Exploring Modified Durum Wheat (*Triticum durum* Desf.*)* Plant Architecture

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Dedication

This Work is lovingly dedicated to my mother, Ayelech Hailemariam, and my father, Metaferia Wolde. Their commitment, support, encouragement, and constant love have sustained me throughout my life.

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Abbreviations

DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization of the United Nations
HI	Harvest Index
t/ha	Ton Per Hectare
Gbp	Gigabase Pairs
TILLING	Targeting Induced Local Lesions In Genomes
RNAi	RNA interference
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CAS9	CRISPR-associated protein 9
RFLP	Restriction Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeat
CAPS	Cleaved Amplified Polymorphic Sequence
NGS	Next Generation Sequencing technologies
GBS	Genotyping-By-Sequencing
Kb	Kilobases
Mb	Megabases
QTL	Quantitative Trait Locus
MBM	Morphological and Biochemical Markers
cM	centiMorgan
Vg	Genotypic variance
Va	variance due to the additive effects
Vd	variance due to the dominance effects
Vi	variance due to the epistatic interactions
H^2	Broad sense heritability
h^2	Narrow sense heritability
σ^2_G	Genotyhaviourpic variance due to additive effects
σ^2_e	Error variance
σ^2_{GEI}	Variance due to Genotype-By-Environment Interaction
Ms_G	Genotypic mean square
Ms_{GEI}	Mean square for the GEI interaction
GEI	Genotype-By-Environment Interaction
GCV	Genotypic Coefficient of Variation
PCV	Phenotypic Coefficient of Variation
ANOVA	Analysis of variance
DH	Double Haploid
RILs	Recombinant Inbred Lines
GWAS	Genome-Wide Association Studies
LD	Linkage Disequilibrium

MAGIC	Multiparent Advanced Generation Inter-Cross
NAM	Nested Association Mapping
NILs	Near Isogenic Lines
SMA	Single-Marker Analysis
SIM	Simple Interval Mapping
CIM	Composite Interval Mapping
LRS	Likelihood Ratio Statistic
LOD	Logarithmic of Odds
PVE	Phenotypic Variance Explained
eQTL	Expression quantitative trait loci
RAM	Root Apical Meristem
SAM	Shoot Apical Meristem
IM	Inflorescence Meristem
SM	Spikelet Meristem
AxM	Axillary Meristem
FM	Floret Meristem
IWPA	Ideal Wheat Plant Architecture
USDA	The United States Department of Agriculture
SS	Supernumerary Spikelet
bh-NILs	*branched head* Near Isogenic Lines
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
F7	The seventh filial generation
IPK14	IPK in 2014
IPK15	IPK in 2015
HAL15	Halle in 2015
GH15	Greenhouse in 2015
°C	Degree Celsius
PH	Potential of hydrogen
°N	Degree North
°E	Degree East
GSR	Global solar radiation
Pt	Precipitation
Tm	Temperature
Hm	Humidity
RCBD	Randomized Complete Block Design
Kg	Kilogram
ha	Hectare
µg	Microgram
µl	Microlitre
pM	picomolar
dNTP	deoxyribose nucleoside triphosphate
U	unit
Taq	*Thermus aquaticus*
5'UTR	The 5' untranslated region

CDS	Coding DNA sequence
3'UTR	The 3' untranslated region
GP	Glume primordium
FP	Floret primordium
TS	Terminal Spikelet
Exp	Expressivity
Pen	Penetrance
RNA	Ribonucleic acid
ng	nanogram
cDNA	complementary DNA
qRT–PCR	Quantitative Real Time Polymerase Chain Reaction
Ct	cycle threshold
E	PCR efficiency
GT	Genotype
POPSEQ	population sequencing
HVA	High-Value Allele
SEM	Standard error of the mean
MITEs	Miniature Inverted-repeat Transposable Elements
TIRs	Terminal Inverted Repeats
TSD	Target Site Duplication
Hap	Haplotype
HF	Haplotype Frequency
GDD	Growing Degree Days
ND	Node Density
NPS	Node Per Spike
PH	Plant Height
PdL	Peduncle Length
PH_wPdL	Plant height without peduncle length
SDW_H	Spike Dry Weight at harvest
SHI	Spike Harvest Index
GA	Grain Area
GL	Grain Length
GW	Seed Width
GPS	Grain Number per Spikelet
GNS	Grain Number per Spike
SL	Spike Length
TKW	Thousands Kernel Weight
addSPS	additional spikelet per spike
totSPS	total spikelet per spike
HD	Heading Date
TN	Tiller Number
FL	Floradur
FL-*bh*-NILs	Floradur-*branched head*- Near Isogenic Lines
M	Mutant

BG	Background Selection
FG	Foreground selection
ER	Embryo Rescue
BC	Backcross
BC1F1	The first backcross generation from F1
BC2F1	The second backcross generation from F1
BC3F1	The third backcross generation from F1
BC4F1	The fourth backcross generation from F1
BC4F2	The fourth backcross generation after Selfing BC4F1
ALF	Automated Laser Fluorescence
RpGR	Recurrent parent Genome Recovery
FT	Free Tillering
DT	De-tillerd
TF	Tiller Fertility
SE	Sieve element
Fam	Family

1 Chapter 1: General Introduction

1.1 Polyploidy and genome evolution in plants

Polyploidization has long been known as a biological barrier to gene flow and instantaneously led to reproductive isolation among related organisms which ultimately results in new species (Mallet, 2007). Even though the term 'species' was first introduced by Carl Linnaeus in the 18th century as a basic unit of biological or taxonomic classification, as such there is no universal definition of species that applies to all organisms (González-Forero, 2009; Mora et al., 2011). However, it has continued to evolve in an attempt to address the values of different species classification criteria (Ladizinsky, 1998).

Among several proposed species concepts, only three of them seemed to have great importance (Ladizinsky, 1998). These are the 'morphological species concept', the 'biological species concept' and the 'evolutionary species concept'. Morphological species, also known as taxonomic species concept, defines species on the basis of shared morphological characteristics. However, one of the weaknesses of morphological species concept was that species with similar morphological characteristics can still be reproductively isolated. Due to such kind of recognizable weaknesses of the morphological species concept, Ernst Mayr proposed the 'biological species concept' (Ladizinsky, 1998; de Queiroz, 2005). According to Mayr, species are breeding population where individuals in the population are capable of interbreeding and are reproductively isolated from another species. However, the biological species concept also came with its own limitations. One of these is the uniparental (single parent) organisms which reproduce clonally through fragmentation and thus, they cannot fulfill the definition of the biological species concept. Then in 1961, Simpson proposed another species concept known as the 'Evolutionary species concept' (Simpson, 1961). Simpson defined species as a lineage characterized by ancestral-descendant populations which can maintain their own identity from other such lineages and have their own evolutionary tendencies.

Regardless of the ambiguity in the definition of species concept, reproductive isolation can lead to genetic isolation (Rieseberg and Willis, 2007) which could be instant or a gradual process (Ladizinsky, 1998). Factors which gradually lead to speciation includes geographical and ecological barriers as well as the modification of reproduction time or behavior (Cracraft, 1983; Ladizinsky, 1998; Sexton et al., 2009). Furthermore, chromosomal rearrangement and sudden change in chromosomal number also another key factor leading to an instant speciation event (Rieseberg, 2001; Faria and Navarro, 2010).

Hence, polyploidization (genome doubling) is one of the driving forces, and thus, a well-established theory of speciation in plants (Adams and Wendel, 2005; Soltis et al., 2015; Wendel et al., 2016). Several cytological events that could lead to polyploidization includes fusion of unreduced gametes from the same or different species, somatic genome duplication, and minor karyotype changes through aneuploidy and/or dysploidy (Ramsey and Schemske, 1998; Stift et al., 2008; De Storme and Mason, 2014). Hence, species undergoing such polyploidization event are reproductively isolated from their ancestral parents due to unbalanced chromosomal complements (aneuploidy) which lead to the sterility of the offspring (Ladizinsky, 1998; Ramsey and Schemske, 2002; Mallet, 2007).

Generally, three types of polyploidization are known. These are autopolyploidy, allopolyploidy and segmental allopolyploidy (Stebbins, 1947; Wendel, 2000; Madlung, 2013). Autopolyploidy arise due to genome duplication within the same species and thus, all genomes are identical or very similar (Stebbins, 1947; Lewis, 1980). Allopolyploidy, by contrast, contains two or more distinct genomes which arise via the hybridization of two different species with a concomitant genome doubling. Segmental allopolyploid carries more than two partially differentiated genomes, which can lead to the formation of both bivalents and multivalents during chromosome pairing (Stebbins, 1947; Madlung, 2013). Crop plants such as potato (*Solanum tuberosum* L.)(2n=4x=48), banana (*Musa sapientum* L.)(2n=3x=33), and Alfalfa (*Medicago sativa* L.) (2n=4x=32) are autopolyploids, while wheat (*Triticum aestivum* L.)(2n=6x=42), coffee (*Coffea arabica* L.)(2n=4x=44), strawberry (*Fragaria x ananassa*)(2n=8×=56), cotton (*Gossypium hirsutum* L.), and canola (*Brassica napus* L.)(2n=4x=38) are all allopolyploids.

Being polyploid has both advantages and disadvantages (Comai, 2005). Among the advantages are heterosis (vigorous as compared to their diploid progenitors) and gene redundancy (ability to withstand deleterious genetic errors or mutations). Among the disadvantages of being polyploidy are the size of the genome and the volume of the cell; the occurrence of spindle irregularities which can lead to the chaotic segregation of chromatids and to the production of aneuploid cells; potential changes or deviations in gene expression; epigenetic instability which may lead to epigenetic gene regulation.

Polyploids generally differ from their progenitors in several morphological, ecological, physiological and cytological aspects (Levin, 1983; Dubcovsky and Dvorak, 2007). These further ensure the competitive advantage of polyploids over their progenitors thereby contributes to the fitness and exploitation of a new niche.

Polyploidization events often accompanied by massive gene silencing, elimination of duplicated genes, and dosage compensation (Feldman et al., 1986; Paterson et al., 2004; Adams and Wendel, 2005; Comai, 2005; Wang et al., 2005). Because allopolyploids carry closely related genomes, there is a risk of the incorrect pairing of the homoeologous chromosome during meiosis which could lead to a reduced fertility (Ma and Gustafson, 2005). To avoid such problem, however, allopolyploids undergo a restricted pairing of homologous chromosomes which is generally known to be as cytological diploidization: an evolutionary process whereby a tetraploid species 'decays' to become a diploid (Wolfe, 2001; Feldman and Levy, 2012). However, the molecular mechanism associated with diploidization is not well understood; but it is believed to be through the accumulation of DNA sequence changes (and/or deletions) between the chromosomes and functional divergence of the duplicated genes through process known as sub- and neo-functionalization (Wolfe, 2001; Adams and Wendel, 2005; Comai, 2005; Hughes et al., 2014). If retained, the individual homoeologous gene pairs may have identical functions, may partition and share the original gene function through a subfunctionalization or they may diverge and develop novel functions through neofunctionalization.

1.2 Wheat genome evolution

According to Darwin's Theory of Evolution, all life is related and has descended from a common ancestor(Hull, 1973). Based on this principle, the diversification of the grass family (Poaceae or also known as Gramineae) from a common ancestor is believed to occur about 55–70 million years ago (Kellogg, 2001). The family contains approximately 10,000 distinct species and about 600 to 700 genera (Kellogg, 2001). About 33% of the earth's land surface is also believed to be grasslands (Shantz, 1954). Among several tribes known in the grass family, the Triticeae tribe containing wheat (*Triticum aestivum*), barley (*Hordeum vulgare L.*) and rye (*Secale cereal*), is the most important tribe from an agricultural point of view.

Wheat came into existence as an allopolyploidization event which occurred twice in wheat. The first allopolyploidization (inter-specific hybridization) occurred some 100,000 years ago between the A-genome progenitor, *Triticum urartu* (2n=2x=14; AA) and the B genome progenitor, *Aegilops speltoides* (2n=2x=14; BB) resulting in tetraploid wheat: *Triticum turgidum* (2n=4x=28; AABB) (Petersen et al., 2006) (Figure 1-1). Hence, *Triticum turgidum* is believed to be an ancestor of the wild and cultivated emmer and durum wheat (*T. turgidum sp. durum*). To date, *T. turgidum sp. durum* is a widely grown species for pasta production.

The second major hybridization event, which occurred about 10,000 years ago, was between *T. turgidum* (2n = 4x = 28; AABB) and goat grass or *Aegilops tauschii* (2n = 2x = 14; DD) (Middleton et al., 2014; Krattinger and Keller, 2016). This gave rise to the ancestral allohexaploid bread wheat, *T. aestivum* (2n = 6x = 42, AABBDD) (Petersen et al., 2006; International Wheat Genome Sequencing, 2014).

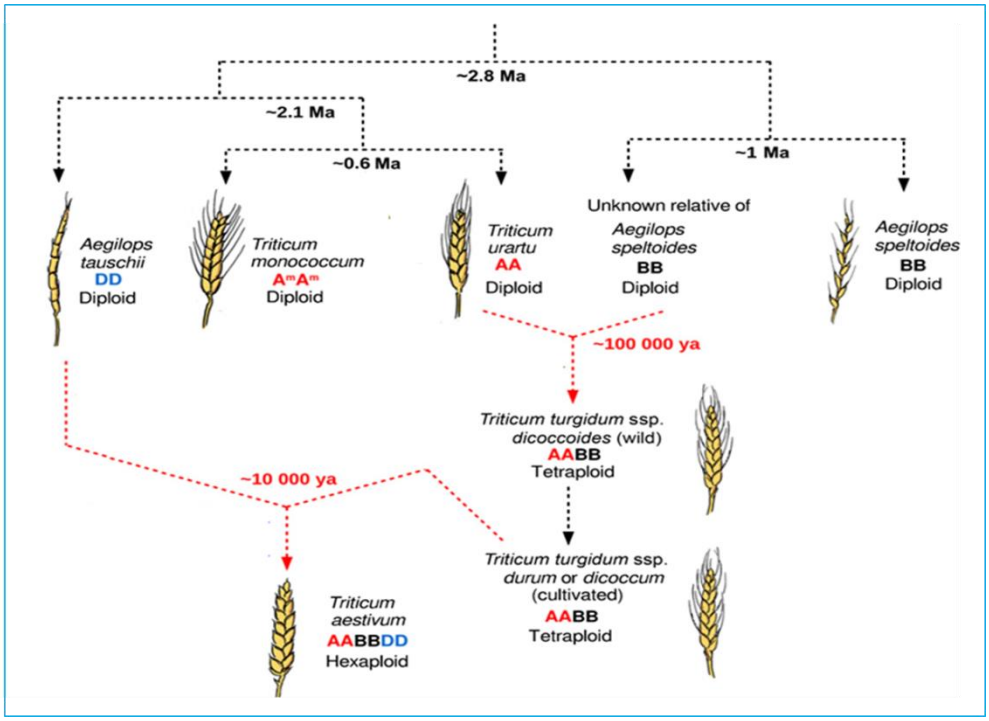


Figure 1-1. Hybridization event and genealogy of the wheat genome
Taken from (Krattinger and Keller, 2016).

Each of the three genomes, i.e. A, B, and D are now called homoeologous genome and coexist in the nucleus with strong pairing behavior of the homologous chromosome sets at meiosis i.e. A with A, B with B, and D with D. This leads to the formation of bivalents, which is a typical characteristic of allopolyploids resulting in the so-called disomic inheritance (Chapman and Riley, 1970; Feldman et al., 1986), while the autopolyploids form multivalents at meiosis and depict polysomic inheritance (Stift et al., 2008; Soltis et al., 2014).

The major gene controlling such homologous chromosome pairing and recombination in wheat has also been identified and named as *Ph1* (Griffiths et al., 2006). *Ph1* ensures that the recombination is happening only between the homologous pairs i.e. avoiding the pairing homoeologous chromosomes. More interestingly, the wheat plant gained the activity of *Ph1* during the polyploidization event, and thus, *Ph1* plays the major role in wheat genome stability (Griffiths et al., 2006)

1.3 Domestication of wheat

Human lifestyle before the start of agriculture relies on wild food through hunting and gathering. Change in the lifestyle of the ancient human came after food production through plant and/or animal domestications which were started about 10,000 years ago during the Neolithic revolution (Haviland et al., 2006). Thus, the Neolithic Revolution has laid the foundation for the transition of human lifestyle from Hunter-Gatherers to sedentary life through food production.

Neolithic Revolution took place in the western part of the Fertile Crescent, known as the Levantine Corridor and then expand to southwest Asia around the Tigris, Euphrates and Jordan rivers and their adjacent hilly flanks, which is believed to be a Cradle of Agriculture and also considered to be the major center for the domestication of several modern day crops (Lev-Yadun et al., 2000; Matsuoka, 2011). Neolithic founder crops that are believed to be domesticated in Fertile Crescent includes diploid einkorn wheat [*Triticum monococcum*], tetraploid emmer wheat [*Triticum dicoccum*], barley [*Hordeum vulgare*], lentil [*Lens culinaris*], pea [*Pisum sativum*]), flax (*Linum usitatissimum*), bitter vetch (*Vicia ervilia*) and chickpea (*Cicer arietinum*) (Brown et al., 2009). Modern day countries included in this region are Israel, Jordan, Lebanon, western Syria, southeast Turkey, Iraq and the western Iran (Salamini et al., 2002; Matsuoka, 2011).

The process of altering unwanted plant traits through genetic selection is called domestication. It is defined as "An evolutionary process whereby humans modify, either intentionally or unintentionally, the genetic makeup of a population of plants or animals, sometimes to the extent that members of the population are unable to survive and/or reproduce without human assistance" (Haviland et al., 2006). Although domestication of crops took place independently to one another, the phenotypic suits associated with domestication were quite similar. These phenotypic suits or packages are associated to yield or productivity in one or the other way and are known as the 'domestication syndrome' (Harlan, 1971; Meyer et al., 2012). Domestication syndrome-related traits include reduced grain shattering, easiness of threshability, weakened seed dormancy, enlarged panicle/spike size, seed color, reduced shoot branching (tillering), large grain/fruit size, and so on.

The domestication of wheat started with the domestication of diploid wheat (*Triticum monococcum*) which gave the domesticated diploid wheat (Einkorn wheat) (Heun et al., 1997; Kilian et al., 2007). Cultivation of the domesticated diploid wheat, however, was replaced by the domesticated polyploid wheat due to the adaptive significance of the polyploid genome as compared to its diploid relative (Salamini et al., 2002; Osborn et al., 2003). Nevertheless, there

were traits that were not entirely up to human need and desire. Therefore, humans started to interfere and select 'best' fitting plants for their own purpose and desire.

One of the major distinguishing feature between wild (*Triticum turgidum* L. ssp. *dicoccoides*; 2n=4x=28; AABB) and domesticated emmer wheat (*Triticum turgidum* L. ssp. *turgidum;* 2n=4x=28; AABB) is the strength of the inflorescence (spike). The spike of wild emmer is known to be weak (brittle) that the spikelets (the building blocks of the inflorescence) can easily drop off (shatter). Of course, shattering was one of the mechanisms for natural seed dispersal in wild plants including wild emmer wheat, and thus, it is a means of survival and generational continuity. However, at the time of domestication, early farmers ('breeders') deliberately selected the non-brittle form to retain the seeds on the spike and maximize seed harvest. Therefore, the acquisition of a non-brittle rachis, which limits the natural seed dispersal mechanisms (shattering) of the wild forms, allows farmers to harvest more grains directly from the plant.

This contrasting feature of the spike form in the wild and domesticated emmer wheat was later known to be controlled by two major domestication-related genes, *brittle rachis 2 (Br-A1)* and *brittle rachis 3 (Br-B1)* located on the short arms of chromosome group 3 (Watanabe et al., 2002). Therefore, a non-brittle rachis (non-shattering spike) was one of the critical early steps in wheat domestication. Recently, mutation in two dominant and complementary barley genes, *Btr1* and *Btr2,* converted the spike from brittle (wild-type) into a tough, non-brittle form promoting grain retention during domestication (Haberer and Mayer, 2015; Pourkheirandish et al., 2015).

Selection of a non-fragile rachis by ancient people was not the only trait modified during domestication; but also traits such as glume tenacity and spike threshability (Sood et al., 2009). After the inter-specific hybridization of *T. turgidum* (2n = 4x = 28; AABB) and the diploid goat grass *Aegilops tauschii* (2n=2x =14; DD), two morphologically distinct hexaploid wheat forms were known (Akerman and Mackey, 1948; Salamini et al., 2002). These are *T. spelta* (which is hulled or non-free threshing wheat) and *T. vulgare* (non-hulled and free-threshing bread wheat). According to the current knowledge, the hulled trait is principally controlled by the Tg (tenacious glume) and the *q* (speltoid) loci (Dvorak et al., 2012). Hence, the free-threshing hexaploid wheat, which is characterized by soft glumes, carries the inactive form of the *Tg* alleles (i.e. *tg*) located on 2A (*Tg-A1*), 2B (*Tg-B1*), and 2D (*Tg-D1*) (Dvorak et al., 2012). The *Q* (square head) gene, which is also required for the free-threshing characteristics of wheat, is located on the long arm of chromosome 5A (Snape et al., 1985; Kato et al., 1998; Simons et al., 2006). Wild and domesticated

emmer and spelt wheat have the recessive q allele, which causes the speltoid spike morphology, tenacious glumes, and brittle rachis in hexaploid wheat (Mackey, 1954; Luo et al., 2000; Simons et al., 2006). The *Q* gene arose due to spontaneous mutation, which had been suggested to have occurred only once to the most primitive allele *q* (Simons et al., 2006). Although other genes are undoubtedly involved, *Q* and *tg* loci are largely believed to be two major known loci connected to the free-threshing ability; and thus contributed to the widespread cultivation of wheat to date.

1.4 Wheat today and tomorrow

Since its domestication, wheat is serving human in all aspects. From our daily bread to biofuel production; wheat is being used in all aspects. The average global wheat consumption is estimated to be 73 kg per capita per annum and can be as high as 166 kg per capita in North African and central Asian countries(Trethowan and van Ginkel, 2009). So, wheat is one of the world's largest and most important food crop (Gustafson et al., 2009). Production wise, in 2013 alone the total world harvest for wheat was estimated to be 713 million tons as compared with 1 billion tons of maize and 745 million tons of rice (FAO, 2013). Durum wheat, which is mainly used for the pasta industry, contributes about 6–8% of the global wheat production (Troccoli et al., 2000).

Since the start of agriculture, spectacular yield increment in wheat has been registered during Green Revolution period in the mid-1960s (Evenson and Gollin, 2003). This was mainly due to the introduction of lodging resistant semi-dwarfing varieties, which enabled farmers to use improved wheat varieties in combination with a higher dose of fertilizer. Since then, a linear increase in yield has been achieved for several decades in almost all top wheat producing countries. However, yield increment seemed to be slowing down; probably touching the biological limit in some of the top wheat-producing European countries such as Germany, UK, and France. This is probably because of the lack of major achievement for increasing yield, especially in the last two decades (Figure 1-2). About 12% of the global wheat production comes from these three countries alone, indicating the requirement for the strategic research re-thinking in order to narrow down the gap between supply and demand.

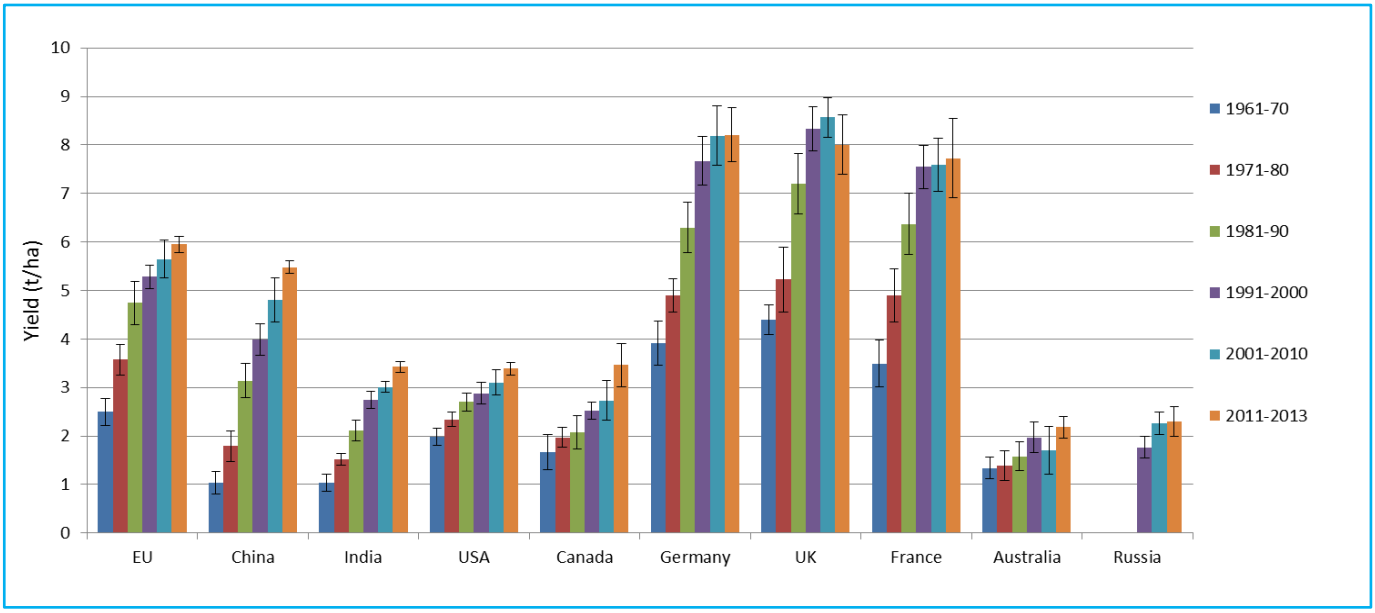


Figure 1-2.Trends of average wheat yield since the Green Revolution.
A 10 years average (±SD) yield per hectare (t/ha) has been computed from data obtained from FAO, 2013 (http://faostat3.fao.org/home/, 2013). EU, European Union; USA, United States of America; UK, United Kingdom

Furthermore, there is a thought that Harvest Index (HI) in wheat is approaching the theoretical maxima (0.6) (Austin et al., 1980); meaning that yield increment only by improving HI is not possible unless new strategic approach is devised.

Although wheat is a mega-environment crop, which is widely grown from the arctic to the tropics and from the lowlands to the highlands (Curtis et al., 2002), production of wheat is under several emerging environmental and disease threats. Rise in temperature as a result of global warming is believed to be one of the threats to the global grain yield production as well as grain nutritional qualities (Gibson and Paulsen, 1999; Myers et al., 2014; Asseng et al., 2015; Lesk et al., 2016). Growing evidence also clearly indicating that wheat is also the most affected crop by the climatic change (Ortiz et al., 2008; Semenov, 2009; Semenov and Shewry, 2011; Trnka et al., 2014). Furthermore, drought and heat stress are also emerging threats to wheat production in several regions including Europe. For example, by 2050 heat stress is expected to be a major threat to winter wheat production in Europe (Semenov, 2009; Semenov and Shewry, 2011). In India, which is the second wheat producing country in the world, wheat production will be vulnerable to heat stress as a result of climate changes (Chatrath et al., 2007; Ortiz et al., 2008). In china, which is the first wheat producing country in the world; and about 85% of which is a winter wheat (He, 2001), production is also under a growing risk of heat stress between heading and maturity (Liu et al., 2014). In Australia, which is also one of the top wheat-producing countries, wheat production is under variable rainfall and often associated with severe drought (Potgieter et al., 2005). In the

USA, about 60 % of the wheat comes from drought susceptible regions of the Great Plains (Paulsen and Shroyer, 2008; Basara et al., 2013). Generally, the effect of high temperature is particularly severe during reproductive and grain-filling phases of wheat (Farooq et al., 2011). Therefore, heat and drought-tolerant wheat varieties are future requirements for wheat production in order to cope up with the eminent global threat.

1.5 Functional genomics in wheat

Grass genome has gone through a complex genome evolution and chromosomal restructuring (Wendel et al., 2016). This has resulted in differences in genome size, chromosome number, and organization. To better understand the genomic interrelationship among the different monocots, genome sequence information plays the major role. So far, genome sequencing of important crops like rice (Goff et al., 2002), maize (Schnable et al., 2009) , sorghum (Paterson et al., 2009), barely (International Barley Genome Sequencing et al., 2012) and model plant Brachypodium (Vogel et al., 2010) has been completed and publically available to accelerate functional genomics in monocots.

The wheat genome is large and highly complex compared to other monocots. The size is estimated to be 17Gbp (Brenchley et al., 2012; International Wheat Genome Sequencing, 2014) which is 63x larger than the genome of Brachypodium, 40x larger than the rice genome, 23x larger than sorghum genome, 7x larger than the maize genome, and 3.3x larger than barley genome. The size of the wheat genome is mainly attributed to its ploidy level (composed of three related diploid sub-genomes) and proliferation of repetitive elements (Smith and Flavell, 1975; Paux et al., 2006). Nevertheless, the first chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) has recently been released (International Wheat Genome Sequencing, 2014) in addition to the draft genome sequence of the A-genome progenitor *Triticum urartu* (Ling et al., 2013).

Besides genome sequence information, high-density linkage maps have now become possible almost in all crops due to high throughput next generation sequencing technologies. Furthermore, the identification of the physical positions (physical map) and integration (anchoring) of genetic markers to the actual physical positions in the genome of several crops facilitates physical mapping and gene tagging for map-based gene cloning approach. However, one of the major challenges in the post-genomic era is the determination of the functions of the genes in the genome. The situation is even more complicated in wheat due to its complex ploidy level of wheat. For instance, identification of mutant phenotypes from gene knockouts is not always

straightforward approach in wheat due to gene dosage effects as a result of homoeologous gene copies from each sub-genomes. However, tools such as RNA-based gene silencing (RNAi) (Travella et al., 2006), Targeting Induced Local Lesions In Genomes (TILLING) (Slade et al., 2005; Kurowska et al., 2011; Chen and Dubcovsky, 2012) are useful functional genomics tools in wheat. Wheat can also benefit from the new genome editing tools such as Clustered, Regularly Interspaced, Short Palindromic Repeats /cas9 (CRISPR/cas9) (Shan et al., 2014; Wang et al., 2014b).

1.6 Introduction to molecular genetic markers

Genetic molecular markers are simply DNA sequences derived from the genome which are used to track genome level molecular events such as meiotic recombinations. A genetic marker is a gene or DNA sequence with a known location on a chromosome which can be associated with a particular gene or trait. So, polymorphisms at the DNA level; for example, due to mutation or sequence alteration, is the basis for the evolution of molecular markers and their vast application. Generally, markers are of three different classes: morphological (phenotypic markers), biochemical (markers from gene products) and molecular (DNA) markers (Tuberosa, 2008). Morphological markers are markers based on the morphology of different structures including organ shape and pigmentation while biochemical or protein markers are markers from gene product i.e. protein or an enzyme. Molecular markers detect variation at the molecular (DNA) level due to sequence variation as a result of deletion, duplication, inversion and/or insertion. Both morphological and biochemical markers were used before the discovery of DNA-derived molecular markers.

1.6.1 History of molecular genetic markers

Molecular genetic markers are the building blocks of genetic maps and are inherited like the Mendelian traits (Schulman et al., 2012). Thus, the first DNA-based molecular genetic marker was RFLP (Restriction Fragment Length Polymorphism) and first used in human Genome mapping (Botstein et al., 1980). RFLP is based on restriction digestion of the genomic DNA and probe hybridization assay to the digested genomic fragments. The probes are generated from single copy DNA loci. RFLP markers are inherited co-dominantly, meaning that it is possible to differentiate between homozygous and heterozygous and are reproducible. Following the human genome mapping, RFLP-based markers were gradually adopted by plant communities (Tanksley et al., 1989). However, the drawbacks of RFLP markers are more pronounced to the genomes characterized by a higher proportion of repetitive elements like wheat (Ranjekar et al., 1976). Nevertheless, RFLP-based genetic linkage maps spanning the different homoeologous group in

wheat were produced as a tool to bridge the gap between classical and molecular genetics (Chao et al., 1989; Gill et al., 1991; Anderson et al., 1992; Devos et al., 1993; Nelson et al., 1995b; Nelson et al., 1995c).

After the discovery of polymerase chain reaction (PCR) by Mullis et al. in 1986 (Mullis et al., 1986), new classes of markers started to evolve (Figure 1-3). Hence, the basis for DNA markers that are used to date is based on the principle of RFLP and/or PCR technology (Tuberosa, 2008).

The first class of such markers is Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990). One can generate such markers by designing pairs of random PCR primers even without knowing DNA sequence of the target species. The PCR product can then be analyzed by electrophoresis. However, RAPD markers are inherited dominantly (the marker is either present or absent) and have limited reproducibility. Hence, because of the technical limitations of RFLP-based markers (Karp et al., 1996) and lack of reproducibility of RAPD markers(Staub et al., 1996), both RFLP and RADP markers were not used widely as universal genotyping tools.

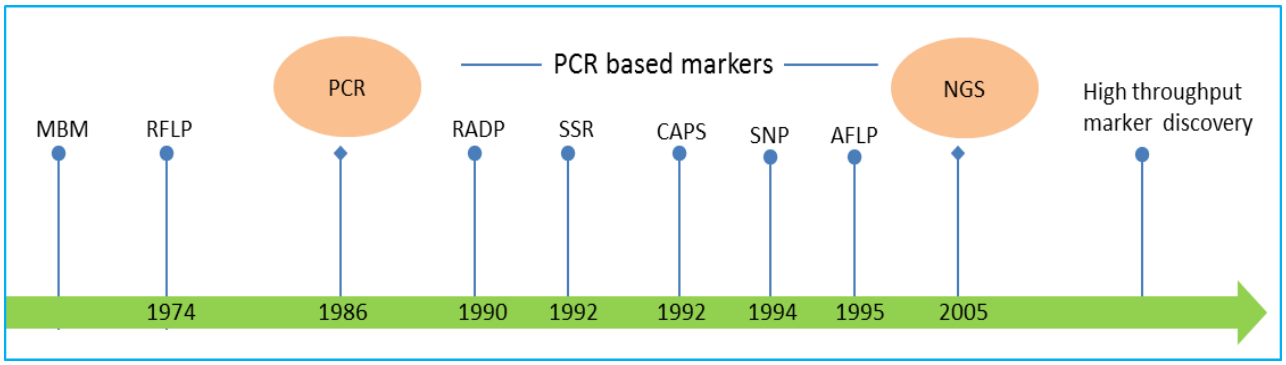


Figure 1-3. Chronological evolution of genetic molecular markers
MBM, Morphological and Biochemical Markers

Another type of PCR-based marker system is Simple Sequence Repeat (SSR). Simple Sequence Repeats (SSRs) are also called microsatellite markers; and are small stretches of DNA sequence consisting of tandemly repeating sequence motifs of length 1-6 bP (Tautz and Schlotterer, 1994; Zietkiewicz et al., 1994). The most common motif includes two alternating bases (di-nucleotides repeats) of type $(CA/GT)_n$, $(CG/GC)_n$ or $(AT/TA)_n$ (Tautz and Schlotterer, 1994; Zietkiewicz et al., 1994; Yang et al., 2015). The basis for microsatellite markers was human and mammalian genomes (Zietkiewicz et al., 1994; Powell et al., 1996). Nevertheless, the plant communities have adopted SSR markers because of the abundance of SSR markers as compared to RFLP and RADP (Akkaya et al., 1992; Wu and Tanksley, 1993; Roder et al., 1995). Despite the fact that SSR markers

are highly informative markers and inherited co-dominantly, marker discovery is time-consuming and expensive. Two strategies are followed to develop SSR markers. The first one is to prepare a probe containing the desired repeat sequence in order to identify the homolog from a DNA library. This method can be used if we don't have genomic sequence information of the target species. The second strategy is to use flanking sequences of a tandemly repeating sequence motif to design primers set for PCR amplification of the target SSR marker from genomic sequence data (Akkaya et al., 1992). To allow visualization of the PCR amplicon (product) on autoradiography on a standard sequencing gel, labeled primers are used. The basis for polymorphism is the differences in a number of repeats and can be detected by gel electrophoresis as well for a particular locus.

Progress towards the development of molecular genetic markers added another new co-dominant genetic marker, known as Cleaved Amplified Polymorphic Sequence (CAPS), to the list of PCR-based molecular genetic markers (Figure 1-3). CAPS markers are a form of genetic markers based on variation in the length of DNA fragments generated by restriction digestion of target PCR products (Akopyanz et al., 1992; Jarvis et al., 1994). CAPS markers are co-dominant meaning that they can easily differentiate between homozygous and heterozygous and are reproducible and easily detected by agarose gel electrophoresis. It is also simple to develop, and also affordable and applicable in positional or map-based cloning. Apart from their own shortcomings, all marker types discussed above were used at different times in crop and animal genetic research fields. Identification and applications of some of these markers, however, are simply laborious. Furthermore, the technology of generating such markers is of low throughput (Agarwal et al., 2008).

Marker discovery has been now completely revolutionized by the advent of Next Generation Sequencing technologies (NGS) and *in silico* marker identification tools (Tang et al., 2006). Single Nucleotide Polymorphism (SNP) markers are part of next generation genetic markers based on single base changes in DNA sequence. They are the most abundant molecular markers in the genome although their occurrence and distribution vary among species and genomic regions of the same species. On average 1 SNP/ 1,900 bp of the human genome (Sachidanandam et al., 2001) , 1 SNP/34-121 bp of maize genome (Ching et al., 2002), 1 SNP/240 bp of barley genome (Ching et al., 2002) and 1 SNPs per/ 250-330 bp of the rice genome(McNally et al., 2006) has been reported. The SNP markers are usually common in non-coding regions of the genome (Agarwal et

al., 2008). SNP detection can be achieved through high-throughput sequencing technologies and analytical (bioinformatics) tools (Tang et al., 2006).

1.6.2 Genotyping-By-Sequencing (GBS)

NGS technologies facilitated the development of high throughput SNP marker discovery based on whole or fraction of genome sequencing approach. However, sequencing and re-sequencing of the entire genome are still costly for routine SNP discovery. Nevertheless, several strategies have been developed which still uses high throughput sequencing technologies with a reduced cost of sequencing. One of these strategies is reduced representation and sequencing approach (Altshuler et al., 2000; Luca et al., 2011). It is a strategy being implemented especially when the goal is to develop markers for genetic and/or physical mapping.

