer. The sample were subject to electrophoresis at 90-100V for 4 hours in 1.0X TBE buffer. The gel was stained with ethidium bromide and visualized under UV light.

3.2.8 Southern Blot Analysis

DNA was isolated from young leaves of putative transgenic plants using the DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) and completely digested with *HindIII*. Based on the construct of the plasmid, Hind III digested genomic DNA will result in a 3.1 Kb fragment in LBA 4404 transformed plants and a 1.8 Kb fragment in EHA 105 transformed plants. Twenty µg of genomic DNA was digested with *Hind* III overnight in a 37 ºC water bath. The digested DNA fragments were electrophoresed on an 0.8% agarose gel in 0.5x Tris-Borate-EDTA (TBE) buffer, and transferred to a nylon membrane by the alkaline transfer method (Reed and Mann, 1985). The [^{32}P]- labeled probes for LBA 4404 transformed plants were made from a 0.5-kb polymerase chain reaction (PCR) product (Primer: 5′-CTG TAG AAA CCC CAA CCC GTG-3′ and 5′-

CAT TAC GCT GCG ATG GAT CCC-3′) containing the *GUS* coding region. The probes for EHA 105 transformed plants were made from a 430 bp PCR product (Primer: 5'- CAT CGTCAACCACTACATCGAG-3' and 5'- CAGCTGCCAGAAACCCAC GTCA-3'). The band was excised from agarose gel and purified using a Pre A gene Kit (Bio-Rad, Hercules, CA). The probe was then labeled with ^{32}P-dCTP using a Random Primed Labeling Kit (Boehringer Mannheim Corporation, Indianapolis, IN) as described by the manufacturer. After hybridization and washing, the blots were exposed to Kodak Biomax MS film at -80 ºC.

3.3 Results and Discussion

3.3.1 Determination of Suitable Kanamycin Concentration in Selection Medium

The use of proper type and concentration of antibiotic in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or plants to survive. Kanamycin has been extensively used as a selective antibiotic in transformation experiments, mainly because several plant transformation vectors include neomycin phosphotransferase II (NPT II) gene as selectable marker. Only transformed cells can grow in the presence of kanamycin. In this experiment, shoot apices were transferred onto a medium containing kanamycin at 0, 30, 50, 75 and 100 mg/l after pre-culturing in MS medium+0.1mg/L kinetin for 3 days. Ten shoot apices were placed in each dish and replicated four times for each concentration. Over a period of three weeks, the number of elongated shoot apices was counted and recorded each week. The results are presented in Figure 3.4. The control (0 mg/L) grew very well in MS media. Shoot elongation was significantly decreased on MS media containing kanamycin. Ten percent of shoot apices survived in MS containing

30mg/L Kanamycin after three weeks. The minimum lethal concentration to kill all the apices in three weeks was 50mg/L. The higher level of kanamycin (100 mg/L and 75 mg/L) killed all the apices within two weeks. Therefore, a concentration of 50mg/L kanamycin was used to select transgenic apices in this research.

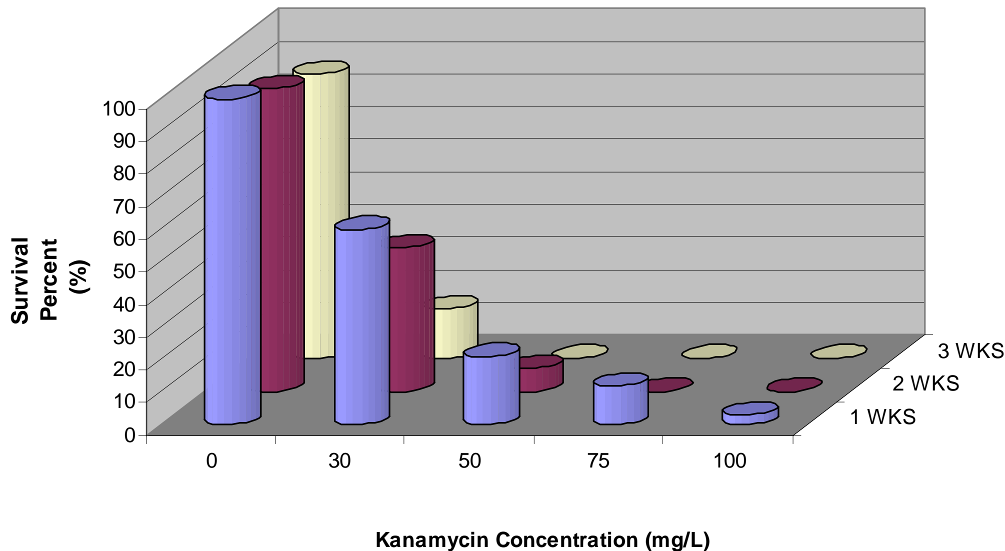

Figure 3.4 Survival rate of shoot apices at different concentrations of kanamycin in 3 weeks

3.3.2 Effect of Inclusion of Acetosyringone During Co-cultivation

Acetosyringone is one of the phenolic compounds secreted by wounded plant tissue and is known to be a potent inducer of *Agrobacterium vir* genes (Stachel *et al.* 1985). Several reports suggest that acetosyringone pre-induction of *Agrobacterium* and/ or inclusion of acetosyringone in the co-cultivation medium can enhance significantly *Agrobacterium* mediated transformation (Yao, 2002; Samuels, 2001; Sunikumar *et al.* 1999). In our experiments, acetosyringone was included at a final concentration of 100 µM during the final stage of *Agrobacterium* growth and during co-cultivation. For the

control treatment, transformation was performed by completely omitting acetosyringone from every step. Ten shoot apices were used in each treatment and the experiment replicated four times. The number of GUS positive apices was recorded after 3 days co-cultivation. The results in table 3.1 show that acetosyringone improved significantly the transformation efficiencies. The mean number of GUS positive apices was 67% higher when acetosyringone was included in the medium. The results suggest that acetosyringone can be used to obtain significant improvements in transformation of cotton. All of the other experiments were performed with acetosytingone treatment during the final stage of *Agrobacterium* growth and during cocultivation.

Table 3.1 Number of GUS positive cotton apices after treatment with 100 uM acetosyringone

Acetosyringone concentration	Rep1	Rep2	Rep3	Rep4	Mean
0 uM	3	2	3	1	2.25^b
100 uM	4	4	4	3	3.75^a

Note: Significant at 0.05 level

3.3.3 Effect of Concentration of *Agrobacterium* and Duration of Co-cultivation

To optimize parameters for efficient transformation, we evaluated different *Agrobacterium* concentrations (absorbance at OD_{600} is 0.2, 0.4, 0.6, 0.8 and 1.0) and duration of co-cultivation (1,2, 3 and 4 days). Twenty shoot apices were placed in each treatment combination with 4 replications. The apices were stained after co-cultivation and the number of GUS positive apices was recorded. The results are presented in Figure 3.5 and show that both *Agrobacterium* concentration and co-cultivation time have a significant effect on transient GUS expression. The highest GUS positive number was observed at OD_{600} 0.6 and co-cultivation for 3 days. The transfer T-DNA from

Agrobacterium to plant cells is a complicated process and it takes time. Co-cultivation with *Agrobacterium* for 1 day was not long enough to maximize the transfer event. The data show that GUS expression rate was always lower in 1 day co-cultivation than 2 days co-cultivation at different *Agrobacterium* concentrations. Increasing the *Agrobacterium* concentration did not always increase the transformation rate. This may be because that having the *Agrobacterium* concentration too high will cause *Agrobacterium* overgrowth problems. The highest observed GUS positive rate was 38%, which occurred at OD_{600} 0.6 and 3 days co-cultivation. These conditions were used in the transformation system.

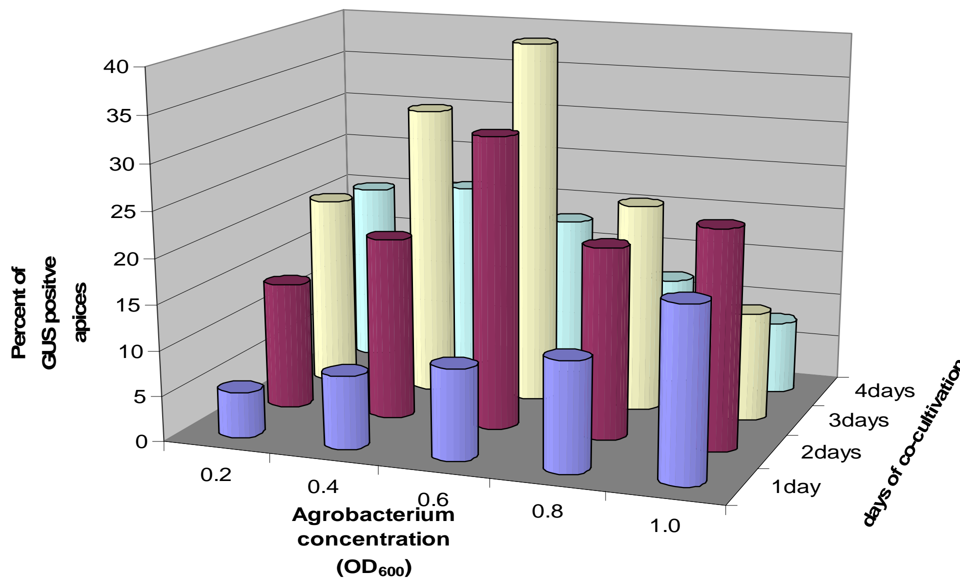

Figure 3.5 Effect of concentration of *Agrobacterium* and duration of co-cultivation

3.3.4 Production of Putative Transgenic Plants

The shoot apices were co-cultivated with *A. tumefaciens* LBA4404 for 3 days. After co-cultivation, the shoot apices were transferred to MS medium with 50 mg/l kanamycin and 200 mg/L timentin. Under kanamycin selection pressure, most of the

shoots appeared to be bleached (Figure 3.6 B), and some of the shoots that were initially green bleached out gradually, leaving only a few green shoots (Figure 3.6 A). Shoot apices were transferred to fresh media every three weeks. After six weeks of selection, surviving shoots were transferred to MS media without kanamycin to induce rooting. Rooting of the transformed shoot apices occurred when they were transferred from kanamycin selection medium to kanamycin free medium. Rooted plantlets were first transferred to Magenta boxes (Figure 3.6 C) for two weeks and then were transferred to soil and grown in a green house. The morphological features of the transgenic plants did not differ from those of non-transgenic plants. Out of a total of 300 *Agrobacterium*-treated shoot apices placed on kanamycin selection, two (0.67%) regenerated plants (T0), grew, and were transferred to soil, reaching maturity after approximately four months (Table 3.2). In contrast, for the 80 apices not treated with *Agrobacterium*, all died on kanamycin selection. Rooting of the transformed shoot apices occurred when they were transferred from kanamycin selection medium no kanamycin free medium (Figure 3.4 E).

