MAPPING AND CHARACTERIZATION OF DWARF, SOFT GLUME AND BRITTLE CULM MUTANTS IN DIPLOID WHEAT

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **MAPPING AND CHARACTERIZATION OF DWARF, SOFT GLUME AND BRITTLE CULM MUTANTS IN DIPLOID WHEAT** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period of July 2004 to January 2010 under the supervision of Dr. H. S. Dhaliwal and Dr. S. K. Tripathi, Professors, Department of Biotechnology and Department of Water Resources Development & Management, respectively, Indian Institute of Technology Roorkee, Roorkee.

The matter in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(MOHD. JAVED ANSARI)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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The PhD Viva-Voce Examination of **Mr. Mohd. Javed Ansari**, Research Scholar has been held on .....

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ABSTRACT

Hexaploid wheat (*Triticum aestivum* L.) being the second most important crop of the world and the single largest traded crop with a global annual production exceeding 650 million tons from an area of 215.26 million hectare. A total of 78.57 million tons of wheat was produced in India in 2009 from an area of 27.6 million hectare. The diploid wheat *Triticum monococcum* L. (einkorn), one of the first crop domesticated by humans in the Fertile Crescent 10,000 - 12,000 years ago which is being still cultivated for its edible seeds in the mountainous area of Germany, Switzerland and Italy, is an ideal material for induced variability which could be characterized and transferred to cultivated wheat. The diploid nature of *T. monococcum* with smaller genome size of 5,700 Mb compared to 17,300 Mb of bread wheat, the existence of a very high level of polymorphism for DNA based markers, conservation of colinearity and synteny with other cereal crops, availability of a large BAC library and resistance against various wheat disease makes this species an attractive diploid model for gene discovery in wheat. The EMS induced dwarf (*Rht22*), soft glume (*sog3*) and brittle (*brc1, brc2 and brc3*) mutants used in the present study were isolated from diploid wheat line *T. monococcum* acc. pau14087. *Rht22* was found to be insensitive to exogenous gibberellic acid (GA_3) application. In histological studies, the cell size of the *Rht22* was found to be reduced due to loss-of-function of the gene in the mutant. The spike of soft glume (*sog3*) mutant was more compact with lax glumes. The seeds in the mature spike could be easily threshed manually. On removal of individual spikelets from the spike, the rachis remained intact whereas in *T. monococcum* the spikelets separate with a wedge of rachis fragments without leaving any intact rachis. Out of three brittle mutants studied morphologically one (*brc1*) had early flowering and early brittleness (at 40-45 days seedling stage) while the other two (*brc2 and brc3*) had late flowering and late brittleness (at 65-70 days seedling stage), indicating that brittleness was development stage specific. All the brittle mutants were sensitive to lodging and had brittleness in all parts of plants including leaves, leaf sheaths, spikes, culms and roots. Biochemical studies of brittle culm mutants indicated that all the brittle mutants had less α-cellulose on secondary cell wall than *T. monococcum* indicating that all of them did not have proper deposition of cellulose microfibrils. Staining by Phloroglucinol-HCL, a lignin specific stain indicated that all the brittle mutants had slight increase in lignin content than the wild type and had very little cellulose deposition on secondary cell wall of sclerenchymatous cells. After treatment with sodium chlorite which removes all hemicellulose and lignin it was found that in brittle

mutants all cells became deformed due to their less cellulose in cell wall whereas in wild type cell shape remained intact. Atomic Force Microscopy studies of surface of brittle and wild type indicated that the surfaces of all brittle mutants were more rough than the wild type In order to map the dwarfing, soft glume and brittle culm mutants, mapping populations were developed by crossing the dwarf (*Rht22*), soft glume (*sog3*) and brittle (*brc1, brc2 and brc3*) mutants with *Triticum boeoticum* acc.pau 5088, a non-brittle, tall and hard threshing wild diploid progenitor of *T. monococcum*. *T. boeoticum* acc. 5088 was chosen as a parent for development of mapping populations on the basis of its high level of polymorphism with *T. monococcum*. Based on the data on recombination frequency, the flanking markers xbarc37 and xbarc113 mapped at distance of 1.9 cM and 10.3 cM, respectively from the *brc1* on chromosome 6AS, flanking markers xcfd62 and xcfd2170 were mapped at distance of 0.8 cM and 2.9 cM, respectively from the *brc-2* on chromosome 3AL, whereas flanking markers xgwm135 and xwmc470 mapped at distance of 2.1cM and 3.9 cM, respectively from the *brc3* on chromosome 1AL. Similarly flanking markers xwmc89 and xbarc107 mapped at distance of 0.9 cM and 0.6 cM, respectively from the *Rht22* on chromosome 4AL and markers xgwm473 and xbarc69 maped at the distance of 1.8 cM and 28.3 cM, respectively from *sog3* on chromosome 7AL. For cloning candidate gene of *Rht22*, gene specific primers were synthesized covering the DELLA domain of gibberellic acid insensitive dwarf mutant gene *Rht-D1* of hexaploid wheat. Amplification was observed in all the dwarf mutants along with *T. moncoccum* and *T. boeoticum*. After sequencing *Rht22* amplicon two bp deletion was found in DELLA domain. PCR primers based on deletion in DELLA motif amplified *Rht22* only which confirmed the two bp deletion in DELLA motif which was also confirmed by DOT MATRIX view. Sequence alignment of *Rht22* shows high similarity with other DELLA domain defected dwarfing gene of hexaploid wheat, barley, mays and rice. Translated protein of *Rht22* shows frame shift in amino acids. 3D of *Rht22* shows high similarity with 3D of *Rht-D1*. Soft glume (*sog3*) mutant mapped on chromosome 7A which is syntenic to 7H chromosome of barley where a naked seed mutant *nud* has been mapped suggesting that *sog3* may be orthologous to *nud* of barley which shows one major ortholog on rice chromosome 6 and one minor ortholog on rice 2 chromosome. Three rice chromosomes, 2, 1 and 5 syntenic to *T. monococcum* 6,3 and 1 respectively with brittle mutants brc1, brc2 and brc3 each had two CesA and CSL orthologs. BLASTN analysis of the six rice CesA and CSL genes identified about five EST contigs with high similarity (bit score ≥200) but none of them was bin mapped.

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Date: **Mohd. Javed Ansari**

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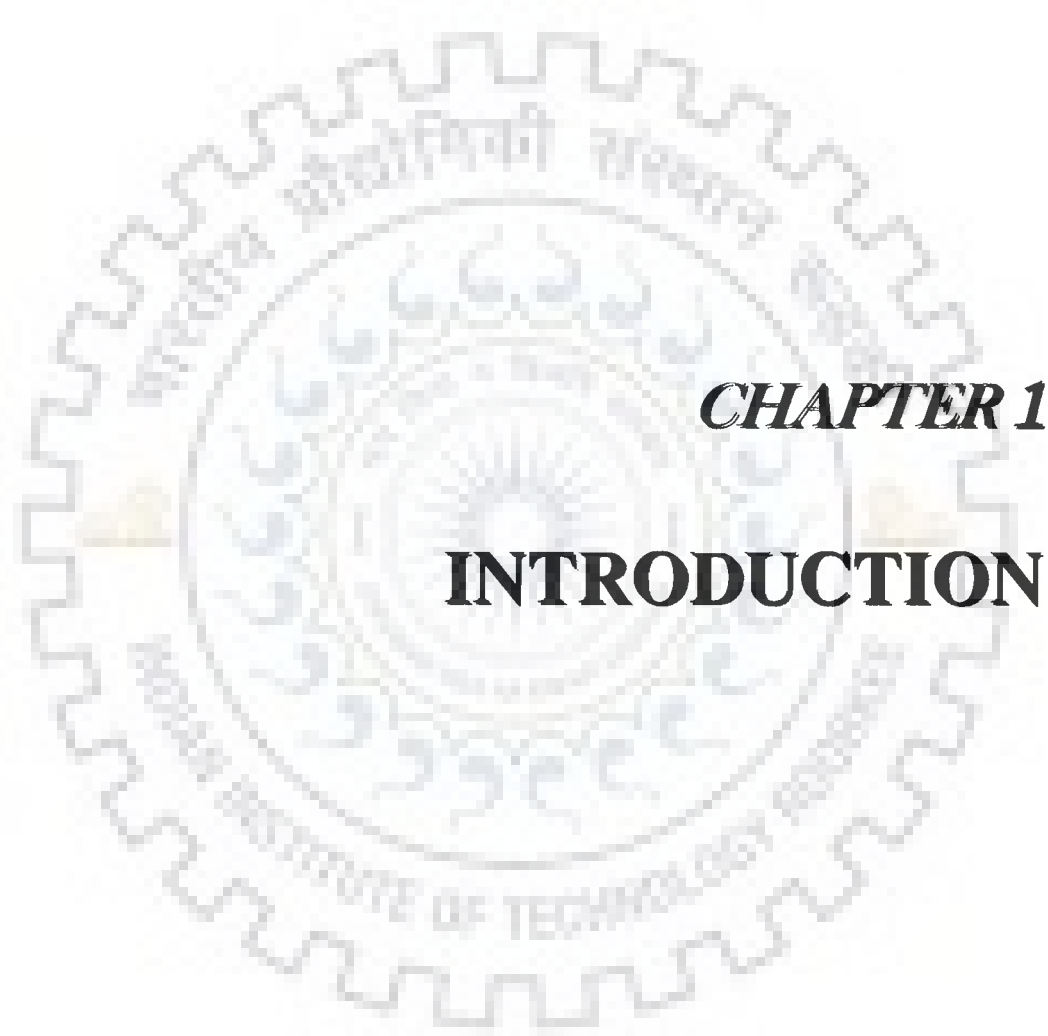
ABBREVIATIONS USED

%	Percentage
µ	micron
µg	Microgram
µm	Micrometer
Bcl	Brittle culm mutants
BLAST	Basic Local Alignment and Search Tool
BLASTN	Nucleotide BLAST
BLASTX	Search protein database using a translated nucleotide query
bp	Base pairs
BSA	Bulk Segregant Analysis
cDNA	Complementary DNA
cM	Centi Morgan
cv.	Cultivated Variety
Cys	Cystine
α-P^{32} dCTP	Radiolabelled P^{32} Cytosine Triphosphate
DNase	Deoxyrinuclease
DEPC	Diethyl Pyrocarbonate
dNTPs	Nucleotide Triphosphates
EMS	Ethyl methane sulphonate
EDTA	Ethylenediaminetetraacetic acid
F_1	First Filial Generation
F_2	Second Filial Generation
F_3	Third Filial Generation
Fig.	Figure
G	Gram
GA	Gibberellic Acid
h	Hour
IRGSP	International Rice Genome Sequencing Project, 2005

Kb	Kilobase pairs (10^3 bp)
kDa	Kilo dalton
L	Litre
LS	Longitudinal Section
Lys	Lysine
M	Molarity
Mb	Megabase pairs (10^6 bp)
Mg	Milligram
Mb	Mega base
min	Minute
Ml	Millilitre
MM	Monococcum mutants
Mm	millimetre
mM	millimole
NCBI	National Centre for Biotechnology Infirmation
NPT	Neomycin Phosphotransferase
°C	°Centigrade
ORF	Open Reading Frame
PAU	Punjab agricultural university
PCR	Polymerase Chain Reaction
ppm	Parts per million
Rht	Reduced Height
RNAi	RNA interference
RiceGE	Rice Functional Genomic Express Database
SDS	Sodium dodecyl sulfate
Sec	Second
SEM	Scanning Electron Microscopy
Sog	Soft glume
SPT	Streptomycin Phosphotransferase
SSC	Sodium Citrate
SSRs	Simple Sequence Repeats
TB	*Triticum boeoticum*

TM	*Triticum monococcum*
TAE	Tris Acetate
TBE	Tris Borate
TE	Tris EDTA
TPase	Transposase
TPR	Tetratrico Peptide repeat
TS	Transverse Section
v/v	Volume/Volume
w/v	Weight/Volume



CHAPTER 1

INTRODUCTION

library and resistance against various wheat disease makes this species an attractive diploid model for gene discovery in wheat (Wicker *et al.* 2001)

Dwarfing or reduced height (*Rht*) genes associated with large increases in yield potential of cereals, have been responsible for ushering Green Revolution since their introduction in wheat and rice breeding programs in 1960s (Evans 1993). The present high yielding wheat varieties possess *Rht-B1b* (syn. *Rht1*) or *Rht-D1b* (syn. *Rht2*) dwarfing genes, which were transferred from the Japanese variety 'Norin10' into a wide range of CIMMYT germplasm before being taken up by other wheat breeding programs worldwide (Gale *et al.*1985).These height-reducing genes located on homoeologous chromosomes 4BS and 4DS interfere with gibberellin (GA) signal transduction (Peng *et al.* 1999). Several GA_3 sensitive and insensitive dwarfing genes have been reported in wheat (Konzak 1988). Other dwarfing genes that do not confer GA insensitivity may be more suitable for reducing final plant height without reducing early plant growth. The GA-responsive dwarfing genes *Rht8* and *Rht9* have been introduced from the Japanese landrace Akagomugi into southern European varieties developed by the Italian wheat breeder N. Strampelli in the 1920s (Lorenzetti 2000). These dwarfing genes do not affect coleoptile length or seedling vigour (Rebetzke *et al.* 1999). Further studies are required to explore the potential of these genes for use in wheat improvement. It will be desirable to identify additional dwarfing gene capable of enhancing yield potential.

Threshability is an important domestication trait in wheat as the free-threshing cultivars could be easily harvested and threshed. All the wild relatives of wheat have tough glumes and hard threshing whereas most of the cultivated wheat varieties are free-threshing with soft glume. Two genetic loci are known to govern the threshability trait in bread wheat. The *Q* gene located on chromosome 5AL and tenacious glume (*Tg*) genes located on homocologous group-2 chromosomes seem to interact to produce a free-threshing spiked (Faris *et al.* 2005). The soft glume (*sog*) gene of diploid wheat and tenacious glume (*Tg*) gene of hexaploid wheat were characterized and mapped on short arm of chromosome $2A^m$ and 2D, respectively (Sood *et al.* 2009).

Cellulose is the most abundant natural polymer in plants which is rendered unfit for feed and industrial utilization due to its undesirable association with lignin (Richmond *et al.* 2000). Tall plants with weak stem are susceptible to lodging resulting in significant yield losses (Zuber 1973). Cellulose is the main determinant of mechanical strength in the stem

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Genetic variability for morphological and economic traits and molecular markers has been extensively used for improvement, construction of high density linkage maps, map based cloning, allele mining, resolution and introgression of QTL, establishing evolutionary and phylogenetic relationship among related species in rice, maize, barley, wheat and rye. Hexaploid wheat (*Triticum aestivum* L.) is being the second most important crop of the world and the single largest traded crop with a global annual production exceeding 650 million tonnes from an area of 215.26 million hectare. A total of 78.57 million tonnes of wheat was produced in India in 2009 from an area of 27.6 million hectare (Kumar *et al.* 2009). Since the discovery of Mendelian principles of genetics in the beginning of 20th century, thousands of varieties of bread and durum wheat have been developed and released for cultivation. There is, however, limited variability in the germplasm of cultivated wheat due to its high ploidy level and peculiar mode of origin involving two steps of hybridization and chromosome doubling resulting in isolation and limited gene transfer from parental species (Kihara 1937, McFadden and Sear 1946). Various attempts to induce useful variability through physical and chemical mutagenesis have not been successful due to its large genome size, higher ploidy level and repetitive DNA (Xue *et al.* 2008).

The diploid wheat *Triticum monococcum* L. (einkorn) was one of the first crop domesticated by humans in the Fertile Crescent 10,000 - 12,000 years ago which is being still cultivated for its edible seeds in the mountainous area of Germany, Switzerland and Italy. It was replaced by tetraploid and hexaploid wheat during the last 5,000 years, and is hence largely ignored by modern breeders .*T. monococcum*, previously considered as the A genome donor, is most closely related to the A genome donor of cultivated wheat, *T. urartu* (Kilian *et al.* 2007). It is tall with tough rachis and hard threshing and mostly single large and bold seed per spikelet. It is therefore, an ideal material for induced variability which could be characterized and transferred to cultivated wheat. The diploid nature of *T. monococcum* with smaller genome size of 5,700 Mb compared to 17,300 Mb of bread wheat, the existence of a very high level of polymorphism for DNA based markers, conservation of colinearity and synteny with other cereal crops, availability of a large BAC

tissue as indicated by the analysis of various mutants in different plant species (Appenzeller *et al.* 2004). Mutants with reduced mechanical strength have been identified in *Arabidopsis*, barley, maize, rice, rye and sorghum (Langham 1940; Kubicka and Kubicki 1988; Kokubo *et al.* 1991; Turner and Somerville 1997; Li *et al.* 2003). No such mutant has been reported in wheat. Mechanical strength in the *brittle culm* mutants has been associated with cellulose content in barley, rice and maize (Kubicka and Kubicki 1988; Kokubo *et al.* 1991, Sindhu *et al.* 2007). About 10 genes encoding cellulose synthase catalytic subunits (*CesA*) have been identified in *Arabidopsis* after complete genome sequencing while 12 genes in maize and 7 in rice (Richmond and Somerville 2000; Dhugga 2001; Appenzeller *et al.* 2004). Mutations in three of the *CesA* genes from *Arabidopsis* resulted in collapsed xylem and reduced mechanical strength of the stem (Turner *et. al.* 1997). Likewise, mutations in the phylogenetically related *CesA* genes from rice resulted in a brittle culm phenotype, indicating the role of these genes in secondary cell wall formation (Tanaka *et al.* 2003). In each case, reduced mechanical strength was correlated with diminished cellulose content. In general, mutations in the *CesA* genes involved in primary wall formation cause severe phenotypic alterations whereas those in secondary wall-forming genes do not alter the apparent phenotype as much as they affect mechanical strength (Li *et al.* 2003; Appenzeller *et al.* 2004).

Among several functional genomics approaches available, EMS mutagenesis is considered to be a very promising tool for high throughput gene discovery and allele mining. Several mutagenic agents have been used to induce point mutations in the genomes of a diverse range of plants (Ahloowalia *et al.* 2004). Of these, ethyl methane sulfonate (EMS) is emerging as the 'mutagen of choice', largely because of its well established mode of action which generates G to A and C to T transitions (Ashburner 1990), and its effectiveness in inducing a high frequency of point mutations in a wide range of organisms in the absence of gross chromosomal abnormalities. The breakthrough in exploiting point mutations for reverse genetics came in 2000, when Claire McCallum and colleagues in Seattle developed a general strategy that they christened 'Targeted Induced Local Lesions in Genomes' or TILLING (McCallum *et al.* 2000, Colbert et *al.* 2001 and Till *et al.* 2003).

EMS mutagenesis in *Triticum monococcum* is a powerful tool for generating knock out mutations for reverse genetics. It facilitates the assigning of biological functions to as yet uncharacterized open reading frames (ORFs) identified in genomic sequences or EST

databases. EMS mutagenesis has been widely used for revealing gene function in *T. monococcum* (Balyan *et al.* 2008). The present investigation deals with the mapping and characterization of EMS induced three brittle culm (*brc1, brc2 and brc3*), one gibberellic acid insensitive dwarf (*Rht22*) and one soft glume mutant (*sog3*) in diploid wheat *Triticum monococcum* using morphological, histological, biochemical and molecular studies.

In gain or loss of function approaches of EMS mutagenesis, it is difficult to find all the knockout mutants or tagged lines for all members of a gene family. In contrast, the RNA interference (RNAi)-induced gene silencing approach can possibly silence multigene families and homoeologous genes in polyploids. It is based on sequence specific RNA degradation thereby silencing one, several or all members of a multigene family in plants (Miki *et al.* 2005).

For the rapid identification of linkage between different PCR based molecular markers and the gene of interest, bulk segregant analysis (BSA) method was suggested by Michelmore *et al.* (1991). In BSA, two bulks (DNA samples) are developed from a segregating population of a single cross. The bulks are then screened for DNA polymorphism and compared against a randomized genetic background of unlinked loci. A marker that differs between the two bulks is expected to be linked to the particular trait. Simple sequence repeats (SSR) or microsatellites are ubiquitous in eukaryotes which are widely used PCR based markers in BSA.

Keeping in view the various aspects of EMS mutagenesis for functional genomics in *Triticum monococcum*, the present investigation was carried out to clone and map a dwarfing gene present in an EMS induced dwarf mutant (*Rht22*) of *Triticum monococcum*. In addition to the dwarf mutant, one EMS induced soft glume viz; *sog3* and three brittle culm (*brc1, brc2 and brc3*) were also characterized in the present study. All the five mutants were isolated from diploid wheat line *T. monococcum* acc. pau14087. This material was obtained from the Punjab Agricultural University Ludhiana.

Approximately 21 dwarf genes have been identified in wheat and there is a need for more new dwarfing genes that can be used to enhance cereal yield. To assign the functions of these putative genes, several approaches have been developed. Among these, EMS mutagenesis has been used widely. Gibberillin-insensitive dwarfing genes *Rht-D1* have already been studied through comparative genomics. The high quality rice genome sequence is serving as a reference for comparative genome analysis in crop plants,

especially cereals. Comparison with hexaploid and diploid wheat showed complex pattern of conserved synteny and colinearity (Singh *et al.* 2007). The sequencing of the wheat genome is in progress and a consortium has been established (www.wheatgenome.org/index.html). Up to date, only about 22 Mb (less than 2% of the wheat genome) has been sequenced. Very large public banks of expressed sequence tags (ESTs), 1,523,865 entries are available in the NCBI (September 2009). A large part of these ESTs (75%) have been used to create 62,121 transcript assemblies or contigs at TIGR (http://www.tigr.org/tdb/e2k1/tae1/). These EST can be used to clone the candidate gene of mutants.

The present investigation was carried out with following broad objectives:

- Phenotypic characterization of dwarf, soft glume and brittle mutants.
- Biochemical and histological characterization of the dwarf and brittle culm mutants.
- Development of mapping populations.
- Bulk segregate analysis of dwarf, soft glume and brittle culm mutants.
- Molecular mapping of mutants.
- Initiate homology search and cloning of dwarfing mutant allele.
- Comparative genomics of wheat and rice.

CHAPTER 2

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

Wheat, rice, and maize are the main sources of carbohydrates in human diet all over the world. These three major cereal crops account for more than 85% of all grain production worldwide and more than half of all the food calories (http://faostat.fao.org/site/567/default.aspx#ancor). Hexaploid wheat (*Triticum aestivum* L.) is the second most important crop of the world and the single largest traded crop with a global annual production exceeding 650 million tonnes from an area of 215.26 million hectare. A total of 78.57 million tonnes of wheat was produced in India in 2009 from an area of 27.6 million hectare (Kumar *et al.* 2009). Hexaploid wheat or bread wheat or common wheat is generally used for making bread, cookies, pastries and noodles whereas durum wheat is used for making pasta and other semolina products. Einkorn, emmer and spelt wheats are today the relic crops of minor importance and cultivated on soil with very poor fertility in some parts of Italy (Nesbitt and Samuel 1996).

Triticum aestivum and *T. durum* are the most important spring cereals of India and grown during November to April. India is today the second largest wheat producer in the whole world. Various studies and researches show that wheat and wheat flour play an increasingly important role in the management of India's food economy. Wheat production is about 75 million tonnes per year in India and it accounts for approximately 12 per cent of the world production. India is the second largest in population; it is also the second largest in wheat consumption after China, with a huge and growing wheat demand. Major wheat growing states in India are Uttar Pradesh, Punjab, Haryana, Rajasthan, Madhya Pradesh, Gujarat and Bihar. Wheat has a narrow geographic land base of production as compared to rice or pulses. Wheat is a temperate crop requiring low temperatures whereas most of the country is subtropical to tropical.

2.1 Domestication of cereals

All cereal crops are the world's primary food source and were domesticated from a diverse array of wild grass species. Despite the independent domestication that occurred in

different continents- Africa (sorghum and millet), Asia (rice), the Near East (wheat, barley, oats and rye) and America (maize), all these cereals have undergone a suite of similar modifications from their wild progenitors (Paterson *et al.* 1995). This common suite of traits is known as ''domestication syndrome" that differentiates most seed and food crops from their wild progenitors (Hammer 1984). Cultivated forms typically have larger grains, more robust plants, more determinate growth or increased apical dominance, loss of natural seed dispersal, loss of seed dormancy, ease of seed removal, changes in photoperiod sensitivity and synchronized flowering (Harlan 1975; Hancock 2004). These changes are the basic requirements for effective agronomic operations like planting, seed harvesting and threshing and obtaining higher grain yields which make the cultivation process practical and rewarding (Harper 1977). Although a common set of characters were targeted by domestication in all cereals, improvement in specific traits in different cereals during the domestication process depended mainly on the crop itself (Doebley *et al.* 2006). In wheat, domestication occurred at all three ploidy levels. At diploid level, *Triticum monococcum* subsp. *monococcum* (2n=2x=14), at tetraploid level, *T. turgidum* subsp. *durum* (2n=4x=28) and at hexaploid level, *T. aestivum* subsp. *aestivum* (2n=6x=42) constitute the cultivated forms. The major traits subjected to selection included loss of spike shattering, loss of tough glumes, increased seed size, reduced number of tillers, change in plant architecture and reduced seed dormancy (Dubcovsky and Dvorak 2007). Mutations at these genetic loci were quickly selected and propagated by the early farmers. These mutant types were more attractive to first farmers than their wild relatives because the mutant plants had determinate growth habit, less number of branches, higher apical dominance and they produced spikes that did not shatter and disperse their seeds before harvest or they had naked seeds that simplified flour milling (Feuillet *et al.* 2007). Selection during domestication for several traits happened differently in wheat at different ploidy levels. As an example, in diploid wheat, presence of large seed accompanied by tough rachis constituted the major improvement over the wild progenitor. Although free-threshing diploid wheat forms with soft glume were available, this trait could not gain importance with farmers due to the pleiotropic effects of soft glume allele on agronomic traits. Whereas in tetraploid and hexaploid wheats, free-threshing forms became very popular as negative effects associated with soft glume alleles were buffered due to their polyploidy (Salamini *et al.* 2002).

2.2 Origin and history of wheat evolution

The family Poaceae is an important grass family for humans being as most people on earth depend on grasses including some major crops like wheat, rice and maize for a major portion of their diet. The family Poaceae contains approximately 10,000 species classified into 600 to 700 genera (Kellogg 2001). Grasses originated almost 55-70 million years ago. All the grasses with basic chromosome number of x = 7 have been included in sub family Pooideae which includes familiar crops like wheat, barley, oats and rye (Kellogg 2001). Phylogenetically, wheat is more closely related to rice (sub family Ehrhartoideae) than to maize and sorghum (sub family Panicoideae) (Kellogg 2001). The genus *Triticum* is comprised of an allopolyploid series at three ploidy levels; diploid, tetraploid and hexaploid. At the diploid level, *Triticum monococcum* L. (2n=2x=14, A^mA^m) and *T. urartu* Tumanian ex Gandilyan (2n=2x=14, A^uA^u) are the two main species. *T. monococcum* further includes two subspecies, the cultivated einkorn wheat *T. monococcum* ssp. *monococcum* and the wild type *T. monococcum* ssp. *aegilopoides* whereas *T. urartu* exists only in the wild form (Van Slageren, 1994). The cultivation of *T. monococcum* ssp. *monococcum* is very limited and it is grown only in mountainous regions of Turkey, Italy and Spain where it is used mainly for animal fodder. Polyploid wheat has two lineages one at tetraploid level and another at hexaploid level. The tetraploid wheats include *T. turgidum* L. (2n=4x=28, AABB), and *T. timopheevii* (2n=4x=28, A^tA^tGG). There are two sub species of *T. turgidum*, *T. turgidum* subsp. *dicoccoides*, which is the wild form and *T. turgidum* subsp. *durum* (durum wheat), which is the cultivated sub species. At the hexaploid level, there are also two species, *T. aestivum* L. (2n=6x=42, AABBDD) (common wheat or bread wheat) and *T. zhukovskyi* (2n=6x=42, $A^mA^mA^tA^tGG$) but all hexaploid *Triticum* species are cultivated and therefore they do not have any wild forms (Van Slageren 1994).

2.3 Genome and origin of hexaploid wheat

Earlier cytogenetic studies suggested that the A genomes of the tetraploids in both evolutionary lineages (*T. turgidum* and *T. timopheevi*) were contributed by *T. monococcum* (Kihara 1924; Lilienfeld and Kihara 1934), but later on it was found out as based on variation in esterase enzyme (Nishikawa 1984) and repetitive nucleotide sequences (Dvorak *et al.* 1988; Dvorak *et al.*1993), that *T. urartu* contributed the A genome to both lineages. Dvorak *et al.* (1993) also suggested that in the second hexaploid lineage (*T. zhukovskyi*),

one set of A genomes was contributed by *T. urartu* and the other by *T. monococcum*. Therefore, *T. zhukovskyi* originated from the hybridization of *T. timopheevi* with *T. monococcum* (Upadhya and Swaminathan 1963). *Aegilops speltoides* is considered as the most probable B genome donor of bread wheat and durum wheat (Sarkar and Stebbins 1956, Kimber and Athwal 1972). Evidence based on karyotype data (Riley *et al.* 1958), C-banding of chromosomes (Friebe and Gill 1996), cytological evidence (Kerby and Kuspira 1988), the geographical distributions of wild populations (Witcombe 1983), and restriction fragment length polymorphism (RFLP) analysis of low-copy and repetitive sequences (Dvorak and Zhang 1990, Talbert *et al.* 1991; Sasanuma *et al.* 1996; Pestsova *et al.* 1998) support the idea that the S genome of *Ae. speltoides* is most closely related to the B-genome of bread wheat. Plasmon analysis has also pointed to *Ae. speltoides* as the B-genome donor (Tsunewaki and Ogihara 1983, Tsunewaki 1991). It is well-established that the D-genome of bread wheat was contributed by *Ae. tauschii* (Kihara 1944, McFadden and Sears 1946). Morphological traits of synthetic hexaploid wheats suggest that the direct D-genome donor was *Ae. tauschii* subsp. *strangulata*. Furthermore, studies of the occurrence of the isozyme α-amylase (Nishikawa *et al.* 1984) and aspartate amino transferase (Jaaska 1980) in common wheat provided additional evidence for subsp. *strangulata* as the direct ancestor of D-genome of bread wheat because these isozymes are typical of subsp. *strangulata* but are rare in subsp. *typica*. It has been suggested that somewhere in the Fertile Crescent area, tetraploid wheat hybridized with *Ae. tauschii* and generated spelt like hulled hexaploid wheats. In the origin of hexploid wheat, the involvement of domesticated tetraploid, *T. turgidum* has been speculated as the distribution of wild tetraploid, *T. dicoccoides* does not overlap with the distribution range of *Ae. tauschii* (Nesbitt and Samuel 1996). Based on the diversity analysis of *Ae. tauschii* gene pools, Dvorak *et al.* (1998) demonstrated that D genome of bread wheat is the most closely related to "strangulata" gene pool in Transcaucasia, Armenia and SW Caspian Iran. Hence, the principal area of origin is the southern Caspian basin. Based on the phylogenetic analysis of the *Acc-1* (plastid acetyl-CoA carboxylase) and *Pgk-1* (plastid 3-phosphoglycerate kinase) genes among *Triticum* and *Aegilops* species, Huang *et al.* (2002) suggested the diploid *Triticum* and *Aegilops* progenitors of the A, B, D, G, and S genomes all radiated 2.5-4.5 million years ago (MYA). The A genome of polyploid wheat was derived from *T. urartu* less than half a MYA (Huang et al. 2002), and *T. aestivum* arose from hybridization of *T. turgidum* and *Ae. tauschii* only 8,000 years ago (Nesbitt and Samuel 1996).

The Fertile Crescent is considered as the birth-place of cultivated wheats about 8,000 to 10,000 years ago. This region spans the modern day Isreal, Jordan, Lebanon, western Syria, and southeast Turkey and along the Tigris and Euphrates rivers into Iraq and western flanks of Iran. Wheat was one of the first crops to be domesticated among all crop plants (Diamond 1997; Moore *et al.* 2000). Pure stands of wild diploid einkorn and wild tetraploid emmer are found in the Fertile Crescent area and may have been harvested and cultivated as such. The first wheat to be domesticated successfully was einkorn wheat, *T. monococcum*. Genetic evidence indicates that einkorn wheat (*T. monococcum*) may have been domesticated from wild einkorn (*T. monococcum* ssp. *aegilopoides)* in the region of the Karacadag mountains in southeast Turkey (Heun *et al.*1997, Dvorak *et al.* 2006). Both wild and cultivated einkorn seed remains have been excavated in the nearby archaeological sites dating from 7500 to 6200 BC.

Wheat is a young polyploid and it shows alterations in genome structure and expression upon polyploidization specifically brought about by important genetic and epigenetic changes (Levy and Feldman 2004). The cyclic translocation involving chromosomes 4A, 5A and 7B, which arose after polyploidization in tetraploid wheat (Naranjo *et al.* 1987; Jiang and Gill 1994a, b) provides an isolated example of gross chromosomal changes upon polyploidy. Evolutionarily, wheat is a highly successful polyploid crop. Its genome is highly stable and displays diploid like chromosome pairing behavior. Gene loss and altered gene expression upon polyploidization has been observed in synthetic allopolyploids of wheat (Kashkush *et al.* 2002; Kashkush *et al.* 2003; He *et al.* 2003; Levy and Feldman 2004).

2.4 Diploid inheritance

Although bread wheat is a polyploid, it is genetically stable and fertile mainly due to its diploid like behavior of chromosome pairing during meiosis. Hexaploid wheat (*T. aestivum*; 2n=6x=42; AABBDD) possess three related ancestral genomes A, B and D. Although these three sub-genomes are closely related and the gene content and order is highly conserved between homoeologous chromosomes, but still only the homologous chromosomes belonging to the same genome, pair at meiosis, and the recombination between homoeologous chromosomes is highly suppressed. This behavior is due to the role of pairing suppressors. The strongest effect on pairing is associated with the *Ph1* (pairing

homoeologous) gene, a single dominant locus on chromosome arm 5BL of wheat (Okamoto 1957; Riley and Chapman 1958; Sears and Okamoto 1958). The *Ph1* locus restricts chromosome pairing and recombination at meiosis to true homologues. Deletions for the *Ph1* locus have been isolated in both hexaploid (*ph1b*) (Sears 1977) as well as tetraploid (*ph1c*) wheat (Giorge 1978). The mutants carrying deletion of the *Ph1* locus exhibit a degree of pairing of related (homoeologous) chromosomes and hence show some multivalent formation at metaphase I of meiosis (Sears 1977). More importantly, the *Ph1* mutants allow pairing between homoeologues chromosomes from related species and genera and thus can aid in transfer of desirable genes from distant related species to cultivated wheats (Sears 1981; Sears 1983). Some studies have suggested that *Ph1* arose upon polyploidization due to the absence of *Ph1* activity in diploid relatives of wheat (Riley *et al.* 1961). The *Ph1* locus has been localized to a 2.5 Mb interstitial region of wheat chromosome 5B containing a structure consisting of a segment of sub-telomeric heterochromatin that inserted into a cluster of *cdc2* (*cdk*)-related genes following polyploidization (Griffiths *et al.* 2006). A second distinct genetic activity affecting homoeologous chromosome pairing was discovered by Riley *et al.* (1961) where *Ae. speltoides* was found to possess a dominant inhibitor of the *Ph1*. Wheat *Ph1* suppressors with major effects have been mapped as Mendelian loci on the long arms of *Ae. speltoides* chromosomes 3S and 7S (Dvorak *et al.* 2006). Understanding the gene structure of *Ph1* and identification of its suppressors has important implications regarding wide transfers of desirable genes from related wild species and genera Einkorn wheat (*T. monococcum* subsp. *monococcum*) is the only cultivated diploid wheat. It has a tough rachis but is non-free-threshing due to the presence of tough glumes. Although a spontaneous free threshing mutant of *T. monococcum* subsp. *monococcum* referred to as *T. sinskajae* has been reported to possess soft glumes (Filatenko and Kurkiev 1975 cited by Gonchariov *et al.* 2002), the free-threshing einkorn wheat could not be used for large scale cultivation due to the association of the soft glume trait with reduced ear length (Salamini *et al.* 2002). A single, recessive gene *sog* controlling *s*oft glume trait in *T. sinskajae* was mapped on the short arm of chromosome $2A^m$ (Taenzler *et al.* 2002). The major genetic factors responsible for glume tenacity and threshability have been located on short arm of homoeologous group-2 chromosomes in wheats of different ploidy levels (Taenzler *et al.* 2002; Jantasuriyarat *et al.* 2004) but their orthologous relationships are not known. The genome size of wheat and its

relative species are given below (www2.le.ac.uk/departments/mathematics/extranet/.../bio/...1/.../file).

Hordeum vulgaris (barley) 2n=2x =14	5,550Mb
Secale cereale (rye) 2n=2x =14	8,280Mb
Triticum monoccocum 2n=2x =14	6,230Mb
Aegilops tauschii 2n= 2x =14	5,010Mb
Ae. Speltoides 2n=2x =14	5,800Mb
T. durum (durum wheat) 2n=4x =28	12,030Mb
T. aestivum (bread wheat) 2n=6x =42	17,330Mb

2.5 *Triticum monococcum* as a model crop

The diploid nature of *T. monococcum* with smaller genome size of 5,700 Mb compared to 17,300 Mb of bread wheat (Bennett and Leitch 1995), the existence of a very high level of polymorphism for DNA based markers (Castagna *et al.* 1994), sequence conservation at orthologous loci (Wicker *et al.* 2003) and availability of a large BAC library (Lijavetzky *et al.* 1999) makes this species an attractive diploid model for gene discovery in wheat

Einkorn wheat was one of the earliest cultivated forms of wheat, alongside emmer wheat (*T. dicoccon*). Grains of wild einkorn have been found in Epi-Paleolithic sites of the Fertile Crescent. It was first domesticated approximately 9000 BP (9000 BP ≈ 7050 BCE), in the Pre-Pottery Neolithic A or B periods. Evidence from DNA finger-printing suggests einkorn was domesticated near Karacadag in southeast Turkey, an area in which a number of PPNB (Pre- Pottery Neolithic B) farming villages have been found (Zohary and Hopf 2000). Its cultivation decreased in the bronze age, and today it is a relict crop that is rarely planted. It remains as a local crop, often for bulgur (cracked wheat) or as animal feed, in mountainous areas of France, Morocco, the former Yugoslavia, Turkey and other countries. It often survives on poor soils where other species of wheat fail.

Archaeological evidence indicates that western agriculture began in the Near East with the remains of founder crops preserved at several excavated sites throughout the region known as the Fertile Crescent (Heun *et al.* 1997; Moore *et al.* 2000; Zohary and Hopf 2000; Gopher *et al.* 2001; Salamini *et al.* 2002). Beginning about 12,000 years ago, the size and morphology of archaeologically preserved einkorn seeds (*Triticum monococcum*, genome

AA with 2n = 14) changed, with the smaller seeds of wild einkorn (*Triticum monococcum ssp. boeoticum*) gradually being replaced at excavated sites by the larger seeds of it's domesticate form (*Triticum monococcum ssp. monococcum*) (Zohary and Hopf 2000) and genetic data for einkorn are consistent with that view (Heun *et al.* 1997; Abbo *et al.* 2006). In crop grasses of the Fertile Crescent—wheat, barley, and rye—domestication is currently thought to mainly involve allele frequency changes at loci governing seed size, rachis stiffness, and bract morphology (Salamini *et al.* 2002), but the mechanisms through which humans evoked those morphological changes are still debated. Evidence from archaeological excavation sites indicates that the process of crop domestication spanned up to 10,000 years and entailed multiple domestication events (Hillman and Davies 1990; Willcox 1999; Kislev 2002; Salamini *et al.* 2002; Tanno and Willcox 2006). Such evidence stands contrary to molecular studies that have suggested a single domestication of each of the Fertile Crescent founder crops (Heun *et al.* 1997; Badr *et al.* 2000; Zohary and Hopf 2000). A third view of domestication suggests that superior varieties emerged in a "core area" and were then dispersed throughout the region, displacing local genotypes (Lev-Yadun *et al.* 2000; Salamini *et al.* 2002; Abbo *et al.* 2006). All of these models predict a reduction of genetic diversity in domesticated forms relative to the wild progenitors through a domestication bottleneck—a reduction in genetic diversity stemming from human selection upon domestication traits—as reported in various domesticated species (Pozzi *et al.* 2004; Doebley *et al.* 2006; Kilian *et al.* 2006). However, distinguishing between reduction of genetic diversity through a domestication bottleneck introduced over 10,000 years ago and reduction through intensive breeding bottlenecks during the last few hundred years is extremely problematic (Kilian *et al.* 2006), and domestication genetic studies of Old World crops that escaped intense breeding are lacking. Einkorn is unique in this respect because it was of limited agricultural use during the last 5,000 years: the crop was largely abandoned as a food source starting in the Bronze Age and intensive breeding was never undertaken (Salamini *et al.* 2002). As a consequence, domesticated einkorn germplasm sampled in remote mountain areas across Europe and the Near East (Perrino *et al.* 1996) should harbor a representative sample of Neolithic genetic variation that was present during domestication in the Fertile Crescent and that was later dispersed by migrating farmers (Nesbitt and Samuel 1996).