For capturing and sequencing a subset (portion) of a genome, two main approaches have been widely used (Luca et al., 2011). The first approach is re-sequencing of a target region which can be selected either by PCR amplification or by hybridization to complementary oligonucleotides (Albert et al., 2007; Okou et al., 2007). However, oligonucleotide libraries significantly add up to the overall costs of sequencing. The alternative approach is, therefore, to produce a reduced representation of the genome through restriction digestion and directly sequencing of the fragments (Baird et al., 2008; Van Tassell et al., 2008). More interestingly, this approach does not require prior sequence information.

Production of genomic fragments with a reduced representation of the genome can be achieved by using methylation-sensitive restriction enzymes (Poland et al., 2012b). This method is widely used to develop SNP markers for mapping population and for genomic selections in breeding particularly for complex genomes like wheat (Poland et al., 2012a; Poland et al., 2012b; Gardner et al., 2014; Huang et al., 2014; Edae et al., 2015; Islam et al., 2015).

GBB is a simple but robust high throughput short-read sequencing approach based on genome complexity reduction algorithms (Elshire et al., 2011; Poland et al., 2012b). A simplified GBS approach is shown in Figure 1-4. Due to the fact the wheat genome is filled with repetitive and transposable elements (more than 80 % of the genome) which cause major problem during genome sequencing and assembly (Smith and Flavell, 1975), the application of methylation-sensitive restriction enzymes systematically exclude the effect of such repetitive elements because of the fact that repetitive elements are heavily methylated (Cantu et al., 2010; He et al., 2011).

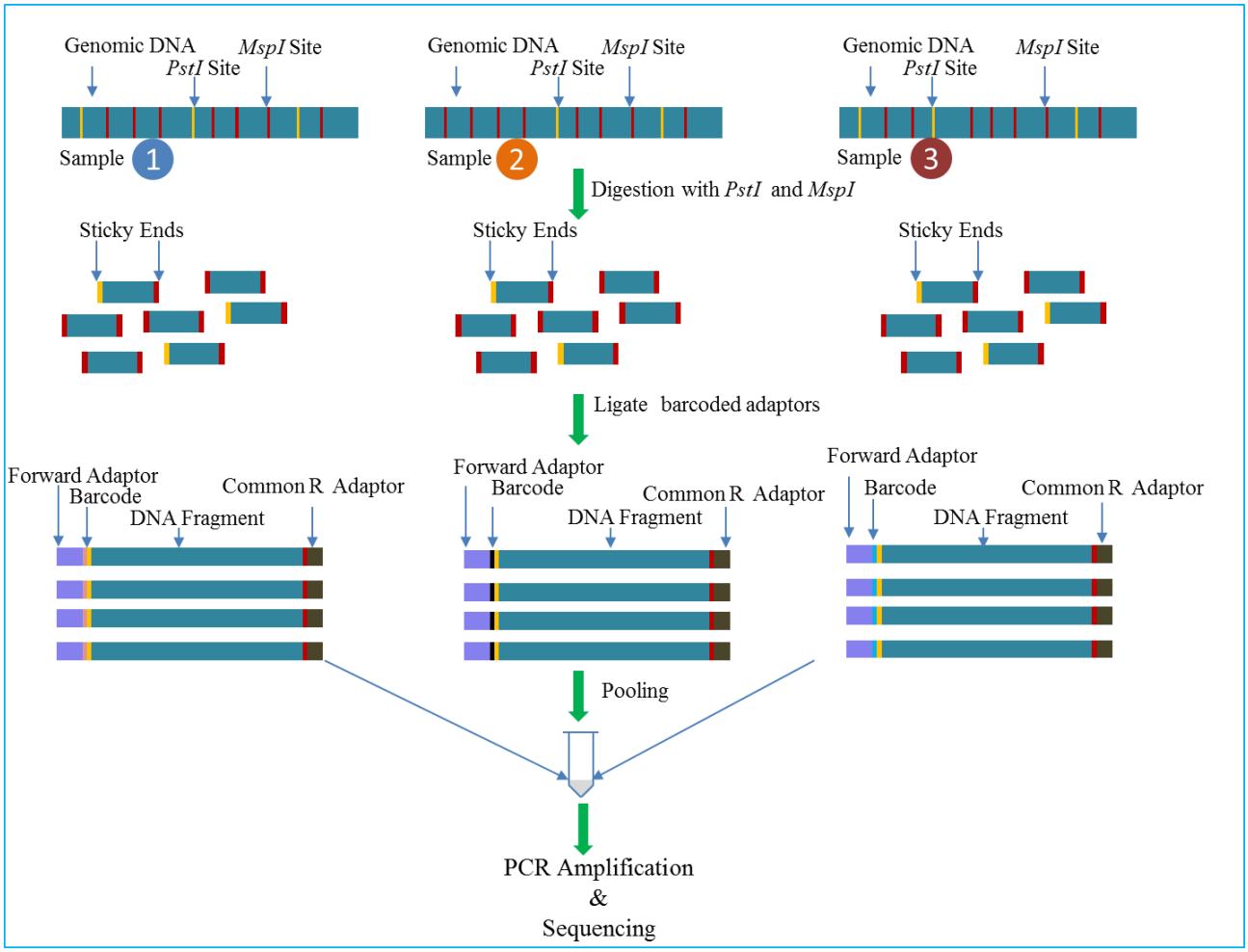


Figure 1-4. The simplified principle of Genotyping-By-sequencing approach.
DNA samples from each individual (sample 1, 2, 3…) are digested using two methylation-sensitive restriction enzymes: *PstI* and *MspI* to generate a reduced representation of the genome i.e. to produce Reduced Representation Library (RRL) which covers a small portion of the genome. For the identification of each fragment, a custom designed barcode adaptor, which is unique for each sample, will be attached (ligated) to the first sticky end. The Illumina standard forward adaptor also ligated during this step. On the other side of the sticky end of each of the fragment, Illumina standard common reverse adaptor will be ligated. After this step, samples will be pooled into a single tube. After completing the remaining steps for Illumina standard library preparation, pooled samples will be sequenced. Based on the barcode sequence, the resulting large file of DNA sequences will be retrieved and assembled into stretches of DNA for each sample. The stretches of DNA sequences readouts will be aligned to a reference genome and SNP markers will be processed thereafter.

1.6.3 Linkage map

The linear order of DNA-derived markers on a chromosome is known as genetic linkage map. The map is assembled based on the meiotic recombination of the mapping population. During prophase of meiosis I, the replicated chromosomes group together, and form a bivalent structure composed of two pairs of sister chromatids. These grouped sister chromatids exchange pieces of chromosomal segments and create new recombination of alleles which ultimately disrupts existing linkage relationship whose effect will be manifested on the progenies. Thus, the progenies

that resemble the parents (parental types) are non-recombinants; while those which don't resemble the parents(non-parental types) are recombinants (Hartwell et al., 2006). Therefore, the first step in linkage mapping is the development mapping population and the generation of molecular markers. Based on this information, recombination frequency can be determined by the following formula:

Equation 1-1. Recombination Frequency (%)

$$\text{Recombination frequency (RF)} = \frac{\text{number of recombinants}}{\text{total number of progenies}} \text{x } 100$$

A genetic linkage map is measured in map units called centimorgans (cM) (Hartwell et al., 2006). Thus, a map unit is equivalent to a 1% frequency of recombination between two loci. Linked genes/loci in each chromosome are considered to be as one linkage group (= a chromosome) and inherited as a group and move as a unit rather than independently during gamete formation. Nevertheless, such linkage relationship of markers on the chromosome cannot be retained permanently; due to recombinational events among loci (Creighton and McClintock, 1931).

Similarly, the actual physical position of such markers/genes can also be assigned to the chromosome as the physical map. The physical map distance is measured in terms of base pair (bp), kilobases (1kb =1,000 bases) or megabases (1Mb = 1 million bases). Although the methods used to construct genetic and physical maps are completely different, the orders of markers/genes in both maps should in principle be the same. However, due to uneven distribution of cross-over events and a non-random gene distribution on the chromosomes; the information depicted by genetic and physical maps are often not the same (Sandhu and Gill, 2002a; Akhunov et al., 2003; Anderson et al., 2003; Erayman et al., 2004; Sidhu and Gill, 2005; Gaut et al., 2007). That is, genetic distance, which is computed from recombination events in terms of map unit (cM), does not necessarily reflect the actual physical distance between markers (measured in terms of millions of base pairs). Nevertheless, integration of the genetic and physical maps facilitates the process of marker development, molecular tagging and cloning of major QTL.

1.6.4 Genetic linkage map of wheat

An enormous progress in sequencing technologies facilitated the process of gene mapping in an unprecedented way. Thus, genetic maps are available for almost all crops, including wheat. Since the discovery of early generation markers until modern day high throughput NGS technologies, several genetic markers were developed for wheat, especially for the hexaploid wheat. For

instance, RFLP-based wheat genetic maps (Chao et al., 1989; Liu and Tsunewaki, 1991; Anderson et al., 1992; Devos et al., 1992; Devos et al., 1993; Deynze et al., 1995; Nelson et al., 1995a; Gill et al., 1996b; Gill et al., 1996a; Blanco et al., 1998; Mingeot and Jacquemin, 1999); SSR-based wheat map (Roder et al., 1998); and high-density SNP-based wheat maps (Wang et al., 2014a; Chapman et al., 2015) can be mentioned. However, no AFLP-based linkage map of wheat was published so far except in wild Emmer Wheat, *T. dicoccoides*, (Peng et al., 2000). Generally, genetic linkage map in wheat is restricted to very few molecular markers namely, RFLP, SSR, and SNP. The genetic maps are also not that dense as compared to other monocots for map-based gene cloning in wheat. However, molecular markers derived from high-throughput NGS technologies have now enormously improved marker development and gene tagging in wheat (Wang et al., 2014a; Chapman et al., 2015).

1.7 The basis for genetic and phenotypic variation

Gene, genotype, and phenotype are the most frequently used terminologies in genetics. They were introduced by Wilhelm Johannsen in 1923 (Johannsen, 1923). He defined 'genes' as particulate units of heredity, 'genotype' as the genetic constitution of an organism and 'phenotype' as an organism's totality of inherited characteristics i.e. the observable physical or biochemical characteristics of an individual. The phenotype of an individual organism is determined by the genotypic constitution and environmental effects. Therefore, phenotypic variations arise due to gene action under certain environment.

1.7.1 Partitioning of the phenotypic variance

Several plant traits are controlled quantitatively; meaning that the trait is affected by several genes/loci. Thus, the effect of an individual locus on the trait(s) is usually small. These types of traits are known as quantitative trait loci (QTL). Besides the effect of the individual locus, expression of a QTL is also sensitive to different factors including the environment (Weinig and Schmitt, 2004; Boer et al., 2007). Therefore, the phenotypic components should be separated into genetic and environmental effects. This will help in estimating the genetic components of the trait by excluding environmental factors. In 1916, Edward East made the first attempt to partition phenotypic variation into it's genetic and environmental components (Neal, 2004). Since then, a general working model was developed to partition the phenotypic measurements into its constituents. Thus, the phenotypic variance can be broken into it's genetic and environmental components by the following general model.

Equation 1-2: Partitioning of phenotypic variance

$$\mathrm{P}ik = \mu + \mathrm{G} + \mathrm{E}k + (\mathrm{GE})ik$$

Where P*ik* = phenotypic value of i^{th} genotype in the k^{th} environment; μ= population mean; G = genotypic value of i^{th} genotype; Ek = environmental effect of the k^{th} environment; and (GE)*ik* = interaction of i^{th} genotype within a k^{th} microenvironment (Nyquist, 1991)

Apart from phenotypic variance, the genetic variance can also be divided into three components. These are additive genetic variance (effect of all alleles affecting the trait), dominance genetic variance (effects between alleles within gene loci) and the interaction component (epistatic interaction between different gene loci that modify the additive effects) (Cockerham, 1954; Kempthorne, 1968; Watkins and Spangelo, 1968). Thus, genotypic variance can be broken into

Equation 1-3: Partitioning of Genetic variance

$$\mathrm{Vg} = \mathrm{Va} + \mathrm{Vd} + \mathrm{Vi}$$

Where Vg= genotypic variance, Va= variance due to the additive effects of the alleles, Vd= variance due to dominance effects between alleles and Vi= variance due to epistatic interactions between alleles controlling the trait (Neal, 2004)

In practice, Vd and Vi are inseparable and are grouped together as a non-additive genetic variance. Hence, the additive genetic variance (Va) is the main cause for the resemblance between parents and their offsprings and thus, between relatives. Partitioning of phenotypic and genetic components to their constituents is required to estimate the heritability of the trait which is used to calculate the genetic component of the phenotypic variance within a group of individuals. Generally, there are two types of heritability: broad sense (H^2) and narrow sense heritability (h^2). H^2 takes into account the different types of genetic variation that may affect the phenotype, while h^2 neglects the contributions of Vd (variance due to the dominant effect) and V_i (variance due to an epistatic interaction) i.e. the non-additive genetic variance and focus only on the additive effects of alleles. Thus, H^2 and h^2 can be calculated by the following general formula.

Equation 1-4: Broad sense heritability

$$H^2 = Vg / (Vg + Ve)$$

$$h^2 = Va / (Vg + Ve)$$

Where H2= broad sense heritability, Vg = genetic variance, Ve = variance due to the environment, Va= variance due to additive effects.

However, the most common method of estimating heritability, especially in plant breeding is calculated by the following formula.

Equation 1-5: Narrow sense heritability

$$h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_{GEI} + \sigma^2_e)$$

Where σ^2_G is genotypic variance due to additive effects, σ^2_{GEI} is variance component due to Genotype- by-environment interaction; σ^2_e is an error variance (Nyquist, 1991; Singh et al., 1993; Holland et al., 2003).

All the variance components shown here can be derived from analysis of variance (ANOVA) Table.

Equation 1-6: Variance components from ANOVA table

$$\sigma^2_G = (Ms_G - Ms_{GEI})/rE;$$

$$\sigma^2_e = Ms_e \text{ and}$$

$$\sigma^2_{GxE} = (Ms_{GEI} - Ms_e)/r$$

Where σ^2G is genotypic variance, σ^2e is an error variance, MsG is genotypic mean square, MsGEI is Mean square for the GEI interaction, r is replication, E is the environment and Mse is error (residual) mean square.

Furthermore, Genotypic and Phenotypic coefficients of variations (GCV and PCV) can also be estimated as the square root of σ^2_G and σ^2_P divided by the mean respectively (Burton, 1952).

1.8 Phenotypic penetrance and expressivity

When we study the relationships between genotype and phenotype, it is important to study the statistical occurrence of the phenotypes in a group of identical genotypes. This is because some identical genotypes do not always show the same/expected phenotype. Hence, phenotypic penetrance and expressivity are used to express the statistical occurrence of the phenotypes in a group of identical genotypes (Griffiths et al., 2000a). If we assume that all the individuals have the same pigment allele *P (*Figure 1-5A*)*; they should have the same potential to produce the phenotype i.e. production of the pigment. However, due to other modifying alleles and environmental effects, pigment production is modified in some of the individuals leading to variation in phenotypic penetrance as well as expressivity. Hence, penetrance measures the proportion of the genotypes showing the expected phenotype (Figure 1-5A). Furthermore, individuals with the same genotype can also show different degree (intensity) of pigment production reflecting what is known as phenotypic expressivity, i.e. intensity at which phenotypic expression differs among individuals of the same genotype (Figure 1-5B). Generally, traits vary in phenotypic penetrance and expressivity; where some of them show complete penetrance and

expressivity; such as the simple Mendelian traits while others show reduced penetrance and expressivity (Hartwell et al., 2011). Among many reasons affecting phenotypic penetrance and expressivity includes the existence of different forms of the gene (alleles), environmental influence (Gene-by-environment interactions), epistatic interactions, and influence of other modifying genes elsewhere in the genome (Griffiths et al., 2000a).

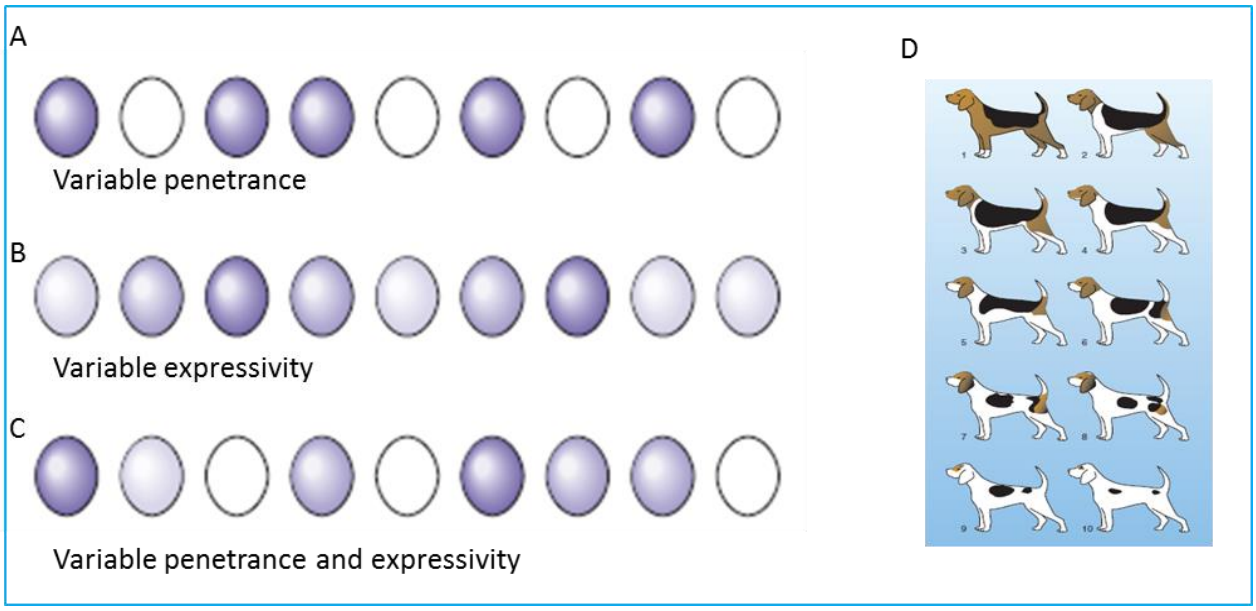


Figure 1-5. Pigment intensity as an example of phenotypic penetrance and expressivity.
(A) Phenotypic penetrance. Each oval represents individuals carrying the same pigment allele (*P*). (B) Variable expressivity. The color intensity reflects variation in phenotypic expressivity. (C) Variable penetrance and expressivity. (D) Variable expressivity showed by 10 grades of piebald spotting in beagles. Each of the dogs has the allele responsible for piebald spots (*SP*). Figures were taken from Griffiths (Griffiths et al., 2000a).

1.9 QTL mapping

The basic method of dissecting and studying complex traits is through QTL mapping. Hence, QTL mapping is the process of identifying the chromosomal region(s) controlling the trait of interest. The process is based on the identification of inherited genetic markers within close proximity (linkage) of the trait of interest. The first QTL analysis was made in 1909 by Nilsson-Ehle who studied kernel (grain) color in wheat (Neal, 2004). He identified three gene loci (QTL) controlling kernel color in wheat. The process of QTL mapping has been further facilitated with the discovery of molecular genetic markers such as RFLP. With the advent of more molecular markers and sequencing technologies, which allowed the construction of robust genetic linkage maps for several species including wheat, QTL mapping and cloning can be performed in a more precise way than ever before.

1.9.1 Principle of QTL mapping

Several loci/genes are involved in a quantitatively controlled trait. The process of identifying the chromosomal location of these loci/genes is QTL mapping. This can be achieved through linkage analysis of the trait with molecular genetic markers. The markers are used as landmarks on each of the chromosomes, where trait-marker linkage can be detected as an indicator for chromosomal location of the QTL controlling the trait. Thus, each marker on the chromosome is used as a sign or reference for locating the QTL. If the marker to the QTL is tightly linked with QTL controlling the trait of interest, then they are said to be linked and are inherited together.

Once the linear order of the molecular markers on each chromosome is determined, which is accomplished through linkage mapping, the basic principle of QTL mapping is to partition the mapping population into groups based on their genotypic score of each marker. Then, statistical computations and genotypic correlation will be performed to determine whether individuals of the given genotype differ significantly as compared to individuals of the other genotype for the trait of interest (Tanksley, 1993). If the difference is significant, then the trait is linked to the marker (locus) used to subdivide the population. The procedure is then repeated for all markers throughout the genome. For example, if the mean of all the tall plants is found to be significantly associated with a particular genetic marker, then the QTL for plant height is linked to that specific marker.

Thus, declaration of QTL is always accompanied with the statistical test (*p*-value) for the trait mean differences between the groups with the tightly linked marker to the mean of the group without the marker. So QTL is to be declared as far as the mean value at specific *P* value (0.05) is significantly different between the two groups. If the difference between these two groups is statistically non-significant, then the markers and QTL are not linked and are said to be randomly inherited. To perform QTL analysis; creation of permanent mapping population such as Double Haploid (DH) and Recombinant Inbred Lines (RILs) are required. Mapping population is critical for the generation of genetic markers as well as measuring the phenotypic data in different environments. Linkage map generation and QTL mapping are performed using computer software designed for this purpose.

1.9.2 Mapping populations

Two major strategies are used to identify QTL: Genome-wide trait linkage analysis and Genome-Wide Association Studies (GWAS) or Linkage Disequilibrium (LD) mapping. Genome-wide linkage analysis (classical QTL mapping) is based on family based (closely related mapping population)

such as F2, DH or RILs. GWAS is mainly based on a diverse set of unrelated individuals or lines drawn from random mating natural populations and takes advantage of historic linkage disequilibrium (LD) between markers to track down the association between marker and trait. Besides the power of dissecting complex traits, both mapping strategies have their own limitations. For example, QTL mapping suffers from two fundamental limitations (Borevitz and Nordborg, 2003; Korte and Farlow, 2013). Firstly, it is limited to the allelic diversity of the parents of the mapping population, and secondly, mapping resolution depends on the number of recombination events in the mapping populations. However, Multi-parent Advanced Generation Inter-Cross (MAGIC) population can be used to improve allelic diversity and recombination within a mapping population (Mackay and Powell, 2007; Cavanagh et al., 2008). The other option which can be used to increase the recombination event in the mapping population is to develop advanced Recombinant Inbred Lines (by inter-crossing randomly selected F2 individuals) before the genotypes are fixed upon selfing (Darvasi and Soller, 1995; Balasubramanian et al., 2009). GWAS or LD mapping can also overcome the two problems of classical QTL mapping mentioned above; but with its own limitations as well (Korte and Farlow, 2013). Hence, different authors have already suggested the complementarity of GWAS to the classical QTL mapping in such a way that first the genetic architecture of a trait can be analyzed using GWAS, and then the result will be used for developing mapping population for the classical QTL mapping approach which still remains to be a powerful mapping method for complex traits (Zhao et al., 2007; Brachi et al., 2010; Mitchell-Olds, 2010; Korte and Farlow, 2013).

Population size and structure are the main factors affecting the result of GWAS (Price et al., 2010; Korte and Farlow, 2013; Tucker et al., 2014). Generally, two types of mapping populations are used for GWAS. These are natural breeding population and family-based mapping population. Natural breeding population includes germplasm from gene bank collections and other elite breeding materials. The family-based mapping populations used in GWAS is of two types: the MAGIC and NAM (Nested Association Mapping) populations (Cavanagh et al., 2008; Yu et al., 2008). MAGIC population is derived from RILs through different combinations of crossing schemes using multiple parents (Cavanagh et al., 2008). Whereas the NAM population, which was originally demonstrated in maize to dissect the genetic basis of complex quantitative traits, requires the initial development of a set of the RILs population from diverse parents (used as a founder) and then crossing them with another common (reference) parent (Yu et al., 2008). Both MAGIC and NAM are also used in classical QTL mapping. The use of MAGIC or NAM population increases both

the precision and mapping resolution for QTL mapping. Although most of the mapping populations are derived from a cross between two or more genetically diverse parents; handling of the progenies in a distinct fashion could result in different types of mapping populations (Figure 1-6) (Singh and Singh, 2015).

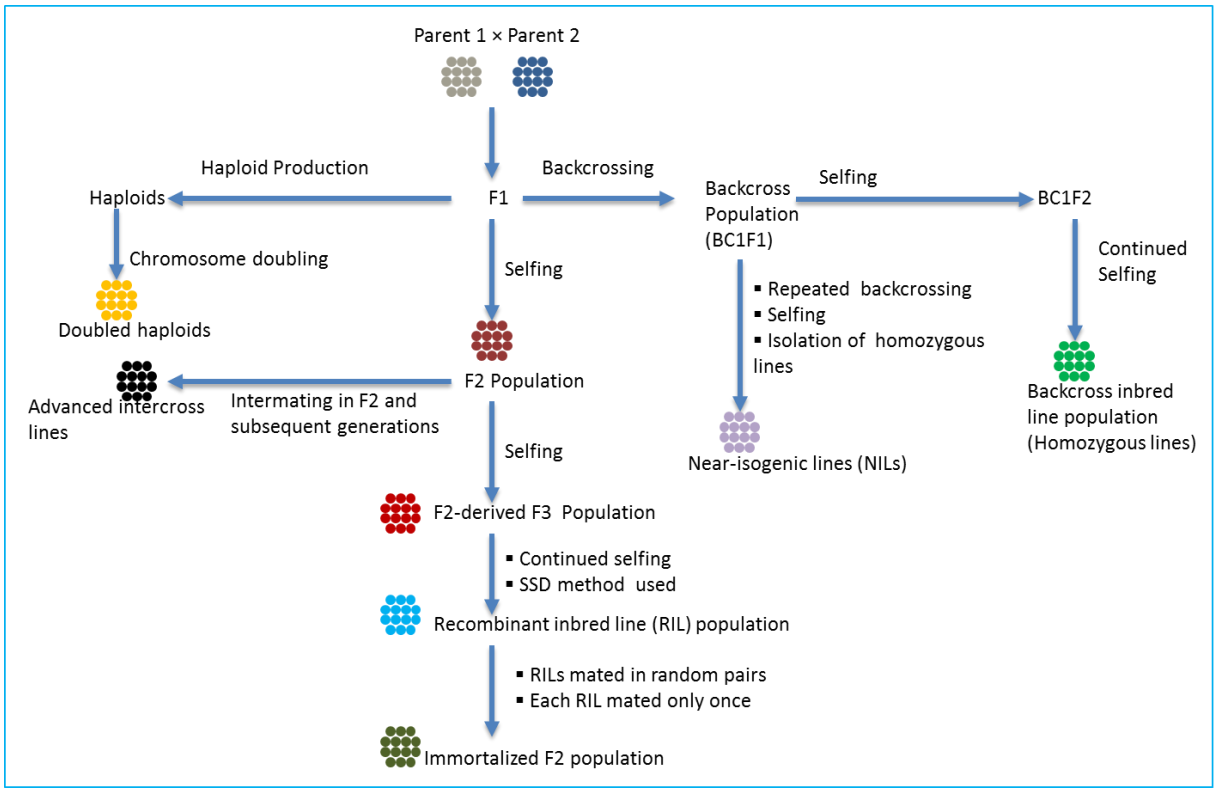


Figure 1-6. Illustration of the development of different bi-parental mapping populations.
Taken from Singh et al., with slight modifications (Singh and Singh, 2015).

Each of these mapping populations possesses their own drawbacks. Hence, careful selection of population type is critical for genetic mapping. Generation of F2 populations is simple and easy, but half of the F2 population are heterozygous and cannot be maintained beyond one generation. So, they are often used for map-based gene cloning and are rarely used for QTL mapping. Alternatively, RILs, Near-Isogenic-Lines (NILs), and DHs are true-breeding (homozygous) lines and hence, they are permanent mapping populations (Figure 1-6). No study has indicated any strict requirement for the number of individuals constituting each of the mapping population. Nevertheless, it is generally recommended to have a large number of individuals in the mapping population as the genetic resolution of the map is directly related to the number of individuals in the population (Collard et al., 2005; Boopathi, 2013b).

1.9.3 Methods of QTL detection

Some of the well-established methods of detecting QTL include Single-Marker Analysis (SMA), Simple Interval Mapping (SIM) and Composite Interval Mapping (CIM) (Collard et al., 2005; Boopathi, 2013a). SMA is the first and simplest technique for detecting QTL. As the name implies, it is the detection of QTL based on a single marker analysis; and it does not require linkage map. The basic principle is to use applications such as Student's *t*-tests, analysis of variance (ANOVA) and linear regression. The purpose is to confirm whether there is a QTL associated with marker based on comparing group phenotypic mean value. This requires splitting of the progenies into two groups based on their genotypes at the specific marker. Some of the disadvantages of SMA is a low power of detecting a QTL farther from the marker and biased estimation of the QTL effects. By overcoming these limitations, another mapping method known as SIM was proposed (Lander and Botstein, 1989). SIM linearly searches the QTL in certain increments across the ordered markers (Linkage map) and statistically test the presence of QTL at the location within the interval. Unlike SMA, SIM requires linkage maps and instead of analyzing single markers, SIM analyzes marker intervals between adjacent pairs of linked markers. The SIM method produces a profile of likely sites for QTL between adjacent linked markers and calculates the probability that an individual has (AA or Aa or aa) at a putative QTL site. The results of the test statistics are typically presented as likelihood ratio statistic (LRS) or logarithmic of odds (LOD) score. There is a one-to-one transformation between LOD and LRS scores, i.e. LRS = 4.6 × LOD score (Boopathi, 2013a). Linkage position with highest LOD/LRS value is used to identify the most likely position of a QTL. Of course, the peak must also exceed a specific significance (threshold) level to declare a QTL (Figure 1-7).The determination of significance thresholds can be obtained through permutation tests (Churchill and Doerge, 1994). During the permutation test, the phenotypic values of the population are shuffled, i.e. all marker-trait associations are broken down several times, while the marker genotypic values are held constant to assess the level of false positive associations to determine the significance level. A typical output from SIM is shown in Figure 1-7.

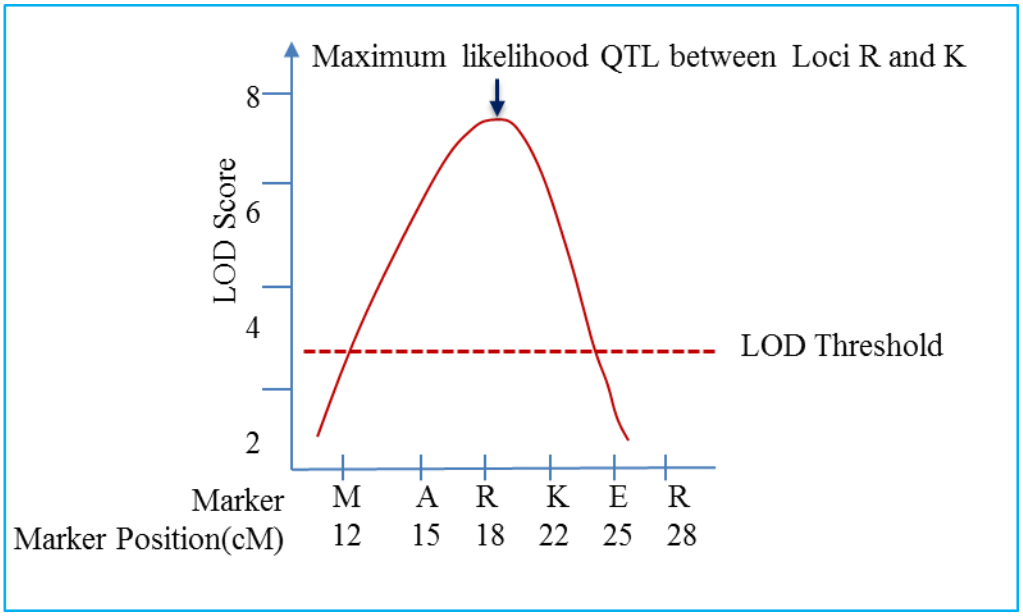


Figure 1-7. Principle of simple Interval mapping (SIM) based on maximum likelihood method

The most popular QTL mapping method is the Composite Interval Mapping (CIM) method (Jansen and Stam, 1994; Li et al., 2007). Because CIM method combines both SMA and SIM with multiple linear regression methods (besides using an adjacent pair of linked markers) it is the most precise and effective method of QTL mapping. The regression step operates in such a way that first it selects markers with highest LRS or LOD score, and then the second most significant marker will be added for testing 2-locus model. If both remain significant in the model, a third significant marker will be added to the model and so on. All non-significant markers are dropped out (Jansen, 1993; Zeng, 1993; Jansen and Stam, 1994; Zeng, 1994). In the end, the model carries all significant markers which are termed as 'cofactors'. Once the cofactors are identified, the whole genome will be scanned using interval mapping to finally identify the QTL.

1.9.4 Estimating QTL effects

Based on the proportion of the Phenotypic Variance Explained (PVE) by a QTL, a QTL can generally be classified as minor or major effect QTL. Although there is no a clear-cut value to designate a QTL as major and minor, a QTL whose effect exceeds 10 % is generally considered as major effect QTL (Boopathi, 2013a). Because plants show phenotypic plasticity as a response to environmental factors, the expression of a QTL is also affected by the environment (El-Soda et al., 2014). In quantitative genetics, it is termed as GEI. Because GEI negatively affects heritability of a trait i.e. the higher the GEI, the lower the heritability of the traits, GEI received much more attention in QTL mapping especially in trait selection for crop breeding. Therefore, to study the joint effect of genotype and environment on phenotypic plasticity and quantify the performance of each

genotype, mapping population needs to be tested across different environments. Apart from the GEI, interactions among loci (epistatic interactions) also contribute to the phenotypic variation of complex trait explained by a QTL. In its simplest definition, epistasis is the interaction between a pair of loci in which the phenotypic effect of one locus depends on the genotype at the second locus(Carlborg and Haley, 2004). Furthermore, some QTL expresses only in a certain environment (none-stable QTL) while others consistently expressed across all environment (stable QTL).

1.9.5 QTL mapping software

A larger number of standalone computer software packages are available for linkage and QTL mapping including R based script driven packages. Most of this software are freely available. However, Joinmap, MapQTL, QGene, and Genstat are commercial software for linkage and QTL mapping. The first QTL mapping program is MAPMAKER which is a command based QTL package (Lander.E, 1987). QTL Cartographer is the most commonly used software for QTL mapping in UNIX and Windows operating systems with user-friendly menu driven packages for QTL mapping (Wang S., 2012). It offers several options from single marker analysis to eQTL and Multiple Interval Mapping. QTL Network is also another user-friendly Windows-based QTL mapping software with an option for mapping QTL epistatic effects and QTL by environment interaction effects. QTL mapping is also available from Genstat with an option of multiple trait analysis for mapping QTL by trait (QTLxTrait) interaction effect(Payne et al., 2014).

1.10 Introduction to Plant Architecture

The complete plant system arises from two major kinds of stem cells: the shoot and root apical meristems. The root apical meristem (RAM) is responsible for the underground part (root system) while the shoot apical meristem (SAM) is responsible for all the aboveground parts. Hence, the fate and determinacy of RAM and SAM affects root and shoot architecture, respectively. After the plant decides to undergo phase transition (from vegetative to a reproductive phase), the final fate of SAM will be determined and thus, the SAM will completely transform into the inflorescence meristem (IM) which is responsible for the development of the complete inflorescence structures. Therefore, RAM, SAM, and IM are the three most important types of meristems that can determine the complete architecture or morphology of plant both below and above the ground. Shoot architecture, which is the three-dimensional organization of the aerial part of a plant, is mainly determined by tiller number, tillering pattern, leaf shape and arrangement, plant height, and inflorescence morphology. Plant architecture, in general, is of high agronomic importance as it determines the suitability of a plant for cultivation and potential grain yield.

1.10.1 Wheat plant architecture

Annual yield increment in wheat is estimated to be less than 1 %, which is far below the required 2.4 % to cope up with the global demand by the year 2050 (Ray et al., 2013). In order to increase wheat yield, different approaches were suggested. One of these was to increase the spike fertility index (Fischer, 2007; Reynolds et al., 2009; Gonzalez et al., 2011; Sreenivasulu and Schnurbusch, 2012). To achieve this, increasing the number of fertile florets (decreasing floret abortion) is a key strategy. However, the main challenge is that the genetic/physiological mechanisms controlling floret and/or primordial abortion (degeneration) is not yet well understood. Hence, raising wheat yield by directly increasing floret fertility does not seem to be an easy task. The alternative approach needs to be sought. One of these is increasing the spikelet number per spike. So, understanding the genetic basis of inflorescence development and architecture in wheat is required. Several studies have indicated that yield potential in wheat is sink limited (Fischer, 1985; Slafer and Savin, 1994; Miralles et al., 2000; Borrás et al., 2004; Miralles and Slafer, 2007), suggesting the necessity of having enlarged spike size and sink activity in wheat. Hence, the *branched head* (*bh*) wheat mutant lines are important genetic materials in this regard (Sharman, 1944).

Spike architecture is among several major traits used to define 'Ideal wheat plant architecture' (IWPA) as defined by C. M. Donald in 1968 (Donald, 1968). He introduced the term 'ideotype', as plant design based on traits that affect performance. He postulated that successful crop 'ideotype' should be a weak competitor relative to the mass (population). Thus he defined IWPA "as short and strong stem (lodging resistant); with few, small, and erect leaves; large and erect ear (multi florets per unit of dry matter of the tops); with awns; and should be a mono/single culm" (Figure 1-8). He argued that the general approach followed by crop breeding programs i.e. breeding approach based on 'defect elimination' should be cautioned; and thus, incorporation of physiological and morphological traits to an 'ideotype' need to be carefully considered in order to develop a less competent (less aggressive) 'ideotype' that can perform well in dense stands without affecting the neighboring plants. For instance, a genotype having efficient light interception capacity in a less crowded or isolated plant community might be less competent in a crowded plant community of the same genotype simply because of the mutual shading effects. Hence, the genotype with higher yield potential might perform less unless optimal conditions such as spacing and weed control are provided. His key point involves the tradeoffs between collective performance (cooperation) and the individual plant competitiveness. When such tradeoffs exist;

nature (natural selection) simply supports individual competitiveness rather than community performance when the two are in conflict (Denison, 2012). For instance, whenever plants of different genotypes compete, natural selection simply favors taller plants, even if they are low-yielder. The low yield associated with tall plants is mainly associated with wasteful resource investment on the stem growth at the expense of grain number compared to shorter plants which invest more on grain yield than investing in stem development.

In addition to being short, Donald suggested that IWPA should have small and fewer leaves with a vertical orientation. This facilitates dense planting and improved light capturing capacity. The number of the leaves is also important because the resource that can be invested for the development of extra leaves can be remobilized to make more grains. He also suggested that the wheat plant should be mono/single culm. Nature, however, favors a wheat plant with more tillers and leaves which spread out, makes a shade and invades the territory of neighboring plants (Denison, 2012).

