

MOLECULAR CHARACTERIZATION OF THRESHABILITY GENES IN WHEAT

by

SHILPA SOOD

B.S., Punjab Agricultural University, Ludhiana, Punjab, India, 2000  
M.S., Punjab Agricultural University, Ludhiana, Punjab, India, 2002

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## Abstract

Threshability is an important agronomic trait in wheat as free-threshing forms facilitate mechanical threshing of grain. All wild relatives of wheat have tough glumes and are non-free-threshing, whereas most cultivated wheats have soft glumes and are free-threshing. Two genetic loci are known to govern the threshability trait in bread wheat. The *Q* gene located on chromosome 5AL and glume tenacity genes located on homoeologous group-2 chromosomes seem to interact to produce a free-threshing phenotype. Although, the *Q* gene was found to be a member of APETALLA 2 (AP2) class of transcription factors, the molecular nature of the tough glume genes remains unknown. In the present study, genetic and molecular characterization of two of the threshability genes in wheat was undertaken. 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<sup>m</sup> and 2D respectively. Comparative mapping of *sog* and *Tg* genes suggested their independent origins. The *sog* gene was mapped in a low-recombination region near the centromere on 2A<sup>m</sup>S. Genomic targeting using deletion bin mapped ESTs assigned the *Tg* gene to a 4.9 cM interval in the distal 16% of short arm of chromosome 2D. In order to find additional markers for fine-mapping the *Tg* gene, macrocolinearity between rice and wheat was explored in the *Tg* region. Although synteny between rice and wheat was found to be conserved in the distal region of chromosome 2DS, the genomic region encompassing the *Tg* gene in wheat showed some rearrangements relative to rice. Molecular characterization of ethyl methanesulfonate-induced free-threshing mutants in two different non-free-threshing backgrounds revealed point mutations as well as variable sized deletions at *Tg* locus. Targeting of *Tg* to the high-recombination gene-rich region in wheat and availability of several genomic resources from the present study will aid in the cloning and further characterization of this important agronomic gene.

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# CHAPTER 1 - REVIEW OF LITERATURE

## Importance of cereals: wheat

Wheat, maize, and rice together constitute the primary source of carbohydrate 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>). Bread wheat is the single largest traded crop with a global annual production exceeding 600 million tons equivalent to about 100 kg grain per capita (Dolezel et al. 2007). Wheat provides more than one-fourth of the calories consumed by humans (FAO, July 2007; <http://faostat.fao.org/>). Majority (>95%) of the wheat crop grown all over the world is common wheat which is used for making bread, cookies, pastries and noodles whereas rest is durum wheat, used for making pasta and other semolina products. Einkorn wheat and other non free-threshing wheats (emmer and spelt) are today relic crops of minor importance (Nesbitt and Samuel, 1996).

The annual wheat production trends in the world and in United States show that there has been an upward trend in wheat production in the last twenty years (Table 1.1), however this accounts for only 34% increase in production in the last twenty years at the world level. Today, the world's population is increasing at the most rapid rate ever. Two hundred people are being added to the planet every minute. Currently the world population is estimated to be 6.7 billion and it has been forecasted that by the year 2042, the world population will increase to almost nine billion people (<http://www.census.gov/ipc/www/idb/worldpopinfo.html>). To feed this growing population, it will require tremendous hike in the food production.

A marked increase in food production has already been experienced in the era beginning in the 1960s due to a widely known phenomenon of 'green revolution' which brought about a tremendous increase in production of food crops especially cereals. Between 1966 and 2000, the population of densely populated low-income countries almost doubled but the food production increased by 125% (Khush, 2001). This significant achievement in world food production was caused by applying advanced technology to the development of high-yielding varieties of cereals. In order to meet the challenges of growing food requirements, a similar green revolution is required again and advanced genetic and genomic technologies will play a major role towards

a more sustainable food production. Dissecting the molecular basis of important agronomic and domestication traits in major food crops like wheat will certainly increase our understanding of the molecular mechanisms underlying the key determination of quantitative and qualitative agronomic traits and to devise new methodologies for increasing yield of popular wheat cultivars.

## **Domestication**

As human societies have evolved, so have the plants in the human environment. The transition from hunting to gathering wild plants and their cultivation, involved increased interaction between humans and plants (Vaughan et al. 2007). About 10,000 to 11,000 years ago, a small group of humans made a shift from hunting and gathering lifestyle to cultivating plants for sustained survival (Tanno and Willcox 2006). The discovery of agriculture caused many changes in human culture-a phenomenon known as Neolithic revolution. The agrarian societies started a sedentary lifestyle which led to stratification in society and development of key technologies (Salamini et al. 2002). But most importantly, the ancient people began a unique plant breeding program of their own. They started to work with the existing variation in the plant germplasm pools and selectively identified and propagated desired variation leading to ultimate fixation of specific alleles of genes that we now know as the ‘domestication genes’. These ancient farmers transformed hundreds of wild plant species into domesticated crops including the most highly productive crops-rice, wheat and maize, on which human survival is dependent today (Doebley et al. 2006).

### ***Domestication traits: targets of selection***

Cereal crops, the world’s primary food source were domesticated from a diverse array of grass species. Despite the independent domestication that occurred in different continents- in 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 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 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 has 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 negative 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 the polyploid nature of the genome (Salamini et al. 2002).

### ***Genetic and molecular dissection of domestication traits***

Various developmental genetic studies have shown that single major genes can turn on complex developmental pathways and mutations in such genes can dramatically alter the phenotype

(Doebley 1993). However, domestication of major cereals involved not only the selection against major genes, but also the accumulation of quantitative trait loci (QTLs) with small genetic effects and in most cases these domestication genes were either known or putative transcription factors (Doebley et al. 2006).

So far in crop plants, eight major domestication genes have been cloned and five of them are quantitatively inherited (for review see Doebley et al. 2006). Among them the maize gene *tb1* was the first domestication QTL to be cloned (Doebley et al. 1997). *tb1* (teosinte branched 1), controls the complex differences in plant architecture between maize and its progenitor, teosinte. It belongs to the TCP family of transcription factors which function in the cell division cycle. The second domestication QTL to be cloned in maize was *tg1* (Wang et al. 2005). The maize domestication QTL *tg1*, which provides naked grains to maize (as opposed to the covered grains of teosinte), is a large effect QTL which segregated as a single Mendelian locus in an isogenic background (Doebley and Stec 1993; Dorweiler et al. 1993). *tg1* belongs to the SBP family of transcription factors and regulates a suit of characters including lignin and silica deposition in cell, organ growth and organ size (Dorweiler and Doebley 1997). The major domestication QTL, *fw2.2* contributing to a tremendous increase in fruit size (almost 30% higher than the wild relative) was cloned in tomato (Frary et al. 2000). Exact molecular function of *fw2.2* is unknown but it acts as a negative regulator of cell division during the development of tomato fruit. In rice, a total of five different QTLs were detected that collectively controlled the shattering trait. Among them, two major QTLs, *sh4* and *qSH1* influencing 68-69% of the genetic variation in grain shattering were isolated. These QTLs belonged to different transcription factor families, where *sh4* showed homology with the MYB3 class of transcription factors and *qSH1* belonged to homeobox containing transcription factors (Konishi et al. 2006; Li et al. 2006).

Some of the major genes that controlled domestication traits are genes that confer photoperiodic flowering in barley (Turner et al. 2005), wheat (Borner et al. 2002) and rice (Yano et al. 2001), the plant height genes in wheat (Borner et al. 1996), rice (Khush 2001) and maize (Sari-Gorla et al. 1999), shattering genes (Li and Gill 2006; Nalam et al. 2006), the free threshing gene *Q* (Faris et al. 2003, Simons et al. 2006) and the tough glume gene *Tg* (Jantasuriyarat et al. 2004) in wheat and seed color genes in rice and wheat (Sweeny et al. 2006; Flintham and Humphrey 1993). Some of these domestication genes have been cloned recently and their molecular nature has been elucidated. *Q* gene of wheat is one such example where it is the first

major domestication gene to be cloned (Simons et al. 2006). It affects a suite of characters including spike type, culm height, spike shattering and glume tenacity (Faris et al. 2003). *Q* is the major gene that gives the square head shape to the wheat spikes. It belongs to the AP2 family of transcription factors which regulates traits related to inflorescence structure and flowering (Simons et al. 2006). Another example is the red pericarp gene, *Rc* in rice where the red grain color is closely associated with seed shattering and dormancy in rice. All wild rice genotypes have red grains while the white grain color appeared to be associated with the domestication syndrome and remains under strong selection in most rice breeding programs. The *Rc* gene encodes a bHLH transcription factor and is a positive regulator of proanthocyanidin, a precursor of anthocyanin pigment (Sweeney et al. 2006). Another single domestication gene recently isolated is the naked caryopsis gene *nud* of barley, which belongs to the ethylene response factor (ERF) family of transcription factors and controls the fusion of hull (lemma and palea) with the caryopsis at maturity (Taketa et al. 2008). Most of domesticated barley types are covered/hulled whereas some cultivars are free-threshing where caryopses are naked (without hull). The *nud* gene has been predicted to function in a lipid biosynthetic pathway where, in naked barley the lack of a lipid layer blocks the hull adhesion and makes the caryopses free-threshing (Taketa et al. 2008).

Among these cloned domestication genes, seven out of eight encode transcription factors that regulate a gene by binding to their DNA. Although transcription factors represent only 5% of the genes in plant genomes (Xiong et al. 2005; Shiu et al. 2005), 88% of the cloned domestication genes belong to transcription factor families. Interestingly, all of these genes belong to a separate class of transcription factors; TCP (*tb1*), SBP (*tga1*), AP2 (*Q*), MYB3 (*sh4*), HOX (*qSH1*), bHLH (*Rc*), ERF (*nud*). The reason why a significant portion of domestication genes are transcription factors is due to the specific role of transcription factors in the plant developmental traits and morphological evolution (Doebley and Lukens 1998; Doebley 2006). Currently, there is limited knowledge available on the detailed functions of each of these domestication genes. Therefore, extensive functional analysis of the cloned genes will advance our understanding of the domestication syndrome and provide an opportunity to transfer knowledge for the domestication of new plants.

### *Genetic nature of varietal differences in plants*

In addition to genes controlling classic domestication traits, many genes controlling differences between varieties of a single crop or important agronomic traits have been clearly identified (for a complete list see Doebley et al. 2006). Some of these genes have been discovered as QTLs, whereas others segregated as Mendelian loci. For understanding morphological and structural traits, there are several examples among the cloned genes especially from rice. The grain number differences between rice varieties are controlled by the *grain number* gene (*gn1*), which encodes an *oxidase/dehydrogenase* that degrades the plant hormone cytokinin (Ashikari et al. 2005). The recently cloned grain weight QTL in rice, *GW2*, which controls rice grain width and weight, encodes a previously unknown RING-type protein with E3 ubiquitin ligase activity, which is known to function in the degradation by the ubiquitin-proteasome pathway (Song et al. 2008). The loss of *GW2* function increased cell numbers, resulting in a larger spikelet hull which accelerated the grain milk filling rate, further resulting in enhanced grain width, weight and yield (Song et al. 2008). Another QTL determining the grain width in rice, *qSW5* (QTL for seed width on chromosome 5), was isolated and a deletion in *qSW5* was shown to cause significant increase in sink size owing to an increase in cell number in the outer glume of the rice flower (Shomura et al. 2008). Although the exact biochemical function of *qSW5* remains to be identified, its importance in rice domestication was unequivocally established (Shomura et al. 2008).

The list of known genes contributing to physiological or biochemical differences between crop varieties is much longer (see the review by Doebley et al. 2006). Following are a few notable examples. The maize *yello1(y1)* gene encodes a kernel specific phytoene synthase that produces yellow kernels with high levels of carotenoids, a precursor for vitamin A synthesis (Palaisa et al. 2003). Grain texture in wheat is determined by a major polyploidization related locus called Hardness (*Ha*). While all the wild diploid species are soft grain textured, the grain hardness in polyploid wheats results from highly conserved mutations in the friabilin components puroindoline a and b of the *Ha* locus (Giroux and Morris 1997; Giroux and Morris 1998; Li et al. 2008). Genetic variation for the seed storage proteins called high molecular weight (HMW) glutenin subunits was responsible for the differences in the elasticity and ultimately on the bread making quality of wheat (Flavel et al. 1989). Most recently, an increase in grain protein content, zinc and iron content in wheat was found to be associated with an increase in the levels of a NAC transcription factor (NAM-B1) controlled by a quantitative trait

locus *Gpc-B1* (Uauy et al. 2006). Understanding the genetic, molecular and biochemical basis of important agronomic and domestication traits is important to elucidate the molecular and cellular pathways in which domestication gene products function and to use such information for further crop improvement.

### ***Domestication bottlenecks***

Agricultural practices of the early farmers led to reduced genetic diversity in domesticated crops. Because early farmers used only a limited number of individuals of the progenitor species for propagation in the next season, much of the genetic diversity in the progenitor was left unused. Moreover, during the domestication process ancient peoples selected and saved only a few seeds from the best plants to grow the next generation. This selective propagation also caused a major genetic bottleneck, which reduced genetic diversity throughout the genome (Doebley et al. 2006). This loss in genetic diversity mainly depends on the population size during the domestication period and the duration of that period (Eyre-Walker et al. 1998). Furthermore, all regions in the genome did not experience the same level of reduced genetic diversity. For genes that did not influence the favorable phenotypes (neutral genes), the loss in diversity of these genes was simply due to the small population size prior to the bottleneck and long duration of the bottleneck (Doebley et al. 2006). However, those genes that affected the desirable phenotypes directly, suffered a major loss in genetic diversity because only the plants carrying favorable alleles for such genes were advanced to the next generation and other alleles were simply eliminated from the population (Wright et al. 2005).

Domestication bottlenecks also resulted in reduced genetic diversity in the modern day wheat cultivars. However, the severity of the bottleneck varied with the ploidy and domestication level of different wheats. A study using 131 restriction fragment length polymorphism (RFLP) loci showed that gene diversity values in cultivated emmer wheat (*T. turgidum* subsp. *dicoccon*) were 58% less than those observed in the wild emmer (*T. turgidum* subsp. *dicoccoides*) across its entire geographic distribution (Luo et al. 2007). Nucleotide diversity in cultivated emmer was also reduced to almost half of its original levels (Dubcovsky and Dvorak 2007). Wheat being a self-pollinated crop, technically should have experienced higher losses in genetic diversity during the domestication as there is lower recombination and movement of alleles within the

population in self pollinators when compared to cross pollinator plant species. However, the loss in nucleotide diversity in self pollinated cultivated emmer was comparable to that observed in cross-pollinating maize and pearl millet (Gaut et al. 1993; Wright et al. 2005). On the other hand, several lines of evidence have indicated the gene flow between wild and cultivated emmer populations which can help explain the comparable loss in nucleotide diversity in wheat relative to maize or pearl millet (Dubcovsky and Dvorak 2007). Another study on nucleotide diversity at 21 loci in 101 individuals belonging to wild, domesticated, cultivated durum and bread wheats, found a loss of 69% in bread wheat and almost 84% in durum wheat (Haudry et al. 2007). This study also indicated that durum wheat experienced a more severe bottleneck during its evolution as compared to bread wheat. After the domestication of durum and bread wheats, selective events during the evolution of land races and modern breeding procedures contributed greatly to the loss in genetic diversity. However, due to recurrent gene flow between populations of hexaploid wheat and wild emmer, the genetic diversity in the A and B genomes of hexaploid wheat was restored to a greater extent as compared to durum wheats (Dubcovsky and Dvorak 2007; Haudry et al. 2007). On the other hand, a study on 18 loci in 321 wild and 92 domesticated lines of einkorn wheat, *T. monococcum* did not reveal any reduction in nucleotide diversity due to the multiple domestication events leading to the evolution of modern cultivated einkorn (Kilian et al. 2007).

Although there has been substantial loss in genetic diversity in the cultivated polyploid wheats, this scenario is most likely going to change as more and more wheat breeding programs are trying to incorporate genetic diversity from related wild progenitors and other species into the cultivated wheat gene pool.

## **Origin and history of wheat evolution**

### ***The evolutionary history of wheat***

The grass family, Poaceae, is of particular interest to humans as most people on earth rely on grasses including major crops like wheat, rice and maize for a major portion of their diet. The grass family contains approximately 10,000 species classified into 600 to 700 genera (Kellogg 2001). According to the data generated by the grass phylogeny working group (GPWG), 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 to which belong the familiar species like wheat, barley, oats and rye (Kellogg 2001). Wheat belongs to the tribe Triticeae along with other temperate grasses like barley oats and rye (Kellogg 1998). 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. ( $2n=2x=14$ ,  $A^m A^m$ ) and *T. urartu* Tumanian ex Gandilyan ( $2n=2x=14$ ,  $A^u A^u$ ) 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* (link) Thell whereas *T. urartu* exists only in the wild form. 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 and as wild species in the mountains surrounding Fertile Crescent. 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* (Zhuk) Zhuk ( $2n=4x=28$ ,  $A^t A^t GG$ ). There are two sub species of *T. turgidum*: *T. turgidum* subsp. *dicoccoides* (Korn.) Thell, which is the wild form and *T. turgidum* subsp. *durum* (Desf.) Husn (durum wheat), which is the cultivated sub species. *T. timopheevii* also has two sub species, *T. timopheevii* subsp. *armeniicum* (Jakubz.), the wild sub species and *T. timopheevii* subsp. *timopheevii*, the cultivated subspecies. At the hexaploid level, there are also two species, *T. aestivum* L. ( $2n=6x=42$ , AABBDD) (common wheat or bread wheat) and *T. zhukovskyi* Menab. & Ericz. ( $2n=6x=42$ ,  $AAA^t A^t GG$ ) but all hexaploid *Triticum* species are cultivated and therefore they don't have any wild forms (Van Slageren, 1994). Although in the wheat lineage, a number of species at all three ploidy levels have been cultivated, the cultivation now is restricted almost entirely to tetraploid durum wheat and hexaploid bread wheat.

### ***Genome donors and origin of wheat***

Early 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, in the studies based on variation in esterase enzyme

(Nishikawa 1984) and variation in repeated nucleotide sequences (Dvorak et al. 1988; Dvorak et al. 1993), it was shown that *T. urartu* contributed the A genome in 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* (Upadhyya and Swaminathan 1963).

*Aegilops speltoides* is considered as the most probable B genome donor of bread wheat and durum wheat. 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  $\alpha$ -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 hexaploid 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 most closely related to 'strangulata' gene pool in Transcaucasia, Armenia and SW Caspian Iran. Hence, the principle area of origin is 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 diverged 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).

### ***History of wheat domestication***

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 Israel, 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). Both wild and cultivated einkorn seed remains have been excavated in the nearby archaeological sites dating from 7500 to 6200 BC.

The important step in the evolution of modern polyploid wheat was the domestication of tetraploid emmer wheat (*T. turgidum* subsp. *dicoccum*) from its progenitor *T. turgidum* subsp. *dicoccoides*. The remains of cultivated emmer have been discovered at several archaeological sites in Syria dating to 7500 BC (Zohary and Hopf, 1993). Unlike the wild progenitor, domesticated tetraploid wheats have a non-brittle rachis but the early domesticated emmer wheat, *T. turgidum* subsp. *dicoccum*, has hulled seeds. The early free-threshing emmer wheat arose by mutation from the cultivated emmer wheats (*T. turgidum* subsp. *dicoccum*) and it was genetically more closely related to Mediterranean and Ethiopian subpopulations of present day domesticated emmer (*T. turgidum* subsp. *durum*) (Dubcovsky and Dvorak, 2007). Emmer was the most important crop in the Fertile Crescent until the early Bronze Age. The archaeological record shows that emmer was domesticated about 10,000 years ago (Willcox 1997). Earlier the

northern part of the Fertile Crescent was suggested as the site of emmer domestication (Ozkan et al. 2002), but the absence of wild emmer populations from many areas in that region demanded more precise identification of the site. After investigating more samples of wild emmer, Mori et al. (2003) concluded that emmer was domesticated in the Karakadag mountains, northeast of Gaziantep in Turkey. In a recent study, Luo et al. (2007) further pinpointed the emmer domestication to the Karacadag mountain region.

Bread wheat is the most recently domesticated wheat. It does not have any wild hexaploid progenitor and therefore it is a farming associated hybrid that has since become the world's leading crop. Bread wheat originated northwest of the Fertile Crescent, in the corridor extending from Armenia in Transcaucasia to the southwest coastal areas of the Caspian Sea in Iran (Dvorak et al. 1998). In this region, the *Ae. tauschii* var. *strangulata* is the predominant type, which evidently hybridized with cultivated emmer to produce *T. aestivum*. It has been speculated that several independent hybridization events might have occurred to create hexaploid wheat and that variation now constitutes the gene pool of bread wheat (Talbert et al. 1998). The first bread wheats may have looked similar to *T. aestivum* ssp. *spelta* found growing in Iran from which free-threshing types were derived by mutation (McFadden and Sears 1946). The European spelt wheats may have been derived secondarily from a hybridization involving *T. compactum* and emmer wheat (Ohtsuka 1998).

## **Polyploidy**

Polyploidy is an evolutionary process whereby two or more genomes are brought together into the same nucleus, usually by hybridization followed by chromosome doubling. As a result, the new polyploid is genetically isolated from its diploid progenitor (s) and a new species is formed. Polyploidy is a prominent speciation process in plants and has been significant in the evolutionary history of vertebrates and other eukaryotes as well. The importance of polyploidy was recognized early in 20<sup>th</sup> century and in the past decades many studies have focused on addressing many aspects of polyploidy speciation and genome evolution. The emerging view is that polyploidy not only provides redundant genes that can diverge in function, but is a condition that can accelerate genomic change. At least 70% of the angiosperm species have undergone a polyploidization event in their evolutionary history (Averett 1980). Many common crop plants,

including coffee, cotton, wheat, oat, tobacco and banana are allopolyploids. Many modern plant genomes harbor evidence of multiple rounds of past polyploidization events which were followed by massive silencing and elimination of duplicated genes (see review by Adams and Wendel 2005).

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 (see review by 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 change 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). The hardness locus (determining grain texture) in wheat demonstrates a beautiful example of evolution of genes and loci after polyploidization (Li et al. 2008). The *Ha* locus contains two puroindoline genes *Pin a* and *Pin b*, where all the diploid wheats contain both these genes and thus have soft textured grains. Upon polyploidization, many wheat lineages lost one or both copies of puroindoline genes independently to produce hard textured grains (Li et al. 2008). The hardness (*Ha*) locus and the high molecular weight glutenin (HMW-*Glu*) locus are two loci in wheat that have been well-studied with regard to elucidating the molecular mechanisms of polyploidy. Sequence analyses of these loci in wheat have pointed out the role of illegitimate recombination as one of the main factors in the evolution of wheat species (Gu et al. 2004; Chantret et al. 2005). Illegitimate DNA recombination occurs between DNA sequences that contain only a few identical nucleotides and thus in contrast with homologous recombination, which requires pairing of two copies of genes or long repeats, illegitimate recombination events require smaller sequence motifs and occur in any genomic region (Kirik et al. 2000; Gregory 2004). Illegitimate recombination leads to various genomic rearrangements such as deletions and inversions and is shown to counteract the effects of genome expansion by transposable element insertion or genomic duplication (Devos et al. 2002; Ma et al. 2004). Retrotransposon movement, point mutations, and epigenetic changes such as cytosine methylation represent additional forces that created gross and specific genomic rearrangements and helped to improve genomic stability and adaptability of the newly formed

allopolyploids such as wheat to facilitate their rapid and successful establishment in nature (Feldman et al. 1997; Shaked et al. 2001; Kashkush et al. 2003; Levy and Feldman 2004).

## Diploid inheritance

Although bread wheat is a polyploid, it is genetically stable and fertile mainly due to its diploid like behavior in chromosome pairing during meiosis. Hexaploid wheat (*T. aestivum*;  $2n=6x=42$ ; genome 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 (megabase) 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.

## **Wheat genetic and germplasm resources**

### ***Genetic stocks***

Common wheat is hexaploid and contains three related sub-genomes (A, B and D). Due to the polyploid nature, the wheat genome is highly buffered and tolerates structural and numerical changes to a greater extent than any diploid species. The plasticity of the wheat genome has allowed various cytogenetic stocks to be developed in wheat. These cytogenetic stocks have been a very important resource for doing the classical as well as molecular genetic analysis for identification of chromosomes or chromosomes regions affecting a specific trait. Lately, these invaluable genetic resources have made an even greater contribution for advancing genomic studies and gene discovery in wheat

### ***Aneuploid stocks***

Aneuploids are special genetic stocks where the plants have a chromosome number that is not exact multiple of the haploid number. The first aneuploid series for all chromosomes in wheat was established by Dr. Ernie Sears at the Missouri Agriculture Experiment Station. The commonly used aneuploid stock in wheat are monosomics, M (in a specific chromosome pair only one homologue is present,  $2n-1$ ; Sears 1954), nullisomic-tetrasomic, NT (one chromosome pair is missing and is compensated by another pair of homoeologous chromosomes,  $2n-2+4$ ; Sears 1966a), ditelosomics, Dt (one chromosome pair is represented by two telosomes for one arm and is missing the other arm,  $2n-2$ ; Sears 1966b) and double-ditelosomics, dDt (one chromosome pair is represented by a pair of telosomes for both arms,  $2n-4$ ; Sears 1978). These aneuploid stocks helped to group all 21 chromosomes of wheat into seven homoeologous groups where each group had one chromosome from the A, B and D genomes (Sears 1966b).

A complete set of NT lines for all 21 pairs of wheat chromosomes has been developed. However, stocks nullisomic for 2A and 4B are maintained as monosomic-tetrasomic lines

because these chromosomes contain major genes for male fertility. Therefore these stocks require cytological screening for identifying nullisomic 2A and 4B plants. Ditelosomic lines lack one pair of chromosome arms. Thirty four of the possible 42 Dt stocks are maintained in the ditelosomic condition, but Dts for arms 2AL, 4AS, 5AS, 2BS, 4BL, 5BS, 5DS, and 7DL are maintained as monotelosomic-ditelosomics because these chromosome arms contain genes essential for survival. Hence, cytological screening is necessary to select for Dts of these chromosome arms. Both NTs and Dts were extensively used for breeding, classical genetics and molecular biological analyses in wheat. The use of these stocks is more pronounced in molecular genetic analysis of wheat as they could be used to quickly locate the DNA markers or sequences to specific chromosomes or chromosome arms without the need for polymorphism.

### ***Deletion lines***

Another invaluable genetic resource in wheat is the set of gametocidal factor (*Gc*) induced chromosome deletion lines. Chromosome deletions in common wheat developed using gametocidal genes were first observed by Tsujimoto and Tsunewaki in 1985. Several *Gc* factors have been identified in different related *Aegilops* species (Tsujimoto and Tsunewaki 1983; Endo and Mukai 1988; Kota and Dvorak 1988; Tsujimoto and Tsunewaki 1988). Plants monosomic for the *Gc* chromosome in wheat produce two types of gametes. Gametes possessing the *Gc* chromosome are normal where gametes lacking the *Gc* chromosome undergo structural chromosome aberrations including deletions depending upon the type of *Gc* factors used (Nasuda et al. 1998). The *Gc* system has been used to develop wheat lines with terminal chromosome deletions (Tsujimoto and Tsunewaki 1988; Endo and Mukai 1988; Tsujimoto and Noda 1989). More than 400 chromosome deletion lines for all 21 chromosomes of wheat have been reported by Endo and Gill (1996). These sub-arm aneuploid stocks are an excellent tool for targeted physical mapping of any gene of interest to a small chromosome bin (Endo and Mukai 1988; Endo and Gill 1996).

### ***Mutant populations***

The ability to induce mutations has been a major driving force in genetics for the past 75 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.

### ***Genomic resources***

In modern day crop genetic research, the availability of advanced genomic tools and technology is a major advantage towards understanding and utilizing the sequence level variation within the

crop species for their agronomic improvement. As more and more plant genomes are being sequenced a plethora of useful information is becoming available for comparative genomics among related plant species and genera (The Arabidopsis Genome Initiative 2000; Goff et al. 2002; Yu et al. 2002; The International Rice Genome Sequencing Project 2005; Jaillon et al. 2007). In cereals where large genome and polyploid state hinders genome sequencing efforts, several large-insert libraries (primarily bacterial artificial chromosome, BAC based) have been developed to aid genomics research. In wheat, genome-specific BAC libraries are available for all ploidy levels (Lijavetzky et al. 1999; Cenci et al. 2003; Akhunov et al. 2005). Cosmid and BAC libraries have also been developed for isolating specific genes (Huang et al. 2003; Ling and Chen 2005).

Genome sequencing is important for understanding the molecular basis of phenotypic variation, accelerating the breeding process and improving the exploitation of genetic diversity to develop new crop varieties with increased yield and improved resistance to biotic and abiotic stresses. But the sequencing of large genomes is capital-intensive, and the assembly of genome sequence is greatly hampered by the presence of significant amount of repetitive DNA (up to 90% in wheat genome, Paux et al. 2006). Therefore it requires development of specific genomic resources to aid the genomic research in such large genome crops as wheat. Due to the polyploid nature of wheat, it can easily tolerate the loss or gain of chromosome or chromosome segments. This has come as an advantage where a technique called ‘chromosome flow sorting’ has been exploited to isolate specific chromosomes and even chromosome arms (Vrana et al. 2000; Kubalaková et al. 2002) by using specific cytogenetic stocks including translocations, deletions, alien additions and ditelosomics (Doležel et al. 2004; Doležel et al. 2007) and develop chromosome-specific BAC libraries (Safar et al. 2004; Janda et al. 2004; Kubalaková et al. 2005; Janda et al. 2006). Such libraries are also available for related cereals like barley and rye (Suchanková et al. 2006; Simková et al. 2008). Recently by using the wheat chromosome 3B specific BAC library resource (Safar et al. 2004), a physical map of the largest wheat chromosome (1 gigabase) has been completed (Paux et al. 2008). This success has paved the way to develop and utilize these highly specialized genomic resources for aiding genomics research and finally genome sequencing of large genome food crops like wheat.



# Molecular mapping in plants

## *Genetic Mapping*

### *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. Various characteristics and usefulness of these molecular marker systems have been summarized in Table 1.2.

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

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).

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 (Tenailon et al. 2001), a recent NSF (national science foundation) funded SNP development project in wheat has already generated 17,174 genome-specific primers (as of May 2006) from EST unigenes and a wheat SNP database has been established to provide information about the primer sequences and the genetic map location of different SNP markers (<http://wheat.pw.usda.gov/SNP/new/index.shtml>). With the increased availability of genome sequence data in wheat, it will become much easier and cost-effective to generate more SNP markers.

### ***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., F<sub>2</sub>, 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>).

## ***Physical Mapping***

### ***Aneuploid stocks***

Aneuploid stocks have been widely used in classical genetic and recent molecular mapping experiments. By using these stocks many agronomic genes have been placed onto specific chromosomes and chromosome arms in wheat (Kerber and Rowland 1974). In the NSF wheat-EST mapping project, the nullisomic-tetrasomic and ditelosomic lines were used to assign individual ESTs to a specific chromosome and chromosome arm ([http://wheat.pw.usda.gov/NSF/progress\\_mapping.html](http://wheat.pw.usda.gov/NSF/progress_mapping.html)). In the genomics era, the aneuploid stocks are being used for the development of chromosome and chromosome arm-specific libraries of polyploid wheats (Janda et al. 2004; Kubalakova et al. 2005; Janda et al. 2006), which are highly useful for conducting wheat genomics research and for developing physical maps of individual chromosomes (Paux et al. 2008).

### ***Deletion mapping***

Deletion lines have been used in the development of cytologically-based physical maps of the 21 chromosomes of wheat (Werner et al. 1992; Gill et al. 1993; Gill et al. 1996a; Gill et al. 1996b; Hohmann et al. 1994; Delaney et al. 1995a; Delaney et al. 1995b). A subset of 109 deletion lines described by Endo and Gill (1996) was used to develop a highly comprehensive physical map of wheat, containing 16,099 mapped expressed sequence tags (EST) (Qi et al. 2003; Qi et al. 2004; <http://wheat.pw.usda.gov/NSF/data>). The information gained out of these lines helped wheat geneticists to discover novel chromosomal structural changes in wheat

(Miftahudin et al. 2004; Qi et al. 2004; See et al. 2006) and the non random distribution of recombination and gene space along the chromosomes (Gill et al. 1993; Faris et al. 2000; Akhunov et al. 2003). Additionally, deletion lines have been a valuable resource for targeting specific genes to physical sub-chromosomal regions in the map-based gene cloning experiments (Faris and Gill 2002; Faris et al. 2003; Dieguez et al. 2006; Lu and Faris 2006; Mateos-Hernandez et al. 2006) and were used extensively for gene discovery and genome analysis in wheat (Gill et al. 1993; Gill et al. 1995; Faris and Gill 2002; Faris et al. 2003; Akhunov et al. 2003; Qi et al. 2004). The deletion lines were also used in physical mapping of wheat microsatellite markers (Sourdille et al. 2004), which has further helped researchers target their genes of interest to specific genomic regions.