The chromosome complement of hexaploid wheat, *Triticum aestivum* L. ($2n = 6x = 42$), consists of three genomes A, B and D. Genetic analysis and gene discovery in hexaploid wheat has been arduous because of large genome size (»17,300 Mb—Bennette and Leitch 1995), abundance of repetitive DNA sequences (SanMiguel *et al.* 2002; Wicker *et al.* 2003) and limited polymorphism (Gale *et al.* 1990). As a result only a few successes of map based gene cloning have been reported in hexaploid wheat. Of the three homoeologous genomes of hexaploid wheat, donors of the A and D genome are well defined. *T. urartu* contributed the A genome (Dvorak *et al.* 1988) and *Aegilops tauschii* Coss. contributed the D genome (McFadden and Sears 1946). *Triticum monococcum* ssp. *monococcum* L. ($2n = 2x = 14$), a diploid A genome species, is domesticated and *T. monococcum* ssp. *aegilopoides* (Link) Thell. (*T. boeoticum*) ($2n = 2x = 14$) is a wild form of *T. monococcum* ssp. *monococcum*. Both, *T. monococcum* ssp *monococcum* (A^m) and *T. monococcum* ssp. *aegilopoides* (A^m) are very closely related to *T. urartu* (A^u) and to the A genome of hexaploid wheat, *T. aestivum* but are reproductively isolated from *T. urartu* (Johnson and Dhaliwal 1976). *T. monococcum* and *T. boeoticum* on the other hand do not show any evidence of differentiation (Dvorak *et al.* 1988) as hybrids between the two species form seven bivalents (Kihara *et al.* 1929; Dubcovsky *et al.* 1996) and viable seeds (Johnson and Dhaliwal 1976). *T. monococcum* was domesticated around 10,000 years ago (Heun *et al.* 1997) and is the only diploid cultivated species of wheat. The A^m and A^u genomes of *T. monococcum* and *T. urartu* diverged some 1.0 MYA (Huang *et al.* 2002) and the A and D genomes diverged some 2.7 MYA (Dvorak and Akhunov 2005). Molecular linkage map of diploid wheat using RFLP markers showed that gene content and order were well conserved between A-genome of wheat and *T. monococcum* except for the chromosomes 4A and 5A that were involved in chromosome interchanges (Dubcovsky *et al.* 1996). Chromosomes 4A, 5A and 7B of wheat were involved in cyclical translocations where chromosome 4A further differed from $4A^m$ of *T. monococcum* by having one pericentric and one paracentric inversion (Devos *et al.* 1995; Mickelson-Young *et al.* 1995). Because of the presence of high-levels of polymorphism and ease of working with single genome, diploid progenitor species of wheat have been used for developing genetic linkage maps (Dubcovsky *et al.* 1996; Boyko *et al.* 1999; Luo *et al.* 2005; Singh *et al.* 2007) that complemented the genome analysis in wheat. Recently, progenitor diploid species were used for gene discovery in wheat by utilizing genomic resources such as BAC libraries of

T. monococcum (Feuillet *et al.* 2003; Yan *et al.* 2003, 2004) and *Ae. tauschii* (Huang *et al.* 2003). The diploid nature of *T. monococcum* with smaller genome size of 5,700 Mb compared to 17,300 Mb of bread wheat (Bennett and Leitch 1995), the existence of a very high level of polymorphism for DNA based markers (Castagna *et al.* 1994), sequence conservation at orthologous loci (Wicker *et al.* 2003) and availability of a large BAC library (Lijavetzky *et al.* 1999) makes this species an attractive diploid model for gene discovery in wheat. The vernalization genes *VRN1* and *VRN2* were cloned in wheat by using the naturally existing variation in *T. monococcum* (Yan *et al.* 2003, 2004). BAC libraries of *T. monococcum* were used for cloning the disease resistance gene *Lr10* (Feuillet *et al.* 2003) and a domestication locus *Q* in wheat (Faris *et al.* 2003; Simons *et al.* 2006). *T. monococcum* was also used to map the genetic factors responsible for earliness *per se* in wheat (Bullrich *et al.* 2002; Valarik *et al.* 2006). *T. monococcum* was used for developing loss of function mutations for a large number of agronomic traits using ethyl methane sulphonate based mutagenesis (Dhaliwal *et al.* 1987). One of the tillering mutant *tin3* from the above EMS mutant collections of *T. monococcum* was mapped on chromosome arm $3A^{m}L$ (Kuraparthy *et al.* 2007) and soft glume gene (*sog*) on chromosome arm $2A^{m}L$ (Sood *et al.* 2009) in Triticeae, further indicating that the *T. monococcum* could be a reference diploid genome for gene discovery in wheat. In addition, A genome linkage map based on diploid wheat will be helpful for selective introgression of desired traits from diploid A genome species to hexaploid wheat. *Triticum boeoticum*, the progenitor of cultivated *T. monococcum*, is sympatric to *T. urartu* over its entire distribution (Johnson and Dhaliwal 1976) and shows immense variability for a number of biotic and abiotic stresses and for productivity traits. All the three species are a rich source of variability for resistance to several diseases like leaf rust (Hussien *et al.* 1997; Bai *et al.* 1998; Anker and Niks 2001), stripe rust (Dhaliwal *et al.* 1993b; Harjit-Singh *et al.* 1998), Karnal bunt (Kuraparthy *et al.* 2000), powdery mildew (Shi *et al.* 1998; Lebedeva and Peusha 2006), partial resistance to cereal aphid (Migui and Lamb 2004), grain protein and carotene content, spikelets per spike, grain weight (Castagna *et al.* 1995), as well as for cereal cyst nematode and many productivity related traits. Molecular linkage map of A genome diploid wheat have been developed using RFLP (Dubcovsky *et al.* 1996) and AFLP markers (Taenzler *et al.* 2002). The available series of simple sequence repeat (SSR) markers have been developed from hexaploid wheat and from *T. urartu* and *Ae. tauschii*, the A and D

genome donors of wheat, respectively (http://www.wheat.pw.usda.gov/GG2/quickquery.). Integration of polymerase chain reaction (PCR) based markers and RFLPs into a single linkage map will not only complement the existing hexaploid wheat map but will also serve as the base map for mapping both qualitative and quantitative traits in diploid A genome wheat, their map-based cloning and for monitoring their introgression into wheat. An integrated molecular linkage map of diploid wheat based on a *T. boeoticum* x *T. monococcum* RIL population (Singh *et.al* 2007). The RIL population will also allow the molecular mapping of agronomically important traits such as resistance to leaf rust, stripe rust, Karnal bunt, cereal cyst nematode, powdery mildew, spikelets per spike, grain weight, tillering and flowering time and for domestication trait like single vs. two seeds per spikelet.

2.5.1 EMS mutagenesis

The ability to induce mutations has been a major driving force in genetics for the past 85 years (Muller 1930). Physical and chemical mutagens have long been successfully used in plant breeding programs to artificially generate genetic variation for the development of new varieties with improved traits such as earliness, reduced height and resistance to diseases. In recent years with the availability of genomic sequence, induced mutants have also become a powerful source for investigation of gene function and expression (McCallum *et al.* 2000; Tor *et al.* 2002; Hecht *et al.* 2007). Various approaches for mutagenesis involving chemical, irradiation, and insertional methods have been developed where each has its own merits for the study of gene function. Among the mutagens that have been used to induce mutations, chemical mutagens administered in various ways have become especially popular. Alkylating agents, such as ethyl methanesulfonate (EMS) are particularly effective to generate point mutations. EMS mutagenesis in plants is the most widely used mutagenesis technique. EMS has high mutagenicity, low mortality rate and ease of use. The chemical principle of EMS mutagenesis is based on its ability to alkylate guanine bases, which results in base mispairing. An alkylated guanine will pair with a thymine base and results primarily in G/C to A/T transitions, which ultimately results in an amino acid change or deletion (Maple and Moller 2007). There are several advantages to EMS mutagenesis compared with other mutagenesis techniques. First, EMS generates a high density of random irreversible

mutations in the genome, which permits saturation mutagenesis without having to screen a large number of individual mutants. Second, EMS mutagenesis not only generates loss-of-function mutants, but can also generate novel mutant phenotypes, which include dominant or gain-of-function proteins owing to alterations of specific amino acids (Kim *et al.* 2006). Large-scale mutagenesis has been carried out in numerous plant systems and several mutant populations have been generated in *Arabidopsis*, rice, maize, wheat, barley, soybean, pea (Till *et al.* 2003; Hirochika *et al.* 2004; Till *et al.* 2004; Caldwell *et al.* 2004; Slade *et al.* 2005; Cooper *et al.* 2008). With the availability of advanced genomic resources and the progress being made in sequencing the wheat genome (Paux *et al.* 2008), importance and demand for these mutant resources will further increase in wheat as valuable tools in reverse genetics analysis to identify novel gene function and expression.

2.5.2 *Agrobacterium tumefaciens* mediated transformation of cereals and T-DNA insertional mutants

Agrobacterium is widely used bacterial genus which is capable of transferring genes to plants. *Agrobacterium tumefaciens* containing a Ti (*tumor inducing*) plasmid is a ubiquitous soil bacterium that induces crown galls on dicot plants. Insertional mutagenesis using maize transposon elements Activator/Dissociation (Ac/Ds), Enhancer/Suppressor mutator (En/Spm; Martienssen 1998), or Agrobacterium T-DNA (Azpiroz-Leehan and Feldmann 1997) have been widely used for generating knockout mutations for reverse genetics and thus revealing the gene function in rice (Chin *et al.* 1999; Puri *et al.* 2009, Kumar *et al.* 2010). T-DNA insertions in the genome are stable while the transposons have the ability to transpose to nearby locations providing a convenient method for mutations within a segment of chromosome (Krysan *et al.* 1999). Insertional mutagenesis in principle provides a more rapid way to clone a mutated gene. As the sequence of the inserted element is known, the gene in which it is inserted can be easily identified using various cloning or polymerase chain reaction (PCR)-based strategies (Bouchez and Hofte 1998). Rice and wheat has been transformed using *A. tumefaciens* mediated transformarion (Dhaliwal *et al.* 2001, Wu *et al.* 2003, Rashid *et al.* 1996, Hensel *et al.* 2009). Several mutants like polyembryo, dwarf culm, oligoculm and seedling lethal generated by T-DNA/Ds insertion in *Oryza sativa*, cv. Basmati 370 and cloned. (Puri *et al.* 2009, Kumar *et al.* 2010).

2.6 Dwarf mutants and dwarfing genes in green revolution

The term "Green Revolution" refers to the huge increases in grain yields after 1960s. It was the outcome of the introduction of new dwarf varieties of wheat and rice for cultivation in the developing world. It was recognized in 1978 by the award of Nobel Peace prize to Dr. Norman E. Borlaug of the International Maize and Wheat Improvement Center (CIMMYT), Mexico that increase in food grain production was a major factor in maintaining per capita food supplies worldwide in the late-twentieth century, despite a doubling in the world population during this time (Evans 1998). Prior to the green revolution, scientists developed high yielding wheat and rice varieties suitable for growing in subtropical and tropical climates. These varieties were higher grain yielder under increased use of fertilizers and pesticides. However, the heavier grain caused the plants to become unstable and prone to lodging during high winds and rain. Dr. N. E. Borlaug introduced dwarfing genes into wheat, giving the plants a stronger and shorter stem that resisted lodging. These genes resulted in the increase in grain yield of both wheat and rice through an improvement in the "harvest index" (the proportion of plant weight in the grain) because a greater proportion of the products of photosynthesis accumulated in the grains rather than in the leaves (http://4e.plantphys.net/article.php?ch=&id=355).

2.6.1 Gibberellic acid biosynthesis

The endogenous phytohormone gibberellins (GA) are a group of the several compounds, which form a large family of tetracyclic diterpenoid phytohormones. GA plays role in the regulation of various growth and developmental processes which are associated with dwarf phenotype in higher plants. Bioactive GAs, such as GA_1 and GA_4, are synthesized from trans-geranylgeranyl diphosphate (GGDP) as shown in Fig. 2.1. (Hedden and Kamiya 1997; Hedden and Phillips 2000). GGDP is converted to the tetracyclic hydrocarbon *ent*-kaurene via *ent*-copalyl diphosphate (CDP) by two kinds of diterpene cyclases in plastids, CDP synthase (CPS) and *ent*-kaurene synthase (KS) *ent*-kaurene is then modified by sequential oxidations to produce GA_{12} via ent-kaurenoic acid. These steps are catalyzed by two membrane-associated Cyt P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The final stage of bioactive GA synthesis, from GA_{13}/GA_{12} to GA_1/GA_4, is catalyzed through two parallel pathways (i.e. early-13-hydroxylation and non-13-hydroxylation pathways) by two soluble 2-oxoglutarate-dependent dioxygenases (2ODDs) in the cytosol, GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox). The bioactive GA_1/GA_4 and their immediate precursors GA_{20}/GA_9 are

inactivated by a third 2ODD, GA 2-oxidase (GA2ox). In some species, GA_9 and GA_{20} are also converted to GA_7 and GA_3, respectively via 2, 3-didehydroGA_9 and GA_5 (Albone *et al.*, 1990). The genes encoding the seven GA metabolic enzymes (CPS, KS, KO, KAO, GA20ox, GA3ox, and GA2ox) have been isolated from various plants (Hedden and Phillips, 2000; Sakamoto *et al.*, 2004). Singh (2003) studied the biochemical effects of exogenous GA_3 on morphologically GA_3 responding and non-responding rice genotypes by the foliar spray of GA_3 at 120 ppm on 20 and 45 days old rice plants. He found that GA_3 responding dwarf genotypes showed marked growth, morphological and biochemical response to GA_3 as compared to non-responding GA_3 genotypes.

2.6.2 Regulation of genes by GA-signaling

GA biosynthesis is a complex process which is governed by GA signal transduction. Hedden and Kamiya (1997) defined GA signal transduction as "the series of biochemical events leading from the perception of the active GA molecule for the final response." The mechanism by which plants use to maintain GA homeostasis (i.e. keeping the concentrations of bioactive GAs within certain limits) involves feedback regulation of GA biosynthesis, as illustrated in Fig. 2.2. A certain GA-receptor present in the cell transfers GA-signal to two different genes A and B. This causes the genes to either switch-off or on so that several GA-dependant responses are carried out.

2.6.3 Gibberellic acid biosynthetic and signaling mutants in plants

The GA-related mutants may be divided into two groups: those that influence GA-hormone levels (hence called GA-synthesis mutants) and those that influence GA-hormone response (hence called GA-response mutants). The GA_3 synthesis mutants respond to the exogenous application of GA with stem elongation and are called GA-sensitive mutants. Most of the GA-sensitive mutants are recessive and involve the loss of wild-type function (Herskowitz, 1987). GA_3 response mutants do not respond to the exogenous application of GA and participate in GA-signaling in plants. The factors responsible for GA-signaling can be broadly divided into positive and negative regulators, depending upon their effects on the final GA-induced responses. Several positive and negative regulators of GA-signal transduction have been identified in several plant species. List of positive and negative regulators of GA-signaling along with their function in different plant species have been summarized in Table 2.1 and Table 2.2.

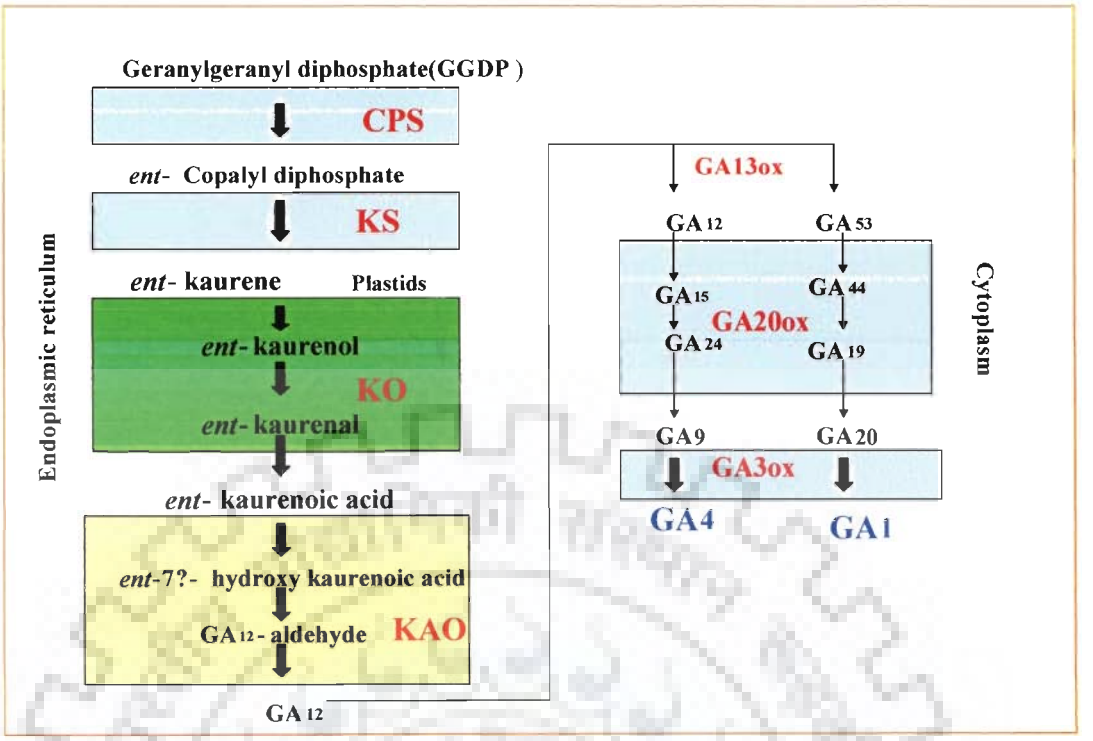


Figure 2.1 GA_3-biosynthetic pathway in plants

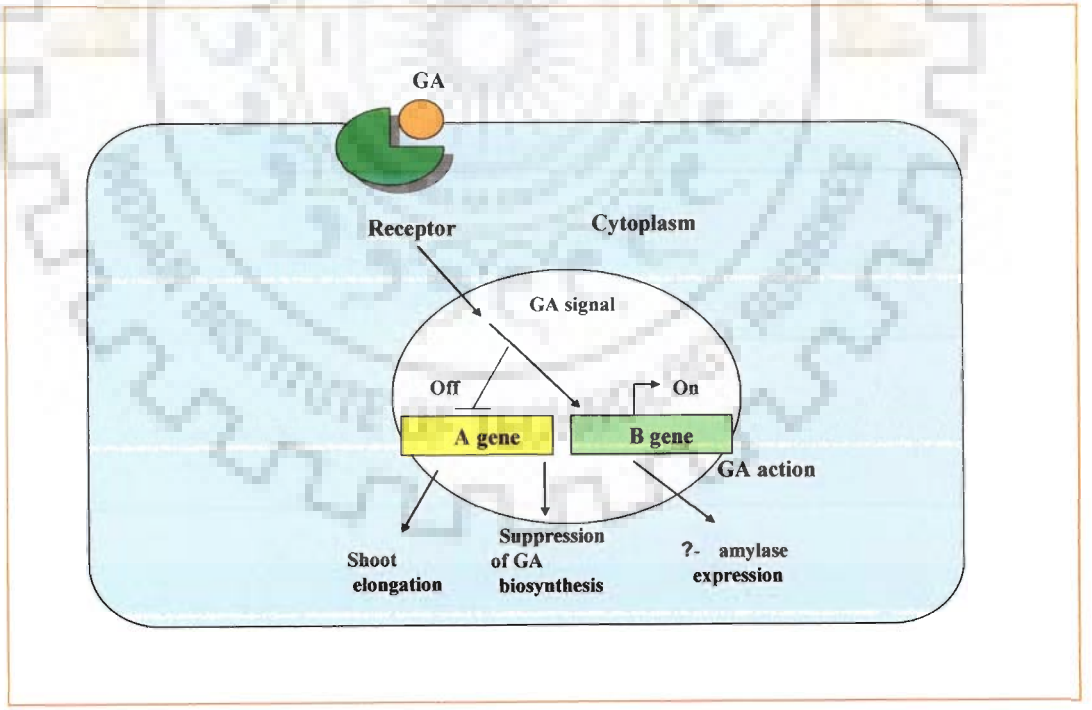


Figure 2.2 Gibberellic acid signaling pathway in plants

Table 2.1 Positive regulators of GA-signaling in different plants

Crop	Gene	Symbol	Function	Reference
Rice	*Dwarf1*	*D1*	Encodes a heterotrimeric G protein α-subunit	Ashikari *et al.* 1999; Fujisawa *et al.* 1999
Rice	*Gibberellin Insensitive Dwarf1*	*GID1*	GA-receptor	Sasaki *et al.* 2001
Rice	*Gibberellin Insensitive Dwarf2*	*GID2*	F-box protein	Sasaki *et al.* 2003
Potato	*Photoperiod Responsive*	*PHOR1*	Growth under short days	Amador *et al.* 2001
Barley	*Ga-Induced Myb Trancription Factor*	*GAMYB*	Activates α-amylase promoter	Gocal *et al.* 2001
Arabidopsis	*Ga-Induced Myb Trancription Factor (Glabrous)*	*GL1*	Initiation and branching of trichomes	Perazza *et al.* 1998
Arabidopsis	*Sleepy*	*SLY*	Seed germination, F-box protein	Mc Ginnis *et al.* 2003; Sasaki *et al.* 2003; Dill *et al.* 2004
Arabidopsis	*Pickle*	*PKL*	Formation of ectopic stipules and meristems in leaves, recessive mutation affects GA-induced differentiation of the seedling primary root.	Oogas *et al.* 1997; Ori *et al.* 2000

Table 2.2: Negative regulators of GA-signaling in different plants

Crop	Gene	Symbol	Mutant phenotype	Reference
Rice	*Slender rice1*	*SLR1*	Recessive, increased growth	Itoh *et al.* 2002; Ikeda *et al.* 2001
Arabidopsis	*Repressor of GA1-3*	*RGA 1-3, GAI*	Semidominant, dwarf	Peng *et al.* 1997; Peng *et al.* 1999; Silverstone *et al.* 1998
Barely	*Slender1*	*SLN1*	Recessive, increased growth	Chandler *et al.* 2002; Gubler *et al.* 2002
Maize	*Dwarf 8*	*D8*	Semidominant, dwarf	Peng *et al.* 1999
Wheat	*Reduced height1and Reduced height 2*	*Rht1, Rht2*	Semidominant, dwarf	Peng *et al.* 1999
Arabidopsis	*Spindly*	*AtSPY*	Recessive, Seed germination reduced	Jacobson and Olszewski 1993
Barley	*Spindly*	*HvSPY*	Recessive slender, resembles wild-type plants that have been treated with GA	Jacobson *et al.* 1996; Robertson *et al.* 1998
Petunia	*Spindly*	*PhSPY*	-Do-	Izhaki *et al.* 2001
Arabidopsis	*Short internode*	*SHI*	Semidominant, Zinc-finger transcription factor	Fridborg *et al.* 1999

The dwarf mutants containing negative regulator are either semi-dominant or recessive. The semi-dominant mutation in *Arabidopsis* (*gai* and *rgal-3*), maize (*d8*), wheat (*Rht1* and *Rht2*) and barley (*Hrt*) cause dwarf phenotype whereas recessive loss-of-function mutations in rice (*slr1*) and barley (*sln1*) cause increased growth. The wild type proteins of semi dominant mutants have a highly conserved DELLA domain which is the target for proteolysis by the ubiquitin-proteasome mediated pathway. The *gai-1* mutant in *Arabidopsis* contains a 51 bp in-frame deletion that results in the loss of 17 amino acids within the DELLA domain. The *gai* was cloned via *Ds*-transposon insertional mutagenesis (Peng *et al.* 1997). SPY shows sequence similarity to Ser (Thr)-*O*-linked N-acetyl glucosamine (O-GlcNAc) which regulates target protein function by glycosylation of serine or threonine residues (Hart 1997).

2.6.4 Dwarfing genes of Wheat

Many genes are associated with a semi-dwarf growth habit of wheat (Ellis *et al.* 2005) which are known as *Reduced height* (*Rht*) genes. Many of the *Rht* genes are dominant or semi-dominant which actively inhibits growth through a gain-of-function mutation. *Rht-B1b* and *Rht-D1b*, having an additive effect on growth, were formerly called *Rht1* and *Rht2* and are being used in many commercial wheat varieties. The *Rht-B1b* and *Rht-D1b* have a series of multiple alleles which cause semi-dwarfing to extreme-dwarfing phenotypic expressions (Ivandic *et al.* 1999). The *Rht-B1b* and *Rht-D1b* dwarfing genes were derived from 'Norin10' 10, which was a semi-dwarf variety bred in Japan and released in 1935 (Gale and Youssefian 1985). Norin 10 was used in United States wheat breeding programs during 1950s for improving lodging resistance in winter wheat under high rates of nitrogenous fertilizer application. A selection from a cross of Norin 10 with the United States variety, Brevor, was high grain yielder and used by Dr. Borlaug in the development of the "Green Revolution" wheat varieties. The wild-type (non mutant) alleles of *Rht-B1b* and *Rht-D1b* were isolated a few years ago (Peng *et al.* 1999) and shown to encode DELLA proteins, which are the components of the GA signal transduction pathway. Peng *et al.* (1999) found that both *Rht-B1b* and *Rht-D1b* dwarfing alleles contained a point mutation which introduced a stop codon into a conserved region known as the DELLA domain, present near the N-terminus of the proteins.

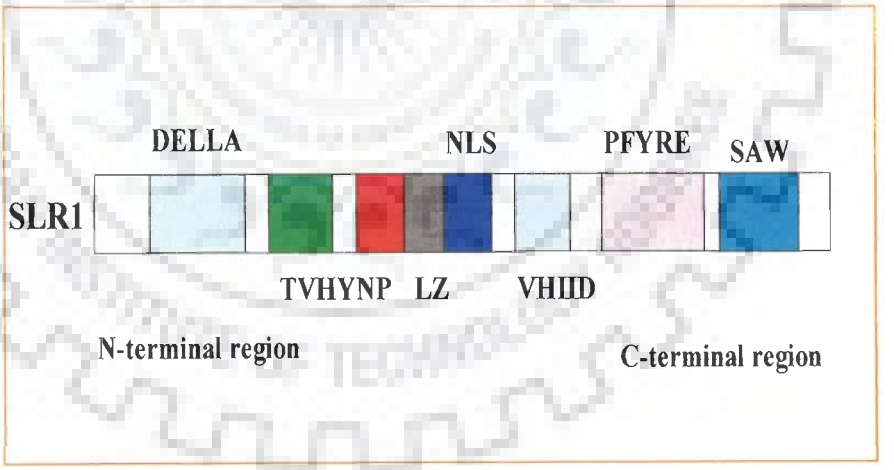
Dwarfing or reduced height (*Rht*) genes have been associated with large increases in the yield potential of cereals and have been a key component of the Green Revolution since

ATGAAGCGGGAGTACCAGGACGCCGGAGGGAGCGGCGGCGGCGGTGGCGGCATGGGCTCGTCCGAGGACAAGATG
ATGGTGTCGGCGGCGGCGGGGGAGGGGGAGGAGGTGGACGAGCTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCT
CCGACATGGCGGACGTGGCGCAGAAGCTGGAGCAGCTCGAGATGGCCATGGGGATGGGCGGCGTGGGCGCCGGCGC
CGCCCCCGACGACAGCTTCGCCACCCACCTCGCCACGGACACCGTGCACTACAACCCCACCGACCTGTCGTCTTGGG
TCGAGAGCATGCTGTCGGAGCTCAACGCGCCGCCGCCGCCCCTCCCGCCCGCCCCGCAGCTCAACGCCTCCACCTCC
TCCACCGTCACGGGCAGCGGCGGCTACTTCGATCTCCCGCCCTCCGTCGACTCCTCCAGCAGCATCTACGCGCTGCGG
CCGATCCCCTCCCCGGCCGGCGCGACGGCGCCGGCCGACCTGTCCGCCGACTCCGTGCGGGATCCCAAGCGGATGCG
CACTGGCGGGAGCAGCACCTCGTCGTCATCCTCCTCCTCGTCGTCTCTCGGTGGGGGCGCCAGGAGCTCTGTGGTGG
AGGCTGCCCCGCCGGTCGCGGCCGCGGCCAACGCGACGCCCGCGCTGCCGGTCGTCGTGGTCGACACGCAGGAGGC
CGGGATTCGGCTGGTGCACGCGCTGCTGGCGTGCGCGGAGGCCGTGCAGCAGGAGAACCTCTCCGCCGCGGAGGCG
CTGGTGAAGCAGATACCCTTGCTGGCCGCGTCCCAGGGCGGCGCGATGCGCAAGGTCGCCGCCTACTTCGGCGAGGC
CCTCGCCCGCCGCGTCTTCCGCTTCCGCCCGCAGCCGGACAGCTCCCTCCTCGACGCCGCCTTCGCCGACCTCCTCCA
CGCGCACTTCTACGAGTCCTGCCCCTACCTCAAGTTCGCGCACTTCACCGCCAACCAGGCCATCCTGGAGGCGTTCGC
CGGCTGCCGCCGCGTGCACGTCGTCGACTTCGGCATCAAGCAGGGGATGCAGTGGCCCGCACTTCTCCAGGCCCTCG
CCCTCCGTCCCGGCGGCCCTCCCTCGTTCCGCCTCACCGGCGTCGGCCCCCCGCAGCCGGACGAGACCGACGCCCTG
CAGCAGGTGGGCTGGAAGCTCGCCCAGTTCGCGCACACCATCCGCGTCGACTTCCAGTACCGCGGCCTCGTCGCCGC
CACGCTCGCGGACCTGGAGCCGTTCATGCTGCAGCCGGAGGGCGAGGAGGACCCGAACGAGGAGCCCGAGGTAATC
GCCGTCAACTCAGTCTTCGAGATGCACCGGCTGCTCGCGCAGCCCGGCGCCCTGGAGAAGGTCCTGGGCACCGTGCG
CGCCGTGCGGCCCAGGATCGTCACCGTGGTGGAGCAGGAGGCGAATCACAACTCCGGCACATTCCTGGACCGCTTCA
CCGAGTCTCTGCACTACTACTCCACCATGTTCGATTCCCTCGAGGGCGGCAGCTCCGGCGGCGGCCCATCCGAAGTCT
CATCGGGGGCTGCTGCTGCTCCTGCCGCCGCCGGCACGGACCAGGTCATGTCCGAGGTGTACCTCGGCCGGCAGATC
TGCAACGTGGTGGCGTGCGAGGGGGCGGAGCGCACAGAGCGCCACGAGACGCTGGGCCAGTGGCGGAACCGGCTGG
GCAACGCCGGGTTCGAGACCGTCCACCTGGGCTCCAATGCCTACAAGCAGGCGAGCACGCTGCTGGCGCTCTTCGCC
GGCGGCGACGGCTACAAGGTGGAGGAGAAGGAAGGCTGCCTGACGCTGGGGTGGCACACGCGCCCGCTGATCGCCA
CCTCGGCATGGCGCCTGGCCGGGCCGTGA

(a)

MKREYQDAGGSGGGGGMGSSEDKMMVSAAAGEGEEV[illegible]LGYKVRASDMADVAQKLEQLEMAMGMGGVG
AGAAPDDSFATHLATDTVHYNPTDLSSWVESMLSELNAPPPPLPPAPQLNASTSSTVTGSGGYFDLPPSVDSSSSIYALRP
IPSPAGATAPADLSADSVRDPKRMRTGGSSTSSSSSSSSLGGGARSSVVEAAPPVAAAANATPALPVVVVDTQEAGIRL
VHALLACAEAVQQENLSAAEALVKQIPLLAASQGGAMRKVAAYFGEALARRVFRFRPQPDSSLDAAFADLLHAHFYES
CPYLKFAHFTANQAILEAFAGCRRVHVVDFGIKQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKL
AQFAHTIRVDFQYRGLVAATLADLEPFMLQPEGEEDPNEEPEVIAVNSVFEMHRLLAQPGALEKVLGTVRAVRPRIVTV
VEQEANHNSGTFLDRFTESLHYYSTMFDSLEGGSSGGGPSEVSSGAAAAPAAAGTDQVMSEVYLGRQICNVVACEGAE
RTERHETLGQWRNRLGNAGFETVHLGSNAYKQASTLLALFAGGDGYKVEEKE GCLTLGWHTRPLIATSAWRLAGP

(b)



(c)

Figure 2.3 Structure of Rht-D1 in wheat (a) Nucleotide sequence (b) Translated AAs (c) Domain

(Source Peng *et al.* 1999)

they were introduced in wheat and rice breeding programs some 40–50 years ago (Evans 1993). Most current wheat varieties contain *Rht-B1b* (syn. *Rht1*) or *Rht-D1b* (syn. *Rht2*), which were transferred from the Japanese variety 'Norin10' into a wide range of CIMMYT germplasm before being taken up by other wheat breeding programs worldwide (Gale *et al.* 1985). These height-reducing genes are located on homoeologous chromosomes 4BS and 4DS and encode proteins involved in gibberellin (GA) signal transduction (Peng *et al.* 1999). By conferring insensitivity to GA, these genes have pleiotropic effects on plant growth, causing reductions in coleoptile length and seedling leaf area (Allan *et al.* 1962; Whan 1976; Rebetzke *et al.* 2001). Other dwarfing genes that do not confer GA insensitivity may therefore be more suitable in reducing final plant height without compromising early plant growth. The GA-responsive dwarfing genes *Rht8* and *Rht9* were introduced from the Japanese landrace Akagomugi into southern European varieties developed by the Italian wheat breeder N. Strampelli in the 1920s (Lorenzetti 2000). These dwarfing genes do not affect coleoptile length or seedling vigour (Rebetzke *et al.* 1999), and are being introduced into some Australian wheat varieties as replacements for the GA-insensitive dwarfing genes (Rebetzke and Richards 2000; Bonnett *et al.* 2001). Other height-reducing genes have been reported by Konzak (1988), who listed 20 *Rht* genes including two additional GA-insensitive genes, *Rht3* (*Rht-B1c*) and *Rht10* (*Rht-D1c*) that are allelic *Rht-B1b* and *Rht-D1b*, respectively. From the remaining *Rht* genes, Ellis *et al.* (2004) identified some genes that reduce adult plant height without affecting early growth.

2.6.5 DELLA protein is a key regulator in gibberellin signaling

Localised in the nucleus, the DELLA proteins are proposed to be transcription factors for GA-signaling in plants (Silverstone *et al.* 2001; Itoh *et al.* 2002). These proteins function as key repressors of GA-signaling by inhibiting the GA-regulated gene expression (Sun and Gubler 2004). These repressors accumulate in the nucleus and are rapidly degraded in response to GA (Silverstone *et al.* 2001, Fu *et al.* 2002). The GA-signals seems to be perceived by N-terminal region of the protein containing the DELLA domain. GA induces degradation of DELLA proteins via the ubiquitin/proteasome pathway (Itoh *et al.* 2003). Mutations within the DELLA domain render these proteins resistant to degradation which results in a GA-insensitive dwarf phenotype (Dill *et al.* 2001; Peng *et al.* 1999). This domain binds to the GA receptor GID1 in a GA dependent manner in rice, and promotes

interaction with the F-box protein GID2. Polyubiquitination of DELLA proteins by the SCF^{GID2} complex, signals the degradation of DELLA proteins by the 26S proteasome pathway. The functional mechanism of DELLA proteins regulation of gene expression and promoting photo morphogenesis was studied by Lucas *et al.* (2008). They found that the *Arabidopsis* nuclear transcription factor PIF4 (encoded by *PHYTOCHROME INTERACTING FACTOR4*) is negatively regulated by DELLA proteins and light photoreceptor phytochrome B.

2.7 Hulled *vs.* free-threshing

All wild wheats are hulled, have tough glumes (husks) that tightly enclose the grains. Glumes, lemma and palea, and grain(s) constitute the spikelet. At maturity the rachis (central stalk of the cereal ear) disarticulates, allowing the spikelets to disperse. *T. aestivum* was the last domesticated wheat species and presumably shares the same mutations that led to the domestication of tetraploid wheat in its A and B genomes. Additionally, the founding population of hexaploid wheat inherited the tenacious glume and brittle rachis genes from *Ae. tauschii* and mutations at these loci presumably occurred and were selected during the cultivation of hexaploid wheat (Salamini *et al.* 2002). In the polyploid wheats, a polygenic system along with modifier genes is known to govern rachis fragility and glume tenacity (MacKey 1966). Rachis fragility is primarily controlled by genes present on the homoeologous group-3 chromosomes (Watanabe and Ikebata 2000; Nalam *et al.* 2006; Li and Gill 2006). All wild wheats have brittle rachis leading to shattering of either the whole spike or individual spikelets (Li and Gill 2006). The First cultivated wheats had a non-brittle rachis (mutant *br* allele) with tough glumes and thus were non-free-threshing. The *Tg* gene controlling glume toughness in wheat is present on short arm of the group-2 chromosomes (Sears 1954; Kerber and Rowland 1974; Chen *et al.* 1999; Simonetti *et al.* 1999; Taenzler *et al.* 2002; Jantasuriyarat *et al.* 2004; Nalam *et al.* 2007). A major modifier gene for domestication related traits (*q* gene) is located on the long arm of chromosome 5A (MacKey 1966; Muramatsu 1986; Faris *et al.* 2002; Faris et al. 2005). Subsequent mutations at these loci during domestication led to the modern free-threshing wheats (genotype *brbrtgtgQQ*). Among these three genes, only *Q* has been cloned and is a member of the APETALA2 family of transcription factors (Simons *et al.* 2006). *Tg* (tenacious glume) was first described by Kerber and Dyck (1969) as an incompletely dominant gene in synthetic

allohexaploid x cultivated wheat crosses. Initial mapping efforts placed *Tg* on the short arm of chromosome 2D of wheat (Kerber and Rowland 1974; Rowland and Kerber 1974).

The glume tenacity genes located in the A-genome have been studied in *T. monococcum*. Einkorn wheat (*T. monococcum* subsp. *monococcum*) is the only cultivated diploid wheat. It has tough rachis but is non-free-threshing due to presence of tough glumes. The major genetic factors responsible for glume tenacity and threshability have been located on short arm of homoeologous group-2 chromosomes in wheats of different ploidy levels. Therefore all three threshability genes, *Sog*, *Tg2* and *Tg* might be homoeoalleles (Taenzler *et al.* 2002; Jantasuriyarat *et al.* 2003), however, none of the studies have tested it experimentally. *Sog* and *Tg*, two of the homoeologous group-2 loci influencing threshability in diploid and hexaploid wheat populations, respectively , however were not found to be true orthologs (Sood *et al.* 2009).

The crop domestication process started ca 5,000–15,000 years ago with the conversion of wild forms (characterized by inflorescences that shatter at maturity into tough fruiting bodies which help protect seeds during natural dispersal) into cultivated forms. These cultivated plants produced non-shattering inflorescences and soft fruiting bodies, which made them suitable for human planting and harvesting. In grasses, mutations at genes controlling several spike-related traits such as rachis fragility (brittle or non-brittle rachis) and glume tenacity (hulled or freethreshing) were selected during domestication and subsequently became fixed in the cultivated populations due to positive selection pressure (Tanksley and McCouch 1997; Feuillet *et al.* 2008).

2.8 Cell wall and Cellulose

In contrast to primary cell walls, plant secondary cell walls are deposited once the cell has stopped expanding. The composition of secondary cell walls varies widely among different species and different cell types. In general, however, they are composed of a complex mixture of lignin, carbohydrates and proteins. Consequently, the formation of a secondary cell wall requires the co-ordinate regulation of a number of complex metabolic pathways. In order to avoid some of the problems encountered with antisense technology, an alternative approach to investigate the function and synthesis of secondary cell wall components involves the characterisation of naturally occurring and chemically induced mutants. For genetic analysis, the study of secondary cell walls rather than primary walls

offers a distinct advantage in that even plants with a very severe alteration in secondary cell wall components still produce viable plants. This is clearly illustrated in the case of cellulose, where severe mutations in primary cell wall cellulose synthesis are often lethal (Arioli *et al.* 1998), whilst plants with mutations in secondary cell wall cellulose deposition grow relatively normally (Turner and Somerville 1997). Given this advantage and the complexity of secondary cell wall deposition relatively few mutants have been identified that are specifically defective in some aspect of secondary cell wall deposition. Secondary cell walls may conveniently be divided as either lignified (woody) or non-lignified. Whilst a number of cell types possess a thick secondary cell wall that does not contain lignin, only two mutants, from cotton fibres (Kohel *et al.* 1993) and *Arabidopsis* trichomes (Potikha and Delmer 1995), have been demonstrated to affect non-lignified secondary cell walls. Lignified cell walls contain lignin, cellulose and non-cellulosic glycans together with a variety of proteins and other minor components. In angiosperms the major cross-linking glycan in lignified secondary cell walls are xylans, which tend to have relatively few substitutions on a linear (1→4) β-D-xylose backbone (Hori and Elbein 1985). In some monocots xylans represent the majority of the secondary cell wall material and are far more abundant than cellulose (Gorshkova *et al.* 1996). Despite this abundance, no report of a secondary cell wall defect has yet been attributed to alterations in secondary cell wall xylans. Consequently, this review is divided into three sections that cover mutations in cellulose synthesis, lignin synthesis and the control of secondary cell wall composition. These simple divisions may themselves cause some problems since it is unclear whether some regulatory mutations affect only a single pathway, such as lignin biosynthesis or all aspects of secondary cell wall synthesis.

2.8.1 Cellulose mutants

Despite the fact that cellulose is the world's most abundant biopolymer, with an estimated 180 billion tonnes being produced annually (Englehardt 1995), the understanding of the mechanisms involved in cellulose biosynthesis in plants is still incomplete. Early biochemical studies of cellulose synthesis in plants yielded only limited success, and this can be attributed to a number of factors. These include the labile nature of the cellulose synthesising complex or a lack of purification of an essential, associated protein or co-factor.

2.8.1.1 Barley mutants

Brittle culm lines of barley were first described on the basis of the physical properties of the culm (Kokubo *et al.* 1989). The maximum bending stress of their culms were found to be less than half the value of normal lines. Cell wall analysis revealed that the maximum bending stress correlated significantly with the cellulose content of the cell walls, but not with non-cellulosic compounds. A more detailed analysis using isogenic brittle lines demonstrated that the cellulose content of the single-gene brittle mutants was less than half that of the corresponding non-brittle line (Kokubo *et al.* 1991). No correlation was found between brittleness and lignin content, demonstrating the importance of the cellulose content in determining the physical properties of the culm.

2.8.1.2 Rice mutants

In rice, several brittle culm mutations have been genetically identified and characterized (Nagato and Yoshimura 1998). For example, *bc1* mutation was characterized by a reduction in cell wall thickness and cellulose content and an increase in lignin level. These findings suggested that the biosynthesis and/or modifications of cell walls are essential for plant mechanical strength and normal cell morphology. Xu *et al.* (2008) described an ethyl methane sulfonate (EMS)-induced rice mutant, *fragile plant 2* (*fp2*), showed morphological changes and reduced mechanical strength. Genetic analysis indicated that the brittle of *fp2* was controlled by a recessive gene. The *fp2* gene was mapped on chromosome 10. Anatomical analyses showed that the *fp2* mutation caused the reduction of cell length and cell wall thickness, increasing of cell width, and the alteration of cell wall structure as well as the vessel elements. The consequence was a global alteration in plant morphology. Chemical analyses indicated that the contents of cellulose and lignin decreased, and hemicelluloses and silicon increased in *fp2*. These results were different from the other mutants reported in rice. Thus, *fp2* might affect the deposition and patterning of microfibrils, the biosynthesis and deposition of cell wall components, which influences the formation of primary and secondary cell walls, the thickness of cell walls, cell elongation and expansion, plant morphology and plant strength in rice.

2.8.1.3 Maize mutants

brittle stalk-2 (*bk2*) in maize, strongly reduces mechanical strength, but the plants look phenotypically normal in the absence of wind pressure (Langham 1940). Stalks of the

maize *bk2* mutant plants have dramatically reduced mechanical strength compared to their wild type siblings (Langham 1940; Coe *et al.* 1988). The other tissues of the *bk2* plants also have reduced mechanical strength. Ching *et al.* (2006) demonstrated that *Bk2* encodes a Cobra-like protein that is similar to the rice Bc1 protein. After biochemical, cytological, and molecular analyses of the maize *bk2* plants showed significantly reduced cellulose content and impaired deposition of secondary cell walls.

2.8.1.4 Cotton mutants

Developing cotton fibre has been an excellent system for studying cellulose synthesis, since single cells develop synchronously on the boll, and after a period of elongation the synthesis of a nearly pure cellulosic cell wall is initiated. Ligon lintless-1 mutant has drastically shortened cotton fibres, but these fibres have extensively thickened cell walls (Kohel *et al.* 1993). Experiments that measured the incorporation of [^{14}C] glucose into crystalline cellulose in both the primary and secondary cell walls demonstrated that the rate of cellulose production was reduced in primary walls, correlating with the reduced rate of fibre elongation (Kohel *et al.* 1993). In the secondary walls, however, there was a five-fold increase in crystalline cellulose production per millimetre of fibre compared to wild-type fibres. Thus the Ligon lintless-1 mutation affects both the growth and development of the cotton fibres along with changes in the formation of cellulose in both the primary and secondary cell walls (Kohel *et al.* 1993).