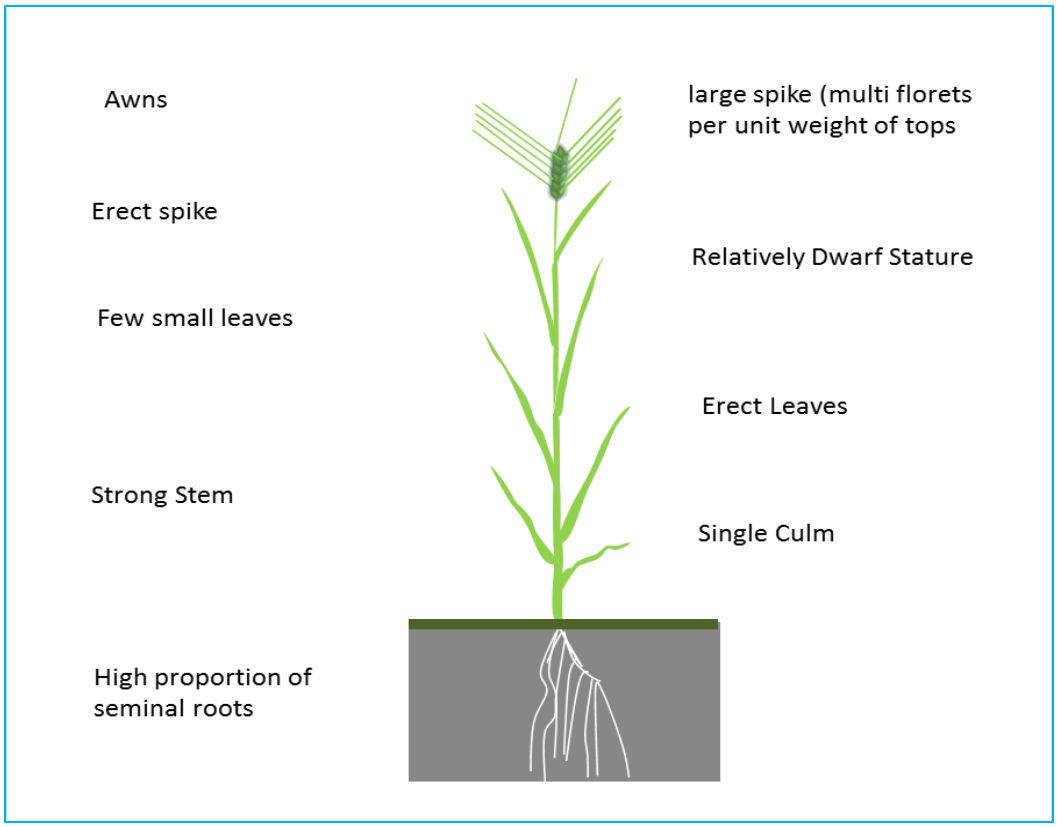


Figure 1-8.Thy hypothetical wheat plant ideotype defined by C.M. Donald in 1968.

1.10.2 Spikelets as a building block of grass inflorescence

Inflorescence development in grasses begins after the SAM is transformed into IM (Tanaka et al., 2013; Kyozuka, 2014). Then, the IM becomes the source of the whole inflorescence. Hence,

Axillary Meristem (AxM) start to emerge which later acquires the identity of either the branch meristem (BM), which reiterates the formation of new meristem for branch formation or acquires the identity of spikelet meristem (SM). Therefore, two forms of grass inflorescences are known. These are the spike and panicle. In spike-type inflorescences, spikelets are attached directly to the inflorescences axis (rachis); like the case of wheat and barley. In a panicle-type inflorescence, due to the higher-order of branch formation from the BM; spikelets are formed on branches like the case of rice panicle and tassel in maize.

So spikelets are the building blocks of grass inflorescence and each of which is embraced by two leaf-like organs called the glumes which protect the delicate floral organs (Figure 1-9). Spikelet number and arrangements (configurations) differ between species (McSteen, 2006; Kellogg et al., 2013). For instance, the rice panicle has several long branches bearing single spikelets and single floret as the terminal unit, while inflorescences of the triticeae (such as wheat and barley) show no branching, having single sessile spikelets per rachis node in a distichous arrangement (Figure 1-9C). In Andropogoneae (the case of maize and sorghum) paired spikelets are the *de facto* arrangement along the inflorescences branches (Vollbrecht et al., 2005).

SM further initiates floral meristem (FM) which will differentiate to produce final floral organs namely, the ovary (carpel), stamen, lodicule, palea and lemma (Figure 1-9B). Unlike flowers in Eudicots, grass florets lack petals and sepals (Ciaffi et al., 2011). Nevertheless, it is strongly believed that the corresponding structures for Eudicot's sepal is lemma and palea in grasses (Lombardo and Yoshida, 2015); while the petals correspond to the grass specific lodicules, which are located at the base of the ovary (Ciaffi et al., 2011; Yoshida, 2012). By swelling and then spreading the palea and lemma, lodicules facilitate pollination.

Among the triticeae, barley has a distinct spikelet architecture. The inflorescence typically develops three spikelet meristems that are arranged alternately at each rachis node. In six-rowed barley, all three spikelets develop three fully fertile florets; whereas, in two-rowed barley, the two lateral spikelets are reduced in size and are normally sterile. Unlike barley, where IM develop indeterminately, the development of the IM in wheat is determinate; meaning that spikelet primordium differentiation ends with the initiation of the terminal spikelet (Bonnett, 1936; Kirby, 1974). First, the IM forms structure called double ridge consisting of lower and upper part. While the upper part acquires SM identity, the lower part, which is an arrested leaf meristem, start to disappear as growth proceeds. Spikelet differentiation starts at the center of the spike (Figure 1-9A) and then proceeds to top and bottom of the spike (Bonnett, 1936).

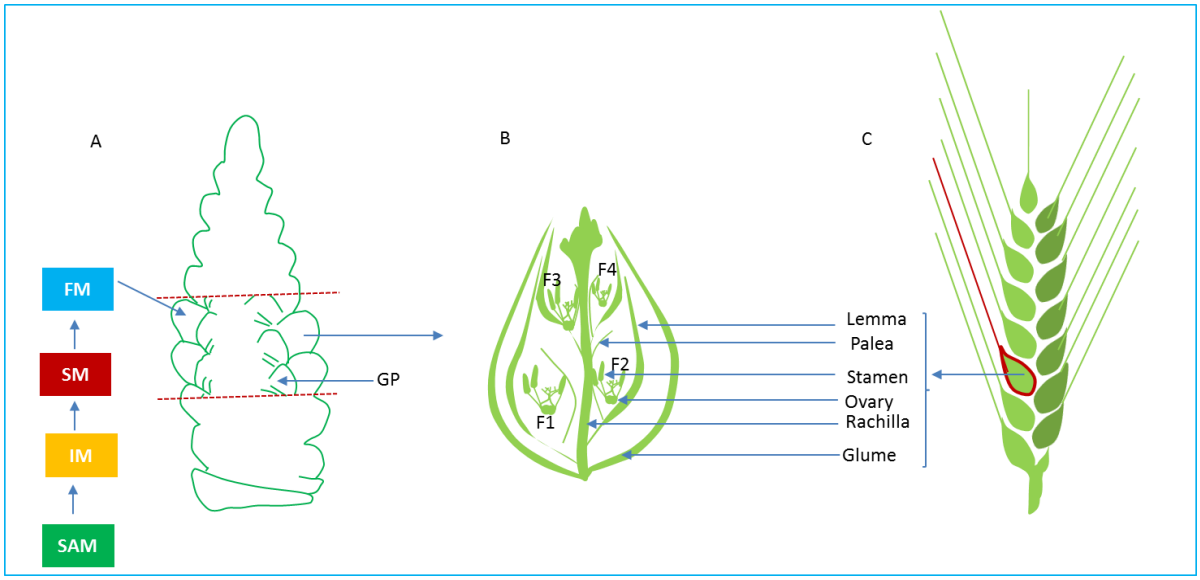


Figure 1-9 Wheat spike and the Inflorescence meristem differentiation.
(A) Wheat Inflorescence meristem differentiation. IM transited from SAM, directly establishes SM as a lateral branch. The SM later differentiates into Glume primordia (GP) and Floral meristems (FM). FM differentiates to form floral organs such as the pistil, stamen, lodicule, palea and lemma (Kirby and Appleyard, 1984), (B) spikelet with indeterminate floret primordial, (C) determinate wheat spike. SAM, Shoot Apical Meristems; IM, Inflorescence Meristems; SM, Spikelet Meristems; GP, Glume primordia; Fm, Floral Meristems; F, Florets

Each spikelet in wheat carries an indeterminate number of floret primordia on the central axis, called the rachilla (Figure 1-9B). In wheat and Brachypodium, florets within each spikelet's rachilla mature acropetally (bottom to top) (Derbyshire and Byrne, 2013). Thus, the most mature and fertile florets are those florets at the bottom of the rachilla, i.e. F1 to F4. Often the most distal florets do not develop further and finally degenerates before anthesis (Kirby, 1974; Ghiglione et al., 2008; Guo and Schnurbusch, 2015; Guo et al., 2016).

1.10.3 Meristem maintenance and determinacy

At the time of phase transitions, maintenance of meristem identity is a critical process to ensure organ identity. Genes controlling such phase transitions and organ identity in grasses, especially rice and maize, have been identified and well characterized (Tanaka et al., 2013). One of these genes which are known in mediating phase transition during SM to FM is the *Branched silkless1 (BD1) (*Colombo et al., 1998; Chuck et al., 2002*).* In subsequent studies, the corresponding orthologous genes from rice, *FRIZZY PANICLE* (*FZP*); Brachypodium, *MORE SPIKELETS1* (*MOS1*); wheat, *FRIZZY PANICLE* (*WFZP*) or *BRANCHED HEAD* (*BH*); and barley, *COMPOSITUM 2 (COM2*) have been identified (Komatsu et al., 2003; Derbyshire and Byrne, 2013; Dobrovolskaya et al., 2015; Poursarebani et al., 2015).

BD1/FZP/MOS1/TtBH/COM2 is a member of the APETALA 2/Ethylene-Responsive Element Binding Factor (AP2/ERF) superfamily of transcription factors widely involved in growth and development, as well as responses to environmental stimuli (Magnani et al., 2004; Mizoi et al., 2012; Licausi et al., 2013).

Originally identified from Arabidopsis, *APETALA2 (AP2)* plays a central role during the establishment of the floral meristem and floral organ identity (Jofuku et al., 1994). The *AP2* genes are distinguished by an essential 68-amino acid motif of a GCC-box binding domain known as the AP2 domain (Allen et al., 1998). The GCC box is an eleven base pair sequence (TAAGAGCCGCC) which is conserved in the promoter region of ethylene-inducible pathogenesis-related genes in plants (Ohme-Takagi and Shinshi, 1995; Hao et al., 1998). Hence, AP2/ERF transcription factors target the GCC motif in the promoter region of the target gene for binding. The AP2/ERF domain contains an N-terminal three-stranded β-sheet for recognition of the target sequence and complex formation; while the C-terminal α-helix domain modulates the specificity (Ohme-Takagi and Shinshi, 1995; Allen et al., 1998). Hence, alteration of amino acid residues in the domain could cause damage in the binding geometry and affects the function of the protein.

Mutations in all of the orthologous genes (*BD1, FZP, MOS1, WFZP/TtBH* and *COM2*) resulted in alteration of SM identity and loss of determinacy, which ultimately affects the subsequent establishment of floral meristem, leading to a higher order of inflorescence branching (Chuck et al., 2002; Komatsu et al., 2003; Derbyshire and Byrne, 2013; Dobrovolskaya et al., 2015; Poursarebani et al., 2015). *bd1* mutant in maize suffers from meristem indeterminacy in the ear and as well as the tassel leading to an abnormal branch formation (Chuck et al., 2002). The rice *fzp* mutants, the transition from SM to FM was severely affected leading to the formation of additional axillary meristems which either arrest or develop into a higher order of branches (Komatsu et al., 2003). In Brachypodium *mos1* mutant produces increased number of axillary meristems which has led to the production of additional spikelets (inflorescence branching). In wheat, the mrs/*bh* mutant clearly shows loss of SM identity leading to spike-branching or supernumerary spikelet (SS) formation (Dobrovolskaya et al., 2015; Poursarebani et al., 2015).

1.10.4 The *branched head* (*bh*) locus of wheat

Although spike-branching is not common in wheat, branched head wheat mutant strains have been known for long by different names, especially in tetraploid wheat, *T. turgidum* (2n=4x=28; AABB) (USDA, 1916; Percival, 1921; Sharman, 1967). Some of the names given to *branched head* wheat mutant strain include ‘Alaska wheat’, ‘Stoner’, ‘Egyptian wheat’, ‘Eldorado’, ‘Jerusalem’,

'Many-Headed', 'Many-spiked', 'Miracle wheat', 'Multiple-headed', 'Mummy', 'Reed', 'Seven Headed', 'Smyrna', 'Syrian', 'Wheat of Miracle', 'Wheat 3000 years old' , 'Wild Goose', 'Eden', 'Forty-to-One', and 'Marvelous'. Later, similar mutant strains were found in hexaploid wheat as well (Koric, 1973). In the early 19's, there were also several claims that the branched head wheat strains outperform normal wheat cultivars in terms of yield. But later, the United States Department of Agriculture (USDA) conducted different yield trials and suggested that the branched head wheat strains (which they later renamed it as 'Stoner wheat') were not an outperforming wheat in terms of yield (USDA, 1916). Since then, different scientific efforts were made to study the branched head wheat mutant strains. Most of these scientific investigations are summarized below.

Meunissier (1918) and Percival (1921) independently indicated that the *bh* locus in tetraploid wheat is a recessive hereditary trait (Meunissier, 1918; Percival, 1921; Masubuchi, 1974).

Sharman (1944) studied the role of day length in the expression of the spike-branching phenotype. He suggested that day length can affect phenotypic expression of spike-branching (Sharman, 1944). He further conducted another study in 1967 and described the *bh* mutant strains as a wheat mutant that can develop miniature side branches with their own lateral and terminal spikelets. He further suggested the position where branching occurs and indicated that branching usually appears from the base of the (spike) (Sharman, 1967).

Rawson and Ruwali (1972) studied yield determination and seed morphology using *bh* wheat lines (Rawson and Ruwali, 1972a). They characterized *bh* wheat as wheat strain carrying a higher number of sessile spikelets which can potentially bear a higher number of grains per spike. They further suggested spikelet sterility and floret abortion in *bh* wheat lines. Finally, they concluded that, although the yield per unit area of *bh* lines was not as high as that of the standard improved wheat varieties, it can offer a radically different concept for yield increment i.e. by increasing spikelet number per spike.

Koric in 1973 studied *bh* in hexaploid wheat and suggested that the *bh* phenotype is controlled by three genes, which he designated as *Ramifera (Rm*), *Tetrastichon* (*Ts*) and *Normalizator* (*Nr*) (Koric, 1973). While the action of *Rm* and *Ts* is complementary in promoting branching, *Nr* acts as a dominant repressor of branching.

Masubuchi (1974) studied the genetic and phenotypic basis of *bh* in hexaploid wheat and described how florets are converted to make up the different types of spike-branching (Masubuchi, 1974).

Pennell and Halloran (1983) suggested the presence of two recessive loci controlling the *bh* phenotype in hexaploid wheat and further suggested the presence of the third locus as a repressor of branching. In a later study, the authors described the phenotype as the appearance of sessile spikelet at a rachis node or in an extended rachilla (Pennell and Halloran, 1984a). Furthermore, the authors also evaluated the effect of time of sowing, temperature, and photoperiod on branching. They suggested that short photoperiods were more conducive for the phenotypic expressivity of supernumerary spikelets. Similarly, they suggested that *bh* lines with a strong vernalization requirement generally tend to have a strong spike-branching phenotype (Pennell and Halloran, 1984a; Pennell and Halloran, 1984b).

Millet (1986) studied spikelet number and heading date using *bh* and regular lines. He suggested the presence of a major gene controlling branching on chromosome 2D (Millet, 1986b).

Kadkol and Halloran (1988) investigated the development and floret fertility using *bh* strain. He concluded that the *bh* strain had a lower number of fertile florets per spikelet as compared to the standard spike. Despite the lowered spikelet fertility, the total grain number per spike was found to be higher for the *bh* strain due to a higher number of spikelet per spike.

Klindworth (1990) studied branching head in tetraploid wheat and identified chromosome 2AS as the bearer of the *bh* locus. He further suggested that branching is inherited quantitatively with the major recessive gene located on chromosome 2AS (Klindworth et al., 1990b).

Peng (1998) also identified the chromosomal location of loci controlling SS which is the appearance of more than two spikelets from the rachis node in hexaploid wheat. He suggested that chromosomes 2D, 4A, 4B and 5A of bread wheat carry the genes for SS with the major effect locus coming from chromosome 2D (Peng et al., 1998).

Sun (2009) studied the inheritance of genes controlling SS in hexaploid wheat. He showed that the inheritance of SS is controlled by two dominant genes having a complementary role. He also suggested the presence of minor effect modifier gene(s) affecting the expression of SS (Sun et al., 2009).

Dobrovolskaya (2009) mapped genes controlling SS in hexaploid wheat and rye using microsatellite markers (Dobrovolskaya et al., 2009). They suggested that the 'Multirow Spike' (*MRS*) phenotype in hexaploid wheat and 'monstrosum spike' (*MO1*) in rye are both controlled by a recessive single locus/allele. The locus controlling the *MRS* phenotype is located on the distal half of 2DS and found to be linked with the microsatellite marker *Xwmc453.*

Using hybrid populations, Aliyeva and Aminov (2011) studied the inheritance of *bh* in tetraploid wheat and suggested that the *bh* locus is controlled by a single recessive gene despite some irregularity against the Mendelian law in F2 generations (Aliyeva and Aminov, 2011).

Li et al. (2012) used SSR markers to identify the first major QTL associated with Triple-Spikelet using Tibetan Triple-spikelet Wheat (Li et al., 2012). He identified a QTL on chromosome 2AS having 33.1% phenotypic variance explained by the QTL.

Haque et al. (2012) mapped plant height and *bh* in tetraploid wheat and re-confirmed that the *bh* locus is located on chromosome 2AS and a microsatellite marker *Xgwm425* flanked the locus proximally (Haque et al., 2012).

Echeverry-Solarte (2014) performed genome-wide dissection of the supernumerary spikelet in hexaploid wheat and found seven QTLs associated with SS. Two of these QTL on2DS and 7BS were found to be a major effect QTL suggesting a polygenic inheritance of SS in hexaploid wheat (Echeverry-Solarte et al., 2014).

Finally, the homoeo-alleles underlying the *bh* locus in tetraploid wheat (Poursarebani et al., 2015) and *MRS* in hexaploid wheat (Dobrovolskaya et al., 2015) have been identified (Dobrovolskaya et al., 2015; Poursarebani et al., 2015). Deletion (frameshift mutation), as well as SNP in the highly conserved AP2/ERF domain of *WFZP-D*, has been identified as the major cause for severe SS formation in hexaploid wheat. Similarly, another deletion which had occurred outside of the AP2/ERF domain of homoeologous gene*, WFZP-A*, has also been linked with SS formation in hexaploid wheat (Dobrovolskaya et al., 2015). In tetraploid wheat, only a single nonsynonymous nucleotide substitution in the AP2/ERF domain (T287C, and hence L96P) of *TtBH-A1* was found to be the only cause for *bh* in tetraploid wheat (Poursarebani et al., 2015).

1.10.5 General objectives of the study

Although the major gene underlying the *bh* locus has been identified recently (Poursarebani et al., 2015), different studies have suggested that spike-branching is a quantitatively inherited trait (Klindworth et al., 1990a; Echeverry-Solarte et al., 2014). Therefore, the goals of the current study are: (i) to map other loci involved in modifying spike-branching in tetraploid wheat and to further quantify the phenotypic effects on the basis of phenotypic penetrance and expressivity (chapter two); (ii) Mendelization of the bh locus by developing *bh* Near Isogeneic Lines (*bh*-NILs) (chapter 3); and (iii) to further test the hypothesis of IWPA in connection with the modified plant architecture using *bh*-NILs (chapter 4).

2 Chapter 2: The Genetic Basis of Wheat Spike Architecture

2.1 Introduction

The size, shape, and orientation of plant body parts are the major factors affecting plant architecture and agronomic performance. Generally, these traits are under the control of genes, hormones and the environmental cues (McSteen, 2009). Therefore, understanding the molecular genetic basis of plant architecture is key for manipulating plant architecture for crop production. So far several genes have been identified controlling plant architectural traits, mainly from rice and maize (Ashikari et al., 2005; Beveridge, 2006; Kurakawa et al., 2007; Ongaro and Leyser, 2008; Kebrom et al., 2013; Tanaka et al., 2013). However, genes controlling wheat plant architecture are largely unknown. The *bh* locus is therefore among the few loci known so far in controlling spike architecture in wheat. Therefore, the *bh* locus is the main focus in this study.

2.2 Objective of the study

Although the *bh* locus and the underlying gene, has been mapped and identified, the enormous variation in the phenotypic penetrance and expressivity of the spike-branching phenotype suggest the presence of other genetic and/or environmental factors modifying the trait. Therefore, the specific objectives of this chapter are to map *bh* loci involved in quantitatively modifying the spike-branching phenotype; estimate the effect of environment on the basis of phenotypic penetrance and expressivity, and study other spike-related traits in connection with the *bh* locus.

2.3 Materials and Methods

2.3.1 Development of the mapping population

In total 146 recombinant inbred lines (RILs) were developed through a single-seed descent (SSD) method from an F2 population derived from a cross between Bellaroi (which is a standard spring durum wheat variety) and TRI 19165 (winter-type *bh* tetraploid wheat) (Figure 2-1). Bellaroi is an Australian high-yielding, disease resistant, and high-quality durum wheat variety suitable for pasta and couscous production in Australia and overseas countries. TRI 19165 is tetraploid wheat accession from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), in Gatersleben, Germany. In addition to spike morphology, Bellaroi and TRI1965 differ in several major traits (Table 2-1). Since the mapping population segregates for the winter/spring growth habit, all RILs with winter growth habit were excluded from the mapping population by growing them in the greenhouse without vernalization i.e. under spring conditions in successive generations until F5. Thus, all the remaining RILs, which can complete their life cycle without

vernalization, are spring type and were used for developing the mapping population. No other selections were made in the population and thus, different alleles from the spring and winter parents were freely segregating in the mapping population.

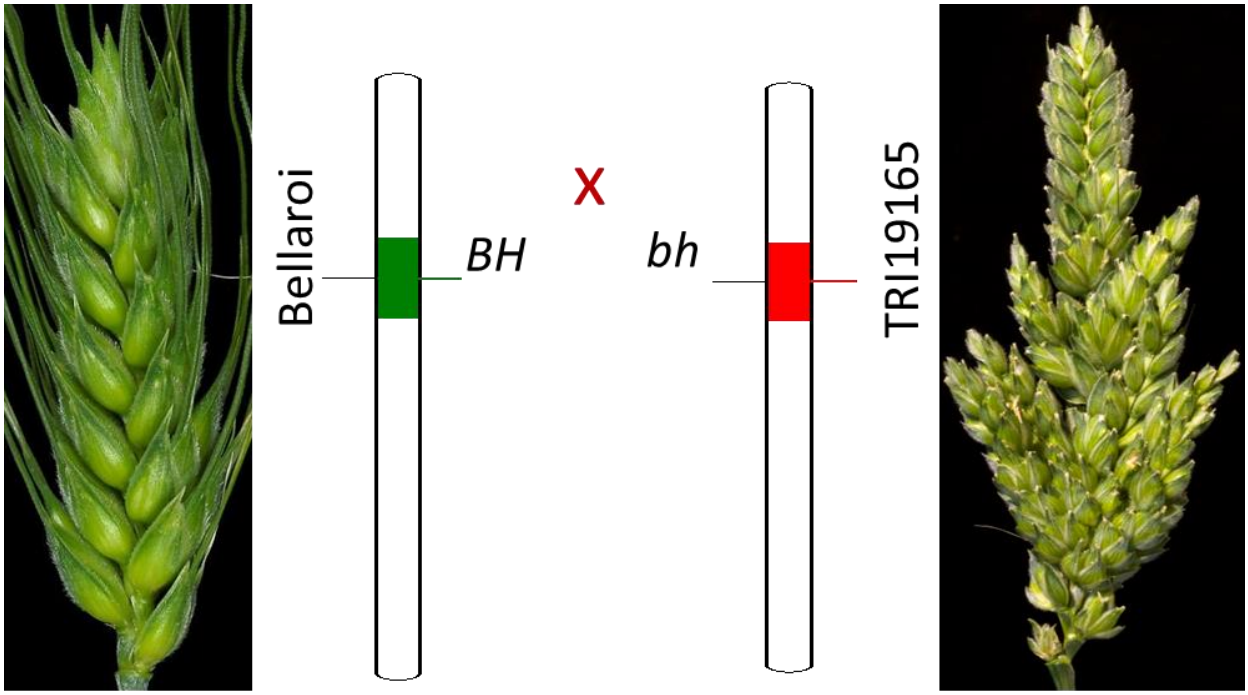


Figure 2-1. Spike architecture of Bellaroi (standard spike, *BH*) and TRI 19165 (branching spike, *bh*).
Awns were trimmed from TRI 19165 for clarity.

Table 2-1. Phenotypic comparison of Bellaroi and TRI 19165

Parent	Ploidy	GrH	PH	Heading	Spike	Awns	Grain Shape	Grain size
Bellaroi	2X=4n= 28	Spring	Semi-dwarf	Early	Standard	Awned	Elongated	Large
TRI 19165	2X=4n= 28	Winter	Tall	Late	Branching	Awned	Spherical	Small

GrH, Growth habit; PH, Plant height

The mapping population was developed at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Germany. After six generations of selfing and seed multiplication (at F6 generation), 146 RILs were field evaluated for two consecutive years (2014 and 2015 growing season) at three different environments (IPK14, IPK15, and HAL15) in Germany. In 2014, the F7 generation was field evaluated in Gatersleben (IPK14), in Germany. Following 2014, the F8 generation was evaluated in two different contrasting environments: Gatersleben (IPK15) and Halle (HAL15) in Germany. See the description of the sites under section 2.3.2, and Table 2-2. Besides the three environments, the F8 generation was also evaluated in the greenhouse in IPK

(GH15). The evaluation in the greenhouse was based on a single plant basis without a replication. The purpose was only to evaluate the spike morphology. It was conducted under controlled long day conditions (16/8 hours day/night and 19/17 ^{0}C day/night temperatures). Right after germination and seedling establishment, plants were vernalized for four weeks at 4 ^{0}C for better plant development and flowering. After four weeks of vernalization, seedlings were hardened for about 15 days at 15/12^{0}C day/night temperatures. Each plant was transplanted to a pot of size 2 litter filled with substrate2 (Klasmann-Deilmann GmbH, 49744 Geeste, Germany), compost and sand with a proportion of 2:2:1 respectively. Each plant received 10g of NPK fertilizer Plantacote® Depot 4M (Wilhelm Haug GmbH & Co. KG Postfach, Düsseldorf, Germany) week after potting. Plants were watered as required. Protection measures against pest and insect were applied as required following the standard procedure for the wheat plant.

2.3.2 Description of the field and growth conditions

IPK14 and IPK15 were located in Gatersleben which is part of the town Seeland located at 51.49° N and 11.16° E in Saxony-Anhalt, Germany. Field evaluation of the F7 RILs in 2014 was conducted in IPK's field station characterized by silty clay soil type having a pH of 7.7. Similarly, the field evaluation in 2015 (IPK15) was conducted in a farmer's field located in Gatersleben. Soil type was clay loam with a pH of 7.1. The field evaluation in Halle in 2015 (HAL15) was conducted at the eastern edge of Halle, which is located at 51.48°N and 11.97°E in the southern part of Saxony-Anhalt. The ground distance between the two testing sites i.e. IPK15 and HAL15 was estimated to be 77 km. The testing site is located inside the teaching and experimental field station (known as Julius-Kühn Field station) of the Agricultural and Nutritional Sciences of the Martin-Luther-University Halle-Wittenberg. This site is among those areas with the lowest precipitation in Germany (Merbach and Deubel, 2007). Generally, the mean precipitation ranges from 460 to 550 mm per annum. The monthly mean temperature, precipitation and growing degree days (GDDs) during the growing season for all locations are presented in Table 2-2. The soil type in Julius-Kühn Field station was classified as Haplic Phaeozem (according to FAO) (Merbach and Deubel, 2007).

Table 2-2. Description of the field conditions.
Monthly mean temperature (°C), precipitation (mm), growing degree days (0C days) and Global solar radiation (w/m2) for the growing season in 2014 and 2015 in Gatersleben and Halle

Year	Location	Month	Tm (^{0}C)	Pt (mm)	GDD (^{0}C days)	Hm (%)	GSR (W/m^2)
2014	Gatersleben (IPK14)	March	6.8	3.9	209	78.40	120.8
		April	11.4	29	343	77.90	169.2
		May	12.9	99.6	399	77.00	191.2
		June	16.3	70.2	488	76.40	222.8
		July	20.3	101.4	629	77.30	208.4
		August	16.6	85.4	515	78.60	173.7
2015	Gatersleben (IPK15)	March	5.2	28.5	161	81.30	105.2
		April	8.5	24.8	256	72.70	189.7
		May	12.8	33.2	395	71.80	220.6
		June	16	30.6	480	73.70	223.4
		July	19.6	86.5	606	73.20	224
		August	20.5	130.9	635	75.00	204.7
2015	Halle (HAL15)	March	5.73	0.97	176	-	-
		April	9.00	1.01	272	-	-
		May	13.18	0.28	416	-	-
		June	16.70	0.92	499	-	-
		July	20.58	3.69	633	-	-
		August	21.36	2.95	658	-	-

GSR, Global solar radiation; Pt, Precipitation; Tm, Temperature; Hm, Humidity

In all locations, the experiment was laid down on a small plot of size of 3.75 m^2 in a Randomized Complete Block Design (RCBD). Three hundred seeds per m^2 were sown in rows at a distance of 20 cm between rows. The experiment was replicated at least two times. Germination of F8 RILs in 2015 was poor (on average 76%) due to the poor seed quality as a result of extended rain during the harvest of F7 generation (sprouting of seeds) in 2014 which led to variable planting densities in 2015 trials. Because TRI 19165 is a winter wheat, three alternatives *bh* wheat accessions (TRI3261, TRI5283, and TRI9652) with spring growth habit were used as mutant checks. Plants in all locations were fertilized according to soil condition. Eleven kg of nitrogen/ha was applied to the experiment conducted in 2014 in IPK (IPK14). Required crop protection measures including weed and pest control have also been applied. The experiment in 2015 (IPK15) also received 22kg of nitrogen /ha besides standard weed and pest control measures. About 111kg of nitrogen/ha was

applied to the experiment in Halle/Saale. Similarly, herbicides and fungicides were also applied in order to control weeds and fungi.

2.3.3 Phenotyping

To phenotype spike-branching, three different approaches were used (Figure 2-2). First, branching was quantified on a whole plot basis using scale 0 to 10, where 0 was assigned to non-branching or wild-type phenotype and 10 when all the plants on the plots show branching (regardless of the branching intensity along the spike). The second strategy was to quantify branching intensity along the spike based on a scale from 1 to 5, where 1 was assigned when the branching intensity along the spike was very weak i.e. occasional occurrence of additional spikelet per spike and 5 was assigned when the spikes show very strong branching. The third approach was counting additional spikelets per spike. For this, about 10-15 spikes were randomly sampled from the middle of each plot from all environments.

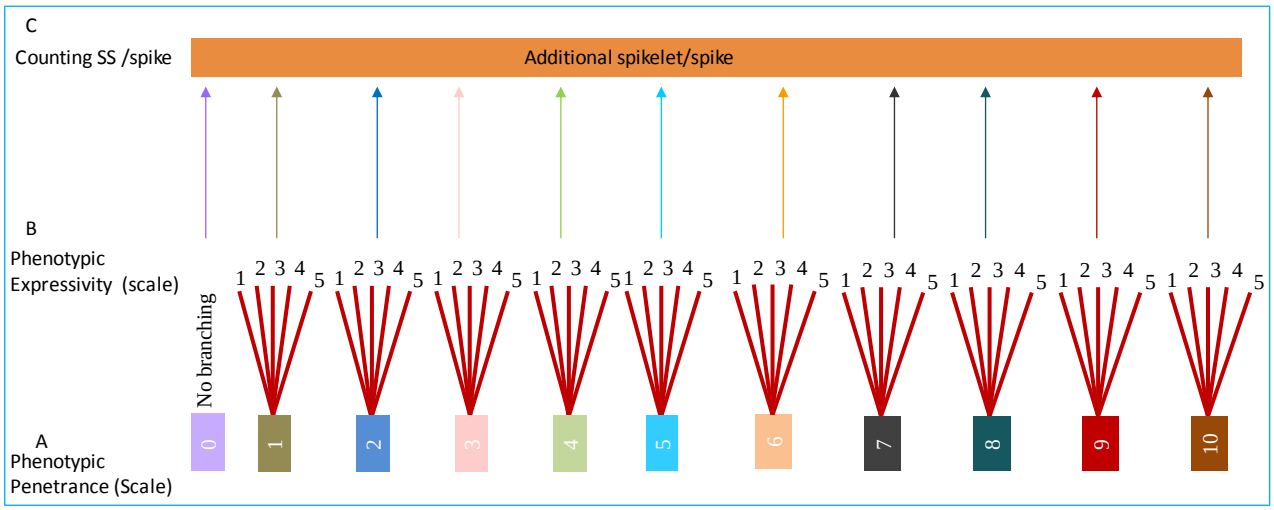


Figure 2-2. The three approaches used to phenotype spike morphology in the field.
(A) Phenotypic penetrance based on the scale on a plot basis.The number indicates the scale from 0 (no plants were branching) to 10 (all plants on the plots were branching). (B) Measuring branching intensity (expressivity) based on a scale from 1 to 5. 1 was given for almost no branching spike while 5 was for the strongly branching spike. (C) phenotyping by counting the added spikelets per spike due to branching.

From the sampled spikes, node number per spike, total spikelets per spike and spike dry weight per spike were taken. Grain numbers per spike, thousand kernel weight, grain length, grain width were also measured and data were used for QTL mapping. The three phenotypic data, namely: grain number per spikelet, node density, and spike harvest index were calculated as follows: Grain number per spikelet was calculated by dividing total grain number per spike to the total number of spikelet per spike (including none fertile spikelets). Similarly, node density was derived by dividing total node number per spike to spike length. Spike harvest index was calculated by

dividing grain weight per spike to the spike dry weight. Besides these traits, Plant height, days to heading, and spike length were also measured. The heading date was recorded following Zadoks scale (Z= 55) (Zadoks, 1985). That is when 50% of the inflorescence comes out of the flag leaf for half of the plants on the plot. Plant height and spike length were recorded close to plant maturity from 5-10 randomly sampled plants from the middle of the plot. Spike length does not include the awns. Grain number and grain related traits were measured using Marvin digital seed analyzer (GAT Sensoric GmbH, Neubrandenburg, Germany).

2.3.4 DNA extraction and genotyping

All the mapping population at F7 generation was grown in the greenhouse (three seeds per pot). Plants were allowed to germinant in the greenhouse at 15^{o}C for about 15 days. After germination and establishment, seedlings were vernalized at 4^{0}C for 4 weeks. After 2 weeks of hardening at 15/12^{0}C day/night temperature; leaf samples were collected and pooled together from three plants for genomic DNA isolation based on the modified CTAB method described by Doyle and Doyle (Doyle JJ, 1990). The final concentration was measured and samples were used for CAPS marker analysis as well as for Genotyping-By-Sequencing (GBS) library preparation following novel two-enzyme genotyping-by-sequencing approach (Poland et al., 2012b).

2.3.5 Development of CAPS marker

Based on the Chinese spring genomic sequence, genome specific primers were designed. All the primers used in this study are listed in Table 2-3. PCR amplifications of both homoeo-alleles of *TtBH (TtBH-A1* and *TtBH-B1)* were separately carried out in a 12.5 µl of reaction volume containing 10 ng of DNA, 1.25 µl of PCR buffer (10X) (Qiagen, Hilden, Germany), 0.5 mM dNTPs, 2.5 µl of Q solution (Qiagen, Hilden, Germany), 1.25 ul of 10pM of each primers, and 0.0625U of Taq polymerase (Qiagen, Hilden, Germany). The PCR conditions for both genes were as follows: initial denaturation step of 95^{o}C/for 3 min , 39 cycles of 95 ^{o}C/30 sec, 62 ^{o}C/30 sec, 72 ^{o}C/1 min and 72 ^{o}C/10 min using the genome specific primers for both alleles (Tafzp_2A_F1: Tafzp_2A_R1; Tafzp_2B_F1: Tafzp_2B_R1, Table 2-3). Specificity of the designed primers was confirmed through sanger-sequencing of each amplicon. For this, PCR products were directly purified using MinElute 96UF PCR purification kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Sequencing of the amplicon was run using BigDye Terminator v3.1 cycle sequencing Kits (Applied Biosystems, USA). Each amplicon was sequenced from both directions. DNA sequence analysis and multiple alignments were made using Geneious software version 6 (Kearse et al., 2012).

The SNP at position 287 (T287C), which has led to amino acid substitution at position 96 (L96P), clearly differentiated standard wheat spike form and the branching mutant in tetraploid wheat (Poursarebani et al., 2015). To develop a diagnostic marker, all possible restriction sites were searched using an online tool, 'NEBcutter' from New England Bio labs (Vincze et al., 2003). Three restriction sites for *BstNI*, (5'...CCWGG...3', W = A or T) including position 287 (T287C), were found. To exclude the remaining two sites, other sets of primers: TdFZP2A_in_F and TdFZP2A_in_R (Table 2-3) were designed. These primers amplify only small fragments (147bp) which include the mutation site (T287C). To maintain genome specificity of these primers (TdFZP2A_in_F: TdFZP2A_in_R), amplicon from the first round genome specific PCR product was used as a template after diluting the amplicon 200x times. Same PCR conditions and settings as before were also used here. After the second round PCR, the amplicon was used for restriction digestion analysis of *BstNI*. Restriction digestion for *BstNI* was as follows*:* 4ul of the PCR product; 5 units of *BstNI* (0.5ul); 0.15ul BSA (Bovine Serum Albumin), 2ul of reaction buffer and 3.35ul of water were mixed and incubated for overnight at 60^0c. Digested product was electrophoresed through a 2% agarose gel.