Table 3.2 Survival of cotton shoot apices after co-cultivation with *Agrobacterium* LBA 4404 and selection with 50mg/L kanamycin

Item	LBA 4404	Shoot apices	Surviving selection	% Established in soil
Co -cultivation	+	300	2	0.67
Control	-	80	0	

3.3.5 Confirmation of Transformation Event

3.3.5.1 Leaf GUS Assay

Histochemical staining revealed that the leaves of these transgenic plants were strongly positive for GUS activity (Figure 3.7 B), suggesting that an integrated GUS gene was expressed at high levels under the control of the 35S promoter of cauliflower mosaic virus (P35S). Leaf samples from non-co-cultivated plants did not stain blue (Figure 3.7

A). Since the GUS construct in LBA4404 (pTOK233) used in the present study contained introns, the observed expression did not come from bacterial contamination.

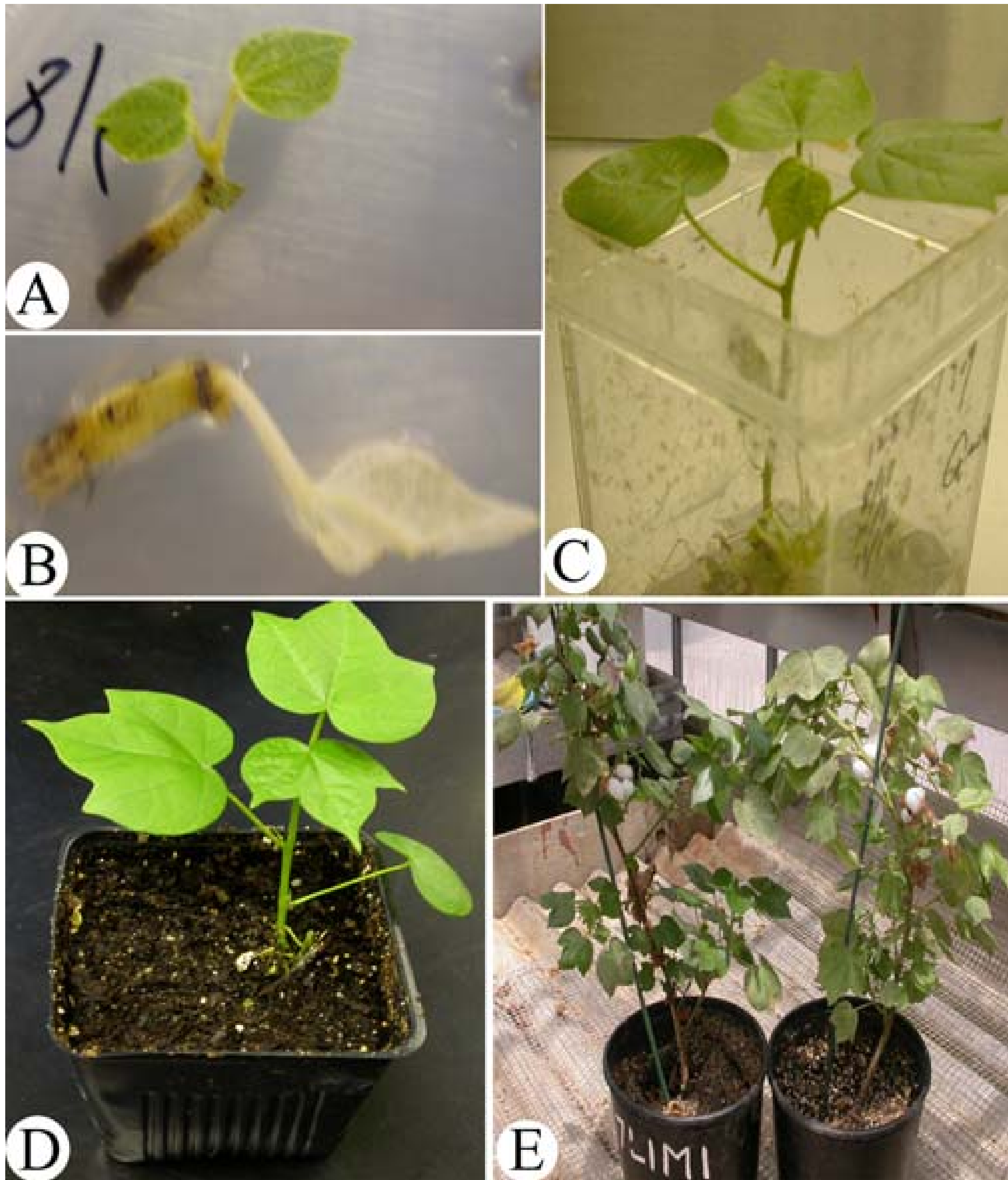

Figure 3.6 Production of putative transgenic plants. A: shoot apex after 3 weeks on selection medium (survival). B: shoot apex after 3 weeks on selection media (bleached). C: Rooted plantlet in Magenta box. D: Regenerated plant in soil. E: Mature regenerated plants in green house.

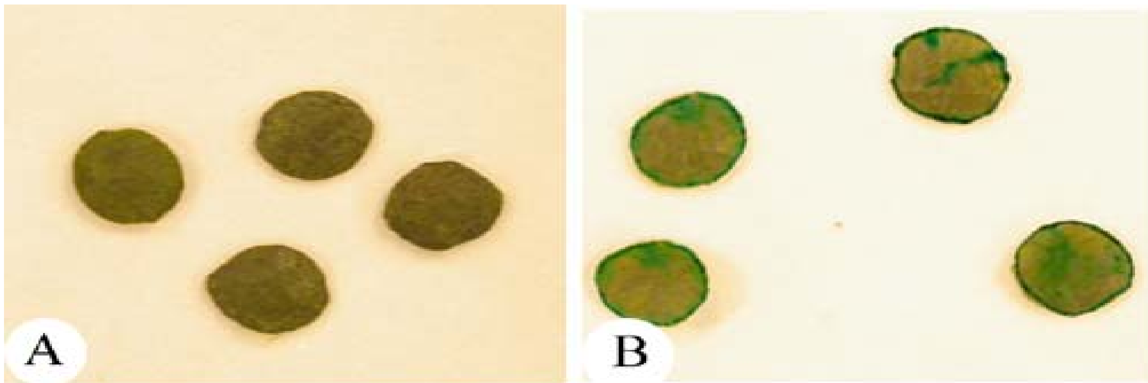

Figure 3.7 Histochemical staining of leaf discs. A: leaf discs from control plant (not treated with *Agrobacterium*. B: leaf discs from putative transgenic plant.

3.3.5.2 Kanamycin Leaf-spotting Test

The putative transgenic plants were tested using a kanamycin leaf-spotting test on the young leaves. Based on the primary experiment of kanamycin leaf test, the concentration of 2% was used in this experiment. Kanamycin solution (2%) plus 0.1 mg/L Tween 20 was painted to fully expanded young leaves. Kanamycin resistance activity in the leaves was variable after one week (Figure 3.8). Leaves of non transgenic plants (control) turned mottle in one week, while leaves from putative transgenic plants did not have the symptom. Plants that were resistant to kanamycin were further tested by PCR and Southern-blot analysis to confirm the transformation event.

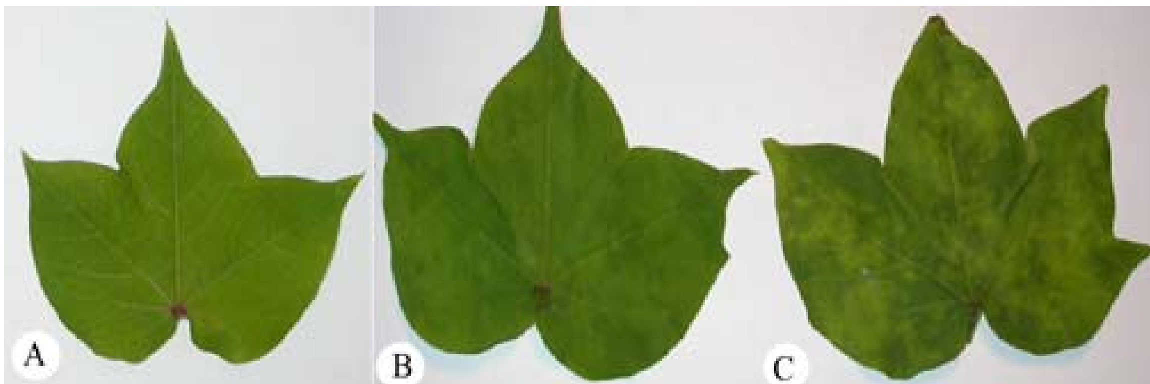

Figure 3.8 Kanamycin leaf spotting test. A: healthy leaf without Kanamycin application. B: leaf from putative transgenic plant, 7 days after Kanamycin application. C: Leaf from non-transgenic plants, 7 days after Kanamycin application

3.3.5.3 PCR and Southern Blot Analysis

DNA isolated from putative transgenic plants, a non-transgenic control plant, and plasmid pTOK233 (isolated from *Agrobacterium* strain LBA4404) was used as template DNA for PCR amplification of the *NPTII* gene (Figure 3.9). The presence of a band at 770 bp in samples from transformed plants (lanes 3, 4) confirmed the integration of the *NPTII* gene. Amplification of this fragment (770 bp) was not observed in non-transformed control plants (lane 2).

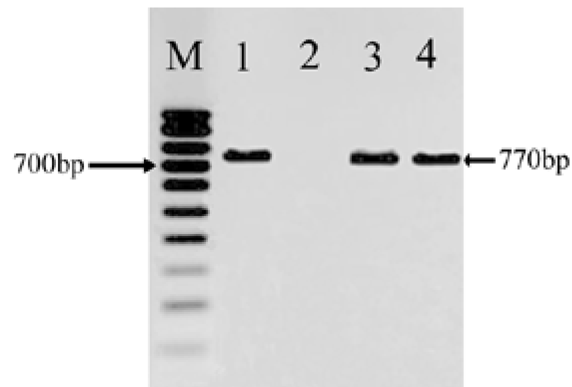

Figure 3.9: PCR analysis of transgenic plants for integration of the NPTII gene. Lanes: M 1Kb marker; Lane 1: Plasmid DNA (positive control); Lane 2: DNA sample from non-transgenic control plant; Lanes 3, 4: DNA samples from putative transgenic plants. Arrow shows the expected 770 bp product.

Southern blot analysis of leaf DNA from transgenic plants, non-transgenic plants and plasmid pTOK233 is presented in Figure 3.10. Hybridization of the GUS probe with a 3.1 Kb fragment was detected in the two transgenic plants. This was consistent with the restriction map of pTOK233, which has two *Hind*III sites, separated by 3.1kb, which flank the 35S-GUS-NOS gene. This result also confirmed the PCR results and indicated integration of the T-DNA region in the transgenic plant genome. No variation in number of copies of the GUS gene was observed between the two transgenic plants examined (Figure 3.8). No hybridization was detected in the non-transgenic control plants.