2.8.1.5 *Arabidopsis* mutants

Although the brittle culm mutants have aided our understanding of the relationship between secondary cell wall cellulose content and physical properties, barley is not an ideal system for molecular genetic investigations. As a result, more recent work was concentrated on the model plant species *Arabidopsis thaliana*, which is far more amenable for molecular genetic studies. The first description of a mutant of *Arabidopsis* deficient in secondary cell wall cellulose synthesis was of the *tbr* (*trichome birefringence*) mutant (Potikha and Delmer 1995). A further series of mutants deficient in secondary cell wall cellulose deposition has been described (Turner and Somerville 1997). These mutations, termed *irregular xylem* (*irx1, 2* and *3*), caused the collapse of mature xylem cells in the inflorescence stems of *Arabidopsis*. These mutants were identified in a screen involving the microscopic examination of cross sections of stems from a chemically mutagenised population. The

collapse of the xylem elements is thought to be due to a weakness in the secondary cell wall of the xylem cells which results in them being unable to withstand the negative pressure generated during water transport up the stem. This collapsed xylem phenotype was also seen in mature hypocotyls and in the primary root and petioles. It was found that the xylem elements initially expanded correctly to attain their normal shape, and these cells only appeared to collapse once they become involved in the transport of water. The *irx* mutants otherwise appeared little different from the wild type, apart from being slightly slower growing and *irx3* showing an inability to maintain an upright growth habit (Turner and Somerville 1997). Upon electron microscopic examination of cell walls from the interfascicular region, where no collapse of the cells is evident, it was found that wild-type cells were evenly thickened around the entire cell and stained in a ubiquitous manner. Walls from *irx1* plants were similar in appearance to the wild type, but were slightly thinner. The *irx2* plants, in contrast, showed a preferential deposition of cell wall material at the corners of the cell, with a decrease in the width of the walls at the midpoint. Some darker-staining regions were observed that appeared to divide the wall into different domains. The walls from *irx3* plants also showed different domains, with darker-staining regions and a very uneven pattern of thickening (Turner and Somerville 1997). As indicated by the inability of *irx3* plants to maintain an upright growth habit, the *irx* mutants demonstrate altered physical properties of their stems. The stiffness of mature stems, as measured by the bending modulus, was decreased in all *irx* mutants, with *irx3* stems displaying only 10% of the rigidity of wild-type stems. The *irx3* plants were also found to have drastically weaker stems, as determined by the maximum stress at yield. There were no apparent differences in the deposition of lignin in *irx1*, *2* or *3* stems compared to the wild type, as determined by diagnostic stains for lignin and by chemical analysis, or in the composition of the non-cellulosic carbohydrate fraction of the walls. There was, however, a considerable difference between the cellulose contents of the stems. At all stages of development, all three mutants showed a large decrease in the total cellulose content of the stem. These differences were greatest in mature stems. The *irx1*, *irx2* and *irx3* stems had 40%, 36% and 18%, respectively, of the total cellulose content of the wild type. Mature hypocotyls showed similarly large changes whereas leaves showed very small, if any, changes in cellulose content. This suggested that only secondary cell wall cellulose production was affected in these mutants (Turner and Somerville 1997). The *irx3* mutation was mapped to the top arm

of chromosome V (Turner and Somerville 1997). An EST that showed sequence similarity to bacterial cellulose synthase (Cutler and Somerville 1997) was found to be tightly linked to *irx*, and genomic clones that contained the gene corresponding to this EST were found to complement this mutation (Taylor *et al.* 1999). Thus, the *irx3* gene encodes a cellulose synthase that is required for cellulose synthesis in secondary cell walls.

2.8.2 Lignin biosynthesis mutants

The manipulation of the lignin content of plant cell walls, both qualitatively and quantitatively, has received much attention (Baucher *et al.* 1998). Lignin itself is a complex aromatic polymer and a major component of plant secondary cell walls (Bacic *et al.* 1988). It provides mechanical strength and support to plant tissue and renders the wall hydrophobic and impermeable to water. Lignin is synthesised by the oxidative polymerization of three monomers, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The structure of lignin varies considerably and depends on the tissue and cell type and on the environmental conditions (Campbell and Sederoff 1996; Campbell and Rogers 2001).

2.8.2.1 Maize mutants

The earliest lignin mutants were identified by a reddish-brown pigmentation of the leaf midrib. Although these mutants have been found in sorghum and millet, they have been best characterised in maize. This class of mutants has been designated *brown midrib* (*bm*) mutants. Further characterisation revealed these mutants had an altered lignin content and composition and were found to have enhanced digestibility properties. To date, 4 independent *bm* loci have been identified (denoted *bm1–4*) in maize but only 3 have been fully characterised (Barrière and Argillier 1993). There has been much commercial interest in the use of *bm* mutants as forage crops since lignin is an indigestible component of the cell wall and limits the breakdown of the wall polysaccharides (Cherney *et al.* 1991). The *bm3* mutation has been extensively studied.

2.8.2.2 Pine mutants

The *cad-n1* mutant in loblolly pine displays a characteristic phenotype of wood with a brown coloration (Mackay *et al.* 1997), similar to the *bm* mutants of maize. Characterisation of the mutant revealed reduced expression of the lignin biosynthetic enzyme CAD whilst the lignin content of the mutant was only slightly decreased. High

levels of coniferaldehyde and dihydroconiferyl alcohol, a monomer not normally associated with the lignin biosynthetic pathway, were identified in *cad-n1* plants. Similarly, biphenyl and biphenyl ether bonds were present in large excess in the mutant. Variations in the lignin composition, therefore, do not appear to disrupt the essential functions of lignin in this mutant (Ralph *et al.* 1997). The *cadn1* plants utilize non-conventional wall phenolics to construct unusual lignins enriched in resistant interunit bonds to compensate for the shortage in normal lignin precursors (Lapierre *et al.* 2000). Commercially, this mutation is particularly useful because it is capable of modulating the lignin composition and, hence, can affect the extractability of the polymer, without compromising the functional properties. The *cad-n1* mutant has also been used to characterize the quantitative effects associated with this allele. Co-segregation analysis indicated that the *cad* locus itself might represent a gene that governs stem growth in pine (Wu *et al.* 1999).

2.8.2.3 *Arabidopsis* mutants

The *fah-1 Arabidopsis* mutant (initially described as *sin1*) displays an increased transparency to UV light and demonstrates a characteristic red fluorescence under UV. Characterisation of the *fah-1* mutant revealed an absence of sinapic acid esters. The transparency to UV light is the result of a lack of sinapoyl malate in the upper epidermis (Chapple *et al.* 1992). In the mutant, the conversion of ferulate to 5-hydroxyferulate is blocked, as determined by *in vivo* radiotracer feeding experiments, precursor supplementation studies and enzyme assays. This step in the general phenylpropanoid pathway is a key reaction in the production of syringyl units. The *fah-1* mutant therefore lacks the sinapic acid-derived components of lignin although the total lignin content remained unchanged. After the isolation of a T-DNA tagged allele, the gene was identified as ferulate 5-hydroxylase (F5H). F5H functions as a cytochrome P450-dependent monooxygenase and exhibits low amino acid sequence homology to other classes of plant P450. F5H was therefore designated as a new family of plant P450, CYP84 (Meyer *et al.* 1996). The ectopic over-expression of F5H abolished tissue-specific lignin monomer accumulation (Meyer *et al.* 1998). Transgenic plants with the cinnamate 4-hydroxylase (C4H) promoter linked to F5H produced syringyl-rich lignin, with a much higher proportion of syringyl units than in any other plant previously reported (Franke *et al.* 2000; Marita *et al.* 1999). The specific control of the monomeric composition of lignin is likely to be

achieved through the engineering of the F5H gene. (Turner and Somerville 1997; Taylor *et al.* 1999, 2000). The mutants are unable to maintain an upright growth habit. Typically, the xylem vessels in these *irx* plants exhibit a collapsed phenotype, which has been attributed to defective secondary cell walls. Biochemical analysis of *irx4* cell walls revealed a 50% reduction in the total lignin content, as determined by histochemical staining, thioglycolic acid assays and solid-state NMR. In contrast, the cellulose and non-cellulosic glycan content of *irx4* cell walls remained unchanged (Jones *et al.* 2001). The most dramatic aspect of the *irx4* phenotype was observed in the ultrastructure of *irx4* secondary cell walls and the consequent effects on the physical properties of the stems. The architecture of the walls in the mutant was drastically altered compared to the wild type with the cell walls having a massively expanded and diffuse appearance. The *irx4* cell walls often occupied a large proportion of the total cell volume. Furthermore, the walls stained unevenly, compared to wild-type cell walls, indicative of modifications in their composition. The diffuse nature of *irx4* cell walls provides clear evidence for the role of lignin in maintaining the structural integrity of the wall and anchoring the components of the wall together. As a consequence of these ultrastructural modifications in *irx4* cell walls, stems from the mutant display significantly altered physical properties with both the strength and stiffness of the stems severely reduced (Jones *et al.* 2001). Phenotypic observations and mapping data suggested that the cinnamoyl-CoA reductase (CCR) gene, an enzyme involved in the penultimate step in the lignin biosynthetic pathway, was a good candidate for the identity of the *IRX4* gene. Further analysis indicated the presence of a mutation within this gene in *irx4* plants and definitive evidence of the identity of IRX4 was achieved by complementation with the wild-type CCR gene (Jones *et al.* 2001). The *irx4* mutant confirms that CCR is an ideal candidate for modifying the total lignin content of secondary cell walls. This mutant has also provided valuable insight into the functional role of lignin within the cell wall and its influence on the mechanical properties of plant tissue.

2.8.3 Regulatory mutants

The mutants described above have proved important in providing insight into the structure and biosynthesis of lignin and cellulose. To date, however, they have only provided information on individual steps in the pathway. At least 17 genes are required to synthesise lignin and as to how the co-ordinate regulation of these individual steps in this

pathway is achieved in a cell-specific manner is an area of intense interest. In addition, the pathways for lignin, cellulose and non-cellulosic glycan biosynthesis must also be regulated in a highly co-ordinated manner to achieve the proper deposition of these polymers during secondary wall formation.

2.8.4 Ectopic lignification mutants

Two mutants, *ectopic lignin deposition* (*elp1*) (Zhong *et al.* 2000) and *ectopic lignification* (*eli1*) (Caño- Delgado *et al.* 2000), appear to alter the normal pattern of lignin deposition and therefore offer an opportunity to examine the spatial control of lignin deposition. The *elp1* mutant in *Arabidopsis* demonstrates altered lignin deposition patterns in the stem. Ectopic deposition of lignin occurs in the walls of pith parenchyma cells in addition to the normal deposition of lignin in the walls of xylem and fibre cells. Lignin appeared to be deposited in regions of parenchyma cells in the pith of both young and mature stems. Furthermore, stems from *elp1* plants had ca. 20% more lignin than wild-type stems and this increase in lignin content was accompanied by an increase in the activities of enzymes involved in the lignin biosynthetic pathway, such as PAL, CCoAOMT and CCR. Ectopic expression of the enzyme CCoAOMT was also identified in pith cells by immunolocalisation. Interestingly, however, this increase in lignification in *elp1* stems was not accompanied by secondary cell wall thickening, suggesting that other secondary cell wall components were unaffected by this mutation. The most likely role of the *ELP1* gene product is as a negative regulator of the lignin biosynthetic pathway that under normal circumstances suppresses lignin deposition in the stem (Zhong *et al.* 2000). In contrast to *elp1*, the primary roots of *eli1* plants demonstrate abnormal lignification patterns with lignin present in cell types not normally lignified, as well as an absence of lignin in cells that are generally lignified, such as the xylem. This ectopic lignification pattern is associated with reduced cell elongation and shorter, thicker primary roots (Caño- Delgado *et al.* 2000). The connection between abnormal cell shape and lignification was further verified by examining previously described mutations with altered cell elongation. Mutants with cell elongation defects, such as *lit*, *rsw1* and *kor*, all show ectopic lignification, suggesting that the ectopic lignification of *eli1* may be a consequence of a cell elongation defect (Caño-Delgado *et al.* 2000). The link between altered secondary cell wall deposition and cell elongation is also suggested by work on the *Arabidopsis* mutant *elongation defective1* (*eld1*). *eld1* plants

exhibit de creased cell elongation, altered xylem development and ectopic deposition of suberin (Cheng *et al.* 2000). Clearly both the *ELI1* and *ELD1* genes will provide valuable information on themechanisms involved in regulating the process of lignification and as such are good candidates for genetically engineering and specifically increasing the lignin composition of cell walls from particular cell types.

2.8.5 Mutants exhibiting decreased secondary cell wall thickening

The *Arabidopsis* mutant *interfascicular fiberless*1 (*ifl1*) was initially described as a mutation affecting the regulation of secondary cell wall deposition (Zhong *et al.* 1997). Whilst the secondary cell wall appears to form relatively normally within the xylem of these plants, the fibres of the interfascicular region appear to undergo little or no secondary cell wall thickening (Zhong *et al.* 1997). Map-based cloning of the *IFL* gene has revealed it to be a homeodomain-leucine zipper protein (Zhong and Ye 1999). Independent work that led to the cloning of the *revoluta* (*rev*) gene (Ratcliffe *et al.* 2000) has demonstrated that *rev* and *ifl* are allelic. The *rev* mutant was initially described as having altered patterns of cell division in the apical meristem (Talbert *et al.* 1995). These observations suggest that the phenotype of the mutation is in fact highly pleiotropic and the reduction in cell wall formation in the interfascicular region may be the consequence of reduced auxin flow from an abnormal apical meristem (Ratcliffe *et al.* 2000). Whatever the initial mode of action of the *REV* gene, understanding how it affects cell wall formation in the interfascicular region is likely to reveal interesting insights into how this process is controlled. In contrast to *ifl/rev*, which appears to disrupt secondary cell wall formation in the interfascicular region, the *gapped xylem* (*gpx*) mutant appears to affect secondary cell wall formation in both the xylem and the interfascicular region. In the xylem, gaps are present in positions where the water-conducting xylem elements normally exist. Since the procambial cells of wild-type and *gpx* plants are indistinguishable, these gaps apparently arise due to a defect in normal xylem element development. In the interfascicular regions, very thick-walled cells are adjacent to cells that contain little or no secondary cell wall. The interpretation of the gapped xylem phenotype stems from considering the difference between the development of xylem elements and the development of interfascicular cells (Turner and Hall 2000). Some differences, such as lignin subunit composition (Chapple *et al.* 1992), exist between secondary cell wall deposition in the xylem and the interfascicular region. The *gpx* mutant,

however, suggests that at least some aspects of the regulation of secondary cell wall formation are regulated in a common manner. In addition, the *gpx* mutant suggests that cell death and secondary cell wall formation in xylem elements are regulated independently, since cell death appears to occur in the absence of a secondary cell wall. This separation of cell death from cell wall formation does not support the idea that the signal for cell death accumulates as an integral part of secondary cell wall formation (Groover and Jones 1999). Whilst some cells in *gpx* fail to form a secondary cell wall, those that do form a secondary wall often form a wall that is considerably thicker than that normally observed in the wild type (Turner and Hall 2000). How these thick-walled cells arise and why only a proportion of the cells form a secondary cell wall remains unclear. It is clear that secondary cell wall mutants provide a complementary approach to antisense/sense strategies as a means of probing the structure, synthesis and assembly of the secondary cell wall. In some areas, such as the regulation of secondary cell wall deposition, analysis of mutants is likely to be the most productive avenue of research. To date, however most secondary cell wall mutants have been caused by mutations in rather obvious targets, such as the biosynthetic pathway of cellulose and lignin biosynthesis. One challenge for the future is to use genetic analysis to reveal completely novel components essential for secondary cell wall biosynthesis

2.8.6 Plant cell wall biosynthesis

Plant cell walls are composed mainly of polysaccharides. Both primary and secondary cell walls contain cellulose and hemicelluloses; primary walls also contain pectins as well as many enzymes and structural proteins, whereas secondary walls contain little protein or pectin, but normally contain lignin (Carpita *et al.* 2000). Among the polysaccharides, cellulose is the most important and normally the most abundant wall component. Cellulose microfibrils are embedded in a matrix that contains other polysaccharides glycoproteins and proteins. Significant progress has been made in defining the structures of the various polysaccharides that make up plant cell walls (Somerville *et al.* 2004). Although cellulose is made at the plasma membrane and deposited directly into the wall (Doblin *et al.* 2003), most other matrix components are made in the Golgi and delivered to the wall in secretory vesicles. The processes and players involved in assembling functional cell walls from components synthesized at different cellular locations are largely unknown.

2.8.6.1 Cellulose

Cellulose is the most abundant biopolymer on earth. Despite its simple structure, omnipresence in the plant kingdom, and ever increasing global importance as industrial raw material, the genetic and biochemical regulation of cellulose biosynthesis continues to be unclear (Joshi and Shawn 2007). Cellulose is ubiquitous among plants in which it constitutes the major polysaccharide of cell walls (Saxena and Brown 2005); it is also considered to be the most abundant biopolymer on Earth. Cellulose is at the core of plant cell walls, where it serves as a scaffold for the binding of other wall components. In the primary wall of higher plants, cellulose microfibrils are about 3 nm in diameter and generally consist of parallel arrangements of 36 β-1,4-glucan chains (Somerville 2006). Membrane-bound cellulose synthase enzyme complexes, the most prolific biomachines in nature, are required for cellulose biosynthesis. These complexes are visible as hexameric rosettes of approximately 25–30 nm in diameter when plant cells are examined using freezefracture electron microscopy (Saxena and Brown 2005). When cytosolic uridine-diphosphoglucose (UDP-glucose) is used as substrate, each rosette subunit is thought to extrude multiple β-1,4-glucan chains that coalesce as microfibrils outside of the plasma membrane). In recent years, significant insight into the molecular details of cellulose biosynthesis has been gained using forward and reverse genetic analyses coupled with advances in plant genomics. The plasma membrane rosettes contain the cellulose synthase catalytic subunit (CESA) proteins that are encoded by the CESA genes (Doblin *et al.* 2002, Scheible *et al.* 2004). Plant genomes typically contain multiple CESA genes as part of a gene family. For example, ten CESA genes are present in *Arabidopsis*, rice has at least nine, and poplar has 18 (Djerbi *et al.* 2005). It is thought that each hexameric rosette comprises six rosette subunits and that each rosette subunit contains six CESA proteins, providing a total of thirty-six CESA proteins per rosette. The idea that there are at least three different CESA proteins in a rosette comes from genetic evidence, which also supports the conclusion that the cellulose in primary cell walls is synthesized by a different set of CESA proteins than that in secondary cell walls. In *Arabidopsis*, CESA1, CESA3, and CESA6 are required for cellulose biosynthesis in primary cell walls (Roberts *et al.* 2004), whereas CESA4, CESA7, and CESA8 are required for cellulose biosynthesis during secondary wall deposition (Somerville 2006). Similar conclusions have been reached in other plants (Joshi *et al.* 2004, Nairn *et al.* 2005), although the numbering system for CESA proteins varies

among plant species CESA proteins are large (around 1000 amino acids) integral membrane proteins that have eight predicted transmembrane domains and a large hydrophilic domain that faces the cytosol. This domain contains the conserved DXD, and D,D,D,QXXRW active site residues that are characteristic of processive β-glycosyltransferases. Another common feature among CESA proteins is a pair of amino-terminal zinc-finger motifs (Taylor *et al.* 2004, Kurek *et al.* 2002). CESA proteins have been shown to form higher-order structures in vitro and in planta. The conserved zinc-finger sequences are thought to act as redox regulated multimerization domains that are involved in the assembly of CESA monomers into rosette complexes (Kurek *et al.* 2002). The redox state of a cell might thus regulate cellulose biosynthesis at the level of assembly of CESA proteins into rosettes. In addition to CESA proteins, cellulose biosynthesis almost certainly requires the action of other proteins, although few of these other necessary proteins have been identified, and very little is known about the biochemical role of those that have been identified. Several strategies have been used to identify additional genes that encode proteins needed for cellulose biosynthesis. Genetic strategies have pinpointed several of these additional genes, including the KORRIGAN gene, which encodes a β-1,4-glucanase (Scheible *et al.* 2004, Kurek *et al.* 2002); CYT1, a gene whose product is involved in the biosynthesis of GDP-mannose; several PEANUT genes, encoding enzymes that are involved in the biosynthesis of glycosyl phosphatidylinositol membrane anchors (Gillmor *et al.* 2005); KOBITO1, a plant-specific gene of unknown function(Pagant *et al.* 2002); and COBRA, which encodes a protein that is essential for microfibril organization (Roudier *et al.* 2005) Alternative strategies are being used to identify additional constituents of cellulose synthase complexes. For example, an epitope-tagged AtCESA7 protein has been used to complement an *Arabidopsis* mutant in which the endogenous AtCESA7 gene is altered, and efforts are being made to purify the protein complex that contains the resulting protein using affinity chromatography (Taylor *et al.* 2004). Another strategy is to identify genes whose regulation is linked to cellulose synthesis. Using this strategy, two groups have identified genes whose expression is coordinately regulated with that of CESA genes (Brown *et al.* 2005, Person *et al.* 2005). Some of the genes are probably involved in events that occur at the same time as cellulose biosynthesis, such as lignin or hemicellulose biosynthesis during secondary wall formation, but other coordinately regulated genes might have a direct role in cellulose biosynthesis. The co-expression strategy is especially powerful when studying the

deposition of tension wood, which consists mainly of cellulose (Anderson *et al.*2006, Bhandari *et al.* 2005); the use of such experimental systems might allow the identification of other cellulose biosynthesis machinery components and even the regulators of cellulose biosynthesis. Reverse genetics has been used to investigate the function of some of these genes (Brown *et al.* 2005) and further studies to reveal their functions are underway. One of the key features of cellulose biosynthesis is that microfibrils are deposited in a controlled orientation; in primary walls their orientation is normally perpendicular to the axis of cell elongation. The orientation of cellulose microfibrils correlates well with the orientation of cortical microtubules, leading to the hypothesis that microtubules somehow control the orientation of microfibril deposition.

2.8.6.2 Hemicelluloses

Hemicellulosic polysaccharides are complex molecules that associate with cellulose microfibrils, providing a cross-linked matrix. Hemicelluloses can be divided into four main classes: (i) xyloglucans (XyG), which contain a heavily substituted β-1,4-glucan backbone; (ii) (gluco)mannans, containing a variably substituted backbone that includes β-1,4-linked mannose (glucose and mannose) residues; (iii) glucuronoarabinoxylans (GAX), containing a substituted β-1,4-linked xylan backbone; and (iv) mixed linkage glucans (MLG), which involve an unsubstituted backbone of glucosyl residues containing both β-1,3- and β -1,4-linkages(Carpita *et al.* 2000). Structural similarities between the β-1,4-linked glucan chains of cellulose and the backbones of the various b-linked hemicellulosic polysaccharides led to the prediction that Cellulose Synthase Like (CSL) genes might encode Golgi-localized glycan synthases that are involved in the biosynthesis of these polysaccharides(Hazan *et al.* 2002, Richnond *et al.* 2001). The CSLs are a family of genes that have sequence similarity to the CESA genes and appear to be present in all plant genomes. Certain CSL subfamilies are common to all plants, whereas other subfamilies are present only in specific groups of plants (Keegstra *et al.* 2006).

2.8.7 Biomass and Paper industry

Grasses provide the majority of calories consumed by humans either directly through the consumption of grains or indirectly through animals feed. Grass cell walls (lignocellulose biomass) are a major source of dietary fiber that provides numerous health benefits beyond simply providing calories (Spiller *et al.* 2001, Harris *et al.* 2006).

Furthermore, grass cell walls are poised to become a significant source of renewable energy because the sugars locked in the polysaccharides of the cell wall can be converted into liquid fuel (e.g. ethanol, butanol) and the entire cell wall can be burned to produce heat or electricity (Ragauskas *et al.* 2006, Perlack *et al.*2005, Service et *al.*2007).

Lignocellulosic biomass consists principally of cell walls, harvested from dedicated bioenergy crops or from dried crop residues, such as sugarcane 'bagasse', or maize (Zea mays), sorghum (Sorghum bicolor) and wheat (*Triticum aestivum*) 'stover'. Lignocellulosic biomass provides a rich source of solar energy trapped as carbohydrate in a broad range of plants unrestricted by climate or geographic location, the conversion of which to biofuel would not impact the price of cereal commodities (Perlack *et al.* 2005, Ragauskas *et al.* 2006). Genetic improvement of cell wall composition and architecture is a goal for two reasons: cell walls constrain cell size and shape and so have a significant role in plant growth, impacting biomass yield. Cell walls are recalcitrant to degradation by microbes to release sugars for fermentation, impacting biomass quality. Plants devote 10% of their genome, 2,500 genes, to construction and dynamic rearrangement of their cell walls during growth (Carpita *et al.* 2001, Yong *et al.* 2005, McCann and Carpita 2005).

Genetic screens for mutants that affect cell wall composition and architecture, either directly or indirectly provide unbiased ways to identify biomass-relevant quality traits, including those resulting from mutations in cell wall-related genes. The brown mid-rib mutants of sorghum are an excellent example of how a defect in lignin structure, which improves forage digestibility by ruminants, can also enhance yields of glucose in screens using commercial cellulases (Vermerris *et al.* 2007). The RILs provide a rich resource to identify QTL and the underlying genes for useful traits, such as plant height, anatomy and architecture, density, mass, and leaf area. Many cell wall related genes can also impact both accumulation of biomass and its degradability. Beyond the obvious modification of lignin content and architecture, increasing carbohydrate content could be accomplished by ectopic expression of transcription factors, such as the NAC and Myb-domain-containing proteins that initiate secondary wall formation (Mitsuda *et al.* 2007, Yang *et al.* 2007) or by coordinately up-regulating expression of the genes of the cellulose synthase complex.

Plant cellulose, nature's most abundant macromolecule is exploited worldwide by the textile and paper industries. The pulp and paper industry is one of the most important industries in the world. It supplies an essential product - paper - to over 5 billion people

worldwide. Paper is made from natural fibers called cellulose. A number of non wood fibers used for making paper. These include agricultural residues such as wheat, barley, oat, rye, and rice straws or sugarcane bagasse. In the past, some of these fibers were commonly used for papermaking, and still are used to some degree in other parts of the world. Globally, 9% of paper fiber comes from fibers other than wood; about 85% of this amount consists of non-wood papers made in China. (Misra 1993).

2.9 Molecular mapping in plants

2.9.1 Molecular markers

Use of molecular techniques for detecting differences in the DNA of individual plants has many applications for crop improvement. DNA-based molecular markers are the most powerful diagnostic tools to detect DNA polymorphism both at the level of specific loci and at the whole genome level. These molecular markers are often associated with specific genes and act as "signposts" to those genes. Such markers, when very tightly linked to genes of interest, can be used to select indirectly for the desirable allele, and this represents the simplest form of marker assisted selection (MAS), which can be used to accelerate the back-crossing of such an allele and in pyramiding several desirable alleles. Markers can also be used for dissecting polygenic traits into their Mendelian components or quantitative trait loci (QTL), thus increasing understanding of the inheritance and gene action for such traits and allowing the use of MAS as a complement to conventional selection procedures.

Molecular markers are also used to probe the level of genetic diversity among different cultivars, within populations and among related species. The applications of such evaluations include varietal fingerprinting for identification and protection, understanding relationships among the units under study, efficiently managing genetic resources, facilitating introgression of chromosomal segments from alien species, and tagging of specific genes. In addition, markers and comparative mapping of various species have been very valuable for improving the understanding of genome structure and function and have allowed the isolation of genes of interest via map-based cloning. Previously DNA based markers were developed either from genomic libraries (RFLPs and microsatellites) or from random PCR amplification of genomic DNA (RAPDs) or both (AFLP). However, recently due to the availability of genomic DNA and cDNA sequences (ESTs) in the public databases marker development has become more direct and cost effective.

RFLPs (restriction fragment length polymorphism) are fragments of restricted DNA separated by gel electrophoresis and detected by subsequent Southern blot hybridization to a radiolabeled DNA probe consisting of a sequence homologous to a specific genomic region. The 20 locus specific DNA probes (0.2 to 2 kb) consist of a sequence of unknown identity or part of the sequence of a cloned gene and are obtained by molecular cloning and isolation of suitable DNA fragments. Fragment length polymorphism is obtained usually by sequence variation generated due to absence or presence of endonuclease recognition sites. DNA probes are constructed from cDNA or genomic libraries. In a polyploid genome like wheat, RFLPs are a useful marker system as it is easy to determine the copy number for any probe sequence.

RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism), STSs (sequence tagged sites), SNPs (single nucleotide polymorphisms), and microsatellites are all PCR-based markers.

RAPD (Williams *et al.* 1990) markers are detected using short (10mer) random oligonucleotides as primers to amplify genomic DNA sequences. RAPDs are scored as dominant markers and show presence/absence polymorphisms. Lack of reproducibility and locus specificity restricted their use in polyploid wheat genetics.

AFLPs are DNA fragments (80-500 bp) obtained from endonuclease restriction, followed by ligation of oligonucleotide adapters to the fragments and selective amplification by polymerase chain reaction (PCR). The PCR-primers consist of a core sequence (part of the adapter), the restriction enzyme specific sequence and 1-3 selective nucleotides. AFLP markers are generally scored as dominant markers. AFLPs (Vos *et al.* 1995) have been widely used in plants for various genetic analyses including genetic mapping. A key advantage of the AFLP technique is a higher degree of polymorphism and reproducibility. Many genetic diversity studies in wheat and related species have been conducted using AFLPs (Heun *et al.* 1997)

Simple sequence repeats (SSRs) or microsatellites markers consist of di-, tri-, or tetranucleotide repeats and DNA sequences flanking the repeats are used as priming sites in PCR reactions. The amplified product contains variable number of repeats depending upon the species and generates length polymorphism. Due to the high variability of number of repeats within a microsatellite even among the members of the same species, microsatellites tend to detect a high degree of polymorphism. SSRs are typically codominant and have high

locus specificity. Hence, they have been used extensively to develop genetic maps in wheat (Roder *et al.* 1998; Somers *et al.* 2004, Singh *et al.* 2007).

STS (sequence tagged sites) markers are usually designed from known sequence in the genomic region of interest. Genomic sequences amplified using STS primers are usually digested with a 4-base cutter enzyme to reveal length polymorphisms.

SNP (Single nucleotide polymorphism) markers are based on single base differences within a given segment of DNA between any two individuals. Usually potential SNPs are identified by sequence alignments of the target sequence among different accessions of the plant material. Although maize has the highest number of SNPs among cereals (Tenaillon *et al.* 2001). Single nucleotide polymorphisms (SNPs) are ideal markers for identifying genes associated with complex diseases for two main reasons. Firstly, SNPs are densely located on the human genome at about one SNP per approximately 500–1,000 base pairs. Secondly, a large number of commercial platforms are available for semiautomated or fully automated SNP genotyping. These SNP genotyping platforms serve different purposes since they differ in SNP selection, reaction chemistry, signal detection, throughput, cost, and assay flexibility (Chunming and Shengnan 2009).

Diversity Arrays Technology (DArT), a novel method to discover and score genetic polymorphic markers. DArT is a sequence-independent, high-throughput method, able to discover hundreds of markers in a single experiment (Kilian *et al.* 2008). DArT markers are typed in parallel, using high throughput platforms, with a low cost per data point. DArT fingerprints will be useful for accelerating plant breeding, and for the characterisation and management of genetic diversity in domesticated species as well as in their wild relatives. DArT successfully developed for rice, barley, wheat and cassava and have produced a dedicated data management and analysis package, a key part of the technology, entirely built from Open Source components. A DArT marker is a segment of genomic DNA, the presence of which is polymorphic in a defined genomic representation. DArT markers are biallelic and behave in a dominant (present vs absent) or co-dominant (2 doses vs 1 dose vs absent) manner (Hurtado *et al.* 2008).

2.9.2 Bulk Segregant Analysis (BSA)

The technique of BSA was first described by Michelmore *et al.* (1991). It has been widely used tool to find DNA based markers linked to target genes. In BSA, two pools or

bulks of DNA samples are contributed equally from 10-20 homozygous individual plants from both extremes. These two bulks should differ for a trait of interest (e.g. tall vs. dwarf for plant height). By making DNA bulks, all loci are randomized, except for the gene of interest. Markers are screened across the two bulks. Polymorphic markers may represent markers that are linked to a gene or QTL of interest. The entire population is then genotyped with these polymorphic markers and a localised linkage map may be generated (Collard *et al.* 2005).

The BSA method is effective method for rapidly mapping major loci and has been used for mapping quantitative (Chalmers *et al.*, 1993) as well as qualitative traits (Michelmore *et al.* 1991). Gottwald *et al.* (2004) used BSA for the fine mapping of a gibberellic acid-insensitive gene *sdw3* in barely on 2HS chromosome. BSA approach has been used in oat to identify random amplified polymorphic DNA (RAPD) marker linked to genes for oat stem rust resistance (Penner *et al.* 1993). Muangprom and Osborn (2004) used BSA to identify molecular markers linked to the dwarf genes by using 18 plants of each phenotypic classes (short and tall or intermediate) from BC_1F_1 population and 92 *Brassica* RFLP probes. Milach *et al.* (1997) mapped the three dwarfing loci available in oat using RFLP markers and identified regions of the genome which contribute to plant height, and verified the utility of BSA and the hexaploid oat RFLP map for mapping dwarfing genes in cultivated oat.

2.9.3 Linkage mapping

Molecular mapping using markers involves the application of molecular techniques to the basic concepts of Mendelian genetics. A mapping function is usually employed to construct the genetic map derived from recombination fraction data because a map based on only recombination fraction data might not provide accurate genetic distances especially for loci that are not tightly linked. Mapping function is a mathematical expression relating observed recombination fraction to map distance expressed in centiMorgans (cM). Kosambi and Haldane are two commonly used mapping functions where Kosambi mapping function assumes presence of interference (i.e. presence of a chiasmata affects the occurrence of another chiasmata in the vicinity) but the Haldane function does not. Two primary requirements for developing a DNA-based genetic linkage map are a mapping population segregating for traits of interest (e.g., F2, backcross, recombinant inbred lines) and a source

of DNA clones for RFLP or a set of primer pairs for PCR-based markers. Molecular marker data along with phenotype data on recombination frequencies is processed using software programs like MAPMAKER for major gene analysis and QTL CARTOGRAPHER and QGENE for quantitative trait analysis and the genetic maps are generated for the initial localization of specific phenotypes of interest or for whole genome analysis.

The availability of high-density genetic linkage maps is a valuable asset as it can facilitate map-based cloning experiments, quantitative trait mapping, marker-assisted breeding and evolutionary studies. The first genetic linkage map based on RFLPs was constructed in humans (Botstein *et al.* 1980). Since then linkage maps using various molecular markers have been developed for many crop species. Molecular maps of wheat using RFLPs and microsatellite markers at all ploidy levels have been developed (Devos *et al.* 1993; Van Deynze *et al.* 1995; Nelson *et al.* 1995; Dubcovsky *et al.* 1996; Röder *et al.* 1998; Blanco *et al.* 1998; Somers *et al.* 2004; (http://wheat.pw.usda.gov/GG2/maps.shtml#wheat).

2.9.4 Molecular genetic maps

A major use of molecular markers is for constructing genetic maps by analyzing the co-segregation of markers and phenotypes or traits in defined populations. These populations come from a variety of sources – F2 populations resulting from narrow or wide crosses, single seeds descent derived recombinant inbred (RI) populations or doubled haploids. The last two have the advantage that they can be permanently maintained. However, in cereals, in many cases only a few markers could be mapped with a specific segregating population due to low level of polymorphism between the parents of the mapping population.

2.9.5 Map-based cloning (MBC)

MBC involves identification of a mutant phenotype for the trait of interest (obtained by mutagenesis or from natural variation) and genetic fine mapping using a large number of progeny plants (Martin *et al.* 1993). This map is then used for chromosome walking or landing, with the help of large-insert DNA libraries or physical maps to isolate the gene (Azhaguvel, *et al.* 2006). Several MBC projects were started in the mid-1990s, and several genes or QTLs for disease resistance or other traits have been isolated in many cereal species (Varshney *et al.* 2006). In wheat some genes have been cloned based on MBC. Leaf

rust resistance genes *Lr10* (Feuillet *et al.* 2003), *Lr21* (Huang *et al.* 2003), Powdery mildew resistance gene *Pm3b* (Yahiaoui *et al.* 2004), major chromosome pairing loci *Ph1* (Griffiths *et al.* 2006), wheat vernailzation genes *VRN1* (Yan *et al.* 2003), *VRN2* (Yan *et al.* 2004), Wheat domestication gene Q (Simons *et al.* 2006), QTLs for resistance to fusarium head blight resistance (Liu and Anderson, 2003), stem rust *Sr2* (Kota 2006) and leaf rust *Lr34* (Krattinger *et al.* 2009).

2.9.6 Comparative genome analysis

Recently, the sequencing of the rice and sorghum genomes (IRGSP, 2005, Paterson *et al.* 2009) as well as the development of a high-density anchored physical map of the maize inbred B73 (Wei *et al.* 2007) provided invaluable tools to compare these three grass genomes. In parallel, the development of large mapped EST collections from the Triticeae allowed *in silico* genome-wide macrocolinearity analyses with these genomes (Devos *et al.* 2000) Comparative genome analyses were first performed between the rice genome, used as a reference and the individual wheat (Sorrells *et al.* 2003, Sorrells *et al.* 2004, La *et al.* 2004, Singh *et al.* 2004), sorghum (Klein *et al.* 2003, Paterson *et al.* 2004), barley (Stein *et al.* 2007, Stein 2007) and maize (Salse *et al.* 2004) genomes. The increased resolution of these analyses revealed additional chromosomal rearrangements within the 30 original ancestral linkage blocks and led eventually to a revision of the 'concentric crop circles' representation (Devos 2005). The evolutionary models that can be deduced from *in silico* genome comparisons rely on the capacity to evaluate with confidence whether two or more genes found in the same order on two chromosomal segments are truly orthologous. So far most of the studies were based on default sequence alignment parameters and were not systematically validated statistically. Very recently, improved sequence alignment criteria and systematic statistical analyses were applied to the latest genome sequences and EST releases of rice, wheat, barley, sorghum and maize to reassess the colinearity between their chromosomes (Salse *et al.* 2008). The results showed that within 50–70 MY of evolution, 70% of the genes retained conserved structural motifs, 40% remained conserved as single copies, while only 20 % remained orthologous. For example, comparisons between the 42654 rice genes and 5003 non-redundant mapped wheat ESTs contigs revealed that 1180 orthologous gene pairs, covering 83.1% and 90.4% of the rice and wheat genomes, respectively, are conserved. A similar comparison between the rice genome sequence and

1411 mapped maize ESTs contigs showed 656 conserved orthologous pairs. Interestingly, 27.2% and 21.8% of these were not found in the expected orthologous position based on the rice gene order, indicating previously unreported rearrangements (inversions, translocations, deletions) in orthologous regions between rice and wheat and between rice and maize, respectively. In addition to genome wide analyses, microcolinearity studies performed at target loci between rice, maize, wheat, barley and sorghum (Lai *et al.* 2004, Swigonowa *et al.* 2005, Chantret *et al.* 2005, Pourkheirandish *et al.* 2007) provided additional evidence for orthologous gene shuffling before and after speciation in each species independently, probably to prevent pairing of homeologous chromosomes.

2.9.7 RNAi silencing as a tool of functional genomics

Besides insertional mutagenesis and EMS mutagenesis, gene silencing is an efficient tool for the determination of gene function (Anand *et al.* 2003; Singh *et al.* 2006b). RNA interference (RNAi) is based on sequence-specific mRNA degradation which follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Baulcombe 2004). In contrast to insertional mutagenesis, RNAi allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids by targeting sequences that are unique or shared by several genes (Miki *et al.* 2005). dsRNA is detected by the host plant genome as aberrant and is cleaved by the action of Dicer-like enzymes into long and short siRNAs (Tang *et al.* 2003). These two classes of small RNAs have distinct RNA silencing functions. The short siRNA (approximately 21-mers) directs post transcriptional signaling via mRNA degradation and the long siRNA (approximately 24-mers) trigger systemic silencing and the methylation of homologous DNA (Hamilton *et al.* 2002).

RNAi is proved to be very efficient in interfering with gene expression in various plant systems such as *Petunia hybrida*, *Arabidopsis thaliana*, *Coffea arabica*, and *Oryza sativa* (Stam *et al.* 1997; Chuang and Meyerowitz 2000; Lee *et al.* 2004; Ogita *et al.* 2004; Miki *et al.* 2005; Isshiki *et al.* 2001). The wide use of this powerful technique reflects its ease of application and the possibilities for genome-wide reverse genetics. When directed against viruses or endogenous genes and transgenes the gene constructs encoding intron-spliced RNA with a self-complementary hairpin (hp) structure have been shown to induce post transcriptional gene silencing with almost 100% efficiency (Smith *et al.* 2000).

CHAPTER 3

MATERIALS AND METHODS

Chapter 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 EMS induced mutants

The EMS induced dwarf (*Rht22*), soft glume (*sog3*) and brittle (*brc1, brc2 and brc3*) mutants used in the present study were isolated in M_2 generation from diploid wheat line *T. monococcum* acc. pau14087 after seed treatment with 0.25% EMS. This material was obtained from the Punjab Agricultural University, Ludhiana (Table 3.1).

Table 3.1 Mutant traits and gene symbol

Mutants ID	Trait	Gene symbol
MM-09	soft glume	***sog3***
MM -13	Brittle	***bcl1***
MM -15	Brittle	***bcl2***
MM -232	Brittle	***bcl3***
MM -21	Semidwarf GA_3 insensitive	NA
MM -24	Semidwarf "	NA
MM -26	Semidwarf "	NA
MM -28	Semidwarf "	NA
MM -29	Semidwarf "	NA
MM -32	Semidwarf "	NA
MM -33	Semidwarf "	NA
MM -34	Semidwarf "	NA
MM -35	Semidwarf "	NA
MM -37	Semidwarf "	NA
MM -38	Semidwarf "	NA
MM -39	Semidwarf "	NA
MM -41	Dwarf GA_3 insensitive	***Rht22***
MM -43	Semidwarf "	NA
MM -46	Semidwarf "	NA

NA: Not available

3.1.1.1 Plant height

It was measured in cm from the ground level to the tip of the terminal spikelet at physiological maturity with the help of measuring scale.

3.1.1.2 Free threshing

The spike of soft glume (*sog3*) mutant was more compact with lax glumes. The seeds in the mature spike could be easily threshed manually. On removal of individual spikelets from the spike, the rachis remained intact in *sog3* whereas in *T. monococcum* the spikelets separate with a wedge of rachis fragments without leaving any intact rachis (Fig. 4.6).

3.1.1.3 Plant brittleness

Brittleness data of individual plant was recorded after 30 days of sowing, after flowering and during harvesting by bending the plant parts. All plant parts of the three brittle mutants could be easily broken by bending.