Similarly, a CAPS marker for *TtBH-B1* was also developed. The developed marker was based on an SNP located -54 bp upstream of the start of the open reading frame. Enzyme *XhoI* (5'...C^TCGAG...3') digests the *TtBH-B1* allele from TRI 19165 but not from Bellaroi. Primers WFZP_2B_F2 and Tafzp_2B_R1 were used to amplify the 5'UTR including the CDS (Table 2-3). Touchdown PCR technique was used for amplification. The conditions were as follows: 95^oC/3 min, 8 cycles of 95 ^{0}C/30 sec, 67-> 60 ^{0}C/30 sec (-1^{0}C in each step), then 36 cycles of 95 ^{0}C/30 sec, 62 ^{0}C/30 sec, 72 ^{0}C/1 min and 72 ^{0}C/10 min. Restriction digestion with *XhoI* was as follows: 4 ul of the PCR product; 5 units of *XhoI* (0.5 ul); 0.15ul BSA (Bovine Serum Albumin), 2ul of reaction buffer and 3.35ul of water were mixed and incubated for overnight at 37^0c. Digested product was analyzed on a 2% agarose gel.

Table 2-3. List of primers used in this study

Name	Sequence (5'-3')	Length	Genome	Location	Amplicon size
WFZP_2B_F1	TGGGCTCTGCCTTCACAATCAG	22	2B	5'UTR	1030 bp
WFZP_2B_R1	GATGAGATGGCGACCTTGG	19			
WFZP_2B_F2	GGCACACAAATCCAAACACA	20	2B	5'UTR + CDS	1608 bP
Tafzp_2B_R1	TCGAGCAATGCCGGTGCATTTGC	23			
Tafzp_2A_F1	AGCCAACCTCACTTCACTTC	20	2A	CDS	946 bp
Tafzp_2A_R1	GAGCAATGCCAGCGCGTCCGT	21			
TdFZP2A_in_F	GACCCGACCACCAAGGAG	18	2A,2B *	CDS	147 bp
TdFZP2A_in_R	GTAGTTGTTGTAGGCGGCGT	20			
Tafzp_2B_F1	GCCAGCCAGCATCACTTCAGTTG	23	2B	CDS	984 bp
Tafzp_2B_R1	TCGAGCAATGCCGGTGCATTTGC	23			
WFZP_2B_R1F	CCAAGGTCGCCATCTCATC	19	2B	Up stream of CDS	
WFZP_2B_F2R	TGTGTTTGGATTTGTGTGCC	20	2B	Up stream of CDS	
TaF_2B _CRR2	CATCATCTGGGAGGCCTG	18	2B	CDS	
wFZP_3UTR_F2	GGACGACCTGGCGTTGAT	18	2B	CDS + 3'UTR	1056 bp
wFZP_3UTR_R3	ATACAAGCCTTGCACCTGCT	20			
qBh_2A_F2	ACGGACGCGCTGGCATTGCTC	21	2A	3‘UTR	157 bp
qBh_2A_R2	TAGGGCACCGAAACAACAAAGC	22			
qBh_2B_F3	ACACTGAAGCAAATGCACC	19	2B	3‘UTR	163 bp
qBh_2B_R3	TAGGGCACCGAAACAAGC	18			

WFZP_2B_R1F, WFZP_2B_F2R, and TaF_2B _CRR2 were designed for sequencing the first two amplicons (1030 and 1608 bp). TdFZP2A_in_F, TdFZP2A_in_R were used to re-amplify the first round PCR for digestion analysis. See text for the detail. TM for each primer sets was 62 0C or performed based on touchdown PCR with final TM set to be 62 0c

2.3.6 Marker generation from the RILs

Markers from RILs were generated by GBS following novel two-enzyme genotyping-by-sequencing approach (Poland et al., 2012b). The simplified illustration of GBS has been shown in the introduction part under section 1.6.2, Figure 1-4. Adapters were trimmed from reads with

cutadapt version 1.8.dev0 (Martin, 2011). Trimmed reads were mapped to the chromosome-shotgun assemblies of bread wheat cultivar Chinese Spring (International Wheat Genome Sequencing, 2014) with BWA mem version 0.7.12 (Li, 2013), converted to BAM format with SAMtools (Li et al., 2009) and sorted with Novosort (Novocraft Technologies Sdn Bhd, Malaysia, http://www.novocraft.com/). Multi-sample variant calling was performed with SAMtools version 0.1.19 (Li, 2011). The command "mpileup" was used with the parameters "-C50 –DV". The resultant VCF file was filtered with an AWK script provided as Text S3 by Mascher et al. (Mascher et al., 2013). Only bi-allelic SNPs were used. Homozygous genotype calls were set to missing if their coverage was below 1 or their genotype quality was below 3. Heterozygous genotype calls were set to missing if their coverage below 4 or their genotype quality was below 10. SNP was discarded (i) if its quality score was below 40, (ii) its heterozygosity was above 20 %, (iii) its minor allele frequency was below 10 %, or (iv) had more than 66 % missing data. Genotype calls were filtered and converted into genotype matrix with an AWK script available as Text S3 of Mascher et al.2013 (Mascher et al., 2013). Chromosomal locations and genetic positions were taken from population sequence (POPSEQ) data (Chapman et al., 2015).

2.3.7 Construction of the genetic linkage map

The genetic linkage map, which is based on recombination values of the mapping population, was constructed using Joinmap 4.0 (Stam, 1993). Theoretically, almost all markers are expected to segregate in 1:1 ratio at F7 generation. Hence, undistorted SNP markers were selected based on goodness-of-fit using a chi-squared test. All the distorted markers were removed from further analysis. However, the wheat vernalization gene, *VRN1,* is located on chromosome 5AL (Yan et al., 2003), almost all the markers on chromosome 5A showed segregation distortion due to the strong selection pressure exerted against vernalization requirement during the development of the mapping population. Regression and maximum likelihood mapping algorithms of the join map software were used for the linkage construction. Linkage group was determined using Haldane's mapping function. The maximum distance of 50 cM was used to determine linkage between two markers. The maps were drawn using Map chart version 2.3 (Voorrips, 2002).

2.3.8 Statistical analysis and QTL mapping

Basic statistical analysis, such as analysis of variance (ANOVA) and correlation analysis were performed using Genstat 17 and SPSS 20 (IBM, 2011; Payne et al., 2014). ANOVA was calculated to check whether there exists significant different among the RILs for measured traits, and to estimate GEI interactions. Heritability was estimated from the variance components obtained

from the ANOVA Table. Data from 146 RILs collected from three environments (IPK14, IPK15, and HAL15) as well as spike morphology data from the greenhouse (GH15), were used for QTL mapping using Genstat 17. First, single environment-single trait linkage analysis was performed in order to map QTL associated with each trait in each environment. A step size of 10 cM, a minimum cofactor proximity of 50 cM, a minimum separation of selected QTL of 30 cM, and Genome-wide significance level (alpha) of 0.05 were used for QTL analysis. Based on the mixed-model approach, the whole genome was scanned first using simple interval mapping (SIM) approach. Then, based on the SIM result, cofactors were selected for composite interval mapping (CIM). The final QTL model was selected using the backward selection on the selected cofactors, where QTL boundaries (lower and upper), QTL effect and phenotypic variance explained (PVE) by QTL were determined. Under the option of a single trait and multiple environments analysis toolbox, similar set up were followed to check whether QTL by environment interaction does exist and if so, to calculate the interaction main effect. Similarly, QTL analysis was conducted using Windows QTL Cartographer, which is also widely used for QTL mapping, following composite Interval Mapping (CIM) algorithms (Wang S., 2012). To control the genetic background while testing a position in the genome, the forward and backward regression method was used as a cofactor. The walking speed chosen for the QTL analysis was 1.0 cM. LOD threshold value for all traits was determined through a permutation test (2000 times). QTL was verified automatically by the program based on the 95% confidence interval. QTL epistatic interaction was mapped using QTL network version 2 (Yang et al., 2008).

2.3.9 Expression analysis

To further check the level of expression of *TtBH* homoeoalleles i.e. *TtBH-A1* and *TtBH-B1*, four different RILs were selected for expression analysis. Expression analysis was aimed at three spike developmental stages, i.e. Glume primordium (GP), Floret primordium (FP), and terminal spikelet (TS) stages. The RILs were selected based on A and B allele combinations using CAPS marker analysis (Table 2-4). Thus, RIL_7769_3_21 (genotype: *aabb*) inherited both mutant alleles (i.e *TtBH-A1* and *TtBH-B1*) from TRI 19165. Therefore, the spike morphology of RIL_7769_3_21 resembles TRI 19165. Phenotypically, RIL_7769_3_21 shows strong spike-branching characteristics across all environments (Table 2-4). RIL_7769_5_6 (genotype: *AABB*), inherited both copies of the wt allele (*TtBH-A1* and *TtBH-B1)* from Bellaroi, and thus, shows no spike-branching (standard spike). Therefore, RIL_7769_3_21 and RIL_7769_5_6 carry parental type alleles. The remaining two lines had the following allele combinations: RIL_7769_3_22 (genotype: *AAbb*), which inherited

the A allele, *TtBH-A1,* from Bellaroi and the B allele, *TtBH-B1*, from TRI 19165. Similarly, RIL_7769_4_38 (genotype: *aaBB*) had the A allele, *TtBH-A1*, from TRI 19165 while its B allele, *TtBH-B1,* was from Bellaroi. Phenotypic penetrance and expressivity of these four RILs are shown in Table 2-4. Spike phenotypic data were compiled from four different environments.

Table 2-4. Phenotypic penetrance and expressivity in selected RILs for expression analysis.

		IPK14		IPK15		HAL15		GH15	
RILs	Genotype	Exp.	Pen.	Exp.	Pen.	Exp.	Pen.	Exp.	Pen.
RIL_7769_3_21	*aabb*	67	100	67	100	47	100	36	100
RIL_7769_5_6	*AABB*	-	-	-	-	-	-	-	-
RIL_7769_3_22	*AAbb*	-	-	-	-	-	-	-	-
RIL_7769_4_38	*aaBB*	0	0	1	15	0	0	0	0

Exp., Expressivity (%); Pen, Penetrance (%). Exp (proportion of addSPS) and Pen (proportion of plants carrying SS) were calculated based on sampled spikes from four different environments

Plants were grown under the following conditions. Seeds from the four lines were grown in 54 Well trays (Well volume of 88cm^3) under controlled long day conditions: 16/8 hours day/night; and 19/17 °C day/night temperatures for 15 days. After 15 days of germination and seedling establishment, plants were vernalized for about four weeks at 4^{o}C. After one week of hardening at 15/12 ^{o}c day/night temperature, all seedlings were transferred to 20/17 ^{0}C day/night temperature and allowed normal development (16/8 hours day/night). Spike development stages (based on Kirby cereal development guide handbook (Kirby and Appleyard, 1984)) were frequently checked after 4 leaf stages and beyond. The whole inflorescence was removed at the right stages by carefully dissecting the plant under a light microscope and collected samples were frozen in liquid nitrogen until RNA isolation. Total RNA was isolated using TRIzol® RNA isolation reagents (Thermo fisher scientific Catalog no. 15596018). After quantification and quality check, the total concentration was adjusted to 50 ng/ul for all samples and the first-strand cDNA was synthesized with oligo (dT) primer and Superscript™ III (Invitrogen, Life Technologies) according to manufacturer's protocol.

Gene-specific primers (for *TtBH-A1* and *TtBH-B1*) i.e. qBh_2A_F2 and qBh_2A_R2 for *TtBH-A1* and qBh_2B_F3 and qBh_2B_R3 for *TtBH-B*) were designed from 3'UTR region the alleles (Table 2-3). Wheat *Actin* gene was used as internal reference gene. Primer sequences for the *Actin* gene were adopted from Distelfeld *et al*.(Distelfeld et al., 2009). All primers for the expression analysis were diluted to final concentration of 0.5pM. Before proceeding to the expression analysis, the first strand cDNA was diluted 100 times in RNA free DEPC treated water. For each reaction 2µl of the

forward primer, 2ul of reverse primer, 1µl cDNA, and 5µl SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK) were used. For each gene, four technical and two to three biological replications were used. The ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, USA) was used for the analysis. The cycle threshold (Ct) values were extracted using SDS2.4 software from the ABI 7900HT Fast Real-Time PCR system. PCR efficiency (E) was calculated by using LinReg PCR (Version 7.5). The expression value was normalized using *Actin* as internal reference gene.

2.3.10 Sequence analysis of the haplotypes

To further analyze sequence variation in *TtBH-B1,* about 49 different tetraploid wheat accessions/varieties were sequenced. Besides the coding sequences, about 1 kb sequences upstream from the start of the open reading frame were sequenced from four modern tetraploid varieties (Bellaroi, Floradur, Tamaroi, and Wollaroi) and from 3 accessions (TRI 19165, TRI27966, and TRI7282). Furthermore, about 750 bp downstream from the stop codon of the gene was sequenced from all 49 accessions/varieties to analyze the sequence variations in the 3' UTR region. Primers used to amplify each of these regions are mentioned in Table 2-3. A touchdown PCR amplification was followed by PCR conditions mentioned earlier. Sequencing of the amplicon was run using BigDye Terminator v3.1 cycle sequencing Kits (Applied Biosystems, USA). Each amplicon was sequenced from both directions. DNA sequence analysis and multiple alignments were made using Geneious software version 6 (Kearse et al., 2012).

2.4 Results

2.4.1 Phenotypic and genotypic analysis of spike-branching in the RILs

The mapping population used in this study was derived from a cross between standard spike form (Bellaroi) and branched head natural mutant variant (TRI19165). In standard wheat spike, spikelets are arranged in a distichous fashion on the main spike axis (rachis) and each rachis node bears only single spikelet (Figure 2-3A). While in the *bh* wheat mutant, the central dogma of single node-single-spikelet cannot be maintained and thus, the spike branches out in the form of mini spike or additional spikelets per rachis node. Spike-branching in the *bh* parent (TRI 19165) of the current mapping population mainly appears in the form of a mini-spike, mainly from the bottom two-third of the spike. The top third of the main spike usually develops normal spikelets without any branch formation (Figure 2-1).

However, three different types of *bh* phenotypes were clearly detected in the mapping population. The first one was the appearance of additional or supernumerary spikelets from the bottom of the spike (Figure 2-3 B&E). The second type of *bh* phenotype was the appearance of mini spike from rachis node (instead of spikelet) especially in the bottom half of the spike (Figure 2-3 C&F), while the third type was extension of the rachilla (Amagai et al., 2015), which is known to be as 'sham ramification' (Figure 2-3D&G). From the 146 mapping population only a single RIL, i.e. RIL_7769_4_78, showed the 'sham ramification' phenotype across all environments (Figure 2-3G). In rare cases, a combination of all the three types might also be seen.

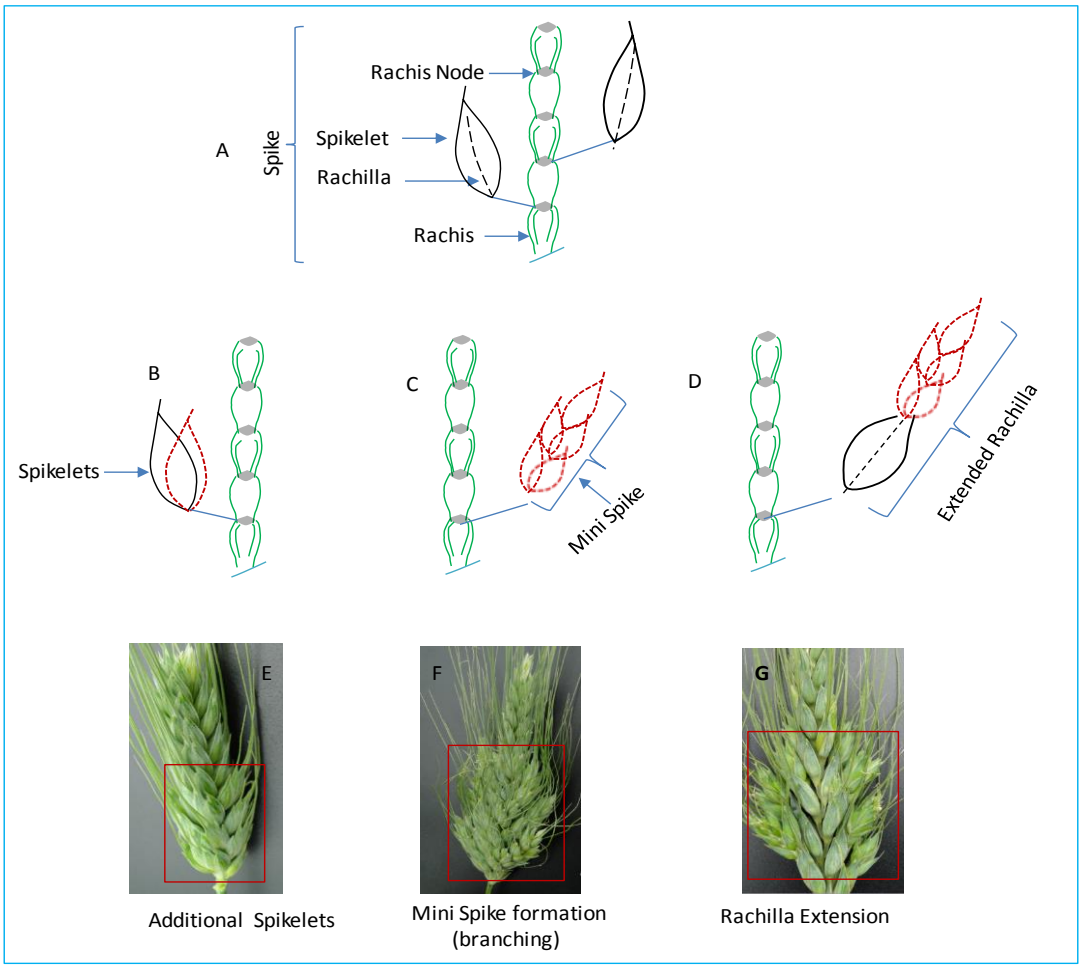


Figure 2-3. Illustration of *bh* phenotype in the mapping population.
(A) Schematics of the standard wheat spike with the distichous arrangement of spikelets. (B), (C), and (D) show the schematics of the three major types of phenotypic classes of the *bh* phenotype in the mapping population. (E), (F), and (G) were photos taken from field grown plants showing the three phenotypic classes.

Although three phenotypic classes of spike-branching were detected in the mapping population, all led to the development of additional spikelets per spike. Therefore, the ultimate effect of the *bh* locus/loci is the development of additional spikelets per spike. Summary of the additional

spikelets per spike (addSPS) from four different environments, as well as the comparison between parent, mutant check, and RILs, is shown in

Table 2-5. Summary of additional and total spikelet per spike across four different environments is shown Figure 2-4.

Table 2-5. Comparison of the parent, mutant checks, and RILs for additional and total spikelets per spike across four different environments.

Trait	Environment	Parent	Mutant Check	RILs(n=146)			
		Bellaroi	(n=3)	Min	Max	Mean	SD
Additional Spikelet/Spike (addSPS)	IPK14	-	32.92	0.00	34.15	7.51	5.66
	IPK15	-	50.83	0.00	35.12	10.40	7.74
	HAL15	-	35.15	0.00	21.58	6.39	4.07
	GH15	-	45.31	0.00	14.50	3.04	4.05
Total Spikelet/Spike (totSPS)	IPK14	13.75	52.37	12.60	51.35	21.02	6.06
	IPK15	14.90	71.57	12.20	54.52	23.82	8.41
	HAL15	12.74	53.78	11.48	39.46	19.78	5.36
	GH15	12.75	69.44	12.00	34.00	20.13	4.89

SD, Standard Deviation

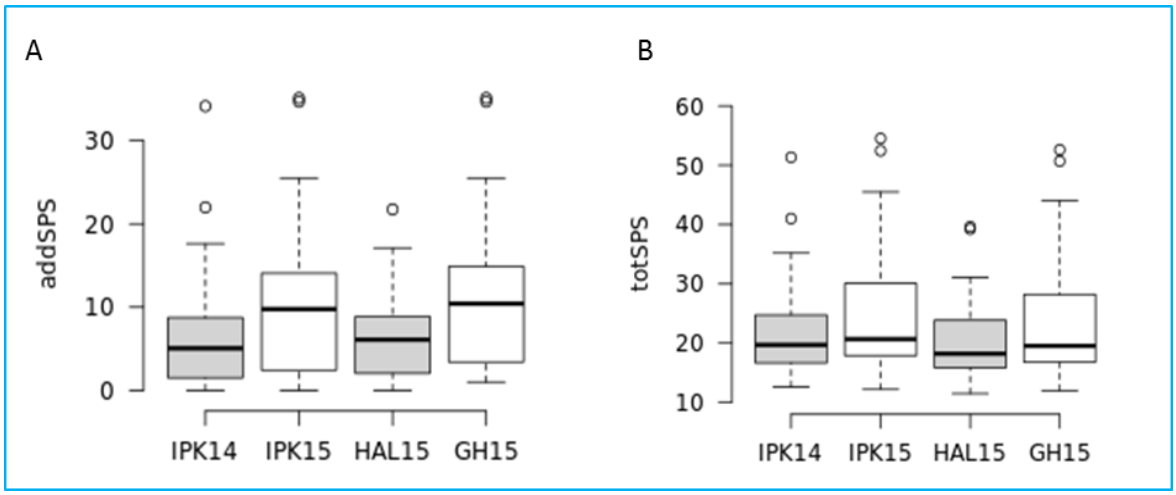


Figure 2-4. Summary of the distribution of additional and total spikelet per spike.
(A) Additional spikelet per spike, (B) total spikelet per spike. The data were compiled from four different environments: IPK14, IPK15, HAL15, and GH15. Solid lines show the median. Small circles are outliers.

2.4.2 Penetrance and expressivity of spike-branching in the RILs

All the RILs used in this study were genotyped (at F7) using the CAPS marker derived from *TtBH-A1* (Supplementary Figure 9-3). Sixty out of 146 (41%) carried the wild-type allele, *BH^t-A1*, from Bellaroi and carry standard spike form (wild type). Seventy-eight RILs (53% of the total RILs) were found to carry the mutant allele, *bh^t-A1*, from TRI 19165. However, not all of them showed the

spike-branching phenotype. Furthermore, the goodness-of-fit (chi- squared test) between the observed and expected number was non-significant, indicating that segregation of the *bh* locus fits the theoretical expected 1:1 segregation ratio.

To further quantify the variation in branching intensity, phenotypic penetrance and expressivity were introduced. Penetrance was used to quantify or measure the proportion of the individuals of the same genotype showing the expected phenotype. This is regardless of the intensity of branching per spike; while expressivity was used to measure or describe the intensity of branching per spike for each genotype carrying the locus. Therefore, the implementation of the three phenotyping approaches (Figure 2-2) was mainly to capture all the phenotypic variations and the associated genetic factors modifying spike-branching in the mapping population. Phenotypic penetrance and expressivity of spike-branching of the 78 RILs carrying the bh^{t}*-A1* allele are shown in Table 2-6. To investigate the role of the homoeoallele, i.e. bh^{t}*-B1*, all the RILs were also genotyped using the CAPS markers derived from *TtBH-B1.* Hence, genotype (GT) of each of the RILs was based on the CAPS marker derived from *TtBH-A1* and *TtBH-B1* (Supplementary Figure 9-3 A and B). Thus, *AABB* indicates that both the A and B alleles (i.e. bh^{t}-A1 and bh^{t}*-B1)* were derived from Bellaroi while *aabb* indicates that both alleles were from TRI 19165. As clearly shown, RILs with genotype, *aaBB* show reduced phenotypic penetrance and expressivity across all the environments as compared to those with genotype *aabb*. However, RIL_7769_4_78 exceptionally showed a 'sham ramification' phenotype with increased penetrance and expressivity (Figure 2-3G). Taking all together, variations in phenotypic penetrance and expressivity suggest a role of the wild-type (wt) allele of *TtBH-B1* from Bellaroi as a suppressor of spike-branching or SS formation.

Table 2-6. Genotypic and phenotypic evaluation of all mutant RILs from three different environments.
Phenotypic expressivity (branching intensity/spike) and penetrance (branching /plot) were based on the scales as indicated in the Materials and Methods section (Figure 2-2). Genotype (GT) of each of RILs was based on allele specific CAPS marker developed from *TtBH-A1* and *TtBH- B1*. IPK14, IPK15, and HAL15 are environments where the mapping population was evaluated.

RILs		AddSPS			Branching intensity/spike			Branching /plot		
	GT	IPK14	IPK15	HAL15	IPK14	IPK15	HAL15	IPK14	IPK15	HAL15
RIL_7769_2_12	*aaBB*	0.00	2.00	2.00	1	1	1	1	1	2
RIL_7769_2_48	*aaBB*	0.40	1.20	1.00	1	1	1	2	2	1
RIL_7769_2_53	*aaBB*	0.00	0.00	1.00	1	1	1	1	1	1
RIL_7769_2_88	*aaBB*	0.00	1.25	0.00	1	1	1	1	1	1
RIL_7769_3_1	*aaBB*	0.00	0.00	0.00	1	1	1	1	1	1
RIL_7769_3_20	*aaBB*	0.20	1.50	1.00	1	2	1	2	4	1
RIL_7769_4_10	*aaBB*	0.00	1.00	1.50	1	1	1	1	1	1
RIL_7769_4_37	*aaBB*	0.00	1.38	0.00	1	1	1	2	1	1
RIL_7769_4_38	*aaBB*	0.00	0.50	0.00	1	1	1	1	1	1
RIL_7769_4_51	*aaBB*	0.20	1.00	1.00	1	1	1	2	1	1
RIL_7769_4_59	*aaBB*	0.00	0.00	1.50	1	1	1	1	1	1
RIL_7769_4_6	*aaBB*	0.10	1.00	0.00	1	1	1	1	2	1
RIL_7769_5_20	*aaBB*	0.00	0.00	0.00	1	1	1	1	1	1
RIL_7769_5_42	*aaBB*	0.00	0.00	0.00	1	1	1	1	1	1
RIL_7783_1_105	*aaBB*	0.65	2.31	1.00	1	1	1	2	3	2
RIL_7783_1_109	*aaBB*	0.00	0.25	1.00	1	1	1	1	1	1
RIL_7769_2_50	*aaBB*	1.70	3.44	2.58	2	3	2	9	7	5
RIL_7769_4_47	*aaBB*	0.00	4.20	2.92	2	3	3	7	10	3
RIL_7783_1_143	*aaBB*	2.10	3.41	3.35	2	2	2	8	9	6
RIL_7783_1_89	*aaBB*	0.00	0.00	0.00	2	1	1	1	3	1
RIL_7783_1_32	*aaBB*	1.50	3.07	2.75	2	2	2	6	8	7
RIL_7783_1_43	*aaBB*	0.00	0.00	0.00	2	1	1	1	1	1
RIL_7783_1_63	*aaBB*	1.35	4.61	3.89	2	2	2	9	8	8
RIL_7769_2_9	*aaBB*	0.90	2.27	2.08	3	2	1	4	7	3
RIL_7783_1_60	*aaBB*	2.40	3.97	2.32	3	3	2	9	8	8
RIL_7769_4_44	*aaBB*	0.05	10.32	9.67	3	4	3	3	10	9
RIL_7769_4_78	*aaBB*	16.40	6.85	4.17	5	5	2	10	9	3
RIL_7783_1_14	*aaBB*	0.15	0.88	0.00	1	2	1	1	4	1
RIL_7783_1_39	*aaBB*	0.00	0.50	2.17	1	1	1	2	1	1
RIL_7783_1_44	*aaBB*	0.30	1.73	1.20	1	2	1	1	5	1
Mean		0.95	1.95	1.60	1.5	1.6	1.3	2.9	3.7	2.4

Table 2-6. Continued…

RILs	GT	AddSPS			Branching intensity/spike			Branching /plot		
		IPK14	IPK15	HAL15	IPK14	IPK15	HAL15	IPK14	IPK15	HAL15
RIL_7769_4_84	*aaBb*	2.75	1.50	3.58	2	2	2	7	5	4
RIL_7769_4_49	*aaBb*	4.05	8.99	6.07	3	3	3	9	8	6
RIL_7769_5_4	*aaBb*	3.00	10.15	9.22	2	3	3	8	8	8
RIL_7783_1_104	*aaBb*	4.30	16.79	9.66	3	3	3	10	9	8
RIL_7783_1_76	*aaBb*	8.60	12.80	9.75	3	4	3	9	5	7
RIL_7783_1_79	*aaBb*	2.80	10.57	7.24	3	3	3	8	7	6
RIL_7769_4_72	*aaBb*	2.50	7.57	10.73	3	4	4	8	6	6
RIL_7783_1_13	*aaBb*	8.55	12.50	7.96	4	4	3	10	7	8
RIL_7783_1_48	*aaBb*	6.80	14.55	9.04	4	5	3	9	5	6
Mean		4.82	10.60	8.14	2.9	3.3	3.0	8.5	6.6	6.7
RIL_7769_1_38	*aabb*	8.45	8.14	6.89	3	3	3	10	10	10
RIL_7769_2_58	*aabb*	7.85	7.77	7.92	3	3	3	10	9	9
RIL_7769_2_80	*aabb*	9.40	11.95	7.85	3	3	3	10	9	9
RIL_7769_3_18	*aabb*	2.08	9.05	6.07	3	3	3	4	6	5
RIL_7769_3_4	*aabb*	8.80	10.07	9.50	3	4	4	10	10	8
RIL_7769_4_17	*aabb*	7.20	12.16	8.15	3	3	3	10	10	10
RIL_7769_4_19	*aabb*	2.55	7.54	4.52	3	4	3	10	8	8
RIL_7769_4_27	*aabb*	7.80	8.75	6.22	3	3	3	10	10	9
RIL_7769_4_54	*aabb*	8.40	11.56	8.51	3	3	3	10	9	9
RIL_7769_4_8	*aabb*	12.40	14.57	7.45	3	4	3	10	10	9
RIL_7769_4_80	*aabb*	0.00	20.96	5.38	3	4	3	3	5	6
RIL_7783_1_107	*aabb*	9.40	15.67	10.60	3	3	3	10	9	9
RIL_7783_1_125	*aabb*	7.85	11.47	7.37	3	3	3	10	10	9
RIL_7783_1_140	*aabb*	7.85	11.21	6.86	3	3	3	10	10	10
RIL_7783_1_144	*aabb*	7.40	10.76	7.69	3	3	3	10	10	10
RIL_7783_1_38	*aabb*	6.25	8.39	5.23	3	3	3	10	10	10
RIL_7783_1_45	*aabb*	10.77	10.69	6.42	3	3	3	10	10	9
RIL_7769_1_34	*aabb*	8.15	15.64	6.51	4	3	3	10	10	10
RIL_7769_2_1	*aabb*	8.75	21.09	10.01	4	4	3	10	10	8
RIL_7769_1_5	*aabb*	18.86	34.69	21.58	5	5	5	10	9	10
RIL_7769_2_17	*aabb*	14.80	16.28	9.28	5	5	4	10	7	7
RIL_7769_3_21	*aabb*	34.15	33.58	14.94	5	5	5	10	10	7
RIL_7769_1_37	*aabb*	3.30	10.88	5.54	2	3	3	9	9	7
RIL_7769_3_2	*aabb*	0.80	5.67	2.97	2	3	2	3	7	8
RIL_7769_5_16	*aabb*	0.35	1.68	1.08	2	2	2	6	6	6
RIL_7783_1_29	*aabb*	2.90	9.06	4.64	2	3	3	9	9	7
RIL_7783_1_34	*aabb*	9.45	15.09	5.74	4	4	3	10	10	9
RIL_7783_1_78	*aabb*	2.75	13.59	7.21	2	3	3	10	9	5
RIL_7769_2_55	*aabb*	8.50	18.17	9.09	4	4	4	10	9	9
RIL_7769_2_90	*aabb*	13.05	19.88	8.70	4	4	4	10	10	10
RIL_7769_3_12	*aabb*	15.15	23.26	10.31	4	4	4	10	10	10

Table 2- 6. Continued…

RILs	GT	AddSPS			Branching intensity/spike			Branching /plot		
		IPK14	IPK15	HAL15	IPK14	IPK15	HAL15	IPK14	IPK15	HAL15
RIL_7769_3_31	*aabb*	2.20	22.83	17.11	4	4	4	10	10	10
RIL_7769_4_2	*aabb*	11.43	24.47	10.43	4	4	4	10	10	10
RIL_7769_5_14	*aabb*	6.30	11.67	7.73	4	4	3	10	8	9
RIL_7769_5_46	*aabb*	12.10	13.36	9.47	4	4	4	10	10	10
RIL_7783_1_100	*aabb*	16.34	20.54	13.70	4	4	4	10	10	10
RIL_7783_1_142	*aabb*	15.40	22.18	10.13	4	4	4	10	9	10
RIL_7783_1_157	*aabb*	3.90	14.98	9.46	4	4	3	10	9	7
RIL_7783_1_86	*aabb*	9.55	14.90	7.79	4	4	4	10	9	8
Mean		8.77	14.71	8.43	3.33	3.56	3.32	9.22	9.02	8.57
RIL_7769_5_10	*AaBB*	0.00	0.00	0.00	0	0	0	0	0	0
RIL_7783_1_70	*AaBB*	0.10	0.75	1.00	1	1	1	1	1	1
RIL_7783_1_91	*AaBB*	0.00	0.00	0.00	0	0	0	0	0	0
Mean		0.03	0.25	0.33	0.33	0.44	0.33	0.33	0.44	0.33
RIL_7769_1_32	*Aabb*	4.65	10.85	6.61	2	3	3	4	6	6
RIL_7769_2_20	*Aabb*	2.05	6.65	4.21	3	2	2	9	8	6
Mean		3.35	8.75	5.41	2.25	2.50	2.33	6.50	7.17	6.17
RIL_7769_4_32	*AaBb*	0.00	0.00	0.00	0	0	0	0	0	0

To further analyze phenotypic expressivity of the spike-branching phenotype, up to 10 spikes per plot were collected from border rows of each plot. The data were compared with samples collected from inside rows from the middle of each plot. The data were collected from F7 RILS grown in 2014 in Gatersleben (IPK14) with two replications. Because, plants in the border rows were well positioned for resources (light, nutrients), the phenotypic difference between these two groups partially reflects the effect of plant competition on the phenotypic expressivity of spike-branching. Hence, border plants showed a higher degree of spike-branching phenotype (higher degree of phenotypic expressivity) as compared to those from the middle part where competition among plants was supposed to be intense (Figure 2-5). This clearly suggests the effect of plant competition (micro-environment) on the phenotypic expressivity of spike-branching phenotype. As more SS were formed, grain number per spike (GNS) also found to be significantly higher for border samples. Similarly, grain number per spikelet (GPS) or spikelet fertility was also significantly improved in the border samples.

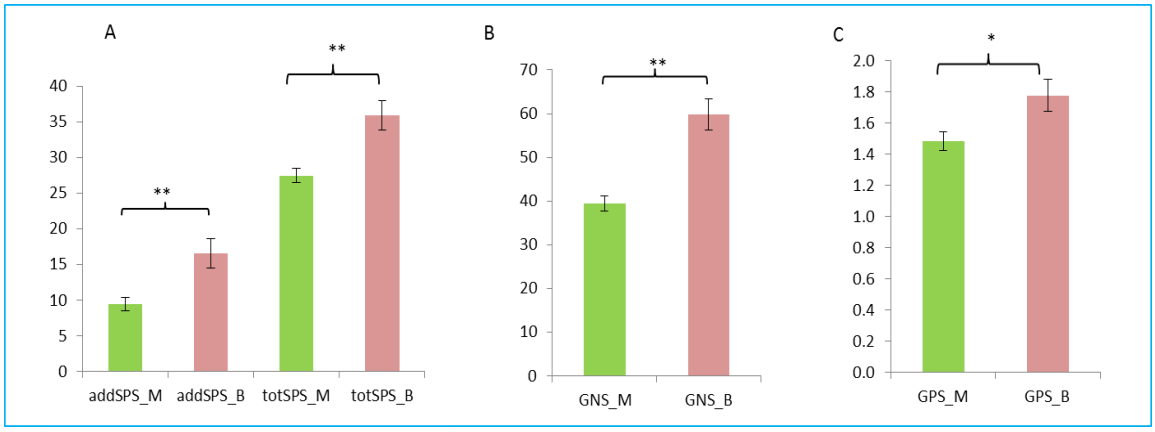


Figure 2-5. Phenotypic plasticity of SS formation.
The comparison was made between field data collected from the border and middle row of each plot. addSPS, totSPS, GPS and GNS were derived from medium to strongly branching RILs (genotype: *aabb;* n=39) from field grown RILs in 2014 (IPK14). (A) Additional and total spikelet per spike, (B) Grain number per spike, (C) Grain number per spikelet. A significance level was calculated based on the unpaired two-tailed student's t-test at P= 0.05. addSPS, additional spikelet per spike; totSPS, total spikelet per spike; GNS, grain number per spike; GPS, Grain number per spikelet M, Middle; B, Border.

2.4.3 Genetic linkage map and identification SS QTLs

From a total of 1268 markers generated from GBS, 996 markers were placed on shotgun contigs anchored genetically based on the information obtained from population sequencing (POPSEQ)(Chapman et al., 2015). See materials and method section for the brief description of marker assembly of the GBS data of the RILs. By removing all the congested markers (those markers located on the same genetic position), 755 markers were selected. These markers were tested for goodness-of-fit based on a chi-squared test for distortion. After removing all the distorted markers, 426 markers were used to construct the genetic linkage map used in this study. The CAPS markers derived from *TtBH-A1* and *TtBH-B1* were also included in the list of markers for the construction of the linkage map. Fourteen linkage groups covering a total genetic distance of 2999.24 cM were created. The average distance between markers was estimated to be 7.04 cM which is sufficient to detect QTL. Distribution of the markers across the linkage group is shown in Table 2-7. The linkage map of all markers is shown in Figure 2-6.