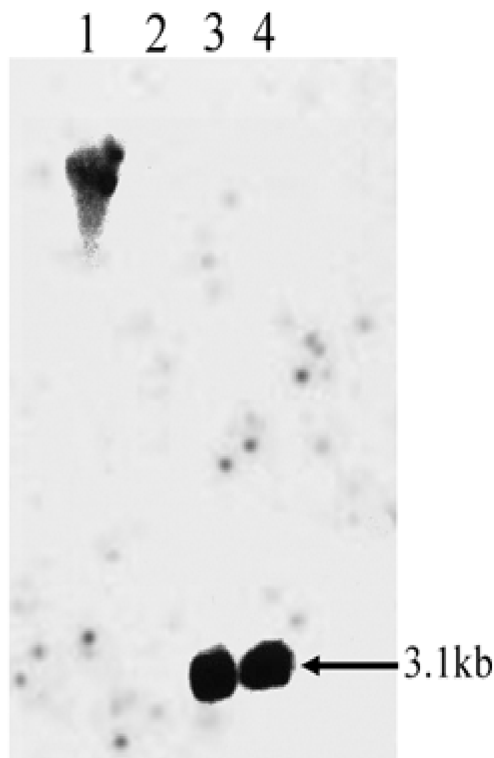

Figure 3.10: Southern blot analysis of transgenic plants for integration of the *GUS* gene. Lane 1: undigested plasmid DNA (positive control); Lane 2: DNA sample from non-transgenic control plant; Lanes 3, 4: DNA samples from putative transgenic plants. Arrow shows the expected 3.1 Kb product.

3.3.6 Production of Herbicide Resistant Cotton

By using the established protocol for cotton transformation, the herbicide resistance *bar* gene was successfully transferred into the cotton genome. A total of 590 shoot apices from variety Coker 312 was co-cultured with *Agrobacterium* strain EHA105 harboring *NPTII* and *bar* genes for 3 days. Under 50 mg/L kanamycin selection pressure, six shoot apices survived and regenerated into plants. The plants were transferred to the greenhouse and allowed to grow to maturity. These plants were considered as putative transgenic plants and were screened for herbicide (Liberty) tolerance and confirmed by PCR and southern blot analysis. The transformation rate in this experiment was about 1%, which is higher than in the previous experiment (0.67%). This may be due to the use of a

different *Agrobacterium* strain in the experiment. It was observed that *Agrobacterium* strain EHA105 grew faster than LBA 4404 in culture. Yao (2002) also reported that EHA105 indeed had a higher transformation rate than LBA 4404 in soybean transformation.

3.3.7 Confirmation of Transformation

The fully expanded young leaves of putative transgenic plants were painted with 0.3 ml/L Liberty plus 0.1 mg/L Tween 20 using a cotton swab. Figure 3.11 demonstrates that leaves from putative plants show resistance to herbicide, while leaves from non - transgenic plants were susceptible to the herbicide Liberty.

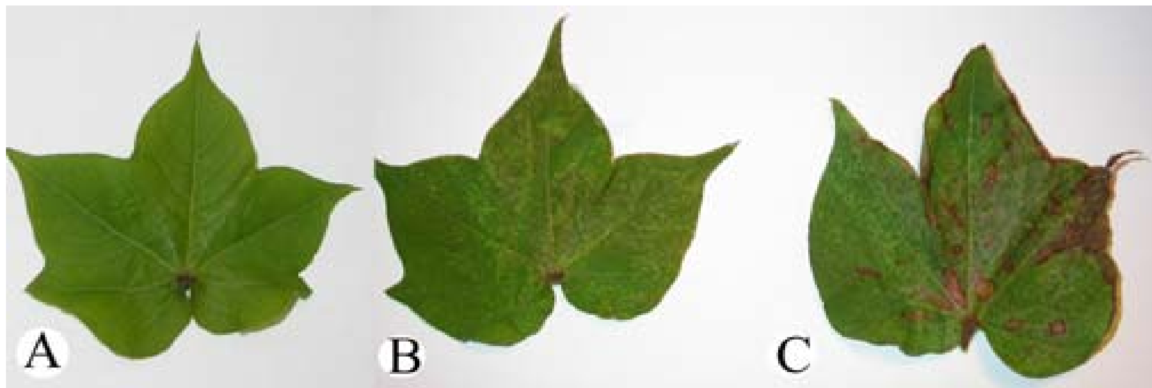

Figure 3.11 Herbicide (Liberty) leaf spotting test. A: healthy leaf without herbicide application. B: leaf from putative transgenic plant, 7 days after herbicide application. C: Leaf from non-transgenic plants, 7 days after herbicide application

PCR and Southern analysis of six putative transgenic plants was carried out to confirm the integration of the *bar* gene into the cotton genome. The results are presented in Figure 3.12 and 3.13. By using a primer specific for *bar* gene, PCR results show the expected 430 kb product in all six putative plants. Those were also confirmed by southern blot analysis (Figure 3.13), all six putative transgenic plants showed the expected band at 1.8 kb. Those results confirm that the *bar* gene was integrated into the cotton genome in these six putative plants.

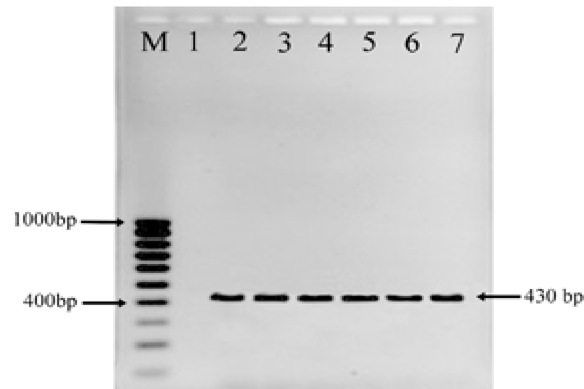

Figure 3.12: PCR analysis of transgenic plants for integration of the *bar* gene. Lane: M 1Kb marker; Lane 1: DNA sample from non-transgenic control plant; Lanes 2-7: DNA samples from putative transgenic plants. Arrow shows the expected 430 bp product

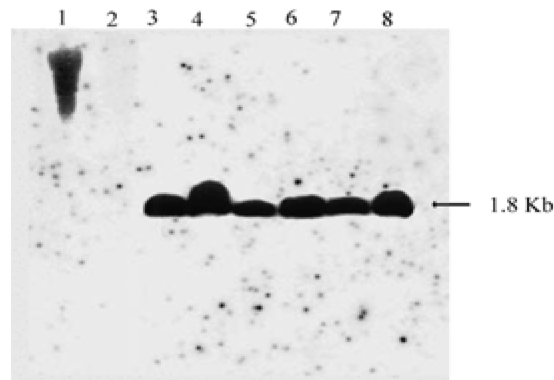

Figure 3.13: Southern blot analysis of transgenic plants for integration of the *bar* gene. Lane 1: undigested plasmid DNA (positive control); Lane 2: DNA sample from non-transgenic control plant; Lanes 3-8: DNA samples from putative transgenic plants. Arrow shows the expected 3.1 Kb product.

3.3.8 Discussions

The development of an efficient transformation system is an important tool for gene manipulation. In this chapter, we optimized a shoot apex based *Agrobacterium* mediated transformation system. The transgenic plants were confirmed via PCR and Southern blot analysis. Pretreated shoot apices were co-cultivated with *Agrobacterium* at concentration of OD_{600} 0.6 for 3 days with addition of 100 µM acetosyringone. Under 50 mg/l kanamycin selection pressure, a total of eight transgenic plants was recovered, in which two plants were transformed by *Agrobacterium* LBA4404 and six were

transformed by *Agrobacterium* EHA 105. The overall transformation rate was 0.9%, which is higher than that of Smith *et al.* (1997) and Zapata *et al.* (1999) (0.8%). It is possible that the slightly higher transformation rate achieved in this study was also due to the slicing of the shoot apex prior to the co-cultivation step. To out knowledge, this is a novel method to facilitate *Agrobacterium* access to germline cells. The plants obtained by the present procedure were phenotypically normal, and in contrast to an embryogenesis-based transformation system, which takes one year or more to obtain fertile plants, we obtained transgenic plants in 5-6 months.

Agrobacterium strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer. The suitability of different strains harboring various plasmids for the transformation of cotton was observed in this experiment. *Agrobacterium* tumefaciens strain EHA 105 was found to be more infective than strain LBA4404 with respect to transformation. Selection of other *Agrobacterium* strains may results in higher transformation rates.

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CHAPTER 4 CHROMOSOMAL ASSIGNMENT OF AFLP MARKERS IN COTTON

4.1 Introduction

Cotton (*Gossypium spp.*) is the world's leading fiber crop and an important source of oil as well. The genus *Gossypium* L. comprises 50 diploid and tetraploid species. Among the four cultivated *Gossypium* species in the world, the American allotetraploid species (*Gossypium hirsutum L.* and *Gossypium barbadense L.*) dominate worldwide cotton production, having almost displaced the old-world diploid cultivars (*Gossypium arboreum L.*and *Gossypium herbaceum L.*) (Lee, 1984). Wild diploid species of the genus *Gossypium* fall into eight different genome types designated A–G and K (Percival *et al*., 1999). All tetraploid species are allopolyploids and probably derive from a single A × D polyploidization event (Endrizzi *et al*., 1985). Variation in ploidy among *Gossypium spp.*, together with a tolerance for aneuploidy in tetraploid cotton species, has facilitated the use of cytogenetic techniques to explore cotton genetics and evolution research. The 26 chromosomes of the tetraploid cotton genome have arbitrarily been numbered 1–13 and 14–26 for the A- and D-related subgenomic groups based on pairing relationships in diploid × tetraploid crosses (Kimber, 1961), respectively. Among 198 mutants identified in cotton, 61 mutant loci have been assembled into 16 linkage groups, 11 of which have been associated with chromosomes using monosomic and monotelodisomic stocks (Endrizzi, *et al*., 1985). Also, aneuploid substitution stocks have been used to assign individual RFLP (Reinisch *et al*., 1994) and SSR (Liu *et al*., 2000) markers to chromosomes or chromosome arms, allowing the assignment of linkage groups to chromosomes.

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting technique capable of detecting several loci in a single PCR reaction (Zabeau, 1993; Vos *et al*., 1995). The AFLP method combines the reliability of RFLPs and the power and sensitivity of PCR-based methods. It can be used to quickly develop linkage maps in plant species and is especially useful for crops with large genomes like cotton (*Gossypium spp.*, 4700cM). The AFLPs have been used for QTL mapping studies in many crops, including rice (Maheswaran *et al*., 1997), barley (Becker *et al*., 1995; Powell *et al*., 1997), and oat (Jin *et al*., 1998), as well as in other crops (Hansen *et al*., 1999; Shan *et al*., 1999).

A genetic map is necessary not only for the reliable detection, mapping and estimation of gene effects of important agronomic traits, but also for further research on the structure, organization, evolution and function of the plant genome. Restriction fragment length polymorphism (RFLP) maps of allotetraploid cotton have been constructed from both interspecific (Reinisch *et al*., 1994, Wright *et al*., 1999, Saranga *et al*., 2001) and intraspecific (Shappley *et al*., 1996, 1998; Ulloa *et al*., 2000, 2002) mapping populations. Of the 705 RFLP loci mapped to 41 linkage groups in the interspecific *Gossypium* populations, the actual chromosome identity of only 14 of the linkage groups was presented (Reinisch *et al*., 1994). A combined RFLP–SSR–AFLP map of tetraploid cotton based on a *G. hirsutum* × *G. barbadense* backcross population was recently reported (Lacape *et al*., 2003). The map consists of 888 loci, including 465 AFLPs, 229 SSRs, 192 RFLPs, and two morphological markers, ordered in 37 linkage groups. Recently, a more saturated genetic map constructed using 3347 markers loci was reported (Rong *et al*., 2004). In all of these genetic maps, aneuploid stocks were

employed to locate markers to individual chromosomes and identify linkage groups to chromosomes. In cotton, monotelodisomic stocks that are hemizygous for one arm provide an easy means to localize genes and marker loci to one arm or the other of a given chromosome (Endrizzi *et al*., 1985; Saha and Stelly 1994). Assignment of RFLP and SSR markers to chromosomes have been reported by Reinisch *et al*. (1994) and Liu *et al*.,(2000) respectively. Information on the assignment of AFLP markers to chromosomes in cotton is not yet available. Here we report our results on the assignment of the AFLP markers to chromosomes in cotton.