3.2 METHODS

3.2.1 Gibberellic acid response

Freshly prepared gibberellic acid (GA_3) at 80ppm and 100ppm concentration was used to grow seedlings of all dwarf mutants along with *T. monococcum* to check the gibberellic acid sensitivity of seedlings of the dwarf mutants (Fig. 4.6). 45 days old plants of dwarf mutants along with *T. monococcum* were also sprayed by 50 ppm and 100 ppm solution of gibberellic acid in the field to check gibberellic acid response and final observations on culm elongation after GA_3 spray were recorded after 10 days of application. The response of GA_3 spray was calculated by the following formula:

$$GA_3 \text{ response}(\%) = \frac{\text{Final height after } GA_3 \text{ spray} - \text{Initial height before } GA_3 \text{ spray}}{\text{Intial height before } GA_3 \text{ spray}} \times 100$$

3.2.2 Chi-square test

χ^2 test for goodness of fit of segregation ratios was carried out at probability $p<0.05$ at one degree of freedom, where 'p' is the probability of deviation of the observed from the expected ratio due to chance alone. χ^2 is the sum of the squared difference between observed and the expected data, divided by the expected data in all possible categories.

3.2.3 Measurements of physical properties

The breaking force and elongation ratio of brittle culms and leaf were measured with a universal strength testing device (Model 7001; ZhongKai, China). To avoid any bias during sampling, the first internodes of culms were used for measurements. The elongation

ratio (%) was defined by the formula 100 × (L1-L2)/L2, where L1 is the length of culm segments at breaking and L2 the original length of the culm segments. Ten culms were used and dried at 30°C for 3 days before measurement. Three internodes above the ground (internodes third, fourth, and fifth) were subjected to a three point Flexural test using a Model 4411 Instron Electromechanical Testing Device (Instron Corp., Canton, MA). The span width of culm and flag leaves between the anchor points was 10 cm. The anvil was vertically driven at a constant speed of 10 cm/min. against the internodal zone, on a horizontally placed culm until it collapsed or snapped. The maximum load to break culm and leaves was used as a measure of strength.

3.2.4 Scanning electron microscopy (SEM)

Surface imaging of leaves and stems of mutants and *T. monococcum* was done using scanning electron microscope. Various steps followed for sample preparation for SEM were as follows:

1. Primary fixation: The sample was immersed in 2.5% glutaraldehyde for two hours at room temperature.
2. Dehydration: 50% ethanol for 5 min., 70% ethanol for 30 min. (Two changes), 90% ethanol for 30 min. (Two changes), 100% ethanol for 30 min. (Two changes), absolute alcohol: amyl acetate (3:1) for 30 min., absolute alcohol: amyl acetate (2:2) for 30 min., absolute alcohol: amyl acetate (1:3) for 30 min. and amyl acetate for 30 min.
3. Critical Point Drying: For 40 min.
4. Mounting: Sample was mounted onto metal stub with double sided carbon tape.
5. Sputter Coating: A thin layer of gold metal over the sample was applied using an automated sputter coater. This process took about 10 min. Spur coated samples were then placed under scanning electron microscope and the surface images of the plant samples were taken at 200X magnification.

3.2.5 Histological observations

For histological examination, leaves and stems were excised and fixed in FAA fixative. 100ml of FAA solution contained ethyl alcohol (50 ml), glacial acetic acid (5 ml), 40% formaldehyde (10 ml) and water (35 ml). FAA fixed leaves and stems were dehydrated in a graded ethanol series (30%, 50%, 70%, 90% and 100%) and xylene. To support the tissues for sectioning, the tissues were embedded in paraffin wax (Sdfine) at 60°C, and

sectioned to 10 µm thickness on a rotary microtome. Ribbons obtained from paraffin sections were mounted on slides, hydrated and dehydrated in a graded ethanol series. The tissues were stained with Phloroglucinol-HCL and toluidine blue O (0.05%, w/v) dye as per the method suggested by Johansen (1940). Phloroglucinol-HCL is a lignin specific dye and gives deep red colour with lignin components of cell wall. Toluidine blue O (TBO) is a metachromatic stain, which produces different colors depending on the polymer to which it adheres. With TBO, the primary walls (ground tissue, phloem subtype of vascular tissue) were stained purple and lignified secondary walls of xylem tracheids and vessels (a subtype of vascular tissue) were stained blue, while some other cells may stain greenish. Transverse sections of stem and leaf of dwarf, brittle mutants and wild type *Triticum monococcum* were observed under a light microscope (Axiostar plus 1169-151, Carl Zeiss Co., Oberkochen, Germany) at different magnifications.

To determine whether the alterations of cellulose and lignin are localized in particular cells, transverse sections of the culms and leaf of wild-type and mutant plants were histochemically stained with Wiesner solution (Phloroglucinol-HCL stain). Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity approximately reflects the total lignin content. The color differences in mechanical tissues, especially in the sclerenchyma cells below the epidermis, between wild-type and mutant plants were clear, indicating an apparent increase in lignin quantity in mutant plants

A Wiesner reaction was performed according to a standard protocol (Strivastava 1966). Fresh hand-cut sections (20 µm thick) from wheat culms were incubated for 2 min in phloroglucinol solution (2% in ethanol:water [95:5, v/v]; Sigma), mounted in 50% HCl, and photographed using a light microscope (Axiostar plus 1169-151, Carl Zeiss Co., Oberkochen, Germany) at different magnifications.

3.2.6 Anatomical structure of leaf

The middle portion of the leaf blades was selected. Transverse sections (20 µm thick) of leaf blades were prepared with a freezing microtome (Leica CM1100 Nubloch, Germany) at 15°C. Parts of the samples were used for histochemical localization of lignin, and the rest were immediately adhered to cover slip prior to drying at room temperature for 1 day and vacuum desiccator for 5 day.

3.2.7 Cell wall isolation for FTIR

The entire shoots of eight-week-old brittle mutant plants were harvested into liquid nitrogen, crushed into a powder in 2-ml Eppendorf centrifuge tubes, and then suspended in 50 mM Tris[HCl], pH 7.0, containing 1% SDS, and heated to 70°C to extract protein and other non-wall components, then collected on a nylon mesh filter (47-µm square, Nitex, Briarcliff Manor, NY), and washed with water. Three stainless steel balls (diameter 5-mm) were added to the tubes and the samples were homogenized at 1200 cycles min^{-1} in a reciprocating shaker (Geno-Grinder, SPEX Certi-Prep) for 10 min. The cell walls from the homogenate were collected on nylon mesh filters, and washed with 50% hot ethanol and then water at 70°C. The cell wall material in water was returned to the 2-ml Eppendorf tube, with three stainless steel balls (diameter 5-mm), and homogenized for second time at 1200 cycles min^{-1} in the reciprocating shaker for 10 min. The cell walls were then collected on the nylon mesh and washed sequentially with water (70°C), 50% (v/v) ethanol (70°C), and water at ambient temperature. The walls were suspended in deionized water and allowed to settle. Aliquots of these wall preparations were then plated on gold-plated IR reflective slides (EZ-Spot, Spectra-Tech) and air-dried.

3.2.7.1 Measurement of cellulose content

Cellulose was assayed according to the methods described previously (Updegraff, 1969). Briefly, the first internode of culms were ground into fine powder in liquid nitrogen. The powder was washed in phosphate buffer (50 mM, pH 7.2) three times, extracted twice with 70% ethanol at 70°C for 1 h and dried under vacuum. The dried cell wall materials were assayed for cellulose content with the anthrone reagent with Whatman 3MM paper as the standard.

3.2.7.2 Measurement of lignin content

Lignin content was also determined by measurement of lignothioglycolic acid (LTGA). Dried cell wall material (15µg) was treated with 0.3 mL thioglycolic acid and 1.5 ml HCl (2 mol L^{-1}) for 4 h at 95°C. After centrifugation at 15,000× *g* for 15 min, the pellet was washed for 3 times using water, and LTGA was extracted using 1.5 ml NaOH (0.5 mol L^{-1}) by incubation with shaking for 16 h at 20°C. The supernatant obtained after

centrifugation and a second supernatant obtained after re-extracting the pellet with 0.4 ml NaOH were mixed and acidified with 0.4 ml concentrated HCl. Lignothioglycolic acid was allowed to precipitate for 4 h at 4°C, recovered by centrifugation at 15,000 × *g* for 20 min, and dissolved in 1 ml NaOH (0.5 mol L^{-1}). The absorbance was measured against NaOH blank at 280 nm. The unit of lignin relative content was *A*280 mg^{-1} cell wall (Musel *et al.* 1997). The values reported were the mean results from triplicate experiments.

3.2.8 Removal of hemicellulose and lignin for SEM analysis

Straw of brittle mutants alongwith wild type was first oven dried at 50°C for 24 hours. The hydrothermally pretreated straw was subsequently delignified by mixing approximately 25 g of dried straw with 800 ml MilliQ water, 40 ml of 98% glacial acetic acid and 20 g of sodium chlorite ($NaClO_2$). The mixture was placed in a water bath at 80°C for 1 hour. The sodium chlorite and acetic acid additions were repeated twice, the second time with the addition of glacial acetic acid only. The reaction was terminated by cooling to 10°C. The holocellulose was isolated by filtration through a glass filter and rinsing with ice-cold MilliQ water, followed by oven-drying at 50°C for 24 hours. For SEM, the straw was lyophilised without prior oven-drying. (Kristensen *et al.* 2008).

3.2.9 Fourier transform infrared spectra

Spectra were recorded using an IR Spectrometer (MIDAC corp.) over the range of 1800–700 cm^{-1} at 4 cm^{-1} resolution in absorbance mode and averaged from 48 scans. Internodal cell wall material were powdered and added to KBr to form a pellet that contained 1% test material.

3.3.1 Mapping of mutants

In order to map the brittle, dwarfing and free threshing mutants, separate mapping populations were developed by crossing all these mutants with *T. monococcum* subsp. monococcum acc. pau14087 as well as *T. boeoticum* acc. 5088 and the F1 hybrids were selfed to develop F2 populations (Fig. 3.1). All the mutants and the F1s were grown in *Rabi* seasons of 2006 and 2007 at the Indian Institute of Technology, Roorkee with the recommended package of practices for wheat.

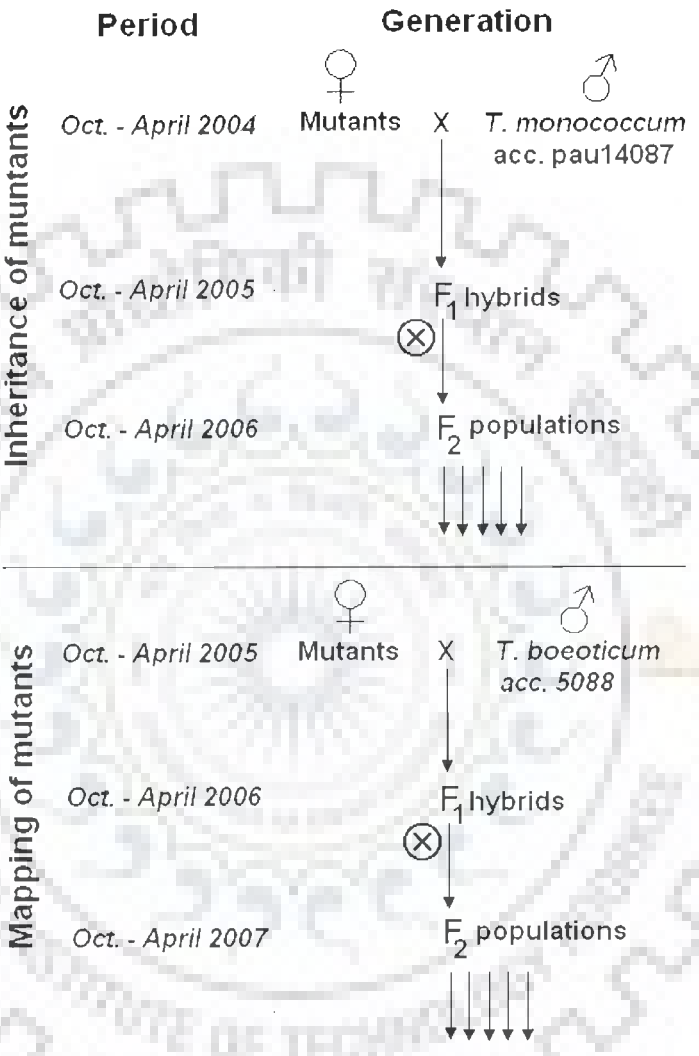


Figure 3.1 Schematic presentation of the development of F2 mapping populations.

3.3.1.1 Molecular Biology Kits

Kit	Supplier
QIAEXII gel elution kit	Qiagen, Valencia, CA, USA QIAquick PCR
purification kit	Qiagen, Valencia, CA, USA
iRIS® kit	IHBT, Palampur, India
Superscript™ II RT kit	Invitrogen- life technologies, CA
RNeasy MinElute cleanup kit	Qiagen, Valencia, CA, USA

3.3.1.2 Enzymes

Taq DNA polymerase	Bangalore Genei, Bangalore
RNase I	Promega Madison, WI, USA

3.3.1.3 Molecular weight markers

100bp DNA ladder	Bangalore Genei

3.3.1.4 Buffers and solutions

10X TBE buffer	1L 108g Tris 55g Boric acid 40 ml of 0.5M EDTA (pH 8.0)
1X TE(Tris-EDTA buffer)	10mM Tris HCl, 1mM EDTA, pH8.0
Plant genomic DNA extraction Buffer	2% Cetyl Trimethyl Ammonium Bromide (CTAB) 50mM EDTA (pH 8.0) 100mM Tris-HCl (pH 8.0) 1.4M NaCl

Autoclaved and 1% β-mercaptoethanol was added before use

5X RNA gel loading buffer	0.2 M MOPS (pH 7.0) 36% Formaldehyde 75% deionized formamide
10X DNA loading dye	0.4% bromophenol blue 0.4% Xylene cyanol FF 50% Glycerol in distilled water

3.3.2 Genomic DNA extraction

Plant DNA of all the F2 plants and the parents were isolated from leaf tissue collected from each plants using the modified CTAB method (Saghai-Maroof *et al.* 1984). About 5-7 gm of young leaves were ground in liquid nitrogen and the powder was quickly transferred to 50ml (Oakridge) tubes. The ground samples were placed in ice or at -20°C. After all the samples were ground, 15 ml of 2X CTAB extraction buffer was added to the powdered samples.

The powder was suspended in the DNA extraction buffer by inverting the tubes gently. The tubes were incubated at 65°C for 30 min. in water bath. The samples were mixed occasionally while maintaining at 65°C. After incubation, 15 ml of chloroform: isoamyl alcohol (24:1) was added and tubes were swirled, till it made an emulsion. The tubes were placed on a rotary shaker for 30 min. and then centrifuged at 10,000 rpm for 10 min. at room temperature. The supernatant was transferred to clean sterile 50-ml Falcon tubes. 5µl RNAse (10mg/ml) was added to each tube and incubated at 37°C in water bath for 1h. Chloroform: isoamyl alcohol extraction and centrifugation steps were repeated after RNAse treatment. The supernatant was transferred to clean sterile 50-ml Falcon tube. About 0.8 volume of chilled isopropyl alcohol was added and the tubes were inverted gently several times. The DNA formed white cotton like precipitates and the good quality DNA floated at top. The floating DNA was hooked out using a sterile hooked pasture pipette. The hooked or pelleted DNA was transferred into clean sterile 2.0ml microfuge tubes and was rinsed with 70 % ethanol. The leftover ethanol was dried completely by inverting microfuge tubes on a blotting paper. Dissolved DNA in 500-800 µl of TE depending upon the quantity of DNA. The tubes were left for few hours at room temperature to allow DNA to dissolve.

3.3.3 Assessment of quantity and quality of DNA

DNA was quantified by agarose gel electrophoresis. In this 0.8 g of agarose was dissolved in 100 ml of 0.5X TBE electrophoresis buffer. Composition of 0.5X TBE buffers is 45 mM Tris base, 45 mM Boric acid and 1 mM EDTA. The mixture was heated till the agarose was completely dissolved, i.e. when the solution becomes transparent and clear. It was cooled down to 60°C with constant stirring. Ethidium bromide was added to a final concentration of 0.5 µg/ml buffer. Then the agarose

solution was poured into an already prepared gel mould with combs and was left for 20-30 minutes for solidification. DNA samples for loading were prepared by adding loading dye to the DNA such that final concentration of loading dye was 1X. When the gel solidified, the DNA samples along with loading dye were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known DNA concentration was also loaded. After loading, the gel was run for about 2 hour at a voltage of 5V/cm. The gel was then visualized under UV transilluminator. Using photo-gel documentation system, the DNA samples under UV light were photographed. The intensity of the fluorescence of each sample was compared with that of the standard marker and then DNA concentration of each sample was ascertained. The quality of the DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (poor quality).

3.3.4 PCR amplification

All the reactions were performed in a MJ Research PTC200 and Eppendorf Master Cycler using 40-80 ng of genomic DNA of each F2 plant with parents in a final volume of 20µl. The final concentration of different components used in the PCR reaction were 1X PCR buffer without $MgCl_2$ (Fermentas), 1.5mM of $MgCl_2$, 100mM of each dNTP, 0.25 µM of each primer, 2.0 µl of home made Taq polymerase (Approx. 2-3 units). The reaction mix was overlaid with a drop of mineral oil and placed in 96 well thermal cycler. Amplifications were performed using following temperature profile:

1.	Initial denaturation at 94°C	4min
2.	Denaturation at 94°C	1min
3.	Annealing at 50-63°C (depending upon primer's melting temperature)	1min
4.	Elongation at 72°C Steps 2-4 were repeated 29 times.	2min
5.	Extension at 72°C	7min
6.	Hold at 12°C	

3.3.4.1 Visualization of PCR products

For 20 µl of amplified products 3.3 µl of 6X loading dye was added so as to bring the final concentration of loading dye to 1X. The PCR products were resolved on 2.5 percent agarose gel or 6% polyacryl amide gel. The agarose gel was prepared in 0.5X TBE

buffer. Ethidium bromide was added at a concentration of 0.5 µg/ml. The gel was run at 5V/cm. The gel was visualized under UV light and photographed using UVP gel documentation system (Model GDS 7600) with GRAB-IT software programme (Annotating grabber 32-Bits). The SSR markers, which were monomorphic in 2.5 percent agarose gel, 6.0 percent polyacrylamide gel with silver-staining procedure (Chen *et a.l* 1999) was used.

3.3.5 Parental Polymorphism

The SSR markers with known primer sequences (Singh *et al.* 2007) were selected from each linkage group, initially at a regular distance of 5-10cM for amplification of parental DNA, positive and negative bulks. At least one polymorphic SSR marker was identified in each 10cM region. The amplified products were scored as polymorphic if amplified band position differed in both parents and monomorphic if amplified band position was same in both the parents.

3.3.6 Bulk Segregant Analyses (BSA)

Two bulks were made from the selected lines of each segregating F_2 mapping populations developed from different crosses. Positive bulks were prepared from homozygous dwarf and gibberellic acid insensitive plants, brittle culm and free threshing (bulk 1) plants, while a common negative bulk (bulk 2) was prepared from 15 RILs of *T. boeoticum* pau5088 x *T. monococcum* pau14087 cross without any mutant. An equal concentration of DNA from these plants was pooled. These two bulks along with the parents were used to identify putative SSR markers linked to the dwarfing, brittle and free threshing gene. The microsatellite markers distinguishing parents and bulks were used to analyze individual plants constituting each bulk and further tested on the F_2 mapping population. Linkage analysis was done using recombination frequency between marker and mutant phonotype at each locus using MAPMAKER (Lander *et al.* 1987, Loncoln *et al.* 1990).

3.3.7 Molecular marker analysis of F2 populations

The DNA of F2 plants was amplified in 96 well plate format that included 90 F2 plants DNA reaction mixture, two parents and water as negative control. Each of 90 F2 plants DNA reaction mixtures was amplified using polymorphic SSR markers. The amplified fragments of F2 were scored as mutant type or *Triticum boeoticum* type or heterozygous. The molecular data obtained for each marker was recorded in an excel sheet.

3.3.7.1 Detection of polymorphism by SSR (Simple Sequence Repeats)

One hundred thirty three diploid wheat SSR or microsatellite primer pairs representing all the 7 chromosomes of *Triticum monococcum* covering both chromosomal arms for initial parental polymorphism survey between *Triticum monococcum* and *Triticum boeoticum* were selected (Singh *et al.* 2007). PCR was carried out according to Singh *et al.* (2007).

3.3.7.2 Resolution of amplified SSR product

2µl of 10X loading dye was added to the 20µl PCR product. The PCR product was loaded on 2.5% LMP high resolution superfine agarose (promega) prepared with 0.5X TBE buffer or on 6% Polyacrylamide Gel. Gels were stained with 1µg/ml ethidium bromide and then visualized under UV light and photographed using UVP Gel documentation system (Model GDS7600) with GRAB-IT software programme (Annotating Grabber 32-Bits).

3.3.8 Linkage analysis

Chi-square test was performed to examine the segregation ratios at the marker loci for deviation from the expected ratios 1:1, and the skewed ness in the population if any was determined. Linkage analysis of polymorphic marker loci was performed with the computer program 'MAPMAKER/EXP version 3.0' (Lander *et al* 1987, Loncoln *et al*, 1990) for F2 lines. We used the mapping function of Haldane (Haldane 1919) because of the independent crossover events in different meiotic phases during the development from F_1 to F_2 generation. Two- point, Three-point and multi-point analyses were used in order to determine the best order of marker loci within the linkage groups. Maximum LOD score of 3.0 and a recombination fraction of 40 were used for identifying linkage groups. The most likely order of markers in each group was determined by using 'order', 'compare' and 'ripple' commands. In case of more than one possible arrangement of linkage groups we chose the one with the smallest genetic linkage distance between the adjacent marker loci of the linkage groups to construct genetic map.

3.3.9 Primers for *Rht22*

The sequence of *RhtD-1* specific primers used for PCR was designed in such a manner that they could amplify the highly conserved DELLA motif present at N terminal of the locus. The *RhtD-1* specific primers used for this purpose were the same as used by Richards *et al.* (2002). Sequence of primers has been shown below:

Forwar primer	DF	5′-CGCGCAATTATTGGCCAGAGATAG-3′
	DF2	5′-GGCAAGCAAAAGCTTCGCG-3′
Reverse Primer	WR2	5′-GGCCATCTCGAGCTGCTC-3′

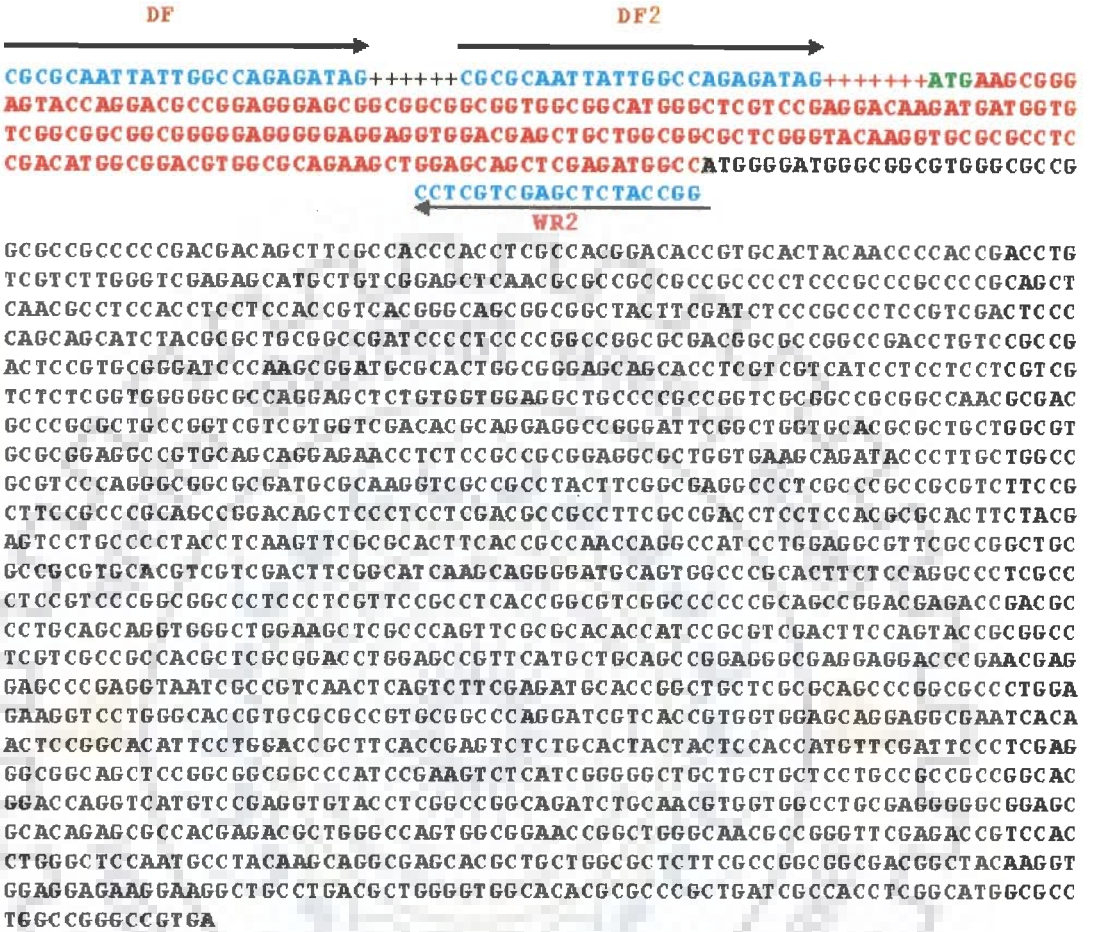


Figure 3.2 Nucleotide sequence of *Rht-D1* showing the PCR primers covering the DELLA domain sequence (Red).

Primer designing was done by using Primer3 programme (http://frodo.wi.mit.edu/) (v. 0.4.0) developed from Whitehead Institute for Biomedical Research. During primer designing certain points were considered to avoid non-specific amplification. Primer designing is always a critical and most important part of any PCR technique. For this, primer length was kept between 17 to 25 nucleotides. Its GC content was fixed around 50%. Care was taken to minimize the long run sequences with any of single nucleotide and primer was designed in such a way that they would not form any secondary structure and would not be complementary.

3.3.9.1 PCR Protocol

Conditions used for the PCR reaction were 95°C for 5 min; 42 cycles of denaturation at 94°C for 30 sec; annealing at 58°C for 30sec; extension at 72^0C for 10 sec.) and a final step of 72 °C for 2 min using PTC-100-programmable thermal controller (MJ Research, USA). The reaction mix (20 µl) for PCR consisted of 100 ng of genomic DNA of both *Rht22* and *T. monococcum*, 4 nmol of dNTPs, 20 pmol each of the forward and reverse primers, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 2 units of *Taq* DNA polymerase.

3.3.9.2 Gel elution and purification of PCR product

The DNA fragment of desired length obtained in PCR reaction was excised from the agarose gel with a clean and sharp scalpel. Three volumes of QX1 (provided in the QIAquick gel extraction kit) was added to 1 volume of gel. The QXII (provided in the QIAquick gel extraction kit) resuspended by vortexing and added to the gel. The gel was incubated at 50°C for 10 min (or until the gel slice was completely dissolved). The sample was centrifuged at 10,000 g for 30 seconds and the supernatant was removed. The pellet obtained was washed with 500µl of QX1 buffer, resuspended by vortexing and centrifuged again at 10000 g for 30 seconds. The pellet obtained was washed with PG buffer (provided in the QIAquick gel extraction kit). The pellet was air dried until it became white. The entire DNA sample was again resuspended in 30µl of MilliQ water with the help of tip and centrifuged for 30 seconds at 4000 rpm. The supernatant was finally collected into a clean tube using pipette.

3.4.1 Sequencing of PCR products

Gel-purified PCR products were sequenced (Ocimum Biosolutions, Hyderabad, India) according to the manufacturer's instructions. It was based on the dideoxynucleotide chain termination method (Sanger *et al.* 1977), by using automatic sequencing machine.

3.4.2 Confirmation of two bp deletion in DELLA motif of *Rht22*

After getting the genomic sequence of wild type and *Rht22* mutant gene, the two bp deletion in dwarf mutant *Rht22* was confirmed using deletion based reverse primers and genome specific forward primers. The gene specific forward primer DF2 was taken as such where as WTM (wild *T. monococcum*) reverse primer was designed using PRIMER3

software in such a way that it would amplify only *Triticum monococcum*. To amplify the product with two bp deletion in *Rht22* reverse primer MTM (mutant *T. monococcum*) was synthesized on the base of two bp deletion. Nucleotide sequence of the gene specific primers are as follows:

Forward primer	DF2	5'-GGCAAGCAAAAGCTTCGCG -3'
Reverse primer	WTM	5'-CATCTCGAGCTGCTCCAGCTTCTG-3'
	MTM	5'-CATCTCGAGCTGCTCCAGCTTCC-3'

Conditions used for the PCR reaction were 95°C for 5 min; 42 cycles of denaturation at 94°C for 30 sec; annealing at 58°C for 30sec; extension at 72^0C for 10 sec. and a final step of 72°C for 2 min using PTC-100 thermal cycler (MJ Research, USA). The reaction mix (20 µl) for PCR consisted of 100 ng of genomic DNA of both *Rht22* and *T. monococcum*, 4 nmol of dNTPs, 20 pmol each of the forward and reverse primers, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and 2 units of *Taq* DNA polymerase.

3.4.3 Bioinformatic analysis

Partial nucleotide sequence of *Rht22* was BLASTed with genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequence alignment with GA_3 Insensitive dwarfing genes of wheat, barley, maize and rice using clustalW. After mapping the brittle mutants on wheat chromosomes, their syntenic rice chromosomes were searched for CesA and CSL genes in order to identify the wheat EST sequences orthologous to the six rice genes involved in cellulose synthesis pathway, the sequences of rice genes were BLASTed against the wheat ESTs available at http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi. Wheat ESTs showing maximum homology (e^{-50} and bit score ≥200) with the each of the six rice genes were further blasted against bin-mapped wheat ESTs/contigs available at http://wheat.pw.usda.gov/GG2/blast.shtml with a view to identify the location of orthologous wheat ESTs on the wheat EST physical maps .

CHAPTER 4

RESULTS

Chapter 4

RESULTS

4.1 Mutant characterization

A field view of the EMS induced mutants received from Punjab Agricultural University, Ludhiana, at the Indian Institute of Technology, Roorkee (Fig. 4.1) and plant morphology of some of the brittle and dwarf mutants studied in detail is shown in Fig. 4.2.

4.2 Gibberellic acid sensitivity of dwarf culm mutants of *Triticum monococcum*

The length of internodes, flag leaf, spikes and seed shape of various dwarf mutants are depicted in Fig. 4.3 and Fig. 4.4. There was higher stem elongation on application of 100 ppm GA_3 than 50 ppm in 45 days old plants in all the mutants and wild type control. *T. monococcum* was highly sensitive to GA_3 with an increase of 25.92 percent height over control. Among 10 dwarf mutants MM-21, MM-37 and MM-41 (*Rht22*) were highly insensitive while other had medium to high sensitivity to GA_3. The seeds of the ten true breeding dwarf *T. monococcum* mutants were germinated in petriplates. Response of dwarf mutants to GA_3 at seedling level was estimated following Yamada (1990). For the GA_3 response at seedling level, the mutants and wild type seedling were grown at 80 ppm of gibberellic acid. Application of GA_3 during germination of various dwarf mutants showed that mutants MM-21, MM-24, MM-35, MM-39 and MM-41 (*Rht22*) were highly insensitive to GA_3 where as *T. monococcum* and other mutants had medium to high sensitivity (Fig. 4.6). The data on GA_3 sensitive and insensitive dwarf mutants at 45 days old plants is given in Table 4.1.

Table 4.1 GA_3 sensitivity of 45 days old dwarf mutants along with *Triticum monococcum*.

Mutant ID	Average plant height (cm)				% Increase in height over control	
	Control	Treated GA_3 (50ppm)	Control	Treated GA_3 (100ppm)	50 ppm	100 ppm
Wild type	84	98	87	102	12.64	25.92
MM-21	54	57	51	53	3.34	3.92
MM-24	75	80	77	86	6.66	11.68
MM-26	76	82	74	84	7.89	13.51
MM-28	67	79	69	80	17.91	15.94
MM-32	74	85	63	79	14.86	25.39
MM-33	59	74	62	76	25.42	22.58
MM-34	66	78	63	77	18.18	22.22
MM-37	74	76	78	82	2.70	5.12
MM-38	51	68	53	64	33.33	20.75
MM-41 (*Rht22*)	53	55	50	52	3.10	3.60

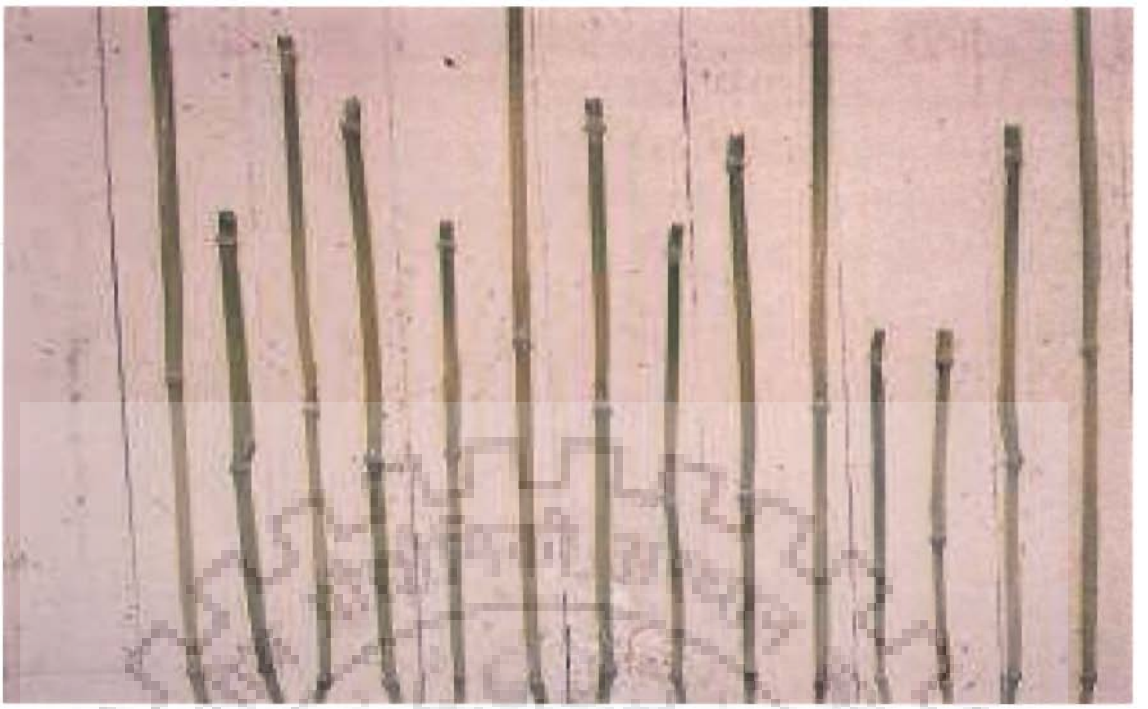
Table 4.2: Morphological traits of GA_3 sensitive and insensitive dwarf mutants of *T. monococcum*

Mutant ID	Second internode	Flag leaf	Spike	Seed	Seedling Sensitivity	GA sensitivity plant
Wild Type	Tall	Long	Long	Slender	Sensitive	Sensitive
MM-21	Semidwarf	Long	Short	Slender	Medium Insensitive	Insensitive
MM -24	Semidwarf	Small	Short	Round	Medium sensitive	Medium sensitive
MM -26	Semi dwarf	Small	Short	Slender	Medium Insensitive	Medium Sensitive
MM -28	Semidwarf	Small	Short	Bold, round	Insensitive	Medium Sensitive
MM -32	Slightly dwarf	Long	Medium Short	Bold, round	Sensitive	Sensitive
MM -33	Semidwarf	Long	Medium Short	Bold, round	Sensitive	Sensitive
MM -34	Semidwarf	Small	Short	Bold, round	Sensitive	Sensitive
MM -37	Semidwarf	Long	Long	Round	Insensitive	Insensitive
MM -38	Semidwarf	Long	Medium Short	Bold, round		Sensitive
MM -39	Semidwarf	Small	Short	Round	Sensitive	Sensitive
MM-41 (*Rht22*)	Semi dwarf	Small	Medium short	Bold, round	Insensitive	Insensitive
MM-43	Semidwarf	Long	Long	slender	Sensitive	sensitive

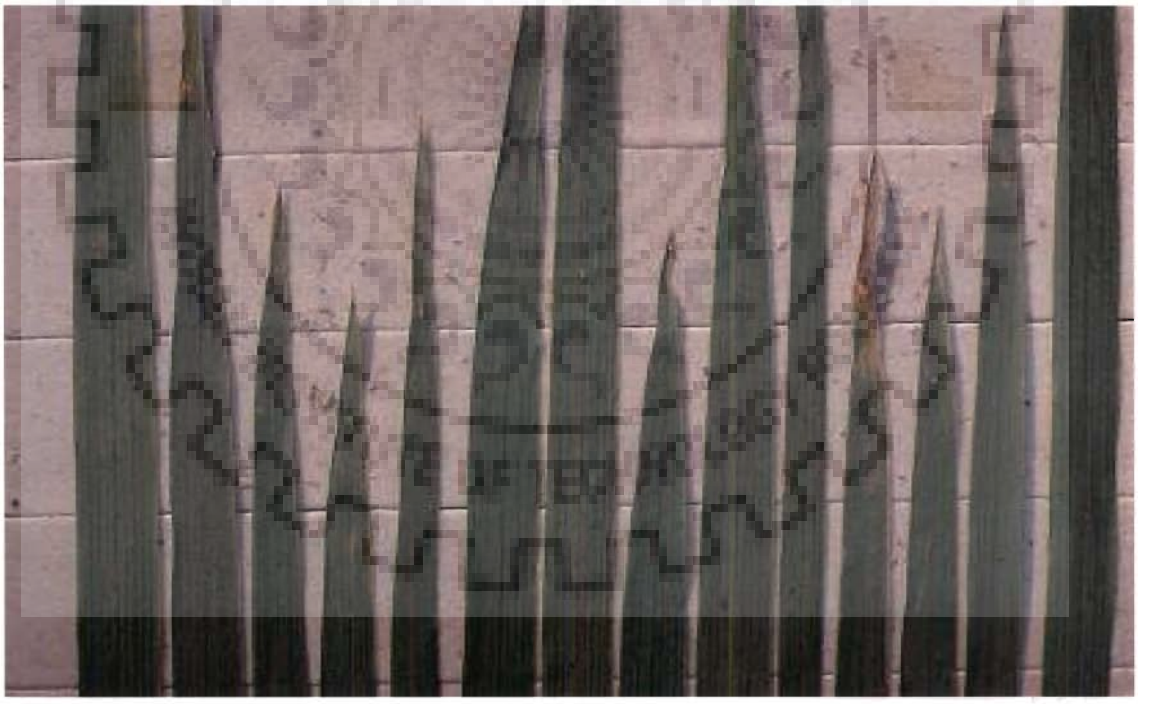
Figure 4.1 A field view of some EMS induced mutants used in the present study. These were isolated from diploid wheat *Triticum monococcum* acc. pau14087 at Punjab Agricultural University Ludhiana.

T. monococcum ***brc1*** ***brc2*** ***Rht22*** ***brc3***

Figure 4.2 A field view of brittle culm and dwarf mutants used in the present study



(a)



(b)

Figure 4.3 Comparative culm and leaf morphology of *Triticum monococcum* and dwarf mutants. (a) Length of second internode (b) Flag leaf.

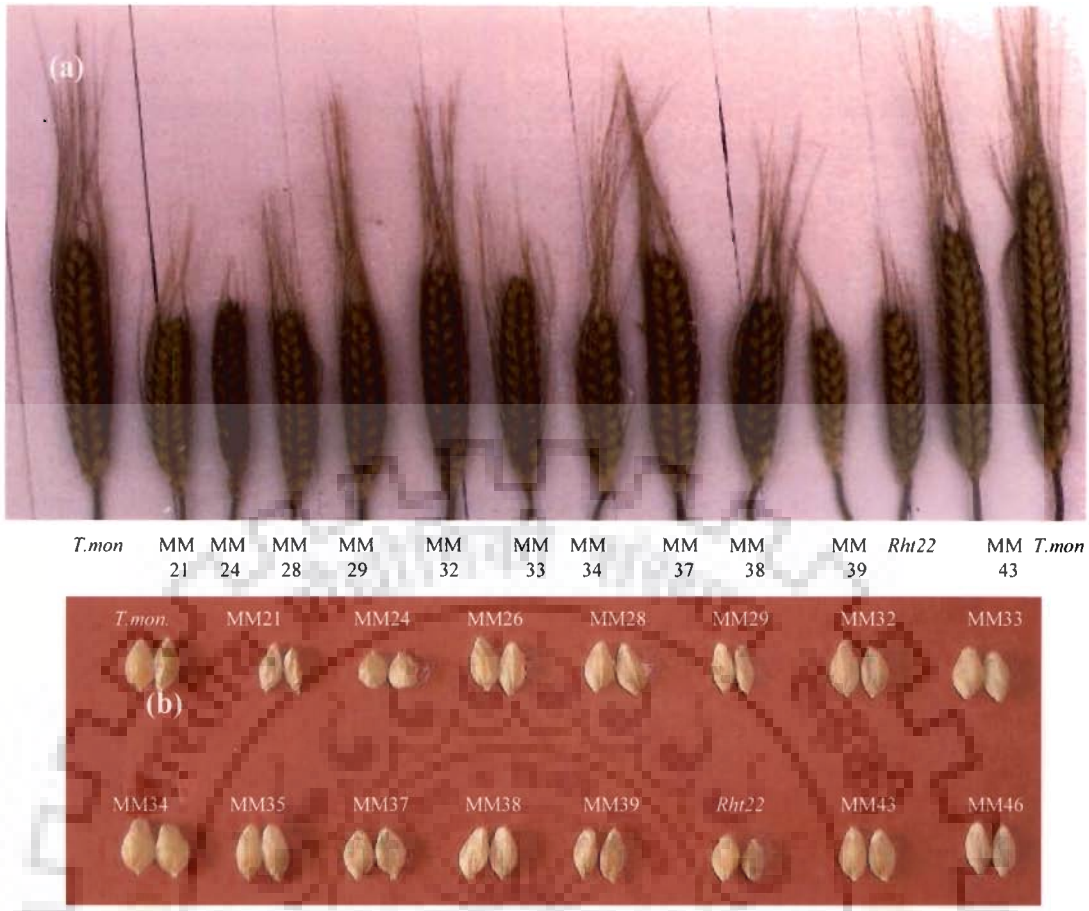


Figure 4.4 Comparative spike and seed morphology of *Triticum monococcum* and dwarf mutants (a) Spikes (b) Seeds

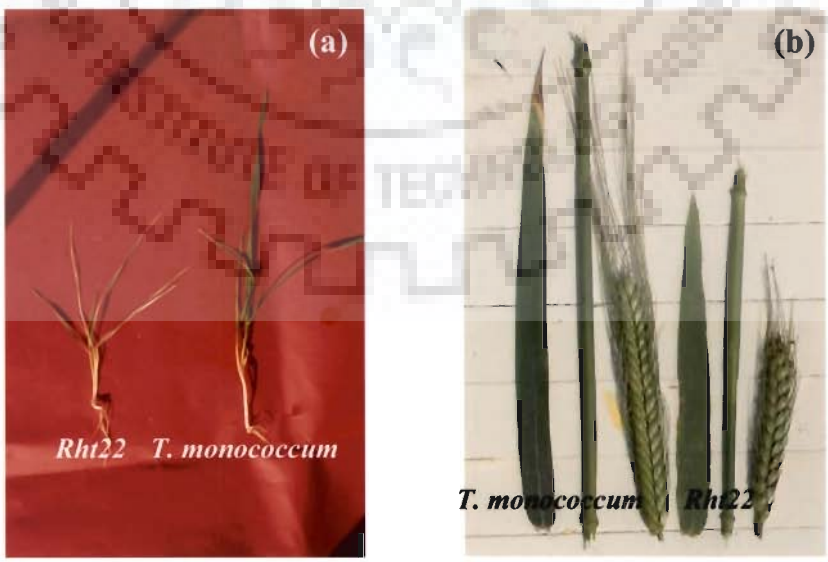


Figure 4.5 Comparative morphology of *Triticum monococcum* and *Rht22* (a) 20 days old seedling (b) A flag leaf, second internode and spike of *Triticum monococcum* (left) *Rht22* (right).