Table 2-7. Distribution of SNP markers across the linkage groups

Linkage Group	Number of markers	Length (cM)
1A	23	157.96
1B	38	231
2A	26	208.65
2B	46	199.97
3A	14	141.93
3B	57	218.95
4A	21	338.01
4B	18	167.64
5A	28	202.5
5B	42	278.57
6A	27	200.83
6B	33	269.45
7A	33	215.75
7B	20	168.04
14	426	2999.24

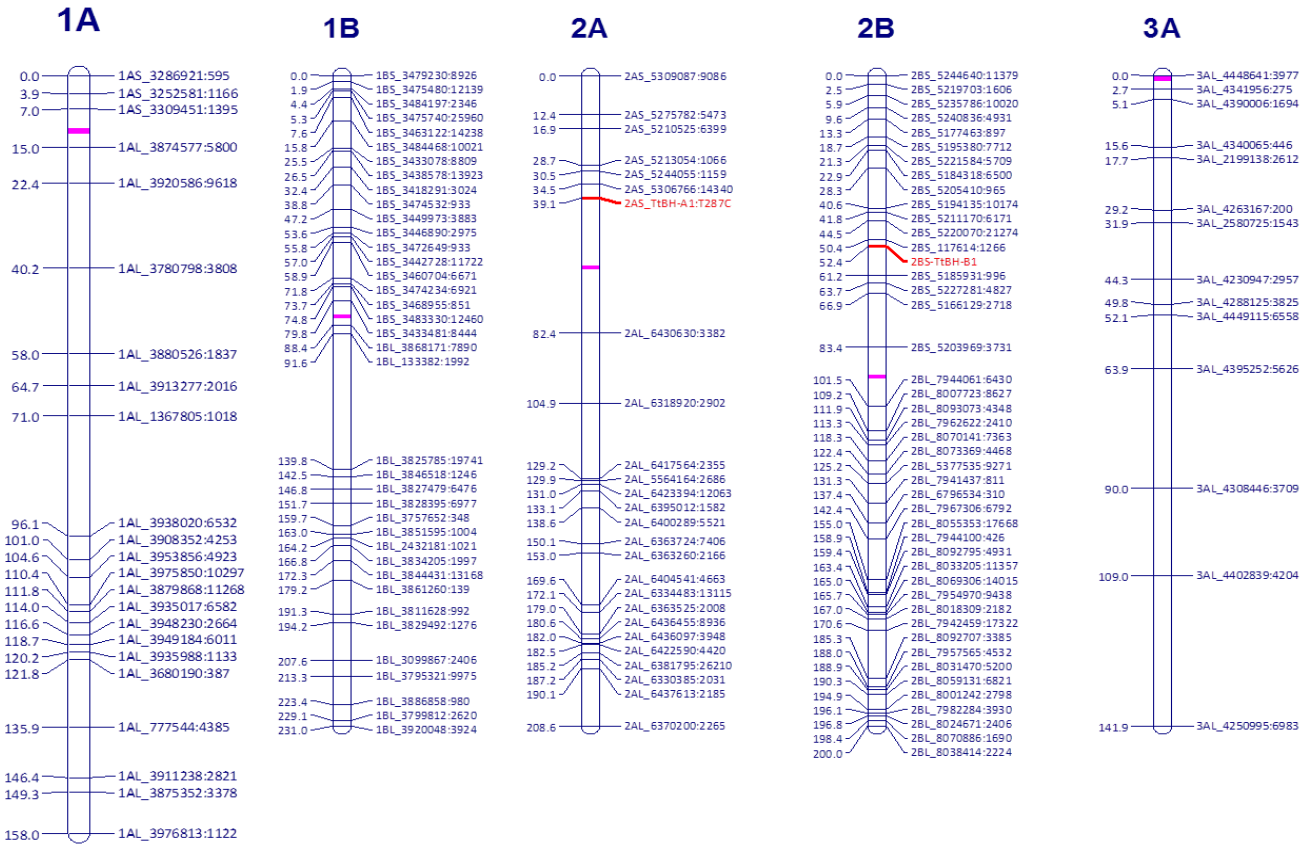


Figure 2-6. Genetic linkage map of markers across 14 linkage groups (chromosomes).
Purple line demarcates the short and long arm of each chromosome. Markers in red fonts on chromosome 2A and 2B are the CAPS marker derived from *TtBH-A1* and *TtBH-B1*, respectively.

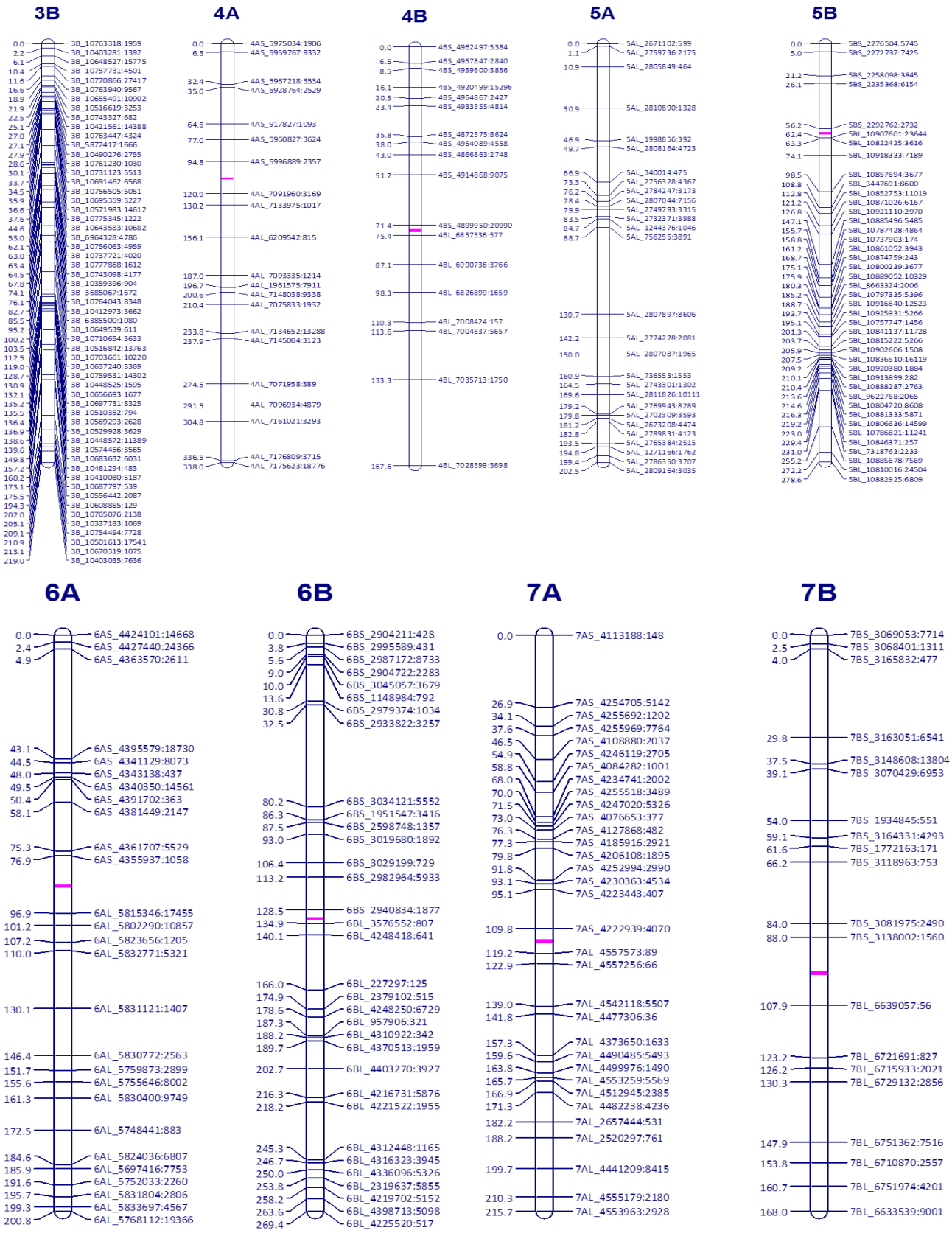


Figure 2-6 Continued…

To identify QTL associated with SS, addSPS from four environments was separately used for mapping following CIM algorithm. In total, three QTL were identified (Table 2-8 and Figure 2-7). Two of which were completely linked to the CAPS marker derived from *TtBH-A1* and *TtBH-B1* (Figure 2-7B). The QTL were named following the regulations of Gene Symbols for Wheat, adopted at the 12th International Wheat Genetics symposium (IWGS, 2013). Accordingly, the QTL identified on the short arm of chromosome 2A had the largest phenotypic effect and was named as *QSS.ipk-2AS* while the other QTL with a medium effect from the short arm of chromosome 2B was named as *QSS.ipk-2BS.* Similarly, the third QTL, which was identified on the short arm of chromosome 1A and had only a minor phenotypic effect, was named as *QSS.ipk-1AS*. Two software (windows QTL cartographer version 2.5 and Genstat version 17) were independently used for mapping (Wang S., 2012; Payne et al., 2014). However, results from both mapping software were identical. Therefore, results from Genstat are shown in Table 2-8 and Figure 2-7. The QTL, QTL confidence intervals, and phenotypic variations explained by each QTL are summarized in Table2- 8.

Table 2-8. Quantitative trait loci linked with supernumerary-spikelet in the RILs.

Trait	QTL	Position(cM)	−log10 (P-value)	Environment	Flanking markers	Position(cM)	PVE(%)	QTL Additive Effect	HVA
SS (addSPS)	*QSS.ipk-1AS*	3.9	4 - 5	IPK15,HAL15	1AS_3286921:595 - 1AS_3309451:1395	0 – 7	5 - 6	1.01 - 1.71	TRI 19165
	QSS.ipk-2AS	39.1	13 - 20	GH15, IPK14, HAL15,IPK15	2AS_5306766:14340 - *	34.5 - 44.76	27 - 32	2.17 - 4.11	TRI 19165
	QSS.ipk-2BS	52.4	11 - 17	GH15, IPK14, HAL15,IPK15	2BS_117614:1266 - 2BS_5185931:996	50.4 – 61.2	22 - 26	1.64 - 3.54	TRI 19165

PVE, Phenotypic Variance Explained; HVA, High-Value Allele; * indicates no flanking marker in the region. See Figure 2-7 for the details.

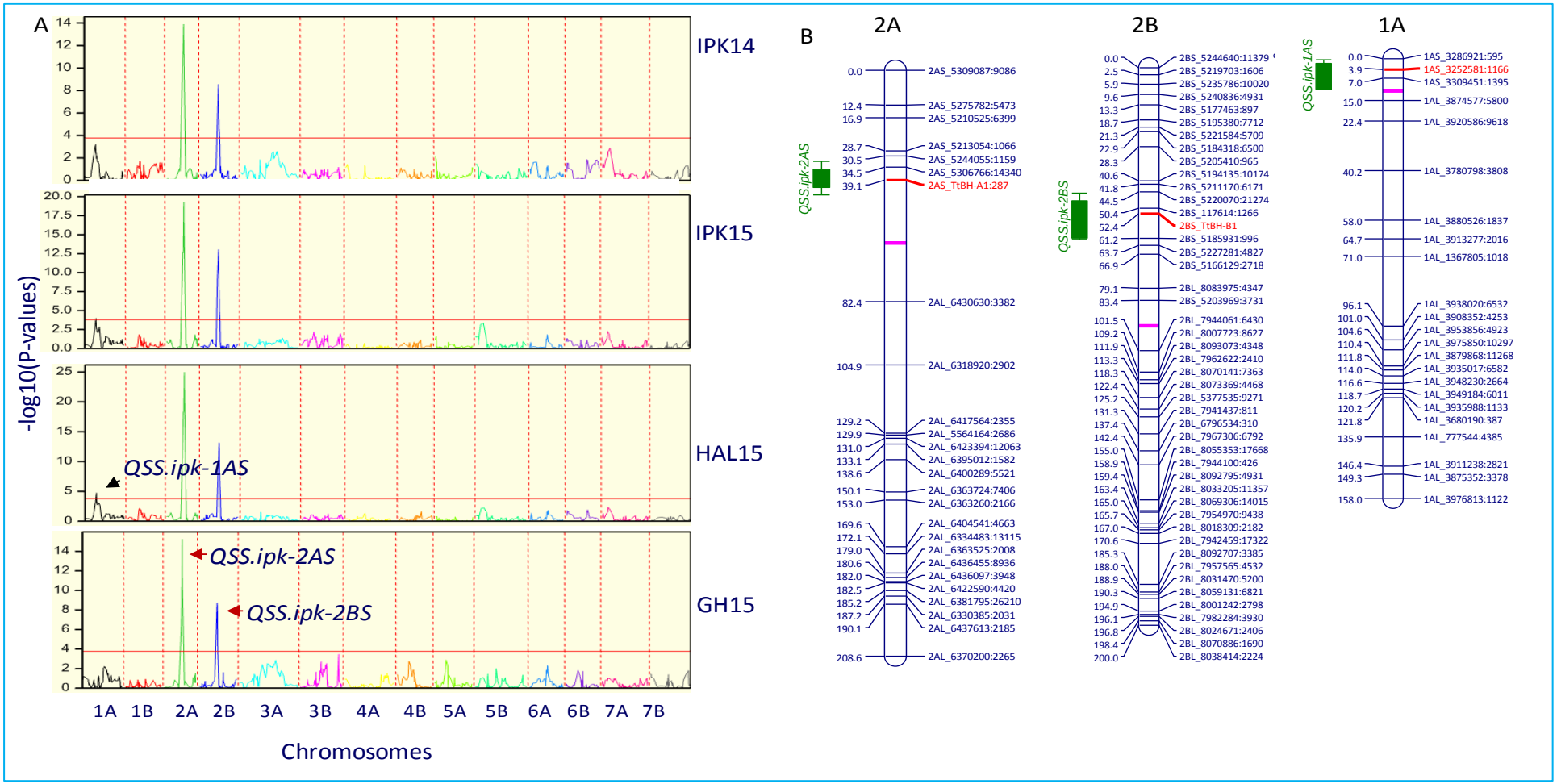


Figure 2-7 Chromosomal location of SS QTL detected from the RILs

(A) Independent detection of SS QTL across four different environments: GH15, IPK14, IPK15, and HAL15. Horizontal lines indicate threshold value which was determined automatically based on genome-wide significance level (alpha) at 0.05. (B) Chromosomal locations bearing SS QTL. Purple line demarcates the short and long arms (centromeric region) of each chromosome. CAPS markers derived from *TtBH-A1* and *TtBH-B1* are shown in red fonts on chromosome 2A and 2B, respectively.

2.4.4 *QSS.ipk-2BS* modifies spike branching

At the beginning of the project, it was not clear whether *TtBH-B1* influences the spike-branching phenotype. Thus, detailed analysis of the genotype versus phenotype was made to find any significant association. If we assume the A and B loci from Bellaroi as *AABB*, and from TRI19165 as *aabb*; and because the mapping population were an F2 derived F7/F8 mapping population, selfing of the F1 heterozygote plants (*AaBb*) result in nine groups of RILs (*AABB, AABb, AaBB, AaBb, Aabb,aaBB, aaBb, Aabb,* and *aabb* (Table 2-9)). The number of RILs comprising each of these nine groups and their corresponding phenotype is shown in Table 2-10.

Table 2-9.Genotype of F2 progenies after selfing the F1 heterozygous.

Gametes	*AB*	*Ab*	*aB*	*Ab*
AB	*AABB*	*AABb*	*AaBB*	*AaBb*
Ab	*AABb*	*Aabb*	*AaBb*	*Aabb*
aB	*AaBB*	*AaBb*	*aaBB*	*aaBb*
ab	*AaBb*	*Aabb*	*aaBb*	*Aabb*

Using the two CAPS markers derived from *TtBH-A1* and *TtBH-B1*, all RILs were genotyped and addSPS was compared among the 9 groups of RILS (Table 2-10).

Table 2-10. Phenotypic comparison of groups of RILS from three locations.

Group	No. of RILS	Spike morphology	AddSPS		
			IPK14	IPK15	HAL15
AABB	30	Non-branching	-	-	-
AAbb	24	Non-branching	-	-	-
AABb	4	Non-branching	-	-	-
AaBb	1	Non-branching	-	-	-
aabb	39	Medium to strongly branching	8.77 ±0.99	14.71 ±1.12	8.43 ± 0.59
aaBB	30	Weakly branching	0.95 ±0.55	1.95 ±0.42	1.6 ± 0.36
aaBb	9	Moderate branching	4.82 ±0.83	10.6 ±1.48	8.14 ± 0.74
AaBB	3	Very weak branching	0.03 ±0.03	0.25 ±0.25	0.33 ±0.33
Aabb	2	Moderate branching	3.35 ±1.3	8.75 ±2.1	5.41 ± 1.2

AddSPS, additional spikelets per spike, which was Mean ± SEM for each location

RILs which have combined both the A and B alleles from TRI 19165, i.e. *aabb*, carry more additional spikelets per spike (up to 15 spikelets per spike) as compared to those carrying only the A allele from TRI 19165 i.e. *aaBB*, which carry less than 2 addSPS (Table 2-10 and Table 2-6). This clearly suggests that: first, the role of allele B from Bellaroi as a suppressor of branching and secondly, it also suggests that the B allele from TRI 19165 might not be fully functional. Suppression effect of the B allele from Bellaroi even completely abolished SS formation in some of the RILs (Table 2-6). As some of these RILs can still occasionally produce SS (of course in a very low intensity), the B allele from Bellaroi did not completely regain the loss-of-function of allele A (from TRI 19165). This is also in a complete agreement with the QTL mapping result where *QSS.ipk-2BS* was found to be medium effect QTL as compared to *QSS.ipk-2AS* (Table 2-8). However, the A wt allele was sufficient to stop SS formation as all RILs carrying the A allele from Bellaroi (genotype: *AAbb*; n=24) did not show SS in all environments; reconfirming the mapping result where QSS.ipk-*2AS* was found to be the major effect QTL (Table 2-8). Therefore, *QSS.ipk-2BS* and *QSS.ipk-2AS* are most likely homoeoloci.

2.4.5 Effect of environment on the penetrance and expressivity of spike-branching

The phenotype of an offspring is not always the product of its genotypic constitution. It is a function of the genotype (G), environment (E) and GEI. Generally, it is described as: P = G + E + GEI. Besides the GEI, Epistatic (loci) interaction could also affect the expression of a QTL. The phenotypic response of a genotype to the environment can be expressed in a so-called 'Norm of reaction' (Griffiths et al., 2000b). GEI occurs when the slope of the 'Norm of reaction', changes across environments, i.e. when there exists change in the rank of the genotypes across different environments (El-Soda et al., 2014). To visualize this, evaluation of identical genotypes at least in two different environments is required. The 'Norm of reaction' for some of the RILs is shown in Figure 2-8. The Figure clearly shows a change in rank of some of the RILS due to phenotypic plasticity of SS formation in different environments. Generally, greenhouse grown plants exhibit reduced branching tendency (expressivity) as compared to field grown plants, which clearly indicates the effect of diverse environment on the expressivity of the phenotype. The effect of environment in the penetrance of SS, however, was less (Figure 2-8).

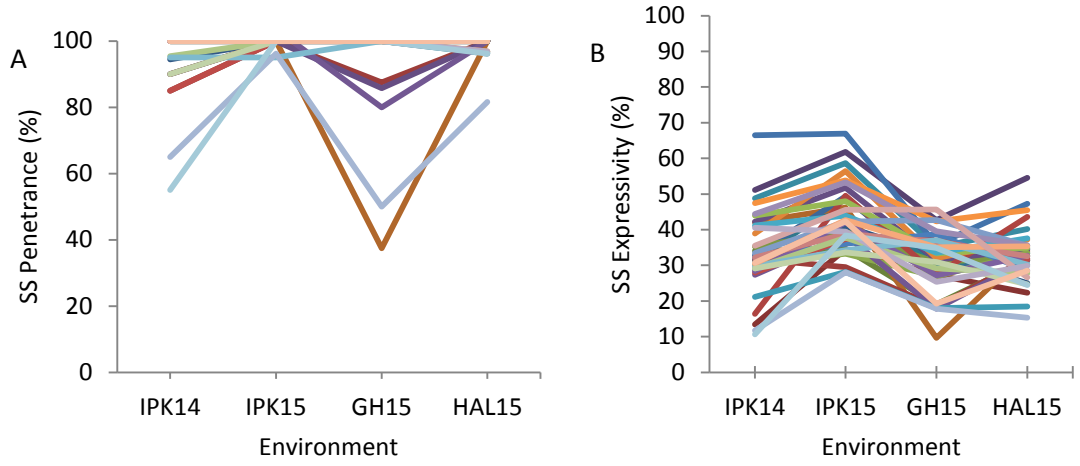


Figure 2-8. A 'Norm of Reaction' for the phenotypic penetrance and expressivity of SS.
(A) SS penetrance, (B) SS expressivity. Penetrance (%) measures the proportion plants per genotype carrying SS (calculated based on spikes collected from field grown plants) and expressivity (%) measures the proportion of additional spikelet per spike. For simplicity, only those RILs showing medium to strong branching were used to plot the graph across the four different environments.

In order to estimate the effect of environment on SS formation, addSPS from all four environments (including Greenhouse) were analyzed for single trait linkage analysis in multiple environments using GenStat 17. QTL by Environment interaction additive effects of the SS is shown in Table 2-11 and Figure 2-9.

Table 2-11. Effect of environment on phenotypic expressivity of supernumerary spikelet

QTL	Position(cM)	Trait	-LOG10(P-value)	Environment	QTLxE Effect	PVE(%)
QSS.ipk-2AS	39.1	addSPS	30.40	IPK14,IPK15,HAL15,GH15	2.2 - 4.2	25.5 - 35.7
QSS.ipk-2BS	52.4	addSPS	24.69	IPK14,IPK15,HAL15,GH15	1.6 - 3.5	15.2 - 20.7

addSPS, additional spikelet per spike; PVE, Phenotypic variation explained; QTLxE, QTL by Environment Interaction

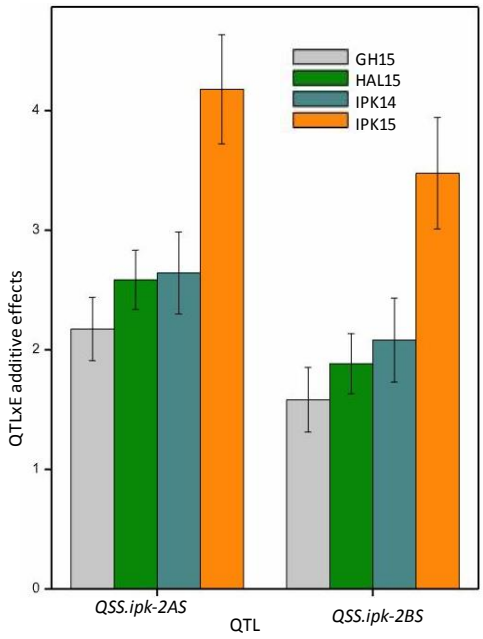


Figure 2-9. SS QTL-by-Environment interaction additive effects.

Although SS formation is affected by the environment (Table 2-11 and Figure 2-9), the genetic component plays the major role (h^2=0.76,Table 2-16). Although most of the genetic variations in quantitatively controlled traits are additive by nature (Mackay, 2014), loci interaction (epistasis) also affects such traits (Carlborg and Haley, 2004). Hence, genome-wide epistatic interaction, which is, of course, a non-linear interaction between loci, was analyzed for SS formation (Table 2-12 & Figure 2-10).

Table 2-12. Epistatic interactions and estimation of the additive-by-additive effect of SS QTL.
A positive value indicates that the parental (TRI 19165) two-locus genotypes have a positive effect and the recombinants had a negative effect. Although no QTL has been mapped on chromosome 5B, the specified interval was found to interact with *QSS.ipk-1AS* and *QSS.ipk-2AS* (Figure 2-10)

QTL Interaction	Additive by Additive (AA) effect	Heritability
QSS.ipk-1AS X QSS.ipk-2BS	0.817	0.018
*QSS.ipk-1AS X 5BS**	-0.522	0.005
QSS.ipk-2AS X QSS.ipk-2BS	1.66	0.062
*QSS.ipk-2AS X 5BS**	-0.606	0.003

5BS* Shows the interval on chromosome 5BS between marker 5BS_2235368:6154 and 5BS_2292762:2732.

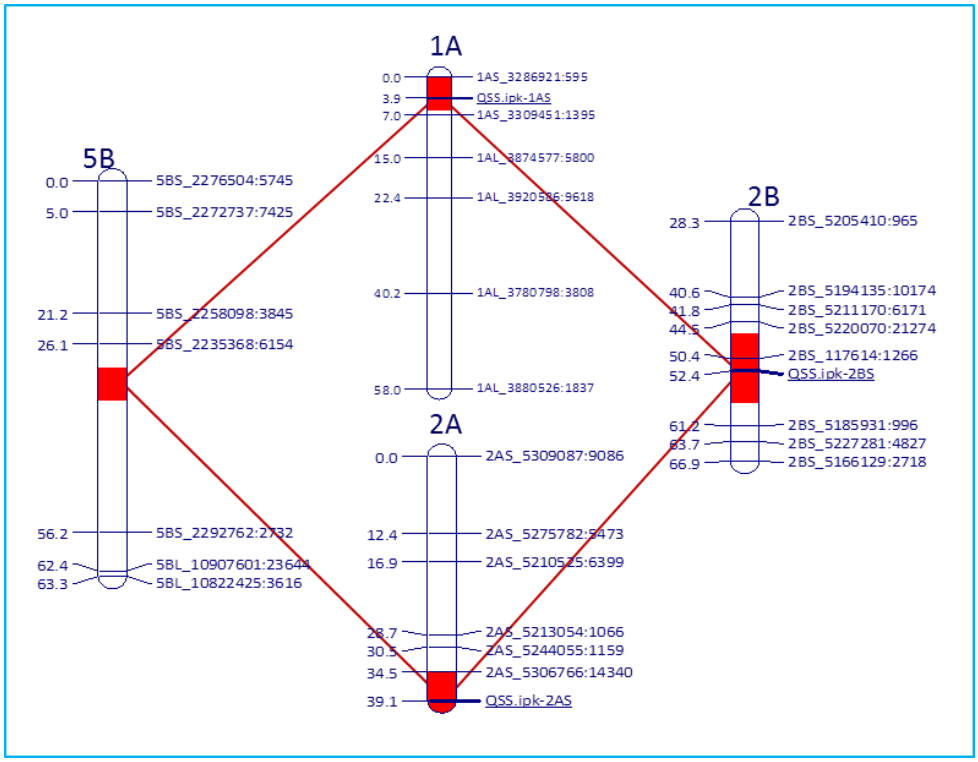
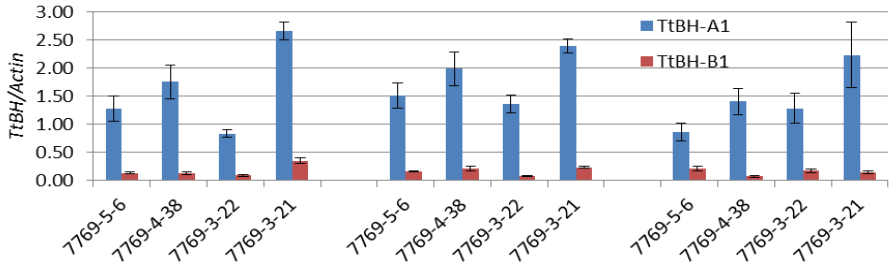


Figure 2-10. Predicted epistatic interaction network among SS QTL.
The red shaded region on chromosome 1A, 2A and 2B show the location of SS QTL (Underlined). The line connects a pair of the interacting loci. Only short chromosomal regions harboring SS QTLs are shown. For chromosome 5B, only the interval is shown as there was no SS QTL mapped to the region.

A positive value indicates that the parental two-locus have a positive effect, meaning that higher additive by additive (AA) effect when both loci come from TRI 19165; while the negative value indicates recombinants with reduced additive by additive effect (AA) due to the buffering effect of 5B locus from Bellaroi.

2.4.6 Measuring transcript levels of *TtBH-A1* and *TtBH-B1*

Transcripts of *TtBH-A1* and *TtBH-B1* were measured at three spike developmental stages. The first stage was at GP where glumes of the spikelets start to differentiate. This stage comes after double ridge and thus it is the earliest stage of spikelet differentiation. The second stage was FP where floral structures such as lemma and palea start to differentiate. The third stage was TS stage where spikelet differentiation stops. The result of the expression analysis is summarized in Figure 2-11.



RIL	GT	GP		FP		TS	
		TtBH-A1	*TtBH-B1*	*TtBH-A1*	*TtBH-B1*	*TtBH-A1*	*TtBH-B1*
RIL_7769_5_6	AABB	1.28 bc	0.14 bc	1.52	0.16 ab	0.86	0.21
RIL_7769_4_38	aaBB	1.75 b	0.13 b	1.99	0.22 a	1.41	0.07
RIL_7769_3_22	AAbb	0.83 c	0.09 c	1.36	0.08 b	1.28	0.17
RIL_7769_3_21	aabb	2.66 a	0.35 a	2.39	0.23 a	2.23	0.14

Figure 2-11.Relative expression of *TtBH-A1* and *TtBH-B1* at GP, FP and TS stages.
The analysis was performed using four selected RILs (7769_3_21; 7769_4_38; 7769_3_22 and 7769_5_6). Genotype of each of the four RILs was as follows: RIL_7769_3_21: *aabb*; RIL_7769_4_38: *aaBB*; RIL_7769_3_22: *AAbb* and RIL_7769_5_6: *AABB*. The relative expression values were mean ± SEM from three biological replicates. Ct values were normalized against the Ct values of the reference gene, *Actin*. Relative expression: $E^{-\Delta Ct}$, where E was the relative PCR efficiency was used to measure the relative expression values (found at the bottom) for both alleles. Letters indicate mean separation among RILs. Means values without letters are not significant among the four RILs. GT, Genotype.

The two parents, Bellaroi and TRI 19165, were not included in the expression analysis because of the difference in growth habit, i.e. Bellaroi is spring type while TRI 19165 is winter. Thus, the four RILs used for transcript analysis were selected based on allele combinations (see Table 2-4). Secondly, there was no control for the background recombinational events which might have direct or indirect effects on the expression of both alleles. Nevertheless, expression of *TtBH-A1* was found to be always significantly higher than *TtBH-B1* in all developmental stages and RILs; clearly indicating that *TtBH-A1* is the major homoeo-allele controlling spikelet meristem identity in tetraploid wheat followed by *TtBH-B1*. This is also in agreement with the result of QTL mapping where *QSS.ipk-2AS* was found to be the major effect QTL followed by *QSS.ipk-2BS* (Table 2-8)

2.4.7 Sequence analysis of *TtBH-B1*

2.4.7.1 Analysis of the upstream region of *TtBH-B1*

The 5'and 3' UTR play a major regulatory role in gene expression. Both regions are transcribed but not translated. The 5' UTR is located at 5' end of all protein-coding genes. It plays a major role in translation initiation (Barrett et al., 2012). Sequence analysis of about 1kb upstream of *TtBH-B1* Open Reading Frame (ORF) from seven different tetraploid wheat accessions/varieties revealed insertion of nested 'Miniature Inverted-repeat Transposable Elements' (MITEs) at the vicinity of

the *TtBH-B1* (Figure 2-12). An online database search revealed that the inserted MITEs are 'MITE: Tourist-2' and 'MITE: Islay Tourist'. MITEs are generally class 2 DNA-mediated nonautonomous transposable elements i.e. they do not have the gene encoding transposase, an enzyme which catalyzes the process of transposition(Feschotte et al., 2002). MITEs are usually less than 500 bp in length and exhibit conserved terminal inverted repeats (TIRs) in very high copy number (about 1,000 –15,000) in eukaryotic genomes (Kikuchi et al., 2003). TIRs are flanked by specific small direct target site duplication (TSD) that yield 2-bp or 3-bp (A + T) rich duplications on transposition (Bureau and Wessler, 1992; Kikuchi et al., 2003; Lu et al., 2012).

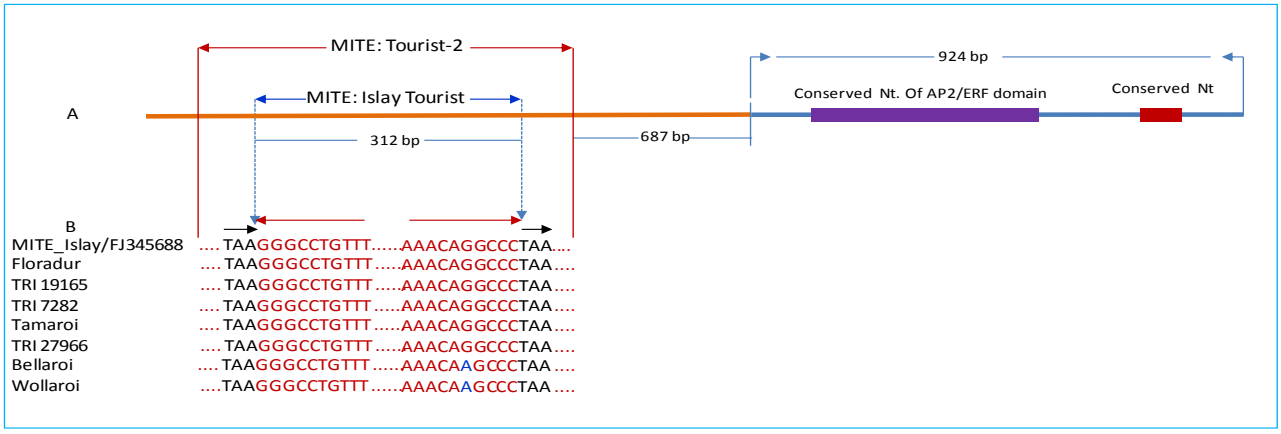


Figure 2-12. Identification of Miniature Inverted-repeat Transposable Element (MITE) in the upstream of *TtBH-B1* ORF. (A) Schematics of *TtBH-B1*. Blue bent arrows indicate the start and stop of the ORF. The orange bar indicates sequenced region (about 1092bP). The vertical red solid line indicates 'MITE: Tourist -2' of length 578 bP. The nested 'Islay Tourist' of size 312 bp is demarcated by the vertical broken blue lines. (B) Sequenced accessions and the identified TIR and the conserved terminal Tri-nucleotide direct repeats (TAA) from the nested 'Islay Tourist'. The TIRs and TSDs are indicated by red and black arrows respectively. The MITE in Bellaroi and Wollaroi carry SNP in the TIR. The dotted line indicates sequences that are not shown here. Complete sequence alignment is shown in Supplementary Figure 9-1 and Supplementary Figure 9-2. Nt, nucleotide, bp, base pair.

Emerging evidence show that MITE families might have spread recently, and some are still actively transposing throughout their host genome (Zhang et al., 2000; Yang et al., 2001; Feschotte et al., 2002; Jiang et al., 2003; Kikuchi et al., 2003; Naito et al., 2006). Because MITE insertions are preferentially located in the vicinity of genes; suggesting that MITEs play important roles in gene regulation and genome evolution. Furthermore, studies have also indicated that MITEs are involved in promoter enhancement and repression of several genes within 1-5 kb upstream of the transcription start site (TSS) (Yang et al., 2001; Yang et al., 2005; Naito et al., 2006; Naito et al., 2009). More interestingly, it has been reported that in the rice genome, MITEs act as an enhancer for cold-inducible nearby genes by providing new binding sites for transcription factors or other regulatory proteins (Naito et al., 2009). In this regards, further study is required to gain insights

into the effect of the MITES identified in this study as the AP2/ERF transcription factors are also widely involved in mediating stress-related responses and developmental programs (Mizoi et al., 2012; Licausi et al., 2013).

2.4.7.2 Analysis of the coding and 3' UTR

The slow expression of *TtBH-B1* might be due to the insertion of the MITE at the very close vicinity of the gene (Figure 2-12). To further analyze sequence variation, 49 different tetraploid wheat accessions/varieties collected from different regions of the world were sequenced for the coding sequence (CDS) including the 3'UTR. Earlier, these accessions/varieties were used to sequence the CDS of *TtBH-A1* (*bh^t-A1* allele) and the result revealed that only single SNP was found to be linked with the spike-branching phenotype. Details of these accessions/varieties can be found at Table S5 of Poursarebani et al. (Poursarebani et al., 2015). In short, 27 accessions were wild type (standard spike) and 22 accessions/varieties were branching carrying the *bh^t-A1* allele. The coding sequence analysis of *TtBH-B1* (*BH^t-B1* allele) from these accessions also revealed five different SNP where three of them were found to be non-synonymous substitutions. The non-synonymous substitutions occurred at position 491 (A491G), 619 (A619T), and 785 (T785C) leading to AA substitutions at position 164 (Y164C), 207 (F207I), and 262 (L262S), respectively (Figure 2-13). Also, see Supplementary Table 9-2 and Supplementary Figure 9-5.

Amino acid alignment of all homologous proteins from grasses showed that Y164C and L262S occurred in non-conserved regions, while F207I occurred in a conserved AA residue (Supplementary Figure 9-5). Furthermore, analysis of the 3' UTR region also revealed one SNP and a 3 bp insertion/deletion (indel) (Table 2-13 and Figure 2-13). The SNP was located at position 994 (G994A) while the indel was located at position 1145, 1146 and 1147. Taking all these structural polymorphisms, all 49 accessions/varieties were grouped into five different haplotype groups (Figure 2-13). These are Hap I-YIL-G-del, Hap II-YIL-G-in, Hap III-YIS-G-del, Hap IV-YIS-G-in, and Hap V-CFS-G-in.

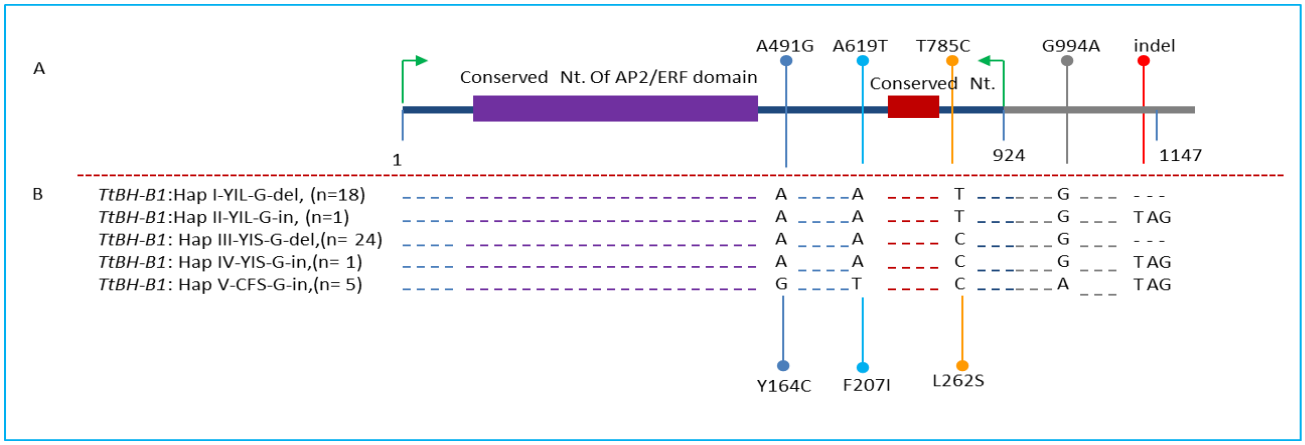


Figure 2-13. Haplotypes based on sequence analysis of the CDS and 3'UTR of *TtBH-B1*.