### ***Chromosome banding and in situ hybridization***

Chromosome banding methods, especially C-banding, permitted a fast and reliable identification of all 21 chromosome pairs of the A, B, and D genomes of wheat and was also used to identify many chromosomes from related species (Gill and Kimber 1974; Gill et al. 1991; Friebe and Larter 1988; Lukaszewski and Gustafson 1983; Friebe and Gill 1996). By combining mitotic chromosome measurements, C-banding offered a reliable estimation of the physical size of the chromosome deletions in Chinese Spring (Endo and Gill 1996) wheat.

The in situ hybridization technique allowed the localization of DNA sequences directly on chromosomes in cytological preparations by using the isotope-labeled probes. Non isotopic in situ hybridization was developed later in plants (Rayburn and Gill 1985). Since then, the in situ hybridization technique has been widely used with varied modifications in the probes and with different levels of resolution.

Genomic in situ hybridization (GISH): GISH uses genomic DNA as a probe and it has varied uses in analyzing chromosome structure, genome evolution, and divergence of allopolyploid species (Bennett et al. 1993; Mukai et al. 1993; Jiang and Gill 1994c). GISH followed by chromosome banding provides a direct and precise method of physical mapping the introgressed alien chromosome segments to a specific wheat chromosome (Friebe et al. 1992; Jiang and Gill 1993).

Fluorescent in situ hybridization (FISH): FISH uses specific DNA probes with fluorochromes for signal detection (Pinkel et al. 1986). Multicolor FISH can allow simultaneous identification of physical order of two or more probes on a chromosome (Mukai et al. 1993).

FISH has been used successfully to determine the physical location and distribution of dispersed or tandemly repetitive DNA sequences on individual chromosomes (Bedbrook et al. 1980; Rayburn and Gill 1985; Lapitan et al. 1986; Anamthawat-Jonson and Heslop-Harrison 1993; Jiang and Gill 1994a) and physical location of multicopy gene families such as the 5S and 18S-26S ribosomal genes (Skorupska et al. 1989; Mukai et al. 1990; Leitch, and Heslop-Harrison 1992; Badaeva et al. 1996). BAC-FISH is a FISH technique where genomic DNA is cloned in a BAC vector and is used as a probe. BAC-FISH has been used to physically map specific DNA sequences and identify individual chromosomes in plants with small genomes, such as rice (Jiang et al. 1995), sorghum (Gomez et al. 1997) and Arabidopsis (Jackson et al. 1998). In wheat, BAC-FISH has been successfully used to identify specific chromosome landmarks (Zhang et al. 2003) and for simultaneous identification of all three genomes of wheat (Zhang et al. 2004). Fiber FISH is a technique which uses extended DNA fibers for physical mapping a specific sequence at a very high resolution (Heng et al. 1992; Parra and Windle 1993). However its use in wheat is yet to be demonstrated.

### ***Large-insert clone contigs***

Physical contig mapping is the arrangement of large-insert clones (Yeast artificial chromosomes, YACs; Bacterial artificial chromosomes, BACs; Plant artificial chromosomes, PAC; cosmids) in a linear array that represents the DNA sequence along the chromosome. These days cosmid clones are mainly being used to develop contigs in smaller genome organisms or in specific regions of interest (Aleksenko et al. 2001; Vanbleu et al. 2004) and although YACs have been extensively used to develop physical maps of individual chromosomes and genomes previously (Zachgo et al. 1996), lately they are being used in combination with more stable BAC vectors (Krzywinski et al. 2004) due to the difficulties in the handling and maintenance of YAC vectors in the lab. More recently, BACs have become the vector of choice for developing large insert clone libraries to construct physical maps mainly because of their large insert capacity (100-300 kb), a low rate of chimera formation, high efficiency of long insert cloning and recovery and stable maintenance of the insert (Woo et al. 1994; Wang et al. 1995).

Genome-wide physical maps provide powerful tools and infrastructure for advanced genomics research of important organisms. They are not only crucial for large-scale genome sequencing (The Arabidopsis Genome Initiative 2000; The International Human Genome Sequencing Consortium 2001; International Rice Genome Sequencing Project 2005), but also

provide powerful platforms required for many other aspects of genome research, including targeted marker development, efficient positional cloning, and high-throughput EST mapping (Zhang and Wu 2001). Whole-genome physical maps have been constructed for various plants e.g. Arabidopsis (Mozo et al. 1999; Chang et al. 2001), rice (Tao et al. 1998; Chen et al. 2002), and soybean (Wu et al. 2004).

In wheat, a NSF funded physical mapping project was initiated with the aim to construct the physical maps of the wheat D genome and to directly estimate the gene distribution across chromosomes in a large genome (for details see <http://wheat.pw.usda.gov/PhysicalMapping/projectdescript.html>). The wheat D-genome was chosen due to its small size (4.5 pg/c) among all three genomes and also because the D-genome of wheat is completely equivalent with the genome of its diploid progenitor, *Aegilops tauschii*, and thus, making it possible to benefit from combined powers of diploid and polyploid genetic analyses. The fingerprinting of BAC clones using SNaPshot labeling kit and sizing of fragments using capillary electrophoresis facilitated high-throughput physical mapping of the D-genome (Luo et al. 2003). Fingerprinting of the wheat D genome using this method has resulted in 13,647 contigs and 4,730 singltons associated with 267,451 BAC/BIBAC clones anchored using ~2000 markers (<http://wheat.pw.usda.gov/PhysicalMapping/>). The same technique was used to fingerprint chromosome 3B specific BAC contigs in wheat, which has recently resulted in the first physical map of an individual chromosome in wheat (Paux et al. 2008).

### ***Comparative mapping***

Comparative maps reveal a common order of genes and markers over large chromosomal tracts among related organisms. Grass genomes show a high levels of similarity in gene content and order even after 65 million years of divergence (Paterson et al. 1995; Kellogg 2001) Early comparative mapping studies using RFLP markers have revealed extensive colinearity among the genomes of cereal crops such as rice, wheat, barley, rye, oat, maize, sorghum, and others (Ahn et al. 1993; Moore et al. 1995; Van Deynze et al. 1995; Devos & Gale 2000). Availability of whole genome sequence of rice (International Rice Genome Sequencing Project 2005) has allowed physical and genetic mapping of various molecular markers in different cereal crops (Devos and Gale 1997; Devos and Gale 2000). Due to the highly conserved synteny among

different cereals, whole genome or chromosome specific comparative maps have been developed for wheat, maize, and sorghum anchored to the sequenced genome of rice (Sorrells et al. 2003; Buell et al. 2005; Salse et al. 2004).

Although the macrocolinearity (at chromosome level) was found to be quite conserved in different comparative mapping studies between wheat and rice, the microcolinearity (at the sequence level) showed variation for different genomic regions. Whereas many studies reported the occurrence of multiple rearrangements in gene order and content (Bennetzen 2000; Feuillet and Keller 2002; Li and Gill 2002; Sorrells et al. 2003; Francki et al. 2004; Lu and Faris 2006) others indicated good levels of conservation in syntenic regions between wheat and rice (Yan et al. 2003; Chantret et al. 2004; Distelfeld et al. 2004; Mateos-Hernandez et al. 2005; Valarik et al. 2006; Griffiths et al. 2006; Kuraparthi et al. 2008). Various small genic rearrangements within the rice genome with respect to another cereal genome can complicate the colinearity analysis but it does not negate the use of rice as a model for large cereal genomes (Bennetzen and Ma 2003). Nonetheless, the rice genome sequence has been successfully used as tool for map-based cloning of genes in wheat in several instances (Yan et al. 2003; Distelfeld et al. 2004; Uauy et al. 2006). Additionally the collinear regions of rice have also been used to generate markers for saturation and high-resolution mapping of target genes in wheat (Liu and Anderson 2003; Distelfeld et al. 2004; Valarik et al. 2006).

Recently a wild cereal, *Brachypodium* has been proposed as new model plant for structural and functional genomics of temperate cereals because of its small genome and the phylogenetic position between rice and *Triticeae* crops (Bossolini et al. 2007). *Brachypodium distachyon* and *B. sylvaticum* are wild grasses that have small genomes (400 Mb) and short reproduction cycle (Draper et al. 2001; Foote et al. 2004). Whereas rice and *Triticeae* lineage diverged almost 50 million years ago (Paterson et al. 2004), *Brachypodium* and wheat diverged about 35-40 million years ago (Bossolini et al. 2007). Thus wheat and *Brachypodium* are considered to be more closely related as compared to wheat and rice. The availability of various genetic and genomic resources such as BAC libraries (Foote et al. 2004; Gu et al. 2006; Hasterok et al. 2006), more than 20,000 ESTs from *B. distachyon* (Vogel et al. 2006) and recent availability of the genome sequence of *Brachypodium distachyon* (<http://www.brachypodium.org/>) has made it an attractive model species for colinearity studies in wheat. However, similar to the case with wheat and rice, the microcolinearity studies involving



Brachypodium and wheat have found mixed results. A high level of macrocolinearity has been observed between Brachypodium and wheat at the *Ph1* region (Griffiths et al. 2006) and *Lr34* region (Bossolini et al. 2007). But at the micro level, some studies have reported perfect gene level conservation between these two species, for example at *Ph1* region of wheat (Griffiths et al. 2006) while others found the sequence level conservation between wheat and rice to be better than wheat and *Brachypodium* such as the *Q* gene region of wheat genome (Faris et al. 2008). Thus the *Brachypodium* genome will definitely aid in the comparative genomic studies involving large genome cereals like wheat and barley but it certainly cannot replace the need for availability of independent genome sequences for these crops.

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**Table 1.1** Wheat production trend in the world and in U.S. over the last twenty years

<b>Market year</b>	<b>World production (million bushels)</b>	<b>U.S. production (million bushels)</b>	<b>U.S. share (percent)</b>
1988	18189.900	1812.200	9.96
1989	19592.628	2036.630	10.39
1990	21627.311	2729.764	12.62
1991	19961.939	1980.155	9.92
1992	20665.985	2466.789	11.94
1993	20511.294	2396.425	11.68
1994	19205.276	2320.990	12.09
1995	19736.370	2182.723	11.06
1996	21358.935	2277.375	10.66
1997	22413.957	2481.450	11.07
1998	21687.828	2547.331	11.75
1999	21559.188	2295.563	10.65
2000	21417.872	2228.175	10.40
2001	21424.449	1947.453	9.09
2002	20896.442	1605.884	7.68
2003	20350.063	2344.763	11.52
2004	22991.936	2158.252	9.39
2005	22812.369	2104.680	9.23
2006	21910.421	1812.053	8.27
2007	22445.704	2066.723	9.21
2008*	24849.073	2462.416	9.91

\* Production values for year 2008 represent the projected values

Source: USDA, Foreign Agricultural Service, Production, Supply, and Distribution Database

<http://www.fas.usda.gov/psdonline/psdHome.aspx>

**Table 1.2** Characteristics and usefulness of molecular marker types for wheat molecular genetics

<b>Use</b>	<b>RFLPs</b>	<b>RAPDs</b>	<b>SSRs</b>	<b>AFLPs</b>
Varietal fingerprinting	Useful	Somewhat useful	Very useful	Very useful
Genetic diversity	Useful	Not useful	Useful	Useful
Gene tagging	Useful	Useful	Useful	Very useful
QTL mapping	Useful	Somewhat useful	Useful	Useful
MAS	Not useful	Not useful	Useful	Somewhat useful
Comparative mapping	Useful	Not useful	Not useful	Not useful
Principle	Endonuclease restriction; southern blot; hybridizations	DNA amplification with random primers	Amplification of simple sequence repeats using specific primers	Endonuclease restriction; use of adapters and specific primers
Types of probes/primers	GenomicDNA, cDNA	Random 9- or 10-mer oligonucleotides	Specific 16- to 30-mer primers	Specific adapter and selective primers
Type of polymorphism	Single base changes; insertions/deletions	Single base changes; insertions/deletions	Change in length of repeats	Single base changes; insertions/deletions
Genomic abundance	High	Very high	Medium	Very high
Level of polymorphism	Medium	Medium	High	High
Inheritance	Co-dominant	Dominant	Co-dominant	Dominant



<b>Use</b>	<b>RFLPs</b>	<b>RAPDs</b>	<b>SSRs</b>	<b>AFLPs</b>
Number loci detected	3-9	1-10	1-3	70-140
Need for sequence information	No	No	Yes	No
Technical difficulty	Medium	Low	Low	Medium/high
Reliability	High	Intermediate	High	Medium/high
Quantity DNA required	10-20 mg	10-50 ng	50-100 ng	100-1000 ng
Use radioisotopes	Yes	No	No	Yes/No
Start-up costs	Medium	Low	Medium	High
Developmental Costs	Medium	Low	High	Medium/high

## CHAPTER 2 - NON-CORRESPONDENCE OF THRESHABILITY GENES SOFT GLUME (*SOG*) AND TENACIOUS GLUME (*TG*) OF WHEAT

### Abstract

Threshability is an important crop domestication trait. The wild wheat progenitors have tough glumes enveloping the floret that make spikes difficult to thresh, whereas cultivated wheats have soft glumes and are free-threshing. In hexaploid wheat, the glume tenacity gene *Tg* along with the major domestication locus *Q* control threshability. The *Q* gene was isolated recently and found to be a member of the AP2 class of transcription factors. Only a few studies have reported on the tough glume trait. Here we report comparative mapping of the soft glume (*sog*) gene of diploid *Triticum monococcum* L. and tenacious glume (*Tg*) gene of hexaploid *T. aestivum* L. using chromosome-specific SSR and RFLP markers. The *sog* gene was flanked by *Xgwm71* and *Xbcd120* in a 7.4-cM interval on chromosome 2A<sup>m</sup>S of *T. monococcum* whereas *Tg* was targeted to a 8.1-cM interval flanked by *Xwmc503* and *Xfba88* on chromosome 2DS of *T. aestivum*. Deletion bin mapping of the flanking markers assigned *sog* close to the centromere on 2AS, whereas *Tg* was mapped to the most distal region on 2DS. Both 2AS and 2DS maps were collinear ruling out the role of chromosome rearrangements for their non syntenic positions. Therefore *sog* and *Tg*, are not true orthologues suggesting the possibility of a diverse origin.

## Introduction

The crop domestication process started ca 5,000 to 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. The cultivated plants can be easily distinguished from their progenitors based on a suite of characters known as ‘domestication syndrome’ (Hammer 1984 as cited by Doebley et al. 2006). Crop plants usually have larger sized grains, increased apical dominance, loss of natural seed dispersal, seed dormancy, photoperiod insensitivity and synchronized flowering as compared to their wild progenitors (Doebley et al. 2006; Dubcovsky and Dvorak 2007). In grasses, mutations at genes controlling several spike-related traits such as rachis fragility (brittle or non brittle rachis) and glume tenacity (hulled or free-threshing) were selected during domestication and subsequently became fixed in the cultivated populations due to positive selection pressure (Tanksley and McCouch 1997).

The ploidy levels of domesticated wheat species include diploid (*Triticum monococcum*,  $2n=14$ ,  $A^m A^m$ ), tetraploid (*T. turgidum*,  $2n=28$ , AABB) to hexaploid (*T. aestivum*,  $2n=42$ , AABBDD). Diploid and tetraploid wheat were independently domesticated from wild relatives ca 13,000 and ca 9,000 years ago, respectively (Nesbitt and Samuel 1995). Hexaploid wheat originated from hybridization of domesticated tetraploid wheat and *Aegilops tauschii* coss. ( $2n=14$ , DD) ca 8,000 years ago (Nesbitt and Samuel 1995). Thus, *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 a brittle rachis leading to

shattering of either the whole spike or individual spikelets (Li and Gill 2006). The brittle rachis trait in ancestral wild forms is conferred by a dominant *Br* allele whereas a mutant *br* allele at the same locus produces a non brittle rachis. The first cultivated wheats had a non-brittle rachis but also tough glumes and were non-free-threshing. The genetic system 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), and the major modifier gene for domestication related traits (*Q* gene) is located on the long arm of chromosome 5A (MacKey 1966). Subsequent mutations at these loci during domestication led to the modern free-threshing wheats.

The major domestication gene *Q* confers the squarehead and free-threshing phenotype in cultivated polyploid wheats (Faris et al. 2002). Additionally, *Q* has a pleiotropic effect on several other domestication related traits including glume shape, rachis fragility, spike length, culm height and glume toughness (MacKey 1966; Muramutsu 1986; Jantasuriyarat et al. 2004; Faris et al. 2005). Expression of the above mentioned traits also, is influenced by genes other than *Q* depending on genetic background (Muramutsu 1986; Luo et al. 2000) thereby suggesting that *Q* could be part of a complex interaction pathway involving different domestication genes in wheat. *Q* is incompletely dominant to the *q* allele and plants with a *Qq* genotype have a spike morphology that is intermediate between speltoid and squarehead. Recently, *Q* has been identified as a member of the APETALA2 family of plant-specific transcription factors (Simons et al. 2006). This gene family is known to regulate a wide range of developmental traits in plants especially those related to inflorescence structure and flowering.

While working on mapping traits derived from *Ae. tauschii* in synthetic allohexaploid X cultivated wheat crosses, Kerber and Dyck (1969) uncovered a genetic system different from the *Q* gene that influenced the free-threshing character. They found that a synthetic allohexaploid produced by crossing a free-threshing tetraploid with hulled *Ae. tauschii* is non-free-threshing despite the presence of the *Q* gene. Later, Kerber and Rowland (1974) showed the D-genome effect was derived from an incompletely dominant allele at the locus *Tg* (tenacious glumes). This gene, derived from *Ae. tauschii* epistatically inhibited the expression of *Q* in the synthetic wheat and, hence, the plants were non-free-threshing. Kerber and Rowland (1974) further concluded that the free-threshing character in hexaploid wheat originated by independent mutations at the *q* and *Tg* loci. Thus, the dominant allele *Q* as well as the recessive allele *tg* must be present in

hexaploid wheats for the complete expression of the free-threshing character. Initial mapping studies using telocentric mapping placed *Tg* on short arm of chromosome 2D (Rowland and Kerber 1974). Molecular mapping of loci influencing the free-threshing trait in the International Triticeae Mapping Initiative (ITMI) recombinant inbred line (RIL) population identified two major quantitative trait loci (QTL) on chromosome 2DS and 5AL (Jantasuriyarat et al. 2004). The QTL mapped in a region on 2DS corresponding to *Tg*, and the QTL on 5AL corresponded to the *Q* gene. This study further suggested that *Tg* has a more pronounced effect on threshability than *Q* (Rowland and Kerber 1974). Nalam et al. (2007) used the ITMI RIL population and reported the presence of two coincident QTL affecting free-threshing habit (threshability and glume tenacity) on short arm of chromosome 2D of wheat and suggested the likely presence of a paralog of *Tg*. This study localized the *Tg* QTL to a 12-cM interval flanked by microsatellite markers using an F<sub>2</sub> population of Chinese Spring and CS-*Ae. tauschii* 2D substitution line.

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. *Tg2* the putative ortholog of *Tg*, is located on 2BS. In tetraploid wheats, *Q* and *Tg2*, along with some minor genes, are required for the complete expression of the free-threshing trait.

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. *T. monococcum* is cultivated only as a relic crop of minor economic importance in some parts of the world. 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). However this free-threshing 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). Taenzler et al. (2002) used *T. sinskajae* to locate the factors controlling glume tenacity in einkorn wheat and identified a single, recessive gene *sog* (soft glume) on the short arm of chromosome 2A<sup>m</sup>.

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. 2004), however, none of the studies have tested it experimentally. In the present study, we characterized *Sog* and *Tg*, two of the homoeologous chromosome group-2 loci influencing threshability in diploid and hexaploid wheat populations, respectively, and investigated the orthologous relationship between these genes.

## Material and Methods

### *Plant material*

*Triticum monococcum* subsp. *monococcum* is non-free-threshing cultivated diploid wheat. Dhaliwal et al. (1987) mutagenized a *T. monococcum* subsp. *monococcum* accession TA4342-95 using ethyl methanesulfonate (EMS) at Punjab Agricultural University, Ludhiana, India. One free-threshing mutant, Tm-9, was obtained. We crossed Tm-9 with *T. monococcum* subsp. *aegilopoides* accession TA4342-96 (non-free-threshing) and developed an F<sub>2</sub> population of 118 individuals. In the present study, we used the Tm-9 X *T. monococcum* ssp. *aegilopoides* population for mapping the soft glume (*sog*) gene. Progeny testing was done by evaluating 12 F<sub>3</sub> plants in each F<sub>2</sub> family to identify the heterozygous individuals.

At the same time an allelism experiment was conducted to find out if Tm-9 and the *T. sinskajae* (*sog*) reported by Tanezler et al. (2002) have the same recessive free-threshing allele. Tm-9 mutant was crossed as a female with the *T. sinskajae* (Taenzler et al. 2002) in fall 2007 at Wheat Genetic and Genomic Resources Center (WGGRC), Manhattan, KS. The F<sub>1</sub> obtained from this cross had wider glumes, short awns and was free-threshing (Fig. 2.1) suggesting Tm-9 presumably harbors the same free-threshing allele (*sog*) as *T. sinskajae*. However, to confirm this result further, segregation of threshability is being studied in the F<sub>2</sub> population.

Although the *Tg* gene in hexaploid wheat was contributed by *Ae. tauschii*, related loci in A and B genomes in hexaploid background also can influence the free-threshing phenotype. Therefore, a population segregating only for the D-genome in hexaploid wheat was developed for mapping the *Tg*. The extracted tetraploid of Canthatch (AABB, 2n=28), was developed by E. R. Kerber (Kerber 1964), is maintained at the WGGRC, Manhattan, KS. An allohexaploid

(TA3419) was synthesized by crossing the extracted tetraploid Canthatch (TA3358) as a female with *Aegilops tauschii* var *meyeri* (TA1599) at WGGRC. The resulting F<sub>1</sub> embryos were haploid (ABD genome) and thus required embryo rescue. Therefore young caryopses were harvested from the crossed spikes, embryos were dissected out and cultured *in vitro* on Murashige and Skoog based tissue culture media (Table 2.1). Plants were regenerated and transplanted in small pots. At the tillering stage (2-3 tillers) the haploid plants were treated with colchicine (0.1%) to double the chromosome number using standard methods (Sood et al. 2003). After the colchicine treatment, old tillers died out but the new tillers had doubled chromosome sectors (AABBDD, 2n=42). Some seeds were set on these plants. These synthetic allohexaploids were grown and phenotyped at maturity for threshability. The synthetic plants had extremely tenacious glumes and were non-free-threshing as expected. This non-free-threshing synthetic (TA3419) and was crossed with free-threshing cultivar Canthatch (TA2987) to develop an F<sub>2</sub> population of 103 individuals. This population segregated for various morphological traits including threshability, foliage waxiness and coleoptile color and was used for mapping *Tenacious glume* (*Tg*) gene. Progeny testing was done using 12 to 16 F<sub>3</sub> plants in each F<sub>2</sub> family.

A set of 11 group-2 deletion lines of Chinese Spring wheat (Endo and Gill 1996) were used to physically localize the threshability genes in a specific deletion bin. Deletion lines used in this study included 2AS-5, 2AL-1, 2BS-1, 2BS-4, 2BS-3, 2BL-2, 2DS-1, 2DS-5, 2DL-3 and two newly characterized deletion lines 2BL-6/2AS and 6BL-5/2BS (Qi et al. 2003).

Individual plants were grown in 1 gallon size (6" top diameter, 7" height and 5" bottom diameter) round pots (Hummert International, Earth City, MO) filled with Scotts Metro Mix 200 (Hummert International, Earth City, MO) in the greenhouse with supplemental lighting for 16 h day and 8 h dark period at temperatures ranging from 18 to 23 °C.

### ***Phenotypic analysis***

Threshability data of individual spikes was recorded after harvest. In the diploid wheat population, spikes of the F<sub>2</sub> plants and their F<sub>3</sub> progenies were scored for glume shape and threshability. Each spike was hand threshed and scored as either non-free- or free-threshing. The method described by Kerber and Dyck (1969) was used for testing threshability. Each spike was placed in a plastic tray with a corrugated rubber bottom lining and rubbed with a wooden block covered with the same rubber material. The threshability of each F<sub>2</sub> plant in the hexaploid

population and the F<sub>3</sub> progenies also was determined by hand rubbing the individual spikes. The *Tg* gene is incompletely dominant (Kerber and Rowland 1974) and, therefore, all the heterozygotes show intermediate threshability. The F<sub>2</sub> genotype was verified by assaying the phenotypes of 12-16 F<sub>3</sub> plants from each F<sub>2</sub> plant.

### ***Molecular marker analysis and genetic mapping***

About 40-60 mg of fresh leaf tissue from the F<sub>2</sub> populations (Tm-9 X *T. monococcum* subsp. *aegilopoides* and TA2987 X TA3419) and parents was used for DNA extractions (Kuraparthi et al. 2007). Extracted DNA was dissolved in TE buffer and quantified using NanoDrop ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Because the chromosome location of *sog* and *Tg* is known (Rowland and Kerber 1974; Jantasuriyarat et al. 2004; Taenzler et al. 2002), chromosome 2A- and 2D-specific microsatellite markers were used for initial mapping based on previously reported maps (Röder et al. 1998; Gupta et al. 2002; Guyomarc'h et al. 2002a and b; Pestsova et al. 2000; Somers et al. 2004; Sourdille et al. 2004). Polymerase chain reaction (PCR) amplifications were performed in 20 µl reactions containing 2.5 µl of 10X PCR buffer, 1.75 µl magnesium chloride (25 mM), 2.0 µl dNTPs (2.5 mM of each dNTP), 1.0 µl each of forward and reverse primer (10 pM/µl), 0.15 µl of *Taq* DNA polymerase (Promega, WI, USA) and 75 ng of DNA in a PTC-200 thermal cycler (MJ research, Waltham, MA, USA). PCR amplification consisted of 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 50, 52, 55 or 60°C depending on the individual primer for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. Amplified PCR products were resolved in 2.5% high-resolution agarose (GenePure HiRes agarose, ISC BioExpress, Kaysville UT, USA) gels with 1X Tris-borate EDTA (TBE) buffer and visualized by ethidium bromide staining. Some of the PCR products were resolved in 6.5% KB<sup>Plus</sup> Gel Matrix (LI-COR) in a LI-COR 4200 DNA sequencer (LI-COR Biosciences, Lincoln, NE, USA) as per the manufacturer's instructions. PCR reactions analyzed in the LI-COR DNA Analyzer were done in a reaction volume of 10 µl. All the forward primers were added with a M13 tail sequence (Schuelke 2000). Fluorescence-labeled M13 primers were used for detection of PCR products in the LI-COR sequencer.



RFLP clones previously mapped to the short arm of the group-2 chromosomes (Devos et al. 1993; Dubcovsky et al. 1996; Nelson et al. 1995, [http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO\\_2.html](http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO_2.html)) were used for physical and genetic mapping of *sog* and *Tg*. All techniques of restriction enzyme digestion, gel electrophoresis, Southern transfer, probe labeling and filter hybridizations were performed as described by Kuraparthy et al. 2007. About 20 µg of DNA was digested with 40 units of an endonuclease in the presence of an appropriate buffer, BSA (0.01 v/v) and RNAase (0.01 v/v) for a total volume of 35 µl. After 16 h incubation at 37°C, the digestion reactions were stopped by adding 8 µl of gel-loading buffer (0.25% bromophenol blue, 30% glycerol in water). Digested product was then resolved in a 0.8% agarose gel made using 1 X TBE (Tris, boric acid and EDTA) on a horizontal gel apparatus for 18 h at 23V. DNA was transferred to Hybond N+ membranes (Amersham Biosciences, GE Healthcare, USA) according to the standard protocols of Sambrook et al. (1989). Prehybridization was done at 65°C for 14–16 h in a solution containing 5 X SSPE (0.15 M NaCl, 0.015 M Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 0.1 M EDTA), 10 X Denhardt's solution (0.2% Ficoll; 2 mg/ml BSA; 2 mg/ml polyvinylpyrrolidone (PVP), and 0.25 mg/ml salmon sperm DNA. The prehybridization solution was replaced the next day with 15 ml of hybridization solution (5 X SSPE, 10 X Denhardt's solution, 1% sodium dodecyl sulphate (SDS), 10% dextran sulphate and 0.5 mg/ml salmon sperm DNA. Probes were labeled with (<sup>32</sup>P) dCTP by the random hexamer method (Feinberg and Vogelstein 1983), purified through Sephadex G50 spin columns, denatured for 4 min, and added to the membranes. After 18–22 h of hybridization, membranes were given a brief wash with 2 X SSC followed by a wash with 2 X SSPE (0.1% w/v SDS) for 30 min, and subsequent two washes for 30 min each with 0.5 X SSPE (0.1% w/v SDS). Hybridizations and all the washes were performed at 65°C. Membranes were then exposed to X-ray film for 3–7 days. Additionally, ESTs (expressed sequence tags) physically mapped in the interstitial deletion bin 2DS1-0.33-0.47 ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_locus.cgi](http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi)) in the National Science Foundation (NSF) wheat EST mapping project were selected for physical mapping of *sog*.

Linkage maps were constructed with MAPMAKER version 2.0 (Lander et al. 1987) for the Macintosh OS. Genetic distances were calculated using the Kosambi mapping function (Kosambi 1944). The 'TRY' and 'RIPPLE' commands were used to add markers to the

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.

## Results

### *Genetic mapping of sog*

In the free-threshing mutant Tm-9 (*sog*), the glumes were softer, longer and broader compared to the wild type *T. monococcum* (*Sog*). In the *sog* mutant plant, the kernels were loosely covered and easy to thresh and the spikes were more compact (Fig. 2.2). The F<sub>1</sub> plants of Tm-9/*T. monococcum* subsp. *aegilopoides* were non-free-threshing suggesting that the mutant soft glume allele (*sog*) is recessive. The F<sub>2</sub> population segregated into 92 non-free-threshing and 26 free-threshing plants indicating monogenic inheritance (Table 2.2). In the F<sub>3</sub> generation, 27 families were homozygous for tough glumes, 57 families segregated and 24 families were homozygous for the free-threshing trait, fitting a 1:2:1 ratio ( $\chi^2$ ,  $P=0.64$ ).

The initial chromosome location of the *sog* gene was determined using chromosome 2A-specific microsatellite markers. A total of 60 microsatellite markers were surveyed for polymorphism between Tm-9 and *T. monococcum* subsp. *aegilopoides* accession 4342-95 and 51 primers amplified at least one fragment suggesting approximately 85% transfer rate of bread wheat microsatellites to *T. monococcum* (Table 2.3, 2.4). Among the 19 microsatellites that showed polymorphism between the parents, 16 produced co-dominant and three gave dominant polymorphism. Eight of the polymorphic microsatellite markers showed linkage with the *sog* gene and were mapped on chromosome 2A<sup>m</sup>S. The microsatellite marker *Xgwm71* was located 3.3 cM from *sog* towards the distal end of the short arm of chromosome 2A<sup>m</sup>. However, we could not place any marker proximal to *sog*. Furthermore, *Xgwm71* amplifies two alleles each in *T. aestivum* and *T. monococcum*; one has been mapped close to the centromere and the other has been mapped distally on the short arm of chromosome 2A maps of bread wheat (Röder et al. 1998; Somers et al. 2004). In the *T. monococcum* population, only one of the alleles of *Xgwm71* was segregating and, thus, the precise genetic location of *sog* on the short arm of chromosome 2A<sup>m</sup> was difficult to determine based on microsatellite markers alone (Fig. 2.3).

Consequently, in order to find flanking marker for the *sog* locus, 62 RFLP markers that were previously mapped on group-2 homoeologous chromosomes were analyzed

([http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO\\_2.html](http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO_2.html), Table 2.6). Parental genomic DNA was digested with six restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Sca*I, *Xba*I) and surveyed for polymorphism. Thirty-five probes detected polymorphism with at least one enzyme (Table 2.3, 2.6). Among the 35 polymorphic probes, 12 RFLP markers were mapped in the *T. monococcum* F<sub>2</sub> population. One RFLP marker *Xbcd120* was mapped 3.5 cM proximal to *sog*. In an effort to physically assign *sog* to a specific chromosome region on short arms of group-2 chromosomes, 14 EST markers physically mapped in the 2DS1-0.33-0.47 deletion bin were utilized ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_locus.cgi](http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi), Table 2.7). ESTs mapped in the interstitial bin were chosen because no prior information was available on the physical location of *sog*. Nine ESTs showed polymorphism among Tm-9 and *T. monococcum* subsp. *aegilopoides* (Table 2.3, 2.7) and three of these were found linked to *sog*. The closest EST marker, *XBE443771*, was mapped 4.3-cM distal to *sog*. As a result, *sog* was placed in a 6.8-cM interval flanked by microsatellite marker *Xgwm71* on distal end and *Xbcd120* on the proximal end (Fig. 2.4). The markers flanking *sog* have been placed in the proximal deletion bin C-2AS5-0.78 (Erayman et al. 2004; Sourdille et al. 2004) on chromosome 2A of wheat, thus assigning *sog* to the proximal 78% of the short arm. In the consensus physical map of group-2 chromosomes (Conley et al. 2004), the closest EST marker to *sog* (*XBE443771*) has been placed in the deletion interval delineated by FL-0.33 and FL-0.47 (FL, fraction length of distance from centromere), hence *sog* was placed in the proximal 50% of the short arm of group-2 homoeologous chromosomes of wheat (Fig. 2.4).