4.2 Materials and Methods

AFLP markers were assigned to cotton chromosome and chromosome arms following a manner described by Lazo *et al*., (1994) for dominant DNA markers. A new interspecific aneuploid, *G. tomentosum* chromosome substitution lines of *Gossypium hirsutum* L., was used in this research. Genetic stocks monosomic for G. *tomentosum* chromosomes 1, 2, 6, 7, 9, 10, 16, 17, 18, 20 and 25 were available for assignment of DNA markers to entire chromosomes. In addition, genetic stocks monotelodisomic for *G. tomentosum* chromosome arms 1*Lo*, 1*Sh*, 2*Lo*, 2*Sh*, 3*Lo*, 3*Sh*, 4*Lo*, 4*Sh,* 5*Lo*, 6*Lo*, 6*Sh*, 7*Sh*, 8*Lo*, 9*Lo*, 10*Lo*, 10*Sh*, 11*Lo*,12*Lo*, 14*Lo*, 15*Lo*, 16*Lo*, 17*Sh*, 18*Lo*, 18*Sh*, 20*Lo*, 20*Sh*, 22*Lo*, 22*Sh*, 25*Lo*, 26*Lo* and 26*Sh* were used. Note that *Lo* is long arm and *Sh* is short arm; that is, monotelodisomic 1*Lo* contains a normal chromosome 1 and a telosome for the long arm of chromosome 1; it is disomic for the long arm but hemizygous for the short arm. Those stocks were obtained from Dr. Saha of the Crop Science Research Laboratory of the USDA ARS at Starkville, MS and evaluated as monosomic or monotelodisomic TM1/*G.tomentosum* F_1s, In each F_1, the “donor genotype” is euploid

G.tomtentosum and the "recipient genotype" is hypoaneuploid *G. hirsutum*, usually a backcross derivative of the accession TM-1. TM1 is an inbred line derived from "Deltapine 14" and is considered the genetic standard of Upland cotton (*G. hirsutum*) (Kohel *et al.*, 1970). A monosomic F_1 substitution stock has a single chromosome from the donor substituted for the corresponding chromosome *pair* of the recipient genotype. Similarly, monotelodisomic F_1 stocks lack alleles from the recurrent parent in the hemizygous chromosome arm from the donor, but carry alleles of the recurrent parent on the opposing arm (either in homozygous or heterozygous condition, depending on the patterns of crossing over).

4.2.1 DNA Isolation

DNA was isolated from plants of TM1, *G. tomentosum*, and all aneuploid genetic stocks. The DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) was used to extract DNA. Fresh young leaves (0.5mg) were ground in liquid nitrogen and used to extract DNA. The protocol was as described in the manufacturer's instructions. An agarose gel method was used to provide information regarding both DNA quantity and quality. The concentration of genomic DNA was estimated by comparing the size and intensity of each sample band with those of a sizing standard, DNA mass ladder (GIBCO). The DNA samples were diluted to a concentration of 20 ng/µL with TE0.1 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to be used as a working solution in AFLP marker analysis.

4.2.2 Amplified Fragment Length Polymorphism Analysis

Thirty primer combinations were used to generate AFLP data (Table 4.1). The generation of the data was performed according to Vos *et al.* (1995) with some modifications. Sample DNA was digested with EcoRI (infrequent cutter with GAATTC

recognition sequence) and MseI (frequent cutter with TTAA recognition sequence) restriction enzymes and oligonucleotide adapters specific to enzyme restriction sites were ligated to the resulting fragments through incubation (150 min, 37 °C) with DNA ligase. This step was carried out on GeneAmp PCR System 9600 (Perkin Elmer). The genomic DNA (20-40 ng) was digested with the restriction endonucleases in a 11 µL reaction containing 3 µL DNA, 3.5 µL enzyme mix, and 4.5 µL adapter mix 43 (Table 4.2). The reaction was incubated at 37 °C for 150 minutes, and then diluted with 89 µL TE0.1.

Table 4.1 Adapters and primers used for pre-amplification and selective amplification of AFLP procedure

Name of Primer/adapter	Sequence (5'-3')
EcoRI adapter	CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA
MseI adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT
EcoRI primer	
E-A	GACTGCGTACCAATTCA
E- AAG	GACTGCGTACCAATTCAAG
E- AAC	GACTGCGTACCAATTCAAC
E-ACA	GACTGCGTACCAATTCACA
E-ACC	GACTGCGTACCAATTCACC
E-AGG	GACTGCGTACCAATTCAGG
E-ACG	GACTGCGTACCAATTCACG
E-ACT	GACTGCGTACCAATTCACT
E-AGC	GACTGCGTACCAATTCAGC
MseI primer	
M-C	GATGAGTCCTGAGTAAC
M-CAA	GATGAGTCCTGAGTAACAA
M-CTT	GATGAGTCCTGAGTAACTT
M-CAC	GATGAGTCCTGAGTAACAC

Table 4.1 *Continue*

M-CAT	GATGAGTCCTGAGTAACAT
M-CTA	GATGAGTCCTGAGTAACTA
M-CTC	GATGAGTCCTGAGTAACTC
M-CTG	GATGAGTCCTGAGTAACTG
M-CAG	GATGAGTCCTGAGTAACAG

4.2.2.1 Pre-Amplification

The pre-amplification reaction (20 µL total volume) consisted of 4 µL diluted(1:10) digestion ligation mixture, 1.0 µL of the EcoRI primer+A (50uM) with 1.0 µL Mse1primer+C (50uM), 0.4 µL dNTPs(10 mM), 1.2 µL MgCl2 (50uM), 0.2 µLTaq polymerase (1 unit), 2.1 µL 10x PCR-buffer, and 10.1 µL water (Table 4.2). The mixture was pre-amplified for 20 cycles (30 seconds denaturation at 94 °C; 60 seconds annealing at 56 °C; 60 seconds extension at 72 °C). After pre-amplification, 10 µL of the reaction was used to run an agarose gel to check the quality of the digestion and the rest (10 µL) was diluted with 190 µL of low TE0.1 to 200 µL, which was sufficient for 40 AFLP-reactions. The diluted reaction mix and the rest of the amplification reaction products were stored at –20 °C.

Table 4.2 Protocol components for digestion and ligation of genomic DNA

Enzyme mix	µL	Adapter mix	µL
10X T4 Ligase buffer	0.350	10X T4 Ligase buffer	0.75
0.5 M NaCl	0.350	0.5 M NaCl	0.75
BSA (1mg/mL)	0.005	BSA (1mg/mL)	0.05
MseI enzyme (10U/µL)	0.050	MseI Adapter (50pmole/µL)	1.00
EcoRI enzyme (20U/µL)	0.250	EcoRI adapter (5pmole/µL)	1.00
T4 DNA Ligase (400 U/µL)	0.0025		
H2O	2.4925	H2O	0.95
Total Volume	3.50	Total Volume	4.50

4.2.2.2 Selective Amplification

Duplex selective amplification was performed using the AFLP protocol developed by LiCor (AFLP Selective Amplification Kit, 2001), and the new Mse1 and IRDye labeled EcoR1 primers comprising three-nucleotide extensions. The reaction components (10.5 µL total volume) included 1.2 µL 10X amplification buffer containing MgCl2, 0.06 µL Taq DNA polymerase [5 units/µL, Promega Inc.], 1.5 µL diluted pre-amplification DNA, 2 µL Mse1 primer containing dNTPs, 0.25 µL IRDye 700 labeled EcoR1 primer-A, and 0.25 µL IRDye 800 labeled EcoR1 primer-B in 0.24 µL deionized water (Table 4.3). The PCR was performed using a touchdown program: 13 cycles of subsequently lowering the annealing temperature from 65 °C by 0.7 °C per cycle while keeping denaturation at 94 °C for 30 seconds and extension at 72 °C for 60 seconds. This was followed by 23 cycles of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 60 seconds. After PCR, 4 µL of Blue Stop Solution was added immediately before storage at –20 °C.

Table 4.3 Reagents used in the pre-amplification step and selective amplification step

Preamplification step	µL	Selective amplification step	µL
10X PCR Buffer	2.1	10X PCR Buffer	1.20
MgCl2(50µM)	1.2	dNTPs (10µM)	
dNTPs (10µM)	0.4	Mse-Primer (containing dNTP)	2.00
Eco-Primer (50µM)	1.0	IRDye700 labeled EcoRIprimer	0.25
Mse-Primer (50µM)	1.0	IRDye700 labeled EcoRIprimer	0.25
Tag (5U/µl)	0.2	Tag (5U/ul)	0.06
H2O	10.1	H2O	5.24
Diluted DNA (after digestion and Ligation)	4.0	Diluted DNA (Pre-Amplified)	1.50
Total Volume	20.0	Total Volume	10.5

4.2.2.3 Electrophoresis and Scoring

Electrophoresis was conduced on an automatic DNA sequencer (Licor 4200 series DNA sequencer). Amplified DNA fragments were separated on a 6% denaturing polyacrylamide gel (LiCor) that included 52.5 g urea, 7.12 g acrylamide, 0.375 g bis-acrylamide, and 1.825 g 20x glycerol. The gels were cast at least 90 minutes before use and pre-run for 30 min just before loading the samples. Pre-running and running electrophoresis steps were performed using 16-bit data collection, 1500 V, 40 W, 40 mA, 45 °C, and 4 X scan speed as recommended by LiCor. The 1X TBE (89mM Tris, 89 mM borate, 2.2 mM EDTA pH 8.3) was used as the running buffer. After the wells were completely flushed with a 20 cc syringe to remove urea precipitate or pieces of gel, 0.8 µL of each denatured sample (denaturation conducted at 94 °C for 3 minutes immediately before loading) was added to a well using an 8-channel Hamilton syringe. Four molecular sizing standards (50-700 bp) were used in designated lanes. The real-time TIFF images were automatically collected and recorded during electrophoresis (Figure 4.1). Loading the same gel twice, each run needed about 3 hours to collect both channel images (700 and 800) resulting in a maximum of four images collected in a single day. The gel images were automatically scored by Saga Generation 2 software with GT & MX modules client version 3.1.0 build 315 (Licor, CA).

4.2.3 Marker Naming

The name of each marker followed the nomenclature of Akash (2003) which consisted of the primer combination followed by the band size (in base pairs) (Table 4.4).