(A) Schematics of *TtBH-B1* ORF and 3' UTR. Green bend arrows demarcate the ORF. Purple and red boxes show conserved nucleotides for the AP2/ERF DNA-binding domain and the C-terminus conserved Nt residues respectively. The three nonsynonymous SNP are A491G (Y164C), A619T (F207I), and T785C (L262S). The two synonymous SNP are not shown here. See Supplementary Table 9-2. (B) The five haplotype groups from 49 different tetraploid wheat accessions/varieties. The dashed line indicates identical nucleotides. The number of accessions/varieties constituting each group is shown in parenthesis. Phenotype and frequency of each of the five haplotype groups are shown in Table 2-13.

Table 2-13. Association of spike morphology with *TtBH-B1* haplotype groups

Haplotype	SNP Position							No. of accessions in the haplotype			HF (%)	
	CDS			3' UTR				WT	Mt	Total	Wt	Mt
	491	619	785	994	1145	1146	1147					
Hap I-YIL-G-del	A	A	T	G	-	-	-	18	0	18	36.7	0.0
Hap II-YIL-G-in	A	A	T	G	T	A	G	1	0	1	2.0	0.0
Hap III-YIS-G-del	A	A	C	G	-	-	-	2	22	24	4.1	44.9
Hap IV-YIS-G-in	A	A	C	G	T	A	G	1	0	1	2.0	0.0
Hap V-CFS-G-in	G	T	C	A	T	A	G	5	0	5	10.2	0.0

Wt, Wilde type; Mt, mutant; CDS, Coding Sequences, HF, Haplotype Frequency.

All the branching accessions (44.9%) were grouped in HapIII-YIS-G-del. Only 4 % of the accession/varieties in this haplotype group show standard spike form; clearly linking Hap III-YIS-G-del with the mutant phenotype (Table 2-13). Most of the wild type accessions (36.7%) were grouped in Hap I-YIL-G-del. No single spike-branching accessions/varieties were grouped in this haplotype group. Interestingly, T785C (L262S) separates these two contrasting haplotypes suggesting that besides the mutation in *bh^{t}-A1*, (L96P), T785C (L262S) from the *TtBH-B1* allele might have contributed for the strong branching phenotype in most of these mutant accessions. Therefore, besides the slow expression of *TtBH-B1*, the three missense mutations, especially T785C (L262S), might have also affected the function of *TtBH-B1* at the post-transcriptional level.

TRI 19165 was grouped in Hap III-YIS-G-del while Bellaroi was grouped in Hap V-CFS-G-in, where two other modern varieties, Wollaroi and Kalka, were also grouped. Floradur was used as a recurrent parent for the development of *bh* Near Isogenic Lines (FL-*bh*-NILs) (Chapter 3).

2.4.8 Effects of *QSS.ipk-2A* and *QSS.ipk-2B* on other spike related traits

Besides spike morphology, other spike-related traits were also studied in the mapping population. Hence, multiple traits linkage analysis was performed to investigate the direct or indirect interaction (pleiotropic) effects of *QSS.ipk-2A* and *QSS.ipk-2B* on other spike related traits. The result from multiple traits analysis is summarized in Table 2-14.

Table 2-14. Effect of the *QSS.ipk-2A* and *QSS.ipk-2B* on other spike-related traits.

QTL	Position (cM)	−log10 (P-value)	Trait Affected	Environment	Effect	PVE (%)	HVA
QSS.ipk-2AS	39.1	16 - 34	GPS	IPK14, IPK15, HAL15	0.41 - 0.53	16.9 - 27.6	Bellaroi
			ND	IPK14, IPK15, HAL15	0.18 - 0.21	2.7 - 3.2	TRI 19165
			NPS	IPK14, IPK15, HAL15	0.20 - 0.37	6.2 -13.5	TRI 19165
			TKW	IPK14, IPK15, HAL15	0.20 - 0.40	3.9 - 15.7	Bellaroi
			GA	IPK14, HAL15	0.15 - 0.31	2.3 - 9.9	Bellaroi
			GL	IPK15, HAL15	0.18 - 0.31	3.2 - 9.7	Bellaroi
			SL	HAL15	0.16	2.6	TRI 19165
			GW	HAL15	0.17	3	Bellaroi
			SHI	HAL15	0.18	3.2	Bellaroi
QSS.ipk-2BS	52.4	6 - 17	GPS	IPK14, IPK15, HAL15	0.24 - 0.34	5.9 - 11.5	Bellaroi
			ND	IPK14	0.17	2.7	TRI 19165
			GW	IPK15	0.18	3.2	Bellaroi

NPS, Node number per spike; SL, spike length; GPS, grain number per spikelet; TKW, thousand kernel weight; SHI, spike harvest Index; GL, Grain Length; GA, Grain Area; GW, Grain width; SL, spike length; PVE, phenotypic variance explained; HVA, High value allele

As shown in Table 2-14, *QSS.ipk-2AS* and *QSS.ipk-2BS* affect spikelet fertility i.e. GPS in all environments. Bellaroi contributed the HVA having an additive genetic effect ranging from 0.41-0.53 GPS. This suggests that although spike-branching leads to addSPS, the *bh* locus is associated with lower spikelet fertility i.e. lower grain number per spikelet. Four groups of RILs were compared for GPS as well as GNS (Figure 2-14 A&B). Medium to strongly branching RILs (genotype: *aabb*) always show poor GPS as compared to the wild-type (genotype: *AABB*) (Figure 2-14 A). The low spikelet fertility was significantly reduced in RILs showing less branching (genotype: *aaBB*). This is clearly due to the effect of the B allele from Bellaroi (Table 2-14). The mean GNS was found to be non-significant between strongly branching and the non-branching group (Figure 2-14 B). This might be due to severe spikelet infertility in the branching group. However, some strongly branching RILs do carry higher GNS (Figure 2-15). For example, some RILs carry more than 60 grain per spike while Bellaroi had a maximum of 40 grains per spike. However, Bellaroi had more GPS (ranging from 2.31 to 2.73 grains) while strongly branching RILs on average carry 1.5 GPS. Furthermore, spikelet infertility was more in spike-branching accessions (used as a check in this study) which usually carry one grain per spikelet; suggesting the higher rate of spikelet infertility associated with spike-branching.

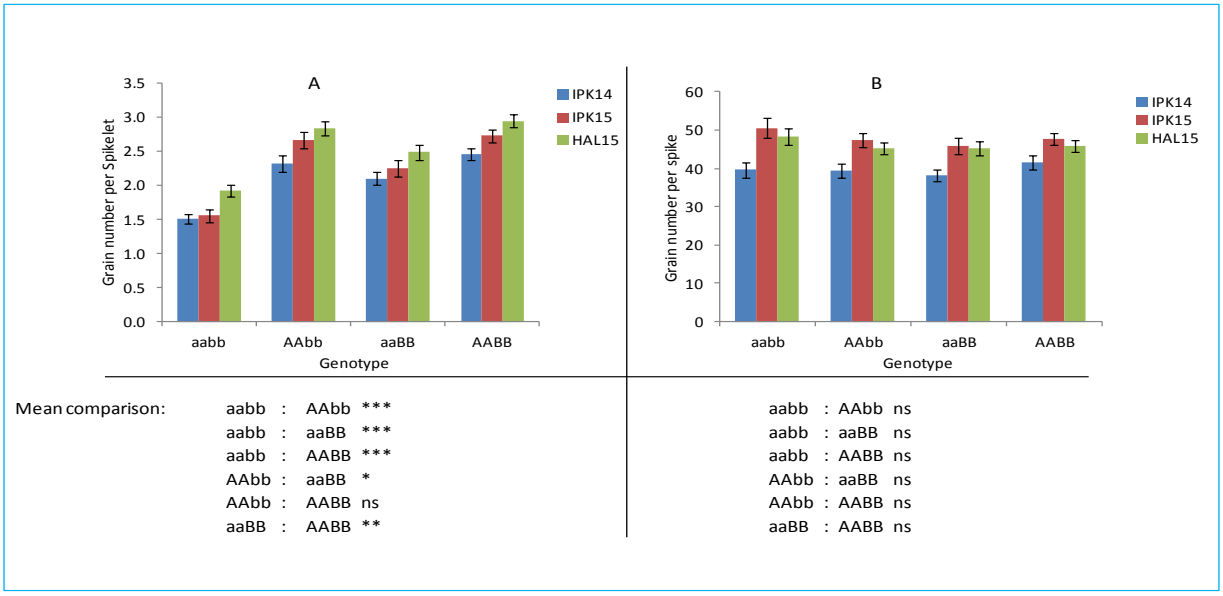


Figure 2-14. Comparison of groups of RILs for spikelet fertility and Grain number per spike.
(A) GPS, (B) GNS. RILs with genotype *aabb* show medium to strong branching (n=39), *aaBB* show weak branching (n=30), *AABB* (n= 30) and *AAbb* (n=24) do not show any branching (wild type). Error bars are mean ± SEM. The mean comparison was made using unpaired two-tailed t-test at p-value 0.05,*; 0.01, **; and 0.001, ***. ns, non-significant.

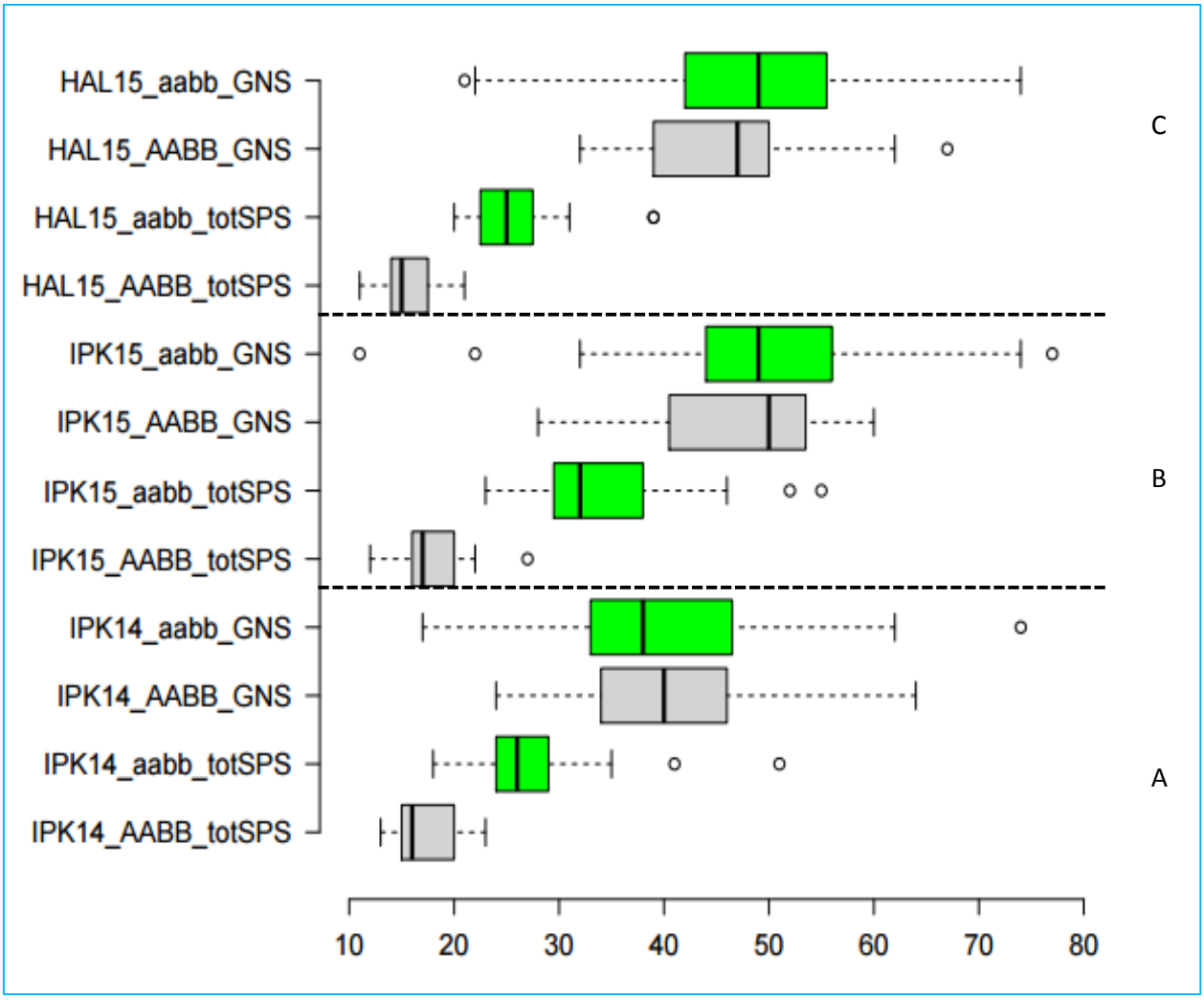


Figure 2-15. Box plot analysis for totSPS and GNS in a strongly branching and non-branching RILs.
Total spikelet per spike and GNS were compared between groups of non-branching RILs (AABB, n=30) and medium to strongly branching RILs (aabb, n=39) in three different environments, IPK14 (A), IPK15 (B), and HAL15(C). Although medium to strongly branching RILs (Green box plot) do carry a higher number of totSPS in all locations and years, GNS depends on the location and year, suggesting GEI effect. The line in the box shows the median and small circles are outliers.

Spike Harvest Index (SHI), which was derived from grain weight and spike dry weight per spike at harvest, was also used as an indicator for the overall spike fertility index. Although not detected in all environments, the *QSS.ipk-2AS* was also found to affect SHI and the HVA came from Bellaroi. Similarly, grain related traits such as TKW also affected by *QSS.ipk-2AS*. Spike length (SL) and Node number per spike (NPS) are also important spike morphological traits affected by *QSS.ipk-2AS* (Table 2-14).

2.4.9 Phenotypic variations of other traits in the mapping population

Bellaroi and TRI 19165 largely differ in several traits (Table 2-1). In total, fifteen traits were studied for two years in three different environments (IPK14, IPK15, and HAL15). Summary of the phenotypic variations between Bellaroi, the three spring type spike-branching mutant lines as well as RILs is shown in Table 2-15. Analysis of the variance components is also shown in Table 2-16. Besides addSPS and totSPS; the mapping population showed enormous variation for GPS, GNS, SL, NPS, ND, SDW at harvest (SDW_H), SHI, HD, PH and grain related traits. The estimated genetic coefficient of variation and narrow sense heritability of these traits is shown in Table 2-16. The genetic coefficient of variation (GCV) ranged from 4.15% for grain width to 138.47 % for SS. Heritability of the traits also ranged from medium for SDW_H (43%) to high for plant height (91%). One of the key agronomic traits for grain yield in wheat is the size of the spike. The SL of Bellaroi ranged from 6.54 to 6.98 cm. Branched head spring wheat accessions (TRI3261, TRI5283, TRI9652), which have similar spike morphology with TRI 19165, had longer spike length ranging from 7.72 cm to 8.64 cm. The RILs showed transgressive segregation of SL ranging from 4.8 cm to 10.92 cm (Table 2-15). GCV and narrow sense heritability (h^2) for SL were 12.67 and 84% respectively. Similarly, the parents and RILs also showed variation in NPS, ND, and SHI. NPS, which is literally the same as spikelet number per spike in a standard wheat spike, also showed high heritability (83%). Although increasing sink size in wheat remained to be one of the targets in wheat breeding, both SDW_H and SHI had low heritability (Table 2-15). Even if branching RILs depict increased sink (spike) size, SHI from these lines was nearly similar with Bellaroi or non-branching RILs; suggesting that the accumulated biomass was not efficiently converted to grain mass. However, the RILs showed transgressive segregation of SDW_H highlighting the possibilities of genomic selection for a better grain yield (Table 2-15). The other traits studied in the mapping population were grain quality related traits such as grain TKW, GL, GW, and GA. The mapping population segregates for these grain related traits as well. TKW from strongly branching RILs was relatively poor as compared to Bellaroi suggesting the effect of spike-branching on grain quality metrics (Table 2-14). This might suggest a direct/ indirect effect of the spike-branching on grain development. Possibly, it might be due to competition for assimilate among spikelets/florets. Heading time was also the other major traits segregating in the population. As the temperature within the growing season can affect growth and development of plants, heading time was converted to growing degree days (GDD), which is based on actual heat accumulation during the growing period (Miller et al., 2001). GDD was calculated by summing the average daily maximum

and minimum temperatures. Thus, average GDD or heading date for Bellaroi ranged from 733 to 839 ^{o}C days (or 68 to 79 days). The RILs also showed a higher degree of variability in heading date (Table 2-15 and Table 2-16). The mapping population also segregates for plant height. Due to *Rht-B1b* (reduced height) allele, Bellaroi is a semi-dwarf ('short') wheat variety (average plant height ranging from 62 cm in Halle to 79 cm in Gatersleben) while TRI 19165 is a traditional tall wheat landrace.

Table 2-15. Phenotypic comparison of the true parent Bellaroi, spring type *bh* mutant checks, and RILs across different environments.

Trait	Environment	Spring Parent	Spring mutant parental type as a check	RILs(n=146)				
		Bellaroi	(n=3)	Min	Max	Mean	SD	CV(%)
Growing Degre Days (GDD)	IPK14	839	955	816	1175	955	90.81	9.51
	IPK15	801	898	749	1106	906	76.33	8.43
	HAL15	733	871	690	1196	868	110.5	12.72
Plant height (cm) (PH)	IPK14	79	150	63	161	113	30.22	26.77
	IPK15	72	137	56	141	99	23.58	23.80
	HAL15	62	112	51	120	85	20.38	23.90
Spike Length (cm) (SL)	IPK14	6.94	7.78	4.80	9.73	6.84	0.81	11.83
	IPK15	6.98	8.64	5.12	10.92	7.89	1.11	14.12
	HAL15	5.54	7.72	4.58	9.21	6.71	0.95	14.21
Node number per spike (NPS)	IPK14	13.75	19.46	12.60	23.85	17.66	2.26	12.81
	IPK15	14.90	20.73	12.20	22.97	18.47	2.14	11.57
	HAL15	12.74	18.63	11.48	22.12	16.60	2.13	12.84
Spike Dry Weight (gm) (SDW_H)	IPK14	2.32	2.37	1.38	3.94	2.48	0.49	19.64
	IPK15	2.89	4.20	1.42	4.97	3.50	0.62	17.61
	HAL15	2.33	2.60	1.29	4.00	2.83	0.46	16.37
Additional Spikelet/Spike (addSPS)	IPK14	-	32.92	0.00	34.15	7.51	5.66	75.40
	IPK15	-	50.83	0.00	35.12	10.40	7.74	74.40
	HAL15	-	35.15	0.00	21.58	6.39	4.07	63.64
Total Spikelet/Spike (totSPS)	IPK14	13.75	52.37	12.60	51.35	21.02	6.06	28.81
	IPK15	14.90	71.57	12.20	54.52	23.82	8.41	35.29
	HAL15	12.74	53.78	11.48	39.46	19.78	5.36	27.08
Grain number/Spike (GNS)	IPK14	32	47	17.10	73.65	39.77	9.52	23.92
	IPK15	40.40	66.92	10.92	84.90	48.50	11.69	24.09
	HAL15	35	56	13.08	74.07	46.52	9.59	20.61
Grain number/Spikelet (GPS)	IPK14	2.31	0.90	0.66	3.48	2.04	0.61	29.76
	IPK15	2.71	0.94	0.36	4.02	2.19	0.76	34.83
	HAL15	2.73	1.06	0.68	4.02	2.48	0.66	26.77

CV describes the amount of variability relative to the mean and calculated by dividing SD to the Mean and x100

Table 2-15 continued ...

Trait	Environment	Spring Parent	Spring parental types as a check	RILs(n=146)				
		Bellaroi	(n=3)	Min	Max	Mean	SD	CV(%)
Spike Harvest Index (SHI)	IPK14	0.69	0.70	0.40	0.87	0.66	0.07	10.25
	IPK15	0.69	0.69	0.28	0.76	0.62	0.09	14.02
	HAL15	0.71	0.71	0.38	0.77	0.67	0.06	8.77
Thousand Grain Weight (TKW)	IPK14	50.55	35.25	25.86	57.54	41.83	5.60	13.39
	IPK15	49.55	43.43	31.23	56.29	45.28	4.78	10.56
	HAL15	47.68	35.99	26.44	52.83	41.49	4.97	11.99
Grain Area (mm^2) (GA)	IPK14	19.40	15.23	14.20	21.70	17.91	1.47	8.20
	IPK15	18.75	16.53	14.70	21.50	18.20	1.19	6.54
	HAL15	18.10	15.00	13.55	20.30	16.79	1.24	7.37
Grain Width (mm) (GW)	IPK14	3.30	3.23	2.85	3.75	3.31	0.18	5.47
	IPK15	3.20	3.23	2.80	3.60	3.27	0.13	4.09
	HAL15	3.10	3.10	2.65	3.50	3.14	0.15	4.92
Grain Length (mm) (GL)	IPK14	7.20	5.82	5.80	7.50	6.64	0.34	5.07
	IPK15	7.25	6.18	6.05	7.65	6.81	0.31	4.59
	HAL15	7.10	5.90	5.60	7.45	6.53	0.32	4.85
Node Density (ND)	IPK14	1.98	2.50	1.75	4.04	2.61	0.37	14.18
	IPK15	2.13	2.40	1.60	3.38	2.38	0.35	14.58
	HAL15	2.30	2.41	1.71	3.44	2.50	0.36	14.22

CV describes the amount of variability relative to the mean and calculated by dividing SD to the Mean and x100

Table 2-16. Mean Squares, Genotypic Coefficient of Variation (GCV), and narrow sense heritability (h^2) of traits studied in the mapping population.
Data were combined from three different environments: IPK14, IPK15, HAL15

Trait	Genotype	Environement	GEI	Residual	GCV (%)	h^2(%)
GDD	47554 **	565496 **	2054 **	209.40	9.58	87
PH	3630 **	59298 **	90.73 **	25.80	24.53	91
SL	5.15 **	119.15 **	0.24 **	0.08	12.67	84
NPS	25.65 **	259.22 **	1.23 **	0.49	11.48	83
ND	0.68 **	3.76 **	0.04 **	0.02	13.00	78
addSPS	183.91 **	401.26 **	13.38 **	4.234	138.47	76
totSPS	239.4 **	1237.48 **	15.28 **	5.64	28.45	78
SDW_H	1.14 **	79.33 **	0.26 **	0.13	13.06	43
SHI	0.02 **	0.24 **	0.004 **	0.020	8.79	53
GNS	481.57 **	6262.12 **	73.21 **	33.74	18.37	56
GPS	2.38 **	14.14 **	0.18 **	0.08	26.93	74
TKW	116.3 **	1289.09 **	20.01 **	6.09	9.34	55
GA	8.38 **	161.37 **	0.87 **	0.33	6.34	68
GL	0.54 **	5.44 **	0.04 **	0.02	4.30	73
GW	0.12 **	2.41 **	0.01 **	0.01	4.15	66

GEI, Genotype-By-Environment Interaction; GCV, Genetic Coefficient of variation; h^2 narrow sense heritability

2.4.10 Multiple trait correlation analysis and mapping

Traits are often correlated. Two factors are believed to be the cause for traits correlation (Lynch and Walsh, 1998). The first one is environmental factors which can modify the expression of different traits in the same or opposite direction. This kind of correlation is termed as environmental correlation. The second factor for trait correlation is due to a genetic factor which is termed as genetic correlation. This will arise when a single gene controls multiple traits which could arise due to the complex nature of biochemical, developmental and regulatory pathways in a biological system. This phenomenon is commonly known as pleiotropy (Lynch and Walsh, 1998). Hence, understanding the relative contribution of environmental and genetic factors of phenotypic correlations is one of the aims in quantitative genetics.

So one can ask: whether there exists pleiotropic relationship among multiple traits? And also whether there exists GEI? Unlike single trait analysis, multiple trait analysis takes into account trait correlation structure for improved statistical power for QTL detection and parameter estimation (Jiang and Zeng, 1995). In this study, first, pair-wise Pearson's correlation coefficient was determined among different traits (Table 2-17). Then, multiple trait analysis (GenStat software version 17) was performed to better understand the genetic correlations among several traits (Table 2-18).

Table 2-17. Pearson's correlation coefficients among traits.
Green shaded boxes show positive correlation while the orange shaded boxes show a negative correlation. Unshaded box shows no significant correlation between the traits. *, ** correlations are significant at 0.05 and 0.01 respectively.

Trait	NPS	addSPS	totSPS	GDD	PH	SL	SDW_H	TKW	GA	GW	GL	GNS	GPS	SHI	ND
NPS	1														
addSPS	.246**	1													
totSPS	.538**	.949**	1												
GDD	.563**	-.078*	.114**	1											
PH	.280**	-.013	.079*	.256**	1										
SL	.485**	.128**	.268**	.266**	.141**	1									
SDW_H	.303**	.129**	.210**	.068*	-.136**	.381**	1								
TKW	-.115**	-.274**	-.276**	-.164**	.099**	.153**	.337**	1							
GA	-.006	-.210**	-.185**	-.025	.139**	.183**	.152**	.819**	1						
GW	.116**	-.106**	-.054	.043	.369**	.033	.069*	.643**	.777**	1					
GL	-.119**	-.212**	-.223**	-.119**	-.090**	.228**	.147**	.681**	.834**	.342**	1				
GNS	.199**	.207**	.244**	.026	-.159**	.148**	.766**	-.159**	-.384**	-.353**	-.274**	1			
GPS	-.386**	-.591**	-.639**	-.122**	-.226**	-.153**	.400**	.112**	-.118**	-.224**	.008	.535**	1		
SHI	-.310**	-.208**	-.281**	-.240**	.101**	-.269**	.012	.115**	-.216**	-.160**	-.134**	.384**	.509**	1	
ND	.377**	.095**	.205**	.206**	.086*	-.613**	-.119**	-.264**	-.204**	.065	-.351**	.035	-.181**	.009	1

GDD, Growing Degree Days; ND, Node Density; NPS,Node Per Spike; PH, Plant Height; SDW_H, Spike Dry Weight at harvest; SHI, Spike Harvest Index;GA, Grain Area;GL, Grain Length; GW, Grain Width; GPS, Grain per Spikelet; GNS, Grain number per spike; SL, Spike Length; TKW, Thousands Kernel Weight; addSPS, Additional Spikelet per Spike; and totSPS, total spikelet per spike

Grain number per spike is one of the key factors determining grain yield in wheat. It depends on spikelet number per spike and GPS. GPS was negatively correlated with addSPS and totSPS; clearly showing the negative effect of branching on GPS (Table 2-17). However, it is positively correlated with SDW_H and SHI. Furthermore, grain metrics such as TKW, GA, GL were negatively affected by spike-branching. Contrary to this, GNS was positively correlated with GPS, addSPS, totSPS, NPS, SL and SDW_H. But negatively correlated with PH and grain related traits. This shows that even though the added spikelets contribute to GNS, the negative correlation of GPS and grain related traits with addSPS can pose a challenge for yield improvement.

In the current mapping population, GPS was mainly affected by the homoeoloci: *QSS.ipk-2AS* and *QSS.ipk-2BS* (Table 2-14). The effect was possibly due to competition as a result of branching. Although GPS and GNS are positively correlated, GNS was not directly linked to these loci. However, mapping identified a QTL for GNS on chromosome 4AL (Table 2-18). UP to 13 % of the phenotypic variance for GNS was explained by *QGNS.ipk-4AL.* Besides controlling GNS, *QGNS.ipk-4AL* was found to affect SDW_H, SL, NPS, GPS, SHI, and grain related traits. Allele from Bellaroi contributed for GNS, GPS, SHI and SDW_H, while TRI 19165 contributed to SL, NPS, TKW, GW, and GA. Based on the annotated POPSEQ contigs, several sugar and hormone, especially auxin, related genes were located in the proximal region (within 1cM) of *QGNS.ipk-4AL* (data not shown). Hence, sugar and/or hormone metabolic gene could be the most likely candidate gene for this QTL. Previously from hexaploid wheat, a sucrose transporter gene *TaSUT1* has been identified on chromosome 4 (Aoki et al., 2002). However; it was not known whether this gene, in fact, resides on the long arm of4A. Nevertheless, *TaSUT1* genes were found to express in developing grains showing the importance of *TaSUT1* during grain development and most likely during grain filling stages. This would increase the confidence that the gene underlying *QGNS.ipk-4AL* is most likely related to sugar metabolism.

Node density is the other important traits affecting spike architecture. In this study, ND was calculated from NPS and SL. So these three traits are genetically correlated. ND was found to be negatively correlated with SL, GPS, and grain related traits; and positively correlated with NPS, totSPS, and HD (Table 2-17). Furthermore, SL was found to positively correlate with NPS, totSPS, GNS, SDW_H and grain related traits; and negatively correlate with ND, GPS, and SHI. NPS was also found to positively correlate with addSPS, totSPS, HD, PH, SL, GNS, and SDW_H; and negatively correlate with GPS, SHI, and grain related traits. Major effect QTL for ND was identified on chromosome 5AL (Table 2-18). The QTL, *QND.ipk-5AL*, was located on chromosome 5AL which is

known to harbor the *Q* gene (Simons et al., 2006). Up to 32 % of the phenotypic variance was explained by this locus. *QND.ipk-5AL* was also found to control HD, PH, SL, NPS, GNS, GPS, and SHI. Bellaroi contributed HVA for ND, HD, and NPS. Allele from TRI 19165 contributed to the SL.

In wheat, grain size and shape are grain quality related traits controlled by distinct genes (Gegas et al., 2010). Nevertheless, both are positively correlated. Hence, increasing in grain size and shape (grain Length, grain width, and grain area or thickness) can directly improve the overall grain weight in wheat, which is a major target in wheat breeding (Su et al., 2011).

Grains from TRI 19165 are usually smaller in size (length) and more or less uniform in shape (this study, and (Rawson and Ruwali, 1972b)). The grains from Bellaroi are standard in shape and size. Thus, the mapping population clearly segregates for grain size and shape as they do for other traits. TKW was found to be positively correlated with GA, GL, GW as well as SDW_H; and negatively correlated with addSPS, totSPS, GNS, ND and HD (Table 2-17). Although numerous quantitative trait loci (QTL) associated with grain size and shape have been identified in wheat (Gegas et al., 2010; Okamoto et al., 2013; Wu et al., 2015; Zhang et al., 2015), only a few genes were identified. The grain size genes, *TaCYP78A5* and *TaGS5*, on chromosome group 2 and 3 (Ma et al., 2016a; Ma et al., 2016b) and grain width gene, *TaGW2,* on chromosome group 6 (Su et al., 2011; Qin et al., 2014) can be mentioned in this regard. In the current mapping population, several loci were identified for grain length, grain width, and grain area (Table 2-18). However, most of them were not consistent across environments; indicating that grain related QTLs are highly sensitive to environmental factors.

The other major plant architectural trait of importance was a plant height. Bellaroi is a semi-dwarf variety carrying *Rht-B1b* while TRI 19165 is a traditional tall wheat landrace. Hence, the mapping population segregates for plant height. PH was found to be positively correlated with NPS, HD, SL, GA, and GW; and negatively correlated with GPS, GNS, and SDW_H (Table 2-17).

Wheat varieties carrying semi-dwarfing or Reduced height (Rht) genes partition more dry matter to spike as compared to their tall counterpart, resulting in increased GPS as well as GNS (Brooking and Kirby, 1981; Youssefian et al., 1992; Miralles et al., 1998). Mapping of plant height identified the major plant height QTL, *QPH.ipk-4BS*, on chromosome 4BS which is known to harbor, *Rht-B1b*. *QPH.ipk-4BS* controls up to 91% of the phenotypic variance in PH in the mapping population. Besides controlling plant height, *QPH.ipk-4BS* also found to control GL, NPS, GPS, SHI, GW, and totSPS (Table 2-18).

As Bellaroi carries the reduced height allele i.e. *Rht-B1b*, comparison was made between RILs carrying *Rht-B1b* (reduced height) and their tall counterpart, *Rht-B1a*, in order to analyze the effect of the reduced height gene on spikelet fertility and grain number per spike (Figure 2-16). In total, 39 RILs (17 carrying *Rht-B1b* allele or reduced plant height, and remaining 22 carrying the corresponding allele, *Rht-B1a*, or tall plant) were selected. As to the spike form, all the 39 RILs carry both alleles, i.e. bh^{t}-*A1* and bh^{t}-*B1,* from TRI 19165. Hence, all of them show medium to strong spike-branching phenotype.

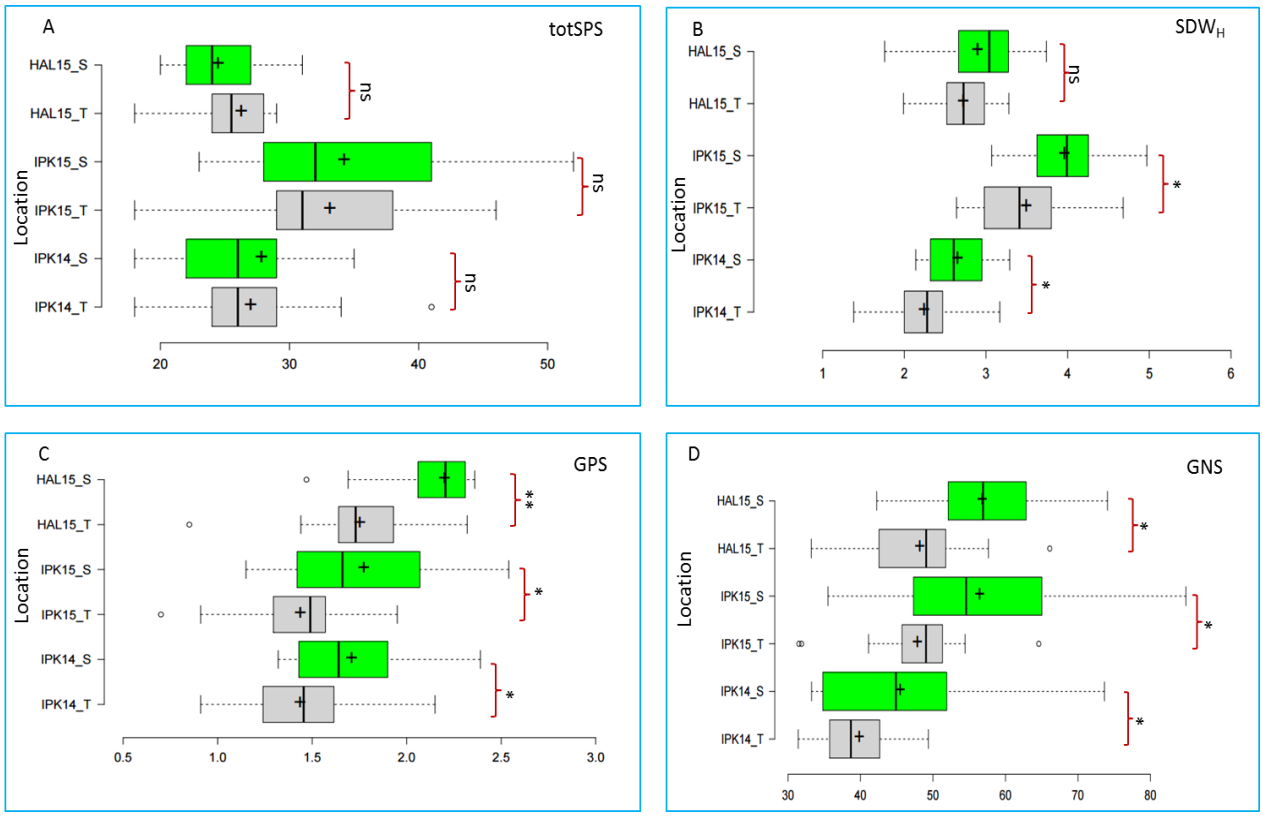


Figure 2-16. Effect of *Rht-B1b* on GPS and GNS in RILs depicting medium to strong spike-branching phenotype. (A) total spikelet per spike (totSPS), (B) spike dry weight at harvest (SDW_H), (C) Grain number per spikelet (GPS) and,(D) grain number per spike(GNS) between semi-dwarf RILs carrying *Rht-B1b* (n=17) and tall RILs carrying *Rht-B1a* (n=22). All the RILs (n=39) show medium to strong spike- branching phenotype. The letters after each location indicate the group i.e. T, tall; S, short; +, indicates sample mean. Significance level between group means was calculated based on unpaired two-tailed Student's t-test at P0.05. ns, non-significant

The total spikelet number did not show a significant difference between RILs carrying *Rht-B1b* (semi dwarf) and *Rht-B1a* (tall) clearly indicating that there was no strong effect of plant height on spike branching (Figure… and Table…). Although spike-branching was found to be negatively correlated with GPS (r= -63.9, Table 2-17), SDW_H and GPS have been significantly improved in the RILs carrying *Rht-B1b* as compared to those carrying *Rht-B1a* suggesting the benefit of combining

the spike branching alleles with the reduced plant height genes (*Rht*) for improving spikelet fertility and grain number per spike (Figure 2-16).

In wheat, heading time is also the most important domestication-related trait. Thus, it was one of the several traits studied in the current mapping population. HD was found to be positively correlated with NPS, PH, SL, ND and totSPS; and negatively correlated with TKW, SHI, GL, and GPS (Table 2-17). Major effect QTL for heading date was identified on chromosome 7BS which has been known to harbor wheat flowering time gene, *TaFT* or *VRN3* (Yan et al., 2006). *QHD-ipk-7BS* consistently appear in all environments controlling about 50 % of the phenotypic variance in the mapping population. Besides controlling HD, *QHD.ipk-7BS* also found to affect PH, SL, NPS, ND, totSPS, SDW_H, GNS, TKW, GA, GL (Table 2-18). Except for TKW, GA, and GL; TRI 19165 contributed the HVA.