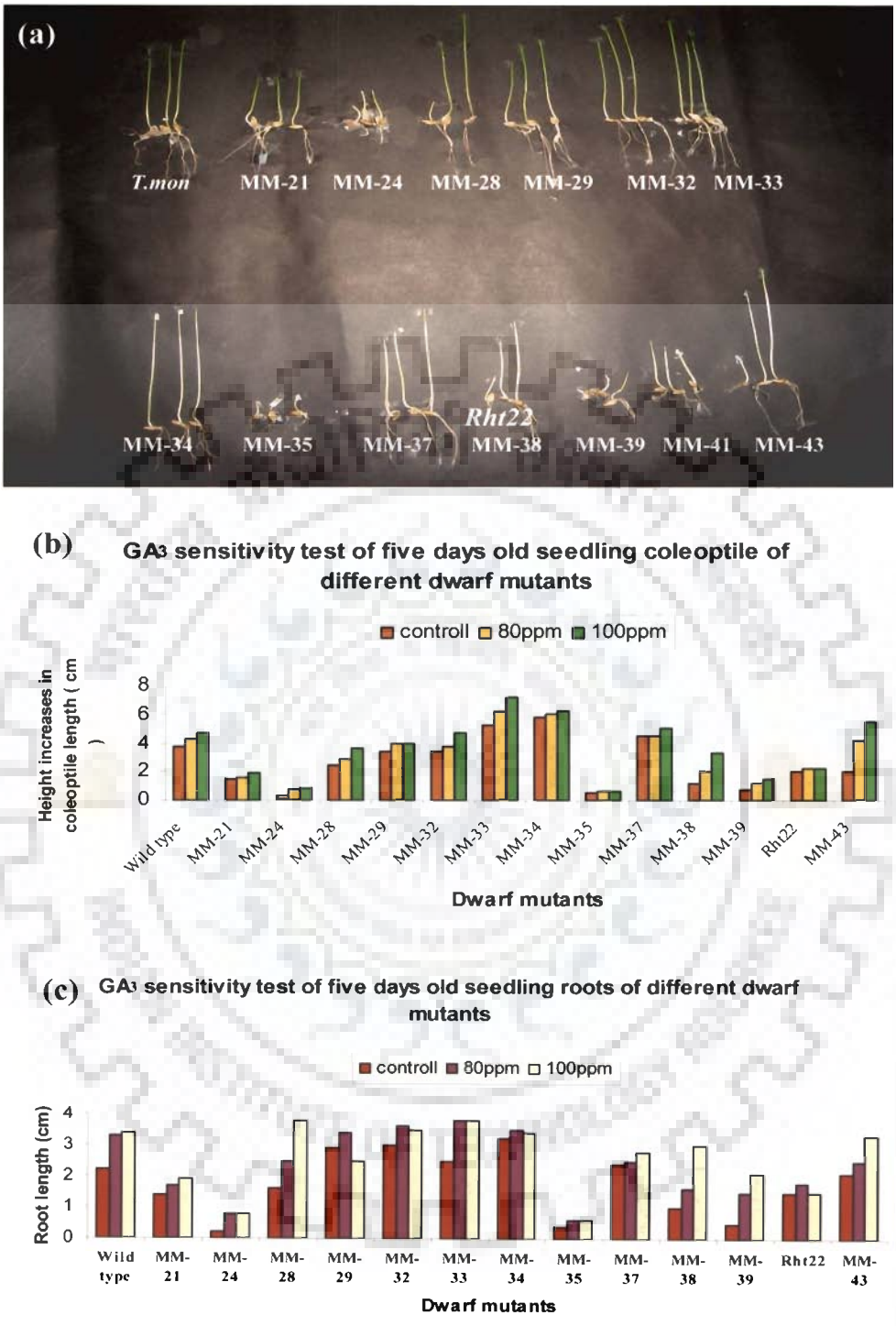


Figure 4.6 GA_3 sensitivity of dwarf mutants at seedling stage (a) Coleoptile length of 5 days old seedlings of *Triticum monococcum* and dwarf mutants during germination on petriplates containing distilled water (control), 80 ppm and 100 ppm GA_3 solution, respectively (b) Comparison of coleoptile length (c) Comparison of root length

Morphological data on dwarf mutants (Table 4.2) shows high variation for internode length, flag leaf, spike length and seed shape besides GA_3 sensitivity at seedling and plant level. MM-21 had thick stem where as MM-39 and MM-41 (*Rht22*) had smallest second internode. Some dwarf mutants had flag leaf as long as *T. monococcum*. Most of the mutants had shorter spike with bold and rounded seeds. Only MM-21 and MM-29 had slender seeds.

4.3 GA_3 insensitive dwarf mutant MM-41 (*Rht22*)

Out of various GA_3 sensitive and insensitive dwarf mutants only one GA_3 insensitive mutant MM-41(*Rht22*) was short listed for mapping and cloning (Fig. 4.5). The height of dwarf mutant *Rht22* was almost half of that of wild type *T. monococcum*. The dwarf mutant had low tillering capacity, small compact spike and slightly smaller and round grains. The mutant plants were photoinsensitive and healthy with broad dark green and smaller leaves. Fig. 4.5 a shows the first, second and third internodes of *T. monococcum* and other dwarf mutants along with *Rht22*. The length of second internode of the *Rht22* was approximately half to that of *T. monococcum*, narrow leaves, high tillering and longer grains as compared to that of the *Rht22*. The grains in *T. monococcum* were longer than that in *Rht22* (Fig. 4.5 b). Some (10-15%) seed sterility was observed in *Rht22* mutant. The dwarf mutant had late flowering as compared to *T. monococcum*. The plant height of *Rht22* was approximately 80 cm at maturity compared to 125 cm of *T. monococcum*. Average number of tillers per plant in *Rht22* was 12 while 18 in *T. monococcum*.

4.3.1 Anatomy of the *Rht22* dwarf mutant

In order to establish as to how the *Rht22* mutant functions in the internode elongation and leaf development, histology of the second internode from 90 days old plants of dwarf mutant and *T. monococcum* was carried out. It is evident from the images taken at different magnifications (Fig. 4.7), that the cells in the mutant plant were packed densely and their cell size was smaller than its wild counterpart. In the transverse section (TS) of second internode (Fig. 4.7 a,b,c and d), there was a difference between the number of layers of cells in dwarf mutant (~16) and *T. monococcum* (~7). In the longitudinal section (LS) of the second internode (Fig. 4.7 e and 4.7 f), the length of cells in certain layers was reduced to half of the wild type plants. Cell density (number of cells per unit area) in the internode was more in dwarf mutant compared with *T. monococcum*. The cell diameter was smaller in

the dwarf mutant and it remained almost same for all the parenchymatous cells as compared to progressive increase in cell diameter from periphery to the center in case of *T. monococcum* stem parenchymatous cells.

4.4 Soft glume mutant (*sog3*)

The spike of soft glume (*sog3*) mutant was tapering towards tip and had lax glumes (Fig. 4.8). The seeds in the mature spike could be easily threshed manually. On removal of individual spikelets from the spike, the rachis remained intact in the *sog3* whereas in *T. monococcum* the spikelets separated with a wedge of rachis fragments without leaving any intact rachis (Fig. 4.8). In the free-threshing mutant *sog3*, the glumes were softer, longer and broader.

4.5 Brittle culm mutants

The brittle culm mutant's plants *brc1, brc2 and brc3* were semi-dwarf with shorter stems, smaller drooping leaves and spreading growth habit as compared with the *T. monococcum.* The leaves, culm and spikes of *brc1, brc2 and brc3* mutants were brittle where as the plants parts did not break on bending in *T. monococcum. brc1* and *brc2* had early brittleness in comparison to *brc3*. Some of the brittle mutants were more sensitive to certain herbicides and wheat disease as compared to wild type *T. monococcum.*

Table 4.3 Morphological characteristics of *T. monococcum* and brittle mutants

Trait	***T. monococcum***	***brc1***	***brc2***	***brc3***
Habit after flowering	Erect	Spreading	Spreading	Spreading
Main culm height (cm)	130	75.4	92.1	98.3
Flag leaf length (cm)	40.6	35.5	36.5	37.5
Numbers of tillers per plant	30	25	27	28
Spikelet per spike	32	22	26	28
Seedling root length (cm)	1.3	1.2	1.2	1.5
Days to 50% flowering	110	90	115	120
Brittleness in leaves, culms, roots and spikes	Absent	Present	Present	Present
Susceptible to herbicide (Topik)	no	yes	yes	no
Susceptible to powdery mildew	no	yes	yes	no

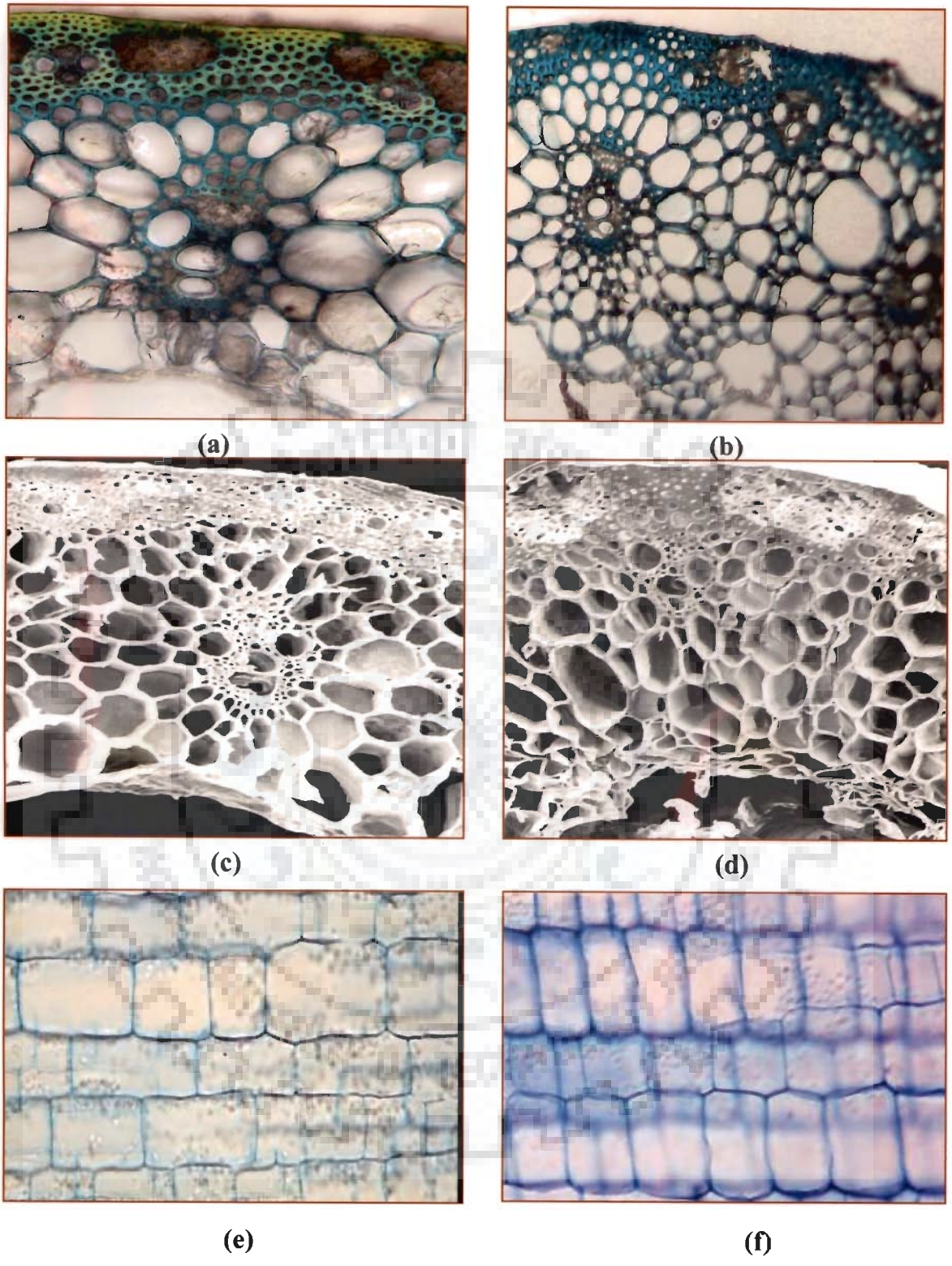


Figure 4.7. Comparative anatomy of *T. monococcum* and *Rht22*. (a) TS of second internode in *T. monococcum* and (b) *Rht22*, at 10X (c) SEM of TS of second internode in *T. monococcum* and (d) *Rht22*, at 10X (e) LS of second internode in *T. monococcum* and (f) *Rht22*, respectively at 40X

Figure 4.8 Comparative morphology of spike of *Triticum monococcum* and *sog3*

4.5.1 The elongation ratios of culm of wild-type and the brittle mutants:

The elongation ratio of the *brc1, brc2 and brc3* culms reduced by ~ 50% compared with that of the wild-type plants (Fig. 4.10 a). The significant reduction in the elongation ratio of the *brc1, brc2 and brc3* mutant indicated that the mutations in *brc1, brc2 and brc3* affected the elasticity that enables plant organs or cells to maintain their proper shapes and positions.

4.5.2 Mechanical strength of brittle mutants

Morphologically, *brc1, brc2 and brc3* mutants plants were clearly distinguishable from *T. monococcum* as they were slightly bent down after flowering, had brittle culms and leaves that could be easily broken by bending (Fig. 4.9). To accurately describe this phenotype quantitatively, the breaking forces required to break the segments of culms or leaves of *brc1, brc2 and brc3* and *T. monococcum*was compared (Fig 4.10 b,c). The force required to break the mutant culms and leaves were decreased to ~70 %, ~75% and ~52% for *brc1*, *brc2* and *brc3,* respectively as compared to the wild type whereas the force required to break the mutant leaves was decreased to 45 to 50 % as compared to wild type.

4.5.3 SEM of Sclerenchymatous cell wall of brittle mutants

Reduction in the mechanical strength of culms and leaves may reflect alterations in cell wall structure, composition, or fiber length. The cell wall morphology was examined with scanning electron microscopy. SEM of transverse sections of second internodes was carried out to see the difference of histological structure of 90 days old plants of *T. monococcum* and brittle mutants *brc1, brc2 and brc3* (Fig. 4.11 and Fig. 4.12).

In wild-type, several layers of sclerenchymatous cells, especially those around the peripheral vascular tissues and under the epidermal layer in culms and leaf veins, provide the mechanical support for the plants. Scanning electron microscopy observations revealed that the wild-type sclerenchyma cell walls were heavily thickened and nearly completely filled up with cell wall materials at the mature stages of culms and leaves (Fig. 4.11 and Fig. 4.12), in striking contrast to those of *brc1, brc2 and brc3* mutant plants where cells had no secondary cell wall thickening. However, no differences in cell length and width were found among *brc1, brc2 and brc3* mutants and wild-type plants. These results suggest that reduction in the mechanical strength of *brc1, brc2 and brc3* plants very likely resulted from defects in thickening of cell wall of the mechanical tissues, such as sclerenchyma. The transverse sections of mature flag leaves through mid rib under scanning electron

microscope showed that the sclerenchymatous cells above and around the vascular bundles had hollow cavities and thin cell walls in *brc1, brc2 and brc3* mutants whereas in the wild type, the sclerenchymatous cells were completely filled with cell wall material (Fig. 4.12).

4.5.4 Brittle mutant's *brc1, brc2 and brc3* plants had an altered cell wall composition

To determine whether the cellular phenotype and the reduced mechanical strength in *brc1, brc2 and brc3* mutant plants resulted from altered cellulose biosynthesis, the α-cellulose contents of mutant and wild-type plants was compared (Table 4.4 and Table 4.5). The amount of cellulose in the cell wall of *brc1, brc2 and brc3* culms was reduced to ~46 %, ~43 % and ~56 % respectively of that in the wild type, suggesting that *brc1, brc2 and brc3* mutants might have directly or indirectly played an important role in the cellulose biosynthesis. As shown in Table 4.4, the Klason lignin of the *brc1, brc2 and brc3* culms increased by ~23 %, ~17 % and ~20 %, respectively compared with that of the wild-type culms. At the same time they also had increase in hemicellulose, ash, silica and silicates and extractives (wax and lipids). Similar differences were found in the cell walls of leaves of wild type and the brittle mutants (Table 4.5).

Table 4.4 Chemical composition of internodal cell wall

Leaf cell wall composition (mg/g)	Wild type	Britle mutant					
	T. monococcum	*brc1*	% change	*brc2*	% change	*brc3*	% change
α-Cellulose	306	147	- 51.97	132	- 56.87	138	- 54.91
Hemicellulose	250	270	+7.41	265	+5.66	269	+7.07
Klason Lignin	160	190	+15.8	162	+1.24	184	+13.05
Ash	122	160	+23.75	188	+35.11	172	+29.07
Silica & Silicates	52	62	+16.13	65	+20	69	+24.64
Extractives	57	68	+16.18	92	+38.05	82	+30.49

Table 4.5 Chemical composition of leaf cell wall

Internodal cell wall composition (mg/g)	Wild type	Britle mutant					
	T. monococcum	*brc1*	% change	*brc2*	% change	*brc3*	% change
α-Cellulose	387	206	- 46.78	168	- 43.41	172	- 56.66
Hemicellulose	273	289	+5.54	314	+13.06	322	+15.22
Klason Lignin	175	228	+23.26	212	+17.46	221	+20.82
Ash	82	126	+34.93	188	+56.39	172	+52.33
Silica & Silicates	47	51	+7.85	49	+4.09	48	+2.09
Extractives	52	68	+23.53	65	+20.00	59	+11.7

Figure 4.9 Brittleness of culm and leaves of *brc1, brc2 and brc3* mutants

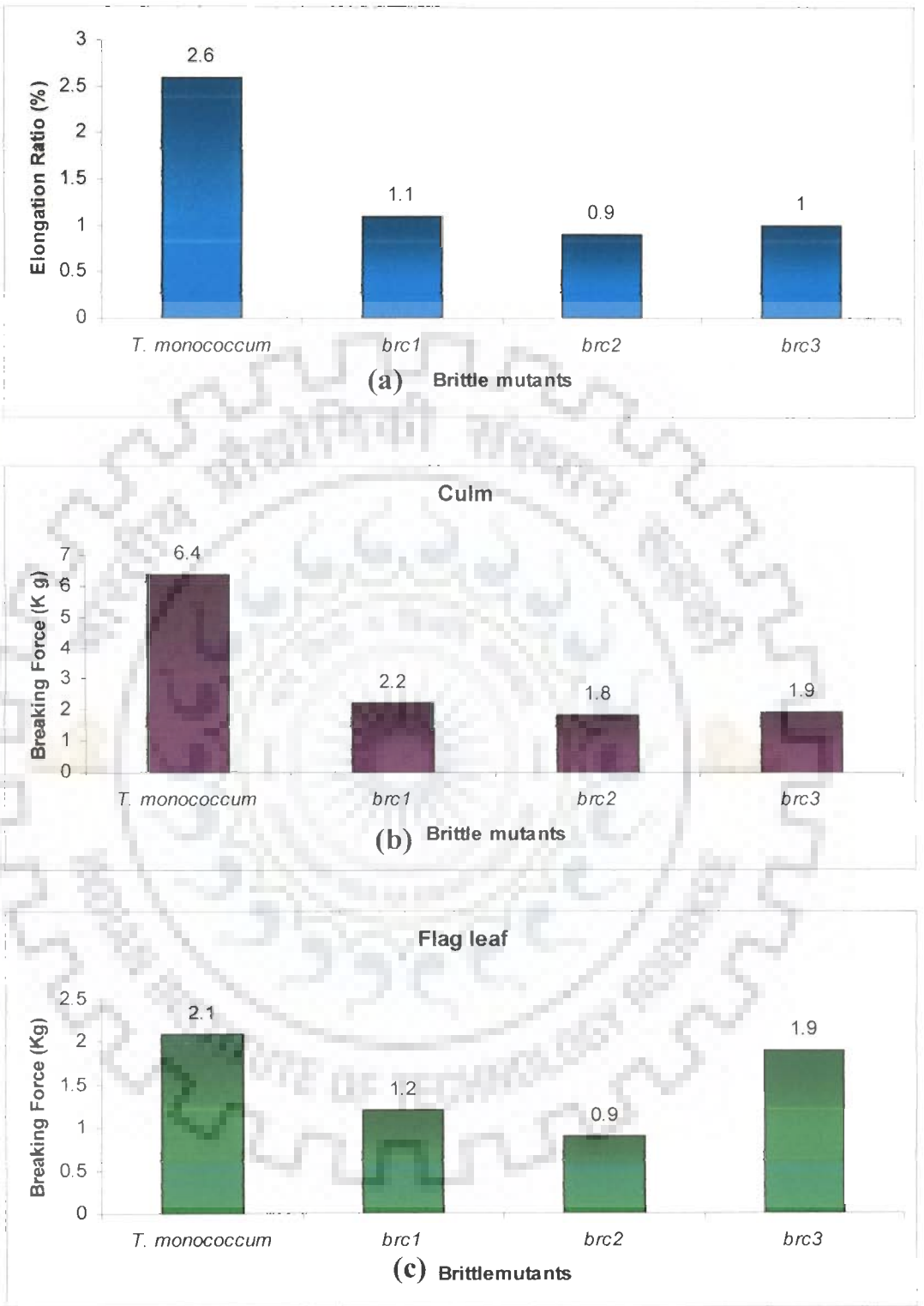


Figure 4.10 Elongation ratio and breaking force of brittle mutants and wild type (a) Elongation ratio of *T. monococcum* and mutants *brc1, brc2 and brc3* (b)Breaking force needed to break culm of *T. monococcum, brc1, brc2 and brc3* (c) Breaking force needed to break flag of *T. monococcum, brc1, brc2 and brc3*

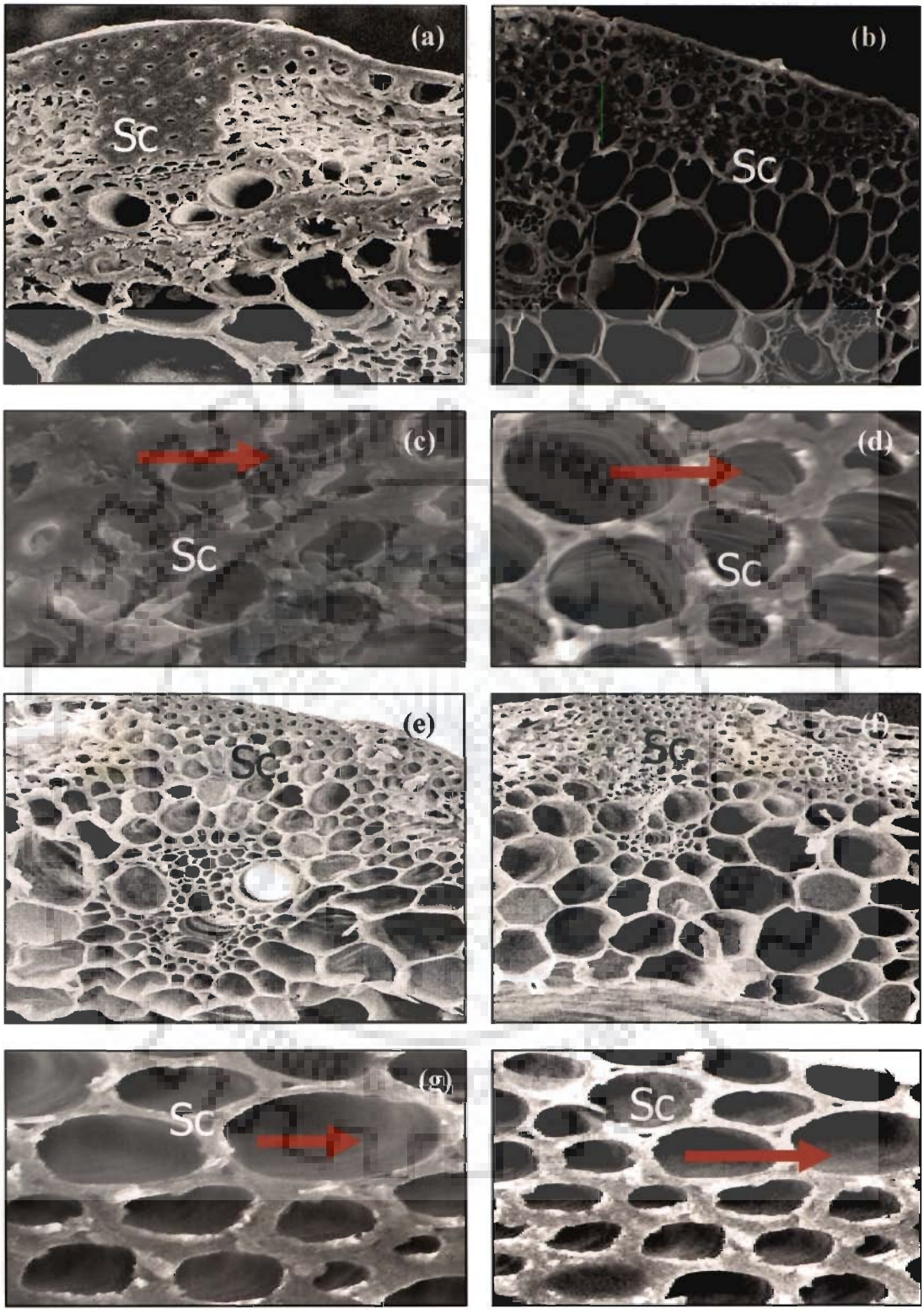


Figure 4.11 SEM of sclerenchyma cells of culm second internode of *T. monococcum* and brittle mutants *brc1, brc2 and brc3*. (a): Surface image of TS of second internode in *T. monococcum* (b) *brc1* (e) *brc2* and (f) *brc3*..(c) sclerenchyma fibers of *T. monococcum* (d) sclerenchyma fibers of *brc1* (g) sclerenchyma fibers of *brc2* (h) sclerenchyma fibers of *brc3*. (500X)

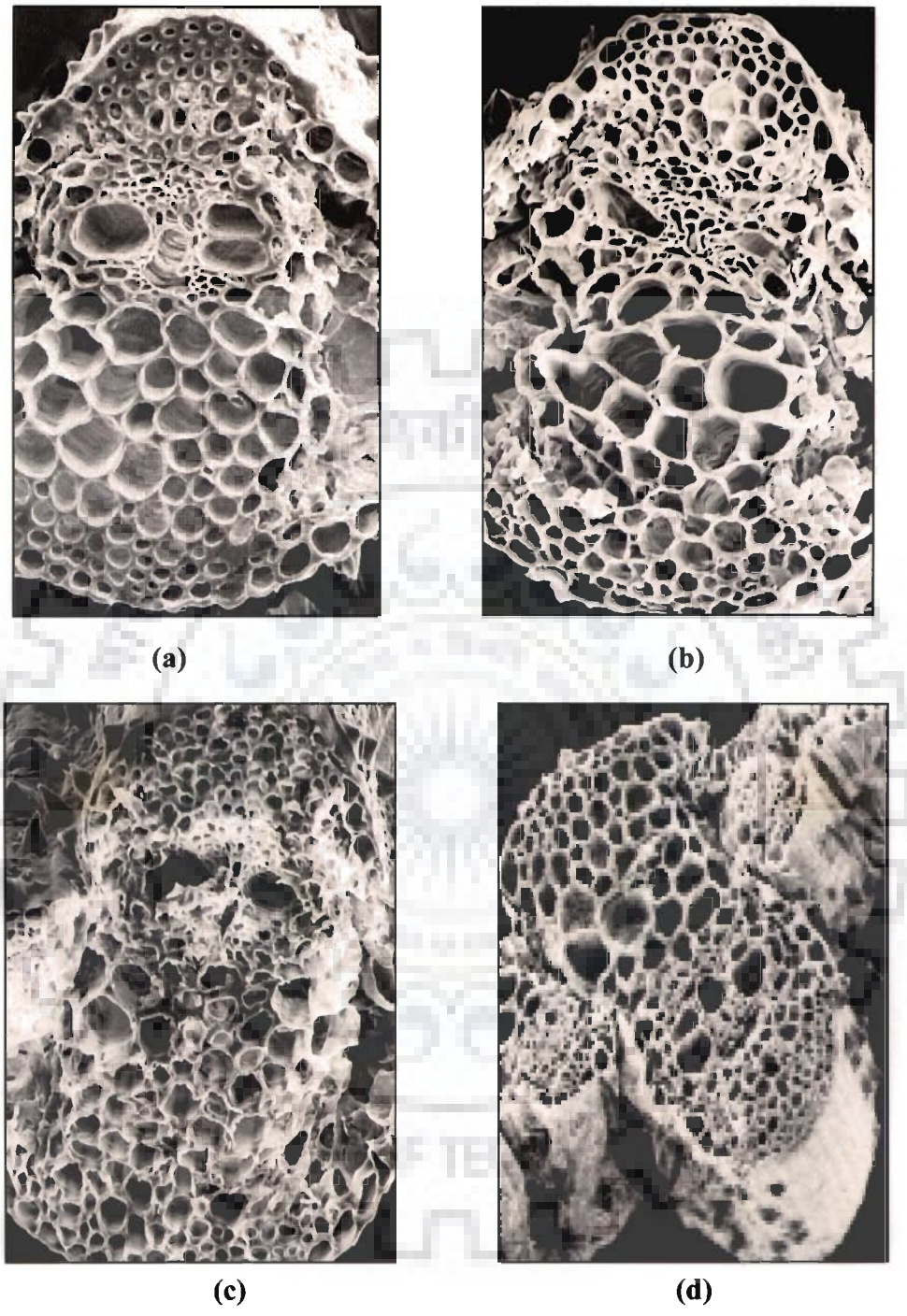


Figure 4.12 SEM of midrib sclerenchyma of *T. monococcum* and Brittle mutants *brc1, brc2 and brc3* (a) Surface image of transverse section of midrib of flag leaf in *T. monococcum* (b) *brc1*. (e) *brc2* and (f) *brc3*. (200X)

4.5.5 Anatomy of leaves and culm of brittle mutants

To determine whether the alterations of cellulose and lignin are localized in particular cells, transverse sections of the culms of wild-type and mutant plants were histochemically stained with Wiesner stain. Wiesner stain is known to react with cinnamaldehyde residues in lignin, and the color intensity approximately reflects the total lignin content. The color differences between wild-type and mutant in mechanical tissues, especially in the sclerenchyma cells below the epidermis and above and around the vascular bundles, (Fig. 4.13 and Fig 4.14) indicated an apparent increase in lignin quantity in mutant plants. On the other hand, Toludine blue stains cellulose, callose, and other glucans greenish yellow (Fig. 4.15) whereas it stains lignin green. Greenish yellow colour were observed in the sclerenchyma cells and vascular bundles in the wild type and green colour in sclerenchyma cells and vascular bundles of *brc1, brc2 and brc3* mutants, demonstrating a significantly high level of ordered cellulose in the sclerenchyma cells and vascular bundles in wild-type plants. In addition the thickness of sclerenchymatous secondary cell walls was more in wild type than in *brc1, brc2 and brc3*. This finding is consistent with the SEM observations indicating that the *brc1, brc2 and brc3* mutants were deficient mainly in the secondary cell walls.

Phloroglucinol staining of TS of flag leaf of *brc1, brc2 and brc3* mutants revealed that only a few sclerenchyma cells were present around the vascular bundle of midrib which replaced by parenchymatous cells in *brc1, brc2 and brc3* whereas in *T. monococcum* all cells around the vascular bundles and below the upper and lower epidermis were sclerenchymatous. In *brc1* and *brc2*, metaxylem vessels deformed and protoxylem vessel completely collapsed which are the major water conducting tissues of in the plants. Higher number of Chloroplasts had been observed in parenchymatous cells around the vascular bundles of leaves of *brc1, brc2 and brc3* than in *T. monococcum* which supports the darker green colour of *brc1, brc2 and brc3* than wild type. Chlorenchymatous cells were elongated in *brc1, brc2 and brc3* and were rounded in *T. monococcum*. Numbers of bulliforms cells were ~2 in *brc1, brc2 and brc3* and ~4 in *T. monococcum* (Fig. 4.14).

4.5.6 Structure of cells in *brc1, brc2 and brc3* mutants and wild type after removal of hemicellulose and lignin

To understand the changes in cell wall structure, cell walls were treated with sodium chlorite and examined by scanning electron microscopy (SEM). SEM revealed that the walls of all mechanical tissue cells of the mutants were thinner than those of the wild type (Fig. 4.16), and cells thin walls became deformed in mutants. In addition to the reduced wall thickness in mechanical tissues cells, the *brc1, brc2 and brc3* mutants also exhibited alteration of the structure of vessel elements. After treatment the primary and secondary cell walls of the wild type remained intact, however, the cell wall structure of *brc1, brc2 and brc3* became unorganized. Hemicellulose is the main component of the middle lamellae and its removal cause a drastic change in the organization of surface microfibrils and uneven surface microfibrils had been observed in *brc1, brc2 and brc3* whereas in wild type surface was smooth with parallel cellulose microfibrils running in a single direction at the same time increased porosity was observed in *brc1, brc2 and brc3* due to disruption of surface cellulose microfibrils (Fig. 4.17). These results indicated that the *brc1, brc2 and brc3* mutations affected the organization of cellulose microfibrils.

4.5.7 Atomic force microscopy of stem surface

Atomic force microscopy of *brc1, brc2 and brc3* mutants along with wild type *T. monococcum* had been done to record the stem surface roughness data and it was found that the stem surface of mutants were rougher than that of wild type *T. monococcum.* It also indicated that mutants had some abnormality in cellulose deposition on stem surface (Fig. 4.18).

4.5.8 FTIR microspectroscopy

The FTIR spectrum of second internodal cell wall material along with wild type is shown in Fig. 4.19. The typical lignin bands at 1650–1250 cm^{-1} and 1200–900 cm^{-1} are clearly visible in *brc1, brc2 and brc3* mutants and not discernible in wild type, which indicates that *brc1, brc2 and brc3* mutants were different from wild type in relation to lignin. The absorption band at 1640 cm-1 is attributed to the absorbed water in the cellulose (Fig. 4.19).

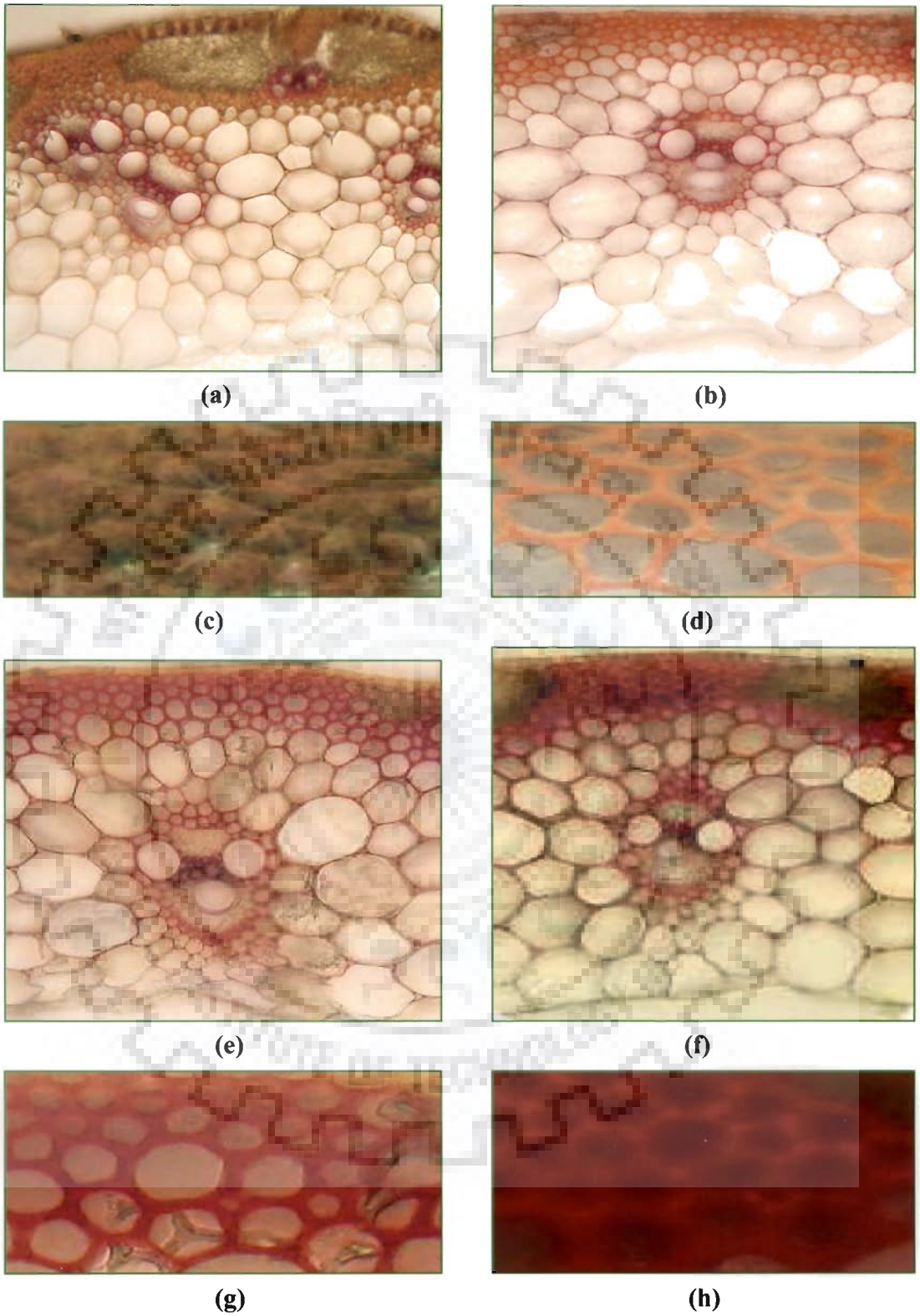


Figure 4.13 Phloroglucinol-HCL (lignin specific) staining of second internode of wild type and mutants (a) T.S of *T. monococcum* (b) T.S of *brc1* (c) Sclerenchyma fibres of *T. monococcum* (d) Sclerenchyma fibres of *brc2* (e) T.S of *brc2* (f) T.S of *brc3* (g) Sclerenchyma Fibres of *brc2* (h) Sclerenchyma Fibres of *brc3*

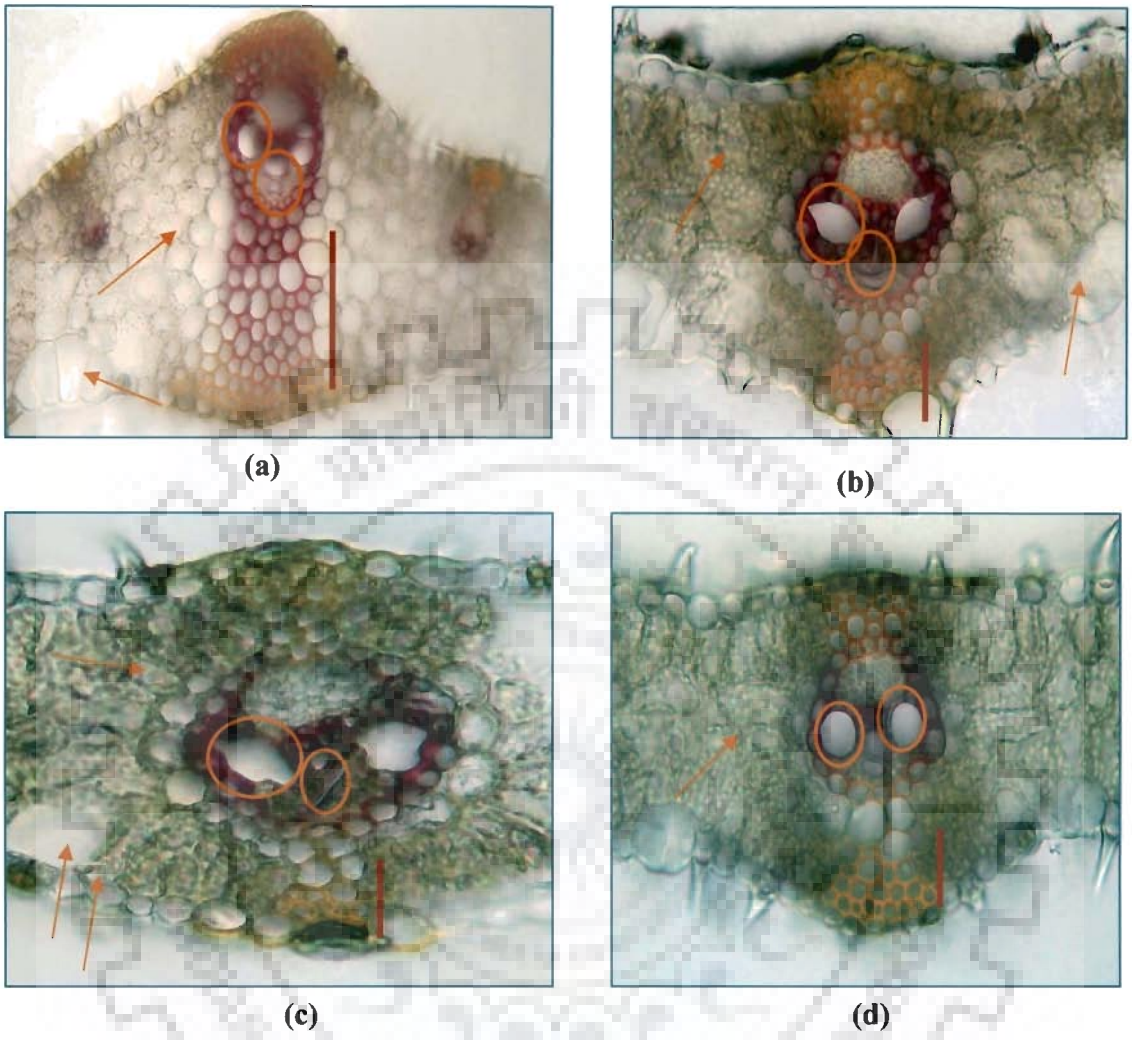


Figure 4.14 Phloroglucinol-HCL (lignin specific) staining of flag leaf sections of wild type and mutants (a) TS through midrib of *T. monococcum* (b) TS through midrib of *brc1* (c) TS through midrib of *brc2* (d) TS through midrib of *brc3*, respectively at 40X magnification.