4.3 Results and Discussion

4.3.1 AFLP Marker Frequency in Cotton

Twenty primer combinations were selected in this research after screening 36 primer combinations by using TM1 and *G. tomentosum.* (Table 4.4). A total of 1556 major AFLP bands was observed; 151 of these (9.68%) were polymorphic. The number of bands generated by individual primer combinations ranged from 52 for C11 (EcoRI+AGG / MseI+CAA) to 106 for C02 (EcoRI+ AAG/MseI+CAA), with a mean of 78 bands. The primer combination EcoR I+AAC/MseI+CTA produced the largest number of polymorphic products (16 in total). There was no correlation between the total number of bands and the number of polymorphic ones. The polymorphism level detected in this study conforms to the results of Akash(2003) (11.2%) and Lacape *et al.* (2003)(11.3%). Also this result is similar to polymorphism revealed in other crops by AFLP: barley (11%) (Becker *et al.*, 1995), and soybean (7.8%) (Young *et al.*, 1999).

Table 4.4 Number of monomorphic and polymorphic (total) and number of AFLP primer combinations between two lines (Pee Dee 2165 and Paymaster 54) of Upland cotton

Name		Selective nucleotides		Number of bands	
Muhanad	Lacape	EcoR1	MseI	Total	Polymorphic
C01	E3M1	AAG	CAA	106	5
C02	E2M8	AAG	CTT	72	11
C03	E1M4	AAC	CAT	102	5
C04	E1M5	AAC	CTA	84	16
C05	E3M6	ACA	CTC	71	5
C06	E3M7	ACA	CTG	82	4
C07	E5M2	ACC	CAC	92	9
C08	E5M3	ACC	CAG	63	4
C10	E1M2	AAC	CAC	75	12
C11	E8M1	AGG	CAA	52	13

Table 4.4 *continued*

Name		Selective nucleotides		Number of bands	
Muhanad	Lacape	EcoR1	MseI	Total	Polymorphic
C13	E6M4	ACG	CAT	96	6
C15	E4M6	ACT	CTC	57	4
C16	E4M7	ACT	CTG	62	3
C17	E7M2	AGC	CAC	75	8
C18	E7M3	AGC	CAG	55	4
C20	E6M2	ACG	CAC	105	5
C25	E3M5	ACA	CTA	58	8
C29	E5M4	ACC	CAT	85	11
C30	E3M1	ACA	CAA	103	11
C31	E4M1	ACT	CAA	62	7
Total				1556	151

4.3.2 Assignment of AFLP Markers to Chromosomes

Since AFLP markers are dominant and the monosomic lines were developed in a TM-1 background, only AFLP markers present in the TM-1 and absent in G.*tomentsum* can be assigned to a chromosome. The monosomic lines will assign markers to a chromosome, while monotelodisomic lines can associate the marker with the short or long arm of a chromosome, and also can confirm the results from the monosomic lines. In this research, 53 markers were assigned to 14 different chromosomes (Table 4.5). Of these, three markers were assigned to whole chromosomes and 50 were assigned to chromosome arms. The number of markers assigned to each chromosome varied from 1 (chromosome 14) to 6 (chromosomes 10). Thirty two markers (60%) were located on the A genome (chromosomes 1-13) and 21 (39%) markers were located on the D genome (chromosomes 14-26). This observation is consistent with the results of Lacape *et al*, (2003) (64% and 34%, respectively, on the A and D genomes). Of these 53 markers, nine were in common with the markers of Akash (2003) population (Paymaster 54 × Pee

Dee 2165), an intraspecific cross (*G.hirsutum* × *G. hirsutum*), and 14 were common with those in Lacape's population ('Guazuncho- 2' × 'VH8-4602'),which is a interspecific cross (*G.hirsutum* × *G.barbadense*) (Lacape *et al*., 2003).

Of all the polymorphic markers found between TM1 and *G. tomentosum*, some could not be assigned to any chromosome. As more aneuploid stocks are developed, the potential exists for locating those markers to a chromosome.

Table 4.5 AFLP markers and its chromosome locations

Marker name	Chromosome	Marker name	Chromosome
C15_204	1*Lo*	C30_292	14*Lo*
C31_97	1*Lo*	C03_193	15*Lo*
C01_164	*2Lo*	C11_78	15*Lo*
C05-111	2*Lo*	C03_70	17
C06_78	2*Lo*	C01_80	17*Sh*
C30_312	2*Lo*	C04_86	17*Sh*
C02_56	2*Sh*	C10_86	17*Sh*
C30_221	2*Sh*	C30_154	18*Lo*
C25_102	3*Lo*	C05-89	18*Sh*
C29_102	3*Lo*	C15-86	18*Sh*
C04_187	4*Lo*	C20_79	18*Sh*
C04_51	4*Lo*	C08_64	22*Sh*
C07_310	4*Lo*	C17_166	22*Sh*
C11_45	4*Sh*	C31_169	22*Sh*
C06_175	5*Lo*	C05-260	25*Lo*
C25_142	5*Lo*	C25_125	25*Lo*
C06_270	7*Sh*	C29_57	25*Lo*
C29_86	7*Sh*	C04_69	26*Lo*
C30_159	7*sh*	C30_179	26*Lo*
C05_64	9	C30_85	26*Lo*
C10_238	9	C04_154	26*Sh*
C15_64	9*Lo*		
C18_292	9*Lo*		
C30_141	9*Lo*		
C31_78	9*Lo*		
C02_71	10*Lo*		
C03_136	10*Lo*		
C30_259	10*Lo*		
C02_96	10*Sh*		
C06_51	10*Sh*		
C16_47	10*Sh*		
C02_112	12*Sh*		

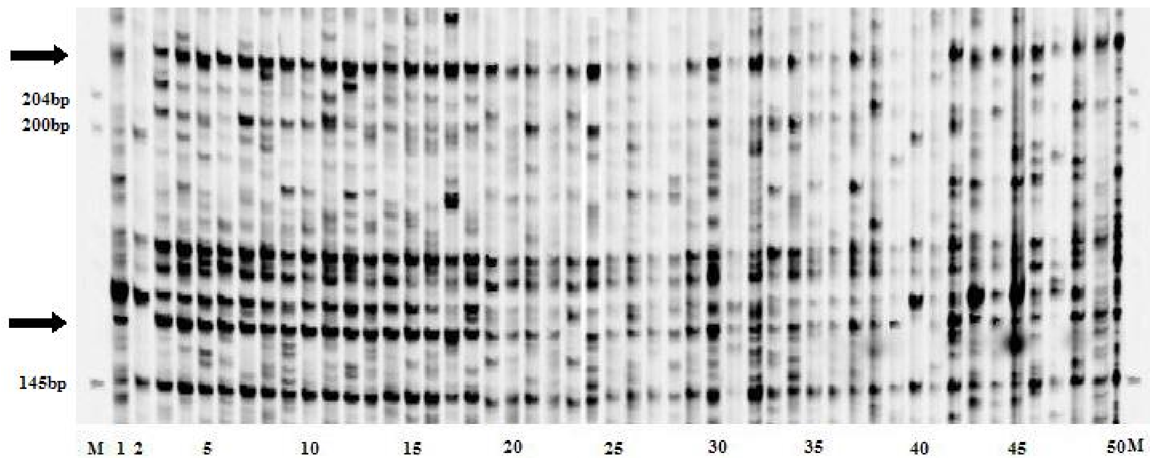

Figure 4.1 AFLP gel image for the primer pair combination EcorI+ACA/MseI+CAA. The DNA samples are: (from left to right) standard size marker, TM1, *G.tomentosum*, Te12*Lo*, H17, Te18*Lo*, H25, Te1*Lo*, Te17*Sh*, Te26*Sh*, Te22*Lo*, Te16*Lo*, H18, Te25*Lo*, Te20*Sh*, H16, Te15*Lo*, Te22*Sh*, Te20*Lo*, Te26*Lo*, Te14*Lo*, Te5*Lo*, Te6*Sh*, Te8*Lo*, Te4*Sh*, Te7*Sh*, Te3*Sh*, H20, Te4*Lo*, H7, Te3*Lo*, Te18*Sh*, Te5*Lo*, Te6*Sh*, Te2*Sh*, Te2*Lo*, Pima 3-79**TM1, H9, H10, H2, TM1, Te9*Lo*, Te1*Sh*, *G.tomentosum*, NTN12-11,T E10*Sh*, H1, Pima 3-97, NTN17-11, Te10*Lo*, Te1*Lo,* standard size marker. Marker C30_159 (bottom arrow) shows polymorphism between TM1 and *G.tomentosum*, and all aneuploid samples present that band except Te7Sh (line 23) indicated that Marker C30_159 is located on short arm of chromosome 7. The Marker C30_207(upper arrow) shows polymorphism between TM1 and *G. tomentosum*, while all the aneuploid F1 stock have that band indicated that Marker C30_207 are likely located on chromosomes where aneuploid were not available.

4.3.3 Association of Linkage Groups to Chromosomes

The polymorphic AFLP markers detected in the aneuploid stock (*G.hirsutum* × *G. tomentosum*) are different from the polymorphic AFLP markers detected in a intraspecific cross (*G.hirsutum* × *G.hirsutum*) by Akash (2003). Only nine of 53 AFLP markers are in common. Based on those common markers, linkage group 15 and linkage

group five were associated with chromosome 10; linkage group 3 and linkage group 28 were associated to chromosome 15 long arm; and linkage groups 1, 10, 16 and 23 were assigned to chromosomes17, 2, 22 and 26, respectively (Table 4.6). There are two common markers on linkage group 5 (C06_51 and C16_47), which confirms that linkage group is located on chromosome 10. In this research, we were unable to associate the remaining 20 groups to chromosomes because of lack of common markers.

Table 4.6 Results of assignment of linkage groups to chromosomes

Linkage group	Reference: marker	Chromosome location
LG15	C01_73	10*Lo*
LG5	C06_51, C16_47	10Sh
LG10	C01_56	2Sh
LG3	C03_193	15Lo
LG28	C11_78	15Lo
LG1	C04_86	17Sh
LG16	C08_64	22Sh
LG23	C04_154	26Sh

4.3.4 Discussions

4.3.4.1 Association of AFLP Markers to Chromosomes

In this research, 53 AFLP markers were assigned to cotton chromosomes and/or chromosome arms using a *G. hirsutum* (TM1) $\times$ *G. tomentosum* aneuploid genetic stocks series. However, the remaining polymorphic AFLP markers could not be assigned to a cotton chromosome. Reasons for this are as follows: 1) The aueuploid genetic series is not complete. We are missing aneuploids for chromosomes 13, 19, 21, 23 and 24. 2) Only polymorphic markers present in *G. hirsutum* and absent in *G. tomentosum* can be associated with chromosomes using aueuploid genetic stocks and 3) Because AFLP

markers are dominant, the assignment is based on presence or absence of a specific band. Sometimes scoring the bands is difficult. The assigned AFLP markers were scattered over the various cotton chromosomes with no apparent clustering pattern. At least one AFLP marker was assigned to each of 16 different cotton chromosomes and 50 markers were localized to 19 different chromosome arms.