### ***Genetic mapping of Tg***

The Canthatch (TA2987) parent is free-threshing with softer and open glumes, whereas the synthetic parent (TA3419) is non-free-threshing with tough glumes that adhere tightly to the kernel (Fig. 2.2). The F<sub>1</sub> plants of this cross had somewhat tough glumes and were intermediate in threshability suggesting that *Tg* is a partially dominant gene. Phenotypic analysis of the F<sub>2</sub> population of 103 individuals gave a good fit for single gene segregation ( $\chi^2$ ,  $P = 0.28$ , Table 2.2). Segregation in F<sub>3</sub> generation also fit the expected 1:2:1 ratio ( $\chi^2$ ,  $P = 0.31$ ). These results confirmed that the free-threshing character of Canthatch is recessive in nature and that a single gene controls glume tenacity and threshability.

Among 49 chromosome 2D-specific microsatellite markers used to screen the parents of the hexaploid population, 25 were polymorphic (Table 2.3, 2.5). Among the polymorphic microsatellite primers, 17 were co-dominant. Ten SSR markers were linked to the *Tg* gene and mapped on the short arm of chromosome 2D. *Xwmc503*, the closest linked microsatellite marker to *Tg*, was located 2.2-cM towards the distal end of chromosome 2D. *Xwmc503* amplified two alleles, both of which were mapped on 2DS but only one of the alleles segregated in the F<sub>2</sub> population (Fig. 2.5). As was the case with *sog* mapping, we were unable to find a closely linked SSR marker for *Tg* on the proximal side. In an attempt to identify flanking marker for the *Tg* locus, 58 previously mapped homoeologous group-2 RFLP markers were chosen (Table 2.6). Parental DNA was digested with the same six enzymes used for mapping *sog*. Nineteen probes were polymorphic between parents with at least one enzyme combination (Table 2.3, 2.6). Nine RFLP markers showed linkage with *Tg*. Two RFLP markers *Xfba88* and *Xfba400* which co-segregated with each other were mapped 5.9 cM proximal to *Tg* on 2DS. Fourteen ESTs physically mapped in the 2DS1-0.33-0.47 deletion bin that were initially used for physical mapping of *sog* were also used to screen the parents of the hexaploid population for polymorphism (Table 2.6). Seven ESTs were polymorphic between Canthatch and the synthetic (Table 2.3, 2.7) and one EST marker, *XBE443771* showed linkage with *Tg*. However, *XBE443771* mapped very proximal to *Tg* on short arm of chromosome 2D (Fig. 2.4). Subsequently, *Tg* was localized in a 8.1-cM interval between markers *Xwmc503* and *Xfba88* (Fig. 2.4). Next, the closest flanking microsatellite marker to *Tg*, *Xwmc503*, was physically mapped in the 2DS3-0.47-1.00 deletion bin on chromosome 2DS (Fig. 2.6). This assigned *Tg* to the distal most deletion bin on chromosome 2DS. Furthermore, by using *Xfba88* as a probe on a set of group-2 deletion lines, *Tg* was mapped in the chromosome deletion bin 2BS-3 (FL 0.84-1.00) on 2B and 2DS-5 (FL 0.47-1.00) on 2D (Fig. 2.7). Thus, in the consensus physical map of group-2 chromosomes of wheat (Conley et al. 2004), *Tg* was targeted to distal 16% of the short arm (Fig. 2.4).

### ***Comparative mapping***

We compared the genetic linkage maps of the *sog* and *Tg* regions constructed using diploid and hexaploid wheat populations respectively (Fig. 2.4). Twenty-one microsatellite, RFLP and EST markers spanning 75.3-cM were mapped on chromosome 2A<sup>m</sup>S in the diploid

map, whereas 20 DNA markers mapped on short arm of chromosome 2D spanning 104.5 cM in the hexaploid map. Both maps shared seven common loci namely *Xbarc124*, *Xfba88*, *Xfba400*, *Xfba272*, *Xpsr130*, *Xcdo405* and *XBE443771*. Although the order of RFLP markers along the 2A<sup>m</sup> and 2D maps was in complete agreement with each other and with the previously published homoeologous group-2 RFLP maps ([http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO\\_2.html](http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO_2.html)), the genetic distance between individual markers varied considerably. In order to facilitate the comparison of recombination frequency, we used markers common between the 2A<sup>m</sup>S and 2DS linkage maps and divided the genetic maps into three major marker intervals. The chromosome region between markers *Xbarc124* and *Xfba88* in the genetic map was designated as interval I, the region between *Xfba400* and *Xfba272* as interval II and the region between *Xfba272* and *Xcdo405* as region III (Table 2.8). We tested the linkage heterogeneity using the *G*-test statistic (Liu 1998) with the hypothesis that true linkage between the marker pairs should be the same for 2A<sup>m</sup>S and 2DS maps. The *G*-statistic indicated that recombination data among given marker pairs is consistent with homogeneity of linkage except for marker pair *Xfba88* and *Xfba272* ( $G=14.253$ ,  $P= 0.0026$ , Table 2.8), which was not unusual because this marker pair spanned 33.5-cM in the 2DS genetic map compared to 9.5-cM in the 2AS map.

Comparative mapping analyses of the *sog* and *Tg* regions revealed that these genes occupy non syntenic regions on homoeologous group-2 chromosomes. This is evident by comparing the map position of threshability genes, *sog* and *Tg* with respect to a common marker such as *Xfba88*, which flanked *Tg* at 5.9 cM on proximal side but was placed 36.1 cM distal to *sog* (Fig. 2.4). Furthermore, physical mapping using a set of CS deletion lines for group-2 chromosomes placed *sog* in the proximal 50% of the short arm relative to *Tg*, which was targeted to the distal most 16% of short arm in the consensus physical map of the group-2 chromosomes (Fig. 2.4).

## Discussion

Threshability is an important domestication trait because free-threshing forms of wheat are essential for cultivation. Various genetic loci are known to influence threshability in wheat.

In the present study, two major threshability genes in wheat, soft glume and tenacious glume were characterized and their orthologous relationship was investigated.

The soft glume gene of diploid wheat, *T. monococcum* was mapped to the short arm of chromosome 2 using wheat microsatellite and RFLP markers. Cultivated *T. monococcum* is non-free-threshing, therefore, an EMS-induced soft-glume mutant, Tm-9, was used to develop a population segregating for threshability. Genetic mapping of *sog* using microsatellite and RFLP markers located it to chromosome 2A<sup>m</sup>S and placed it in a 6.8-cM interval flanked by marker loci *Xgwm71* and *Xbcd120* both of which have been mapped near the centromere on group-2 chromosomes of wheat (Röder et al. 1998; Somers et al. 2004; Nelson et al. 1995). Taenzler et al. (2002) assigned the *sog* gene to chromosome 2A<sup>m</sup> using AFLP (amplified fragment length polymorphism) markers. Although AFLP markers are reproducible across different genotypes when using same primer combinations (Alonso-Blanco et al. 1998), they are more useful when integrated to already existing microsatellite or RFLP linkage maps (Meudt and Clarke 2007) especially for the purpose of enriching the genetic map or a specific genomic region with markers. Also AFLPs produce multiple amplicons which are difficult to assign to a specific chromosome region unless the polymorphic AFLP fragment is converted into a sequence tagged site, STS or RFLP marker (Faris et al. 2002). Thus, because of the nature of markers used, they could not target *sog* to a specific region of the chromosome. We used chromosome-specific RFLP and EST markers to assign *sog* to a specific deletion-bin based on the physical map of chromosome 2A of wheat. Therefore, this is the first report to precisely locate *sog*, the threshability gene in diploid wheat, to a specific chromosome region in wheat.

The major determinant of threshability in hexaploid wheat, *Tg*, was localized to an 8.1-cM interval flanked by *Xwmc503* and *Xfba88* on chromosome 2DS. Two previous studies (Jantasuriyayrat et al. 2003 and Nalam et al. 2007) aimed at QTL mapping of genetic factors responsible for glume tenacity and threshability in hexaploid wheat, also identified major QTL on chromosome 2DS using recombinant inbred lines of ITMI population. Although both studies measured glume tenacity and percent threshability as separate traits, the QTL location for glume tenacity coincided with the QTL for percent threshability in the linkage map (Jantasuriyayrat et al. 2003; Nalam et al. 2007). These QTL were closely associated with the microsatellite marker *Xgwm261* on chromosome 2DS. In our 2DS linkage map, *Xwmc503*, the closest flanking marker to *Tg* was mapped 0.5-cM proximal to *Xgwm261*. Considering the fact that QTL usually occupy

large chromosomal regions, the glume tenacity QTL (Jantasuriyarat et al. 2003; Nalam et al. 2007) and *Tg* gene might represent the same locus. However, mapping of the glume tenacity QTL (*Tg1*) in a CS/CS2D F<sub>2</sub> population localized *Tg1* to a 12-cM region between markers *Xwmc112* and *Xbarc168* (Nalam et al. 2007). *Xwmc112* and *Xbarc168* were non-polymorphic and not mapped in our population but *Xwmc112* has been located 8.0-cM proximal to *Xwmc503* on chromosome 2DS in the published consensus microsatellite map for chromosome 2DS (Somers et al. 2004). In our linkage map, *Tg* was mapped 2.2-cM proximal to *Xwmc503*, suggesting that *Tg* and *Tg1* may represent the same genetic locus. Alternatively, two coincident genetic factors may control threshability and glume tenacity on 2DS as has been indicated by Nalam et al. (2007). The genetic background (minor loci in D genome) also may have had an effect on the phenotype, which further affected the mapping results. Consequently, further research is needed to confirm the relationship between *Tg* and *Tg1*.

Various molecular mapping and comparative mapping studies within the *Triticeae* have established that gene synteny is well conserved among the homoeologous group-2 chromosomes in wheat (Devos et al. 1993; Nelson et al. 1995). Homoeologous chromosomes are essentially collinear except for some minor rearrangements (Conley et al. 2004; Devos et al. 1993). A threshability gene is found on the short arm of each homoeologous group-2 chromosome and all the threshability genes (*sog*, *Tg2* and *Tg*) may be orthologues (Jantasuriyarat et al. 2004; Simonetti et al. 1999; Taenzler et al. 2002). However, experimental evidence in support of this hypothesis is lacking. In the present study, we compared the map positions of *sog* and *Tg* using homoeologous group-2-specific RFLP markers and found these genes to be non-orthologous. The *sog* and *Tg* genes occupy different positions on the genetic map of short arm of homoeologous group-2 chromosome as demonstrated by RFLP marker *Xfba88*, which flanks *Tg* at 5.9-cM on the proximal end of chromosome 2DS and is placed 36.1-cM distal to *sog* on chromosome 2A<sup>m</sup>S. The RFLP marker, *Xfba88*, also has been mapped in the deletion bin 2BS-3 (FL 0.84-1.00) on chromosome 2B of wheat, which targets *Tg* to the distal 16% of the short arm in the consensus physical map of the group-2 chromosomes. The marker locus, *XBE443771*, which is genetically mapped 4.3-cM distal to *sog* on 2A<sup>m</sup>S and 80.7-cM proximal to *Tg* on 2DS in this study, has been physically mapped in deletion bin 2DS-1 delineated by FL0.33 and FL0.47 in the consensus physical map of group-2 chromosomes of wheat (Conley et al. 2004)

and thus placing *sog* in the proximal half of the short arm of group-2 chromosomes. This clearly suggests that *sog* and *Tg* genes are not orthologues.

Comparing the map distances between the same loci on chromosome 2A<sup>m</sup> and 2D also indicated the local variability in recombination frequencies between these linkage maps. Significantly higher recombination was observed between *Xfba88* and *Xfba272* marker loci in the chromosome-2D map as compared to the chromosome-2A<sup>m</sup> genetic map. The most likely reason for a greater genetic distance in the *Xfba88* and *Xfba272* marker interval may be that the parents of both populations (both diploid and hexaploid levels) are biologically different with respect to recombination in this region. This assumption is supported by a higher *G*-statistic value for this marker pair and also by the fact that marker interval lengths in the corresponding regions on the 2D map developed in this study are quite similar to those from other mapping studies involving cultivated x synthetic wheat crosses (Nelson et al. 1995; Korzun et al. 1998; Pestsova et al. 2000).

Uneven distribution of genes and recombination in the wheat genome has been widely documented (Gill et al. 1996; Sandhu et al. 2001; Akhunov et al. 2003). Most wheat genes are present in clusters that occur frequently in the distal areas of the chromosomes (Gill et al. 1996). Higher rates of recombination also have been observed in the distal regions (Werner et al. 1992; Akhunov et al. 2003). In this study the major threshability gene in hexaploid wheat, *Tg*, has been localized in the most distal deletion bin of chromosome 2DS. Moreover, in the consensus physical map of group-2 chromosomes, *Tg* is targeted to the distal 16% of the short arm between breakpoints FL0.84 and FL1.00 (Fig. 3), implying that *Tg* lies in a high-recombination region. Several studies in wheat have demonstrated that despite the large genome of wheat ( $1.6 \times 10^{10}$  bp), genes present in regions of high-recombination are amenable to map-based cloning (Feuillet et al. 2003; Huang et al. 2003; Yan et al. 2003, 2004; Simons et al. 2006). Thus isolating *Tg* using a positional cloning approach should be feasible. Because the deletion bin location of *Tg* is known, specific deletion-bin mapped wheat ESTs ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_locus.cgi](http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi)) can be readily utilized to identify markers closely linked to *Tg*. Furthermore, a genome wide analysis of mapped wheat ESTs has shown large blocks of conserved collinearity between wheat and rice genomes (Gale and Devos 1998; Sorrells et al. 2003). Distal and proximal regions of wheat chromosome arm 2S are homoeologous to rice chromosomes 4 and 7, respectively (Sorrells et al. 2003) and it has been well-established that



synteny between wheat and rice in this region is highly conserved (Ahn et al. 1993; Sorrells et al. 2003; Conley et al. 2004). Therefore, rice genomic sequence information can be used as an additional source of markers to enrich the *Tg* region to aid in its cloning.

On the contrary, *sog*, the threshability gene in diploid wheat, has been genetically mapped close to the centromere (our results, Röder et al. 1998; Nelson et al. 1995). Based on a study by Erayman et al. (2004), 29 percent of the wheat genome is presumed to be gene-rich region and these regions have been divided into 18 major and 30 minor gene-rich regions (GRRs). RFLP markers flanking *sog*, *Xrz395* and *Xbcd120* have been placed in GRR 2S0.5 (FL 0.47-0.57) on chromosome 2S, which physically places *sog* in the proximal half of the chromosome arm. This GRR spans a physical length of 21 Mb and has a recombination frequency of 1.5 Mb/cM, thus emphasizing that *sog* is located in a relatively low-recombination region. Efforts to isolate *sog* using map-based cloning approach therefore might be ineffective.

Comparative mapping analysis of QTL regions corresponding to seed size, spike disarticulation and day length-insensitive flowering in sorghum, rice and maize led to the hypothesis that domestication traits among cereals might represent repeated selection on the same underlying genes or genomic regions and suggested convergent domestication (Paterson et al. 1995). However, our results on comparative mapping of the *sog* and *Tg* gene regions in wheat do not support this hypothesis. Wheat, barley, rice, maize and sorghum are major cereals crops that are not only evolutionary related (Kellogg 2001) but also share a high degree of gene conservation (Gale and Devos 1998). Among these cereal crops, two *Tg*-related genes that control the ease of threshability have been reported. In maize, *tgal* (*teosinte glume architecture1*), which causes the stony fruitcase appearance in progenitor teosinte (Dorweiler et al. 1993; Wang et al. 2005) and in barley the naked caryopses gene, *nud* that is responsible for hulled/hullless spikes (Taketa et al. 2008) have been isolated. The wheat tough glume gene, *Tg*, maps in a region (chromosome 2S) that is syntenic to maize chromosomes 2 and 7 (Ahn et al. 1993; Gale and Devos 1998) whereas the maize tough glume gene, *tgal*, is located on chromosome 4 of maize (Dorweiler et al. 1993). Additionally, the maize *tgal* orthologue in wheat maps to the group-7 chromosomes of wheat (Wanlong Li, personal communication). Similarly, the *nud* gene is mapped on chromosome 7H of barley, which is homoeologous to wheat group-7 chromosomes. Thus, *tgal* of maize and *nud* of barley are non orthologous to the wheat group-2 chromosome threshability genes. Similar results have been reported for shattering

genes in wheat where *Br1* does not have any orthologue in syntenic regions of barley, rice, maize or sorghum (Li and Gill 2006).

Thus, it is conceivable that the wheat threshability genes (genetic loci on group-2 chromosomes) do not share an ancestral relationship with related genes in other cereals and they originated after the separation of the wheat lineage from the last common cereal ancestor. Additionally, because *sog* and *Tg* also are non orthologous (present study), these genes might not be derived from a common ancestral gene but arose by independent mutations in A- and D-genome diploid progenitors after their evolution from a common parent 2.5-4.5 million years ago (Huang et al. 2002). Another possibility could be that the *sog* and *Tg* are homologous genes but occupy non orthologous positions on the homoeologous chromosome arms. The putative mechanism for such a phenomenon has been described for explaining non-orthologous relations among different cereal genomes where ancient gene duplication in the common ancestor is followed by the loss of one gene copy in one species and the loss of the second copy in the other species (Ware and Stein 2003). One such example of a duplication/ deletion event has been provided by the comparative analysis between genomes of the Triticeae species and those of rice, sorghum and maize in the *Sh2/Al* region (Li and Gill, 2002). This region contains five genes, *Sh2*, *X1*, *X2* and two tandem copies of *Al*, in rice, sorghum and maize and is orthologous to long arm of group-3 chromosome region in Triticeae species. Among these genes, *X1* was duplicated twice during Triticeae evolution with copies on chromosome 3L, 7L and 3S followed by deletion of 3' portion of the original *X1* ortholog (chromosome 3L). However, the *X1* paralog (on chromosome 7L) retained a high degree of homology to the original *X1* gene of rice, sorghum and maize (Li and Gill 2002).

In addition to the major tough glume genes on group-2 chromosomes of wheat, some minor genes and modifiers also are involved in determining the threshability trait. The QTL affecting percent threshability have been identified on chromosome 6A and 6D (Simonetti et al. 1999; Jantasuriyarat et al. 2004), suggesting that loci other than those on group-2 chromosomes and chromosome 5A (*Q* gene) also may be involved in the control of threshability trait in wheat. On the other hand, *Secale cereale* L., *Thinopyrum elongatum* (Host) D. R. Dewey and several *Aegilops* species carry the tough glume genes on the group-2 chromosomes as has been demonstrated by their chromosome-2 addition and substitution lines in wheat (Friebe et al. 1999; Cheng and Murata 2002; Dvorak 1980). The monosomic and disomic additions of chromosome

VI of *Th. elongatum* (homoeologous to group-2 chromosomes of wheat) to *T. aestivum* resulted in plants with tenacious glumes (Dvorak and Knott 1974; Dvorak 1980). Therefore, the threshability trait seems to be under the control of several major and minor genes and will involve an in-depth genetic analysis to elucidate the pathways relating to various threshability genes in wheat. The precise physical and genetic mapping of threshability genes and exploring their orthologous relationship is the first step towards isolating these genes and understanding their genetic interactions.

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**Figure 2.1** Spike phenotypes of free-threshing mutants in diploid wheat (Tm-9 and TA139) and their hybrid



Tm-9

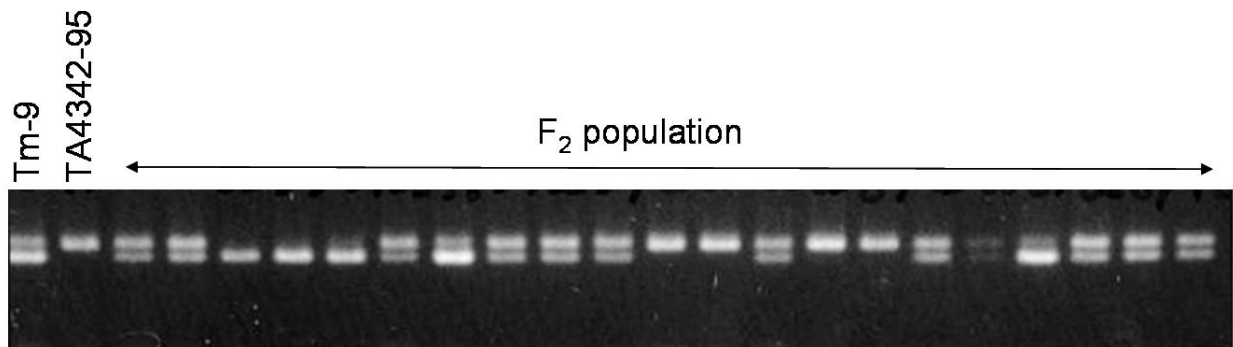
F<sub>1</sub>

TA139

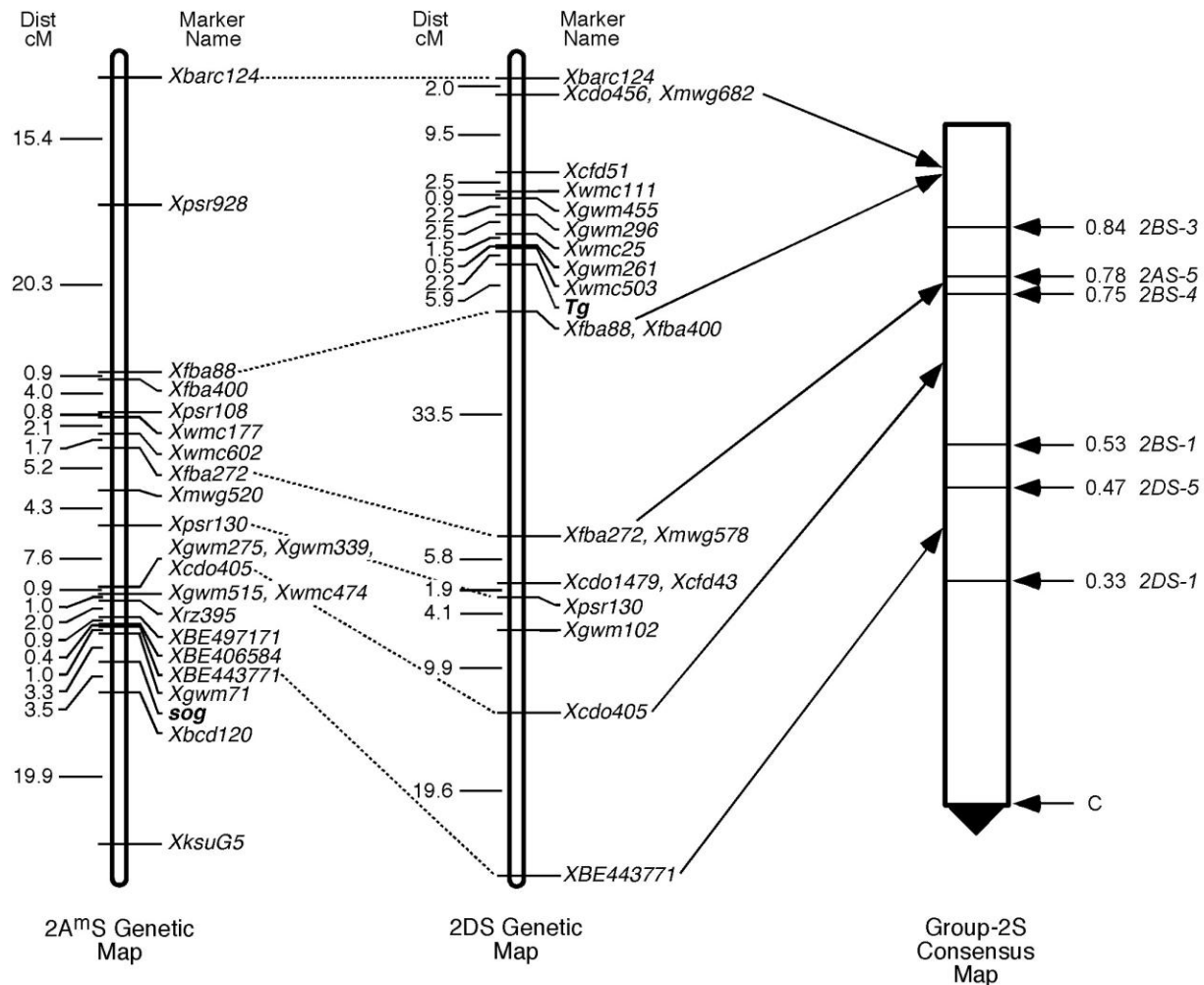
**Figure 2.2** Phenotypes of contrasting alleles of threshability genes *sog* and *Tg* in diploid and hexaploid wheat respectively. Comparison of spike shape between a. non-free-threshing *T. monococcum* (*Sog*) and free-threshing mutant Tm-9 (*sog*) and b. non-free-threshing synthetic wheat (*Tg*) and the free-threshing cultivar Canthatch (*tg*). Spikelets showing the glume morphology differences in c. *T. monococcum* (*Sog*) and Tm-9 (*sog*) and d. synthetic wheat (*Tg*) and cultivar Canthatch (*tg*)



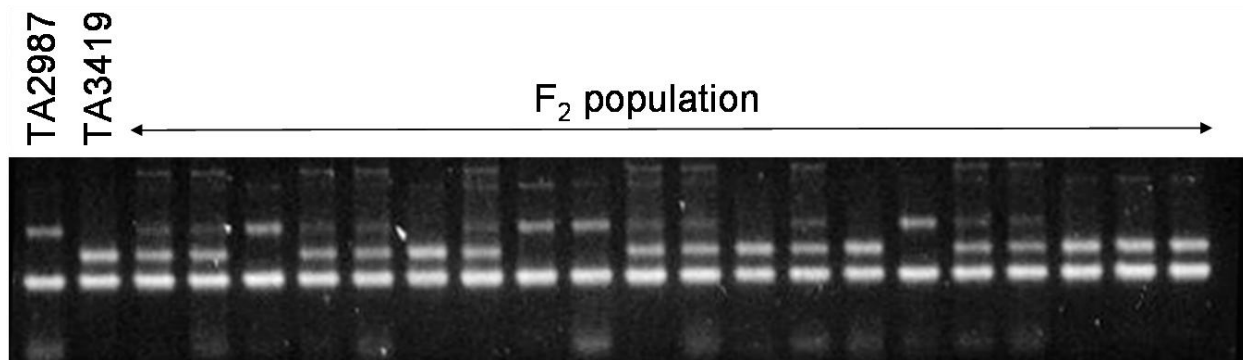
**Figure 2.3** Molecular mapping of threshability gene in diploid wheat F<sub>2</sub> population using wheat microsatellite markers. An agarose gel electrophoresis image showing segregation of microsatellite marker flanking *sog* gene, *Xgwm71* in selected individuals of F<sub>2</sub> population



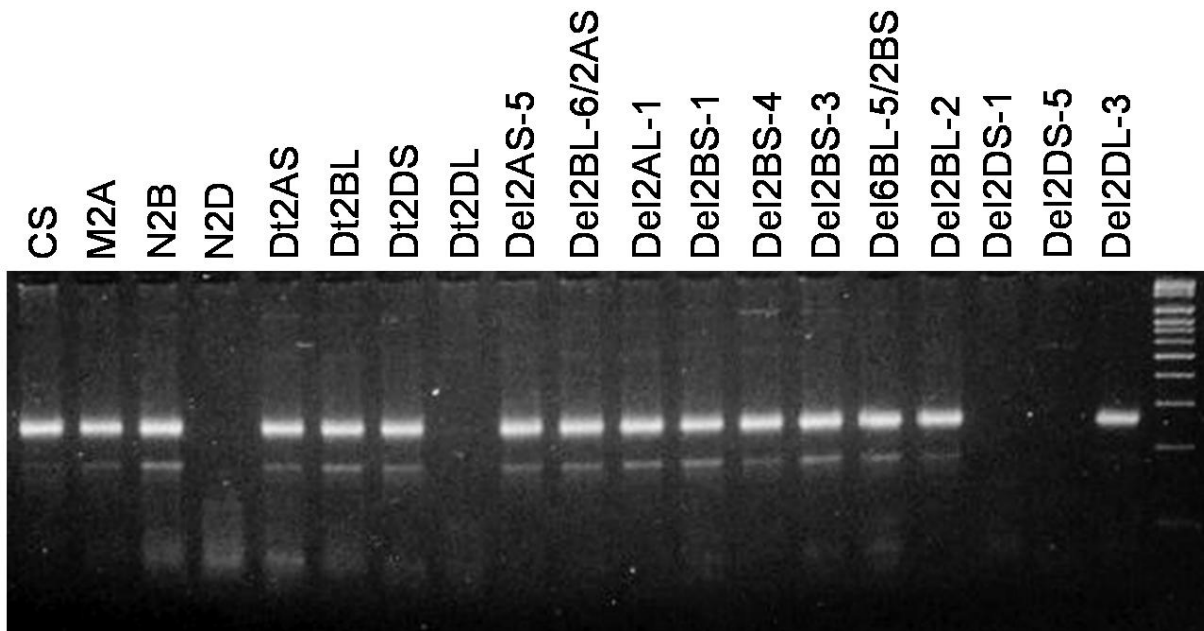
**Figure 2.4** Genetic and physical mapping of threshability genes *sog* and *Tg*. Each map is oriented top to bottom from telomere towards centromere. Common marker loci between the two genetic maps are connected by dotted lines. The threshability loci *sog* and *Tg* are represented in bold. Map distances are given in cM. The centromere on chromosome 2A<sup>m</sup> map is located between marker loci *Xbcd120* and *XksuG5*. The co-segregating markers have been placed next to each other on the genetic maps. The group-2S consensus physical map has been redrawn based on Conley et al. 2004. Each section of consensus physical map represents a bin delimited by deletion breakpoints expressed as fraction of the arm length from the centromere. The breakpoints of various deletions, along with their FL values, are marked by arrows on the right of the consensus map. Black solid lines indicate the deletion bin location of RFLP and EST markers in the consensus physical map that are common between 2A<sup>m</sup> and 2DS genetic maps



**Figure 2.5** Molecular mapping of threshability gene in hexaploid wheat F<sub>2</sub> population using microsatellite markers. An agarose gel electrophoresis image showing segregation of microsatellite marker flanking *Tg* gene, *Xwmc503* in selected individuals of F<sub>2</sub> population

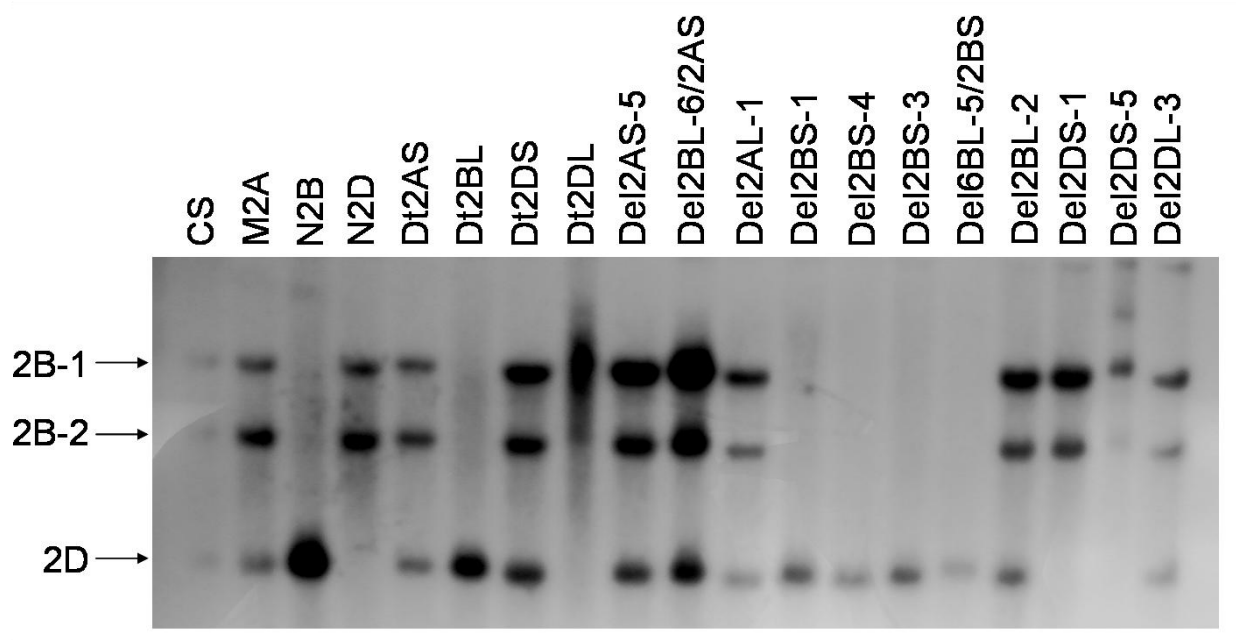


**Figure 2.6** Deletion bin based physical mapping of tenacious glume gene, *Tg* of hexaploid wheat using flanking markers. The agarose gel electrophoresis image showing differential amplification of microsatellite marker, *Xwmc503* in the aneuploid lines (monosomic, M; nullisomic, N; ditelosomic, Dt; Deletion lines, del) for group-2 chromosomes of Chinese Spring wheat