Table 2-18. QTL identified from Genome-wide multiple trait analysis

QTL Name	Position	-LOG10(P)	Flanking markers	Position	Trait	location	QTL Effect	PVE (%)	HVA
QGW.ipk-1AL	96.1	6.162 - 6.675	1AL_1367805:1018 - 1AL_3908352:4253	81.01 - 101.01	GW	IPK14,IPK15	0.281 - 0.43	7.9 - 18.5	TRI 19165
					SL	IPK14,IPK15	0.158 - 0.212	2.5 - 4.5	Bellaroi
					TKW	IPK14,IPK15	0.212 - 0.307	4.5 - 9.5	TRI 19165
					GA	IPK14,IPK15	0.193 - 0.268	3.7 - 7.2	TRI 19165
					GNS	IPK14	0.204	4.2	Bellaroi
QGW.ipk-1BL	139.8	3.602	1BL_133382:1992 - 1BL_3846518:1246	117.2 -144.4	GW	IPK15	0.189	3.6	TRI 19165
					SHI	IPK15	0.154	2.4	Bellaroi
QSDW.ipk-2AL	129.9	4.046	2AL_6417564:2355 - 2AL_6423394:12063	112.9 - 135.9	ND	HAL15	0.155	2.4	TRI 19165
					SDW_H	HAL15	0.169	2.9	TRI 19165
QND.ipk-2AL	82.4	3.97	* - 2AL_6318920:2902	64.9 - 92.2	ND	IPK15	0.311	9.7	TRI 19165
					SL	IPK15	0.141	2	Bellaroi
					TKW	IPK15	0.212	4.5	Bellaroi
QSdL.ipk-2AL	131.3	6.92	2BL_5377535:9271 - 2BL_6796534:310	126.2 - 138.1	PH	HAL15	0.15	2.2	TRI 19165
					GL	HAL15	0.248	6.2	TRI 19165
QSHI.ipk-2BL	142.4	3.82	2BL_6796534:310 - 2BL_8055353:17668	137.01 - 147.01	ND	IPK15	0.188	3.5	Bellaroi
					SHI	IPK15	0.196	3.8	TRI 19165
					TKW	IPK15	0.175	3	TRI 19165

GW, Grain Width; SL, Spike Length; TKW, Thousands Kernel Weight; GA, Grain Area; GNS, Grain number per spike; SHI, Spike Harvest Index; ND, Node Density; SDW_H, Spike Dry Weight at harvest; PH, Plant Height; GL, Grain Length;HVA, High-Value Allele; PVE, Phenotypic Variation Explained

Table 2-18. Continued…

QTL Name	Position	-LOG10(P)	Flanking markers	Position	Trait	location	QTL Effect	PVE (%)	HVA
QGNS.ipk-4AL	120.9	10.49 - 21.41	* - 4AL_7133975:1017	105.7 - 130	GNS	IPK14,IPK15,HAL15	0.229 - 0.361	5.3 -13	Bellaroi
					GPS	IPK14,IPK15,HAL15	0.225 -0.317	5.1 -10	Bellaroi
					GA	IPK14,IPK15,HAL15	0.203 - 0.273	4.1 - 7.5	TRI 19165
					SDW_H	IPK15,HAL15	0.192 - 0.235	3.7 -5.5	Bellaroi
					GW	IPK14,IPK15,HAL15	0.205 - 0.350	4.2 - 12.3	TRI 19165
					SHI	IPK15	0.187	3.5	Bellaroi
					SL	IPK15,HAL15	0.197 -0.212	3.9 - 4.5	TRI 19165
					TKW	IPK14,HAL15	0.227 - 0.275	5.1 - 7.6	TRI 19165
					NPS	IPK15, HAL15,IPK14	0.269 - 0.342	7.3 - 11.7	TRI 19165
QPH.ipk-4BS	51.2	88.37 - 97.5	4BS_4866863:2748 - 4BS_4899950:20990	47.9 - 55	PH	HAL15,IPK15,IPK14	0.895 - 0.953	80.1 - 90.8	TRI 19165
					GW	IPK15,IPK14,HAL15	0.155 - 0.262	2.4 - 6.9	TRI 19165
					SHI	IPK15,IPK14	0.229 - 0.258	5.3 - 6.7	TRI 19165
					NPS	IPK15,IPK14	0.142 -0.198	2 - 3.9	TRI 19165
					GL	IPK15,HAL15	0.215	4.6	Bellaroi
					GPS	IPK14	0.17	2.9	Bellaroi
					totSPS	IPK14	0.16	2.6	TRI 19165

GNS, Grain number per spike; GPS, Grain per Spikelet; GA, Grain Area; SDW_H, Spike Dry Weight at harvest; GW, Grain Width; SHI, Spike Harvest Index; SL, Spike Length; TKW, Thousands Kernel Weight; NPS, Node Per Spike; PH,Plant Height; GL, Grain Length; totSPS, total spikelet per spike; HVA, High-Value Allele; PVE, Phenotypic Variation Explained

Table 2-18. Continued…

QTL Name	Position	-LOG10(P)	Flanking markers	Position	Trait	location	QTL Effect	PVE (%)	HVA
QND.ipk-5AL	142.2	9.43 - 14.24	5AL_2807897:8606 - 5AL_2807087:1965	140.2 - 151.2	GNS	IPK15	0.186	3.4	TRI 19165
					HD	IPK14	0.146	2.1	Bellaroi
					ND	IPK14,HAL15,IPK15	0.479 - 0.565	23 - 31.9	Bellaroi
					PH	IPK14,IPK15,HAL15	0.102 - 0.154	1 -2.4	TRI 19165
					SHI	IPK15	0.24	5.8	TRI 19165
					SL	HAL15,IPK14,IPK15	0.452 - 0.536	20.4 - 28.7	TRI 19165
					NPS	IPK14, HAL15	0.184 - 0.62	3.4 - 7.0	Bellaroi
QND.ipk-5AL	66.9	4.27	5AL_2808164:4723 - 5AL_2756328:4367	52.2 - 73.6	ND	HAL15	0.205	4.2	Bellaroi
QNPS.ipk-5BL	210.4	3.33	5BL_10913899:282 - 5BL_9622768:2065	196.1 - 213	ND	IPK14	0.151	2.3	TRI 19165
					NPS	IPK15	0.262	6.9	TRI 19165
QGA.ipk-6AL	96.9	4.39	* - 6AL_5802290:10857	86.01 - 106.01	GPS	IPK14	0.144	2.1	Bellaroi
					ND	IPK14	0.149	2.2	TRI 19165
					GA	IPK14	0.163	2.7	Bellaroi
					TKW	IPK14	0.162	2.6	Bellaroi
QSHI.ipk-6BS	93.046	3.93	6BS_2598748:1357 - 6BS_3029199:729	92.8 - 108.9	GPS	IPK15	0.145	2.1	TRI 19165
					GL	IPK15	0.172	3	Bellaroi
					SHI	IPK15	0.334	11.1	TRI 19165

GNS, Grain number per spike;HD, Heading Date; ND, Node Density; PH, Plant Height; SHI, Spike Harvest Index; SL, Spike Length; NPS, Node Per Spike; GPS, Grain per Spikelet; GA, Grain Area; TKW, Thousands Kernel Weight; GL, Grain Length; HVA, High-Value Allele; PVE, Phenotypic Variation Explained;HVA, High-Value Allele; PVE, Phenotypic Variation Explained

Table 2-18. Continued…

QTL Name	Position	-LOG10(P)	Flanking markers	Position	Trait	location	QTL Effect	PVE (%)	HVA
QSDW.ipk-7BS	84	4.67	7BS_3118963:753 - 7BS_3138002:1560	84 - 97	GNS	HAL15	0.232	5.4	TRI 19165
			-		SDW_H	HAL15	0.308	9.5	TRI 19165
QHD.ipk-7BS	29.8	17.05 - 23.74	7BS_3165832:477 - 7BS_3148608:13804	17.4 - 33.3	HD	HAL15,IPK15,IPK14	0.631 - 0.71	39.8 - 50.4	TRI 19165
					SL	IPK14, IPK15,HAL15	0.256 - 0.367	6.5 - 13.4	TRI 19165
					NPS	IPK15,HAL15,IPK14	0.398 - 0.509	15.9 - 25.9	TRI 19165
					totSPS	IPK14,HAL15	0.181 - 0.204	3.3 - 4.2	TRI 19165
					SDW_H	HAL15,IPK15	0.216 - 0.25	4.6 - 6.2	TRI 19165
					PH	HAL15	0.118	1.4	TRI 19165
					ND	IPK14	0.203	4.1	TRI 19165
					GNS	IPK14	0.273	7.5	TRI 19165
					TKW	HAL15,IPK14	0.154 - 0.329	2.4 - 10.8	Bellaroi
					GA	IPK14	0.231	5.3	Bellaroi
					GL	IPK14	0.18	3.2	Bellaroi
					SHI	IPK15	0.269	7.3	Bellaroi
QSL.ipk-4AL	256.2	3.89 - 4.04	4AL_7145004:3123 - 4AL_7071958:389	251.9 - 271.2	ND	IPK15, IPK14,HAL15	0.357 - 0.374	12.7 - 14	Bellaroi
					PH	IPK14	0.132	1.7	Bellaroi
					SL	IPK15, HAL15,IPK14	0.421 -0.448	17.8 - 20.1	TRI 19165

GNS, Grain number per spike; SDW_H, Spike Dry Weight at harvest; HD, Heading Date; SL, Spike Length; NPS, Node Per Spike; totSPS,total spikelet per spike; PH, Plant Height; ND, Node Density; TKW, Thousands Kernel Weight; GA, Grain Area; GL, Grain Length; SHI, Spike Harvest Index; ;HVA, High-Value Allele; PVE, Phenotypic Variation Explained;HVA, High-Value Allele; PVE, Phenotypic Variation Explained

2.5 Discussion

2.5.1 *QSS.ipk-2AS* and *QSS.ipk-2BS* are homoeoloci

Mapping of SS resulted in three QTL controlling SS in tetraploid wheat Table 2-8 and Figure 2-7. However, only two of them: *QSS.ipk-2AS* and *QSS.ipk-2BS* were found to be the major and medium effect QTL consistently appearing in all environments. The gene underlying *QSS.ipk-2AS* has already been identified (Poursarebani et al., 2015). *TtBH-A1* derived CAPS marker also linked *QSS.ipk-2AS* to *TtBH-A1* (Figure 2-7B); indicating that *QSS.ipk-2AS* is actually controlled by *TtBH-A1.* Similarly, *TtBH-B1* derived CAPS marker also found to be linked with *QSS.ipk-2BS,* regardless of the fact that the role of *TtBH-B1* has not been reported before.

Tetraploid wheat (2n = 4x =28, AABB) carries homoeologous gene sets from its genome progenitors; *Triticum urartu* (2n = 2x = 14; AA) and *Aegilops speltoides* (2x= 2n = 14; BB). Although each gene from the sub-genomes is assumed to be expressed, their function can be altered or silenced in a non-random way. This creates unequal contribution from the homoeologous gene pairs; leading to genome asymmetry towards the control of various traits in wheat (Kashkush et al., 2002; He et al., 2003; Feldman et al., 2012). This makes wheat as an excellent model for studying the effect of genome asymmetry and trait control (Feldman et al., 2012). For instance, spike-branching in hexaploid wheat, known as SS or multi-row spikelet (MRS) (Echeverry-Solarte et al., 2014; Dobrovolskaya et al., 2015), is controlled by the homoeoalleles: *WFZP-D* and *WFZP-A* (homoeologous to *TtBH-A1*) with the major effect coming from *WFZP-D* followed by *WFZP-A* (Dobrovolskaya et al., 2009; Dobrovolskaya et al., 2015). *WFZP-B* was reported to be silent due to the inserted MITE in the promoter region. Although the same MITE has been found in tetraploid wheat (Figure 2-12), *TtBH-B1* is expressed in all of the spike development stages analyzed (Figure 2-11), though to a much lower extent than *TtBH-A1*. Expression analysis further showed that *TtBH-A1* is the most expressed allele in all spike development stages of tetraploids, clearly validating the mapping result where *QSS.ipk-2AS* and *QSS.ipk-2BS* were found to be major and medium effect QTL, respectively (Table 2-8). It is important to note that the MITE contributes to the allelic diversity of genes by providing regulatory sequences and thereby diversifying the expression of homoeoalleles in different tissues (Feschotte et al., 2002). In this regard, further study is required to gain insights into the effect of the MITES identified in this study.

Although sequence variation in the ORF and 3' UTR region of TtBH-B1 resulted in 5 different haplotype groups, single SNP (T785C) separated the wild type haplotype, Hap I-YIL-G-del, from mutant haplotype, Hap III-YIS-G-del, suggesting the connection of T785C (L262S) with spike-

branching (Table 2-13). Therefore, besides the slow expression, the function of *TtBH-B1* might also be affected at a post-transcriptional level similar to its homoeoallele *TtBH-A1*, where only a single amino acid change at position 96 (L96P) is responsible for spike-branching in tetraploid wheat (Poursarebani et al., 2015). Thus, the higher phenotypic penetrance and expressivity of all RILs carrying both alleles from TRI 19165 (genotype: aabb, Table 2-10) is due to the reduction or loss-of-function of *TtBH-B1* besides the loss-of-function of *TtBH-A1*.

To further study the *bh* locus, the development of *bh*-NILs was one of the main targets of the current study (chapter 3). Although the major target of the project was to introgress bh^{t}*-A1* into an elite cv. Floradur, few NILs were found to carry donor fragment flanking *QSS.ipk-2BS* (Table 3-4), suggesting that these NILs (Fam1 plants) seemed to have combined both alleles. Interestingly, phenotypic analysis at BC3F2 generations also shows that these NILs (Fam1 plants) had 2 extra addSPS as compared to those NILs carrying only *QSS.ipk-2AS* (Table 3-2), clearly indicating the additive effects of the homoeoallele.

Taking all together: first, through QTL mapping of SS where both SS QTL were linked with gene derived CAPS marker (Figure 2-7), secondly, group based phenotypic analysis of RILs carrying different combinations of the A and B alleles from Bellaroi and TRI 19165 i.e. *aabb* vs *aaBB* (Table 2-10), thirdly, through expression analysis at different spike developmental stages (Figure 2-11), and fourthly, through analysis of NILs (Fam1 plants) which have combined both homoeoalleles (Table 3-2), and finally through haplotype analysis (Table 2-13), it is concluded that *QSS.ipk-2BS* is the homoeolocus of *QSS.ipk-2AS* and thus; the gene underlying *QSS.ipk-2BS* is, in fact, *TtBH-B1*.

2.5.2 Mutation in the *BH* locus converts the unbranched wheat spike to branching

Modifying inflorescence architecture has a tremendous effect for improving grain yield. Hence, detail molecular understanding of the inflorescence architecture is mandatory, especially in wheat. As spikelets are the building blocks of grass inflorescence; the size and determinacy of the IM and SM largely affect inflorescence architecture and thus directly controls the number of spikelets and grain number per plant. However, the genetic and molecular mechanisms governing inflorescence development and floral organ identity in wheat is largely unknown. The central role of *TtBH* and the orthologous genes from other grasses: *BD1/FZP/MOS1/COM2* is to suppress inflorescence branching and to facilitate phase transition from SM to FM (Chuck et al., 2002; Komatsu et al., 2003; Derbyshire and Byrne, 2013; Dobrovolskaya et al., 2015; Poursarebani et al., 2015). Even though *BD1/FZP/MOS1/TtBH/COM2* suppresses inflorescence branching, the

relationship of *BD1/FZP/MOS1/TtBH/COM2* with other floral organ identity gene is still unknown. However, emerging evidence show that *FZP* is a major negative regulator of *RFL/APO2* (the rice homolog of Arabidopsis *LFY* gene) to determine the transition from panicle branching to spikelet formation (Bai et al., 2016). *RFL/APO2*, which is required for the class C gene activity, mediates the patterning of rice inflorescence architecture whose expression is required for the initiation of the branch primordium formation (Kyozuka et al., 1998; Bai et al., 2016). *RFL* is predominantly expressed in young panicles and promotes branching (Kyozuka et al., 1998). In wheat, *WFL* (the wheat homolog of the Arabidopsis *LFY* gene) is also found to express in young spike suggesting its role in spikelet development (Shitsukawa et al., 2006). This clearly suggests that *TtBH* might also function similarly in wheat.

Although spike development in wheat is determinate; such spike determinacy has been lost in *bh* wheat mutant strains. Therefore, the main spike develops several axillary mini-spikes from the base of the main spike, which leads to spike-branching or SS formation; or in some cases, an extension of the rachilla which carries a complete spikelet instead of florets (Figure 2-3D&G). Aiming at studying the mechanisms that regulate inflorescence architecture in wheat and the relationship with ploidy level; Shitsukawa et al., (2009) and Poursarebani et al., (2015) studied the standard wheat spike and the *bh* spike using light and scanning electron microscopy. The target was to compare floret development and the timing of the initiation of floral primordia (Shitsukawa et al., 2009; Poursarebani et al., 2015). They found that the inflorescence of *bh* appears to be normal in the early phases of inflorescence development. However, the difference comes later when the SM of *bh* mutants starts to elongate; during which an ectopic branching start to emerge after the initiation of GP. Ectopic meristems start to develop from ectopic branches of the axis alternately on opposite sides which give rise to another round of SM. The authors suggested that the ectopic florets (from ectopic spikelets) had a normal morphology having normal stamens and carpel. Other studies also suggested that spikelet initiation in *bh* mutants extends beyond TS, which is usually not the case in standard wheat spike. This indicates that primordia for the additional spikelets appear indeterminately (Rawson and Ruwali, 1972c; Kadkol and Halloran, 1988). The authors further indicated that spikelets from the indeterminate rachilla also appear lately as compared to the regular spikelet, suggesting that spikelet and floral development is not completely synchronized in *bh* wheat mutants. This might be the main cause for the high rate of spikelet infertility during branching (Table 2-17).

2.5.3 *BH* controls sink size in wheat

Sink size is generally believed to be yield limiting factor in crops (Marcelis, 1996; Yu et al., 2015) including wheat (Rawson, 1970; Slafer and Savin, 1994; Borrás et al., 2004; Miralles and Slafer, 2007; Foulkes et al., 2011). This suggests that more has to be done for manipulating sink strength and activity in wheat (Reynolds et al., 2001; Reynolds et al., 2005; Reynolds et al., 2009). Modern wheat varieties seem to have reached the ceiling for the Harvest Index (HI), which is believed to be 0.6 (Austin et al., 1980). Hence, more research is needed to raise sink size and sink activity in wheat. Due to increased spike biomass as a result of branching, *QSS.ipk-2AS* and *QSS.ipk-2B* control sink size in wheat. Furthermore, *QSS.ipk-2AS* and *QSS.ipk-2BS* also found to affect spikelet fertility (Table 2-14). The negative correlation of spike-branching and spikelet fertility (Table 2-17) indicates that there might be competition between spikelets and/or florets for assimilate. This indicates that there is source limitation in wheat or, even if the source is not limiting, the sink isn't able to utilize the available source simply due to a problem related to sink activity (architecture) or a problem related to both source and sink. Therefore, the general thought that grain yield in wheat is sink limited need to be critically re-assessed if spikelet infertility during spike-branching is in fact due to source limitations.

Most of the sink-source manipulation studies in wheat have been made by removing (defoliating) the source and/or sink components (Ma et al., 1990; Slafer and Savin, 1994; Borrás et al., 2004); meaning that none of them have used mutant genetic material whose source- sink balance was affected in a genetically controlled way. In this regard, utilization of the *bh* or *bh*-NILs for studying source-sink relationship is important as the sink size in *bh* or *bh*-NILs is genetically controlled.

So far very few genes were identified, which are known to operate in the areas of sink size enhancement in crops. The major target genes in this regard, however, are genes related to sucrose synthase or invertase gene families (Sturm, 1999; Ruan et al., 2010). Overexpression of cell wall invertase, an enzyme which catalyzes the cleavage of sucrose to glucose and fructose, is required for carbon partitioning to the sink tissue (Cheng et al., 1999) and was associated with increased grain size, grain number, and starch content due to enhanced carbon partitioning during the grain-filling phase in maize (Li et al., 2013). Similarly, another cell wall invertase gene, *GRAIN INCOMPLETE FILLING 1* (*GIF1)* was also found to control grain-filling and yield in rice (Wang et al., 2008). Combining these genes together with the *bh* allele(s) might alleviate the problem associated with grain weight and related traits which were found to negatively correlate with branching.

However, very few information are available concerning the cell wall invertase in wheat (Ji et al., 2010; Ma et al., 2012; Webster et al., 2012). Nevertheless, two sucrose synthase genes (*TaSus1 and TaSus2*), which are located on chromosome group 7 and 2, respectively, were associated with gain yield and TKW; and were a major target of selection in wheat breeding (Hou et al., 2014). Therefore, partitioning of more carbon to the developing grain could improve TKW, which correlate negatively with the totSPS or GNS (Table 2-17).

2.5.4 *QSS.ipk-2AS* and *QSS.ipk-2B* affect spikelet fertility in wheat

GNS and GPS are the major factors controlling grain yield in wheat (Alvaro et al., 2008; Green et al., 2012). Based on the multi-trait analysis, *QSS.ipk-2AS* and *QSS.ipk-2B* affect spikelet fertility in wheat (Table 2-14). About 27.6 % of phenotypic variance for GPS was found to be controlled by *QSS.ipk-2AS*. The contribution of *QSS.ipk-2B* was also significant (about 11.5%). The allele from Bellaroi contributed to the higher grain number per spikelet. GPS also found to be negatively correlated with SS formation (Table 2-17) suggesting the magnitude of floret abortion or sterility during spike-branching, especially when branching is intense. This could be attributed to different factors.

The first most likely factor could be competition among the spikelets/florets for limited resources which has been generally taken as an established thought in wheat (Rawson and Evans, 1970; Hanif and Langer, 1972; Langer and Hanif, 1973; Kirby, 1988; Youssefian et al., 1992). The introduction of reduced plant height genes (*Rht* genes) since the Green Revolution has significantly improved assimilate partitioning to the developing floret primordia thereby improving the number of fertile florets that can reach anthesis and set grain (Siddique et al., 1989; Youssefian et al., 1992; Slafer and Miralles, 1993; Miralles et al., 1998). Therefore, the combined effect of *Rht* and *bh* might be used to develop new wheat ideotype characterized by sturdy plant stature (lodging resistant) with branching spike (strong sink size).

The other factor which might have contributed to the lowered spikelet fertility is related to the time at which SS develops. An earlier study suggested that the appearance of SS lags behind the standard spikelets/florets suggesting the indeterminacy as well as the unsynchronized development of SS /floret during branching. This, of course, needs further study. Generally, the dynamics of floret primordia generation and degeneration determines fertile florets and thus grain number per spikelet in wheat (Kirby, 1988; Ferrante et al., 2013; Guo et al., 2015b; Guo and Schnurbusch, 2015). Although both spikelet and floret primordia are indeterminate in the spike-

branching mutant lines, generally it is the survival rate which can determine fertile floret and grain number per spikelets. Out of 8 or more floret primordia per spikelet that are formed during floret initiation phase, only 2-5 floret primordia are known to survive and produce grains (Kirby, 1988; Guo et al., 2015b; Guo and Schnurbusch, 2015). Hence, raising wheat yield through directly increasing floret fertility does not seem to be an easy task. However, increasing spikelet number per spike would be an alternative approach.

2.5.5 Phenotypic plasticity of spike-branching

Because of the involvement of genetic and environmental factors, the details of the genetic machinery underlying phenotypic plasticity are very complex. It has been suggested that there is a relationship between the rapidity of plant organ development and the degree at which this structure can be affected by environmental factors (Pigliucci, 2001). Several studies have also been conducted on the developmental aspect of phenotypic plasticity in plants (Diggle, 1993; Pigliucci, 1997; Pigliucci, 2001).

Under normal circumstances, inflorescence (spike) development in wheat is determinate; meaning that no additional spikelets appear after the initiation TS. In this case, phenotypic plasticity might arise due to the variation in development and fertility of the spikelets instead of spikelet number *per se.* Contrary to this, the phenotypic plasticity during spike-branching (SS formation) could arise from the variation in spikelet number itself beside the development and fertility of each spikelet. So, environmental factors and the indeterminate nature of spike-branching are the main factors for the phenotypic plasticity in spike-branching wheat lines.

Plants normally compete for limited resources such as for nutrients, water and light energy (Weiner, 1986; Aerts, 1999; Craine and Dybzinski, 2013). One of the major factors which can trigger such competition is light energy. When plants compete for light energy, they display a ‘shade avoidance syndrome’ where they are forced to adjust their shoot architecture in order to win the competition (Nagashima and Hikosaka, 2011; Pierik and de Wit, 2014). This requires an accelerated growth to bring their leaves higher above the vegetation to capture the sunlight. Under such circumstances, plants strengthen apical dominance thereby inhibiting tillering and inflorescence branching (Weiner, 1986; Smith and Whitelam, 1997). Plants under ‘shade avoidance syndrome’ also undergo accelerated flowering and early seed production (Salter et al., 2003).

Significant differences in the expressivity of SS between border and middle row plants clearly highlighted the role of competition in phenotypic penetrance and expressivity of spike-branching or SS formation (Figure 2-5). QTL mapping of data obtained from border and middle row plants did not result in any new SS QTL; suggesting the phenotypic variation (plasticity) in the expressivity of spike-branching between the middle and border plants is controlled by environment factors.

It has also been suggested that SS formation is sensitive to temperature and photoperiod (Percival, 1921; Sharman, 1944; Pennell and Halloran, 1984a; Pennell and Halloran, 1984b). Pennell and Halloran suggested that low temperature and short photoperiod are conducive environmental factors for the expressivity of spike-branching. This is most likely associated with the duration of spike development phase during which spike length and thus, spikelet number are determined (Rawson, 1970; Fischer, 1985). Hence, combined with optimum temperature, a longer spike development phase is critical for increasing spikelet number in wheat. Accelerated growth during this period can significantly reduce spikelet and grain number in wheat (Wall and Cartwright, 1974; Fischer, 1985; Rawson and Richards, 1993). This clearly suggests the importance of spike growth phase for spikelet number and spikelet fertility in wheat. Thus, extending spike growth phase might also mitigate the synchronization problem associated with spikelet development during branching; for instance, by developing photoperiod sensitive NILs in winter wheat genomic background.

2.5.6 Flowering time and spike architecture in wheat

The life cycle of wheat is predominantly controlled by complex interactions between environment and three sets of major flowering-related genes; namely vernalization, photoperiod and *earliness per se* genes (Yan et al., 2003; Yan et al., 2004; Yan et al., 2006; Beales et al., 2007; Lewis et al., 2008; Gawroński et al., 2014).

Among the genes that have played a major role in shaping wheat plant in terms of heading (flowering) time is *Ppd1* (Worland et al., 1998; Beales et al., 2007). Although the primary effect of *Ppd1* is to accelerate flowering, it has the major role in the adaptability of wheat in a broad range of environments ((Worland et al., 1998; Snape et al., 2001; Royo et al., 2015). Wheat can be further classified as photoperiod sensitive and insensitive (day neutral). Photoperiod sensitive wheat varieties need exposure to long day to flower while the insensitive one is day neutral and flower early both under long and short day conditions (Beales et al., 2007; Yan, 2009). Early

flowering of the insensitivity group is due to early activation of the flowering pathway (due to misexpression of *Ppd1)* regardless of day length (Beales et al., 2007). *Ppd1* has a negative pleiotropic effect on plant development by reducing the vegetative (such as tillering) and inflorescence primordia (such as the floret) across different environments (Worland et al., 1998). As a consequence, plants will be shorter, having smaller ears and fewer tillers. This might even have a stronger effect on photoperiod insensitive varieties which flowers early as compared to the sensitive ones. Although no direct relationship has been detected between spike-branching or SS formation and the heading time, *QHD.ipk-7BS* might also play the indirect role of affecting expressivity of spike-branching or SS formation. This is because; *QHD.ipk-7BS* was found to have a positive effect on NPS (spikelet number per spike), totSPS, SL, SDW_H, GPS, and grain weight indicating the effect of flowering time on spike-related traits (Table 2-18).

Recently, it has also been shown that the insensitive allele of *Photoperiod-1 (Ppd-1)*, which promotes early flowering, had a major inhibitory effect on paired spikelet formation (a different form of sessile additional spikelets arising from the same node) by regulating the expression of *FLOWERING LOCUS T* (*FT*) (Boden et al., 2015). Under short-day conditions, day length sensitive *Ppd-1* NILs and *FT-B1* mutants (*ft-B1* lines), which flowers late, were associated with increased number of paired spikelet formation (increased total spikelet per spike) which is a clear indication that spikelet development phase is a key factor in controlling spikelet number in wheat. Earlier studies have also indicated that flowering time genes control inflorescence architecture by controlling the size and a number of organs produced (Xue et al., 2008; Endo-Higashi and Izawa, 2011; Weng et al., 2014).

The role of carbohydrate in the regulation of plant architecture, flowering time and crop yield (Satoh-Nagasawa et al., 2006; Wahl et al., 2013; Nuccio et al., 2015) is attracting lots of interests and thus, becoming a hot topic in crop research. In maize, inflorescence branching is regulated by three *RAMOSA* (*RA*) genes (Vollbrecht et al., 2005) and one of which, *RA3,* was found to encode a T6P phosphatase, an enzyme which converts trehalose 6-phosphate (T6P) to trehalose indicating the importance of sugar in controlling inflorescence architecture. Overexpression of rice T6P in developing maize ears using floral promoter increased the level of sucrose in ear spikelets leading to increased kernel number and harvest index (Nuccio et al., 2015). Furthermore, the loss-of-function of *TREHALOSE-6-PHOSPHATE SYNTHASE 1* (*TPS1*), which is also an enzyme involved in the biosynthesis of trehalose 6-phosphate (T6P) from UDP-glucose and glucose 6-phosphate, resulted in late flowering in Arabidopsis (Wahl et al., 2013). Therefore, a deeper understanding of the link

between flowering and carbohydrate metabolic genes is important for improving inflorescence architecture, especially in connection with shifting plant phenology due to climate change (Craufurd and Wheeler, 2009; Wang et al., 2015). Recently, the flowering time gene in barley (barley *FT1*) was found to be co-regulated with genes know to be involved in nutrient transport, carbohydrate metabolism, and cell cycle regulation suggesting the role of *FT1* in altering the source-sink relationships in barley (Digel et al., 2015). Therefore, sink size (spikelet/floret number in wheat) and flowering time are important traits to understand floret fertility and sink strength in wheat, especially in connection with spike-branching.

3 Chapter 3: Development of Floradur *bh*-NILs (FL-*bh*-NILs)

3.1 Introduction

In order to study and characterize quantitatively inherited loci, separation of the locus from other background loci is required. This is what is known as 'Mendelization' of the locus so that the locus is inherited in a simple Mendelian fashion (Alonso-Blanco and Koornneef, 2000; Salvi and Tuberosa, 2005). Mendelization of a QTL is therefore accomplished by developing Near-Isogenic Lines (NILs). NIL development requires several steps of backcrossing to the recurrent parent. In order to further facilitate the process, molecular genetic markers are used in each generation of backcrossing for tracking the locus and selecting individuals with a higher proportion of the recurrent parent genome.

During NIL development, two types of marker selection are common. These are the foreground (FG) and background (BG) selections. Foreground selection is a process of tracking the locus/gene of interest based on markers derived from the gene itself or another marker that is linked to the gene. Whereas background selection is the application of molecular genetic markers for selecting individuals with a higher proportion of the recurrent parent genome recovery. Background selection is also helpful to lower the number of rounds of backcrossing; for example from six to three/four (Frisch et al., 1999).

To reduce the drag over effect from the donor genome, several rounds of backcrossing might be needed which of course take more time. However, the time needed for the generation of NILs can be further shortened by applying embryo rescue (ER) technique. Soto-Cerda *et al.* used an embryo rescue technique and Marker Assisted Backcrossing (MAB) to successfully introgress aluminum resistant gene, *HvAACT1,* in barely in a very reasonable timeframe (Soto-Cerda et al., 2013).

After reasonable rounds of backcrossing, NILs are fixed by selfing the last generation and selecting homozygous individuals.The NILs generated in this way are nearly identical to the recurrent parent except for the introgressed segment and other minor genomic segments from the donor (hence the name 'near-isogenic lines'). To estimate the introgressed segment as well recurrent parent genome recovery, NILs need to be genotyped using any genotyping platforms such as GBS.

3.2 Objective of the study

Three QTL controlling SS formation were identified from the mapping population derived from a cross between Bellaroi and TRI 19165 (Table 2-8). Two of these QTL (*QSS.ipk-2AS* and *QSS.ipk-2BS*) were major and medium effect QTL, respectively, controlling SS formation. Besides controlling SS

formation, *QSS.ipk-2AS* and *QSS.ipk-2BS* were also found to affect spikelet fertility and other spike traits. Furthermore, *QSS.ipk-2AS* and *QSS.ipk-2BS* also control spike/sink size in wheat. Therefore, the main objective of developing NILs is to introgress or Mendelize the *bh* loci into a completely new genomic background i.e. cv. FLORADUR (FL) in order to single out and further study the effect of the *bh* loci. Furthermore, FL-*bh*-NILs will be an excellent genetic material to further dissect source-sink relationship in wheat. They can also be used as excellent genetic materials for gene pyramiding for creating the IWPA as defined by CM Donald in 1968 (Donald, 1968).

3.3 Materials and Methods

3.3.1 Plant Materials

A German standard spike and commercial elite durum wheat variety, cv. FLORADUR was used as a recurrent parent. Spike-branching mutant, TRI 19165 was used as a donor parent for the bh^{t}-*A1* allele.

3.3.2 Backcrossing Schemes

The parents were grown side by side for the crossing scheme under greenhouse conditions. For physiological synchronization of the male and female parents, the pollen parent (TRI 19165) was grown at different time series after the female parent (Floradur). Emasculation (removal of the anthers) from Floradur was initiated when half of the ear emerged from the flag leaf. All young florets were surgically removed by leaving the bottom two florets. Terminal and very young spikelets were completely removed. After removing anthers, the whole spike was covered with bags to avoid any pollen cross-contamination. The plants developed further for another 3 to 6 days until pollination. After pollination, the spike was covered again to protect any pollen cross-contamination. Then the pollinated florets were allowed to develop normally in the greenhouse. F1 seeds were collected and re-grown in the greenhouse for BC1F1 (Figure 3-1).

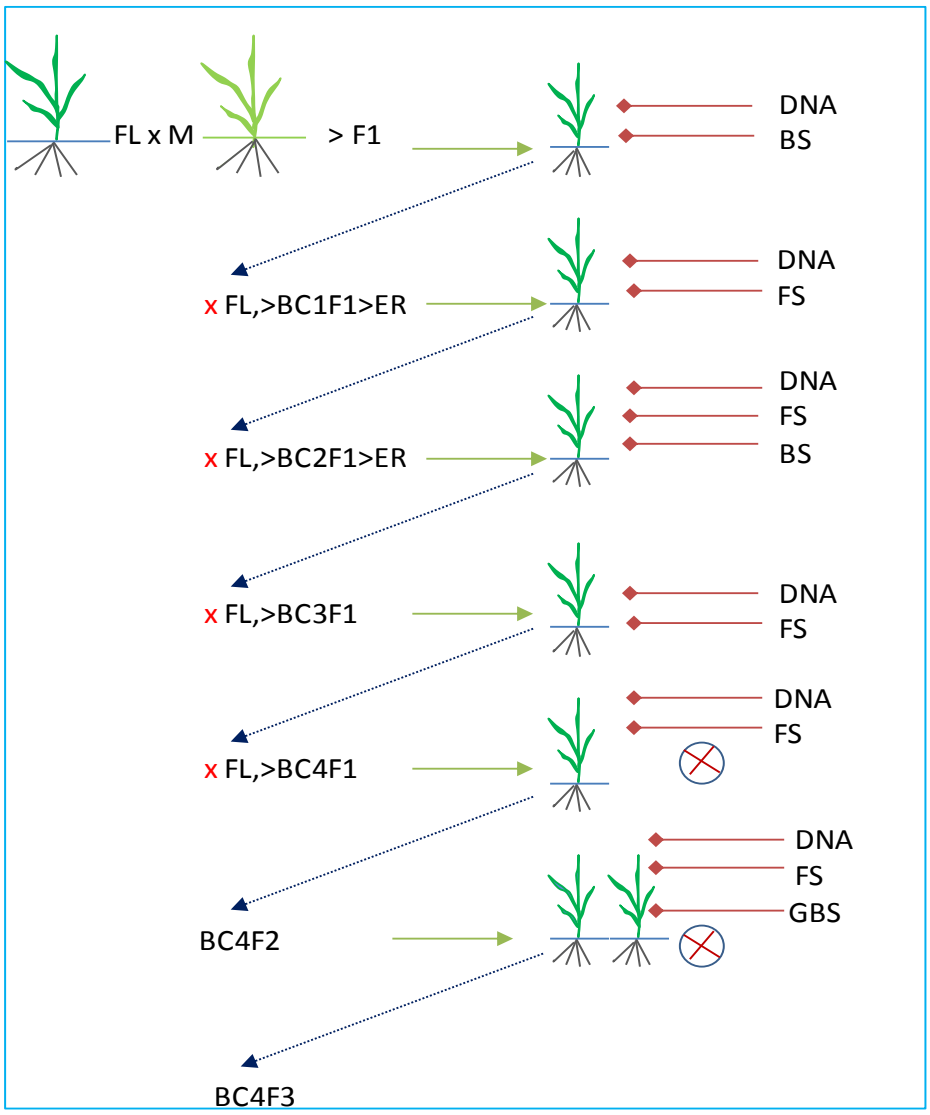


Figure 3-1. Backcrossing scheme for the development of FL-*bh*-NIL.
FL, Floradur; M, mutant (TRI 19165); F1 the first filial generation; BS, Background Selection; FS, Foreground selection; BC1F1, the first backcross generation of F1; ER, Embryo Rescue; BC2F1, the second backcross generation of F1; BC3F1, the third backcross generation of F1; BC4F1 , the fourth backcross generation of F1;Circle with a cross show selfing ; BC4F2 , the fourth backcross generation obtained by Selfing BC4F1 and GBS , Genotyping-BY-Sequencing.