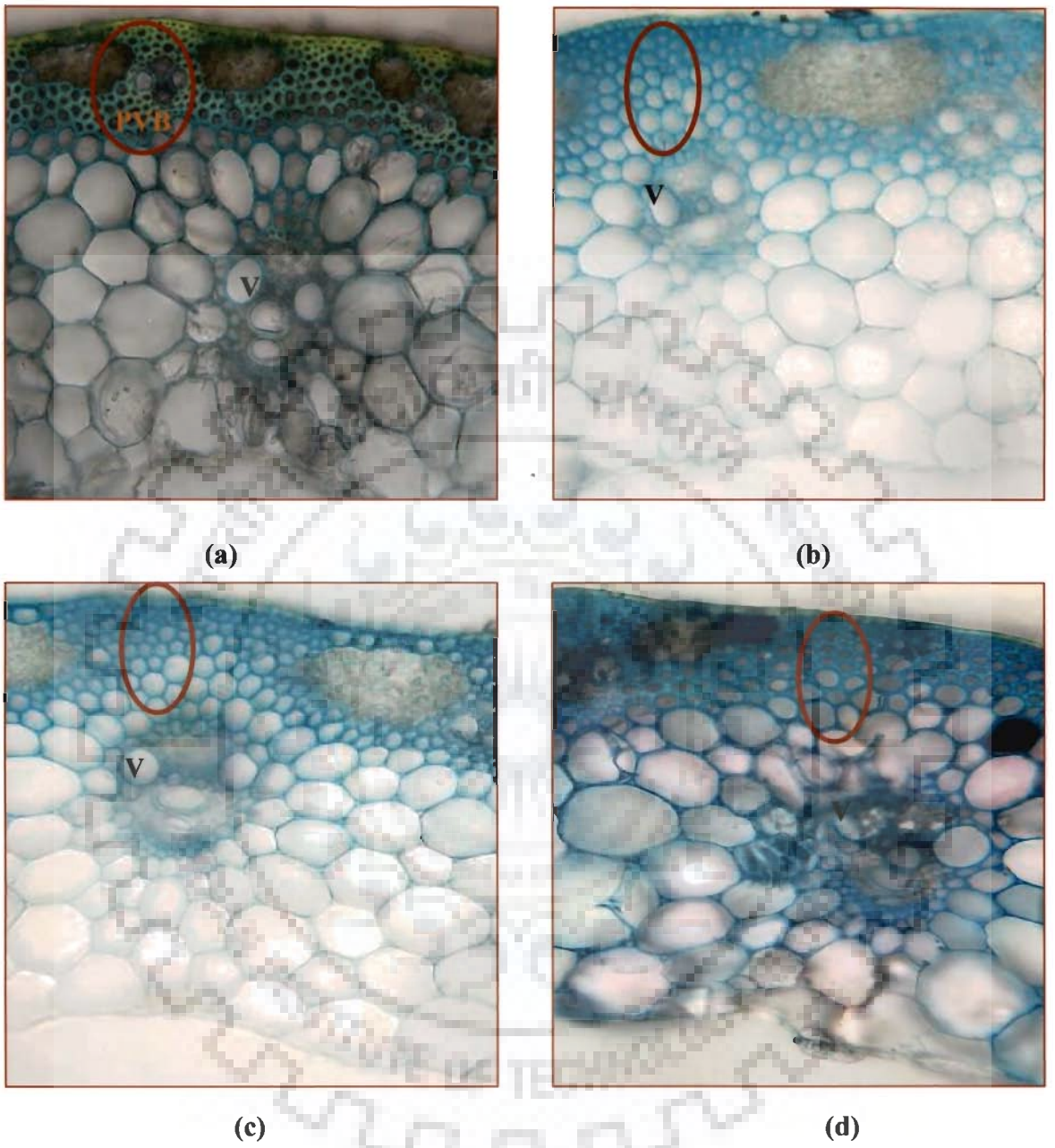


Figure 4.15 Toludine blue (multi chromatic staining) of second internode (a) T.S of second internode of *T. monococcum* (b) T.S. of *brc1* (c) T.S. of *brc2* (d) T.S. of *brc3* PVB denotes primary vascular bundle, V denotes Xylem vessel

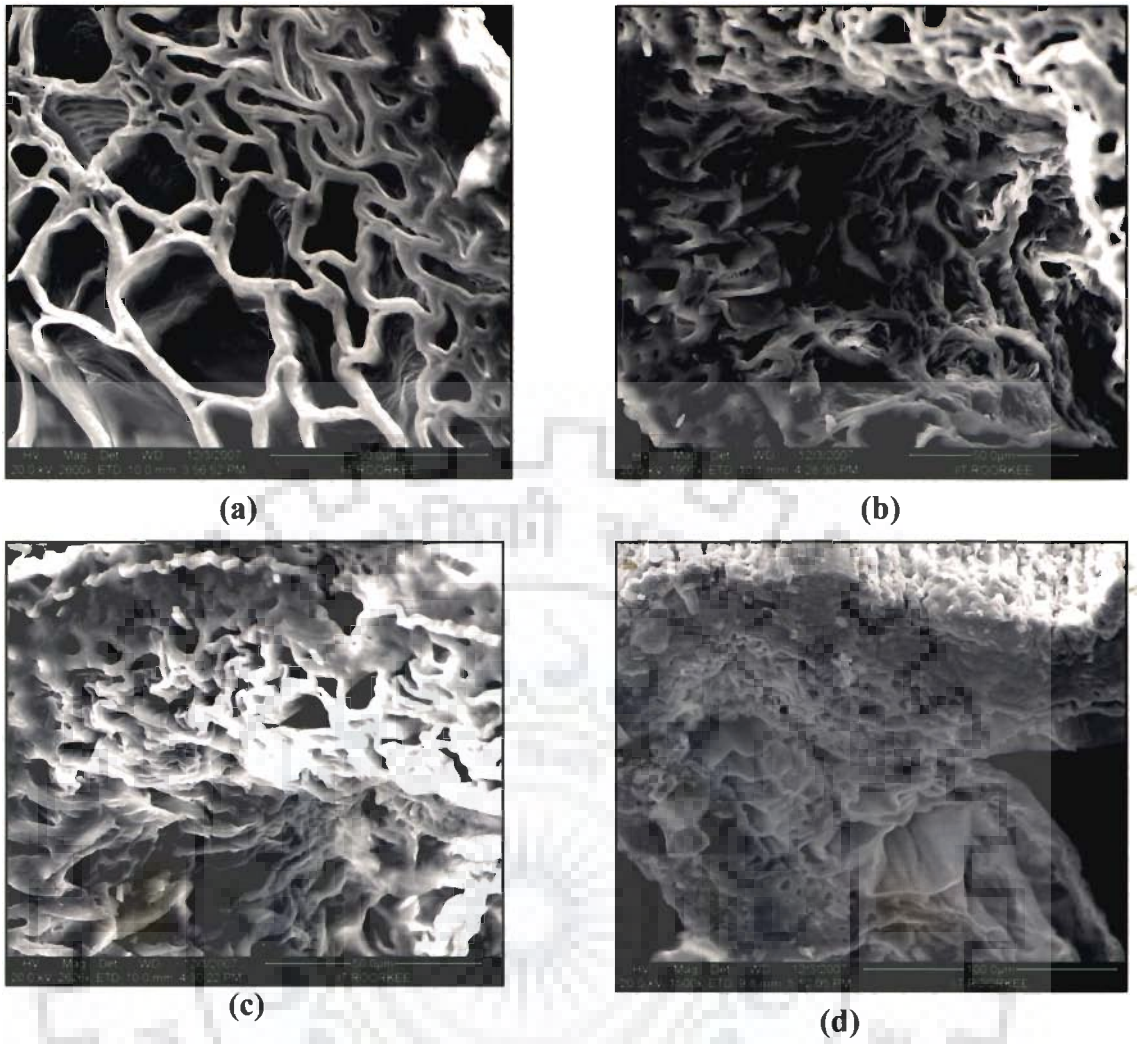


Figure 4.16 SEM after removal of lignin and hemicellulose (a) T.S of *T. monococcum* (b) T.S of *brc1* (c) T.S. of *brc2* (d) T.S of *brc3* (1500X)

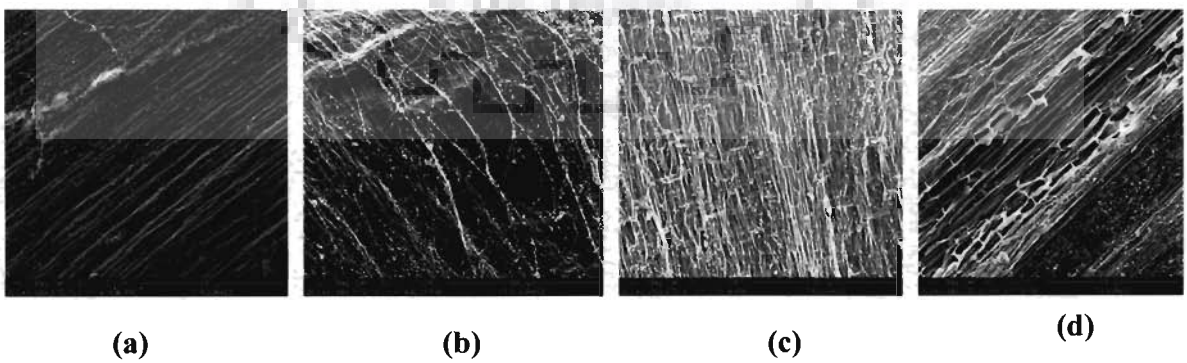


Figure 4.17 SEM after removal of lignin and hemicellulose (a) Surface of *T. monococcum* (b)Surface of *brc1* (c) Surface of *brc2* (d) Surface of *brc3* (300X)

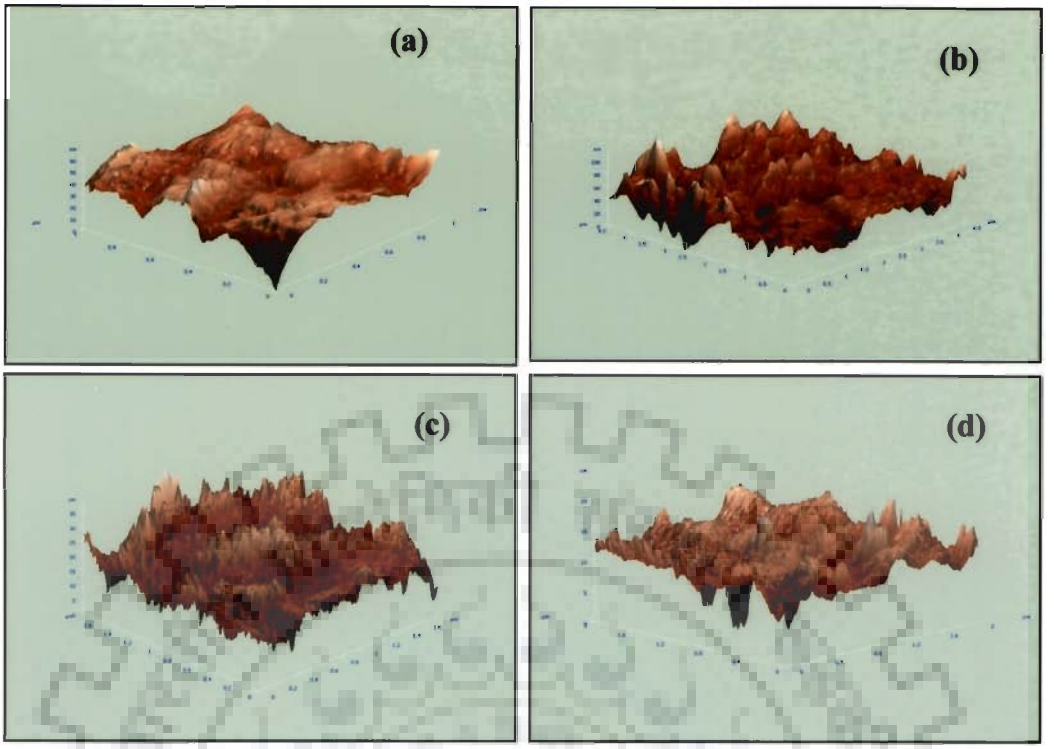


Figure 4.18 AFM studies of the stem surface of (a) *T. monococcum* (b) *brc1*(c) *brc2* (d) *brc3*

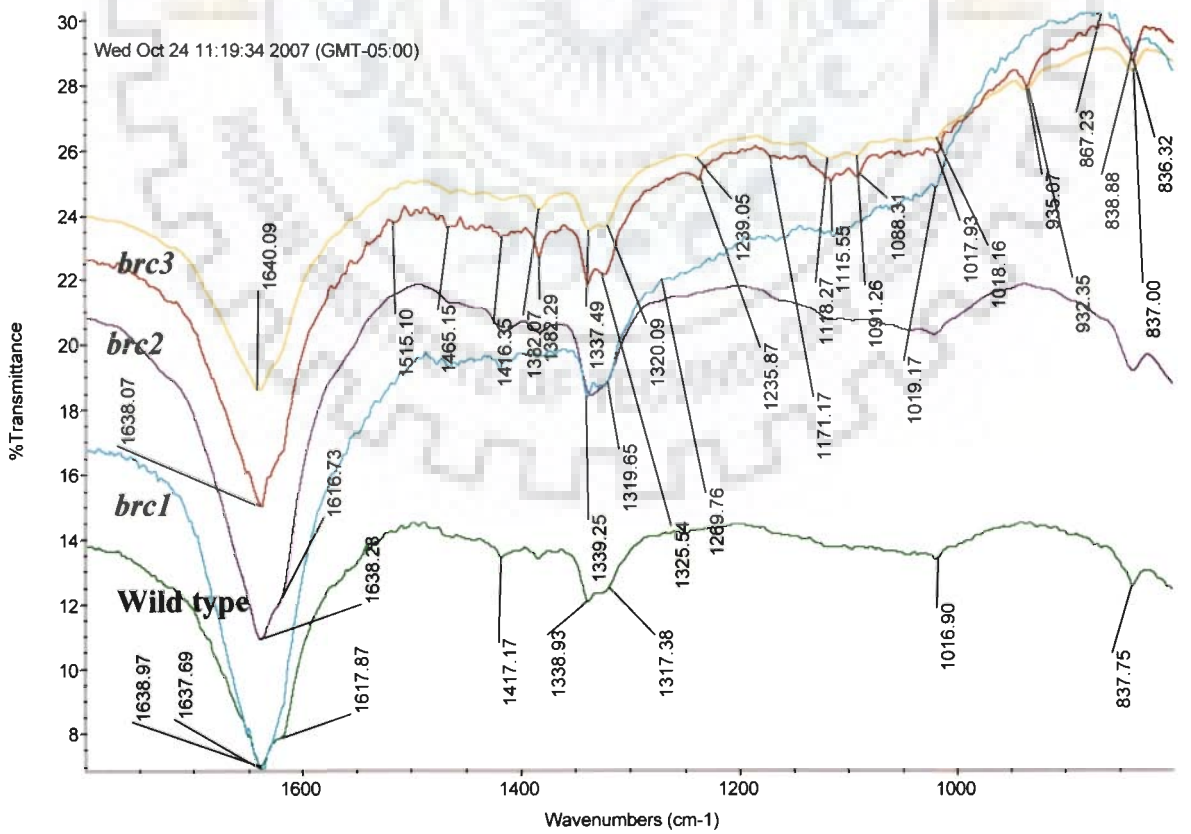


Figure 4.19 FTIR spectrum of second internodal cell wall material

4.6 Inheritance and mapping of *Rht22* dwarf mutant

Inheritance of dwarfing mutant was studied by crossing *Rht22* with its wild type *T. monococcum*. In order to tag the dwarfing gene, a mapping population was developed by crossing the GA-insensitive dwarf mutant *Rht22* with *T. boeoticum*, a tall and GA_3 sensitive wild type. The F_1 plants obtained from both the crosses were tall and sensitive to GA_3 spray indicating that the dwarfness and gibberellic acid sensitivity was recessive. 167 F_2 plants from *Rht22* X *T. boeoticum* cross and 200 F_2 plants from *Rht22* X *T. monococcum* cross were scored for plant height and sensitivity to exogenous GA_3 spray. The percentage increase in height after GA_3 treatment among the 167 F_2 plants segregated into 125 GA-sensitive: 42 GA-insensitive with a good fit to the expected 3:1 ratio (Table 4.7). The tall: dwarf and GA-sensitive *vs.* GA-insensitive plants in F_2 of *Rht22* X *T. monococcum* cross segregated in 3:1 ratio for both height and GA-sensitivity (Table 4.6), indicating the presence of recessive and monogenic mode of inheritance for the trait. All the dwarf plants were also GA_3 insensitive indicating the pleiotropic effect of the mutant controlling both the traits. Cut-off point for grouping the segregating F_2 plants for plant height and GA-responsiveness in both of the crosses was in the range of parental value, i.e., for dwarfness it was 85 cm. and for the height of mutant sensitivity to gibberellic acid was 30.5%. The average plant height of dwarf mutant, *T. boeoticum* and *T. monococcum* at flowering stage was 80cm, 140 cm, and 130 cm, respectively.

Table 4.6 F_2 segregation for response to GA_3 treatment and plant height in crosses of *Rht22* with *T. boeoticum* and *T. monococcum*.

Trait	Cross (*Rht22* X *T. monococcum*)					
	Number of plants					
	Total	Tall	dwarf	Ratio	χ^2 value at 1 *df*	
Plant height** (cm)					Calculated	Table
	200	149	51	3:1	0.870	3.84
	Cross (*Rht22* X *T. boeoticum*)					
	Number of plants					
% increase in height after GA spray*	Total	GA sensitive	GA insensitive	Ratio	χ^2 value at 1 *df*	
					Calculated	Table
	167	125	42	3:1	0.964	3.84

* Cut-off point for % increase in height after GA_3 spray for classification of GA_3 insensitive and GA_3 sensitive plants was 30.5% as that for *T. monococcum*.

** Cut-off point for plant height for classification of dwarf and tall plants was 85 cm, the average height of dwarf mutant.

4.6.1 Screening for parental polymorphism:

A total of 133 SSR markers uniformly distributed across $7A^m$ chromosomes were selected (Annexure II) as based on the SSR maps of *T. monococcum* X *T. boeoticum* RIL population (Singh *et al.* 2007) for parental polymorphism survey.

4.6.2 Bulk segregant analysis (BSA)

All the 167 F_2 plants obtained from *Rht22* X *T. boeoticum* cross were scored for to 50% flowering and tiller number. Normal frequency distribution for these traits in F_2 population validated the population to be used for bulk segregant analysis (BSA) for mapping the dwarfing gene. For bulk segregant analysis (BSA) positive bulk was made from 15 F2 dwarf plants (*Rht22*) from respective F2 population directly while a common negative bulk included 15 RILs of *T. monococcum* X *T. boeoticum* RIL population of PAU Ludhiana (Singh *et al.* 2007), developed from the same two parents without any mutation.

4.6.3 Identification of SSR markers linked to dwarfing gene

In BSA, the SSR markers xwmc89 and xbarc106, located on chromosome 4A at a physical distance of 0.9 cM and 0.6 cM, respectively, showed polymorphism between bulks (Fig. 4.20) for plant height. The amplification pattern obtained clearly indicated the association of these markers with *Rht22* gene. These markers were further used on debulked plants of positive bulk (Fig. 4.21). Only 2/15 plants were heterozygous for xwmc89 marker suggesting close linkage of the marker with the dwarfing mutant.

4.6.4 Mapping of the dwarfing mutant

A total of 167 individual F_2 plants were genotyped with the two microsatellite markers viz., xwmc89 and xbarc106 located on chromosome 4A (Fig. 4.22). Co-segregation analysis of individual markers and the genotypes of 167 individual F_2 plants was carried out with the help of recombination frequency between marker at each locus Based on the data on recombination frequency, the markers xwmc89 and xbarc106 mapped at distance of 0.9cM and 0.6cM, respectively from the *Rht22* mutant (Fig. 4.23) on chromosome 4A.

Figure 4.20 Identification of putative linked SSR marker xbarc106 for dwarfing mutant *Rht22* through bulked segregant analysis (BSA): Lane 1: *Rht22*, 2: *T. boeoticum* 3: common negative bulk and 5: Positive bulk of *Rht22,* 4,6,7, 8 are positive bulks of other mutants

Figure 4.21 PCR of debulks of positive bulk F2 plants using putatively linked marker(a) xwmc89 and (b) xbarc106 Lane:1: *Rht22*, 2: *T. boeoticum*, 3-17: debulks

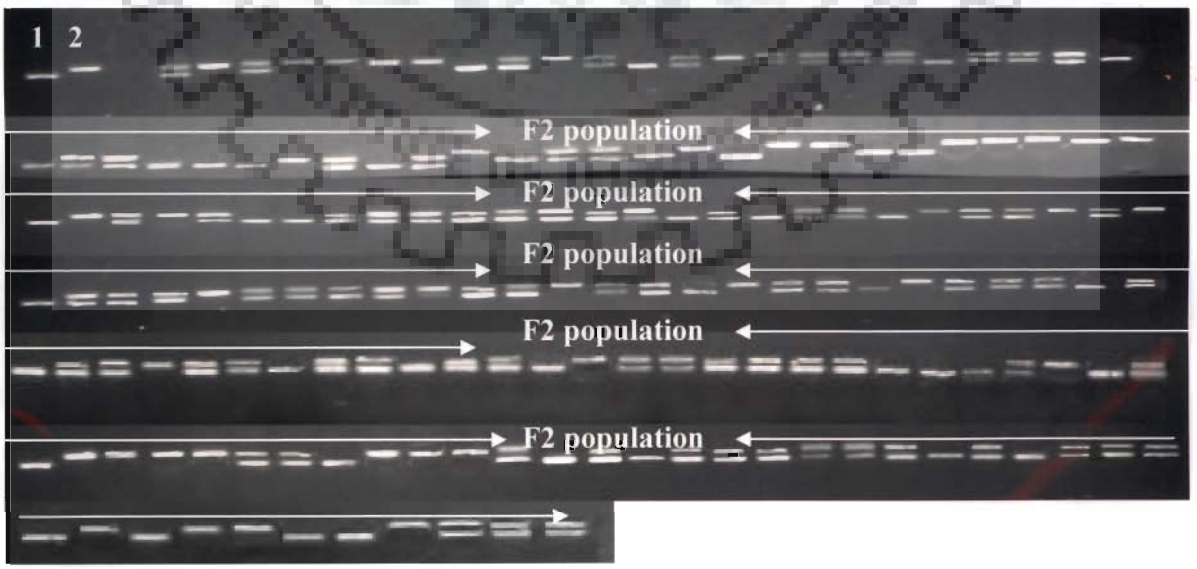


Figure 4.22 PCR analysis of 167 F2 plants of *Rht22 /T. boeoticum* with marker xbarc106 Lane1: *Rht22*, 2:*T. boeoticum* 3.Negative control

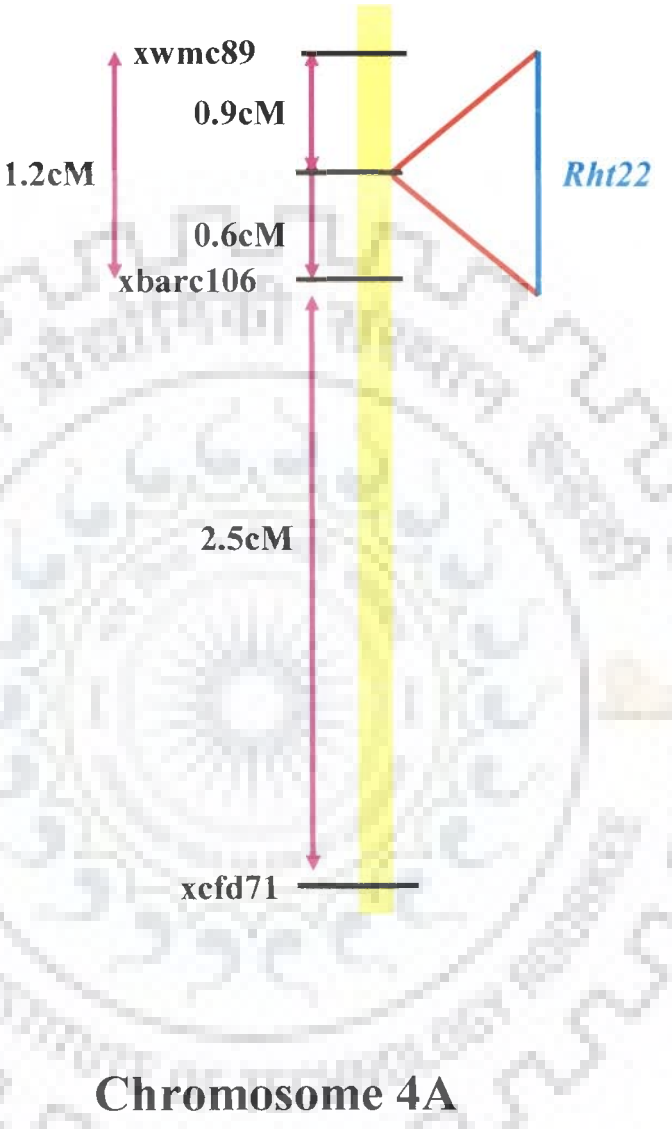


Figure 4.23 The location of the dwarfing mutant *Rht22* gene in the molecular linkage map on chromosome 4AL

4.7 Inheritance and mapping of *sog3* mutant

In order to established the inheritance of the soft glume mutant, the mutant was crossed with its wild parent *T. monococcum* and the F1 was advanced to F_2. The morphology of F_1 obtained from the cross was the same as the wild type indicating that *sog3* mutant was recessive. The F_2 plants (170), segregated for free threshing and hard threshing (hulled). The hulled (129): free threshing (41) plants in F_2 segregated in 3:1 ratio. The segregation ratio of the parental phenotypes had a good fit to the expected 3:1 ratio (χ^2 0.790) confirming the monogenic recessive inheritance of *sog3* mutant. In order to map the *sog3* mutant, a mapping population was developed by crossing the *sog3* mutants with *Triticum boeoticum* acc.pau 5088, a hulled diploid wheat line.

4.7.1 Bulk segregant analysis (BSA)

170 F_2 plants obtained from *sog3* X *T.boeoticum* cross were scored for hulled vs free threshing. For bulk segregant analysis (BSA) positive bulks were made from 15 F2 soft glume plants (*sog3*) from respective F2 population directly while the common negative bulk included 15 RILs of *T. monococcum* X *T. boeoticum* RIL population of PAU Ludhiana (Singh *et al.* 2007). In BSA, the SSR markers xgwm473 and xbarc69 located on chromosome $7A^mL$ showed polymorphism between bulks (Fig. 4.24). The amplification pattern in debulk of F2 plants of positive bulk clearly indicated the association of these markers with *sog3* mutant (Fig. 4.25).

4.7.2 Mapping of the *sog3* by genotyping of F_2 population

A total of 170 individual F_2 plants were used for genotyping with the two microsatellite markers viz., xgwm473 and xbarc69 located on chromosome $7A^mL$ (Fig. 4.25 and Fig. 4.26). Co-segregation analysis of individual markers using the marker and mutant genotype of 170 individual F_2 plants was carried out with the help of recombination frequency between marker at each locus. Based on the data on recombination frequency, the marker xgwm473 and xbarc69 mapped at distance of 1.8cM and 28.3cM, respectively from the *sog3* mutant on chromosome $7A^mL$. The TRY and RIPPLE commands were used to add markers to the 60 framework map and check the final marker order. Markers were ordered at a minimum LOD score of 3.0 with the exception of some co-segregating or very closely linked markers (Fig. 4.27).

4.8 Inheritance and mapping of *brc1, brc2 and brc3* brittle mutants

In order to check the inheritance of all the brittle mutants, the mutants were crossed with its wild parent *Triticum monococcum* and the generation was advanced to F_2. The F_1s obtained from three crosses were non brittle like wild type indicating that all the brittle mutants were recessive. The F_2 plants when grown in field segregated for brittleness. The ratio of brittle and non brittle F_2 plants of crosses segregated in 3:1 ratio for brittle culm trait confirmed that the brittle mutants were monogenic recessive.

4.8.1 Bulk segregant analysis

133 polymorphic SSR primers which exhibited polymorphism between *T. monococcum and T. boeoticum* were used for BSA. For Bulk segregant analysis (BSA) positive bulks were made by 15 F2 brittle plants from each of the F2 populations directly along with the common bulk from 15 RILs. The markers xbarc37 and xbarc117 located on chromosome 6AS showed polymorphism between bulks of *brc1* (Fig. 4.28), markers xcfd62 and xcfa2170 located on chromosome 3AL showed polymorphism between bulks of *brc2* (Fig. 4.31), whereas markers xgwm135 and xwmc470 located on chromosome 1AL showed polymorphism between bulks of *brc3* (Fig. 4.34). Genotyping of the debulked F2 plants with the putatively linked markers (Fig. 4.29, Fig. 4.32, and Fig. 4.35) also confirmed their close linkage with the respective mutants.

4.8.2 Mapping of the *brc1, brc2* and *brc3* by genotyping of F2 populations:

To map the *brc1, brc2 and brc3* mutants, F2 mapping populations were developed by crossing the brittle (*brc1, brc2 and brc3*) mutants with *Triticum boeoticum* acc. pau 5088, a non brittle and tall diploid wheat line.

Table 4.7 Segregation of F_2 plants for brittleness in crosses with (*brc1, brc2 and brc3x T. monococcum*).

Mutants	Cross (Mutants × *T. boeoticum*)					
	Total plants	Non brittle	brittle	Segregation ratio	$\chi 2$ value	$\chi 2$ value $_{tab}$
brc1	250	188	62	3:1	0.941	3.84
brc2	250	187	63	3:1	0.941	3.84
brc3	185	139	46	3:1	0.966	3.84

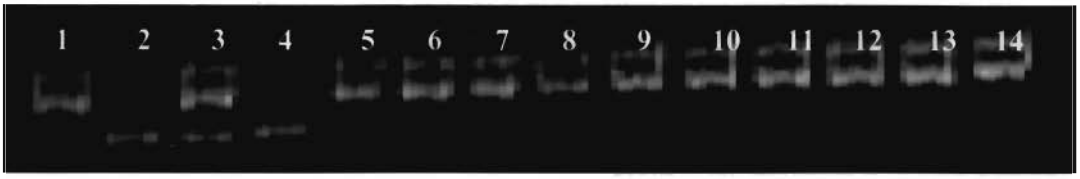
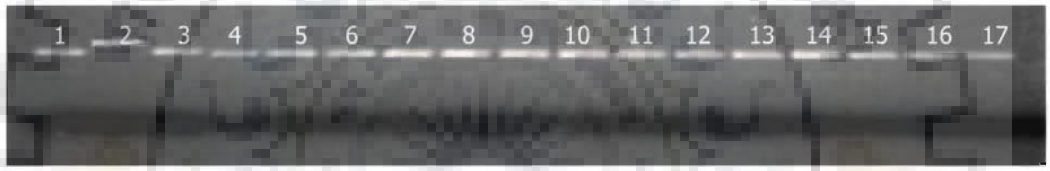
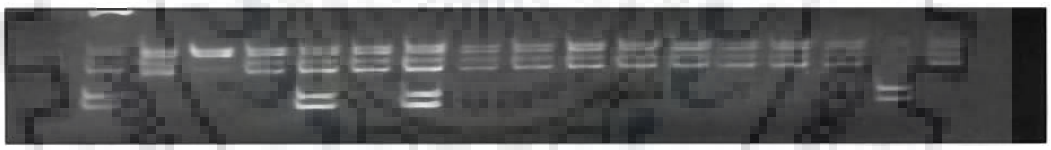


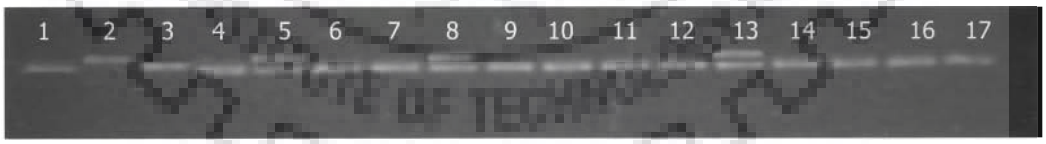
Figure 4.24 Identification of putative linked SSR marker xgwm473 for soft glume mutant *sog3* through bulked segregant analysis (BSA) on PAGE: Lane 1: *T. boeoticum*, 2: *sog3* 3: Negative bulk and 4: Positive bilk of *sog3,* Lane 5,6,7, 8,9,10,11,12,13,14 are positive bulk of other mutants



(a) xgwm473



(b) xwmc96



(c)xbarc69

Figure 4.25 PCR of debulks of positive bulk F2 plants using putatively linked marker on agarose gel. (a) xgwm473, (b) xwmc96 and (c) xbarc69 Lane:1: *sog3*, 2: *T. boeoticum*, 3-17: debulks

Figure 4.26 PCR genotyping of 170 F2 population of *sog3/T.boeoticum* with marker xgwm473 Lane1:*sog3*, 2:*T. boeoticum*

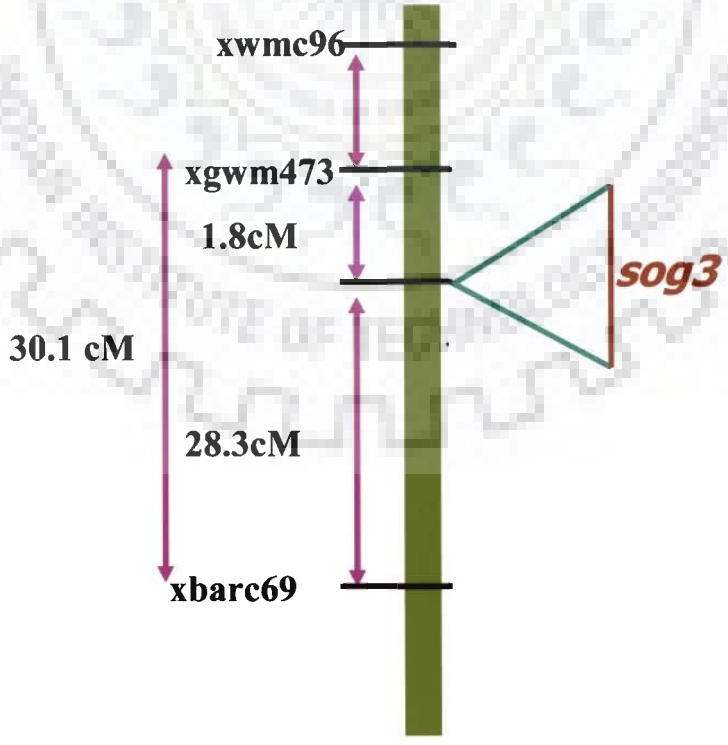


Figure 4.27 Mapping of the *sog3* mutant on chromosome 7AL

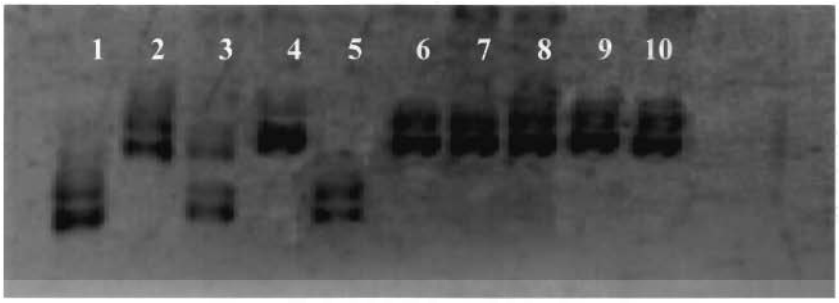


Figure 4.28 Identification of putative linked SSR marker xbarc37 for brittle culm mutant *brc1* through bulked segregant analysis (BSA): Lane 1: *brc1*, 2: *T. boeoticum* 3: Negative bulk and 5: Positive bulk of *brc-1* 4,6,7,8,9,10 are positive bulks of other mutants

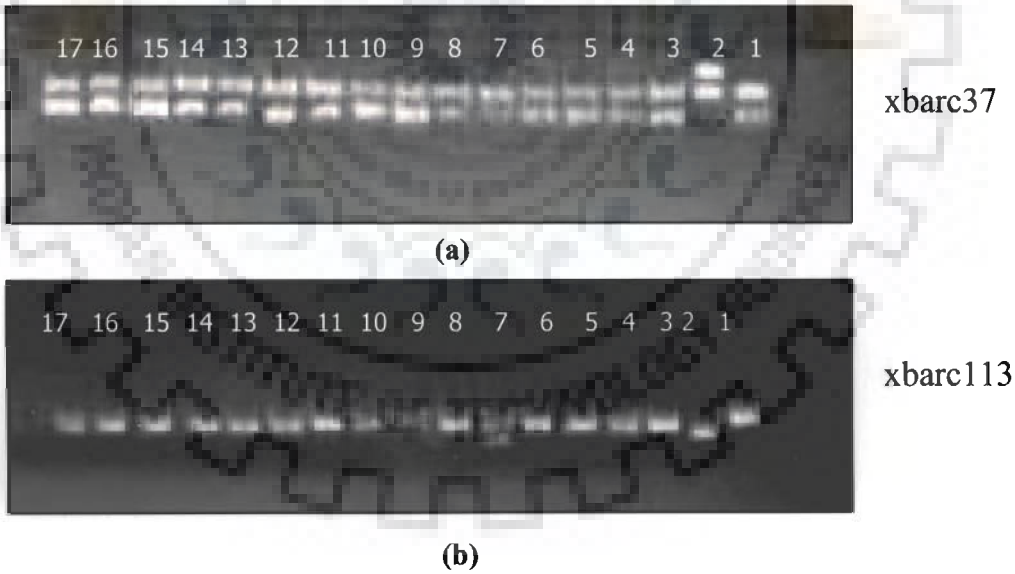


Figure 4.29 PCR of debulks of positive bulk F2 plants using putatively linked marker (a) xbarc37, (b) xbarc113 and Lane:1: *brc1*, 2: *T. boeoticum*, 3-17: debulks

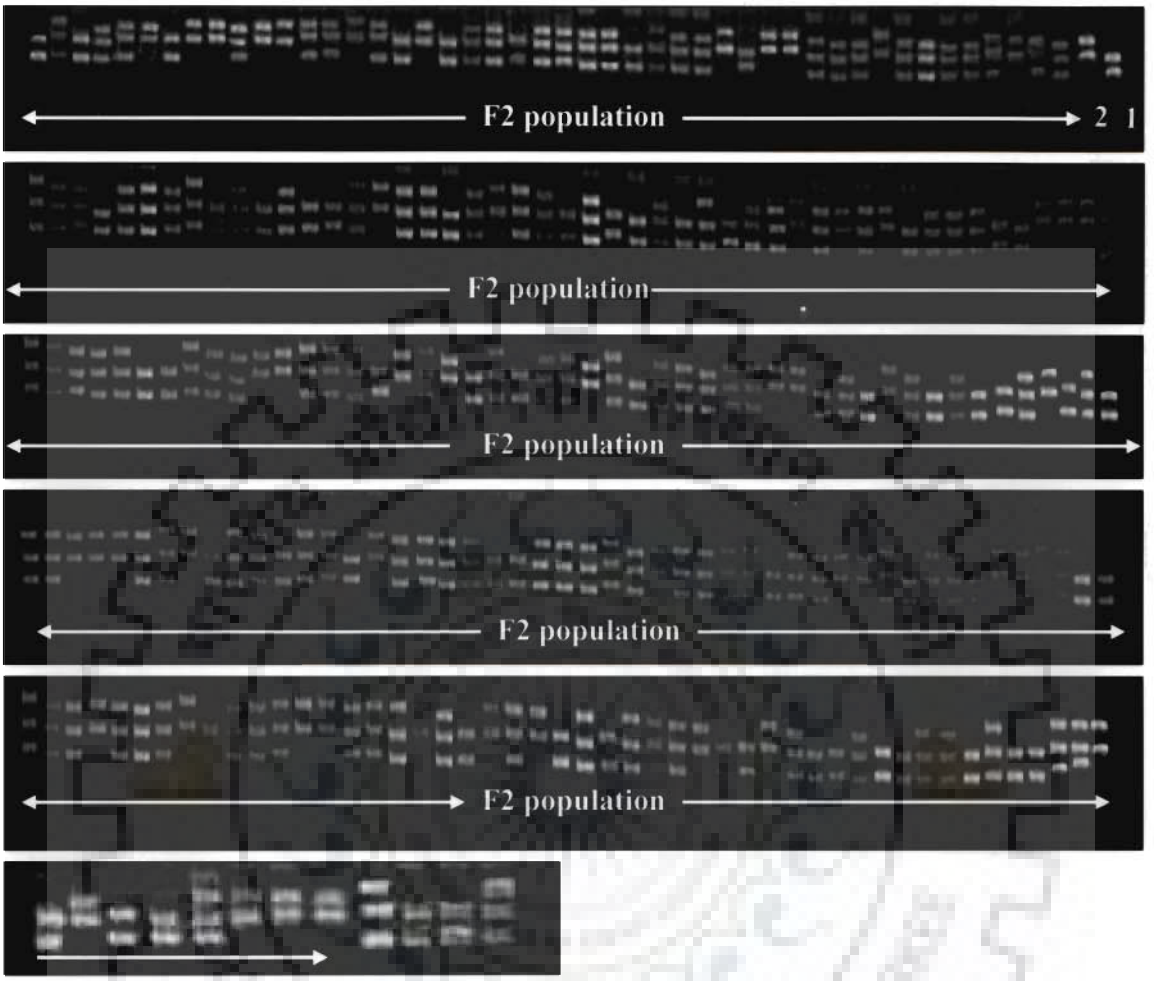


Figure 4.30 PCR genotyping of 250 F2 plants of *brc1/T. boeoticum* with marker xbarc37 on PAGE Lane1:*brc1*, 2:*T. boeoticum*

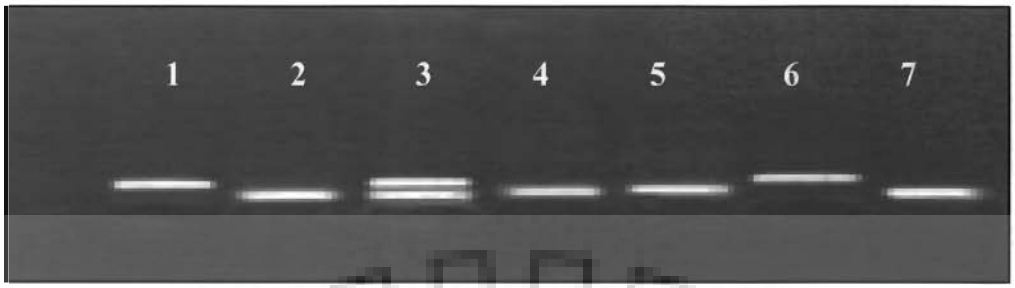


Figure 4.31 Identification of putative linked SSR marker xcfd62 for brittle culm mutant *brc2* through bulk segregant analysis (BSA): Lane 1: *brc2*, 2: *T.boeoticum* 3: Negative bulk 6: Positive bulk of *brc2* and 4,5,7, are positive bulk of other mutants