**Figure 2.7** Deletion bin based physical mapping of *Tg* by mapping flanking RFLP marker *Xfba88* in Chinese Spring (CS) group-2 aneuploid stocks. Autoradiograph image showing the hybridization pattern of RFLP probe FBA88 on Monosomic (M), Nullisomic (N), ditelosomic (Dt) and deletion lines (Del) of group-2 chromosomes. The chromosome specific alleles are indicated by arrows on the left side of the image



**Table 2.1** Recipe for embryo rescue medium used to grow the haploid embryos derived from Canthatch and Synthetic wheat crosses

Components			
ddH <sub>2</sub> O	300 ml	600 ml	1200 ml
MS FeEDTA*	5 ml	10 ml	20 ml
MS Sulfates	5 ml	10 ml	20 ml
MS Halides	5 ml	10 ml	20 ml
MS P, B, Mo	5 ml	10 ml	20 ml
B5 Vitamins**	5 ml	10 ml	20 ml
NH <sub>4</sub> NO <sub>3</sub>	0.825 g	1.65 g	3.3 g
KNO <sub>3</sub>	0.95 g	1.90 g	3.8 g
Kinetin (1 mg/ml stock)	1 ml	2 ml	4 ml
Sucrose	15 g	30 g	60 g
pH with KOH	5.7	5.7	5.7
Final volume	500 ml	1000 ml	2000 ml

\* MS indicates Murashige and Skoog salts based tissue culture media. The FeEDTA salt has to be added first while preparing the media. After adding all the listed components, 1.0 g of Gelrite is added per 500 ml of media and autoclaved for 20-25 min

\*\* Indicates Gamborg B5 vitamins

**Table 2.2** Segregation of threshability alleles in the diploid and hexaploid wheat populations

	Generation	Total no. of plants	Observed ratio <sup>c</sup>	Expected ratio	$\chi^2$	<i>P</i> -value <sup>d</sup>
Tm-9 X TA4342-95 (Diploid wheat)	F <sub>2</sub>	118	92: 26	3:1	0.553	0.46
	F <sub>3</sub>	111 <sup>a</sup>	27: 60: 24	1: 2:1	0.89	0.64
TA2987 X TA3419 (Hexaploid wheat)	F <sub>2</sub>	103	82: 21	3:1	1.17	0.28
	F <sub>3</sub>	102 <sup>b</sup>	26: 57:19	1: 2:1	2.36	0.307

<sup>a,b</sup> Seven families from the diploid wheat F<sub>2</sub> population and one family from hexaploid F<sub>2</sub> population could not be phenotyped in F<sub>3</sub> due to lack of sufficient seeds

<sup>c</sup>Plants were categorized as non-free-threshing (NFT): free-threshing (FT) in F<sub>2</sub> and as NFT: segregating: FT in F<sub>3</sub> generation

<sup>d</sup>  $\chi^2$  values were tested at a 0.05 level of significance

**Table 2.3** Polymorphism data for molecular markers used to screen diploid and hexaploid wheat parents for genetic and physical mapping of *sog* and *Tg* genes respectively

Molecular marker type	Number of markers	Polymorphic markers	Percent polymorphism	Codominant markers	Dominant markers
<i>In diploid wheat:</i>					
Microsatellite	60	19	36.5	16	3
RFLP	62	31	50.0	22	9
EST	14	9	64.3	7	2
<i>In hexaploid wheat:</i>					
Microsatellite	49	25	51.0	17	8
RFLP	58	19	32.7	14	5
EST	14	7	50.0	6	1

**Table 2.4** Polymorphism data for chromosome 2A-specific microsatellite markers in diploid wheat parents

<b>Microsatellite marker</b>	<b>Amplification*</b>	<b>Number of bands amplified</b>	<b>Polymorphism**</b>	<b>PCR amplicon detection platform</b>
<i>Xbarc124</i>	A	>2	P	Agarose
<i>Xbarc138</i>	A	>2	P	Agarose
<i>Xbarc201</i>	A	>2	NP	LICOR
<i>Xbarc208</i>	A	>2	NP	LICOR
<i>Xbarc212</i>	A	>2	NP	Agarose
<i>Xcfa2043</i>	A	>2	NP	Agarose
<i>Xcfa2058</i>	A	1	NP	LICOR
<i>Xcfa2164</i>	A	>2	NP	Agarose
<i>Xcfa2263</i>	A	>2	NP	Agarose
<i>Xcfd2</i>	A	1	NP	Agarose
<i>Xcfd36</i>	A	2	NP	Agarose
<i>Xcfd50</i>	A	2	P	Agarose
<i>Xgwm10</i>	A	>2	NP	Agarose
<i>Xgwm102</i>	A	1	NP	LICOR
<i>Xgwm122</i>	A	2	P	LICOR
<i>Xgwm210</i>	A	1	NP	Agarose
<i>Xgwm249</i>	A	1	NP	Agarose
<i>Xgwm265</i>	A	>2	NP	Agarose
<i>Xgwm275</i>	A	>2	P	LICOR
<i>Xgwm294</i>	A	>2	NP	Agarose
<i>Xgwm296</i>	A	2	NP	Agarose
<i>Xgwm311</i>	A	1	P	Agarose
<i>Xgwm312</i>	A	1	P	LICOR

<b>Microsatellite marker</b>	<b>Amplification*</b>	<b>Number of bands amplified</b>	<b>Polymorphism**</b>	<b>PCR amplicon detection platform</b>
<i>Xgwm328</i>	A	1	P	LICOR
<i>Xgwm339</i>	A	1	P	LICOR
<i>Xgwm356</i>	A	1	P	LICOR
<i>Xgwm359</i>	A	1	NP	Agarose
<i>Xgwm372</i>	A	>2	NP	Agarose
<i>Xgwm425</i>	A	2	NP	Agarose
<i>Xgwm445</i>	NA	-	-	Agarose
<i>Xgwm448</i>	A	2	P	Agarose
<i>Xgwm47</i>	A	>2	NP	Agarose
<i>Xgwm473</i>	A	>2	NP	Agarose
<i>Xgwm497</i>	NA	-	-	LICOR
<i>Xgwm512</i>	A	2	NP	Agarose
<i>Xgwm515</i>	A	2	P	Agarose
<i>Xgwm558</i>	A	2	P	LICOR
<i>Xgwm614</i>	A	1	NP	Agarose
<i>Xgwm636</i>	A	1	NP	LICOR
<i>Xgwm71</i>	A	2	P	Agarose
<i>Xgwm95</i>	A	>2	NP	Agarose
<i>Xwmc109</i>	NA	-	-	LICOR
<i>Xwmc149</i>	NA	-	-	LICOR
<i>Xwmc177</i>	A	1	P	Agarose
<i>Xwmc181</i>	NA	-	-	LICOR
<i>Xwmc261</i>	A	>2	NP	Agarose
<i>Xwmc296</i>	NA	-	-	LICOR
<i>Xwmc382</i>	NA	-	-	LICOR
<i>Xwmc407</i>	A	1	P	LICOR

<b>Microsatellite marker</b>	<b>Amplification*</b>	<b>Number of bands amplified</b>	<b>Polymorphism**</b>	<b>PCR amplicon detection platform</b>
<i>Xwmc453</i>	NA	-	-	LICOR
<i>Xwmc455</i>	A	1	NP	LICOR
<i>Xwmc474</i>	A	2	P	LICOR
<i>Xwmc522</i>	A	1	NP	LICOR
<i>Xwmc598</i>	NA	-	-	LICOR
<i>Xwmc602</i>	A	1	P	LICOR
<i>Xwmc63</i>	A	1	NP	LICOR
<i>Xwmc632</i>	A	>2	NP	LICOR
<i>Xwmc667</i>	A	>2	P	LICOR
<i>Xwmc702</i>	A	1	NP	LICOR
<i>Xwmc728</i>	A	1	NP	LICOR

\* A = amplified loci, NA = non-amplified loci

\*\*P = polymorphic loci, NP = non-polymorphic loci

**Table 2.5** Polymorphism data for chromosome 2D-specific microsatellite markers in hexaploid wheat parents

<b>Microsatellite marker</b>	<b>Chromosome location</b>	<b>Number of bands amplified</b>	<b>Polymorphism*</b>	<b>PCR amplicon detection platform</b>
<i>Xbarc124</i>	2DS	1	P	Agarose
<i>Xbarc159</i>	2DL	>2	P	Agarose
<i>Xbarc168</i>	2DS	>2	NP	Agarose
<i>Xbarc219</i>	2D	>2	NP	Agarose
<i>Xbarc292</i>	2DL	1	NP	LICOR
<i>Xbarc297</i>	2DS	>2	NP	Agarose
<i>Xbarc352</i>	2DS	>2	P	Agarose
<i>Xbarc59</i>	2DS	>2	NP	Agarose
<i>Xcfa2040</i>	2DS	>2	NP	Agarose
<i>Xcfd116</i>	2DS	1	P	Agarose
<i>Xcfd160</i>	2DS	1	NP	Agarose
<i>Xcfd161</i>	2DL	1	P	Agarose
<i>Xcfd175</i>	2DS	1	P	LICOR
<i>Xcfd193</i>	2DL	>2	NP	Agarose
<i>Xcfd25</i>	2DS	1	NP	Agarose
<i>Xcfd255</i>	2DS	1	NP	Agarose
<i>Xcfd36</i>	2DS	1	NP	Agarose
<i>Xcfd43</i>	2DS	1	P	Agarose
<i>Xcfd51</i>	2DS	>2	P	Agarose
<i>Xcfd53</i>	2DS	1	NP	Agarose
<i>Xcfd56</i>	2DS	>2	P	Agarose
<i>Xcfd65</i>	2DS	1	P	Agarose
<i>Xcfd77</i>	2DS	1	NP	Agarose
<i>Xgdm107</i>	2DS	1	NP	Agarose



<b>Microsatellite marker</b>	<b>Chromosome location</b>	<b>Number of bands amplified</b>	<b>Polymorphism*</b>	<b>PCR amplicon detection platform</b>
<i>Xgdm19</i>	2DS	>2	P	Agarose
<i>Xgdm5</i>	2DS	>2	NP	Agarose
<i>Xgdm77</i>	2DS	1	NP	Agarose
<i>Xgdm102</i>	2DS	>2	P	LICOR
<i>Xgwm157</i>	2DL	1	P	Agarose
<i>Xgwm210</i>	2DS	1	NP	Agarose
<i>Xgwm261</i>	2DS	1	P	Agarose
<i>Xgwm296</i>	2DS	1	NP	Agarose
<i>Xgwm30</i>	2D	1	P	Agarose
<i>Xgwm301</i>	2DL	1	P	LICOR
<i>Xgwm311</i>	2DL	>2	P	Agarose
<i>Xgwm320</i>	2DL	>2	NP	Agarose
<i>Xgwm349</i>	2DL	>2	P	Agarose
<i>Xgwm382</i>	2DL	>2	P	Agarose
<i>Xgwm455</i>	2DS	>2	P	Agarose
<i>Xgwm484</i>	2DS	1	NP	Agarose
<i>Xgwm515</i>	2DS	>2	NP	Agarose
<i>Xgwm539</i>	2DL	1	P	Agarose
<i>Xgwm608</i>	2DL	>2	NP	Agarose
<i>Xwmc111</i>	2DS	>2	P	LICOR
<i>Xwmc112</i>	2DS	>2	P	LICOR
<i>Xwmc166</i>	2DS, 7BS	>2	NP	Agarose
<i>Xwmc167</i>	2DS	>2	P	Agarose
<i>Xwmc25</i>	2DS	>2	P	Agarose
<i>Xwmc453</i>	2DS	1	NP	LICOR

\* *P* = polymorphic loci, *NP* = non-polymorphic loci

**Table 2.6** Detailed polymorphism analysis data for homoeologous chromosome group-2 specific RFLP markers used for genetic and physical mapping of threshability genes *sog* and *Tg* of diploid and hexaploid wheat respectively

<b>RFLP marker</b>	<b>Polymorphism in diploid wheat*</b>	<b>Restriction enzyme</b>	<b>Polymorphism in hexaploid wheat</b>	<b>Restriction enzyme</b>
<i>Xabg356</i>	NP	-	NP	-
<i>Xbcd102</i>	P	<i>HindIII</i>	P	<i>DraI</i>
<i>Xbcd111</i>	NP	-	P	<i>XbaI</i>
<i>Xbcd1119</i>	P	<i>DraI, ScaI, XbaI</i>	NP	-
<i>Xbcd120</i>	P	<i>EcoRI, EcoRV, ScaI</i>	NP	-
<i>Xbcd1709</i>	NP	-	ND	-
<i>Xbcd175</i>	P	<i>EcoRI</i>	P	<i>XbaI</i>
<i>Xbcd18</i>	NP	-	NP	-
<i>Xbcd260</i>	NP	-	NP	-
<i>Xbcd855</i>	NP	-	NP	-
<i>Xcdo1376</i>	NP	-	NP	-
<i>Xcdo1417</i>	P	<i>DraI</i>	NP	-
<i>Xcdo1479</i>	NP	-	P	<i>DraI</i>
<i>Xcdo370</i>	NP	-	NP	-
<i>Xcdo405</i>	P	<i>EcoRI, XbaI</i>	P	<i>EcoRV</i>
<i>Xcdo456</i>	P	<i>HindIII</i>	P	<i>HindIII</i>
<i>Xcdo57</i>	P	<i>DraI, ScaI,</i>	NP	-
<i>Xcdo64</i>	P	<i>DraI, EcoRI</i>	NP	-
<i>Xcmwg763</i>	ND	-	ND	-
<i>Xfba272</i>	P	<i>EcoRI, ScaI</i>	P	<i>XbaI</i>
<i>Xfba341</i>	NP	-	P	<i>ScaI, XbaI</i>

<b>RFLP marker</b>	<b>Polymorphism in diploid wheat*</b>	<b>Restriction enzyme</b>	<b>Polymorphism in hexaploid wheat</b>	<b>Restriction enzyme</b>
<i>Xfba359</i>	NP	-	NP	-
<i>Xfba38</i>	NP	-	NP	-
<i>Xfba400</i>	P	<i>EcoRI</i>	P	<i>DraI, EcoRI, ScaI, XbaI</i>
<i>Xfba65</i>	P	<i>EcoRI, EcoRV</i>	NP	-
<i>Xfba88</i>	P	<i>EcoRI, ScaI</i>	P	<i>ScaI, XbaI</i>
<i>Xfbb274</i>	P	<i>EcoRV</i>	NP	-
<i>Xfbb279</i>	P	<i>DraI</i>	NP	-
<i>XKsuD18</i>	P	<i>DraI, XbaI</i>	NP	-
<i>XKsuD22</i>	NP	-	P	<i>EcoRI</i>
<i>XKsuD8</i>	NP	-	NP	-
<i>XKsuE16</i>	P	<i>EcoRI, EcoRV, ScaI, XbaI</i>	P	<i>EcoRV</i>
<i>XKsuF11</i>	NP	-	P	<i>EcoRV</i>
<i>XKsuF2</i>	P	<i>DraI</i>	NP	-
<i>XKsuG30</i>	P	<i>DraI, ScaI</i>	NP	-
<i>XKsuG5</i>	P	<i>EcoRV, ScaI</i>	NP	-
<i>XKsuG57</i>	P	<i>EcoRV</i>	P	<i>EcoRI, ScaI, XbaI</i>
<i>XKsuH8</i>	NP	-	NP	-
<i>XKsuM1</i>	NP	-	NP	-
<i>Xmwg2067</i>	NP	-	NP	-
<i>Xmwg520</i>	P	<i>DraI, EcoRI, EcoRV, ScaI</i>	NP	-
<i>Xmwg578</i>	P	<i>EcoRV, DraI</i>	P	<i>XbaI</i>
<i>Xmwg64</i>	NP	-	NP	-
<i>Xmwg682</i>	P	<i>HindIII</i>	P	<i>HindIII</i>

<b>RFLP marker</b>	<b>Polymorphism in diploid wheat*</b>	<b>Restriction enzyme</b>	<b>Polymorphism in hexaploid wheat</b>	<b>Restriction enzyme</b>
<i>Xmwg858</i>	NP	-	NP	-
<i>Xmwg878</i>	NP	-	NP	-
<i>Xpsr100</i>	P	<i>EcoRI</i>	NP	-
<i>Xpsr107</i>	NP	-	NP	-
<i>Xpsr108</i>	P	<i>DraI, XbaI</i>	P	<i>DraI</i>
<i>Xpsr112</i>	NP	-	NP	-
<i>Xpsr130</i>	P	<i>EcoRV, HindIII</i>	P	<i>EcoRV</i>
<i>Xpsr131</i>	P	<i>DraI, EcoRI, EcoRV, ScaI</i>	NP	-
<i>Xpsr146</i>	NP	-	NP	-
<i>Xpsr390</i>	P	<i>DraI, XbaI</i>	NP	-
<i>Xpsr575</i>	P	<i>EcoRI, ScaI</i>	NP	-
<i>Xpsr899</i>	ND	-	NP	-
<i>Xpsr912</i>	P	<i>EcoRI, HindIII</i>	NP	-
<i>Xpsr928</i>	P	<i>EcoRI, HindIII</i>	NP	-
<i>Xrz395</i>	P	<i>EcoRV</i>	NP	-
<i>Xrz444</i>	P	<i>HindIII, XbaI</i>	NP	-
<i>Xrz69</i>	NP	-	P	<i>EcoRI, XbaI</i>
<i>Xwg405</i>	P	<i>DraI, ScaI</i>	NP	-

\* The polymorphism is scored for different RFLP markers as *P* = polymorphic, *NP* = non-polymorphic, *ND* = not determined (due to poor hybridization signal)

**Table 2.7** Detailed polymorphism analysis data for EST markers used for physical mapping of threshability genes *sog* and *Tg* of diploid and hexaploid wheat respectively

<b>EST marker</b>	<b>Polymorphism in diploid wheat*</b>	<b>Restriction enzyme</b>	<b>Polymorphism in hexaploid wheat</b>	<b>Restriction enzyme</b>
<i>XBF475019</i>	P	<i>EcoRI, EcoRV, HindIII</i>	P	<i>XbaI</i>
<i>XBE470848</i>	P	<i>EcoRV, ScaI, XbaI</i>	NP	-
<i>XBE406584</i>	P	<i>HindIII, ScaI</i>	P	<i>ApaI</i>
<i>XBE497590</i>	NP	-	P	<i>XhoI</i>
<i>XBE497171</i>	P	<i>EcoRV</i>	NP	-
<i>XBF200812</i>	NP	-	NP	-
<i>XBF473388</i>	P	<i>EcoRI</i>	NP	-
<i>XBE488732</i>	NP	-	P	<i>XhoI</i>
<i>XBE443771</i>	P	<i>EcoRV, XbaI</i>	P	<i>BglII</i>
<i>XBE446513</i>	NP	-	P	<i>XhoI</i>
<i>XBF146002</i>	P	<i>ApaI</i>	NP	-
<i>XBF293463</i>	P	<i>DraI</i>	NP	-
<i>XBG263521</i>	NP	-	NP	-
<i>XBF484705</i>	P	<i>DraI, EcoRV, ScaI</i>	P	<i>DraI</i>

\* The polymorphism is scored for different EST markers as *P* = polymorphic, *NP* = non-polymorphic

**Table 2.8** Recombination frequency distribution between different molecular marker intervals in the genetic linkage maps of short arm of chromosome 2A<sup>m</sup> and 2D in relation to location of threshability genes *sog* and *Tg* respectively

Chromosome interval region on linkage maps	Molecular markers encompassing the interval	Genetic distance in the given marker interval (cM)		<i>G</i>	<i>P</i> -value <sup>1</sup>
		2A <sup>m</sup> S map	2DS map		
I	<i>Xbarc124-Xfba88</i>	35.7	29.7	1.856	0.603
II	<i>Xfba88-Xfba272</i>	9.5	33.5	14.253	0.0026*
III	<i>Xfba272-Xcdo405</i>	17.1	21.8	0.244	0.9702

<sup>1</sup> *P*-values are obtained by testing linkage heterogeneity between marker pairs using the *G*-test statistic. Significant values are indicated by asterisk.

# CHAPTER 3 - TARGETED EST MAPPING AND MACROCOLINEARITY ANALYSIS AT THE TENACIOUS GLUME LOCUS OF WHEAT WITH RICE

## Abstract

Threshability in wheat is an important agronomic and domestication trait. The *tenacious glume* (*Tg*) gene is the major determinant of threshability in hexaploid wheat. *Tg* is an incompletely dominant gene that has been located on chromosome 2DS using wheat microsatellite and RFLP markers in a 8.1 cM interval. In the present study we targeted *Tg* to a 4.9 cM interval using physically mapped wheat ESTs from the distal most deletion bin of chromosome 2DS. The closest flanking EST marker, *XBE518031* was placed 2.7 cM proximal to *Tg*. In order to utilize the rice genomic sequence information for isolating *Tg*, we studied the wheat-rice synteny in the *Tg* region. Synteny was found to be conserved in the chromosomal region distal to *Tg* whereas the colinearity between rice and wheat broke down around the *Tg* region indicating the possibility of some chromosomal rearrangements in wheat with respect to rice. BAC contigs in the *Tg* region in D-genome as well as in the newly sequenced *Brachypodium distachyon* genome were identified. For precise determination of the chromosomal region harboring *Tg* locus, free-threshing mutants were generated in the non-free-threshing synthetic wheat background. Seven independent free-threshing mutants for *Tg* gene in two diverse synthetic wheat backgrounds were obtained. Characterization of mutants revealed a range of informative genotypes, which included variable size deletions and point mutations. Possibility of generating more markers in *Tg* region and strategy to isolate *Tg* has been discussed.

## Introduction

The domestication of cultivated plant species from their wild ancestors represents one of the most important events in human history, allowing early human populations access to greater food resources than were previously available (Diamond 2002). Cultivation of major cereals, the world's primary food, likely started about 10,000 years ago (Tanksley and McCouch, 1997; Salamini et al. 2002; Tanno et al. 2006). These food crops were domesticated from wild species during the transition from nomadic hunter-gatherers to life in agrarian societies. During domestication ancient humans subjected several key traits to selection which included increase in number of seeds, change in seed size and shape, change in plant architecture, adaptation to different photoperiods, loss of seed dormancy and loss of seed shattering (Konishi et al. 2006).

The genetic changes responsible for traits that differentiate domesticated plants from their wild ancestors are referred to as the domestication syndrome (Hammer 1984 as cited by Doebley et al. 2006). In wheat, as in other cereals, a primary component of this syndrome was the loss of spike shattering, preventing the grains from shattering by wind and facilitating harvesting. The chromosome location of genes controlling shattering in diploid wheat, *Triticum monococcum* is unknown but in tetraploid wheat, *T. turgidum* subsp. *durum*, shattering is determined by the *Br* (brittle rachis) loci on short arms of chromosomes 3A and 3B (Nalam et al. 2006).

Another important trait for wheat domestication was the loss of tough glumes, converting hulled wheat into free-threshing hullless forms (Salamini et al. 2002; Dubcovsky and Dvorak, 2007). All the wild wheats are non-free-threshing as the hard glumes enclosing seeds in the spikelet protect them from any physical damage in the natural environments. During the domestication of wheat from wild to cultivated form, early farmers selected for the free-threshing variants in the populations so as to ensure easy recovery of grain. Selection for free-threshing forms happened independently at every ploidy level in wheat. However, the free-threshing forms of *Triticum monococcum* did not gain much importance due to the negative pleiotropic effect of soft glume trait on spike compactness and low fertility (Salamini et al. 2002; Pozzi et al. 2004; Dubcovsky and Dvorak, 2007).

The primary determinants of free-threshing habit in wheat are recessive mutations at the *Tg* (tenacious glume) loci (Jantasuriyarat et al. 2004) accompanied by modifying effects of the dominant mutation at the *Q* locus and mutations at minor loci (Simonetti et al. 1999;



Jantasuriyarat et al. 2004). Recent cloning of the *Q* gene, which also controls several other traits including spike type, spike shape, culm height and glume keeledness (Faris et al. 2003), showed that it encodes an AP2-like transcription factor (Simons et al. 2006) which are commonly involved in regulating inflorescence related traits. The mutation that gave rise to the dominant *Q* allele is the same in tetraploid and hexaploid free-threshing wheats, suggesting that *Q* only originated once most likely in a tetraploid, which upon hybridization to *Aegilops tauschii* produced hexaploid, *T. aestivum* which over time evolved into modern day free-threshing durum and bread wheats (Faris et al. 2005).

The tenacious glume gene in hexaploid wheat, *Tg* was derived from *Ae. tauschii*. It is partially dominant to *tg* and the plants with genotype *Tgtg* have intermediate threshability. These genetic loci on group-2 chromosomes are thought to interact with *Q* gene to produce free-threshing forms. In hexaploid wheat, the *Tg* gene is known to inhibit the expression of *Q* in synthetic hexaploids (Kerber and Rowland 1974). Thus the recessive gene *tg* as well as *Q* must be present for complete expression of free-threshing trait in the hexaploid wheats (Kerber and Rowland 1974). The *Tg* related genes in wheat have been assigned to the short arm of homoeologous group-2 chromosomes (Kerber and Rowland 1974; Simonetti et al. 1999; Taenzler et al. 2002; Jantasuriyarat et al. 2004; Nalam et al. 2007; Sood et al. unpublished results). However, similar to the shattering genes of wheat (Li and Gill 2006), there seems to be some evidence that *Tg* homoeoalleles on group-2 chromosomes also do not occupy orthologous positions on the homoeologous chromosomes (Sood et al. unpublished results, chapter 2). Furthermore, the wheat orthologues of *Tg* related genes in other cereals such as barley and maize also do not map in the genomic locations syntenic to *Tg* genes (Sood et al. unpublished results) and thus challenging the convergent evolution of domestication loci in grass genomes (Paterson et al. 1995).

The *Tg* gene has been mapped to short arm of chromosome 2D in hexaploid wheat in studies involving cultivated and synthetic wheat crosses (Kerber and Dyck 1969; Jantasuriyarat et al. 2004; Nalam et al. 2007; Sood et al. unpublished results). A likely presence of a paralog of *Tg* on the short arm of chromosome 2D has also been suggested (Nalam et al. 2007). Later studies have targeted *Tg* to the distal most chromosome bin on 2DS and suggested the feasibility for its isolation using map-based cloning (Nalam et al. 2007).

Expressed sequence tags (ESTs) are an important genomics resource in large genome crops like wheat where genome sequence information is not yet available. More than one million ESTs from wheat and related species have been generated by the national science foundation (NSF) funded wheat EST project and other public and private entities so far ([http://www.ncbi.nlm.nih.gov/dbEST/dbEST\\_summary.html](http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) updated October 3, 2008). Furthermore, by using the wheat chromosome deletion lines, more than 16,000 ESTs were localized to specific chromosome deletion bins by the NSF EST project (Qi et al. 2004). Although the wheat genome has not yet been sequenced, the availability of the EST resource has greatly advanced genomic research in wheat. These ESTs have proven to be a valuable resource for genome analysis, identification of candidate genes for traits of interest, predicting biological function of genes and comparative genomics (Lu and Faris, 2006). By using wheat ESTs for the comparative mapping studies, genomic regions of interest in wheat were targeted to specific chromosome segments (Liu and Anderson 2003, Distelfeld et al. 2004) which further led to the map-based isolation of those genes (Uauy et al. 2006).

The availability of molecular markers and molecular maps in plant genomes allowed researchers to conduct comparative mapping studies, which involve the comparison of order and content of genes and molecular markers along chromosomes of related species. In grasses, it has been shown that there is a great degree of genetic colinearity at the chromosome (macro) level (Gale and Devos 1998; Devos and Gale 2000). Early studies comparing marker colinearity among members of the Poaceae including wheat, rice, barley, maize, rye and oats revealed remarkable conservation at chromosome level across these cereal genomes (Ahn et al. 1993; Moore et al. 1995; Van Deynze et al. 1995 a, b; Devos & Gale 2000). While the genomes of closely related species barley and wheat were colinear across most of the genome, those of more distantly related grasses such as rice, maize and oats could be divided into homologous linkage blocks that corresponded to segments of barley and wheat genomes (Ahn et al. 1993; Van Deynze et al. 1995 a, b).

With its compact genome (~400Mb, Yu et al. 2002) and high chromosome level conservation among other cereals such as wheat and barley, rice has been used extensively as a model genome for structural and functional characterization of genes in large genome grasses (Feuillet and Keller 2002). The availability of genome sequence of rice (International Rice Genome Sequencing Project 2005) further allowed specific sequence level comparisons of rice

with wheat and barley (Faris et al. 2008). Whereas many studies reported the occurrence of multiple rearrangements in gene order and content due to deletions, duplications and other rearrangements (Bennetzen 2000; Feuillet and Keller 2002; Li and Gill 2002; Sorrells et al. 2003; Francki et al. 2004; Lu and Faris 2006) others indicated good levels of conservation in syntenic regions between wheat and rice (Yan et al. 2003; Chantret et al. 2004; Distelfeld et al. 2004; Mateos-Hernandez et al. 2005; Valarik et al. 2006; Griffiths et al. 2006; Kuraparthi et al. 2008a, b). Hence, it has been suggested to practice caution while using rice genome as an anchor to isolate genes in large genome cereals like wheat and barley (Bossolini et al. 2007). Nonetheless, the rice genome sequence has been successfully used as a tool for map-based cloning of genes in wheat in several instances (Yan et al. 2003; Distelfeld et al. 2004; Uauy et al. 2006). Additionally the colinear regions of rice have also been used to generate markers for saturation and high-resolution mapping of target genes in wheat (Liu and Anderson 2003; Distelfeld et al. 2004; Valarik et al. 2006).

Mutants are another important genetic resource that has always been an important raw material for genetic and genomic studies involving gene characterization, isolation validation and function analysis. Various mutagens such as fast neutrons, gamma radiation or ethyl methanesulfonate (EMS) have been used to generate mutants in genome-wide or gene-specific mutagenesis studies in wheat (Roberts et al. 1999; Slade et al. 2005; Spielmeyer et al. 2008). However, alkylating agents, especially EMS have been very effective in generating specific mutants with small deletions or point mutations (Greene et al. 2003) because they form adducts with nucleotides, causing them to mispair with their complementary bases thus introducing base changes after replication (Haughn and Sommerville, 1987). EMS mutagenesis results in high point mutational densities with only low levels of chromosomal breaks that would cause aneuploidy, reduced fertility and dominant lethality (Greene et al. 2003). Several recent studies have demonstrated the usefulness of mutants containing deletions and point mutations for the locus under study in the large and polyploid genome of wheat. By using the interstitial deletion mutants, the *Lr34/Yr18* region in hexaploid wheat was physically targeted to a genetic interval of 0.5 cM (Spielmeyer et al. 2008). The pairing homologue (*Ph1*) gene of wheat was also targeted to a 140 kb section of the syntenic region in rice with the help of deletion mutants lacking *Ph1* gene (Griffiths et al. 2006). The sequence analysis of several mutants with point mutations helped in validation of *Q* gene of wheat (Simons et al. 2006).

Previously we reported on the molecular mapping of tenacious glume gene, *Tg* in hexaploid wheat using microsatellite and RFLP markers. The *Tg* gene was targeted to 8.1 cM interval on short arm of chromosome 2D (Sood et al. 2009, chapter 2). Here, we report on enrichment of the *Tg* region with more markers using physically mapped ESTs and comparative study of colinearity between rice and wheat in the *Tg* region.

## **Material and Methods**

### ***Plant material***

For mapping the EST markers, an F<sub>2</sub> population of 103 individuals derived from a cross between Canthatch (TA2987) and synthetic wheat (TA3419) was used. This population was advanced to the F<sub>6</sub> generation (118 individuals) using the single seed descent method. The F<sub>6</sub> population was used to construct homozygous free-threshing and non free-threshing bulks for marker analysis. The F<sub>6</sub> population segregated as 62 non free-threshing and 52 free-threshing plants which gave a good fit for single gene segregation ( $\chi^2=0.876$ ,  $P=0.35$ ). Additionally, another F<sub>2</sub> population of 2320 plants segregating for D-genome loci was used to identify recombinants in the *Tg* region. This population was derived by crossing a bread wheat cultivar Prelude (TA2988) with synthetic wheat (TA8051). The synthetic allohexaploid was derived by crossing extracted tetraploid Prelude (TA3359; AABB genome; 2n=4x=28) with *Aegilops tauschii* (TA1604; DD genome; 2n=2x=14) at WGGRC, Manhattan, KS. Approximately 360 free-threshing recessive F<sub>3</sub> plants derived from the TA2988 X TA8051 population were used for fine-mapping of the *Tg* gene.