4.3.4.2 Association of Linkage Groups to Chromosomes

A low frequency (9/53) of common AFLP markers was found between the aneupolid stock and the intraspecific cross used in this research. However, a higher frequency (14/53) of common markers was found between the aneupolid stock (*G. hirsutum* × *G. tomentosum*) with an interspecific cross (*G. hirsutum* × *G.barbadense*). Further research by using another set of aneuploids (*G .hirsutum* × *G. barbadense*) is ongoing, these will detect more common markers and confirm the results of this research.

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CHAPTER 5 IDENTIFICATION OF QUANTITATIVE TRAIT *LOCI* FOR YIELD AND YIELD COMPONENT TRAITS IN UPLAND COTTON

5.1 Introduction

Cotton (*Gossypium hirsutum* L.) is the world's major natural source of textile fiber and the second largest oilseed crop. The objectives of most cotton improvement and breeding programs are to increase lint yield and to produce more uniform, longer and stronger cotton fiber. Cotton lint yield is probably best understood in terms of its constituent components. Fiber or lint yield in cotton is determined by two major components, i.e., the number of seeds produced per acre and the weight of fiber produced on the seed (Lewis, 2003).

$$\text{Yield} = \frac{\text{No. of Seeds}}{\text{Acre}} \times \frac{\text{Weight of Fiber}}{\text{Seed}},$$

While the No. of seeds per acre can be divided into:

$$\frac{\text{No. of Seeds}}{\text{Acre}} = \frac{\text{No. of Plants}}{\text{Acre}} \times \frac{\text{No. of Bolls}}{\text{Plant}} \times \frac{\text{No. of Seeds}}{\text{Boll}},$$

and weight of fiber per seed can be divided into:

$$\frac{\text{Weight of Fiber}}{\text{Seed}} = \frac{\text{No. of Fiber}}{\text{Seed}} \times \frac{\text{Weight}}{\text{Fiber}},$$

The number of fibers per seed and weight per fiber can be estimated using lint index, Fiber Length (UHM), Fiber Uniformity (UI) and Micronaire as following.

$$\frac{\text{No. of Fiber}}{\text{Seed}} = \frac{100{,}000 \times \text{Lint Index}}{\text{UHM} \times \text{UI} \times \text{Micronaire}}$$

$$\frac{\text{Weight}}{\text{Fiber}} = \text{Length (UHM)} \times \text{Micronaire}.$$

All of these traits including yield and fiber quality traits, contribute to the lint yield of cotton. Cotton yield traits have continuous phenotypic distributions which imply that many genes with relatively minor effects, termed quantitative trait loci (QTL), control those traits. With the advent of molecular marker techniques as well as the availability of saturated DNA marker maps it is now possible to identify and locate loci (genes) controlling complex traits like fiber yield and its contributing components (Paterson *et al.*, 1988). The association of molecular markers with desirable quantitative traits should contribute to the discovery of genetic variability and aid in the selection of desirable parents and progeny through marker assisted breeding. The first cotton linkage map, reported by Reinish et. al. (1994) was constructed using 705 RFLP (restriction fragment length polymorphism) markers from an interspecific cross (*G. hirsutum* × *G. barbadense*). After that, several linkage maps were reported based on both interspecific and intraspecific crosses (Table 5.1). Recently, a more saturated genetic map which was developed using 3347 markers was reported (Rong, *et al.*, 2004). The availability of saturated molecular maps (Lacape *et al.*, 2003; Reinisch, *et al*. 1994; Rong, *et al*., 2004) has made it possible to elucidate the inheritance pattern of quantitative trait loci (QTL). The identification of QTL controlling lint yield and yield components and their association with molecular makers has been the focus of our research. QTL analysis of cotton traits (lint yield and fiber quality) have been reported by several researchers (Jiang *et al.*, 1998; Akash, 2003; Ulloa *et al*., 2002; Zhang *et al*., 2003) and in their research, lint yield was divided into bolls per plant, boll weight and lint percentage. In this research, we dissect the yield components into a more detailed level as described above.

Table 5.1 Reported linkage maps for tetraploid cotton

Cross	Mapping population	Molecular markers	Genome coverage	Number of linkage groups	Reference
G. hirsutum × *G.hirsutum*	F2	RFLP	43	05	Shappley, 1994. Shappley, *et al.* 1996
G. hirsutum × *G.hirsutum*	F2	RFLP	865	31	Shappley, *et al.* 1998a, 1998b
G. hirsutum × *G.hirsutum*	F2	RFLP	700	17	Ulloa and Meredith, 2000
G. hirsutum × *G.hirsutum*	F2	RFLP	1503	47	Ulloa, *et al.* 2002
G. hirsutum × *G.hirsutum*	F2:3	AFLP	1773	28	Akash, 2003
G. hirsutum × *G. barbadense*	F2	STS (2584)	4908	26	Rong, *et al.* 2004
G. hirsutum × *G. barbadense*	F2	RFLP	4675	41	Reinisch, *et al.* 1994 Wright, *et al.* 1999
G. hirsutum × *G. barbadense*	F2	RAPD, AFLP	521.5	11	Altaf, *et al.* 1997
G. hirsutum × *G. barbadense*	F2	RFLP	856	18	Brubaker, *et al.* 1999
G. hirsutum × *G. barbadense*	F2	RFLP	3664	26	Jiang, *et al.* 2000
G. hirsutum × *G. barbadense*	F2	RFLP, SSR	3315	43	Zhang, *et al.* 2002
G. hirsutum × *G. barbadense*	F2	RAPD, SSR	1058	28	Ulloa, *et al.* 2000
G. hirsutum × *G. barbadense*	F2	RFLP, RAPD, SSR	1337	8	Zuo, *et al.* 2000

5.2 Materials and Methods

5.2.1 Plant Materials

One hundred thirty eight $F_{2:3}$ progeny lines were developed from an intraspecific cross of *G. hirsutum* (Paymaster 54 and Pee Dee 2165). Paymaster 54 was bred by the private sector for high yield performance; Pee Dee 2165 was bred for high fiber quality and released as a parent for improvement of fiber quality by the USDA-ARS and South Carolina AES (Culp and Harrell, 1979). These two parents were selected on the basis of a pervious study (Lu and Myers, 2002). The $F_{2:3}$ population was planted in May, 2002 in two different field environments (Dean Lee Research Station in Alexandria and Central Research Station in Baton Rouge). The $F_{2:3}$ seeds were planted in single-row plots, 5 m long, spaced 1 m apart with seed sown by hand, 15 cm apart. At each station, two replications of the entries, arranged in an incomplete block design, were used to evaluate agronomic traits.

5.2.2 Phenotypic Traits Measurement

Cotton lint yield and yield components data along with fiber quality data were collected as described by Muhanad (2003). Fiber quality trait (length, strength, uniformity and micronaire) were measured by HVI at the LSU cotton Fiber Lab in Baton Rouge, LA. Yield components data including lint yield (LY), bolls per plant (B/P), seed number per boll (S/B), number of fibers per seed (F/S) and mean weight per fiber (W/F) were estimated as described in the introduction.

5.2.3 Linkage Analysis

The linkage map of Paymaster 54 × Pee Dee 2165, developed earlier using 143 AFLP (amplified fragment length polymorphisms), was used. The map length of this

population is approximately 1773.2 cM which provides coverage of 37.7% of the cotton genome (Akash, 2003).

5.2.4 QTL Analysis

Summary statistics, normality tests, correlation analysis and path analysis were carried out for all traits by using PROC UNIVARIATE and PROC CORR in SAS V9.0 (SAS 1988). The mean value across the replicates was used for QTL analysis of each trait. The association between phenotype and marker genotype was investigated using single-point analysis (SPA), interval mapping (IM) and composite interval mapping (CIM) methods. All analyses were carried out using QTL cartographer (Wang, *et al*., 2004). A significance level of 0.05 was used in SPA analysis and a threshold LOD of 2.00 was used in IM and CIM analysis.

5.3 Results and Discussions

5.3.1 Summary Statistics and Normality Test of Traits

Summary statistics and normality test results are presented in Table 5.2. A large amount of variation for all traits studied was detected. Lint yield and number of fiber per seed were the most variable traits, showing more than four-fold differences among the 138 plants of the $F_{2:3}$ population studied. Three to four-fold differences were detected for seed number per boll and bolls per plant. The least variable trait was weight per fiber, which showed only a 45% (approximate) difference between the lowest and highest values in the $F_{2:3}$ population. All traits except for bolls per plant ($P<0.001$) showed normal distribution (Figure 5.1). The mean values of the trait (bolls per plant) that did not show normal distribution was converted using a log transformation for QTL analysis, as

previously described (Jiang *et al.*, 1995). After log transformation, the trait showed a normal distribution (P=0.6483).

Table 5.2 Summary statistics and normality tests for yield and yield component traits

Traits	N†	Mean	STD‡	Range	Pr<W+
Lint Yield (LY)	122	81.62	8.327	36.32-151.44	0.4979
No. of fiber per seed (S/F)	124	11916.11	3643.08	4291-20324	0.2556
Weight per fiber(W/F)	125	3.96	0.238	3.05-4.47	0.0540
Seed No. per boll (S/B)	137	26.53	5.133	14-40	0.9466
Bolls Per Plant (B/P)	123	10.54	3.161	5.37-22.0	0.0001
Log of bolls per plant*	123	2.31	0.288	1.68-3.09	0.6483

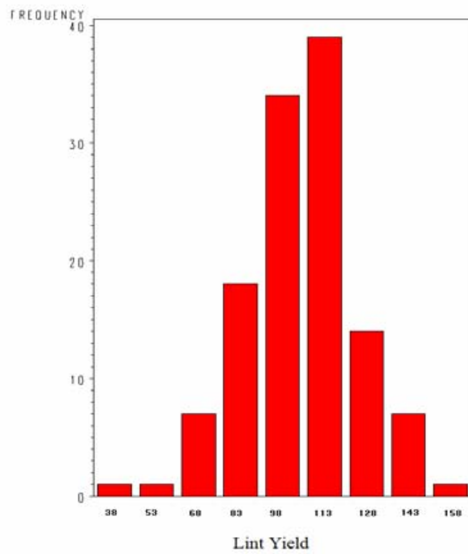
Note: † Number of lines ‡Standard deviation, * After log transformation +test for normality

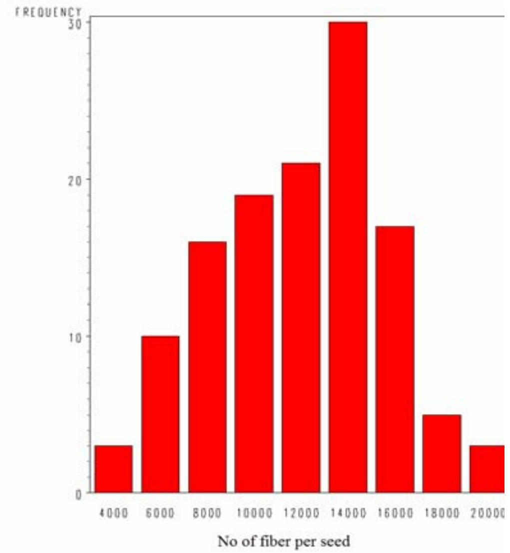
5.3.2 Traits Correlations

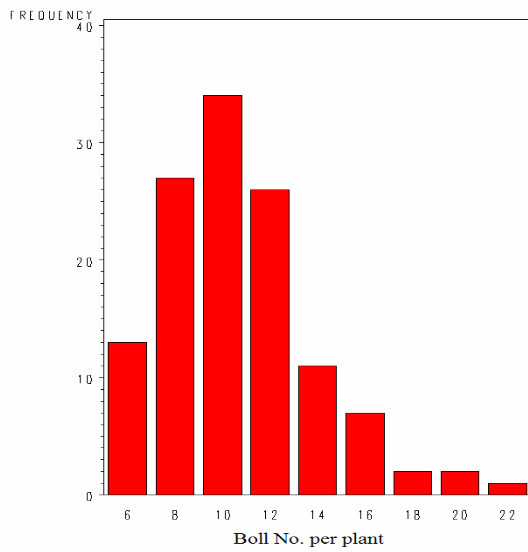
Correlation analysis indicated that yield component traits were positively associated with lint yield (Table 5.3). Lint yield was significantly correlated with bolls per plant, weight per fiber and number of fiber per seed. The highest correlation was observed between lint yield and number of fiber per seed ($r=0.59$, $P<0.01$), followed by lint yield and bolls per plants ($r=0.29$ $P<0.01$). However, an insignificant correlation was found between lint yield and seed number per boll. A positive correlation was detected between bolls per plant and mean weight per fiber ($r=0.31$, $p<0.01$).