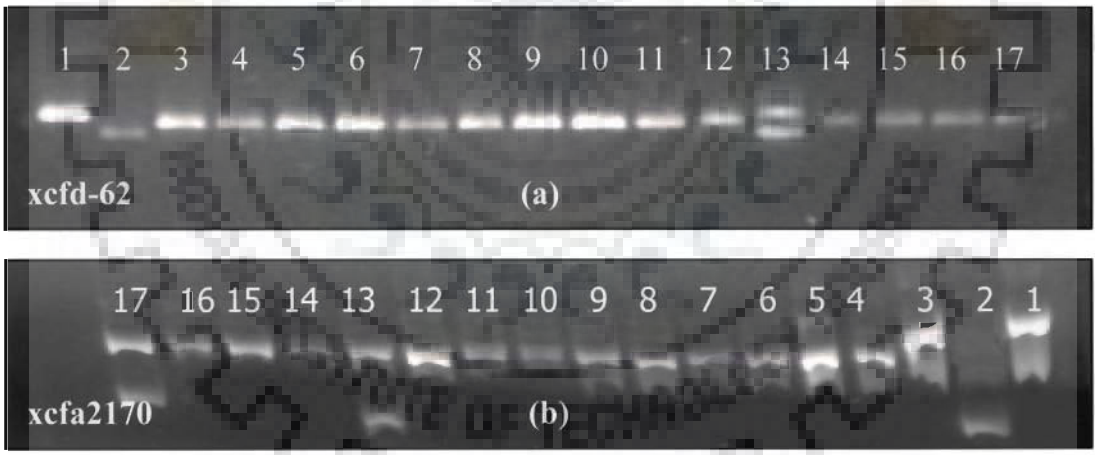


Figure 4.32 PCR genotyping of debulks of positive bulk F2 plants of *brc2* using putatively linked marker (a) xcfd62, (b) xcfa2170 and Lane:1: *brc2*, 2: *T. boeoticum*, 3-17: debulks

Figure 4.33 PCR analysis of 250 F2 plants of *brc2/T.boeoticum* with marker xcfd62 on PAGE Lane1: *brc2*, 2:*T. boeoticum*

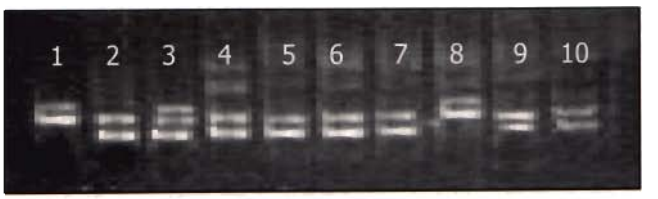
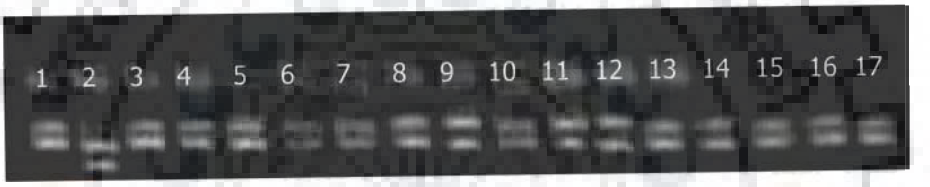
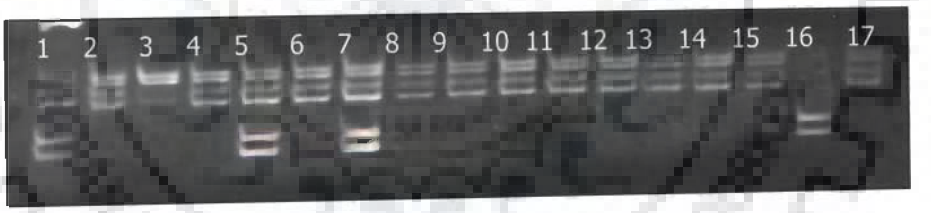


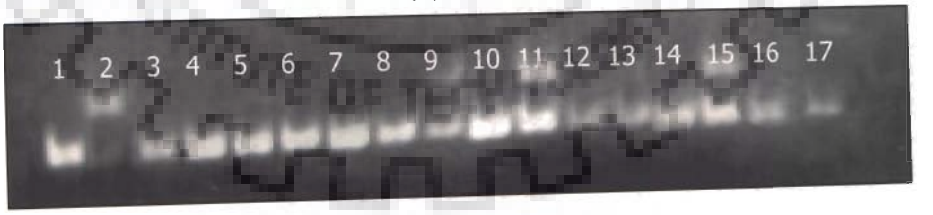
Figure 4.34 Identification of putative linked SSR marker xgwm135 for brittle culm mutant *brc3* through bulk segregant analysis (BSA): Lane 1: *brc3*, 2: *T.boeoticum* 3: Negative bulk and 8: Positive bilk of *brc3*, 4,5,6,7, 9,10 are positive bulks of other mutants



(a) xgwm135



(b) xwmc470



(c) xcfd59

Figure 4.35 PCR of debulks of positive bulk F2 plants of *brc3* using putatively linked marker (a) xgwm135, (b) xwmc470 and (c) xcfd59 Lane:1: *brc2*, 2: *T. boeoticum*, 3-17: debulks

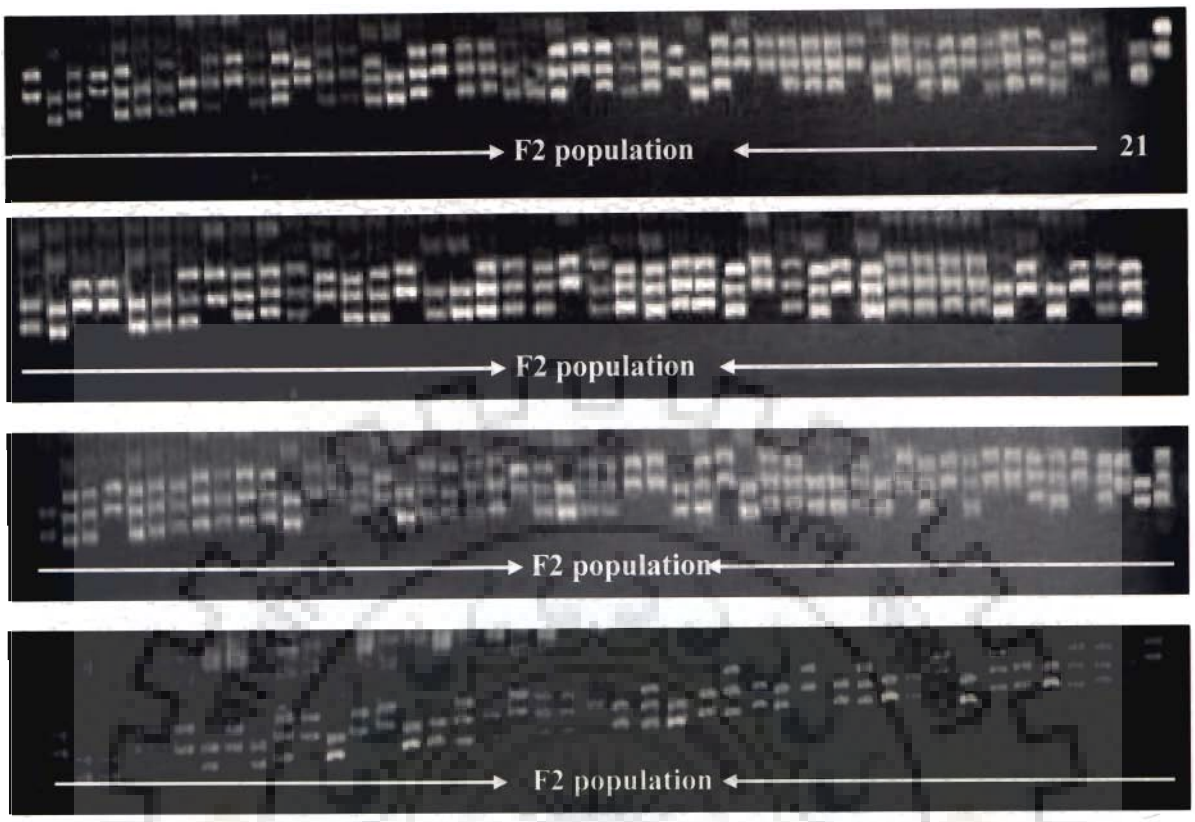


Figure 4.36 PCR analysis of 185 F2 plants of *brc3/T.boeoticum* with marker xgwm135 on PAGE Lane1:*brc3*, 2:*T. boeoticum*

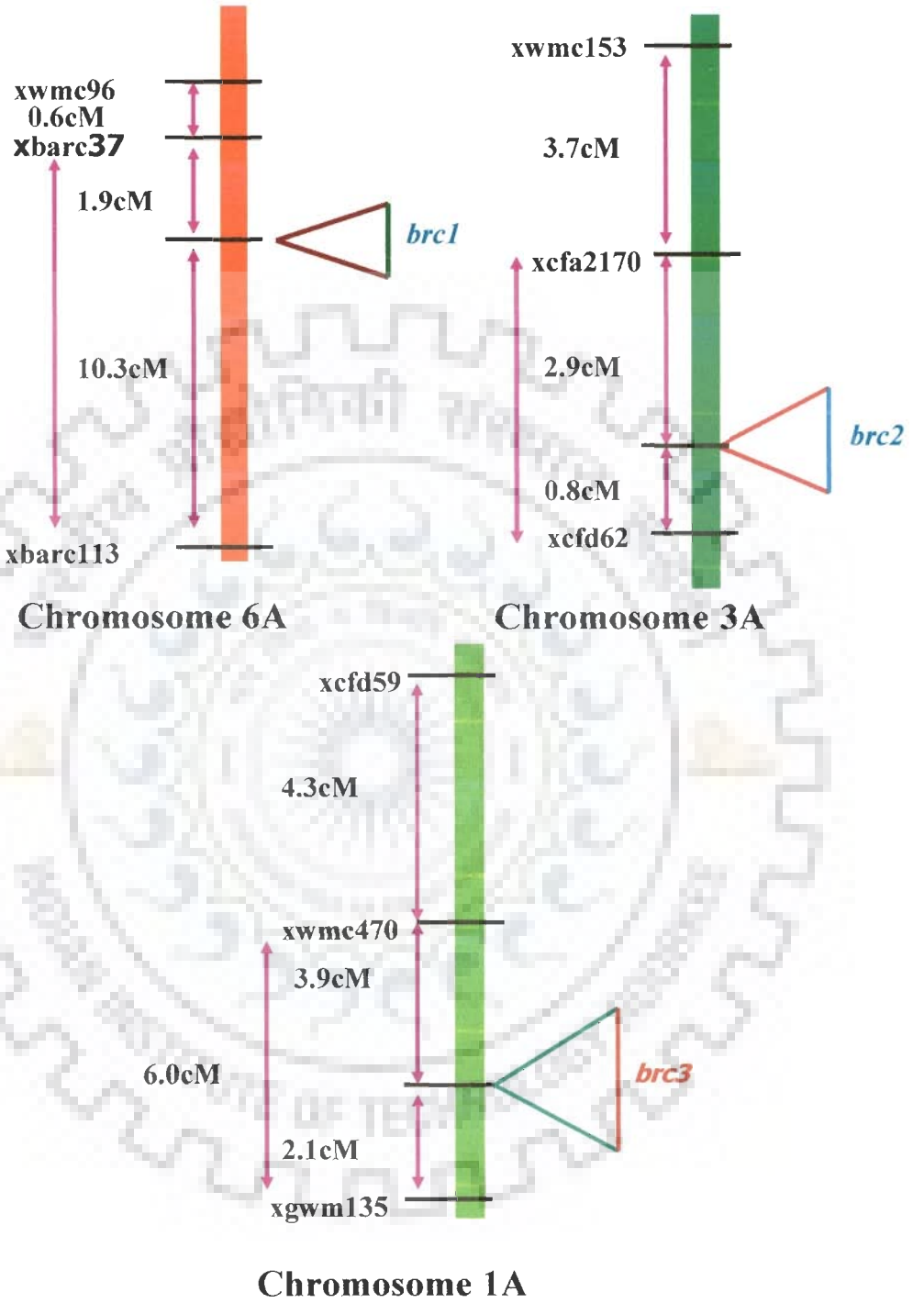


Figure 4.37 Mapping of ***brc1, brc2 and brc3*** mutant on chromosome 6AS, 3AL and 1AL, respectively

A total of 250, 250 and 185 individual F_2 plants were used for genotyping of *brc1, brc2 and brc3* F2 populations (Fig. 4.30, Fig. 4.33 and Fig. 4.36). Co-segregation analysis of individual markers xbarc37 and xbarc113 for *brc1* located on chromosome 6AS, xcfd62 and xcfa2170 for *brc2* located on chromosome 3AL and xgwm135 and xwmc470 for *brc3* located on chromosome 1AL using the marker and mutant genotypes of F_2 plants was carried out with the help of recombination frequency between marker at each locus. Based on the data on recombination frequency, the marker xbarc37 and xbarc113 were mapped at distance of 1.9cM and 10.3cM, respectively from the *brc1* gene on chromosome 6AS, marker xcfd62 and xcfa2170 mapped at distance of 2.9cM and 0.8cM, respectively from the *brc2* gene on chromosome 3AL and marker xgwm135 and xwmc470 were mapped at distance of 3.9cM and 2.1cM, respectively from the *brc3* mutant on chromosome 1AL (Fig. 4.37).

4.9 Homology search and PCR based cloning of *Rht22*

Two orthologous wheat dwarfing genes *Rht-B1b* and *Rht-D1b* have been cloned (Peng *et al.* 1999). They are also orthologous to the *Arabidopsis GA1* gene, a de-repressible modulator of gibberellic acid (GA) response (Peng *et al.* 1997). These genes have mutation in DELLA conserved domain. Since the gene for GA_3 insensitive mutant *Rht22* has been mapped on 4A, so for cloning the candidate gene of *Rht22,* gene specific primers were synthesized covering the DELLA domain of *Rht-D1b* gene as below.

- DF (5′-CGCGCAATTATTGGCCAGAGATAG-3′),
- DF2 (5′-GGCAAGCAAAAGCTTCGCG-3′),
- WR2 (5′-GGCCATCTCGAGCTGCTC-3′)

4.9.1 PCR amplification with DF2+WR2 Primers and sequencing of amplicon

Primers DF2+WR2 amplified the product in all the dwarf mutants along with wild type *Triticum monococcum* and the size of all the products was ~270 bp (Fig. 4.38). Amplicon from PCR reaction with DF2 and WR2 were run on 2% agarose gel eluted and purified using the Qiagen QIAEXII gel elution kit (USA). The PCR products were eluted in 30µl of water. About 80 ng of the purified PCR-amplified DNA was used for cycle sequencing under the conditions specified by the manufacturer (Ocimum Biosolutions, Hyderabad, India). The sequencing results with DF2 and WR2 showed 271 bp sequence in wild type (Fig. 4.39) and in other dwarf mutants except *Rht22*. In *Rht22* the product was 269 bp with 2 bp deletion (Fig. 4.40).The sequences then used to query the NCBI Gene Bank by using BLASTN and BLASTX programs (Altschul *et al.*, 1990, 1997).

4.9.2 Confirmation of two bp deletion in DELLA domain of *Rht22*

The following PCR primers were synthesized to confirm 2 bp deletion:

- DF2 (5'-GGCAAGCAAAAGCTTCGCG-3'),
- WTM (5'CATCTCGAGCTGCTCCAGCTTCTG-3')
- MTM (5'-CATCTCGAGCTGCTCCAGCTTCC-3')

Primer DF2 and WTM were synthesized such a way that they would amplify only wild type and primers DF2 and MTM will amplify only mutant type *Rht22*. Primers DF2 and MTM based on deletion in DELLA domain amplified *Rht22* only which confirmed the two bp deletion in DELLA domain (Fig. 4.41). When these primers were applied on homozygous tall plant as well as 10 plants of positive bulk of *Rht22* X *T. boeoticum* population, DF2+WTM amplified only negative debulks of homozygous tall plants. And no amplification was observed in the positive debulked of homozygous dwarf plants. With the DF2+MTM primer combination, amplification observed was only in the positive debulks of homozygous dwarf plants of the population (Fig 4.42) clearly showed the association of 2 bp EMS induced deletion in DELLA domain with the GA_3 insensitive mutant *Rht22*.

4.9.3 Bioinfomatic analysis of *Rht22* sequence

The amplified sequence of *Rht22* was analyzed for alignment with the database using BLASTN and its maximum alignment was found with GA_3 insensitive *Rht-D1* sequence of hexaploid wheat with 97% identity and maximum sequence coverage among all the available hits so it is supposed to have function similar to *Rht-D1* of wheat. It also showed similarity with *H. vulgare* transcription factor *SLN1*, *Zea mays DWARF8* and *Oryzae sativa* DELLA protein SLR1 gene (http://blast.ncbi.nlm.nih.gov/Blast.cgi), all of which had mutations in DELLA domain (Fig. 4.43). Multiple sequence alignment of DELLA domain containing nucleotide sequence from *T. monococcum, Rht22, H. vulgare, Zea mays* and *O. sativa* was further analyzed by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). This multiple sequence alignment showed that most of the sequences were conserved in different species as shown by asterisk. It was also confirmed that the two bp deletion occurred in *Rht22* with respect to wild type *T. monococcum* and the deletion was in the DELLA domain (blue gap in Fig. 4.44). DOT MATRIX analysis of *T. monococcum* and *Rht22* as shown in Fig. 4.45 indicated that most of the sequences were similar between them with some deviation in the end of the sequences as broken line which corroborates the multiple sequence alignment.

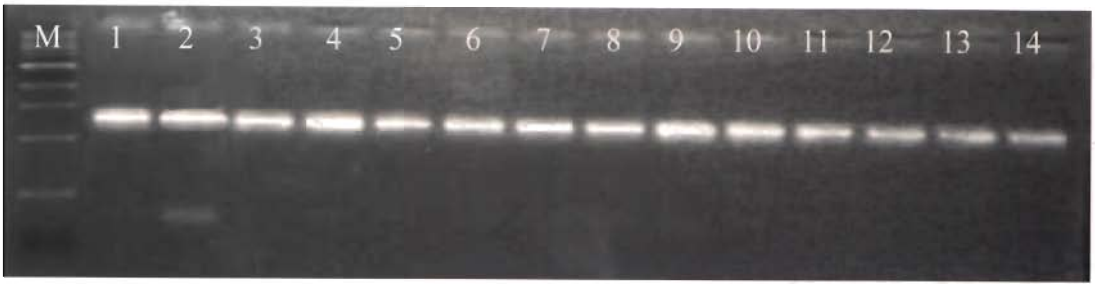


Figure 4.38 PCR amplification with DF2+WR2 primer combination in wild type *T. monococcum* and Other dwarf mutants. Lanes: M: 100bp ladder. 1: *T. monococcum*, 2- MM-21, 3- MM-*24*, 4- MM-26, 5- MM-28, 6- MM-29, 7- MM-32, 8- MM-33, 9- MM-34, 10- MM-37, 11- MM-39, 12-*Rht22* 13- MM-43,14- MM-46

DF2

GGCAAGCAAAAGCTTCGCGATTATTGGCTAGGTAGAGAGCGAGGTAGCTCGCT
CGCGGCGAGGATCATGAATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGG
TGGCGGTGGTGGCGGCATGGGCTCGTCCGAGGACAAGATGATGGTGGGGTCGG
CGGCGGCGGGGGAGGGGGAGGAGGTGGACGAGCTGCTGGCGGCGCTCGGGTA
CAAGGTGCGCGCCTCCGACATGGCGGACGTGGCGCAGAAGCTGAGCAGCTCGA
GATGGCC

WR2

Figure 4.39 Sequence of PCR product of DF2+WR2 primers in *T. monococcum* (271bp)

DF2

GGCAAGCAAAAGCTTCGCGATTATTGGCTAGGTAGAGAGCGAGGTAGCTCGCT
CGCGGCGAGGATCATGAATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGG
TGGCGGTGGTGGCGGCATGGGCTCGTCCGAGGACAAGATGATGGTGGGGTCGG
CGGCGGCGGGGGAGGGGGAGGAGGTGGACGAGCTGCTGGCGGCGCTCGGGTA
CAAGGTGCGCGCCTCCGACATGGCGGACGTGGCG--GAAGCTGAGCAGCTCG
AGATGGCC

WR2

Figure 4.40 Sequencing product of DF2+WR2 primers in *Rht22* (269bp)

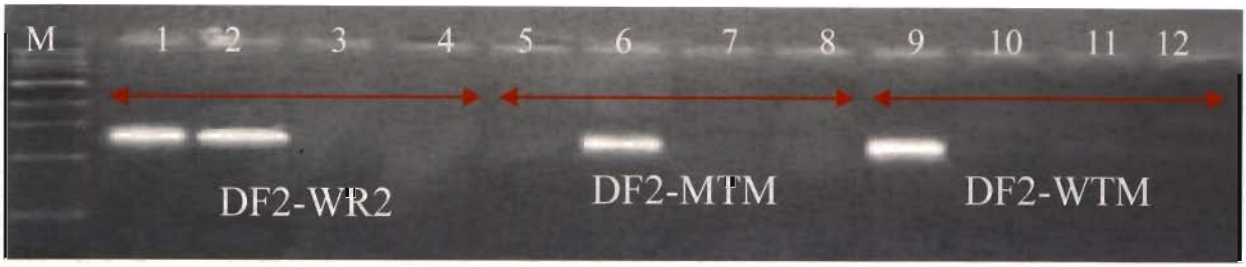


Figure 4.41 Confirmation of two bp deletion in *Rht22* mutant gene Lane: M: 100bp ladder.Lane1,5,9: (*T. monococcum*), Lane 2,6,10: (*Rht22*), Lane 3,7,11: (WL711) and Lane 4,8,12: (PBW343)

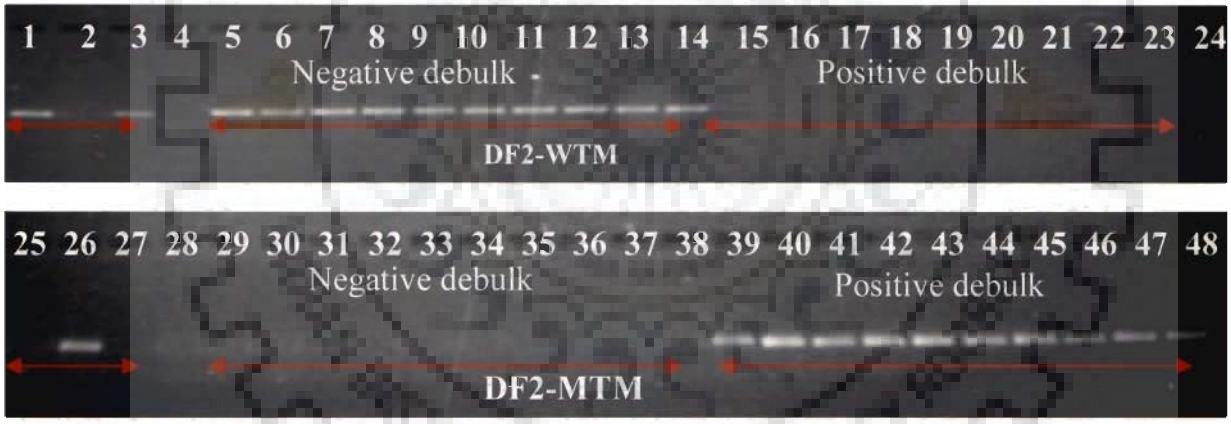


Figure 4.42 PCR amplification with DF2+WTM and DF2+MTM primer combination in positive and negative debulk F2 plants of *Rht22* X *T. boeoticum*.Lane1, 25: (*T. monococcum*), Lane 2, 26 (Rht22), Lane 3, 27: (*T. boeoticum*), Lane 4, 28(Control), Lane 5-14 and 29-38: Negative debulk, Lane 15-24 and 39-48: (Positive debulk)

```
>  emb|AJ242531.1|  Triticum aestivum rht-D1a gene for gibberellin
     response modulator
Length=1872

 Score =  334 bits (370),  Expect = 1e-88
 Identities = 195/201 (97%), Gaps = 3/201 (1%)
 Strand=Plus/Plus

Query  71   ATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGGTGGCGGTGGTGGCGGCATGGGCTCG  130
            ||||||||| ||||||||||||||||||||||||| ||||| ||||||||||||||||||
Sbjct  1    ATGAAGCGGGAGTACCAGGACGCCGGAGGGAGCGGCGGCGGCGGTGGCGGCATGGGCTCG  60

Query  131  TCCGAGGACAAGATGATGGTGGGGTCGGCGGCGGCGGGGGAGGGGGAGGAGGTGGACGAG  190
            |||||| |||||||| |||||  |||||||||||| |||||||||||||||||||||||||
Sbjct  61   TCCGAGGACAAGATGATGGTG---TCGGCGGCGGCGGGGGAGGGGGAGGAGGTGGACGAG  117

Query  191  CTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCTCCGACATGGCGGACGTGGCGCAGAAG  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118  CTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCTCCGACATGGCGGACGTGGCGCAGAAG  177

Query  251  CTGGAGCAGCTCGAGATGGCC  271
            |||||||||| |||||||||||
Sbjct  178  CTGGAGCAGCTCGAGATGGCC  198


>gb|AF460219.1| Hordeum vulgare subsp. vulgare nuclear transcription
factor SLN1 gene, complete cds, Length=4098

Score =  376 bits (416),  Expect = 3e-101
 Identities = 248/273 (90%), Gaps = 6/273 (2%)
 Strand=Plus/Plus

Query  1     GGCAAGCAAAAGCTTC--GCGATTATTGGCTAGGTAGAGAGCGAGGTAGCTCGCTCGCGG  58
            |||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1697  GGCAAGCAAAAGCTTCCCGCGATTATTGGCTAGGTAGAGAGCGAGGTAGCTCGCTCGCGG 1756

Query  59    CGAGGATCATGAATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGGTGGCGGTGGTGGC  118
            ||||||||||    |||||||||||||||| ||| |||||||| || || ||||
Sbjct  1757  CGAGGATCATGA----AGCGCGAGTACCAGGACGGCGGCGGGAGCGGCGGTGGGGGTGAT  1811

Query  119   GGCATGGGCTCGTCCGAGGACAAGATGATGGTGGGGTCGGCGGCGGCGGGGGAGGGGGAG  178
            |  |||| |||||  |||||||||||||||| |||| ||| ||||||||||||||||
Sbjct  1813  GAGATGGGGTCGTCGAGGGACAAGATGATGGTGTCGTCGTCGGAGGCGGGGGAGGGGGAG  1871

Query  179   GAGGTGGACGAGCTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCTCCGACATGGCGGAC  238
            |||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||||
Sbjct  1873  GAGGTGGACGAGCTGCTGGCGGCGCTCGGGTACAAGGTGCGGGCGTCCGACATGGCGGAC  1931

Query  239   GTGGCGCAGAAGCTGGAGCAGCTCGAGATGGCC  271
            ||||||||||||||||||||||||||||||||
Sbjct  1933  GTGGCGCAGAAGCTGGAGCAGCTCGAGATGGCC  1965
```

```
>gb|AF413159.1|Zea mays cultivar CML5 DWARF8 gene, partial cds Length=2641

 Score =  223 bits (246),  Expect = 5e-55 Identities = 173/207 (83%), Gaps
= 12/207 (5%) Strand=Plus/Plus

Query  71    ATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGGTGGCGGTGGTGGCGGCATGGGCTCG  130
             ||||||||||||||||| |||||||| |||||||| |||||      || |||||||||
Sbjct  895   ATGAAGCGCGAGTACCAAGACGCCGGCGGGAGCGGCGGCGG------CGACATGGGCTCC  948

Query  131   TCCGAGGACAAGATGATGGTGGGGTCGGCGGCGGCGGGGGAGGGGGAGGAGGTGGACG--  188
             ||| |||||||||||||| || | ||||||  || ||||||  |||||||| |||||
Sbjct  949   TCCAAGGACAAGATGATGGCGGCGGCGGCGGGAGCAGGGGAGCAGGAGGAGGAGGACGTG  100

Query  189   ----AGCTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCTCCGACATGGCGGACGTGGCG  244
                 |||||||||| |||||||||||||||||  | || || ||||||||||| |||
Sbjct  1009  GATGAGCTGCTGGCCGCGCTCGGGTACAAGGTGCGTTCGTCGGATATGGCGGACGTCGCG  106

Query  245   CAGAAGCTGGAGCAGCTCGAGATGGCC  271
             |||||||||||||||||||||||||
Sbjct  1069  CAGAAGCTGGAGCAGCTCGAGATGGCC  1095

>dbj|AB262980.1| Oryza sativa Japonica Group SLR1 gene for DELLA
protein, complete cds  Length=2487
GENE ID: 4333860 Os03g0707600 | Os03g0707600 [Oryza sativa Japonica Group]
(10 or fewer PubMed links)

 Score =  217 bits (240),  Expect = 2e-53
 Identities = 172/207 (83%), Gaps = 12/207 (5%)
 Strand=Plus/Plus

Query  71    ATGAAGCGCGAGTACCAGGACGCCGGAGGGAGCGGTGGCGGTGGTGGCGGC------ATG  124
             |||||||||||||||| || ||||| |||||| | ||||| ||  || ||      |||
Sbjct  216   ATGAAGCGCGAGTACCAAGAAGCCGGCGGGAGCAGCGGCGGCGGGAGCAGCGCCGATATG  275

Query  125   GGCTCGTCCGAGGACAAGATGATGGTGGGGTCGGCGGCGGCGGGGGAGGGGGAGGAGGTG  184
             || |||| | |||||||| |||||| ||||       ||||||||||||| |||||| ||
Sbjct  276   GGGTCGTGCAAGGACAAGGTGATGGCGGGG------GCGGCGGGGGAGGAGGAGGACGTC  329

Query  185   GACGAGCTGCTGGCGGCGCTCGGGTACAAGGTGCGCGCCTCCGACATGGCGGACGTGGCG  244
             |||||||||||||||||||||||||||||||| | ||||||||||| ||||| |||
Sbjct  330   GACGAGCTGCTGGCGGCGCTCGGGTACAAGGTGCGGTCGTCCGACATGGCCGACGTCGCG  389

Query  245   CAGAAGCTGGAGCAGCTCGAGATGGCC  271
             |||||||||||||||| |||||||||
Sbjct  390   CAGAAGCTGGAGCAGCTGGAGATGGCC  416
```

Figure 4.43 Alignment of the DF2-WR2 sequence with *Triticum aestivum, Hordeum vulgare, Zea mays* and *Oryza sativa* using BLASTN.

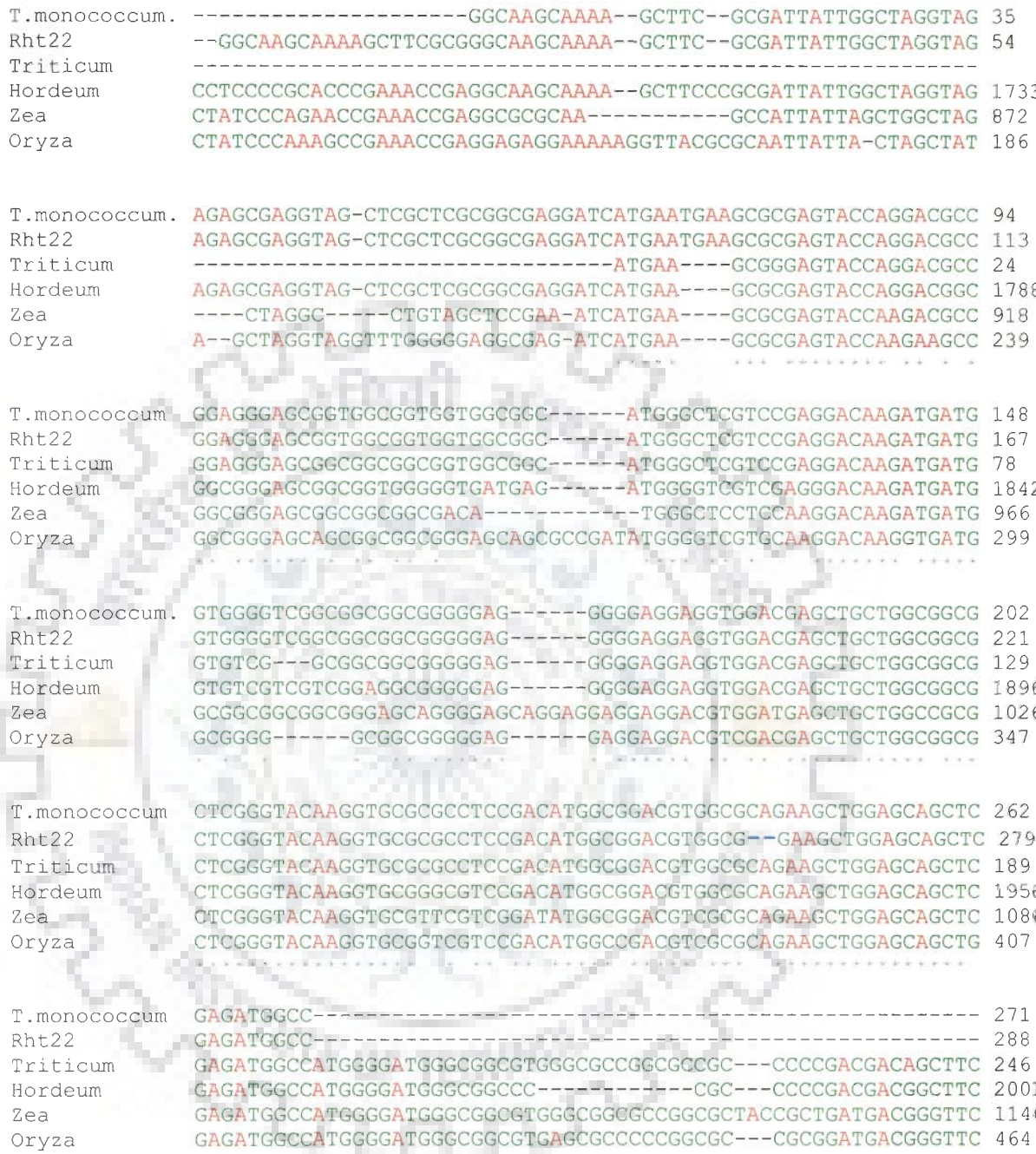


Figure 4.44 Multiple sequence alignment of DELLA domain containing nucleotide sequences from *T. monococcum, Rht22, T. aestivum, H. vulgare, Zea mays* and *O. sativa.* Conserved nucleotide residues in all the six sequences*.

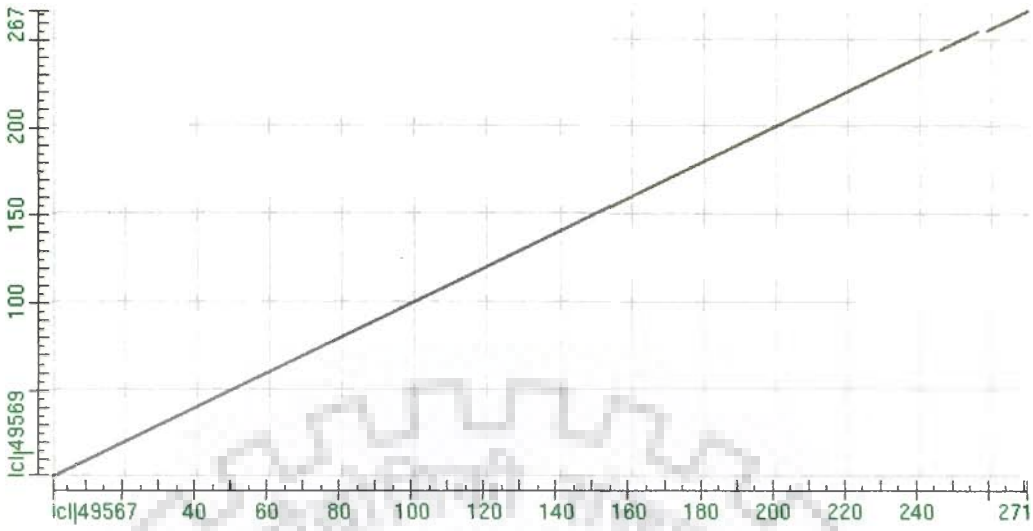


Figure 4.45 DOT MATRIX view of sequences of *T. monococcum* and *Rht22* indicating two bp deletion in *Rht22*.

>Translated *Rht22*

MKREYQDAGGSGGGGGGMGSSEDKMMVGSAAAGEGEE
VDELLAALGYKVRASDMADVAEAGASRW

>Translated wild type

MKREYQDAGGSGGGGGGMGSSEDKMMVGSAAAGEGEE
VDELLAALGYKVRASDMADVAQKLEQLEMA

Figure 4.46 Translated protein sequence using expasy tool of *Rht22* and wild type *T. monococcum*

```
 >lcl|65293 wild type Length=67
Score = 108 bits (271), Expect = 5e-30, Method: Compositional matrix
adjust.
Identities = 58/59 (98%), Positives = 59/59 (100%), Gaps = 0/59 (0%)

  Rht22 1 MKREYQDAGGSGGGGGGMGSSEDKMMVGSAAAGEGEEVDELLAALGYKVRASDMADVAEAGASRW  6
          MKREYQDAGGSGGGGGGMGSSEDKMMVGSAAAGEGEEVDELLAALGYKVRASDMADVA+
Wild typ1 MKREYQDAGGSGGGGGGMGSSEDKMMVGSAAAGEGEEVDELLAALGYKVRASDMADVAQKLEQLEMA67
```

Figure 4.47 Sequence alignment of translated products of wild type and *Rht22* showing amino acid frameshift variation (red)

```
>sp|Q9ST59.1|RHT1_WHEAT  RecName: Full=DELLA protein RHT-1;
AltName: Full=Reduced height
protein 1; AltName: Full=Protein Rht-B1/Rht-D1
 emb|CAB51555.1|  gibberellin response modulator [Triticum
aestivum]
Length=623

 Score = 93.6 bits (231),  Expect = 7e-18
 Identities = 66/67 (98%), Positives = 66/67 (98%), Gaps = 1/67 (1%)
 Frame = +2

Query 71 MKREYQDA------EDKMMVGSAAAGEGEEVDELLAALGYKVRASDMADVAQK 250
         MKREYQDAGGSGGGGGGMGSSEDKMMV SAAAGEGEEVDELLAALGYKVRASDMADVAQK
Sbjct 1  MKREYQDAGGSGGGGGGMGSSEDKMMV-SAAAGEGEEVDELLAALGYKVRASDMADVAQK  59

Query 251 LEQLEMA  271
          LEQLEMA
Sbjct 60  LEQLEMA  66
```

Figure 4.48 Sequence alignment of translated products of *T. monococcum* and *Rht-B1/Rht-D1*

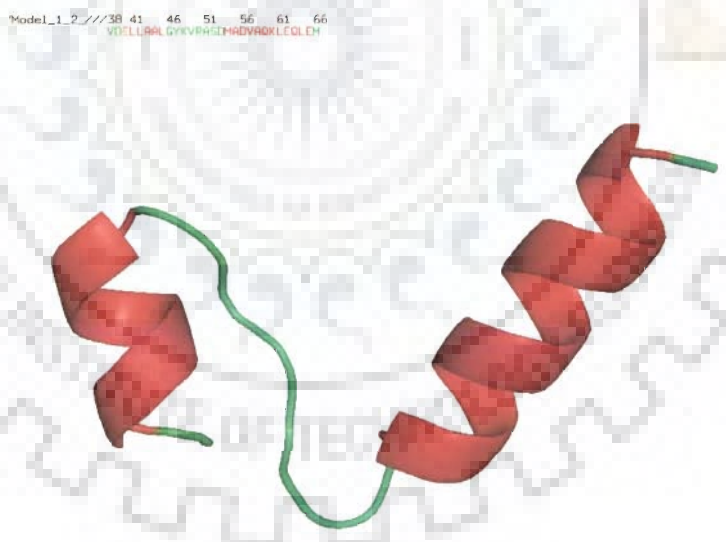


Figure 4.49 3D model built based on template based on template **2zsiB** (1.80 Å) (structural basis of gibberellin(GA4)-induced della recognition by the gibberellin receptor in *Arabidopsis thaliana*) sequence identity [%]: 82.759

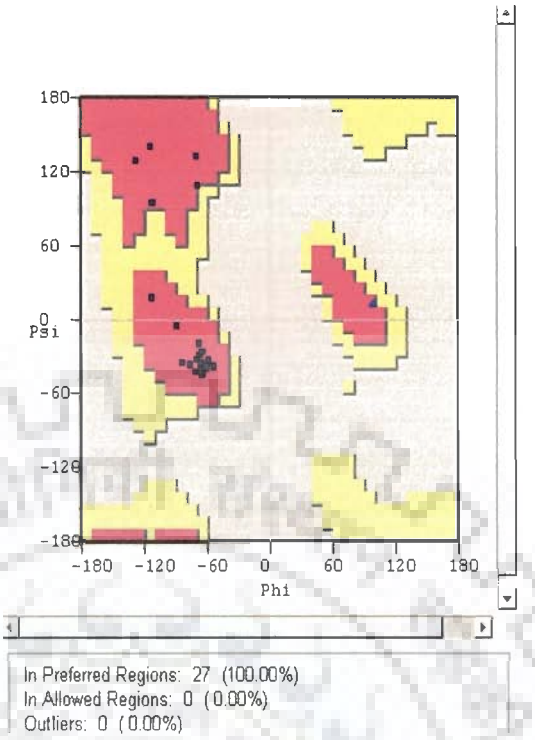


Figure 4.50 3D structural validation on the basis of Ramachandran Plot.

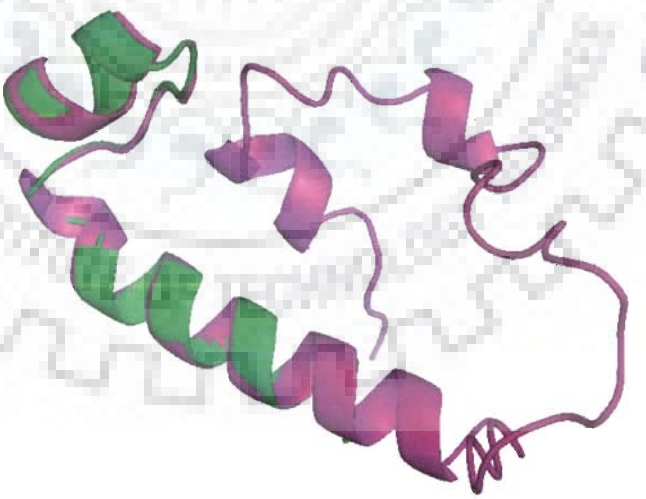


Figure 4.51 3D Modelled structured, magenta = *T. aestivum* Rht-B1/Rht-D1 DELLA motif and green = *T. monococcum* translated DELLA motif executive: RMS = 0.628 (193 to 193 atoms). So belonging to same family of proteins.