### ***Ethyl methanesulfonate treatment***

Loss-of-function mutants are essential to verify function and expression of cloned genes. The chemical mutagen, EMS (Ethyl methanesulfonate) is known to produce point mutations and small deletions (Chang et al. 1993). Such mutants form the ideal material to target genes to a specific physical region on the chromosome and to study the functional domains within the gene. Therefore, we designed an EMS mutagenesis screen of non free-threshing synthetic wheat genotypes (TA3419, TA3418) to isolate free-threshing mutants in the synthetic wheat

background. EMS mutagenesis procedure was modified from Williams et al. (1992). Approximately 1500 seeds of TA3419 and 1000 seeds of TA3418 were pre-soaked in 0.05 M phosphate buffer ( $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ ) for 8 hours and then treated with 0.40% and 0.35% (v/v) solution of EMS in the same buffer for TA3419 and TA3418, respectively, for 16 h at 20°C. Treated seeds were washed in running tap water for 1 minute to remove the excess EMS solution from the surface. Seeds were dried briefly on the paper towels and immediately followed by seeding in the root trainers with regular soil mix in greenhouse. The  $M_1$  plants were maintained at 20-23°C under supplemental lighting with 16 h light and 8h dark cycle.

Parents of *Tg* mapping populations and individual plants in  $F_2$  and  $F_6$  populations were grown in round pots (1 gallon, 6" top diameter X 7" height X 5" bottom diameter) in regular soil mix. The EMS mutagenesis populations were grown in root trainers (1" square X 6" deep) (Hummert International, Earth City, MO, USA). All plants were kept in the greenhouse with supplemental lighting for 16 h day and 8 h dark period at temperatures ranging from 18-24 °C.

### ***Phenotypic Analysis***

Threshability data of individual spikes was recorded after harvest. The method described by Kerber and Dyck (1969) was used for testing threshability. Each spike was placed in a plastic tray with a corrugated rubber bottom lining and rubbed with a wooden block covered with the same rubber material. For the mutant populations, phenotyping was done in  $M_2$  generation. In the  $M_2$  generation, twelve plants were grown per  $M_2$  family for both the mutant populations and tested for threshability. All  $M_2$  progenies were harvested individually. The spikes were hand judged for threshability while harvesting, and data for probable free-threshing plants was recorded. The threshability of these free-threshing spikes was confirmed further by using the corrugated rubber bottom trays. In the  $M_3$  generation, 6-8 plants from each free-threshing  $M_2$  plant were tested for threshability. The selected free-threshing  $M_3$  plants were selfed for another generation to grow  $M_4$  individuals.

### ***Molecular marker analysis and genetic mapping***

30-40 mg of fresh leaf tissue was collected and ground in liquid nitrogen. DNA was isolated using SDS (sodium dodecyl sulfate) method for large-scale DNA isolation as described

by Kuraparthi et al. (2007). Extracted DNA was dissolved in TE buffer and quantified using NanoDrop ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). PCR (polymerase chain reaction) amplification and agarose gel electrophoresis for wheat microsatellite markers was performed the same way as described in chapter 2.

*Tg* was physically assigned to the distal most deletion bin 2DS5-0.47-1.00 on chromosome 2DS of wheat (Sood et al. 2009; Chapter 2). We utilized the data generated by national science foundation (NSF) wheat EST physical mapping project and selected ESTs previously mapped in 2DS5-0.47-1.00 deletion bin on chromosome 2DS ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_locus.cgi](http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi), Table 3.1). Bulk segregant analysis (Michelmore et al. 1991) was used to identify ESTs closely linked to the *Tg* gene. Due to the small size of the F<sub>2</sub> population, number of free-threshing and non free-threshing homozygous individuals was less and therefore, homozygous DNA bulks were derived from two different populations. In the F<sub>2</sub> population derived from Canthatch and synthetic wheat cross, same amount of DNA from 18 homozygous individuals each were pooled to create a non-free-threshing (T1) and free-threshing (S1) bulks, respectively. In the F<sub>6</sub> population from the same cross, DNA from 24 homozygous non-free-threshing and free-threshing individuals each were pooled together to constitute bulks T2 and S2 respectively. To ensure the homogeneity of DNA samples in each bulk, DNA from homozygous individuals in F<sub>2</sub> and F<sub>6</sub> populations were quantified using NanoDrop ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Parents and the bulk DNA was digested using nine restriction enzymes (*Apa*I, *Bgl*III, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Sca*I, *Xba*I and *Xho*I) and screened for polymorphism.

### ***Linkage analysis***

Recombination values were calculated by MAPMAKER version 2.0 (Lander et al. 1987) for the Macintosh OS and a genetic linkage map was constructed based on LOD scores greater than 3.0. Map distances were calculated using the Kosambi mapping function (Kosambi 1944).

### ***Wheat-rice synteny***

Comparative genomic analysis using physically and genetically mapped ESTs with the rice BAC/PAC sequence was used to establish the synteny and macrocolinearity in the genomic

region encompassing *Tg* gene of wheat. Full length cDNA sequences or tentative consensus (TC) sequences of the mapped ESTs were extracted using The Institute of Genomic Research (TIGR) wheat gene index TaGI release 11.0 (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat>; verified October 15, 2008). These sequences were subjected to BLASTx searches against the NCBI nonredundant (nr) database. A significant match was declared on the basis of a minimum 40% amino acid identity for at least half of the length of the TC or EST sequence and an *e* value of less than  $e^{-11}$ . The hit with the lowest *e* value was considered the corresponding protein when several matches were found. TC sequences were further used to search the rice genome database (<http://rice.plantbiology.msu.edu/blast.shtml>; verified October 15, 2008) using BLASTn (Atschul et al. 1997) to identify syntenic rice BAC/PACs. In case of unavailability of TC or full-length cDNA sequence for a particular EST, the EST sequence was used as such for BLASTn searches against rice database. Sequences in the target region of rice genome were also used as queries in BLASTn searches of the wheat EST database (<http://blast.jcvi.org/euk-blast/index.cgi?project=tael>) to identify additional ESTs that are potentially linked to *Tg* gene and to develop STS (sequence tagged site) markers for enriching the *Tg* region with additional markers. TC sequences were also subjected to tBLASTx searches against rice genomic sequences using Gramene (Ware et al. 2002) for confirming the homology between wheat and rice in the *Tg* region. For BLASTn, the threshold limit for significant matches was at least 65% nucleotide identity for at least half of the TC or EST sequence but not less than 150 bases, and an *e*-value of less than  $e^{-20}$ . For tBLASTx searches, significance was declared when there was at least 40% amino acid identity over at least half of the TC sequence, but no less than 150 amino acids and an *e*-value of less than  $e^{-11}$ .

STS primer design was done using Primer3 software (<http://frodo.wi.mit.edu/>) and amplicons of 150–300 bp were selected. Polymerase chain reaction (PCR) amplifications were performed in 25  $\mu$ l reactions with 2.5  $\mu$ l of 10 $\times$  PCR buffer, 1.5  $\mu$ l of magnesium chloride (25 mM), 2.0  $\mu$ l of deoxyribonucleotide triphosphates (dNTPs) (2.5 mM each dNTP), 1  $\mu$ l each of forward and reverse primer (10 pmol/ $\mu$ l), and 100 ng of DNA in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). Primer annealing temperatures ranged from 50 to 60°C depending on the individual primer. All PCR products were resolved in 1% agarose gels with 1 $\times$  Tris-borate EDTA (TBE) buffer and visualized using ethidium bromide staining. After verifying the fragment sizes of the PCR products from EST–STS markers in agarose gels, fragments were

eluted using a NucleoTrap® Gel Extract Kit (BD Biosciences Clontech, Palo Alto, CA, USA) as per the manufacturer's instructions. Purified PCR product was quantified using a NanoDrop ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) and ligated to pGem-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturer's instructions. For all PCR products, excess salts were removed by drop dialysis using 0.025 µm dialysis membranes (Millipore, Billerica, MA, USA). The ligated mixture was transformed into competent cells of *Escherichia coli* strain DH10B (Invitrogen, Carlsbad, CA, USA) by electroporation using a Cell-Porator (Life Technologies, Invitrogen). The transformation products were then mixed with SOC medium and incubated in a shaker at 37°C for 1 h. Almost 8–15 µl of the incubated mixture was inoculated on Luria–Bertani (LB) media containing X-gal and carbenicillin. Eight to ten white colonies of each transformant were selected and grown in liquid LB containing 50 mg/ml carbenicillin for 8–12 h. Plasmid DNA was isolated from three well-grown cultures for each transformant using a Qiagen Plasmid Mini Kit (Qiagen, Santa Clarita, CA, USA) as per the manufacturer's instructions. Plasmid inserts were sequenced and positive clones were identified through sequence alignment using bl2seq of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>), and positive clones were PCR amplified using the standard M13 primer (forward: 5'-GGAAACAGCTATGACCATGA-3' and reverse: 5'-TTGTAAAACGACGGCCAGTG-3'). The PCR product was purified using QIAquick PCR Purification kit (Qiagen) as per the manufacturer's instructions. About 50 ng of purified PCR product was used for as an RFLP probe for parental polymorphism screening. All techniques of restriction enzyme digestion, gel electrophoresis, Southern transfer, probe labeling and filter hybridizations were performed as described in chapter 2.

## Results

### *Molecular mapping of Tg*

A total of fifty-eight ESTs previously mapped in the distal most deletion bin on chromosome 2DS (2DS5-0.47-1.00) were utilized for testing the polymorphism between the parents of *Tg* population. The selection for these single copy ESTs was done based on the hybridization pattern (autoradiograph images) made available through the NSF EST physical mapping project. Bulked segregant analysis (BSA) of homozygous non free-threshing and free-



threshing bulks was used to identify closely linked markers to the *Tg* gene. ESTs were examined for polymorphism among the parents and the non free-threshing and free-threshing bulks. Nineteen ESTs showed polymorphism between Canthatch and synthetic with one or more restriction enzymes (Table 3.1). Out of these, nine ESTs were mapped in the *Tg* region on chromosome 2DS (Fig. 3.1). The map of the genomic region under investigation consisted of 19 molecular markers including previously mapped six microsatellites and four RFLP markers (chapter 2) and nine ESTs (present study). The EST marker *XBE518031* identified diagnostic polymorphic alleles between the non free-threshing and free-threshing bulks (Fig. 3.2) and mapped 2.7 cM proximal to the *Tg* gene. *XBE518031* helped place *Tg* in a 4.9 cM region on chromosome 2DS (Fig. 3.1).

EST mapping in the present study further targeted *Tg* to the distal most deletion bin 2DS5-0.47-1.00 on chromosome 2DS. The closest flanking EST to *Tg*, *XBE518031* was also physically mapped in the distal most deletion bin 2BS3 (FL0.84-1.00) ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_locus.cgi](http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi)) on short arm of chromosome 2B. Because the deletion bin 2S-0.84-1.00 of consensus map corresponded to deletion bin 2BS3-0.84-1.00 of chromosome 2B (Delaney et al. 1995), the *Tg* gene was actually mapped to the distal 16% of the short arm of group-2 chromosomes.

### ***Wheat-rice synteny***

In order to leverage the rice genome sequence information for map-based cloning of the *Tg* gene, colinearity between the wheat genomic region containing *Tg* and the rice genome was studied at the macro level. Macrocolinearity was determined using one RFLP and nine EST markers spanning 35 cM and encompassing *Tg* on chromosome 2DS. Comparative genomic analysis of these genetically mapped ESTs and RFLPs with the rice genome sequences showed that, of the 10 marker sequences, only one (*XBF201415*) had no significant similarity to rice genomic sequence. BLASTx searches of the NCBI nr database using the EST and RFLP sequences in the *Tg* region revealed that all the sequences had significant similarity to known or putative proteins (Table 3.3). *XBF201415* was the only EST that detected no similar sequence in rice but was assigned a putative protein function (cytochrome P450 monooxygenase). Among the nine ESTs, five had significant homology to the colinear rice genomic sequence (Table 3.2;

Fig. 3.3) and three EST markers (*XBE518031*, *XBE490444*, *XBE517877*) including the proximal flanking marker to *Tg* (*XBE518031*), showed high homology to non-colinear rice sequences (Table 3.2, 3.3).

With the exception of *XBF201415* which did not detect a significant similarity with any rice genomic region, six markers in the distal 16.5 cM of the genetic map that detected similar rice sequences had colinearity with short arm of rice chromosome 4 (Fig. 3.3). The order of the four distal EST markers in wheat chromosome 2DS with respect to rice chromosome 4 was conserved except for the EST *XBE499212*. This EST has been physically targeted to 5.72 Mb region on the short arm of rice chromosome 4 ([http://rice.plantbiology.msu.edu/cgi-bin/ordered\\_bac.pl?chr=4](http://rice.plantbiology.msu.edu/cgi-bin/ordered_bac.pl?chr=4)). Whereas *XBE498396*, which mapped 0.5 cM distal to *XBE499212* in the genetic map on chromosome 2DS of wheat, had been physically targeted to 0.61 Mb region on short arm of rice chromosome 4 and *XBE444541* which was mapped 1.5 cM proximal to *XBE499212* in the 2DS genetic map, had been physically assigned to 0.35 Mb region on rice chromosome 4 (Fig. 3.3). Therefore, *XBE499212* seemed to have been translocated in the middle of *XBE498396* and *XBE444541* in syntenic wheat region as compared to rice. Additionally, *XBE499212* had two copies in the wheat genome physically mapped on short arm each of the of homoeologous group-2 chromosomes ([http://wheat.pw.usda.gov/cgi-bin/westsql/map\\_image.cgi?i=UMN113BE499212.jpg](http://wheat.pw.usda.gov/cgi-bin/westsql/map_image.cgi?i=UMN113BE499212.jpg)), therefore, it might be possible that the genetically mapped allele of *XBF201415* in wheat is not the true rice orthologue and in fact it represents a duplication event that happened in wheat after the divergence of the wheat and rice lineage.

The 6.9 cM region proximal to *Tg* on the chromosome 2DS genetic map was a mosaic of similarities to different rice chromosomes (Fig. 3.3). The closest proximal EST marker to *Tg*, *XBE518031*, showed similarity to rice chromosome 3 whereas the EST markers *XBE490444* and *XBE517877* (proximal to *XBF518031*), displayed sequence homology to chromosomes 10 and 7, respectively (Fig. 3.3).

At the macro level, the *Tg* locus was flanked by EST, *XBF484829* on distal side, which was syntenic to rice chromosome 4 and on the proximal side by EST, *XBE518031*, which showed similarity to rice chromosome 3 (Fig. 3.3). Therefore, the colinearity between the wheat *Tg* region and rice did not appear to be conserved at the macro level and therefore by using the

physically and genetically mapped EST markers we could not flank the *Tg* locus in the rice genome.

The EST marker *XBE518031*, which was closely linked to *Tg* in the genetic map, showed high sequence homology to the rice BAC OSJNBb0015I02 that was mapped on long arm of chromosome 3 (Table 3.3). All EST markers distal to the *Tg* locus were syntenic to rice chromosome 4 and *XBE518031* was the only closest EST marker to *Tg* on proximal side but it showed homology to rice chromosome 3. As a result, we decided to use the gene sequences within BAC OSJNBb0015I02 for further comparative mapping analysis and genomic targeting of *Tg* and in a hope to flank the *Tg* locus in rice. The rice BAC OSJNBb0015I02 is 122.8 kb and contains 20 annotated genes with putative known and unknown functions. Annotated gene sequences from this BAC were used as queries in BLASTn searches of the wheat EST database (<http://blast.jcvi.org/euk-blast/index.cgi?project=tae1>). Fourteen STS primers were designed from wheat EST sequences that showed similarity to annotated gene sequences within BAC OSJNBb0015I02 (Table 3.4, 3.5). However only nine of these primers sequences gave amplification in wheat genomic DNA (Table 3.4) and were therefore used for probe development. The parents of the F<sub>2</sub> population, TA2987 and TA3419, were tested for polymorphism using these STS primers. The parents were digested with six restriction enzymes; *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Xba*I and *Sca*I. Two probes, *XSTS-FT\_27* and *XSTS-FT\_38* gave polymorphism using one or more enzymes between TA2987 and TA3419. However, upon mapping these markers, they did not show linkage with *Tg* and therefore were not mapped on chromosome 2DS. This result further suggested the divergence of chromosomal region encompassing *Tg* in wheat relative to rice.

### ***Identification of a contig in D-genome and Brachypodium distachyon***

Although the polyploid genome of wheat has not been sequenced yet, the availability of genomic resources such as large insert bacterial artificial chromosome (BAC) libraries in progenitor genomes and physical maps of the D-genome have proven to be great resources for fine-mapping genes and generating contigs in the genomic regions of interest. The closest flanking EST marker, *XBE518031*, was used to identify a contig in the BAC based physical map of D-genome donor of wheat, *Aegilops tauschii* (<http://wheatdb.ucdavis.edu:8080/wheatdb/>). It

identified a contig ctg6299 consisting of 73 fingerprinted overlapping BAC clones of *Aegilops tauschii* with a contig length of 370 kb. The EST marker *XBE518031* identified two BAC clones, HD077D24 and HI09018 in the contig ctg6299, suggesting it might contain two copies in the genome (Fig. 3.4). These individual BAC clones will be fingerprinted and end-sequenced to generate more markers for genetic mapping to span the *Tg* locus on the distal end. Also it will help to further identify the minimum tiling path to extend the BAC contig length in the *Tg* region.

The US Department of Energy Joint Genome Institute (JGI) has recently completed a “checkpoint assembly” of the *Brachypodium distachyon* line Bd21 whole genome shotgun sequence that covers the genome to an approximate 4x depth. BLAST searches were done using the flanking EST *XBE518031* to identify a contig in the *Tg* region from the first draft of genome sequence of *Brachypodium* (<http://blast.brachybase.org/>). The EST identified a 1 kb contig region super\_3: 21931984-21932983 kb with a good level of confidence (*e*-value=1e-35). This region in *Brachypodium* genome can be exploited to develop more markers for targeted mapping in the *Tg* region in wheat and to study the colinearity with *Brachypodium* for further contig development and isolation of *Tg*.

### ***Characterization of free-threshing mutants***

Among the 1295 M<sub>1</sub> plants derived from EMS mutagenesis of synthetic wheat (TA3419), only 358 plants produced 12 or more seeds whereas among the 852 M<sub>1</sub> plants derived from EMS mutagenesis of synthetic TA3418, 351 families had more than 12 seeds. Therefore 6-12 plants from 358 M<sub>2</sub> families of TA3419 mutant population (06-41) and 351 families of Ta3418 population (06-40) were grown in fall 2006 and spring 2007, respectively, and scored for threshability at maturity. Five free-threshing mutant M<sub>2</sub> families were identified in the 06-41 mutant population (Table 3.6) and three free-threshing M<sub>2</sub> families were identified in the 06-40 mutant population (Table 3.7). About 4-6 M<sub>3</sub> plants from each free-threshing mutant were grown to maturity and tested for threshability to confirm the phenotype of the mutants. One free-threshing mutant family (07-91-956-3) derived from TA3419 was highly sterile and did not produce any seed and was not included in further analysis. All other seven free-threshing mutants belonging to four different families in the TA3419 background and five free-threshing

mutants belonging to three different families in the TA3418 background were easy to thresh and had comparatively softer glumes (Fig. 3.5; Fig. 3.6).

All the free-threshing mutants were characterized using the molecular markers genetically mapped on chromosome 2DS in the present study and in previous studies (Somers et al. 2004). One free-threshing mutant (07-91-55-3) showed deletion of most of the markers in the *Tg* region and in the region distal to *Tg* (Table 3.8). In this mutant, there appeared to be a deletion involving at least the distal 20% of the short arm of chromosome 2D including the *Tg* locus (Fig. 3.7). Three free-threshing mutants belonging to one family (07-91-1045) showed deletion of markers flanking *Tg* (*Xwmc503* and *XBE518031*) (Table 3.8, Fig. 3.7, Fig. 3.8). All other free-threshing mutants did not show any deletions for the molecular markers being tested (Table 3.8, Table 3.9, Fig. 3.9) indicating that they might contain point mutations at the *Tg* locus or that the size of the deletion is too small to be detected with the current resolution of markers flanking *Tg*.

### ***Shuttle mapping***

During the domestication process, wheat experienced several domestication bottlenecks which led to the lower variability in the cultivated wheat germplasm (Dubcovsky and Dvorak 2007). Therefore in gene mapping studies, often times it is difficult to obtain sufficient polymorphism in one population unless using highly divergent parents. Thus, many studies targeted at fine-mapping and gene isolation in wheat tend to utilize more than one mapping population (Dubcovsky et al. 1998; Huang et al. 2003; Faris et al. 2002; Bullrich et al. 2005). In order to find more recombinants in the *Tg* region to aid in fine-mapping and eventually cloning of *Tg*, we utilized an additional F<sub>2</sub> population (Prelude, TA2988 x synthetic, TA8051) segregating for D-genome related traits including threshability.

However, severe segregation distortion in the *Tg* region in this population was observed. The population segregated for threshability as 360 free-threshing plants and 1960 non free-threshing plants. The observed genetic ratio for the phenotype showed significant deviation from the expected ratios ( $P < 0.05$ ). We selected only the free-threshing recessive F<sub>2</sub> plants and decided to do the progeny testing for verifying phenotype. Six plants from each F<sub>2</sub> line were grown and scored for threshability at maturity. Most of the free-threshing F<sub>2</sub> lines stayed true to

phenotype (242 lines) but almost one third of the lines (118) showed some kind of discrepancy in the scoring of phenotype. Therefore these lines were removed from the final molecular analysis.

Four of the microsatellite markers previously mapped in *Tg* region on chromosome 2DS (present study; chapter 2, Nalam et al. 2007), *Xgwm261*, *Xgwm455*, *Xwmc112* and *Xwmc503* were polymorphic between the parents, TA2988 and TA8051, and were therefore mapped in the free-threshing recessive genotypes. Three microsatellite markers clustered within 6.0 cM on chromosome 2DS showed significant deviation from the expected segregation ratios due to deficiency of TA8051 homozygotes and heterozygotes and with an excess of TA2988 homozygotes ( $P < 0.05$ ). When genetically mapped these molecular markers gave a skewed distribution of map distances relative to each other and to the *Tg* locus than expected (Fig. 3.10).

## Discussion

In the present study, based on EST mapping, we have targeted the tenacious glume gene of hexaploid wheat, *Tg* to a 4.7 cM interval flanked by *Xwmc503* and *XBE518031*. The closest EST marker to *Tg* has been mapped 2.7 cM. By far, this is the smallest genetic interval any study has targeted for genetic mapping of *Tg* (Jantasuriyarat et al. 2004; Nalam et al. 2007). Furthermore, our results also helped target *Tg* to the distal most 16% of the chromosome arm which is estimated to be almost 0.84  $\mu\text{m}$  in length (Gill et al. 1991). This distal most region on the short arms of group-2 chromosomes also showed high density of physically mapped EST compared to the proximal neighboring region (Conley et al. 2004) indicating that it is a gene-rich region and therefore *Tg* should be amenable to map-based cloning

The important aspect of the present study is the analysis of macrocolinearity in the *Tg* region of wheat relative to rice. The presence of large blocks of collinear markers among different grass sub-families have well-established the conserved synteny between wheat and rice (Ahn et al. 1993; Gale and Devos 1998; Devos and Gale 2000; Keller and Feuillet 2000; Moore et al. 1995; Van Deynze et al. 1995a, b). The conservation of gene order within the grass family provides a unique opportunity to transfer information from the completely sequenced genome of rice to other grass species. The comparative analysis of wheat and rice genomes have been investigated several times by studying the syntenic relationship of wheat ESTs with the rice genomic sequence (Sorrells et al. 2003; Conley et al. 2004; Franki et al. 2004; Hossain et al.

2004; La Rota and Sorrells 2004; Linkiewicz et al. 2004; Munkvold et al. 2004; Peng et al. 2004) but in all these cases there was no prior information about the genetic order of ESTs within the deletion bins, where the use of genetically resolved EST loci within chromosome bins can reveal more information regarding the length and organization of syntenic units between wheat and rice. In the present study, comparative mapping using nine genetically mapped ESTs spanning 35 cM allowed us to determine the macro level colinearity between wheat and rice in the genomic region of tenacious glume gene of wheat on chromosome 2DS.

Genome synteny is much more complicated than previously thought (for a review, see Delseny 2004). In general, colinearity among the wheat genomes is better in the proximal regions of the chromosomes than in the distal regions (Akhunov et al. 2003a). The ends of chromosomes seem to be particularly rich in colinearity exceptions. This increase seems to be associated with the higher gene density and higher rates of recombination observed in the telomeric regions of the large genomes of the Triticeae species (Akhunov et al. 2003b). High recombination rates were also associated with a higher frequency of colinearity interruptions among wheat homoeologous chromosomes in the distal regions relative to centromeric regions (Akhunov et al. 2003a). Comparative genomic analysis at the whole-genome level between wheat and rice has indicated an increase in the divergence of gene sequences physically located at or near the telomeric ends of wheat chromosomes (See et al. 2006). However, the wheat homoeologous group-2 chromosomes have been shown to be highly conserved relative to syntenic rice genomic regions (Sorrells et al. 2003; La Rota and Sorrells 2004). The group-2 chromosomes especially chromosome 2DS of wheat is syntenic to two rice chromosomes where distal part of wheat 2DS is syntenic to chromosome 4 of rice and the proximal regions are syntenic to rice chromosome 7 (Sorrells et al. 2003; Conley et al. 2004). Similar results were obtained in the present study. The synteny between rice and wheat in the *Tg* region was conserved especially in the distal regions of the chromosome. Except for one EST, *XBE499212* which showed duplication in wheat relative to rice, all other ESTs distal to *Tg* were syntenic to chromosome 4 of rice. However, some local rearrangements were observed in wheat relative to rice in the region proximal to *Tg* where the closest proximal EST to *Tg*, *XBE518031* was syntenic to chromosome 3 of rice and the EST proximal to *XBE518031* was syntenic to chromosome 10 of rice. Furthermore, two of polymorphic STS markers derived from the BAC

syntenic to *XBE518031* did not show linkage to *Tg*. Hence, there is a strong possibility that the *Tg* region is highly diverged in wheat relative to rice.

Wheat and rice diverged ~50 million years ago from a common ancestor (Paterson et al. 2004) and since that time they have evolved independently. Furthermore *Tg* like gene has not been characterized in rice. Additionally, it has already been established that even *Tg* and *sog*, two of the threshability genes located in homoeologous genomes of wheat itself are not orthologues and also *Tg*-like genes of wheat are non-orthologous to related genes in other cereals like barley and maize (see chapter 2). In this case therefore, it is likely that *Tg* is specific to wheat lineage and originated after divergence of wheat and rice. Another possibility could be the presence of extensive rearrangements including duplications, deletions, inversions and translocations etc. in the *Tg* region of wheat relative to rice. A similar example was demonstrated in a study on tandem repeats of the glutathione *S*-transferase genes in wheat mapped on wheat group-6 chromosomes. The orthologue of the most-conserved copy of the glutathione *S*-transferase gene was found on rice chromosome 10 rather than chromosome 2, which had previously been identified as the most syntenic region for wheat chromosome 6 (Xu et al. 2002). A similar example of duplication and deletion events in *Sh2/Al* region was provided by a comparative analysis among different grass genomes (Li and Gill 2002). The genetic map of EST markers on chromosome 2DS and its anchoring to the rice genomic sequence is the first step in characterizing this important region in cereals

Recently, a wild cereal, *Brachypodium*, has been proposed as a new model plant for structural and functional genomics of temperate cereals because of its small genome and the phylogenetic position between rice and *Triticeae* crops (Bossolini et al. 2007). The availability of BAC libraries (Foote et al. 2004; Gu et al. 2006; Hasterok et al. 2006), ESTs (Vogel et al. 2006) and genome sequence (<http://www.brachypodium.org/>) has made it an attractive model species for colinearity studies in wheat. Whereas high level of macrocolinearity has been observed between *Brachypodium* and wheat at the *Ph1* region (Griffiths et al. 2006) and *Lr34* region (Bossolini et al. 2007), the microcolinearity studies have found mixed results. Perfect gene level conservation between *Brachypodium* and wheat was found at the *Ph1* region of wheat (Griffiths et al. 2006) while the *Q* gene region in wheat was more conserved between wheat and rice compared to *Brachypodium* (Faris et al. 2008). The identification of a syntenic region in *Brachypodium* is the first step towards utilizing this genomic resource. In the present study, we



identified a contig region in the *Brachypodium* genome for the *Tg* region of wheat. The wheat D genome is the smallest of the three wheat genomes (4.5 pg/c) and is equivalent to the genome of its progenitor *Aegilops tauschii*. We have also identified a contig in the *Tg* region in D-genome of *Ae. tauschii*. Due to the smaller genome size, simple inheritance and availability of large genomic resources in diploid progenitor species of wheat, they have been successfully used as vehicles for cloning the genes in polyploid wheat (Huang et al. 2003; Faris et al. 2003; Feuillet et al. 2003). Hence, the availability of the BAC library for the D-genome (Akhunov et al. 2005) and the *Brachypodium* genome resource will provide a starting point to generate more markers in the *Tg* region in order to fine-map and clone the *Tg* locus in wheat.

The distal region on the short arm of chromosome 2D of wheat contains many genes related to important agronomic and domestication traits. The dwarfing gene, *Rht8* that is known to be highly effective in many Mediterranean countries (Worland and Law 1986 as cited by Korzun et al. 1998) has been mapped on chromosome 2DS (Korzun et al. 1998). *Rht8* is gibberellic acid sensitive and has a easily scorable phenotype unlike the more popular ‘Norin 10’ genes for reduced height, *Rht1* and *Rht2* (Korzun et al. 1998). The *Rht8* gene has been mapped 0.6 cM proximal to microsatellite marker *Xgwm261* on chromosome 2DS in a genetic map of bread wheat cultivar ‘Cappelle-Desprez’ and ‘Cappelle-Desprez with the 2D chromosome substituted by another cultivar ‘Mara’ (*Rht8* carrier) (Korzun et al. 1998). Where *Tg* is mapped 2.7 cM proximal to *Xgwm 261* in the genetic map developed in present study. Another important agronomic gene mapped in the same region is the leaf rust resistance gene, *Lr22*. The *Lr22* gene represents one of the ‘undefeated’ rust resistance genes for which virulence has not been reported so far (Hiebert et al. 2007). *Lr22* has been mapped 2 cM proximal to a microsatellite marker *Xgwm296* on chromosome 2DS in an F<sub>2</sub> population (Hiebert et al. 2007), and *Tg* is mapped 6.7 cM proximal to *Xgwm296* in our genetic map.

Another gene in vicinity of *Tg* is the domestication-related semi-dominant gene, *Ppd-D1* which is the major source of the photoperiod insensitivity in wheat (Beales et al. 2007). In a comparative mapping study, *Ppd-D1* was mapped more than 30 cM proximal to RFLP marker, *Xpsr130* (Borner et al. 1998) which has been mapped almost 47 cM proximal to *Tg* gene (Chapter 2). Furthermore, it has been suggested that *Ppd-D1* gene of wheat and *Ppd-H1* gene of barley lie in syntenic regions, although even after the recent cloning of the barley homeologue (Turner et al. 2005), *Ppd-D1* has not been isolated yet (Beales et al. 2007). Thus, in a 10-15 cM

interval surrounding *Tg*, three agronomically important genes have been mapped on 2DS, especially *Lr22* and *Rht8* genes (~4.0 cM) Therefore, the extensive genetic mapping in *Tg* region in the present study will benefit studies aimed at isolation of these genes. The presence of several important genes in the vicinity of *Tg* also suggests that it is a highly important genomic region and seems to be directly involved in domestication and superior agronomic selection during the evolution of wheat. Co-localization of domestication related genes has also been reported in other cereals like rice, where seed shattering, red pericarp and seed dormancy genes are closely linked and constitute a domestication block (Ji et al. 2006). Hence, the cloning of cluster of domestication related genes will offer new insights into the identity, function and evolution of genes contributing to domestication syndrome in cereals.

Segregation distortion is the deviation of observed genetic ratios from the expected Mendelian ratios of a given genotypic class within a segregating population. Distorted segregation ratios may result from gametophytic competition resulting in preferential fertilization, or abortion of the male or female gametes or zygotes (Lyttle 1991). Distorted segregation ratios of genetic markers have been often observed in progeny of inter and intra specific hybrids (Lyttle 1991). Mechanisms of segregation distortion have been less studied in plants as compared to human and other animal systems, however genomic regions harboring markers with segregation ratios that do not conform to the expected Mendelian ratios are encountered often and have been reported in many crop species. Deviations from expected Mendelian ratios have been observed in maize (Bentolila et al. 1992; Pereira et al. 1995), barley (Graner et al. 1991; Heun et al. 1991), pearl millet (Liu et al. 1996) and rice (Causse et al. 1994; Xu et al. 1997). We also observed segregation distortion in one of the populations being used for identification of recombinants in *Tg* region. Since this population represents a wide cross and is segregating for D genome only. It is suggestive that the segregation distortion loci are located on the D genome. Several segregation distortion loci have been reported in the genome of diploid progenitor of wheat, *Ae. tauschii*. These loci were mapped to chromosomes 1D, 3D, 4D and 7D (Faris et al.1998). The exact mechanism of segregation distortion is not known but it has been suggested that preferential transmission of one of the gametes due to gametophyte incompatibilities or the action of gametocidal chromosomes in wheat (as reported for other *Aegilops* species; Mann 1975; Endo 1990) is possible. Because of the presence of segregation

distortion in the *Tg* region in TA2988 and TA8051 derived population, we were unable to utilize it for further study on fine-scale mapping of *Tg*.