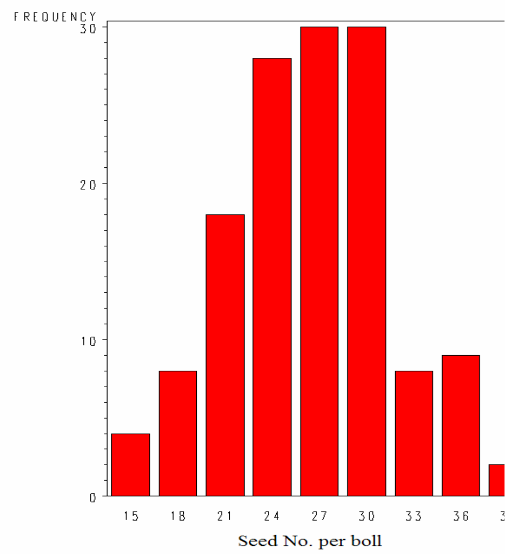
5.3.3 Path Analysis of Yield Components

Path analysis of yield components to lint yield was performed in SAS; the results are listed in Table 5.4. The analysis revealed that components with the highest correlation to lint yield also had the largest direct effects on yield. Of the yield components, number of fibers per seed exerted the largest direct influence on yield









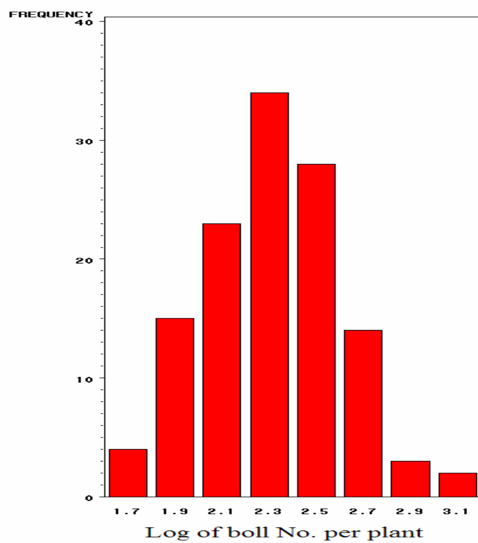


Figure 5.1 Frequency distribution for lint yield and yield components. Log of boll No. per plant was the log transformation of boll No. per plant data

(P=0.61886). The direct effect of bolls per plant, number of seeds per boll and average weight per fiber were similar (about 0.2). For the indirect effect, bolls per plant have the largest indirect effect to lint yield through other components. Number of fiber per seed and number of seed per boll have a negative indirect effect to lint yield through other components.

Table 5.3 Correlation coefficients among traits in an intraspecific cross of $F_{2:3}$ cotton population

Traits	LY	F/S	W/F	B/P	S/B
LY	1.000	0.5870**	0.1993*	0.2936**	0.0413
F/S		1.000	-0.1204	0.1018	-0.1670
W/F			1.000	0.312**	0.0646
B/P				1.000	-0.1563
S/B					1.000

Note: * Significant at 0.05 level; ** Significant at 0.01 level

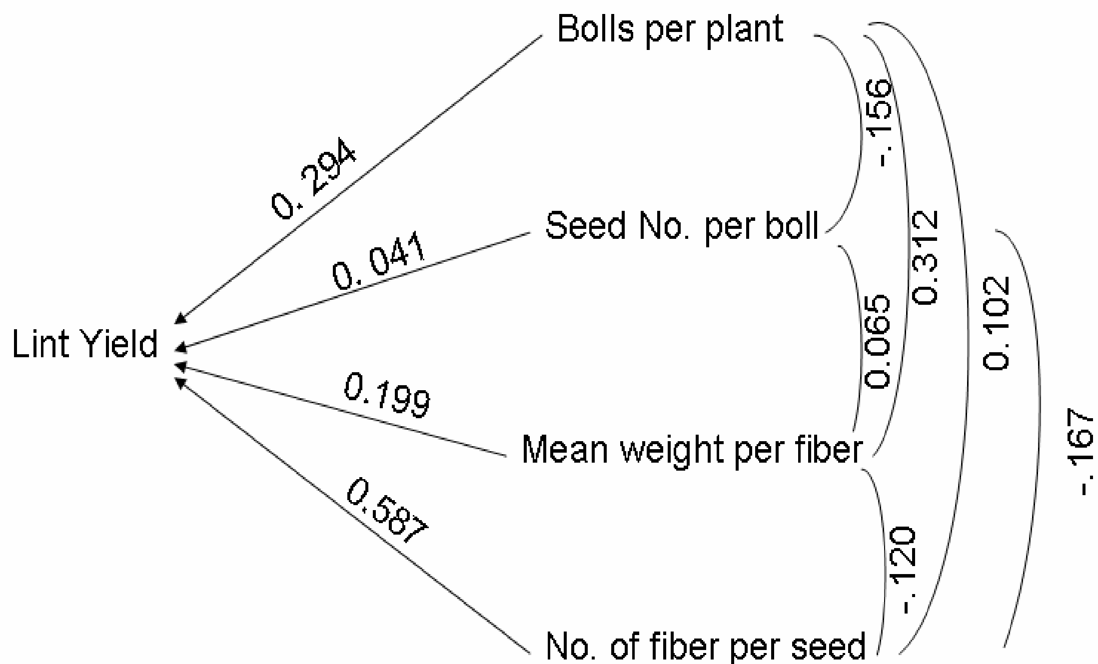


Figure 5.2 Path diagram of cotton yield and yield component tratis

Table 5.4 Path analysis of yield components to lint yield in a $F_{2:3}$ population of an intraspecific cross of *G. hirsutum*

Pathway		Path coefficient
Bolls per plant $\rightarrow$ Lint Yield		
Direct effect		0.19211
Indirect effect via		
Mean weight per fiber		0.06379
No. of fiber per seed		0.06298
Seed No. per boll		-0.02524
Correlation coefficient r	=	0.2936
Mean weight per fiber $\rightarrow$ Lint Yield		
Direct effect		0.20302
Indirect effect via		
Bolls per plant		0.06036
No. of fiber per seed		-0.07454
Seed No. per boll		0.01043
Correlation coefficient r	=	0.1993
No. of fiber per seed $\rightarrow$ Lint Yield		
Direct effect		0.61886
Indirect effect via		
Bolls per plant		0.01956
Mean weight per fiber		-0.02445
Seed No. per boll		-0.02698
Correlation coefficient r	=	0.5870
Seed No. per boll $\rightarrow$ Lint Yield		
Direct effect		0.16157
Indirect effect via		
Mean weight per fiber		-0.03001
No. of fiber per seed		0.01311
Bolls per plant		-0.10333
Correlation coefficient r	=	0.04134
Residual effect^{+}	=	0.7302

Note: + Calculated based on formula from Kang, M.S. (1992)

5.3.4 QTL Analysis of Lint Yield

Seven markers were detected that were associated putative QTL influencing lint yield by SPA method (Table 5.5), with one and two QTL detected by IM and CIM methods, respectively (Table 5.6). The variation explained by these individual QTL ranged from 12.36% to 14.87% as determined by IM and CIM 27% of the variation was explained by two main QTL which were detected by CIM methods. These results agree with Ulloa and Meredith (2000) where two QTL associated with lint yield explained about 25% phenotypic variance in an intraspecific $F_{2:3}$ population. At least one QTL which has a negative additive effect (-3.024 or -0.2801) on linkage group 21 (C14-053-C17-054) was detected by all three methods. One QTL which has a positive additive effect (0.2891) was detected by both SPA and CIM on linkage group 11 (C01-106-C16-147), but was not detected by IM methods. Using the same population, Akash (2003) identified a QTL which was associated with lint weight per boll on the same linkage group (LG21: C14-053-C17-054).

Table 5.5 AFLP markers that were associated with putative QTL influencing lint yield by using single point analysis

Method	LG	Marker	F	P
SPA	1	C17-161^{+}	4.775	0.0306
	1	C19-112	4.0750	0.0455
	11	C06-106	3.9921	0.0477
	11	C16-147	5.4228	0.0214
	21	C14_053	9.494	0.0025
	21	C15-061	11.331	0.0010
	21	C17-054	6.160	0.0143

Note: $^{+}$ Names follows Akash's dissertation, 2003

Table 5.6 AFLP markers that were associated with putative QTL influencing lint yield by using interval mapping (IM) and composite interval mapping(CIM)

Method	QTL	LG	position	A	d	lod	PVE[+]
IM	1	21	63.47	-3.024	0.0000	2.4989	14.75
CIM	2	11	14.01	0.2891	0.7407	2.1889	14.87
		21	63.47	-0.2801	-.00024	2.5158	12.36

Note: + Percent of variance explained

5.3.5 QTL Analysis of Bolls per Plant

Five markers were identified that were associated with putative QTL influencing bolls per plant by SPA method (Table 5.7). The IM method did not detect any QTL, however, one QTL was detected by CIM which was also detected by SPA (Table 5.8). The QTL was located on linkage group 19 at position 28.4cM (marker interval: C14-191-C01-118). Variation explained by this QTL are 8.56%. The additive and dominance effects of this QTL are -0.4.23 and -0.7081, respectively.

Table 5.7 AFLP markers that were associated with putative QTL influencing bolls per plant by using single point analysis

Method	LG	Marker	F	P
SPA	1	C20-028	4.1998	0.0424
	10	C02-056	4.0910	0.0451
	19	C14-191	4.3063	0.0399
	19	C06-118	9.1863	0.0029
	21	C14-053	4.4669	0.0364

Table 5.8 AFLP markers that were associated with putative QTL influencing bolls per plant by using interval mapping (IM) and composite interval mapping(CIM)

Method	QTL	LG	position	A	D	lod	PVE
IM	0						
CIM	1	19	28.4	-0.4023	-0.7081	2.6874	8.56

5.3.6 QTL Analysis of Number of Fiber per Seed

Thirteen markers located on seven linkage groups were identified that were associated with putative QTL influencing the number of fiber per seed by SPA (Table 5.9). IM detected four markers that are located on three different linkage groups. Only two QTL were detected by CIM (Table 5.10). The two QTL located on linkage group three and five were detected by all three methods. The variation explained by individual QTL ranged from 4.49% to 20.53%. About 25% of the variation was explained by the two main QTL detected by all three methods. Three out of four QTL detected by IM have a negative additive effect. One QTL located on linkage group three (position 2.01 cM) had a positive additive effect (1894.19). The CIM method also gave similar results (additive effect is 2016.2).