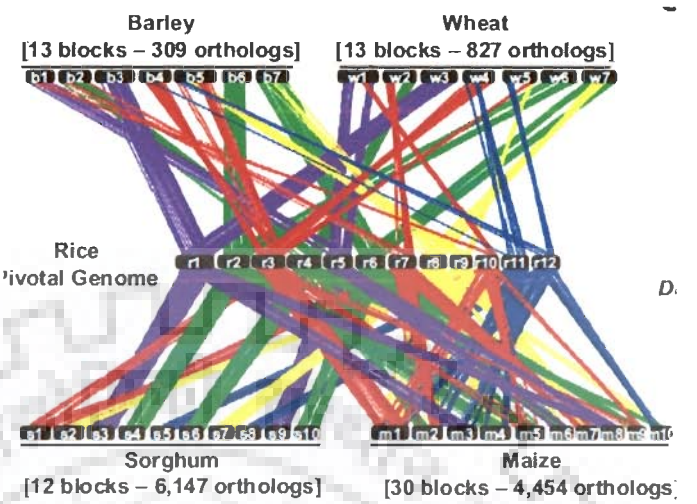


Figure 4.52 Synteny among cereals chromosomes (Source: Salse *et al.* 2009)

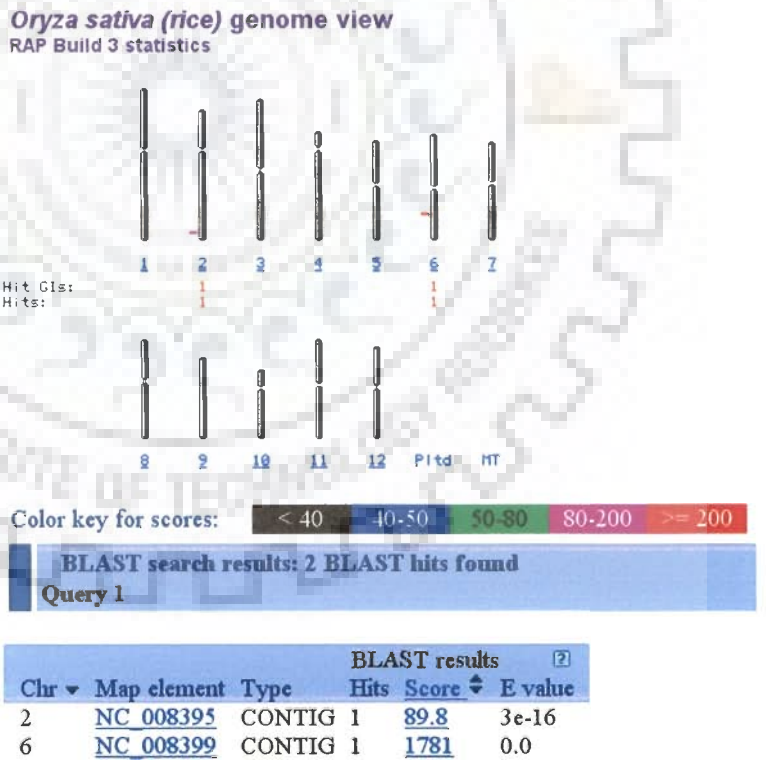


Figure 4.53 Synteny of nud gene of barley with rice genome shows one major hit on 6RL and one minor on 2RL (shown in Red).

The *Rht22* and *T. monococcum* amplified sequence was computationally translated into protein using Expasy (http://www.expasy.ch/tools/dna.html) and the translated protein was further analyzed by amino acid wise sequence alignment which showed that most of the AAs residues were identical from the beginning while there was frameshift mutation after the deletion as shown in red color in Fig. 4.47. Sequence of *Rht22* translated protein was BLASTed using BLASTP which showed the first hit with DELLA protein of *Rht-B1/Rht-D1* supporting our results of mutation in DELLA domain of *Rht22* (Fig. 4.48). The 3D of Rht22 translated protein was built using Swiss model (http://swissmodel.expasy.org/) based on template **2zsiB** (Fig. 4.49). All the residues of the built model came in the preferred region of Ramachandran plot (Fig. 4.50). This showed that there was correct φ and ψ relationship for all the residues of the protein. The built model of Rht22 was superimposed with the built model of *Rht-B1/Rht-D1* (Fig. 4.51) showed that the residues were aligned with 0.628 Å deviation, which clearly indicated that both the protein belonged to the same protein super family at the structural level.

4.9.4 BLAST search of rice genes and wheat ESTs

Soft glume (*sog3*) mutant mapped on chromosome 7AL. which is syntenic 7HL of *H. vulgare* (Salse *et al.* 2009). A naked caryopsis mutant (*nud*) has been mapped on 7HL (Taketa *et al.* 2008) suggesting that *sog3* may be orthologous to *nud* of barley. *nud* BLASTed with rice genome database found two hits, one major hit on chromosome 6RL with 72% homology and the other one minor hit on 2RL chromosome with 78% homology (Fig. 4.52 and 4.53).

The homology of wheat chromosome 6A, 3A and 1A with Rice chromosome 2R, 1R and 3R has already been known (Salse *et al.* 2009). On the basis of this homology we searched orthologous genes of cellulose synthase and related genes on corresponding rice chromosome as detailed of these genes was already reported (Sado *et al.* 2009, Joshi *et al.* 2007). Six genes of cellulose synthesis pathway were found on syntenic rice chromosomes two genes on each chromosome. Positions of these genes on rice chromosome were found through NCBI data base. Six individual rice genes involved in cellulose synthesis pathway BLASTed against the available wheat ESTs, led to the identification of one wheat EST each showing high similarity (bit score ≥200) against each of the six rice genes (Table 4.8). The identified wheat ESTs were used as querry sequences in BLAST search against the bin-

mapped wheat EST/contigs. However, none of the six EST sequences showed similarity with the bin-mapped wheat ESTs/contigs.

Table 4.8 Rice CesA and cellulose synthase like genes and their maximum homology with corresponding wheat EST.

S. No.	Rice gene	Rice gene ID	Rice chromosome	Homologous to wheat chromosome	Wheat EST matched with Rice genes
1	CESA4	LOC_OS01G54620.1	1	Group 3	BQ579118
2	CSLC1	LOC_OS01G56130.1	1	Group 3	TC299623
3	CSLA1	LOC_OS02G09930.1	2	Group 6	TC282597
4	CSLE2	LOC_OS02G49332.1	2	Group 6	TC330457
5	CESA1	LOC_OS05G08370.1	5	Group 1	TC280199
6	CSLC7	LOC_OS05G43530.1	5	Group 1	CJ635129

CHAPTER 5

DISCUSSION

Chapter 5
DISCUSSION

In hexaploid wheat various attempts to induce useful variability through physical and chemical mutagenesis have not been very successful due to its large genome size, higher ploidy level and repetitive DNA (Xue *et al.* 2008). The diploid wheat, *T. monococcum* with smaller genome size of 5,700 Mb compared to 17,300 Mb of bread wheat (Bennett and Leitch 1995), the existence of a very high level of polymorphism for DNA based markers (Castagna *et al.* 1994), sequence conservation at orthologous loci (Wicker *et al.* 2003), availability of a mapping populations (Dubkovsky *et al.* 1996, Röder *et al.* 1998 and Singh *et al.* 2007) and large BAC library (Lijavetzky *et al.* 1999) makes an attractive diploid model for gene discovery in wheat.

Mutants for various traits available in *T. monococcum* can be easily mobilized to A genome of durum and hexalpoid wheat through recombination (Singh *et al.* 2007). EMS mutagenesis in *T. monococcum* can give a wide spectrum of mutants with distinct phenotypes (Dhaliwal *et al.*1987) which can be used for functional genomics and allele mining through TILLING (Kim *et al.* 2006; Slade *et al.* 2005). With the availability of advanced genomic resources and International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org/news.php), importance and demand for these mutants will further increase in wheat as valuable tools in reverse genetics analysis to identify novel gene function and expression.

In the present study, five independent EMS induced mutants of *T. monococcum* namely MM-41 (*Rht22*), MM-09 (*sog3*), and three brittle culm mutants MM-13, MM-15 and MM-232 (*brc1, brc2* and *brc3,* respectively) were used for their phenotypic and molecular characterization. All the five EMS induced mutants were monogenic recessive. Such mutants probably could not have been recovered in polyploid wheat because of orthologous loci on other genomes unless and until multiple mutants are induced at all loci or certain loci had been silenced during evolution. It was possible for Slade *et al.* (2005) to get a hexaploid wheat line with amylopectin through waxy mutants in all the three genomes. The vernalization genes *VRN1* and *VRN2* were cloned in wheat by using the naturally existing variation in *T. monococcum* (Yan *et al.* 2003, 2004). The tillering mutant *tin3* and

soft glume mutant *sog* from the EMS mutant collections of *T. monococcum* (Dhaliwal *et al.*1987) were mapped on chromosome arm $3A^mL$ and $2A^mS$, respectively (Kuraparthy *et al.* 2007 and Sood *et al.* 2009) to identify the tillering and soft glume genes in Triticeae. It further indicates that the *T. monococcum* could be a reference diploid genome for gene discovery in wheat. In addition, high density linkage maps (Dubkovsky *et al.* 1996; Röder *et al.* 1998 and Singh *et al.* 2007) of diploid wheat will be helpful for identification of desired traits from diploid A genome species and their transfer to hexaploid wheat.

Among ten EMS induced dwarf mutants characterized for GA_3 sensitivity only three mutants were GA_3 insensitive while the other were GA_3 sensitive. About 21 GA_3 sensitive and insensitive dwarf mutants have already been isolated and mapped in wheat (http://www.shigen.nig.ac.jp/wheat/komugi/genes/symbolClassList.jsp). The only GA_3 insensitive mutant MM-41 (*Rht22*) investigated in detail was similar to other DELLA domain defective GA_3 insensitive dwarf mutants of hexaploid wheat, barley, maize and rice. Mapping of this dwarf mutant to $4A^mS$ indicates that it could be orthologs to *Rht-B1* and *Rht-D1* of hexaploid wheat, which have been mapped to 4BS and 4DS chromosomes of wheat, respectively. Several differences were found in the histological sections of the internode and leaf of *Rht22* and *T. monococcum*. Shorter internodes, small seed size, reduced cell size and low tillering ability in *Rht22* indicated the pleiotropic effect of the inactivated gene. Most of the dwarfing genes exhibit their pleiotropic effects for tillering, grain size, spike type and other characters (Youssefian *et al.* 1992). The dwarf mutant can be used for breeding in hexaploid wheat because of its favorable effects for lodging resistance as no such mutant has been identified for the a genome.

Partial sequence of *Rht22* shows its homology with *Rht-D1a* gene for gibberellin response modulator of hexaploid wheat, *SLN1* nuclear transcription factor of *Hordium vulgare*, *DWARF 8* gene for *Zea mays* and *SLR 1* of rice. All these mutants have mutation in DELLA domain and show GA-insensitive dwarf phenotype. Dot matrix and BLASTN of partial sequence of *T. monococcum* and *Rht22*, cover the DELLA domain showing two bp deletion in *Rht22*. Sequence alignment of partial translated protein from nucleotide sequence covering DELLA domain showed variation in amino acid sequence of *Rht22* after deletion due to frame shift. Localised in the nucleus, the DELLA proteins are reported to be the transcription factor for GA-signaling in plants (Silverstone *et al.* 2001; Itoh *et al.* 2002). These proteins function as a key repressor of GA-signaling by inhibiting the GA-regulated

gene expression (Sun and Gubler 2004). These repressors accumulate in the nucleus and are rapidly degraded in response to GA (Silverstone *et al.* 2001; Fu *et al.* 2002). The GA-signals seems to be perceived by N-terminal region of the protein containing the DELLA domain. GA induces degradation of DELLA proteins via the ubiquitin/proteasome pathway (Itoh *et al.* 2003). Mutations within the DELLA domain render these proteins resistant to degradation which results in a GA-insensitive dwarf phenotype (Dill *et al.* 2001; Peng *et al.* 1999). 3D model built based on template structural basis of GA4 induced DELLA recognition by the gibberellin receptor in *Arabidopsis thaliana* shows complete homology of partial 3D helix and loop of Rht22 with Rht-D1 of hexaploid wheat indicating that they belong to the same super family of protein and this structural validation is also supported by Ramachandran plot.

The only free threshing mutant of *T. monococcum* with soft glume investigated in this study MM-09 (*sog3*), was distinct from the previously mapped tenacious or soft glume mutants of *T. aestivum* and *T. monococcum* (Nalam *et al.* 2007, Salamini *et al.* 2002, Sood *et al.* 2009), as the *sog3* mapped on 7AL, is not orthologous to those. Furthermore the soft glume, sog3 mutant in addition to free threshing also has tough rachis which remains intact like that of free threshing wheat with Q gene. In *T. monococcum* with tough rachis, rachis breaks as wedge with individual spikelets and no intact rachis remains. It may be orthologous to the barley *Nud* locus which is responsible for hulled caryopsis through some lipid deposition below hull and pericarp (Taketa *et al.* 2008). Nud gene sequence after BLASTing with rice genome data base show one major hit on rice 6RL and one minor hit on 2RL chromosomes.

Although a spontaneous free threshing mutant of *T. monococcum* subsp. *monococcum* referred to as *T. sinskajae* has been reported to possess soft glumes (Filatenko and Kurkiev 1975 cited by Gonchariov *et al.* 2002), the free-threshing einkorn wheat could not be used for large scale cultivation due to the association of the soft glume trait with reduced ear length (Salamini *et al.* 2002). A single, recessive gene *sog* controlling soft glume trait in *T. sinskajae* was mapped on the short arm of chromosome $2A^m$ (Taenzler *et al.* 2002). Sood *et al.* (2009) by comparative mapping with other cereals mapped soft glume (*sog*) region of diploid *Triticum monococcum* L. and tenacious glume (*Tg*) region of hexaploid *T. aestivum* on chromosome $2A^mS$ and 2DS, respectively. It is noteworthy to

report that the spikes in *sog3* were as long as that of *T. monococcum* and not reduced in size as the other soft glume mutants.

In polyploid wheats, a polygenic system along with modifier genes is known to govern rachis fragility and glume tenacity (MacKey 1966). Rachis fragility is primarily controlled by genes present on the homocologous group-3 chromosomes (Watanabe and Ikebata 2000; Nalam *et al.* 2006; Li and Gill 2006). All wild wheats have a brittle rachis leading to shattering of either the whole spike or individual spikelets (Li and Gill 2006). The first cultivated wheat had non-brittle rachis (mutant *br* allele) with tough glumes and thus was non-free-threshing. The *Tg* gene controlling glume toughness in wheat is present on short arm of the group 2 chromosomes (Sears 1954; Kerber and Rowland 1974; Chen *et al.* 1999; Simonetti *et al.* 1999; Taenzler *et al.* 2002; Jantasuriyarat *et al.* 2003; Nalam *et al.* 2007). A major modifier gene for domestication related traits (*q* gene) is located on the long arm of chromosome 5A (MacKey 1966; Muramatsu 1986; Faris *et al.* 2002; Faris *et al.* 2005). Subsequent mutations at these loci during domestication (Salamini *et al.* 2002) led to the modern free-threshing wheats (genotype *brbrtgtgQQ*). Among these three genes, only *Q* has been cloned and is a member of the APETALA2 family of transcription factors (Simons *et al.* 2006).

In the tetraploid wheat, Simonetti *et al.* (1999) characterized the genetic loci influencing glume tenacity/threshability. They studied the free-threshing habit in the RIL population derived from a *T. turgidum* subsp. *durum* X *T. turgidum* subsp. *dicoccoides* cross and found four QTL influencing the threshability trait. These QTL were associated with chromosomes 2BS, 5AL, 5AS, and 6AS. The 2BS and 5AL QTL corresponded to the homoeologous genes *Tg* and *Q* of hexaploid wheats, respectively, where *Tg2* the putative ortholog of *Tg*, was located on chromosome 2BS. Furthermore, this study suggested the complexity of free-threshing trait in tetraploid wheat where major genes, *Tg2* and *Q* along with several minor genes are required for the complete expression of the free-threshing trait. A free threshing naked caryopsis mutant in barley (*nud*) has been mapped on chromosome 7HL, which is homoeologous to wheat group-7 chromosomes. The *sog3* on $7A^{m}L$ of *T. monococcum* may be orthologous to *nud* of barley.

All the three brittle mutants' *brc1*, *brc2* and *brc3* were defective in cellulose synthesis and deposition in secondary cell wall of sclerenchyma cells. In all the brittle mutants cellulose of secondary cell wall was reduced by 50 to 55% compared to wild type

T. moncoccum with slight increase in lignin and no appreciable change in hemicellulose, silicates and ash. Comprehensive mechanical strength, histological, biochemical, SEM, AFM and FTIR analyses of culms and leaves of all the brittle mutants supplemented and complemented the findings that the mutants had defective cellulose synthesis/deposition on the secondary cell walls. All plants parts of the mutants were highly brittle indicating that the cellulose in secondary cell wall is the main component for straw strength and not the lignin in *T. monococcum* as often thought of.

The plant cell wall is the major component of mechanical support to cells, tissues, and the entire plant body. Sclerenchyma cells having both a primary wall and a thick secondary wall provide major mechanical support in non-elongating regions of the plant body (Carpita and McCann 2000). Cell walls delimit the boundaries of individual cells; the shapes of individual cell walls determine cell morphology and whole plant morphology (Burk *et al.* 2001). Cell walls contain different substances to suit its function, for example, cellulose usually constitutes 20–30% of the dry weight of the primary wall and 40–90% of the secondary cell wall (Taylor *et al.* 1999). Lignin and hemicellulose are the other two important contents in the cell wall. Many mutants defective in plant strength have been isolated and characterized. The barley *brittle culm* showed reduced mechanical strength and cellulose content (Kokubo *et al.* 1989, 1991), indicating a correlation between the cellulose content and the plant mechanical strength (Li *et al.* 2003). The rice classic mutant *bc1* with a decreased level of cellulose has a defect in a COBRA-like protein that may function in the development of secondary cell walls (Li *et al.* 2003). In maize, Maize *Brittle stalk 2* also have decreased level of cellulose with a defect in COBRA- like protein (Sindhu *et al.* 2007). Similarly, in *Arabidopsis*, the *irregular xylem* mutants (*irx1* to *irx3*) have shown cellulose synthesis defects in secondary walls, and decreased stiffness of mature stems (Turner and Somerville 1997) indicating the direct role of cellulose in maintaining cell morphology.

The brittle mutants *brc1, brc2* and *brc3* have been mapped to different chromosome of wheat (6A, 3A and 1A, respectively) suggesting that there could be multiple cellulose synthesizing and deposition genes in diploid wheat like that of rice and *Arabidopsis* (Sado *et al.* 2009). In a complete sequence of *Arabidopsis thaliana* genome it has been estimated that, of approximately 27000 Arabidopsis genes, approximately 15% are dedicated to the processes of cell wall synthesis, modification, assembly and degradation, and correct regulation of these processes during growth and development (Carpita *et al.* 2001).

Therefore, the cell wall is one of the most complicated and the least-understood plant cell structures. Mutants defective in mechanical strength have been proven to be valuable for identifying genes involved in the biogenesis and modification of cell walls (Li *et al.* 2003; Tanaka *et al.* 2003; Taylor *et al.* 2003; Zhong *et al.* 2002). Wall polysaccharides and glycoproteins have tremendous structural complexity, and therefore plants require large families of glycosyltransferases (GTs) to facilitate their biosynthesis. Based on sequence similarities and the existence of certain motifs, hydrophobic clusters and their catalytic specificity (Rosen *et al.* 2004), GTs have divided into 91 families (http://www.cazy.org/CAZY/). Among these, both cellulose synthase active subunits (CESA) (Somerville, 2006) and CESA-like proteins (CSL), former are responsible for synthesizing cellulose and the latter are believed to be responsible for the formation of glycan backbones in the endoplasmic reticulum (ER) or Golgi apparatus (Burton *et al.* 2006; Dhugga *et al.* 2004).

Grasses provide the majority of calories consumed by humans either directly through the consumption of grains or indirectly through animals fed a diet of grains and forage. Grass cell walls are a major source of dietary fibers that provide numerous health benefits beyond simply providing calories (Spiller *et al.* 2001; Harris *et al.* 2006). Furthermore, grass cell walls are poised to become a significant source of renewable energy because the sugars locked in the polysaccharides of the cell wall can be converted into liquid fuel (e.g. ethanol, butanol) and the entire cell wall can be burnt to produce heat or electricity (Ragauskas *et al.* 2006; Perlack *et al.*2005; Service et *al.*2007).

Each of three rice chromosomes 2, 1 and 5 syntenic to wheat chromosomes 6, 3 and 1 carry gene for cellulose synthesis for the brittle mutants had at least two CesA and CSL orthologs (Salse *et al.* 2009). Six genes of cellulose synthesis pathway on syntenic rice chromosome were BLASTed against the available wheat ESTs. The identified wheat ESTs were further BLASTed with the bin-mapped wheat EST/contigs. However, none of the six EST sequences showed similarity with the bin-mapped wheat ESTs/contigs. This may be attributed to the limited number of bin-mapped wheat ESTs available in Graingene databases (http://wheat.pw.usda.gov/GG2/blast.shtml). Therefore, physical mapping of the six wheat ESTs showing homology with the six rice genes involved in the cellulose biosynthesis pathway will need to be carried out to identify their bin-position and development of "perfect" markers, if possible, during future studies.

FUTURE PROSPECTIVE

To study their expression in cultivated wheat, all these mutants can be easily mobilized to durum and than bread wheat cultivars using *T. durum* as a bridging species. The functional marker for *Rht22* and closely linked flanking markers for other mutants can be used for their MAS. The A^m genome is most closely related the A^u genome of *T. urartu,* the A genome donor of cultivated wheats (Johnson and Dhaliwal 1976). The introgression of the mutant alleles can occur through recombination without any linkage drag. A number of genes for resistance against wheat disease have been transferred from *T. monococcum* to cultivated wheats (Singh *et al.* 2007). The transfer of semi dwarfing gene *Rht22* will be of special interest as there is no dwarfing gene of A genome deployed in cultivated wheat. A number of GA_3 sensitive and insensitive mutants identified and genes controlling them remain to be mapped and cloned. There is lot of interest among wheat breeders to deploy GA_3 sensitive dwarfing genes in cultivated wheats instead of the usual GA_3 insensitive *Rht-B1b* and *Rht-D1b*, because *Rht-B1b* and *Rht-D1b* reduce the plant height as well as coleoptiles length, thus they are not suitable for wheat cultivation in rain fed area requiring deeper seed placement. Cultivars with GA_3 sensitive dwarfing gene (*Rht8*) are relatively ideal candidate for growing in dry land since it significantly reduce the plant height and are likely to have longer coleoptiles length for seedling emergence from deep sowing and early vigor of plants for weed smothering. GA_3 sensitive dwarfing gene (*Rht8*) has already been deployed by Italian and Australian wheat breeders.

The soft glume mutant *sog* of *T. sinskajae* could not be exploited in *T. monococcum* due to its deleterious pleotrophic effects (Salamini *et al.* 2002). The spikes were highly compact with significantly reduced harvest index. The *sog3* not only has soft glume and intact rachis but the spike are as long as that of *T. monococcum* with normal seeds. Such a mutant can be exploited for free threshing in cultivated *T. monococcum* or at least in TILLING and mapping populations involving *T. monococcum.* The work to clone a full length *Rht22* gene is in progress. However substantial work for map based cloning of *sog3* and brittle mutants through development of large mapping population, fine mapping of the interval between the flanking markers, genome walking, identification of BAC with candidate gene and gene validation remains to be taken up.

Brittleness is one of the most important agronomic traits that affect not only grain production but also the usefulness of cereal straws as animal forage. As an important player

regulating culm brittleness, *brc1, brc2* and *brc3* (and their orthologs in other cereals) could make a significant contribution to the future improvement of wheat crop. It will be desirable to clone and over express the wild type CesA or CesA like genes to be able to get higher cellulose synthesis for stronger straw, higher cellulose/lignin ratio for commercial exploitation in pulp and paper industry as well as in biofuel industries.

CHAPTER 6

REFERENCES

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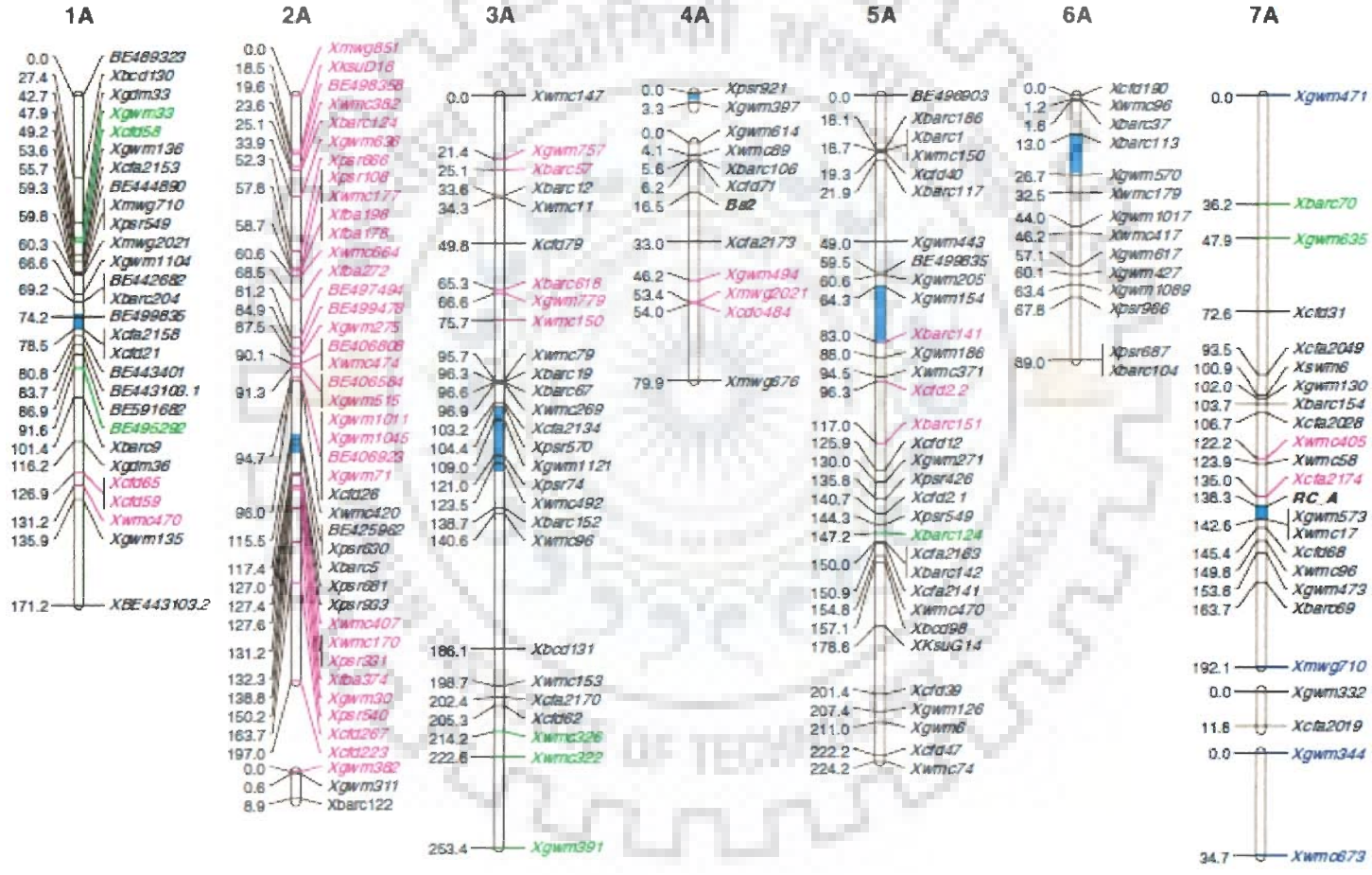
CHAPTER 7

ANNEXURES

Annexure I

Molecular linkage map of diploid A genome wheat develop using RIL population of cross *T. monococcum* x *T. boeoticum*



Annexure II

Polymorphic Microsatellite markers used in the study (Singh *et al.* 2007)

Marker	Chromosome	Forward Primer	Reverse Primer
gdm33	1A	GGCTCAATTCAACCGTTCTT	TACGTTCTGGTGGCTGCTC
gwm33	1A	GGAGTCACACTTGTTTGTGCA	CACTGCACACCTAACTACCTGC
cfd58	1A	AATGGGCCTTTAAGAGCAAAA	AGGGGTGAAAGGTTGGAGAC
gwm136	1A	GACAGCACCTTGCCCTTTG	CATCGGCAACATGCTCATC
cfa2153	1A	TTGTGCATGATGGCTTCAAT	CCAATCCTAATGATCCGCTG
gwm1104	1A	-	-
barc204	1A	CGCAGAAGAAAAACCTCGCAGAAAAACC	CGCAGTGTATCCAAATGGGCAAGC
cfa2158	1A	TTTCGTCTTCAAAATGCACTG	TGGTAGCTTACAAAGGTGCG
cfd21	1A	CCTCCATGTAGGCGGAAATA	TGTGTCCCATTCACTAACCG
barc9	1A	GCGGTCGGTGTCTCCAGTTTTTTTATCA	GCGACATGCGGACAGTATTTAAATTTC
gdm36	1A	ATGCAAAGGAATGGATTCAA	CAAATCCGCATCCAGAAAAT
cfd65	1A	AGACGATGAGAAGGAAGCCA	CCTCCCTTGTTTTTGGGATT
cfd59	1A	TCACCTGGAAAATGGTCACA	AAGAAGGCTAGGGTTCAGGC
wmc470	1A	ACTTGCAACTGGGGACTCTC	TCCCCAATTGCATATTGACC
gwm135	1A	TGTCAACATCGTTTTGAAAAGG	ACACTGTCAACCTGGCAATG
wmc382	2A	CATGAATGGAGGCACTGAAACA	CCTTCCGGTCGACGCAAC
barc124	2A	TGCACCCCTTCCAAATCT	TGCGAGTCGTGTGGTTGT
gwm636	2A	CGGTAGTTTTTAGCAAAGAG	CCTTACAGTTCTTGGCAGAA
wmc177	2A	AGGGCTCTCTTTAATTCTTGCT	GGTCTATCGTAATCCACCTGTA
wmc664	2A	CAGTCAGTGCCGTTTAGCAA	AGCTTTGCTCTATTGGCGAG
gwm275	2A	AATTTTCTTCCTCACTTATTCT	AACAAAAAATTAGGGCC
wmc474	2A	ATGCTATTAAACTAGCATGTGTCG	AGTGGAAACATCATTCCTGGTA
gwm515	2A	AACACAATGGCAAATGCAGA	CCTTCCTAGTAAGTGTGCCTCA
gwm1011	2A	-	-
gwm1055	2A	-	-
gwm71	2A	GGCAGAGCAGCGAGACTC	CAAGTGGAGCATTAGGTACACG
cfd26	2A	TCAAGATCGTGCCAAATCAA	ACTCCAAGCTGAGCACGTTT
wmc420	2A	ATCGTCAACAAAATCTGAAGTG	TTACTTTTGCTGAGAAAACCCT
barc5	2A	GCGCCTGGACCGGTTTTCTATTTT	GCGTTGGGAATTCCTGAACATTTT
wmc407	2A	GGTAATTCTAGGCTGACATATGCTC	CATATTTCCAAATCCCCAACTC
wmc170	2A	ACATCCACGTTTATGTTGTTGC	TTGGTTGCTCAACGTTTACTTC
gwm30	2A	ATCTTAGCATAGAAGGGAGTGGG	TTCTGCACCCTGGGTGAT
cfd267	2A	GTGCGTCGTGTAGCAGCTC	CTCTCTGTCGTCCAGGTCGT
cfd223	2A	AAGAGCTACAATGACCAGCAGA	GCAGTGTATGTCAGGAGAAGCA
gwm382	2A	GTCAGATAACGCCGTCCAAT	CTACGTGCACCACCATTTTG
gwm311	2A	TCACGTGGAAGACGCTCC	CTACGTGCACCACCATTTTG
barc122	2A	CCCGTGTATATCCAGGAGTG	CAGCCCTTGTGATGTGATG
gwm757	3A	-	-
barc57	3A	GCGACCACCTCAGCCAACTTATTATGT	GCGGGGAGGCACATTCATAGGAGT
barc12	3A	CGACAGAGTGATCACCCAAATATAA	CATCGGTCTAATTGTCAATGTA
wmc11	3A	TTGTGATCCTGGTTGTGTTGTGA	CACCCAGCCGTTATATATGTTGA
cfd79	3A	TCTGGTTCTTGGGAGGAAGA	CATCCAACAATTTGCCCAT
barc618	3A	AGTTGCCGCTTCTTTTCATTTTT	AGAGGTCCATTTTTCGTCCTTTGAC
gwm779	3A	-	-
wmc150	3A	CATTGATTGAACAGTTGAAGAA	CTCAAAGCAACAGAAAAGTAAA
wmc79	3A	CATCAATGCATATGGCTGAAAT	AAAAGTTGTCATGAGCGAAGAA
barc19	3A	GCGACCCGAGTAGCCTGAA	GGTGGACCATTAGACGCTTACTTG
barc67	3A	GCGGCATTTACATTTCAGATAGA	TGTGCCTGATTGTAGTAACGTATGTA
wmc269	3A	GCACCTTCTAACCTTCCCCAGC	CCCTAATCCAGGACTCCCTCAG
cfa2134	3A	TTTACGGGGACAGTATTCGG	AAGACACTCGATGCGGAGAG
gwm1121	3A	-	-
wmc492	3A	AGGATCAGAATAGTGCTACCC	ATCCCGTGATCAGAATAGTGT
barc152	3A	CTTCCTAAAATCGGGCAACCGCTTGTTG	GCGTAATGATGGGAGTGGCTATAGGGCAGTT
wmc96	3A	TAGCAGCCATGCTTAGCATCAA	GTTTCAGTCTTTCACGAACACG
wmc153	3A	ATGAGGACTCGAAGCTTGGC	CTGAGCTTTTGCGCGTTGAG
cfa2170	3A	TGGCAAGTAACATGAACGGA	ATGTCATTCATGTTGCCCCT
cfd62	3A	CAAGAGCTGACCAATGTGGA	ACGGCGGTGAGATGAG
wmc326	3A	GGAGCATCGCAGGACAGA	GGACGAGGACGCCTGAAT
wmc322	3A	CGCCCCACTATGCTTTG	CCCAGTCCAGCTAGCCTCC
gwm391	3A	ATA GCG AAG TCT CCC TAC TCC A	ATG TGC ATG TCG GAC GC
gwm397	4A	TGTCATGGATTATTTGGTCGG	CTGCACTCTCGGTATACCAGC
gwm614	4A	GATCACATGCATGCGTCATG	TTTTACCGTTCCGGCCTT
wmc89	4A	ATGTCCACGTGCTAGGGAGGTA	TTGCCTCCCAAGACGAAATAAC

barc106	4A	GCCCTCAAATAATTACGCCAATCCCTATG	GCGTCAAGATCAGAAGGCATCCTATTATTG
cfd71	4A	CAATAAGTAGGCCGGGACAA	TGTGCCAGTTGAGTTTGCTC
cfa2173	4A	GACATACTCCGGCGTTGAAT	TTCCCAGGACATCCTTCTTG
gwm494	4A	ATTGAACAGGAAGACATCAGGG	TTCCTGGAGCTGTCTGGC
gwm186	5A	GCAGAGCCTGGTTCAAAAAG	CGCCTCTAGCGAGAGCTATG
barc1	5A	GCGATGCTTTTGCCTTGTTTCAG	GCGGCCCCTTTGACTCTTCATAG
wmc150	5A	CATTGATTGAACAGTTGAAGAA	CTCAAAGCAACAGAAAAGTAAA
cfd40	5A	GCGACAAGTAATTCAGAACGG	CGCTTCGGTAAAGTTTTTGC
barc117	5A	TCATGCGTGCTAAGTGCTAA	GAGGGCAGGAAAAAGTGACT
gwm443	5A	GGGTCTTCATCCGGAACTCT	CCATGATTTATAAATTCCACC
gwm205	5A	CGACCCGGTTCACTTCAG	AGTCGCCGTTGTATAGTGCC
gwm154	5A	TCACAGAGAGAGAGGGAGGG	ATGTGTACATGTTGCCTGCA
barc141	5A	GGCCCATGGATAATTTTTGAAATG	CAATTCGGCCAAAGAAGAAGTCA
barc186	5A	GGAGTGTCGAGATGATGTGGAAAC	CGCAGACGTCAGCAGCTCGAGAGG
wmc371	5A	GGAAACCAAGGCAGCAGTCA	CCAGGGCTACTTCAGCCAGG
cfd2	5A	GGTTGCAGTTTCCACCTTGT	CATCTATTGCCAAAATCGCA
barc151	5A	TGAGGAAAATGTCTCTATAGCATCC	CGCATAAACACCTTCGCTCTTCCACTC
cfd12	5A	GTTACCCAAACCTGCCCTTT	CTACGAGTCGGGATCAGCAT
gwm271	5A	CAAGATCGTGGAGCCAGC	AGCTGCTAGCTTTTGGGACA
barc124	5A	TGCACCCCTTCCAAATCT	TGCGAGTCGTGTGGTTGT
cfa2163	5A	TTGATCCTTGATGGGAGGAG	CATCATTGTGTTTACGTTCTTTCA
barc142	5A	CCGGTGAGAGGACTAAAA	GGCCTGTCAATTATGAGC
cfa2141	5A	GAATGGAAGGCGGACATAGA	GCCTCCACAACAGCCATAAT
wmc470	5A	ACTTGCAACTGGGGACTCTC	TCCCCAATTGCATATTGACC
cfd39	5A	CCACAGCTACATCATCTTTCCTT	CAAAGTTTGAACAGCAGCCA
gwm126	5A	CACACGCTCCACCATGAC	GTTGAGTTGATGCGGGAGG
gwm6	5A	CGTATCACCTCCTAGCTAAACTAG	AGCCTTATCATGACCCTACCTTT
cfd47	5A	TGACCATGTCATGTTTTATACCACT	TGGACTACATGTCAAGCACAAA
wmc74	5A	AACGGCATTGAGCTCACCTTGG	TGCGTGAAGGCAGCTCAATCGG
cfd190	6A	CAATCAGAAGCGCCATTGTT	CCCTGATGTTTTCTTTTTCTCC
wmc96	6A	TAGCAGCCATGCTTAGCATCAA	GTTTCAGTCTTTCACGAACACG
barc37	6A	CAGCGCTCCCCGACTCAGATCCTT	GCGCCATGTTTCTTTTATTACTCACTTT
barc113	6A	GCGCACAACAACGGACACTTAACAATT	GGGACTCATTTAGCTTCTACTCGCCATTA
gwm570	6A	TCGCCTTTTACAGTCGGC	ATGGGTAGCTGAGAGCCAAA
wmc179	6A	CATGGTGGCCATGAGTGGAGGT	CATGATCTTGCGTGTGCGTAGG
gwm1017	6A	-	-
wmc417	6A	GTTCTTTTAGTTGCGACTGAGG	CGATGTATGCCGTATGAATGTT
gwm617	6A	GATCTTGGCGCTGAGAGAGA	CTCCGATGGATTACTCGCAC
gwm427	6A	AAACTTAGAACTGTAATTTCAGA	AGTGTGTTCATTTGACAGTT
gwm1089	6A	-	-
barc104	6A	GCGCTTCCAAGGCTTAGAGGCT	GCGAGCATCAATAATTGAGAAATACATAGA
gwm471	7A	CGGCCCTATCATGGCTG	GCTTGCAAGTTCCATTTTGC
barc70	7A	GCGAAAAACGATGCGACTCAAAG	GCGCCATATAATTCAGACCCACAAAA
gwm635	7A	TTCCTCACTGTAAGGGCGTT	CAGCCTTAGCCTTGGCG
cfd31	7A	GCACCAACCTTGATAGGGAA	GTGCCTGATGATTTTACCCG
cfa2049	7A	TAATTTGATTGGGTCGGAGC	CGTGTCGATGGTCTCCTTG
swm6	7A	CTTACACGG ACCATGTGCAGA GG	AGGGAGTGGATGAACAAAGTGTG
gwm130	7A	AGCTCTGCTTCACGAGGAAG	CTCCTCTTTATATCGCGTCCC
barc154	7A	GTAATTCCGGTTCCACTTGACATT	GGATGGGCAGCTTCAAGGTATGTT
cfa2028	7A	TGGGTATGAAAGGCTGAAGG	ATCGCGACTATTCAACGCTT
wmc405	7A	GTGCGGAAAGAGACGAGGTT	TATGTCCACGTTGGCAGAGG
wmc58	7A	ATCGGCGGTGAAGCTGATAAGT	TGAAAcAGGGGAATCAGAGGGT
cfa2174	7A	ACGGCATCACAGGTTAAAGG	GGTCTTTGCACTGCTAGCCT
gwm573	7A	AAGAGATAACATGCAAGAAA	TTCAAATATGTGGGAACTAC
wmc17	7A	ACCTGCAAGAAATTAGGAACTC	CTAGTGTTTCAAATATGTCGGA
cfd68	7A	TTTGCAGCATCACACGTTTT	AAAATTGTATCCCCCGTGGT
wmc96	7A	TAGCAGCCATGCTTAGCATCAA	GTTTCAGTCTTTCACGAACACG
gwm473	7A	TCATACGGGTATGGTTGGAC	CACCCCCTTGTTGGTCAC
barc69	7A	AGGCGGCGGTCGTGGAACA	GCGTACCGAGAAGTGATCAAGAACAT
gwm332	7A	AGCCAGCAAGTCACCAAAAC	AGTGCTGGAAAGAGTAGTGAAGC
cfa2019	7A	GACGAGCTAACTGCAGACCC	CTCAATCCTGATGCGGAGAT
gwm344	7A	CAAGGAAATAGGCGGTAACT	ATTTGAGTCTGAAGTTTGCA
wmc673	7A	AGGAAACAAGAGTGTGTGTGGG	AGGAATAAGGACTCGCAAAACG

PUBLICATIONS

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Paper and poster presentations:

- Poster presented and abstract published in International Conference on Plant Genomics and Biotechnology: Challenges & Opportunities. October 26-28, 2005, **Ansari, M. J.,** Singh, K., Chhuneja, P. and Dhaliwal, H. S. Isolation and characterization of induced brittle mutants in diploid wheat (*Tritcum monococcum*). Raipur. (Abstract) p53. pp 200-201.

- Abstract published in international conference "Botany and Plant Biology 2007". July 7-11, (2007), **Ansari, M. J.,** Singh, K., Chhuneja, P. and Dhaliwal, H. S. Isolation and characterization of brittle culm mutants of diploid wheat (*Triticum monococcum*) affecting mechanical strength of wheat tissues by altering the composition and structure of secondary cell walls. Hilton chikago, Illinois (U.S.A.). (Abstract ID: 30).

- Poster presented in "National biotechnology Conferenc on Current Trends and Future Perspectives, 2-3 September 2006. **Ansari, M. J**, and Dhaliwal, H.S. Study of some EMS induced mutants in *T. Monococcum*" Department of Biotechnology, IIT Roorkee and Indian Federation of Biotechnologists (IFB-India).