The first step towards isolating *Tg* will require identification of more markers and their genetic and physical mapping in the *Tg* region in order to saturate the region. The already identified BAC clones in the D genome contig in this study will be a starting point. BAC end sequencing for selected BACs identified by closely linked EST marker *XBE518031* can generate enough information to design more markers and map them in *Tg* region. Using this approach a contig spanning *Tg* in the D-genome can be developed which will help towards its isolation. Otherwise, genomic information from *Brachypodium* can also be utilized. The non-polymorphic and unmapped ESTs from the distal most deletion bin on chromosome 2DS can be utilized to design additional primers and generate more markers for fine-mapping *Tg*. The STS markers thus generated can help flank *Tg* region in rice. Additionally, AFLP markers can be used to identify tightly linked markers in *Tg* region. In order to do the fine-mapping, a much larger segregating population will be required. For this purpose, the RIL population from TA2987 X TA3419 cross can be used. Additionally, there is an F<sub>2</sub> population available for another cultivated X synthetic cross (TA2987 X TA3418) which can be used to get additional polymorphism for markers in *Tg* region. The recombinants in this population in the *Tg* interval can be identified using the flanking markers and can be used for fine mapping the *Tg* region. Furthermore, the availability of free-threshing mutants in two different synthetic wheat backgrounds with variable deletion sizes will be extremely useful for physical targeting of *Tg* to a smaller interval and for further fine-mapping studies. The free-threshing mutants with point mutations will be highly useful for validation and functional analysis of *Tg* after its cloning.

*Tg* is an important domestication gene and its cloning and characterization will definitely aid towards increasing our knowledge about the molecular nature of domestication genes. *Tg* is known to interact with *Q* gene to determine the free-threshing phenotype in hexaploid wheat and since the *Q* gene has already been cloned (Simons et al. 2006), it becomes highly important to isolate *Tg* in order to study its epistatic interaction with *Q* at the molecular level. Majority of the domestication genes isolated so far belong to different families of transcription regulators (Doebley et al. 2006), therefore, it is plausible that *Tg* is also a transcription regulator and it functions upstream of *Q* in the developmental pathway leading to free-threshing phenotype. Recently the first physical map of longest wheat chromosome became available (Paux et al.

2008). Hence, with the improved genomic technologies, the sequencing of large genome crops like wheat is not too far in future. All the genomic resources available in wheat and the comparative genomic information among different plant genomes will hopefully help in isolating this important agronomic and domestication gene in wheat.

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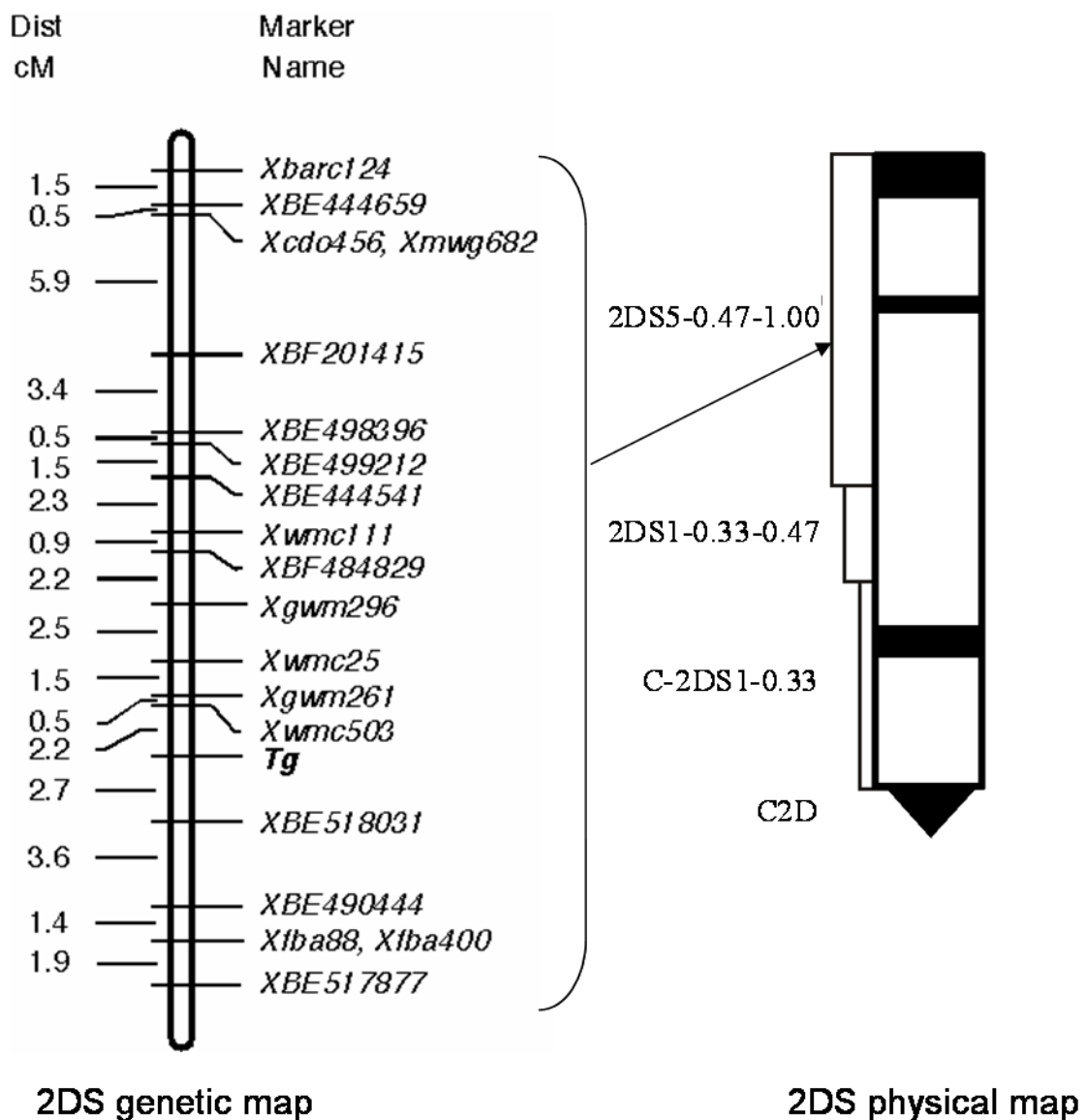
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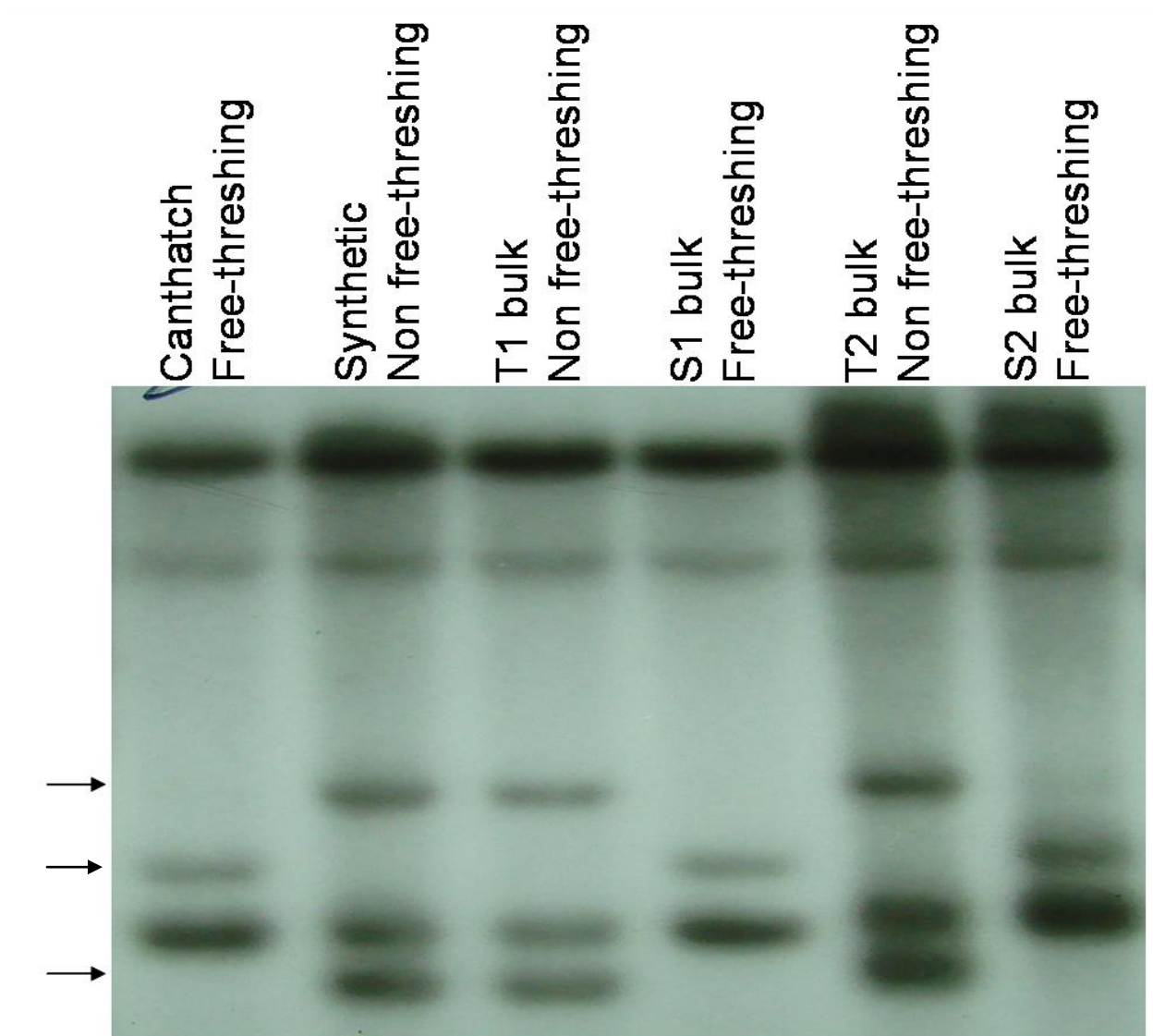
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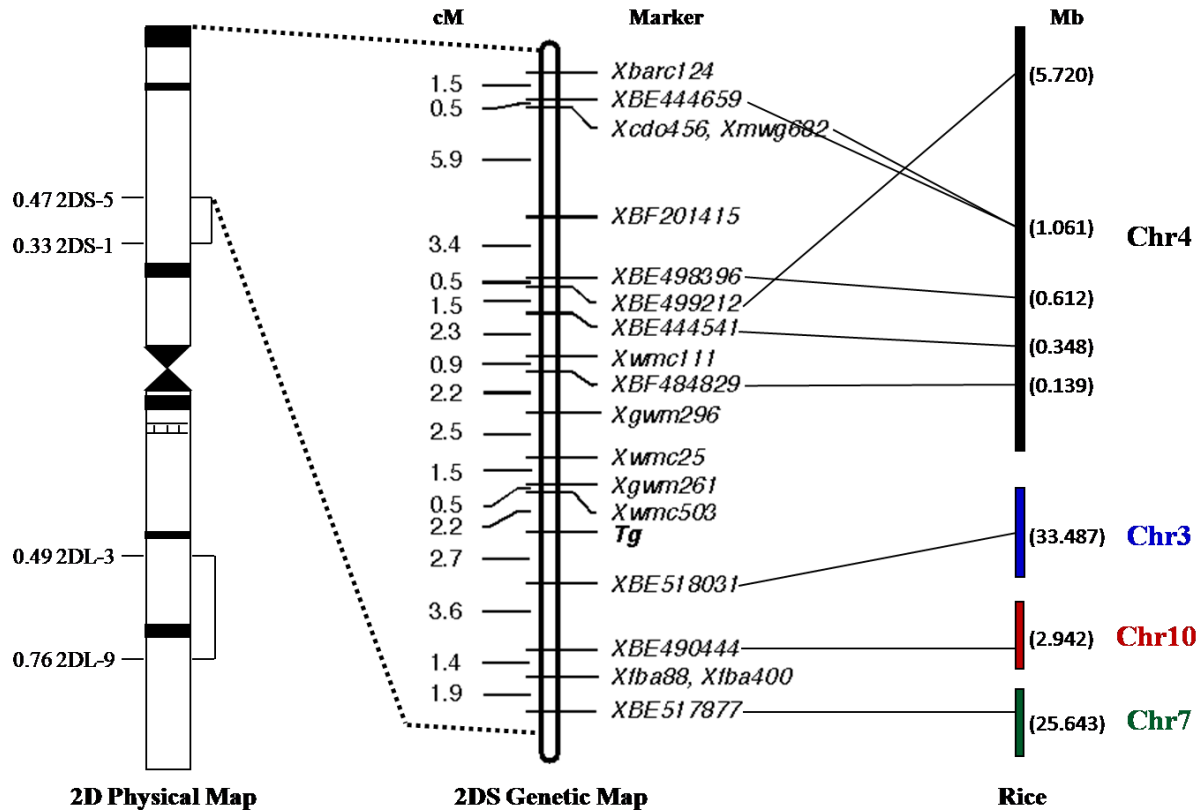
**Figure 3.1** Genetic and physical targeting of *Tg* gene using wheat ESTs in an F<sub>2</sub> population of 103 individuals derived from Canthatch X synthetic wheat cross. The maps are oriented top to bottom from telomere towards centromere. Genetic map distances are in cM. Cosegregating markers are separated by comma on the genetic map. Threshability locus, *Tg* is represented in bold. Each section of physical map represents a bin delimited by deletion breakpoints expressed as fraction of the arm length from the centromere. The breakpoints of various deletions, along with their fraction length (FL) values, are marked by arrows on the left of the physical map



**Figure 3.2** Molecular mapping of tenacious glume gene *Tg*, in hexaploid wheat using bulk segregant analysis. Southern hybridization pattern of *Eco*RI digested genomic DNA of parents, and bulks from homozygous free-threshing and non free-threshing  $F_2$  individuals (18 plants per bulk) and  $F_6$  individuals (24 plants per bulk) derived from Canthatch X synthetic cross using the probe BE518031. The RFLP markers diagnostically polymorphic between non free-threshing and free-threshing bulks are indicated by arrows.

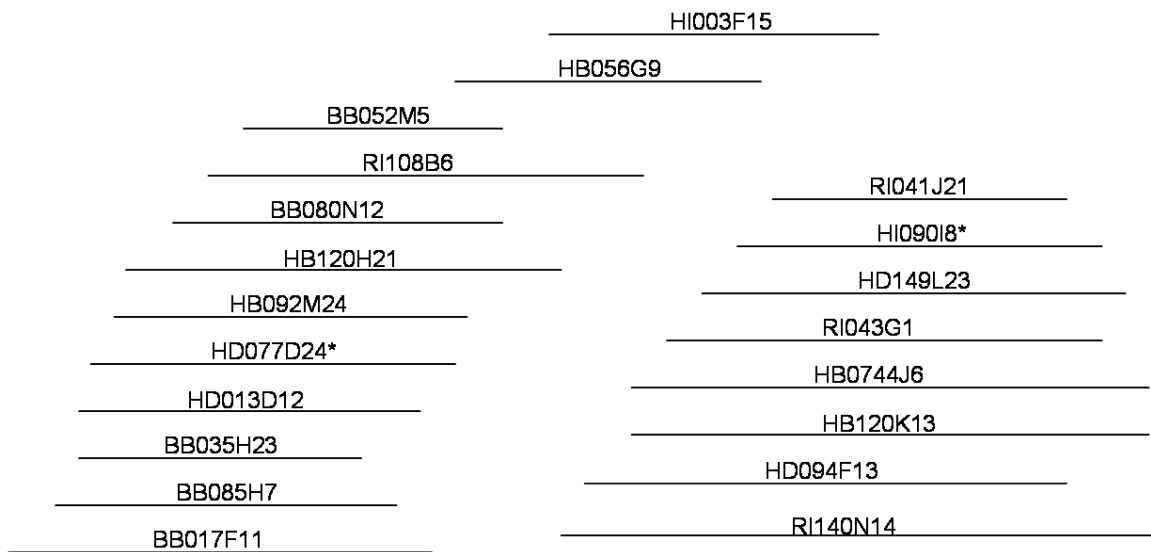


**Figure 3.3** Comparative genomic analysis of the genetically mapped wheat EST markers encompassing the *Tg* region with physical map of rice chromosomes. The genetic map of chromosome 2DS is oriented from telomere towards centromere. A physical map of the wheat chromosome 2D is shown on the left for reference of the genomic region evaluated in this study. Rice chromosomes harboring sequences and significant similarity to ESTs are indicated to the right and the megabase positions of the significant hits along the rice chromosomes are indicated in parentheses.





**Figure 3.4** Identification of a D-genome contig showing overlapping BACs for physical mapping of *Tg* using *Ae. tauschii* physical map. The BACs with asterisk were identified by flanking EST marker *XBE518031*.



**Figure 3.5** Spike phenotypes of free-threshing mutants of synthetic wheat, TA3419. The spikes are in following order, TA3419, 07-91-55-3-1, 07-91-111-3-3, 07-91-111-12-1, 07-91-593-3-6, 07-91-1045-6-4, 07-91-1045-7-3 and 07-91-1045-11-2

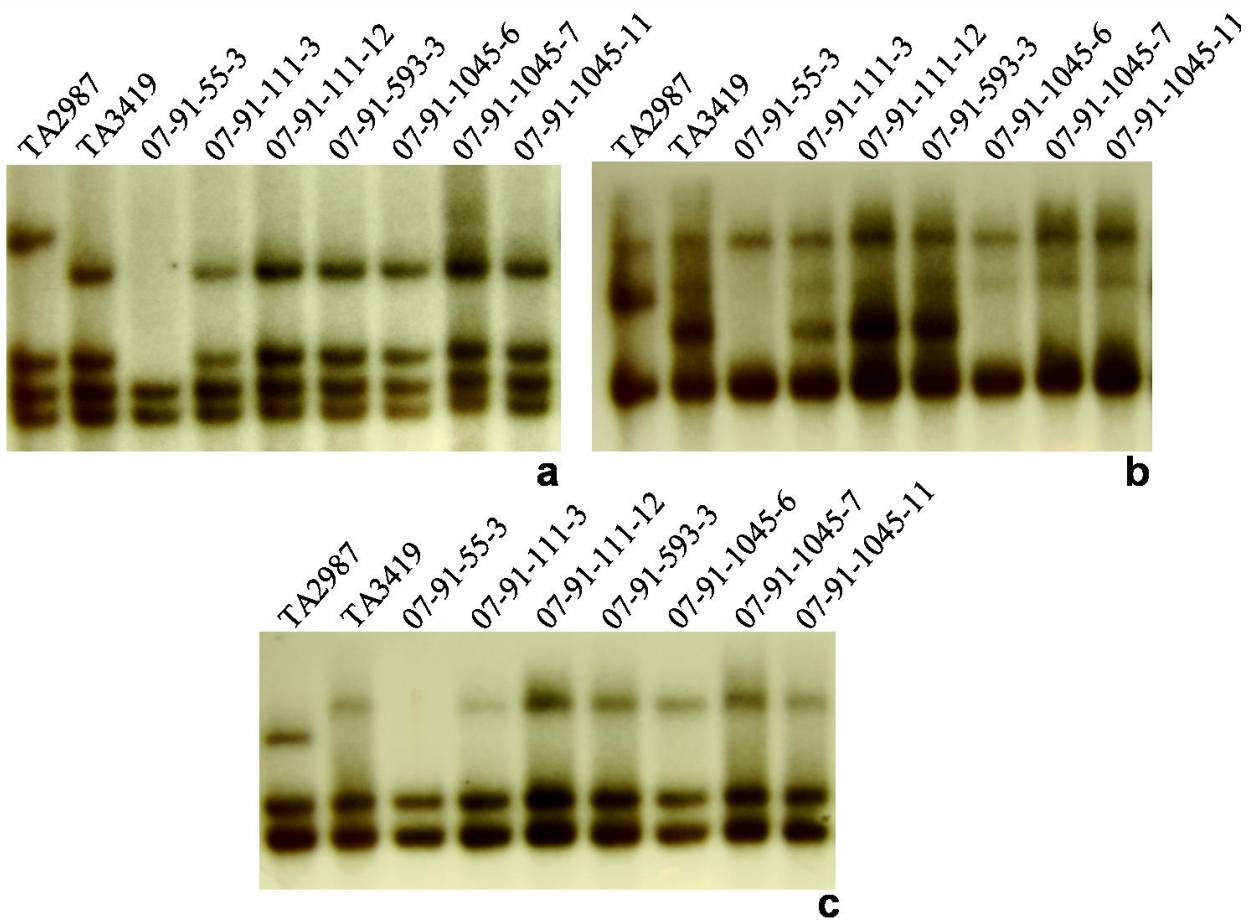


**Figure 3.6** Spike phenotypes of free-threshing mutants of synthetic wheat, TA3418. The spikes are in following order; TA3418, 07-350-507-2C, 07-350-507-3F, 07-350-519-2A, 07-350-519-3F and 07-350-624-8A

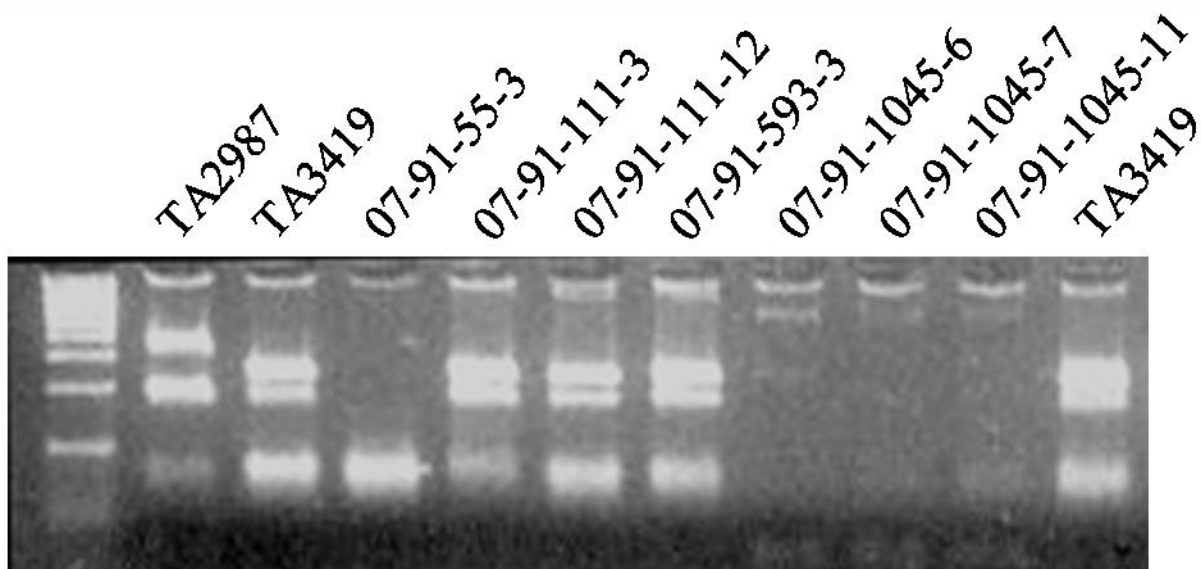


**Figure 3.7** EST analysis of free-threshing mutants derived from synthetic wheat, TA3419

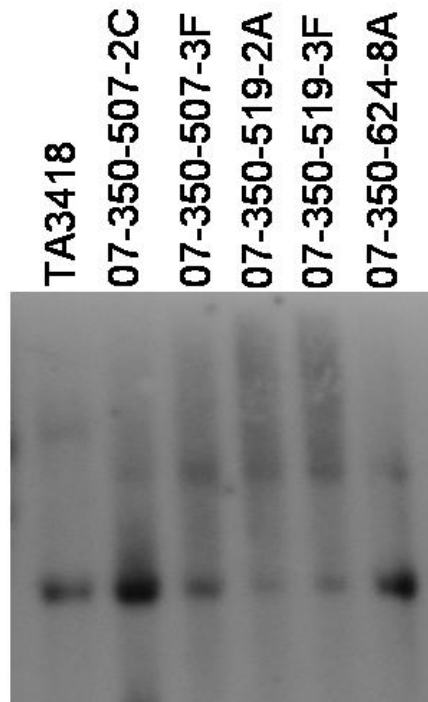
- a. Southern hybridization pattern of *EcoRV*-digested genomic DNA of free-threshing cultivar, TA2987, TA3419 and the free-threshing mutants in TA3419 background using probe BE444541 mapped 12.1 cM distal to *Tg*
- b. Southern hybridization pattern of *EcoRI*-digested genomic DNA of free-threshing cultivar, TA2987, TA3419 and the free-threshing mutants in TA3419 background using probe BE518031 mapped 2.7 cM proximal to *Tg*
- c. Southern hybridization pattern of *HindIII*-digested genomic DNA of free-threshing cultivar, TA2987, TA3419 and the free-threshing mutants in TA3419 background using probe FBA88 mapped 7.7 cM proximal to *Tg*



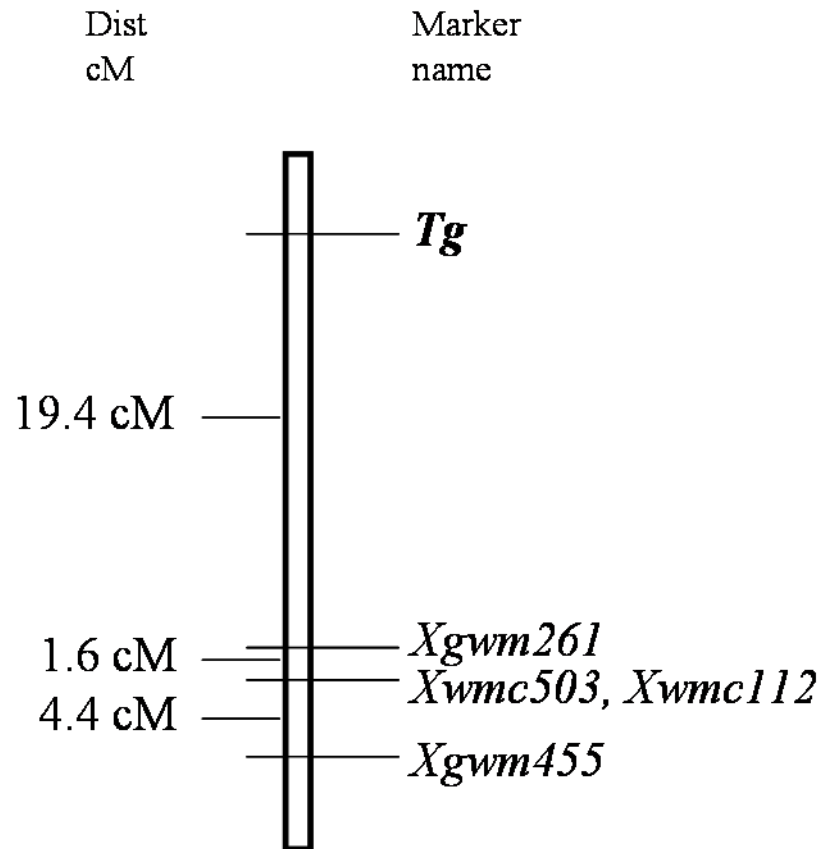
**Figure 3.8** Microsatellite marker analysis of free-threshing mutants derived from TA3419. Agarose gel electrophoresis image showing amplification in free-threshing cultivar, TA2987, TA3419 and the free-threshing mutants using the closest flanking marker *Xwmc503*, mapped 2.2 cM distal to *Tg*. First lane contains 100 bp DNA size standard.



**Figure 3.9** EST analysis of free-threshing mutants derived from synthetic wheat, TA3418. The autoradiograph image showing hybridization pattern in TA3418 and the free-threshing mutants in TA3418 background using probe BE444659 which is mapped 23.7 cM distal to *Tg*.