Table 5.9 AFLP markers that were associated with putative QTL influencing number of fiber per seed by using single point analysis

Method	LG	Marker	F	P
SPA	1	C12-254	10.1612	0.0018
	1	C14-100	7.8230	0.0058
	2	C12-251	11.7477	0.0008
	3	C04-056	26.6701	0.0000
	3	C05-049	11.6961	0.0008
	4	C11-334	17.0130	0.0001
	4	C01-536	15.8988	0.0001
	4	C20-175	18.6321	0.0000
	4	C12-258	15.5500	0.0001
	5	C06-051	13.1517	0.0004
	5	C08-338	12.3070	0.0006
	15	C02-073	8.2342	0.0043
	22	C12-230	9.9505	0.0020

Table 5.10 AFLP markers that were associated with putative QTL influencing number of fiber per seed by using interval mapping (IM) and composite interval mapping(CIM)

Method	QTL	LG	position	a	d	lod	PVE
IM	4	3	0.01	1975.09	0.000	5.5909	20.53
		4	63.1	-2582.3	0.000	3.716	12.29
		4	110.6	-2324.9	0.000	3.927	17.81
		5	14.7	-1200.7	0.000	2.5434	6.71
CIM	2	3	0.01	2016.2	-177.4	5.003	19.2
		5	14.7	-1004.6	-1667.2	2.530	4.49

5.3.7 QTL Analysis of Mean Weight per Fiber

Seventeen markers located on six linkage groups were associated with putative QTL influencing mean weight per fiber by SPA method (Table 5.11), four and two QTL were detected by IM and CIM methods, respectively. One main QTL located on linkage group 16 (position 0.01 cM) was detected by all three methods. The variation explained by individual QTL ranged from 7.2% to 21.8%. All QTL detected by the IM methods had a negative additive effect. One QTL located on linkage group 24 (position 2.01 cM) has a positive additive effect (0.1794).

Table 5.11 AFLP markers that were associated with putative QTL influencing average weight per fiber by using single point analysis

Method	LG	Marker	F	P
SPA	1	2	7.912	0.0056
	1	3	4.377	0.0383
	1	5	7.587	0.0067
	1	6	6.282	0.0134
	1	10	9.539	0.0024
	1	11	6.728	0.0105
	2	9	7.919	0.0056

Table 5.11 continue

Method	LG	Marker	F	P
	2	7	6.856	0.0098
	5	1	4.923	0.0282
	5	2	8.210	0.0048
	12	1	10.99	0.0012
	12	2	15.987	0.0001
	13	1	11.187	0.0011
	13	3	3.873	0.0317
	16	1	17.029	0.0001
	16	2	12.188	0.0006
	16	4	13.697	0.0003

Table 5.12 AFLP markers that were associated with putative QTL influencing average weight per fiber by using interval mapping (IM) and composite interval mapping(CIM)

Method	QTL	LG	position	a	d	lod	PVE
IM	4	12	6.01	-0.2592	0.000	3.0731	21.8
		13	0.01	-0.1487	0.000	2.0335	7.2
		16	0.01	-0.2502	0.000	3.6437	20.5
		16	22.1	-0.2531	0.000	2.6119	19.8
CIM	2	16	0.01	-0.2268	-0.2893	3.8555	16.26
		24	2.01	0.1794	0.0187	2.6095	9.36

5.3.8 QTL Analysis of Seed Number per Boll

Four markers located on two linkage groups were identified and were associated with putative QTL influencing seed number per boll by SPA (Table 5.12). No QTL was detected by either IM or CIM methods. This may be due to the low significant difference in this trait.

5.3.9 Discussions

A comparison of results obtained from SPA, IM and CIM in this study

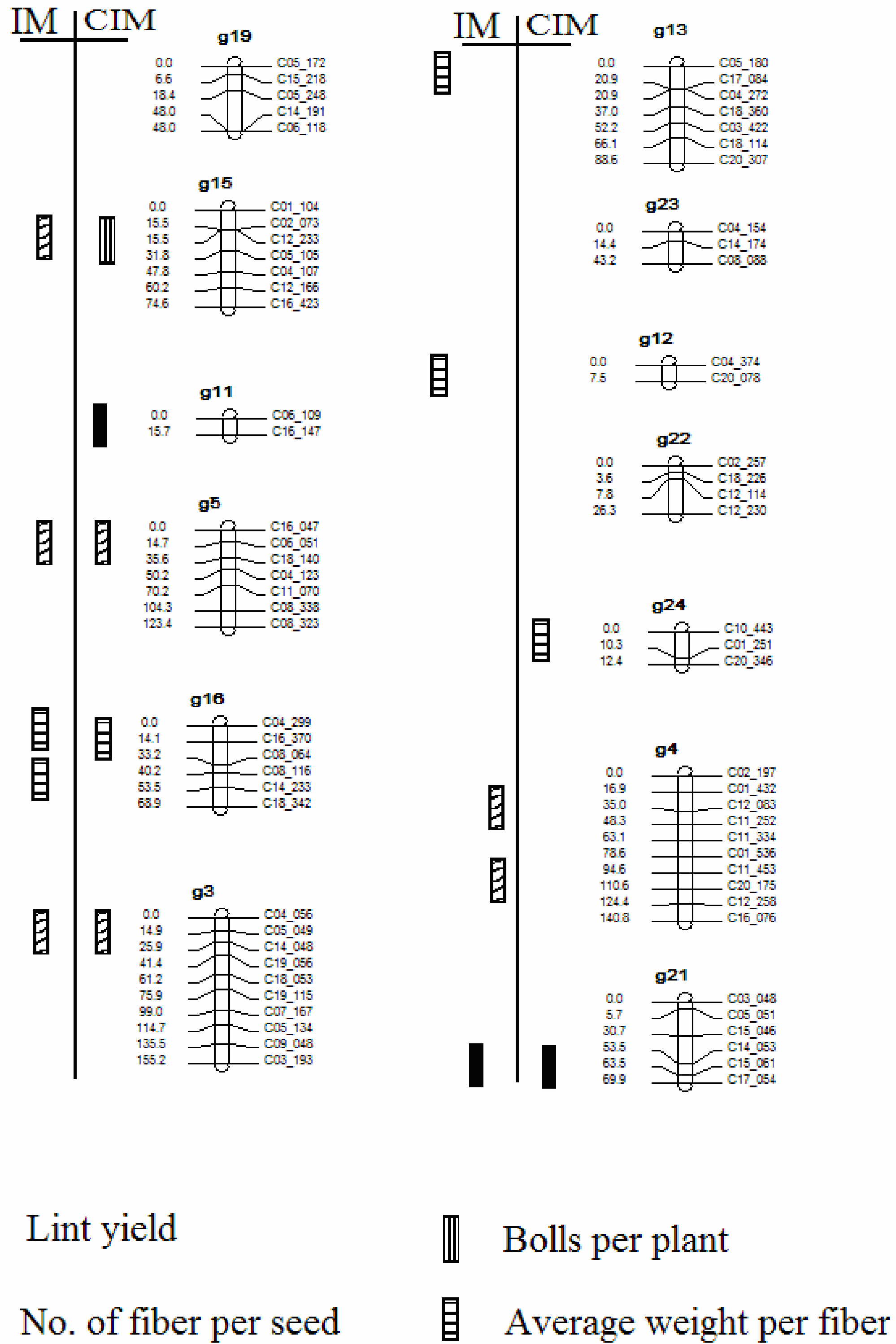


Figure 5.3 A comparison of QTL positions for Upland cotton lint yield and yield components

demonstrated that these three methods identified the same QTL most of the time. The F values from SPA were converted to LOD scores (method described by Champoux et. al., (1995) to compare results obtained from IM and CIM (Table 5.11). Interestingly, the corresponding LOD for p value 0.05 and 0.001 in SPA analysis was 0.84914 and 1.47395, respectively, both of them are less than 2.0; the corresponding P value for LOD 2.0 and 2.5 in IM analysis was 0.00276 and 0.00083, respectively. In view of these findings, the common practice of reporting QTL detected by SPA at $P \leq 0.05$ is likely to detect numerous false positives. If the converted LOD criteria in SPA analysis are used the numbers of QTL identified using SPA agreed most closely with those of IM. The CIM estimates the position of the QTL differently than SPA or IM, and by identifying multiple QTL that simultaneously affect a trait and extracting the variance associated with them, this analysis eliminated some of the loci that meet the significance criteria with the other analyses. Therefore, some of the QTL that appeared to be significant in SPA and IM fell below the assigned significance threshold with CIM. For example, QTL located on linkage group 12 and 13 for mean weight per fiber trait were detected by IM, but was not detected by CIM.

In this study, a total of 47 markers was detected. Those markers were associated with yield and yield component traits. Nine and sveen QTL were detected by IM and CIM methods, respectively. Four QTL were detected by all three methods. CIM analysis detected fewer QTL (seven) than IM (nine), while five QTL were exclusively detected by IM, and three QTL were only identified by CIM. Different number of QTL detected by IM and CIM has been previously reported (Moncada *et al*., 2001; Zhang *et al*., 2001; Akash, 2003). A range of small to medium proportions of the trait phenotypic variance

(6.71 to 28.76%) explained by QTL was common in our study and supports a model for quantitative inheritance for all the agronomic traits studied (Lande and Thompson, 1990; Ulloa and Meredith, 2000).

Table 5.13 F and P value in SPA analysis and it's corresponding LOD score

F value	P value	LOD	F value	P value	LOD
1	0.31906	0.21833	14	0.00027	2.98659
2	0.15955	0.43588	15	0.00017	3.19432
3	0.08550	0.65263	16	0.00010	3.40135
4	0.04746	0.86860	17	0.00006	3.60765
3.910	**0.05**	**0.84914**	18	0.00004	3.81326
5	0.02695	1.08380	19	0.00003	4.01815
6	0.01556	1.29823	20	0.00002	4.22235
6.822	**0.01**	**1.47395**	21	0.00001	4.42586
7	0.00910	1.51189	22	0.00001	4.62868
8	0.00538	1.72480	23	0.00000	4.83081
9	0.00320	1.93695	24	0.00000	5.03227
9.398	**0.00276**	**2.0**	25	0.00000	5.23305
10	0.00193	2.14835	26	0.00000	5.43316
11	0.00116	2.35901	27	0.00000	5.63260
11.67	**0.00083**	**2.5**	28	0.00000	5.83138
12	0.00071	2.56893	29	0.00000	6.02951
13	0.00043	2.77812	30	0.00000	6.22698

Note: sample size used for calculation is 138

5.4 References

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