**Figure 3.10** Genetic map of the *Tg* region on chromosome 2DS using microsatellite markers in an F<sub>2</sub> population derived from TA2988 and TA8051. The map is oriented centromere towards telomere from top to bottom



**TA2988 X TA8051 map**

**Table 3.1** Polymorphism analysis of wheat ESTs mapped in the distal most deletion bin (2DS5-0.47-1.00) of short arm of chromosome 2D

<b>EST marker</b>	<b>Polymorphism*</b>	<b>Marker type</b>	<b>Restriction enzyme</b>
<i>XBF201415</i>	P	Dominant	<i>Bgl</i> III
<i>XBF291618</i>	P	Codominant	<i>Xba</i> I
<i>XBF210235</i>	NP	-	-
<i>XBE444659</i>	P	Codominant	<i>Bgl</i> III
<i>XBF484829</i>	P	Codominant	<i>Eco</i> RV, <i>Eco</i> RI
<i>XBF483620</i>	NP	-	-
<i>XBF428792</i>	NP	-	-
<i>XBF474943</i>	NP	-	-
<i>XBE443449</i>	NP	-	-
<i>XBF474637</i>	NP	-	-
<i>XBG263447</i>	NP	-	-
<i>XBE445242</i>	NP	-	-
<i>XBE498640</i>	NP	-	-
<i>XBG604650</i>	NP	-	-
<i>XBE426431</i>	NP	-	-
<i>XBG262864</i>	NP	-	-
<i>XBE444297</i>	P	Dominant	<i>Xba</i> I
<i>XBE500206</i>	NP	-	-
<i>XBE499478</i>	NP	-	-
<i>XBE518306</i>	P	Codominant	<i>Eco</i> RI
<i>XBE497586</i>	P	Codominant	<i>Xho</i> I
<i>XBE518031</i>	P	Codominant	<i>Sca</i> I, <i>Dra</i> I, <i>Eco</i> RI
<i>XBE446435</i>	NP	-	-
<i>XBE443026</i>	NP	-	-
<i>XBE426229</i>	NP	-	-
<i>XBE499671</i>	NP	-	-



<b>EST marker</b>	<b>Polymorphism*</b>	<b>Marker type</b>	<b>Restriction enzyme</b>
<i>XBE444541</i>	P	Codominant	<i>HindIII</i>
<i>XBE446339</i>	P	Codominant	<i>XhoI</i>
<i>XBE443747</i>	NP	-	-
<i>XBE445628</i>	NP	-	-
<i>XBE499648</i>	NP	-	-
<i>XBF478837</i>	NP		-
<i>XBE497622</i>	P	Codominant	<i>ScaI</i>
<i>XBE406474</i>	P	Codominant	<i>EcoRI</i>
<i>XBE498730</i>	NP	-	-
<i>XBE404601</i>	NP	-	-
<i>XBE498252</i>	NP	-	-
<i>XBE404682</i>	NP	-	-
<i>XBE497393</i>	NP	-	-
<i>XBE406808</i>	P	Codominant	<i>EcoRV, BglII</i>
<i>XBE407000</i>	NP	-	-
<i>XBE494262</i>	NP	-	-
<i>XBE498608</i>	NP	-	-
<i>XBE498683</i>	P	Dominant	<i>BglII</i>
<i>XBE498396</i>	P	Codominant	<i>EcoRI, EcoRV, XbaI</i>
<i>XBE490444</i>	P	Dominant	<i>DraI, EcoRI, EcoRV, BglII</i>
<i>XBE499212</i>	P	Codominant	<i>BglII, EcoRI, HindIII, ScaI</i>
<i>XBE517877</i>	P	Dominant	<i>DraI, XbaI</i>
<i>XBE495012</i>	NP	-	-
<i>XBE474998</i>	NP	-	-
<i>XBF200862</i>	NP	-	-
<i>XBE497616</i>	NP	-	-
<i>XBE500725</i>	NP	-	-
<i>XBE428609</i>	P	Codominant	<i>EcoRV</i>
<i>XBE404434</i>	NP	-	-

<b>EST marker</b>	<b>Polymorphism*</b>	<b>Marker type</b>	<b>Restriction enzyme</b>
<i>XBF474313</i>	NP	-	-
<i>XBG607045</i>	NP	-	-
<i>XBE426384</i>	NP	-	-

\* *P* = polymorphic; *NP* = Non-polymorphic

**Table 3.2** Predicted proteins of RFLP and EST markers based on BLASTx searches of NCBI database and the chromosome assignments of corresponding rice orthologues based on the best BLASTn and tBLASTx hits to rice genomic sequences

GenBank	Marker	TC	NCBI BLASTx		Rice BLASTn		Rice tBLASTx	
			Putative protein	<i>e</i> value	Rice chromosome	<i>e</i> value	Rice chromosome	<i>e</i> value
BE444659	<i>XBE444659</i>	TC303991	Hypothetical protein ( <i>O. sativa</i> )	1.0e-117	4	1.8e-121	4	3.7e-103
AA231914	<i>Xcdo456</i>	TC332153	Hypothetical protein ( <i>O. sativa</i> )	4.0e-145	4	2.7e-101	4	6.9e-84
BF201415	<i>XBF201415</i>	N/A	cytochrome P450 monooxygenase	2.0e-43	N/S		N/S	
BE498396	<i>XBE498396</i>	TC304187	Unknown protein ( <i>Z. mays</i> )	1.0e-142	4	2.6e-94	4	6.5e-98
BE499212	<i>XBE499212</i>	TC315334	Hypothetical protein ( <i>O. sativa</i> )	0	4	2.3e-202	4	1.4e-165

GenBank	Marker	TC	NCBI BLASTx		Rice BLASTn		Rice tBLASTx	
			Putative protein	<i>e</i> value	Rice chromosome	<i>e</i> value	Rice chromosome	<i>e</i> value
BE444541	<i>XBE444541</i>	TC317182	Unknown protein ( <i>Z. mays</i> )	9.0e-137	4	4.0e-55	4	1.1e-89
BF484829	<i>XBF484829</i>	TC307733	Unknown protein ( <i>Z. mays</i> )	1.0e-133	4	1.6e-50	4	4.4e-38
BE518031	<i>XBE518031</i>	TC322151	Putative zinc finger protein ( <i>O. sativa</i> )	2.0e-146	3	6.6e-105	3	1.6e-101
BE490444	<i>XBE490444</i>	TC314021	Hypothetical protein ( <i>O. sativa</i> )	2.0e-39	10	8.3e-49	10	7.6e-29
BE517877	<i>XBE517877</i>	TC278954	Hypothetical protein ( <i>O. sativa</i> )	3.0e-97	7	8.3e-116	7	1.1e-67

**Table 3.3** Wheat-rice syntenic relationships of the wheat ESTs and RFLP markers mapped in the region encompassing the *Tg* gene on wheat chromosome 2DS

Wheat		Syntenic relationship with rice			
EST/TC	Marker	BLASTn			
		<i>e</i> value	Rice BAC/PAC	Rice chromosome	Physical position on chromosome 4 (bp)
TC303991	<i>XBE444659</i>	1.8e-121	OSJNBa0020P07	4	1061224- 1225520
TC332153	<i>Xcdo456</i>	2.7e-101	OSJNBa0020P07	4	1061224- 1225520
BF201415	<i>XBF201415</i>	N/S	-	-	-
TC304187	<i>XBE498396</i>	2.6e-94	OSJNBb0060M15	4	612762- 729044
TC315334	<i>XBE499212</i>	2.3e-202	OSJNBb0003A12	4	5720189- 5884607
TC317182	<i>XBE444541</i>	4.0e-55	OSJNBb0004G23	4	348959- 431312
TC307733	<i>XBF484829</i>	1.6e-50	OSJNBa0082I13	4	139972- 214230
TC322151	<i>XBE518031</i>	6.6e-105	OSJNBb0015I02	3	-
TC314021	<i>XBE490444</i>	8.3e-49	OSJNBa0031A07	10	-
TC278954	<i>XBE517877</i>	8.3e-116	P0594D10	7	-

**Table 3.4** Wheat-rice synteny based STS markers used for targeting the tenacious glume gene

<b>Marker</b>	<b>Source</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>	<b>TA (°C)</b>	<b>Fragment size (bp)</b>
<i>XSTS-FT_27</i>	CK163283	CTCGGGATCAAGGTCAACAT	CTCGTGCTCACGTGCATACT	52	150
<i>XSTS-FT_30</i>	TC302624	TGCACTTCCAGTTCAACACC	CGGAACTGGACTTTGAGGAG	52	187
<i>XSTS-FT_31</i>	BJ283108	GGCGTCGATGTCTTCGTTAT	CGTCGGAGAGGTGTTTGTCT	52	205
<i>XSTS-FT_34</i>	TC316528	GCCAGTGACGCTAAAGAACC	ACCCAGTCTCGACCATGC	54	185
<i>XSTS-FT_35</i>	TC353898	TGTGCGTCTTCTACCTCGTG	TATCCTTCGCCTAGCTCCAA	54	211
<i>XSTS-FT_36</i>	TC315501	TTGAGCCACAGATACGCAAG	GAGCCAACCTCTTCCAACAGC	52	160
<i>XSTS-FT_38</i>	CV759387	CCTTCATCGCACACTCTCAA	TCATGGACAACCAACCAGAA	52	233
<i>XSTS-FT_39</i>	TC284553	AGTCTTGGAAGGCAGGGAAT	CCACTGAAAGCTCCAAAAGC	52	217
<i>XSTS-FT_40</i>	TC312858	ACTACCACATCGGCCAGAAC	ACCGACCTGACTCTTGCAGT	52	185

**Table 3.5** Wheat ESTs with sequence homology to annotated gene sequenced within the syntenic rice BAC OSJNBb0015I02 that flanks the tenacious glume gene of wheat and the putative functions of rice orthologous genes within the BAC

<b>Wheat EST</b>	<b><i>e</i>-value</b>	<b>Locus identifier</b>	<b>Putative function</b>
CK163283	1.5e-73	LOC_Os03g58940	Putative lipid binding protein
CJ702151	1.1e-103	LOC_Os03g58960	Palmitoyltransferase ZDHHC20 putative, expressed
CV773987	2.9e-88	LOC_Os03g58970	Expressed protein
DR739544	6.7e-74	LOC_Os03g58980	Germin like protein subfamily T member 2 precursor, putative, expressed
BJ283108	4.9e-25	LOC_Os03g58990	Cupin family protein
CV767933	1.0e-74	LOC_Os03g59010	Germin like protein subfamily T member 1 precursor, putative, expressed
CK165858	3.0e-158	LOC_Os03g59020	T-complex protein 1 subunit theta, putative, expressed
CD870081	2.0e-59	LOC_Os03g59030	UDP-rhamnose rhamnosyltransferase, putative, expressed
CK166248	1.6e-167	LOC_Os03g59040	Squalene synthetase, putative, expressed
CJ628495	2.6e-136	LOC_Os03g59050	ATP-dependent RNA helicase ded1, putative, expressed
CK209492	1.3e-156	LOC_Os03g59060	OsPP2Ac-2-Phosphatase 2 <sup>a</sup> isoform 2 belonging to family 2, expressed

<b>Wheat EST</b>	<b><i>e</i>-value</b>	<b>Locus identifier</b>	<b>Putative function</b>
CV759387	3.9e-78	LOC_Os03g59070	Phosphoric ester hydrolase, putative, expressed
CJ681760	1.0e-127	LOC_Os03g59080	Acyl-activating enzyme 18, putative, expressed



**Table 3.6** Pedigree information of homozygous free-threshing mutants derived from synthetic allohexaploid, TA3419

<b>M<sub>2</sub> Pedigree number (fall 2006)</b>	<b>M<sub>3</sub> plant number (spring 2007)</b>	<b>M<sub>4</sub> plant number (summer 2007)</b>	<b>Phenotype</b>
06-41-55	07-91-55-3	-3-1	Free-threshing
06-41-111	07-91-111-3	-3-3	Free-threshing
	07-91-111-12	-12-1	Free-threshing
06-41-593	07-91-593-3	-3-6	Free-threshing
06-41-956	07-91-956-3*	-	Free-threshing, dark glumes and stem, low fertility
06-41-1045	07-91-1045-6	-6-4	Free-threshing
	07-91-1045-7	-7-3	Free-threshing
	07-91-1045-11	-11-2	Free-threshing

\* In M<sub>3</sub> generation six plants were grown from 07-91-956-3 line but none of the plants produced seed

**Table 3.7** Pedigree information of homozygous free-threshing mutants derived from synthetic allohexaploid, TA3418

<b>M<sub>2</sub> Pedigree number (spring 2007)</b>	<b>M<sub>3</sub> plant number (fall 2007)</b>	<b>M<sub>4</sub> plant number (spring 2008)</b>	<b>Phenotype</b>
07-350-507	-2	-2-C	Free-threshing, reduced height, low fertility
	-3	-3-F	Free-threshing, reduced height, low fertility
07-350-519	-2	-2-A	Free-threshing
	-3	-3-F	Free-threshing
07-350-624	-8	-8-A	Free-threshing

**Table 3.8** Genotypic characterization of homozygous free-threshing M<sub>4</sub> mutants derived from synthetic allohexaploid, TA3419 using molecular markers flanking tenacious glume gene, *Tg* on short arm of chromosome 2D

Molecular markers	07-91 pedigree (M <sub>4</sub> generation)						
	55-3-1	593-3-6	111		1045		
			3-3	12-1	6-4	7-3	11-2
<i>Xbarc124</i>	-	+	+	+	+	+	+
<i>XBE444541</i>	-	+	+	+	+	+	+
<i>XBF484829</i>	-	+	+	+	+	+	+
<i>Xwmc503</i> *	-	+	+	+	-	-	-
<i>XBE518031</i> *	-	+	+	+	-	-	-
<i>Xfba88</i>	-	+	+	+	+	+	+
<i>Xfba400</i>	-	+	+	+	+	+	+
<i>Xwmc112</i>	+	+	+	+	+	+	+
<i>Xcfd25</i>	+	+	+	+	+	+	+

\* *Xwmc503* and *XBE518031* flank *Tg* in a 4.9 cM interval on the genetic map of 2DS

**Table 3.9** Genotypic characterization of homozygous free-threshing M<sub>4</sub> mutants derived from synthetic allohexaploid, TA3418 using molecular markers flanking tenacious glume gene, *Tg* on short arm of chromosome 2D

Molecular markers	07-350 pedigree (M <sub>4</sub> generation)				
	507		519		624
	2C	3F	2A	3F	8A
<i>XBE444659</i>	+	+	+	+	+
<i>XBE201415</i>	+	+	+	+	+
<i>XBE498396</i>	+	+	+	+	+
<i>XBE444541</i>	+	+	+	+	+
<i>Xgwm296</i>	+	+	+	+	+
<i>Xgwm261</i>	+	+	+	+	+
<i>Xwmc503*</i>	+	+	+	+	+
<i>XBE518031*</i>	+	+	+	+	+
<i>XBE517877</i>	+	+	+	+	+

\* *Xwmc503* and *XBE518031* flank *Tg* in a 4.9 cM interval on the genetic map of 2DS

# **Appendix A - PHENOTYPIC CHARACTERIZATION AND MICROSATELLITE ANALYSIS OF AWNED MUTANTS OF CHINESE SPRING WHEAT**

## **Abstract**

Awning is an important agronomic trait in cereals. Awned wheat varieties produce significantly higher yields compared to awnless varieties under dry conditions. Awning is controlled by three dominant inhibitors in wheat; *Hd*, *B1* and *B2* located on chromosomes 4AS, 5AL and 6BL respectively. Additionally awn promoter genes on homoeologous group-2 chromosomes have also been mentioned in literature but no loci has been genetically characterized so far. In the present study, we characterized sixteen homozygous awned mutants derived from independent EMS and fast neutron mutagenesis screens of awnleted bread wheat cultivar Chinese Spring. Four mutants had hooded (*Hd*) awn phenotype, seven showed tipped 1 (*B1*) and five showed tipped 2 (*B2*) awn phenotype. One hundred and three wheat microsatellite markers were used to genotype the awned mutants and study the nature of deletions. None of the awn mutants showed major terminal deletions or loss of entire chromosome arm in the genomic regions under study. Two hooded awn mutants, 05-249 and 05-258 were found to have an interstitial deletion putatively spanning the *Hd* locus on chromosome 4AS. Another interstitial deletion was found in proximity of the centromeric region in the short arm of chromosome 2A in a tipped-1 awn mutant, 05-255, suggesting the possibility of deletion of an awn promotion gene. Although, further research is required to address the co-segregation of awn phenotype and the deletions reported in the awn mutants in this study, these mutants will advance our knowledge in understanding the complex interaction among different genetic loci for such an important agronomic trait.

## Introduction

Awns are the long spines or ‘beard’ on the ears of wheat. Technically, awns are the extensions of lemma in a wheat spikelet where lemma and palea are the structures that enclose the developing seed in the ear. Awnedness is considered as an important adaptation trait in wild plants as awns aid in the dispersal of the seed. In wild wheats the seed dispersal unit is characterized by two pronounced awns that balance the unit as it falls (Elbaum et al. 2007). Powered by the humidity cycle, awns propel the seed into the ground to ensure better germination and survival (Elbaum et al. 2007). All wild relatives, land races and most of the modern wheat cultivars possess awns. Over time, several wheat breeding programs have led to the development of awnleted (awnless) cultivars to suit the needs of regional breeding programs. Related cereals like barley, oats and rye also have awned spikes.

Wheat awns play a major role in maintaining good yield under drought conditions. Many characteristics of awned varieties make them suitable for cultivation under dry conditions such as higher surface area compared to flag leaf blade (McDonough and Gauch 1959), better light interception and CO<sub>2</sub> uptake, shorter pathway for assimilate movement from awns to the developing kernel (Evans et al. 1972) and their low tendency to senesce compared to flag leaf. Therefore in wheat, presence of awns has been found to double the rate of net ear photosynthesis (Evans and Rawson 1970). Hence, even though awns have only a limited effect on yield in wet climates (Vervelde 1953), their effect is much more significant under dry conditions where awned cultivars surpass awnleted or de-awned wheats in yield (Miller et al. 1944, Vervelde 1953, Grundbacher 1963). Because awns are photosynthetically active in wheat and they have direct vascular linkage with the developing spike, lack of awns can potentially reduce the total photosynthetic contribution to developing kernels (Weyhrich et al. 1995). Further the reduced photosynthate supply could translate into lower kernel weight and test weight (Olugbemi et al. 1976) and possibly reduced grain yield (McKenzie 1972).

Although there are many advantages of having awned spikes, in certain cases awnleted (awnless) wheat varieties have been shown to perform better. For example, awnleted varieties are preferred for forage production in graze-out or forage-only management systems, where they pose less mouth and eye irritation to livestock (Martin et al. 2003) where these varieties give producers the added flexibility to defer their decision to use a standing crop either for grain

production or for hay or late-season grazing. Furthermore, some studies have correlated awnedness with susceptibility to abiotic and biotic stresses. Research done at CSIRO (Commonwealth Scientific and Industrial Research Organization) has indicated negative correlation between awns and pre-harvest sprouting of wheat ears. The Australian study showed that wheat varieties lacking awns are far less susceptible to sprouting damage than the wheat varieties with awns (<http://www.csiro.au/index.asp?type=mediaRelease&id=Prprawnless&xml=rel...>). On a parallel note, Snijders (1990) reported the linkage between Fusarium head blight (FHB) resistance and awnedness in a study on winter wheat infected with *Fusarium culmorum* and presence of awns was shown to enhance the development of the FHB disease (Mesterhazy 1995). However later on it was demonstrated that the linkage between FHB and awnedness could be easily broken in certain genotypes (Ban and Suenaga 2000) and development of FHB resistant awned cultivars was easily attainable (Buerstmayr et al. 2000).

Genetic control of awnedness in wheat is considered simple and only a few genes are known to account for the differences between awned and awnleted varieties. In bread wheat, three dominant awning inhibitors have been described (McIntosh et al. 1998): *Hd* (Hooded), *B1* (Tipped 1) and *B2* (Tipped 2). In the hooded mutant type (*hd*), awn length of the main tiller is reduced and the awns are often bent at the base in a characteristic way. In addition glumes are inflated and awns of late tillers are either reduced to a hook or bent around themselves to form a close spiral. The tipped-1 mutants (*b1*) have very short awn tips at the base and the middle of the ear but they increase in length in the apical quarter and may sometime reach 1 cm. These awn tips are usually straight and not bent at the base. The other tip awned mutants (*b2*) are quite different. Generally, the length of the tips does not vary all along the ear, maybe occasionally close to the center of the spike but not at the apex. Length rarely exceeds 6mm and if awns are curved, they are never curved around themselves as hooded wheats (Watkins and Ellerton 1940). Wheat genotypes carrying the three recessive alleles *hd*, *b1* and *b2* in homozygous conditions are fully awned.

Assignment of these three genes to chromosomes and chromosome arms was initially done by using aneuploid (nullitetrasonic and ditelosomic) lines of cultivar Chinese Spring (CS) (Sears 1954). *Hd* is located on short arm of chromosome 4A, *B1* on long arm of chromosome 5A and *B2* on long arm of chromosome 6B (Sears 1954, 1966). Additionally, some awn promoting

genes have been reported on group-2 chromosomes (Sears 1954) though no gene symbols have yet been recognized for these genes. CS is an awnletted variety but the group-2 disomic addition lines of CS wheat ( $2n=44$ ) are awned. Therefore it has been suggested that presence of eight copies of group-2 chromosomes lead to awned phenotype (Dvorak 1980; Friebe et al. 1999). In this context, various epistatic effects and complex interactions have also been proposed among the three awn inhibitor genes (McIntosh et al. 1998).

Attempts have been made to identify molecular markers linked to *Hd*, *B1* and *B2* genes by various groups. In a QTL (quantitative trait loci) mapping study, QTL for three traits related to awning (awn length at base, the middle and the top of the ear) in wheat were studied in a double haploid population derived from cultivars ‘Courtot’ (awned) and ‘Chinese Spring’ (awnletted) (Sourdille et al. 2002). Two major QTLs were detected in this population. The first QTL was located on chromosome arm 4AS near *Xfba78* locus. This QTL explained around 39.7% of the mean variability. The second QTL was located on chromosome arm 6BL, close to locus *Xwmc182*. This locus explained on an average 20.8% of the variation for the trait. These QTLs on 4AS and 6BL were shown to correspond with the *Hd* and *B2* locus respectively. Although no QTL was detected for the *B1* locus on chromosome 5AL in this population due to the identical allelic constitution of Chinese Spring and Courtot at this locus, several other studies have reported mapping of *B1* locus. Kato et al. (1998) mapped *Vrn1*, *Q* and *B1* genes using RFLP (restriction fragment length polymorphism) markers in a single-chromosome substitution recombinant line population developed from CS (Cappelle-Desprez 5A) and CS (*Triticum spelta* 5A). The *B1* locus was located distal to the 5A/4A chromosome breakpoint at the end of long arm of chromosome 5A where an RFLP marker, *Xpsr164* was mapped 57.3 cM proximal to *B1*. Additionally *B1* locus has been associated with some of the disease resistance genes mapped on chromosome 5AL. In a Fusarium head blight (FHB) QTL mapping study in European winter wheats, one of the three FHB QTLs on 5A was reportedly flanked by *B1* at the distal end of chromosome (Gervais et al. 2003). Similarly in a stripe rust resistance characterization study, *B1* was mapped 12.2 cM proximal to *Yr34* gene and 8.2 cM distal to *Xgwm410* in a double haploid (DH) population derived from WAWHT2046/Carnamah (Bariana et al. 2006).

The awn inhibitors genes have been physically assigned to specific regions on the chromosomes using deletion lines of CS wheat. By phenotyping the 4A, 5A and 6B chromosome deletion lines the *Hd*, *B1* and *B2* genes were physically targeted to the deletion bins



4AS-3 (fraction length, FL-0.76-1.00), 5AL-10 (FL-0.57-0.78) and 6BL-5 (FL-0.40-1.00) respectively (Sourdille et al. 2002). The fraction length values indicate the distance of the deletion breakpoint from the centromere.

Induced mutant populations in plant systems are a great genetic resource and play a major role in gene discovery through forward as well as reverse genetic screens (Till et al. 2003; Multani et al. 2003). Among the chemical mutagens, EMS (ethyl methanesulfonate) is the chemical of choice because it has high mutagenicity, low mortality rate and ease of use. The cloning of EMS induced mutant alleles has shown that chemical mutagenesis generally results in single nucleotide changes or very small deletions (Mita et al. 1988; Chang et al. 1993). In Arabidopsis, it has been well-established that EMS generally causes G/C to A/T base transitions almost 99% of the time (Greene et al. 2003). Several EMS populations have been developed as a community resource in various plant systems like barley, rice, maize, soybean and Arabidopsis (Hirochika et al. 2004; Till et al. 2003; Till et al. 2004; Cooper et al. 2008; Caldwell et al. 2004).

On the other hand, ionizing mutagens such as fast neutrons are thought to produce double strand DNA breaks that are then repaired by mechanisms which are not clearly understood but could be similar to repairing lesions caused by T-DNA introgression (Shirley et al. 1992). Therefore, fast neutron treatment potentially causes much larger deletions (Men et al. 2002) and the deleted chromosomal regions can be potentially determined by the use of RFLP (restriction fragment length polymorphism) or PCR (polymerase chain reaction) markers (Faris and Gill 2002) by comparing wild-type and deletion DNAs and identifying restriction fragment or PCR-amplified polymorphisms associated with the deletion and ultimately the mutation.

In an EMS and fast neutron mutagenesis screen at Wheat Genetic and Genomic Resources Center (WGGRC), Manhattan, KS, several awned mutants of Chinese Spring were identified (Kuraparthi V, WGGRC, unpublished data). Awned mutant characterization was undertaken in an attempt to identify novel genes/ loci affecting awning or new alleles for already known awning genes. Thus, in the present study we characterized a set of awned mutants of Chinese Spring wheat morphologically, used chromosome-specific wheat microsatellite markers to fingerprint the mutants at known loci and studied the type of mutations in the awned mutants.

## **Materials and Methods**

## ***Plant material***

In addition to Chinese Spring, sixteen homozygous awned mutants in Chinese Spring (CS) background were used for this study. Eight homozygous mutants at M<sub>6</sub> generation were selected from a fast neutron mutagenesis experiment. Another 8 homozygous mutants at M<sub>4</sub> generation were selected from an ethyl methanesulfonate (EMS) mutagenesis screen. The detailed pedigree information for the CS awned mutants is given in Table A.1.

Aneuploid and deletion lines of CS were grown for genotypic evaluations. Aneuploid lines included; monosomic-tetrasomic line (one of the missing chromosome is partially compensated by an extra pair of homoeologous chromosomes) for chromosome 2A (M2AT2B), nullisomic-tetrasomic line (a pair of missing chromosomes is partially compensated by an extra pair of homoeologous chromosomes) for 2B (N2BT2D), 2D (N2DT2A), 4A (N4AT4B), 5A (N5AT5B) and 6B (N6BT6A); ditelosomic lines (specific chromosome arm pair is missing) for 2AS, 2BL, 2DS, 2DL, 4AS, 5AL, 6BS and 6BL; monotelosomic line (specific chromosome arm is missing) for 2AS, 5AS and deletion lines (specific chromosome segment is missing) for 2AS (2AS-5), 4AS (4AS-3, 4AS-4) and 6BL (6BL-5).

## ***Generation of mutants***

Mutants were generated using EMS and fast neutron bombardment in Chinese Spring (CS) background. Because most of the genetic and genomic stocks in wheat are available in CS, it was used as the starting material for mutagenesis to aid the characterization and genetic and genomic targeting of newly identified mutant loci.

EMS mutagenesis: Mutants were generated and evaluated at WGGRC, Manhattan, KS (Kuraparthi V, unpublished results). The EMS mutagenesis procedure was adapted from the protocol described by Williams et al. (1992). Chinese spring seeds were pre-soaked in 0.05 M phosphate buffer (pH 8) for 8 h and then soaked for 16 h in a 0.4% (v/v) solution of EMS in the same buffer. During the treatment EMS solution containing seeds was aerated by gentle agitation on the shaker. After 16 h of soaking, treated seeds were rinsed under running tap water for 1-2 minutes to get rid of excess EMS solution from seed surfaces and seeded immediately in the root-trainers (Hummert International, Earth City, MO, USA) in greenhouse. M<sub>1</sub> plants were grown in the greenhouse at 13-18°C diurnal temperatures with a 16 h day and 8 h night cycle.

About 12 M<sub>2</sub> seedlings from each M<sub>1</sub> spike were grown and phenotyped for awning and various other morphological characters. For the present study the homozygous awned mutant plants at M<sub>4</sub> generation were selected.

Fast neutron mutagenesis: Mutagenesis experiment was done at Plant Breeding Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, International Atomic Energy Agency Laboratories, Seibersdorf, Austria by Andrea Kodym (Kodym and Afza 2003). Chinese spring seeds were treated by fast neutron bombardment at a dose of 5 Gy. The M<sub>1</sub> seed was grown in the greenhouse at WGGRC, Manhattan, KS. Phenotyping was done starting at M<sub>2</sub> generation. Homozygous plants with awned spikes at M<sub>6</sub> generation were used for the present study.

### ***Phenotyping***

Chinese spring is awnless and thus plants with awn phenotype were easily identified from the mutagenesis screen and selected for the initial analysis. CS is known to have all three dominant awning inhibitor genes, *Hd* (hooded), *B1* (tipped 1) and *B2* (tipped 2). These genes in recessive form exhibit specific awn phenotype. All 16 awned mutants were phenotyped for the hooded, tipped 1 and tipped 2 gene phenotypes (Sourdille et al. 2002). The awned mutants were grown for three seasons in the greenhouse (2003-2005) and one season in the field plots (summer 2004). Awnedness was evaluated when plants were at flowering stage.

### ***DNA isolation***

About 2-3 cm of young leaf section was taken from 3 week old seedlings. DNA was isolated using mini-prep isolation procedure. Plant tissue was ground using liquid nitrogen with a small glass rod. About 700 µl of pre-warmed (60°C) extraction buffer (50 mM EDTA, 0.1 M Tris-HCl, 0.5 M NaCl, 0.84% SDS) (pH 8.0) was added to the ground tissue. The samples were incubated for 20-30 min at 60-65°C and 700 µl of chloroform: iso-amyl alcohol (24:1) mixture was added. Tubes were centrifuged at 13,000 rpm for 10 min and 500 µl of supernatant was transferred to a new tube. DNA was precipitated by adding 1 ml of ice-cold 95% ethyl alcohol. Tubes were centrifuged at 13,000 rpm for 2 min. DNA pellet was washed using 70% ethyl alcohol and later 1 ml of 70% ethyl alcohol was added to the tubes. Once again tubes were centrifuged at 13,000 rpm for 1 min. DNA pellet was air dried and dissolved in 150 µl of 1X TE

buffer (Tris-EDTA). Quantification of DNA was done using the NanoDrop ND-1000 UV-VIS spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). DNA concentration was adjusted to 25ng/μl.

### ***Microsatellite analysis and genetic mapping***

The awning inhibitor genes are located on chromosomes 4A, 5A and 6B therefore microsatellite markers mapped on these chromosomes were selected using the previously published microsatellite linkage maps (Röder et al. 1998; Guyomarc'h et al. 2002a and b; Somers et al. 2004). Additionally, some role of homoeologous group-2 chromosomes in affecting awnedness has also been reported in literature (Sears 1954; Dvorak 1980), thus we selected microsatellite markers from chromosomes 2A, 2B and 2D also to genotype the awned mutants. Because most of the microsatellite markers have been physically mapped in the deletion bins using CS deletion lines (Sourdille et al. 2003), at least one microsatellite marker per deletion bin was selected for each of the chromosomes in most cases to get complete coverage on the chromosome. Polymerase chain reaction (PCR) amplifications were performed in 20 μl reactions containing 2.5 μl of 10X PCR buffer, 1.75 μl magnesium chloride (25mM), 2.0 μl dNTPs (2.5 mM of each dNTP), 1.0 μl each of forward and reverse primer (10 pM/μl), 0.15 μl of *Taq* DNA polymerase (Promega, WI, USA) and 75 ng of DNA in a PTC-200 thermal cycler (MJ research, Waltham, MA, USA). PCR amplification consisted of 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 50, 52, 55 or 60°C depending on the individual primer for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. Amplified PCR products were resolved in 2.5% hi-resolution agarose (Gene Pure HiRes Agarose, ISC BioExpress, UT, USA) gels with 1X Tris-borate EDTA (TBE) buffer and visualized by ethidium bromide staining. Linkage map for the target loci was constructed using MAPMAKER version 2.0 (Lander et al. 1987) for Macintosh operating system and genetic distance was calculated using Kosambi mapping function (Kosambi 1944).

## **Results**

### ***Phenotypic Characterization***

Sixteen homozygous awned mutants of CS wheat were characterized based on their awn phenotype (Fig. A.1). Mutants were classified into three categories based on the phenotype of three major awn inhibitor genes (Table A.2). Four mutants (05-249, 05-252, 05-257 and 05-258) had curved awns which were bent towards the base and therefore these mutants were referred as hooded phenotype. Among these four mutants, 05-249 and 05-252 were generated using fast-neutron treatment and 05-257 and 05-258 were generated using EMS. Seven mutants (05-248, 05-250, 05-251, 05-254, 05-255, 05-256 and 05-263) had spikes with variable awn length. The length of the awns was smaller near the base and middle of the spike but increased significantly towards the top portion. In some cases the spike had short awns with small length variation throughout the spike. Such mutants were referred to as tipped-1 phenotype. Out of these seven mutants, five (05-248, 05-250, 05-251, 05-254 and 05-255) were generated through fast-neutron mutagenesis and two (05-256 and 05-263) were from EMS mutagenesis screen. Third category included tipped-2 awned mutants where awn length was more or less uniform through out the spike and awns were much longer. Five of the mutants (05-253, 05-259, 05-260, 05-261 and 05-262) under study were classified as tipped-2 phenotype. 05-253 was a fast-neutron mutant whereas other tipped-2 mutants were derived from EMS treatment. Awnedness was scored over four different seasons and the phenotype expression of the awned mutants was found to be uniform throughout. This observation helped confirm the homozygosity of the mutants and ruled out the effect of environment on the awned phenotype in the selected mutants.

### ***Genotyping using microsatellite markers***

One hundred and thirteen microsatellite markers from six chromosomes were used to genotype the awned mutants of Chinese spring (Table A.3). Mutants were screened using 15-20 microsatellite markers per chromosome. The distribution of microsatellite markers over the length of each chromosome in most cases was uniform (Fig. A.2, A.3 and A.4). This allowed estimation of large deletions in the chromosome regions of interest. No big deletions involving whole chromosome arm were detected using the microsatellite markers in any of the chromosomal regions under study. All awned mutants were screened using the microsatellite marker *Xwmc182* associated with the awned QTLs on 6BL (Sourdille et al. 2002), but none of

the mutants showed any deletion polymorphism for *Xwmc182*. Even the mutants with tipped-2 phenotype (Fig. A.1) did not show any deletion for *Xwmc182* (data not shown).

Overall, three independent awned mutants showed deletion of different microsatellite markers (Table A.4). The microsatellite markers *Xcfa2026* mapped on 4AS showed lack of amplification in the mutants 05-249 and 05-258. *Xcfa2026* amplified two alleles in Chinese spring (CS) and both the alleles were deleted in mutants 05-249 and 05-258 (Fig. A.5). Using the CS nullitetrasonic line and the ditelosomic lines for chromosome 4A it was confirmed that both the alleles of *Xcfa2026* were in fact located on 4AS. Using chromosome 4AS deletion lines, 4AS-3 and 4AS-4 it was further established that the missing alleles were mapped in the region of *Hd* locus (Fig. A.5). Another microsatellite marker, *Xcfa2256* also failed to amplify in the same set of awned mutants (05-249 and 05-258) as *Xcfa2026* (Fig. A.6). *Xcfa2256* amplified only one allele in CS and which was deleted in 05-249 and 05-258 mutants. Physical mapping using Chinese Spring deletion lines placed *Xcfa2256* in the distalmost deletion bin (4AS3-0.76-1.00) on short arm of chromosome 4A (Fig. A.6).

However, in the available bread wheat microsatellite linkage maps there was no information available on genetic distance between *Xcfa2026* and *Xcfa2256* markers. Thus, in order to map *Xcfa2026* and *Xcfa2256* genetically, we used a recombinant inbred line (RIL) population of 118 individuals derived from a cross of CS X CSDIC4A (4A chromosome of CS substituted by 4A of *T. dicoccoides*). Both *Xcfa2026* and *Xcfa2256* were polymorphic between CS and CSDIC4A and were scored as dominant loci. Linkage mapping placed *Xcfa2026* and *Xcfa2256*, 9.7 cM apart on chromosome 4AS. This indicated that almost 10 cM region between the markers has been deleted in the mutants 05-249 and 05-258.

The *Hd* gene has been physically located in the deletion bin 4AS3-0.76-1.00 (Sourdille et al. 2002) where both microsatellite markers *cfa2026* and *cfa2256* have also been physically mapped in the same deletion bin. Furthermore, mutants 05-249 and 05-258 have hooded awn phenotype i.e. the awns are small and bent towards the base (Fig. A.7). Therefore it is conceivable that the region between the markers *cfa2026* and *cfa2256* contains the *Hd* gene and has been deleted in these mutants.

Another awned mutant, 05-255 showed deletion for two of the microsatellite markers *Xgwm515* and *Xgwm425* (Fig. A.8). *Xgwm515* amplified three loci in CS, one has been mapped on chromosome 2DS, another one on chromosome 2AS (Röder et al. 1998) and the chromosome

location of third locus is unknown. The locus mapped on chromosome 2AS was deleted in the mutant 05-255. The chromosome location of *Xgwm515-2A* allele was established using the mono-tetrasomic line of chromosome 2A (Fig. A.8). Another marker, *Xgwm425* also amplified three PCR products in CS where the locus mapped on chromosome 2AS (*Xgwm425-2A*) was deleted in the mutant 05-255 (Fig. A.9). In addition to *Xgwm515* and *Xgwm425*, 19 other microsatellite markers (previously mapped on chromosome 2AS) were amplified in 05-255 awned mutant however consistent lack of amplification was only observed for markers *Xgwm515* and *Xgwm425*. This suggested that there might be an interstitial deletion involving the region encompassed by microsatellite markers *Xgwm515* and *Xgwm425* in mutant 05-255.

## Discussion

Awning is an important agronomic trait and extensive research has been done to understand their role in the agronomic sustenance of wheat plant under diverse climatic conditions. However, only a limited number of genetic studies have been done to identify, characterize and locate different genetic factors responsible for controlling awnedness. In the present investigation we identified location of a novel awn promoter gene on chromosome 2A and identified and characterized two independent mutants for hooded awn type.

EMS and fast neutrons, two mutagenic agents used to generate awned mutants in the present study differ widely with respect to their mode of action. Where EMS generally causes point mutations (Greene et al. 2003) or small deletions (Mita et al. 1988; Chang et al. 1993), fast neutrons are known to cause much larger deletions (Men et al. 2002). The genotyping analysis of CS awned mutants with 113 wheat microsatellite markers surprisingly did not identify any large deletions involving whole chromosome arms or terminal regions of chromosome arms in the genomic regions being studied in any of the awned mutants. However, we did find similar sized interstitial deletions spanning almost 10 cM in two different awned mutants and a much smaller interstitial deletion in another mutant. Two microsatellite markers, *cfa2026* and *cfa2256* detected a putative deletion for *Hd* locus on chromosome 4A in an EMS mutant and probably a similar sized deletion in a fast-neutron mutant. Another two microsatellite markers, *Xgwm515* and *Xgwm425* identified a smaller deletion on chromosome 2AS in a fast-neutron mutant. Thus, in our pool of awned mutants, we could only identify small interstitial deletions in the genomic

regions of interest. Although we did not recover any large terminal deletions in the fast neutron generated awned mutants, there is a fair probability of presence of point mutations for the awned EMS mutants. However, we were unable to detect point mutations due to the low-resolution of our detection platform.

Two Chinese Spring (CS) mutants (05-249, 05-258) with hooded awn mutation that showed deletion polymorphisms compared to wild type CS were recovered in this study. These mutants showed amplification for all sixteen microsatellite markers located on chromosome 4AS except for markers *Xcfa2026* and *Xcfa2256*. The microsatellite markers *Xcfa2026* and *Xcfa2256* represent a genetic interval of 9.7 cM (present study) and have been physically targeted to distal most deletion bin 4AS3-0.76-1.00 on short arm of chromosome 4A (Sourdille et al. 2004). The *Hd* locus has also been located in the same chromosomal region on chromosome 4AS (Sourdille et al. 2002) thus, the deletion interval spanned by microsatellite markers *Xcfa2026* and *Xcfa2256* might represent the deletion of *Hd* locus. However, in order to confirm this result, further research needs to be done. An F<sub>2</sub> population segregating for *Hd* locus by crossing CS with CS *Hd* mutants (05-249 and 05-258) can be developed and the co-segregation of deletion (identified by *Xcfa2026* and *Xcfa2256*) with the hooded awn phenotype can be studied. If there is co-segregation, it will indicate that the observed deletion contains the *Hd* gene and these deletion mutants can be further utilized for genetic and genomic dissection of *Hd* region.

The uneven distribution of recombination has been well-documented for wheat where distal gene rich regions show high recombination (Gill et al. 1996a; Gill et al. 1996b; Akhunov et al. 2003). Recombination in wheat centromeric and peri-centromeric regions is reported to be very low (Ref?). The average relationship between genetic and physical distance in wheat, based on a genome size of 16,000 Mb and a genetic map of 3700 cM, is about 4.4 Mb/cM (Faris et al. 2000) and the recombination rates can vary from 118 kb/cM in gene rich regions to 22,000 kb/cM for gene poor regions in wheat (Gill et al. 1996a). Other studies on gene rich regions have identified recombination rates ranging from 20 kb/cM (in 1DS of *Aegilops tauschii*; Spielmeier et al. 2000) to 260 kb/cM (in 5A<sup>m</sup>S of *T. monococcum*; Tranquilli et al. 1999). The hooded (*Hd*) gene lies in the distal 22% of the short arm of chromosome 4A, assuming that the chromosomal region surrounding *Hd* is a gene rich region and an average recombination rate for the gene rich regions is 118 kb/cM (Gill et al. 1996a), the ~10 cM deletion interval in awned mutants, 05-249 and 05-258 will translate into an approximate physical size of 1200 kb.



The CS awned mutant, 05-255 showed deletion polymorphism for microsatellite markers *Xgwm425* and *Xgwm515*, which are genetically mapped in the region of suppressed recombination near centromere (Röder et al. 1998; Somers et al. 2004). Therefore, it is difficult to estimate the size of the chromosome region encompassed by the markers *Xgwm425* and *Xgwm515* and thus the size of the deletion in mutant 05-255. However, as these microsatellite markers are mapped in the centromeric region which is generally gene poor with low level of recombination (up to 22,000 kb/cM; Gill et al. 1996a), the physical distance between these markers and hence the size of deletion might be much bigger.

Awning seems to be a complex trait and in addition to the three major inhibitors controlling awning, there seems to be several minor genes involved. These minor genes can be awning suppressors or awning promoters. Various observations have been done on awnleted cultivar, Chinese Spring (CS) wheat (allelic constitution: *Hd B1 B2*) for the major awning inhibitor genes. Phenotype analysis of CS aneuploid lines has revealed a lot of information on minor genes affecting awning in wheat. Short arms of chromosome 2A and 2D are known to carry awn promotion genes (Sears 1954). Monosomics of homoeologous group-2 chromosomes have short awns whereas trisomics and tetrasomics exhibit longer awns (Sears 1954). It has been observed in disomic addition lines of Chinese spring wheat that presence of eight doses of group-2 chromosomes causes awning (Dvorak 1980; Friebe et al. 1999; BS Gill, personal observation). Thus, suggesting a dosage effect of the awn promotion genes located on group-2 chromosomes. Furthermore, nullisomic line for chromosome 3D is awned (Sears 1954) and ditelosomic lines for chromosome arms 3AS and 6DL also exhibit awning (personal observation), thus indicating the possible presence of an awn suppressor gene on chromosome 3D, 3AL and 6DS. Additionally, genetic background seems to play at least some role in the expression of awned character as demonstrated by the awnedness of hemizygotes for *B2* locus (Tsujimoto 2001). Hemizygote for *B2* (monosomic 6B) in CS background is awnless whereas F<sub>1</sub> of CS (*B2*) and Norin-26 (*b2*) is awned (Tsujimoto 2001). All these observations suggest that there is a complex interaction network among various major awn genes and several minor genes scattered through the genome and somehow these genes seem to function in a dosage dependent manner. Therefore it will require much detailed study to characterize different genes affecting the awning phenotype in wheat. For this purpose, the CS awned mutants reported in the present study will serve as an excellent genetic material.

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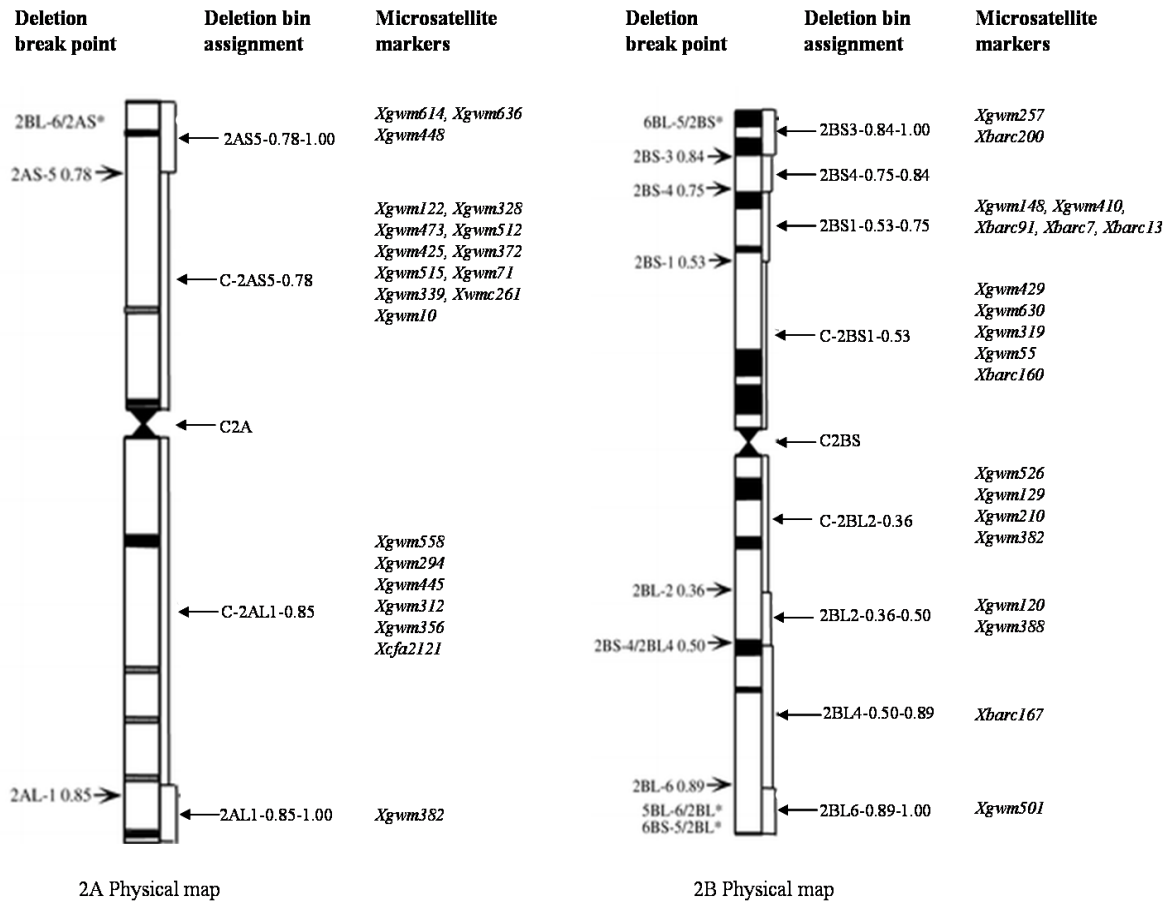
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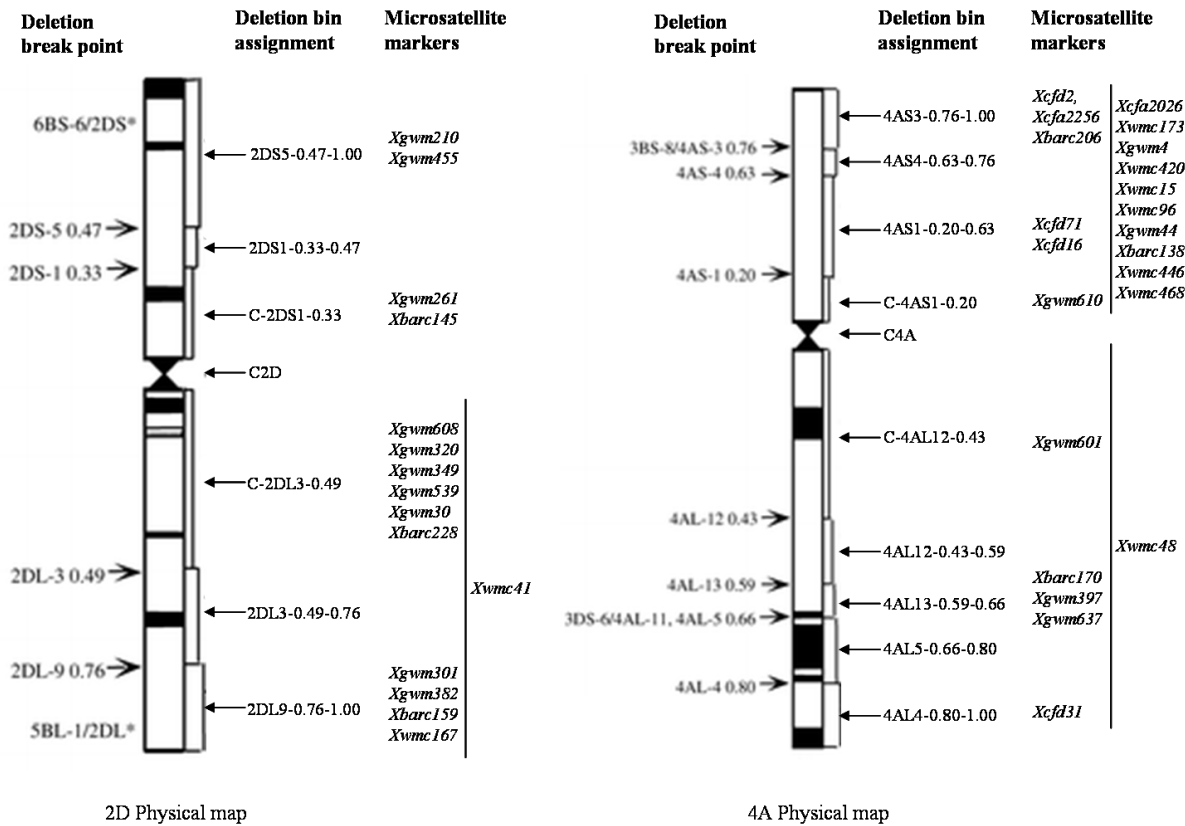
**Figure A.1** Awn phenotypes of Chinese spring (CS) mutants. The spikes have been arranged in the following order: Chinese spring; Top row: Fast neutron generated awned mutants, 05-248, 05-249, 05-250, 05-251, 05-252, 05-253, 05-254 and 05-255; bottom row: EMS generated awned mutants, 05-256, 05-257, 05-258, 05-259, 05-260, 05-261, 05-262 and 05-263



**Figure A.2** Deletion-bin based physical maps of chromosomes 2A and 2B (Qi et al. 2004) showing the distribution of wheat microsatellite markers used to genotype CS awned mutants

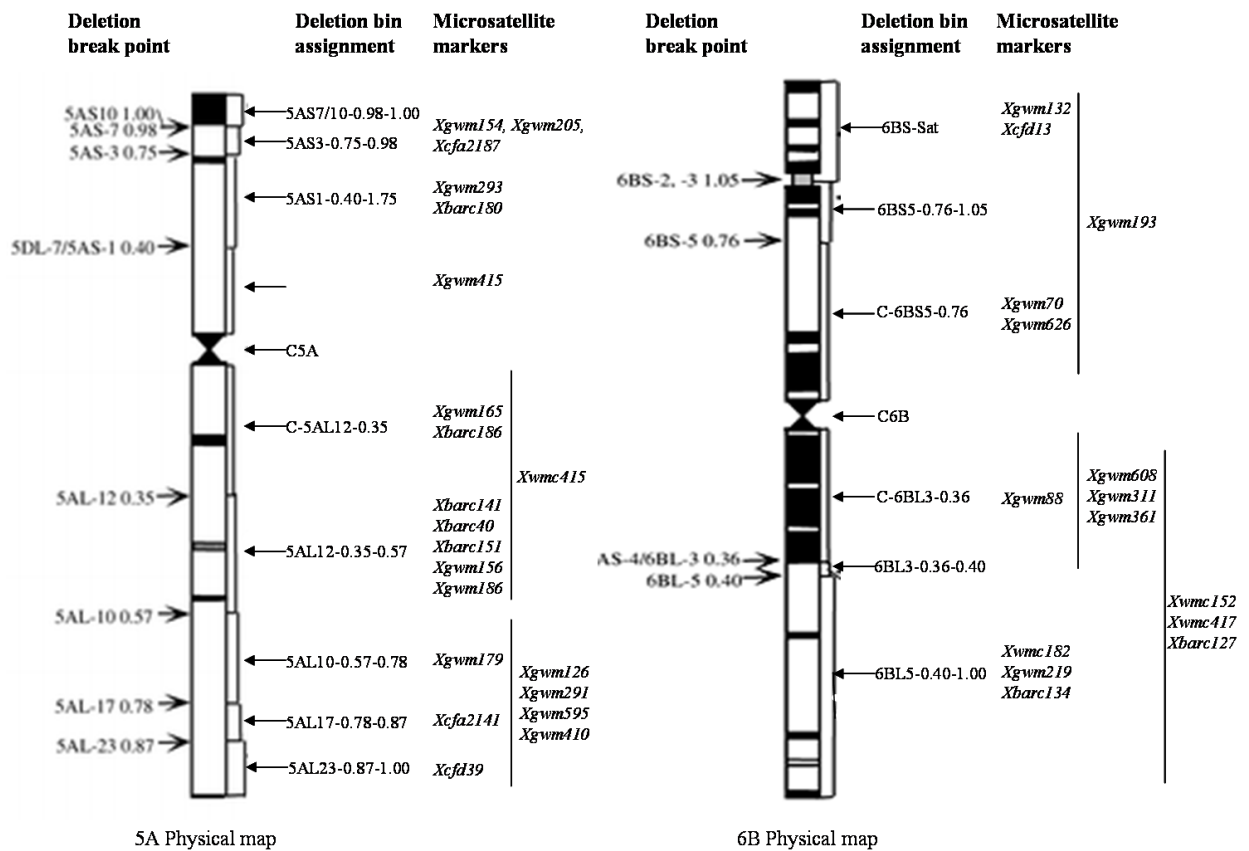


**Figure A.3** Deletion-bin based physical maps of chromosomes 2D and 4A (Qi et al. 2004) showing the distribution of wheat microsatellite markers used to genotype CS awned mutants

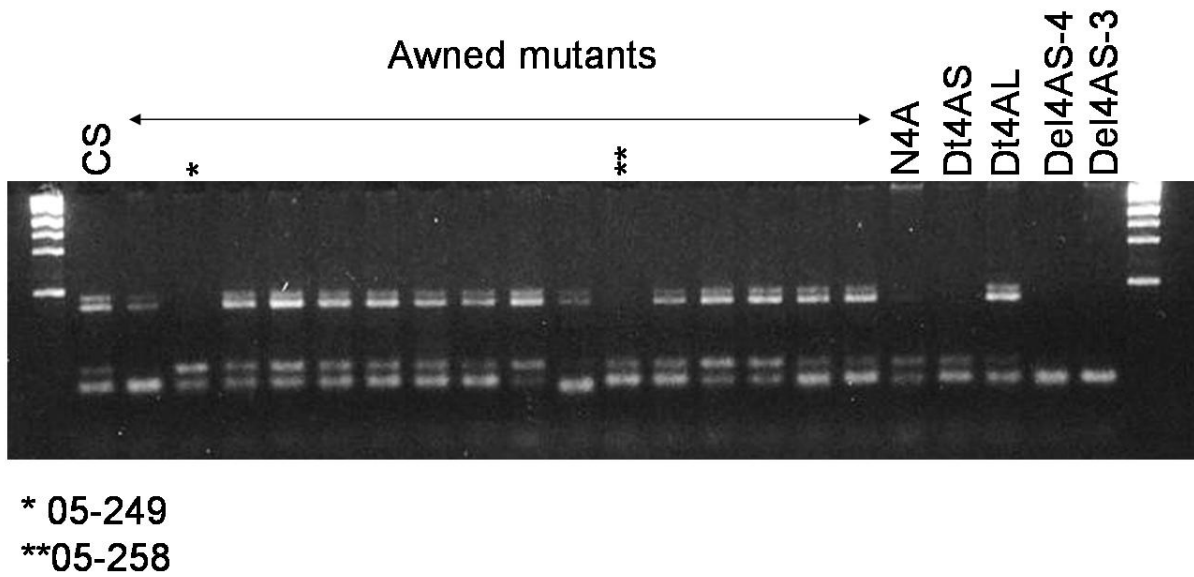




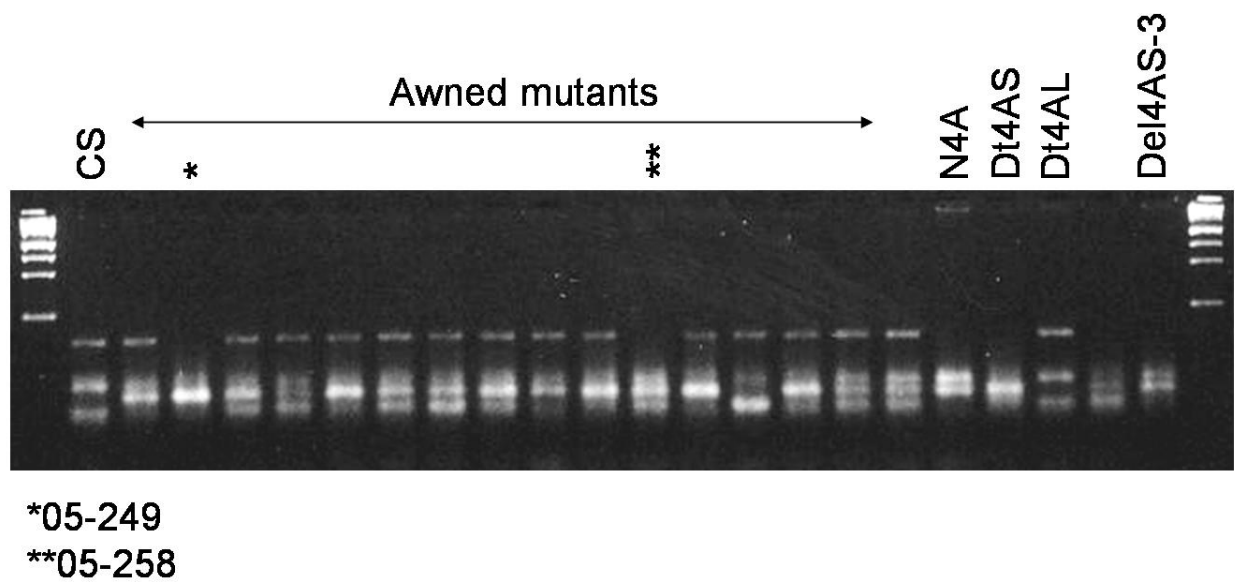
**Figure A.4** Deletion-bin based physical maps of chromosomes 5A and 6B (Qi et al. 2004) showing the distribution of wheat microsatellite markers used to genotype CS awned mutants



**Figure A.5** Microsatellite marker analysis of CS awned mutants. Amplification pattern of microsatellite marker *Xcfa2026* mapped on chromosome 4AS in CS, the awned mutants (05-248 through 05-263) and chromosome 4A aneuploid stocks. The first and the last sample contain 1 kb DNA size standard



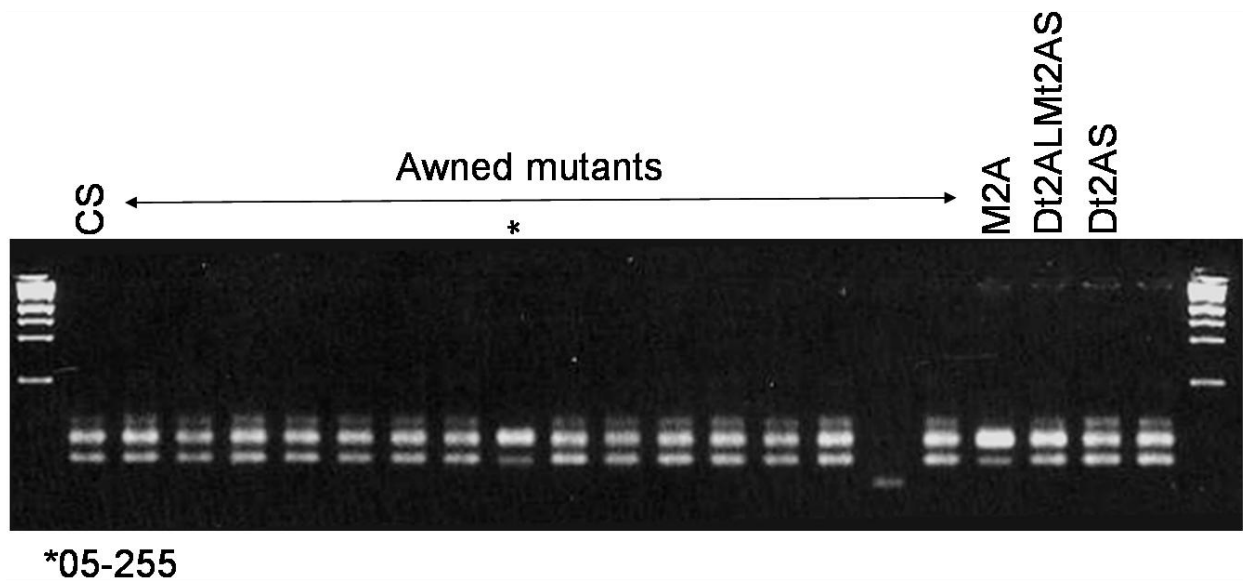
**Figure A.6** Microsatellite marker analysis of CS awned mutants. Amplification pattern of microsatellite marker *Xcfa2256* mapped on chromosome 4AS in CS, the awned mutants (05-248 through 05-263) and chromosome 4A aneuploid stocks. The first and the last sample contain 1 kb DNA size standard



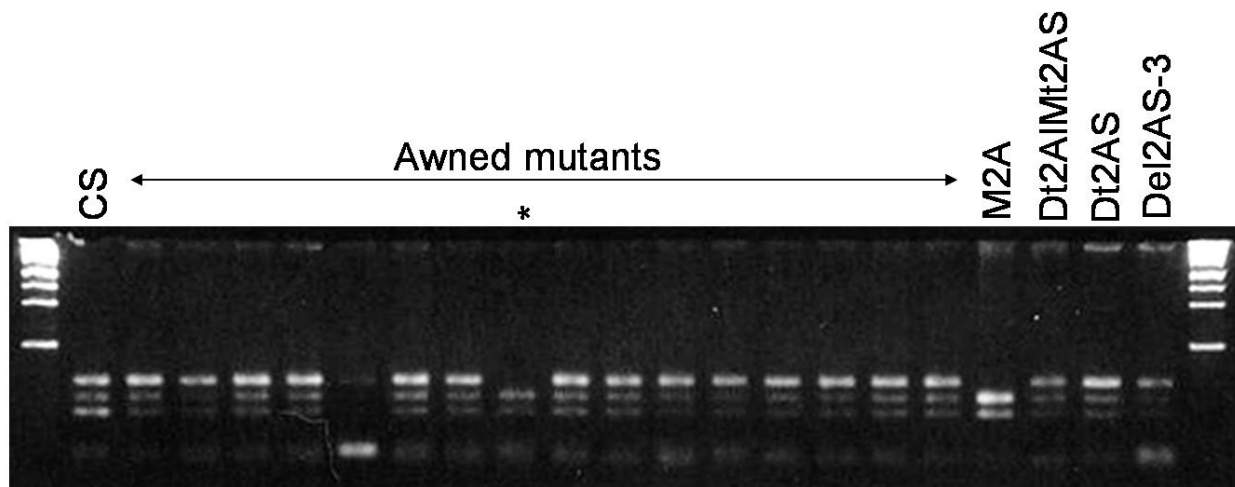
**Figure A.7** Awn phenotypes of Chinese spring hooded mutants in comparison to Chinese spring aneuploids for chromosome 4A



**Figure A.8** Microsatellite marker analysis of CS awned mutants. Amplification pattern of microsatellite marker *Xgwm515* mapped on chromosome 2AS in CS, the awned mutants (05-248 through 05-263) and chromosome 2A aneuploid stocks. The first and the last sample contain 1 kb DNA size standard



**Figure A.9** Microsatellite marker analysis of CS awned mutants. Amplification pattern of microsatellite marker *Xgwm425* mapped on chromosome 2AS in CS, the awned mutants (05-248 through 05-263) and chromosome 2A aneuploid stocks. The first and the last sample contain 1 kb DNA size standard



\*05-255

**Table A.1** Pedigree information for the homozygous awned mutants of Chinese spring (CS)

<b>Method of mutagenesis</b>	<b>Season-1 (Greenhouse, Manhattan, KS)</b>	<b>Season-2 (Field plot, Aberdeen, ID)</b>	<b>Season-3 (Greenhouse, Manhattan, KS)</b>	<b>Season-4 (Greenhouse, Manhattan, KS)</b>
<b>Fast Neutron*</b>	03-400-3	272	04-185-3	05-248
	03-400-9	273	04-185-8	05-249
	03-400-14	281	04-185-13	05-250
	03-400-15	282	04-185-14	05-251
	03-400-19	284	04-185-16	05-252
	03-400-21	286	04-185-18	05-253
	03-400-43	298	04-185-32	05-254
	03-400-44	299	04-185-33	05-255
<b>EMS*</b>	03-399-48-5	233	04-184-20	05-256
	03-399-57-6	236	04-184-23	05-257
	03-399-60-6	237	04-184-25	05-258
	03-399-185	259	04-184-66	05-259
	03-399-214	263	04-184-74	05-260
	03-399-215	264	04-184-75	05-261
	03-399-216	265	04-184-76	05-262
	03-399-222	267	04-184-78	05-263

\* The Fast neutron awned mutants were at M<sub>6</sub> generation in season-1 evaluations and the EMS mutants were at M<sub>3</sub> generation

**Table A.2** Phenotypic characterization of awned mutants of Chinese spring wheat

<b>Pedigree Number</b>	<b>Awn phenotype</b>	<b>Additional information</b>
05-249, 05-252, 05-257, 05-258	Hooded	Awns are bent towards the base
05-248, 05-250, 05-251, 05-254, 05-255, 05-256, 05-263	Tipped-1	Awn length varies along the spike and increases towards the tip
05-253, 05-259, 05-260, 05-261, 05-262	Tipped-2	Awn length is uniform along the spike and awns are longer



**Table A.3** List of microsatellite markers used for genotyping the awned mutants of Chinese spring

<b>Chromosome</b>	<b>Number of microsatellite markers used</b>	<b>Microsatellite markers</b>
2A	21	<i>Xgwm614, Xgwm636, Xgwm448, Xgwm122, Xgwm328, Xgwm473, Xgwm512, Xgwm425, Xgwm372, Xgwm515, Xgwm71, Xgwm339, Xwmc261, Xgwm10, Xgwm558, Xgwm294, Xgwm445, Xgwm312, Xgwm356, Xcfa2121 and Xgwm382</i>
2B	20	<i>Xgwm257, Xbarc200, Xgwm148, Xgwm410, Xbarc91, Xbarc7, Xbarc13, Xgwm429, Xgwm630, Xgwm319, Xgwm55, Xbarc160, Xgwm526, Xgwm129, Xgwm210, Xgwm382, Xgwm120, Xgwm388, Xbarc167 and Xgwm501</i>
2D	15	<i>Xgwm210, Xgwm455, Xgwm261, Xbarc145, Xgwm608, Xgwm320, Xgwm349, Xgwm539, Xgwm30, Xbarc228, Xwmc41, Xgwm301, Xgwm382, Xbarc159 and Xwmc167</i>
4A	21	<i>Xcfd2, Xcfa2256, Xcfa2026, Xwmc173, Xgwm4, Xwmc420, Xwmc15, Xcfd71, Xgwm601, Xgwm397, Xcfd31, Xwmc96, Xcfd16, Xgwm44, Xbarc138, Xbarc170, Xwmc48, Xgwm610, Xwmc446, Xwmc468 and Xgwm637</i>
5A	21	<i>Xgwm154, Xgwm205, Xcfa2187, Xgwm293, Xbarc180, Xgwm415, Xgwm165, Xbarc186, Xwmc415, Xbarc141, Xbarc40, Xbarc151, Xgwm156, Xgwm186, Xgwm179, Xgwm126, Xgwm291, Xgwm595, Xgwm410, Xcfa2141 and Xcfd39</i>
6B	15	<i>Xgwm132, Xcfd13, Xgwm193, Xgwm70, Xgwm626, Xgwm88, Xgwm608, Xgwm311, Xgwm361, Xwmc152, Xwmc417, Xbarc127, Xwmc182, Xgwm219 and Xbarc134</i>

**Table A.4** Genotyping of Chinese spring awned mutants using wheat microsatellite markers

<b>Awned mutant</b>	<b>Microsatellite marker deleted</b>	<b>Phenotype classification</b>
05-248	-	Tipped-1
05-249	<i>Xcfa2026, Xcfa2256</i>	Hooded
05-250	-	Tipped-1
05-251	-	Tipped-1
05-252	-	Hooded
05-253	-	Tipped-2
05-254	-	Tipped-1
05-255	<i>Xgwm515, Xgwm425</i>	Tipped-1
05-256	-	Tipped-1
05-257	-	Hooded
05-258	<i>Xcfa2026, Xcfa2256</i>	Hooded
05-259	-	Tipped-2
05-260	-	Tipped-2
05-261	-	Tipped-2
05-262	-	Tipped-2
05-263	-	Tipped-1