DNA was isolated from all F1 plants and tested for polymorphism using Gatersleben wheat microsatellite markers: *xgwm155* (chr.3A) and *xgwm95* (chr.2A) (Roder et al., 1998). PCR conditions and fragment analysis were conducted as described by Röder(Roder et al., 1998). PCR product was analyzed using Automated Laser Fluorescence (ALF) sequencer (Amersham Biosciences, Sweden) using short gel cassettes as described by Röder. An external standard with four fragments of size 73, 122, 196, 231 bp was used for fragment size estimation. Fragment sizes were calculated using the computer program 'Fragment Analyzer' Version 1.02. Allele size from *xgwm155* was estimated to be 128 bp and 143 bp for Floradur and TRI 19165, respectively. For *xgwm95*, Floradur had 123 bp while TRI 19165 had 128bp allele size. All true-bred heterozygous

F1 plants were backcrossed with Floradur following the same procedure described earlier (Figure 3-1). Immature embryos were rescued from all BC1F1 plants. ER was initiated after 21 days of pollination. Immature grains were removed from the mother plant and rinsed in 70% alcohol for 1 minute. After rinsing the grains with sterilized water, the samples were washed with a washing buffer containing 20% hypochlorite and tween20. After seven minutes of shaking, samples were repeatedly washed with sterilized water. The embryo was surgically removed in the laminar hood and carefully placed on B5 medium on Petri dish (Duchefa biochemie, Haarlem, The Netherlands) for about 72 hours at 24 ^{0}C under dark conditions in a growth chamber. The calli were then transferred to fresh new media in a magenta box and allowed to grow under long day conditions (16 hrs. light and 8 hrs. dark) for about 10-12 days at 24^{0}C. Seedlings were then transplanted in pots of size 2 liter filled with substrate2 (Klasmann-Deilmann GmbH, 49744 Geeste, Germany), compost and sand with a proportion of 2:2:1, respectively. Plants received all the standard treatments for wheat including fertilizers and pesticides. DNA was extracted from these plants and FG selection was made using allele-specific CAPS marker derived from *TtBH-A1* (see section 2.3.5). All heterozygous plants were selected and backcrossed with Floradur following the same procedure described earlier. Similarly, following the same procedure, ER was applied after 21 days of pollination. DNA from BC2F1 plants was extracted for FG and BG selection.

3.3.3 Background selection

BG selection was performed on 56 BC2F1 plants. Markers were first checked on the two parents for polymorphism. The final markers were selected in such a way that polymorphism can be easily detected on an agarose gel. About 47 genome specific wheat microsatellite markers were used (Supplementary Table 9-3). PCR conditions, as well as fragment analysis of each polymorphic marker, was as described earlier in section 3.3.2. Genome recovery was calculated using the following formula:

Equation 3-1: Recurrent Parent Genome Recovery

$$\mathrm{RpGR} = \frac{\mathrm{HO} + (\mathrm{HE}/2)}{\text{Total Markers}} * 100$$

Where RpGR is the Recurrent parent Genome Recovery, HO is the number of homozygous markers (FL alleles); HE is the number of heterozygous markers (alleles).

Five plants with higher RpGR were selected and backcrossed with Floradur to make five families of the BC3F1 plants (Figure 3-1). BC3F1 grains were kept on the mother plant until harvest (no ER at this stage). Grains from BC3F1 were harvested and kept in a cold room (4 ^{0}C) for about 4 weeks to break down seed dormancy for a better germination. Then, BC3F1 grains were grown in the greenhouse. DNA was isolated, marker analysis (FG selection) was performed and heterozygous plants were selected and backcrossed with Floradur to make the next generation of plants (BC4F1) (Figure 3-1). BC4F1 grains from each family were again grown in the greenhouse. DNA was isolated, marker analysis (FS) was performed and heterozygous plants were selfed to produce BC4F2 grains (Figure 3-1). BC4F2 grains grown again in the greenhouse and DNA were isolated from each plant for final genotyping. On the basis of the expected segregation ratio at BC4F2 (1:2:1), all *bh*-homozygous and heterozygous plants (75 % of BC4F2 plants) were kept for further analysis and genotyping (67 plants in total) using the GBS approach in order to estimate the size of the introgressed segment as well as calculate the RpGR.

3.3.4 Field evaluation and phenotyping

The main target for field evaluation was to study FL-*bh*-NILs for spike morphology and associated traits. Thus, some plants from each of the five family plants were selfed at BC3F1 generation to select homozygous BC3F2 plants. Grains from selfed BC3F1 plants were randomly picked from each of the five plant families and were grown in 96 well trays under greenhouse conditions. DNA was extracted from each of these plants for marker screening and selection of homozygous plants. All plants were then directly transplanted in the field characterized by silty loam soil with 10 cm distance between plants and 20 cm between rows. All homozygous plants were phenotyped for Spike Length (SL), Plant height (PH), Heading Date (HD), Node number per Spike (NPS), additional spikelets per spike(addSPS), total spikelet per spike(totSPS), Tiller number (TN), Spike Dry weight at harvest (SDW_H), grain number per spike (GNS), grain number per spikelet (GPS), thousands kernel weight (TKW), grain area (GA), grain width (GW), and grain length (GL).

3.4 Result

3.4.1 Foreground and background selection of heterozygous plants

The target of this study was to introgress the *bh*^{t}-*A1* allele into cv. Floradur. Therefore, F1 plants derived from Floradur x TRI 19165 were backcrossed four times to Floradur (Figure 3-1 and Table 3-1). This has been accomplished by selecting heterozygous plants using the *TtBH-A1* CAPS marker developed earlier. Heterozygous plants selected in each generation (Table 3-1), gel picture from

TtBH-A1 CAPS diagnostic marker used for the FG selection (Figure 3-2A), gel picture from the gel based microsatellite marker screening for the BG selection (Figure 3-2B), selected BC2F1 plants based on BG selection using 47 wheat microsatellite markers (Figure 3-2C), and homozygous BC3F2 plants (Figure 3-2D) are shown in (Figure 3-2. Based on BG selection (RpGR analysis), five single plants i.e. P-69-4 (F1), P-103-16 (F2), P-114-27 (F3), P-126-45(F4) and P-170-50 (F5) were selected to constitute five different plant families of the BC3F1 generation. Detail phenotypic analysis of these five plant families at BC3F2 generation is presented in Table 3-2.

Table 3-1. Number of heterozygous plants selected in each generation based on *TtBH-A1* CAPS marker

Generation	No. of heterozygous plants selected	Technique used to advance the generation
BC1F1	15	Embro rescue
BC2F1	56	Embryo rescue
BC3F1	49	Grain
BC4F1	27	Grain

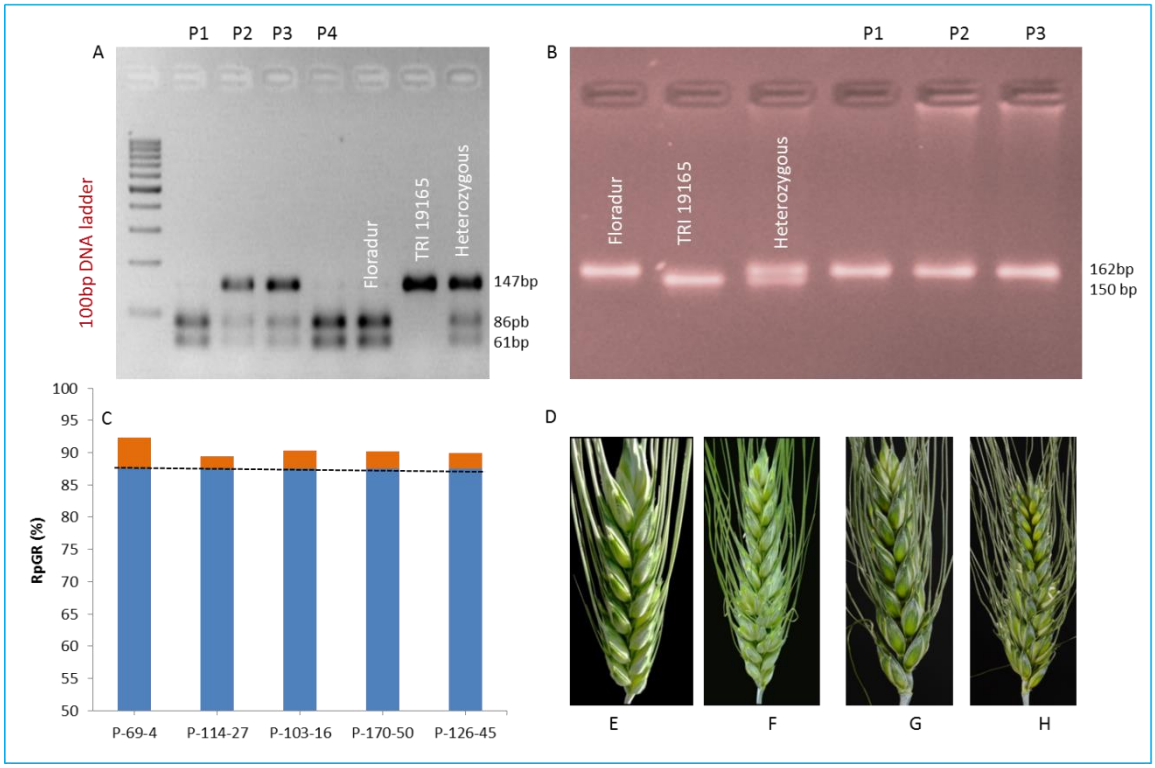


Figure 3-2. Foreground and background selection of five BC2F1 family plants.

(A) *TtBH-A1* CAPS diagnostic marker for FG selection, (B) Gel based wheat microsatellite marker screening for the BG selection (x*gwm1128* is shown as an example), (C) Five selected BC2F1 plants based on RpGR using 47 wheat microsatellite markers. The broken horizontal line shows the theoretical expected RpGR at BC2F1 generation (87.5%). The orange bar shows the actual RpGR attained by each of the five plant families. (D) Spike morphology of homozygous BC3F2 plants along with the recurrent parent FL. The photos were taken from field (E and F) and from greenhouse (G and H) at BC3F3 generation

3.4.2 Whole-genome genotyping of BC4F2 plants

After selfing of BC4F1 plants, BC4F2 plants were expected to segregate for the target locus with the expected Mendelian segregation ratio of 1:2:1. After excluding all the homozygous wild-type plants, 67 BC4F2 plants (23 homozygous and 44 heterozygous) were genotyped using the GBS as described in section 2.3.6. In total, 799 polymorphic SNP markers were used to genotype all the 67 BC4F2 plants. Distribution of the markers across the linkage groups (chromosomes) is shown in Table 3-2. Genetic position of the markers was according to the POPSEQ linkage map (Chapman et al., 2015). The complete linkage map of all 799 is shown in Supplementary Figure 9-6

Table 3-2. Distribution of polymorphic SNP markers across the chromosome

Chromosome	Number of Markers	Length (cM)
1A	63	117.88
1B	58	113.81
2A	40	92.52
2B	62	111.51
3A	61	171.06
3B	63	121.91
4A	67	156.68
4B	50	102.13
5A	59	111.97
5B	71	155.0
6A	45	98.82
6B	54	97.87
7A	64	135.63
7B	42	118.55
14	799	1705.34

Based on these markers, RpGR was calculated for all 67 BC4F2 plants. Best 20 NILs whose RpGR was greater or equal to 94 % were selected for further analysis (Table 3-3). As the main target of developing *bh*-NILs was to Mendelize the *bh^{t}-A1* allele (i.e. *QSS.ipk-2AS*) and to introgress it into the Floradur background*;* indeed all the selected plants carried *bh^{t}-A1*. Based on the QTL mapping result, *QSS.ipk-2BS* is also involved in controlling SS formation (chapter 2, section 2.5.1.). Detailed analysis of the five different plant families at BC4F2 generation showed that the first family, Fam1, plants also carry the donor (TRI 19165) fragment containing marker 2BS_5185931:996 (2BS_5198792:13914), which was flanking *QSS.ipk-2BS* at close proximity (see Figure 2-7). This suggests that the donor fragment might also carry *QSS.ipk-2BS.* Graphical genotyping showed that three Fam1 family plants: P-69-4-4-127, P-69-4-4-121, and P-69-4-4-122 were heterozygous for marker 2BS_5198792:13914 (2BS_5185931:996), while P-69-4-4-126 was homozygous (Table 3-5). Phenotypic data analysis at BC3F3 generation also clearly showed that the Fam1 plants had 2

more additional spikelets per spike as compared to other families, indicating the additive effect of *QSS.ipk-2BS* for increasing spikelet number per spike (Table 3-6).

Table 3-3. RpGR based on markers from 2A, 2B, and whole genome for the selected BC4F2 plants.

Family	Plant ID	Expected RpGR at BC4F1	Calculated RpGR (%)			Zygosity (*TtBH-A1*)	Zygosity (*TtBH-B1*)
			Chr. 2A	Chr. 2B	Whole Genome		
Fam1	P-69-4-4-127	96.88	90.43	97.86	97.74	+/-	+/-
Fam1	**P-69-4-4-126**	96.88	91.88	95.67	97.10	+/-	**+/+**
Fam1	P-69-4-4-121	96.88	89.92	95.94	96.38	+/-	+/-
Fam1	**P-69-4-4-122**	96.88	82.05	97.28	96.37	**+/+**	**+**/-
Fam2	**P-103-16-34-31**	96.88	95.28	98.08	97.77	**+/+**	-/-
Fam3	P-114-27-73-69	96.88	96.47	99.47	96.92	+/-	-/-
Fam3	P-114-27-68-178	96.88	96.22	99.25	96.51	+/-	-/-
Fam3	P-114-27-68-176	96.88	92.70	99.41	96.42	+/-	-/-
Fam3	P-114-27-73-181	96.88	96.03	99.47	96.32	+/-	-/-
Fam3	P-114-27-73-192	96.88	96.22	98.82	96.31	+/-	-/-
Fam3	P-114-27-73-189	96.88	95.21	97.33	95.58	+/-	-/-
Fam3	P-114-27-73-71	96.88	95.47	97.76	95.21	+/-	-/-
Fam3	P-114-27-73-61	96.88	99.12	98.99	95.19	+/-	-/-
Fam3	P-114-27-68-53	96.88	95.47	98.40	95.02	+/-	-/-
Fam3	P-114-27-73-62	96.88	93.39	96.63	94.56	+/-	-/-
Fam4	P-126-45-103-81	96.88	96.35	99.57	95.27	+/-	-/-
Fam5	P-170-50-121-107	96.88	89.92	86.81	97.34	+/-	-/-
Fam5	**P-170-50-121-103**	96.88	89.92	89.05	97.24	**+/+**	-/-
Fam5	P-170-51-130-115	96.88	98.55	99.15	96.14	+/-	-/-
Fam5	**P-170-50-121-99**	96.88	87.53	80.40	95.11	**+/+**	-/-

+/+, mutant homozygous; +/-, heterozygous; -/-, wild-type homozygous. Zygosity of *TtBH-B1* of each plant was determined from the graphical genotyping as shown in Table 3-5.

3.4.3 Estimation of the introgressed segment

During the development of NILs, the drag over effect from the donor parent is expected. This is mainly due to less chance of recombination event as the introgressed fragment size is getting smaller beyond a certain level. Suppression of recombination event in the genome can also leave donor fragments elsewhere in the genome of the recurrent parent. Hence, the introgressed donor segment has been estimated in all BC4F2 plants. Generally, the size of the introgressed segment in NILs ranged from 2.28 - 37.58 cM (Table 3-4). Fam1 plants carry the smallest donor fragment (2.3 cM). Graphical genotyping also clearly showed that the F1 family plants do carry donor fragments from the long arm of chromosome 2A closer to the centromere (Table 3-5 and Figure 3-3A). Besides carrying *QSS.ipk-2AS,* the F1 family plants also carry donor fragment of size 3.5 cM,

presumably carrying *QSS.ipk-2BS.* Introgressed donor segments in selected F1 family plants are shown in Figure 3-3.

Table 3-4. Estimation of introgressed segment for selected BC4F2 plants

Family	Plant ID	Chromosome 2A			Chromosome 2B		
		Proximal Marker (cM)	Distal marker (cM)	Interval (cM)	Proximal Marker (cM)	Distal marker (cM)	Interval (cM)
Fam1	P-69-4-4-122	50.11	52.39	2.28	54.6	58.1	3.5
Fam1	P-69-4-4-121	50.11	52.39	2.28	54.6	58.1	3.5
Fam1	P-69-4-4-126	50.11	52.39	2.28	54.6	58.1	3.5
Fam1	P-69-4-4-127	50.11	52.39	2.28	54.6	58.1	3.5
Fam2	P-103-16-34-31	39.84	52.39	12.55	-	-	-
Fam3	P-114-27-68-176	14.81	52.39	37.58	-	-	-
Fam3	P-114-27-68-178	14.81	52.39	37.58	-	-	-
Fam3	P-114-27-68-53	29.60	52.39	22.79	-	-	-
Fam3	P-114-27-73-181	14.81	52.39	37.58	-	-	-
Fam3	P-114-27-73-189	14.81	52.39	37.58	-	-	-
Fam3	P-114-27-73-192	29.60	52.39	22.79	-	-	-
Fam3	P-114-27-73-61	50.11	52.39	2.28	-	-	-
Fam3	P-114-27-73-62	19.36	52.39	33.04	-	-	-
Fam3	P-114-27-73-69	14.81	52.39	37.58	-	-	-
Fam3	P-114-27-73-71	14.81	52.39	37.59	-	-	-
Fam4	P-126-45-103-81	19.35	52.39	33.04	-	-	-
Fam5	P-170-50-121-103	42.11	52.39	10.28	-	-	-
Fam5	P-170-50-121-99	42.11	52.39	10.28	-	-	-
Fam5	P-170-50-121-107	42.11	52.39	10.28	-	-	-
Fam5	P-170-51-130-115	42.11	52.39	10.28	-	-	-

(-) indicates no donor segment

Table 3-5. Graphical genotyping of selected Fam1 plants and the introgressed donor segments

Marker 2BS_5185931:996 (2BS_5198792:13914) was used to link the position of SS QTL (based on F7 mapping population) to the donor fragment detected in Fam1 plants. 'A' represents Floradur allele; 'B' represents TRI 19165 allele; H represents heterozygous; * shows 'no marker in close proximity '

SS QTL Mapped in F7 mapping population			FL-*bh*-NILs (Fam1 family Plants)							
Name	Flanking Markers	Position	Marker	Position	Floradur	TRI 19165	P-69-4-4-122	P-69-4-4-126	P-69-4-4-121	P-69-4-4-127
			2AS_5203328:4893	49.0	A	B	A	A	A	A
			2AS_5296611:2363	50.1	A	B	A	A	A	A
QSS.ipk-2AS	2AS_5306766:14340 - *	52.4 - *	2AS_5272493:9637	52.4	A	B	B	H	H	H
			2AL_4305348:1350	58.1	A	B	A	H	A	H
			2AL_6396518:3194	59.2	A	B	H	A	H	B
			2AL_6371388:8162	61.5	A	B	A	A	A	A
			2AL_6405678:3964	66.1	A	B	A	A	A	A
			2BS_2330340:2355	50.1	A	B	A	A	A	A
			2BS_5179549:4574	50.7	A	B	A	A	A	A
			2BS_5185477:4076	51.8	A	B	A	A	A	A
QSS.ipk-2BS	2BS_117614:1266	54.64	2BS_2711893:5446	54.6	A	B	A	A	A	A
	2BS_5185931:996	55.2	2BS_5198792:13914	55.2	A	B	H	B	H	H
	(2BS_5198792:13914)		2BS_5162356:2993	55.8	A	B	H	B	H	H
			2BS_5166129:2718	56.9	A	B	H	B	H	H
			2BS_5202174:713	58.1	A	B	A	A	A	A

Note that marker positions are based on the POPSEQ genetic position

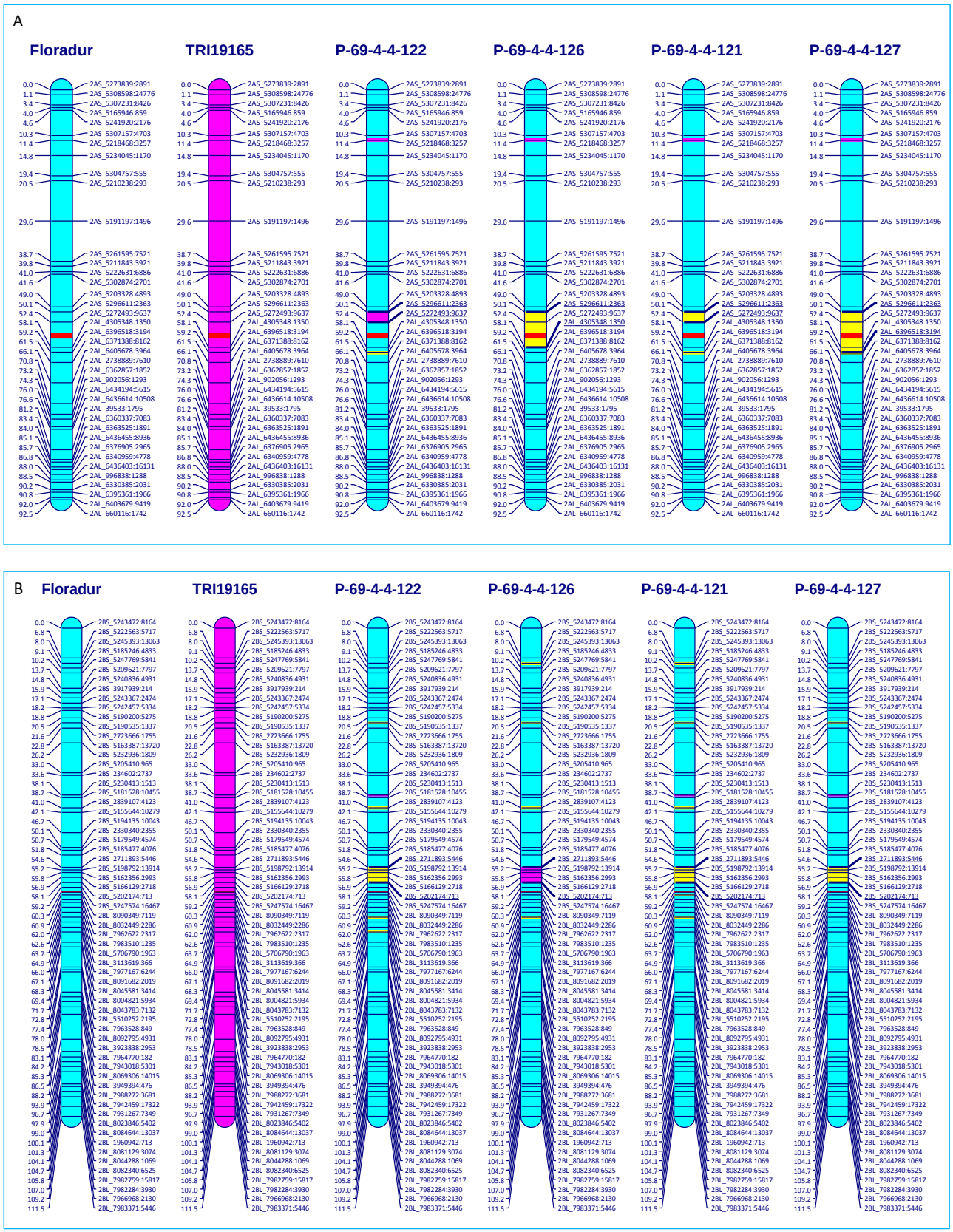


Figure 3-3. Graphical display of introgressed segments of selected Fam1 plants.
(A) Chromosome 2A carrying *QSS.ipk-2AS*. (B) Chromosome 2B carrying donor fragment; presumably carrying *QSS.ipk-2BS*. Markers flanking the introgressed donor segments for both chromosomes are underlined. Blue color depicts Floradur, purple for TRI 19165, yellow for heterozygous. Red line demarcates the short and long arms of each chromosome (centromeric region).

3.4.4 Phenotypic analysis of homozygous BC3F2 plants

Summary of the spike and spike-related traits from 25 homozygous BC3F2 plants, comprising five different families, is shown in Table 3-6. On average, homozygous plants were found to carry 3.42 to 5.10 additional spikelets per spike. The phenotypic penetrance, which was calculated based on the proportion of spikes showing SS (branching phenotype) per plant, ranged from 61.50 to 90.12 %, while expressivity (the proportion of additional spikelet per spike) ranged from 11.83 to 19.92 %. Although SS is a quantitative trait controlled by at least three QTL, i.e. *QSS.ipk-2AS*, *QSS.ipk-2BS* and *QSS.ipk-1AS* (Chapter 2, Table 2-8), *QSS.ipk-2BS* and *QSS.ipk-1AS* were not considered when the development of NILs was initiated. However, after genotyping of all 67 BC4F2 NILs, the Fam1 plants P-69-4-4-121, P-69-4-4-122, P-69-4-4-126, and P-69-4-4-12 were found to carry donor fragment of size 3.5 cM that possibly carries *QSS.ipk-2BS (bh^t-B1* allele; Table 3-5 and Figure 3-3B). Phenotypic analysis in BC3F2 plants showed that these Fam1 plants carry more addSPS, NPS as well as totSPS as compared to Fam2, Fam3, and Fam5 plants carrying only *QSS.ipk-2AS*, clearly indicating the additive effect of both alleles on the expressivity of SS formation (Table 3-6).

Reduced expressivity of SS formation in BC3F2 NILs removed some of the trade-off effects associated with strong branching and spikelet fertility as well as grain weight (Table 2-17). Interestingly, GNS has increased due to the formation of SS (Table 3-6). Hence, controlled expression of SS formation can be targeted in wheat breeding for increasing grain yield with acceptable grain quality. Furthermore, phenotypic data from BC3F2 also revealed some of the pleiotropic effects of the *bh^t-A1* allele, such as SL and NPS (Table 2-14), which are also positively associated with GNS (Table 2-17). Due to branching, FL-*bh*-NILs are also characterized by having a genetically controlled enlarged sink size. The NILs show up to 23.6 % increment in SDW_H (Table 3-6). As sink size is thought to be a yield limiting factor in wheat, the developed NILs will be an excellent genetic material to further assess the source-sink relationship in wheat.

The heading date (HD) was one of the several traits studied in BC3F2 plants. Besides having more addSPS (totSPS), the Fam1 plants were late in heading (Table 3-6). The HD of individual plants within the family ranged from 958 to 1165 ^{0}C days (average of 1060 ±24 ^{0}C days). Graphical genotyping showed that two of these plants, i.e. P-69-4-4-122 and P-69-4-4-127, were found to carry the donor fragment from chromosome 7BS, where the heading date QTL, *QHD.ipk-7BS* (close to *TtFT1-B*), was mapped earlier in the mapping population (Table 2-18). TRI 19165 contributed an allele for lateness (delayed heading), suggesting that these Fam1 plants carry *QHD.ipk-7BS* from TRI 19165 (Supplementary Figure 9-7).

Table 3-6. Summary of spike and spike-related traits from homozygous BC3F2 plants.
The data were generated from 25 different homozygous BC3F2 plants comprising four different plant families: Fam1, Fam2, Fam3, and Fam5. Fam4 was excluded from the analysis due to less number of plants (n=2). Mean ±SEM from all 25 plants as well as the recurrent parent Floradur, and three different spring type mutant checks (replacement for TRI 19165) are included. Number of plants for each group is indicated in the parenthesis. A significance level was calculated based on the unpaired two-tailed Student's *t*-test between the average mean value of all NILs (n=25) and recurrent parent Floradur (n=10) at $P_{0.05}$, * and 0.01,**.

Trait	BC3F2 Families					Floradur (n=10)	Mutant check (n=10)
	Fam1 (n=9)	Fam2 (n=6)	Fam3 (n=4)	Fam5 (n=6)	Mean(n=25)		
SL(cM)	7.34 ±0.14	7.16 ±0.1	7.17 ±0.09	7.17 ±0.08	7.24 ±0.06	6.9 ±0.08 *	9.12 ±0.11
PH(cm)	74 ±2.09	63 ±12.69	77 ±2.96	78 ±1.35	72.56 ±3.2	68 ±7.68 ^{ns}	132 ±3.12
HD	1060 ±24.39	995 ±37.67	927 ±19.5	932 ±16.89	992 ±17	959 ±32.29 ^{ns}	1111 ±23.75
NPS	16.93 ±0.26	15.7 ±0.27	15.43 ±0.88	15.85 ±0.31	16.14 ±0.22	14.47 ±0.2 **	23.04 ±0.29
addSPS	5.1 ±0.45	3.41 ±0.44	3.37 ±0.97	3.42 ±0.41	4.02 ±0.3	0	60 ±1.44
totSPS	22.04 ±0.53	19.11 ±0.43	18.8 ±1.73	19.27 ±0.56	20.15 ±0.45	14.47 ±0.2 **	83 ±1.38
Pen%	84.18 ±3.89	90.12 ±4.63	71.75 ±15.43	61.5 ±6.95	77 ±3.88	0	100 ±0
EXP %	19.92 ±1.53	16.32 ±2.21	14.07 ±4.6	11.83 ±2.17	16.18 ±1.27	0	67 ± 2.47
TN	14.25 ±1.58	7.5 ±0.56	10 ±0.91	12 ±1.44	11.3 ±0.84	9.8 ±1.27 ^{ns}	8.2 ±2.5
SDW	2.97 ±0.13	3.14 ±0.15	3.02 ±0.36	2.85 ±0.1	3 ±0.08	2.54 ±0.07 **	3.73 ±0.22
GNS	44.08 ±1.73	48.42 ±2.5	47.25 ±5.43	39.65 ±2.32	44.57 ±1.4	39.57 ±0.91 *	67.74 ±5.61
GPS	2 ±0.07	2.53 ±0.1	2.5 ±0.07	2.05 ±0.09	2.21 ±0.06	2.74 ±0.17 **	0.97 ±0.14
TKW(g)	50.04 ±0.94	47.55 ±0.83	48 ±0.81	54.78 ±1.45	50.25 ±0.76	50.74 ±2.89 ^{ns}	41.1 ±1.49

SL, Spike Length; PH, Plant Height; HD, Heading Date; NPS, Node Per spike; addSPS, additional spikelet per spike; totSPS, total spikelet per spike; Pen, Penetrance; EXP, Expressivity; TN, Tiller Number; SDW, spike Dry Weight; GNS, Grain Number per Spike, GPS, Grain Number Per spikelet; TKW, Thousand Kernel Weight

3.5 Discussion

Wild relatives of several domesticated crops carry important alleles related to yield, drought and disease resistance, which could be transferred to an elite cultivar through backcrossing (Villareal et al., 1995; Fedak, 1999; Zamir, 2001; Placido et al., 2013; Wendler et al., 2015). It can also be used to develop modern cultivars with desirable traits through gene pyramiding or combining several genes into a single genomic background (Liu et al., 2000; Barloy et al., 2007; Fukuoka et al., 2015). Thus, marker-assisted backcrossing (MAB) is a powerful tool for introducing the gene of interest for functional analysis. It is especially more useful for monocots (grasses), which are often recalcitrant to transgenic approach.

The current study mainly aimed at introgressing *QSS.ipk-2AS* from TRI 19165 into the German elite durum wheat variety Floradur. After four generations of backcrossing of the F1 hybrids obtained by crossing Floradur and TRI 19165, *QSS.ipk-2AS* was successfully introgressed into Floradur genomic background, which is a semi-dwarf modern durum wheat variety used for pasta making industry. Twenty different NILs carrying the *QSS.ipk-2AS* with a donor fragment size ranging from 2.3 to 37 cM were identified at the BC4F2 generation (Table 3-4). Previous mapping result showed that the *bh^t-A1* allele has been physically mapped to one of the gene-rich regions of chromosome 2A (Dobrovolskaya et al., 2009; Poursarebani et al., 2015), suggesting that the region is most likely amenable for recombinational events. Different studies in wheat have suggested that recombination, which is known to be a non-random event and unevenly distributed across chromosomes, is higher in gene-rich regions (Gill et al., 1993; Faris et al., 2000; Sandhu and Gill, 2002b; Sidhu and Gill, 2005). Thus, one or two further backcrossing may shorten the introgressed segment bellow 2.3cM.

Significant phenotypic difference between Floradur and homozygous BC3F2 NILs was obtained under field conditions for SL, NPS, GPS, SDW_H (Table 3-6). Although TRI 19165 is known for strong spike-branching (both in terms of phenotypic penetrance and expressivity), SS formation in the BC3F2-NILs is mainly restricted to the development of additional spikelets per spike from the base of the spike, suggesting that although the locus/loci were successfully introgressed into the target genome of Floradur, phenotypic expressivity and penetrance of SS formation is highly reduced in a Floradur background (Figure 3-2 D).

One of the most probable reasons for the reduction in expressivity and penetrance of SS formation is the genome background effect. Floradur is an elite durum wheat variety which has been developed through wheat breeding. Thus, all spike-related traits have gone through constant

selection during trait improvement, and hence, spike-related loci might exert a strong branch suppression effect. Furthermore, SS formation and expressivity are also under the influence of yet unknown environmental factors either positively or negatively.

For several years, yield increment in wheat mainly depended on improving the harvest index (Austin et al., 1980). However, in order to further increase grain yield in wheat other option were also suggested (Reynolds et al., 2009; Foulkes et al., 2011; Parry et al., 2011; Reynolds et al., 2011). Increasing grain yield by increasing spikelet number could be also another alternative option. Even though spike-branching negatively affects spikelet fertility and grain weight, controlled expression of spike-branching i.e. by reducing the branching intensity; one could solve the tradeoff between strong spike-branching and spikelet fertility and associated grain traits (Table 2-17). Thus, because of the reduction of branching intensity (expressivity) in the FL-*bh*-NILs, the trade-off between strong spike-branching and spikelet fertility affecting GPS and TKW has been significantly reduced (Table 3-6) highlighting the possibility of using the TRI 19165 alleles for increasing spikelet number and grain yield in wheat.

Sink strength is believed to be a critical yield limiting factor in wheat (Fischer, 1985; Slafer and Savin, 1994; Richards, 1996; Borrás et al., 2004; Reynolds et al., 2007; Foulkes et al., 2011). Improving sink size is, therefore, one of the major targets for increasing grain yield in wheat. In this regard, FL-*bh*-NILs would be an ideal wheat ideotype with increased sink size to further study source-sink relationship and grain yield determination in wheat. Besides having increased sink size, FL-*bh*-NILs are semi-dwarf carrying the reduced plant height gene (most likely, *Rht-B1b*), which make FL-*bh*-NILs interesting genetic materials to study the combined effect of both loci on spike architecture and floret fertility in wheat. Furthermore, having already combined *Rht* and *bh* loci, FL-*bh*-NILs could also be used as the first genetic material for gene pyramiding towards the development and testing of IWPA as defined by CM Donald (Donald, 1968).

4 Chapter 4: Towards the Development of Ideal Wheat Plant Architecture

4.1 Introduction

Plant architecture is defined as the three-dimensional configuration of all plant body parts. Traits which define shoot architecture include tiller number, tiller angle, leaf number, leaf erectness, plant height and inflorescence architecture (Figure 4-1). Although plant architecture is affected by different environmental factors including light, temperature, and nutritional status; it is the genetic component that predominantly controls plant architecture. Although several genes have been identified in this regard, especially in rice (Springer, 2010; Lu et al., 2013; Tanaka et al., 2013), only a few are known in wheat. Nevertheless, hypothetical wheat plant architecture has been proposed by CM Donald in 1968 (Donald, 1968). Plant height and spike architecture were some of the key traits used by Donald to define IWPA. Hence, reduced height (*Rht*) and the branching head (*TtBH/WFZP*) genes play important role in creating and further testing IWPA. Furthermore, being mono/single culm was also another trait used by Donald to define IWPA. Although the underlying gene has not yet been identified, the *tiller inhibition (tin)* locus will also be another addition to the gene list towards creating the proposed wheat architecture.

The main focus of this study was spike architecture in tetraploid wheat. According to Donald, IWPA should also have a 'large' and fertile spike. Several other studies have also suggested that grain yield in wheat is rather sink-limited suggesting the need to have an enlarged, and fertile spike (Ma et al., 1990; Slafer and Savin, 1994; Borrás et al., 2004).

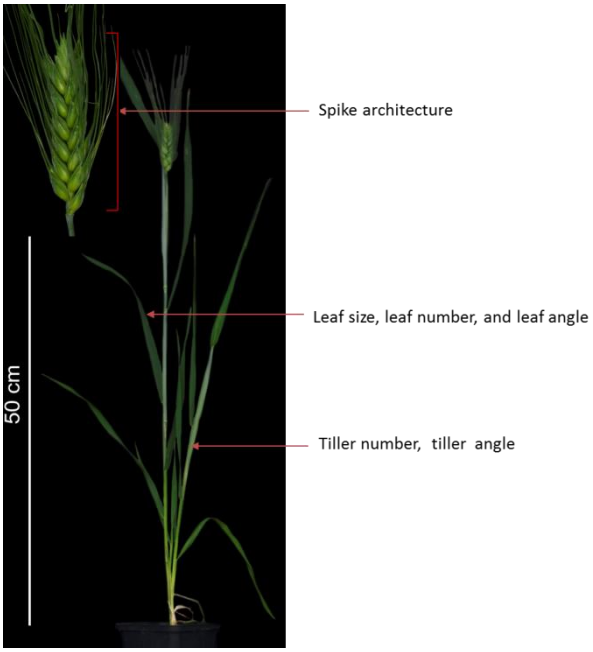


Figure 4-1. Traits used to define shoot architecture of the wheat plant

4.2 Objectives

The objectives of this chapter are: firstly, to study the effect of source-sink manipulation on spikelet fertility between spikelets sharing rachis node using FL-*bh*-NILs (at BC3F3 generation). Secondly, to explore the effect of mono/single culm on wheat spikelet fertility by phenocopying the mono/single culm characteristic of the *tin* wheat mutant through de-tillering of FL-*bh*-NILs; and thirdly, to propose a hypothetical model for assimilate distribution in wheat spikelet/floret.

4.3 Materials and Methods

The experiment was laid down under controlled greenhouse conditions. Grains from each of the five homozygous BC3F2 family plants (Table 3-6) were used for this study. Seven seeds per family were germinated in 96 well trays under controlled long day conditions: 16/8 hours day/night and 19/17 ^{0}C day/night temperatures for 15 days. After 15 days of seedling establishment, plants were vernalized for about four weeks at 4^{0}C. After one week of hardening at 15/12 ^{0}C day/night temperature, each seedling was transferred to a pot of size 0.5 liter filled with substrate2 (Klasmann-Deilmann GmbH, 49744 Geeste, Germany), compost and sand by a proportion of 2:2:1 respectively. Each plant received 10g of NPK fertilizer called Plantacote® Depot 4M (Wilhelm Haug GmbH & Co. KG Postfach, Düsseldorf, Germany) week after potting. The experiment was laid down in three replicates (35 plants for each replicate) for both treatments: free-tillering (FT) and De-tillering (DT). Tillers were removed four times a week (every the other day interval). Each plant/pot was randomly arranged and received all the standard treatments (light, water, fertilizer, disease and pest controls) equally. Traits such has tiller number(TN), HD, PH, peduncle length (PdL), SL, SDW_H, NPS, addSPS, totSPS, GNS, TKW, GA, GL and GW from the main culm were recorded from both treatments. All grain related traits from SS and standard adjacent spikelet sharing the same rachis node (Figure 4-2) were analyzed separately. The aim was to make a positional comparison of grain development and spikelet fertility between SS and the standard adjacent spikelet. This will give a better understanding of the variation in spikelet fertility in wheat.

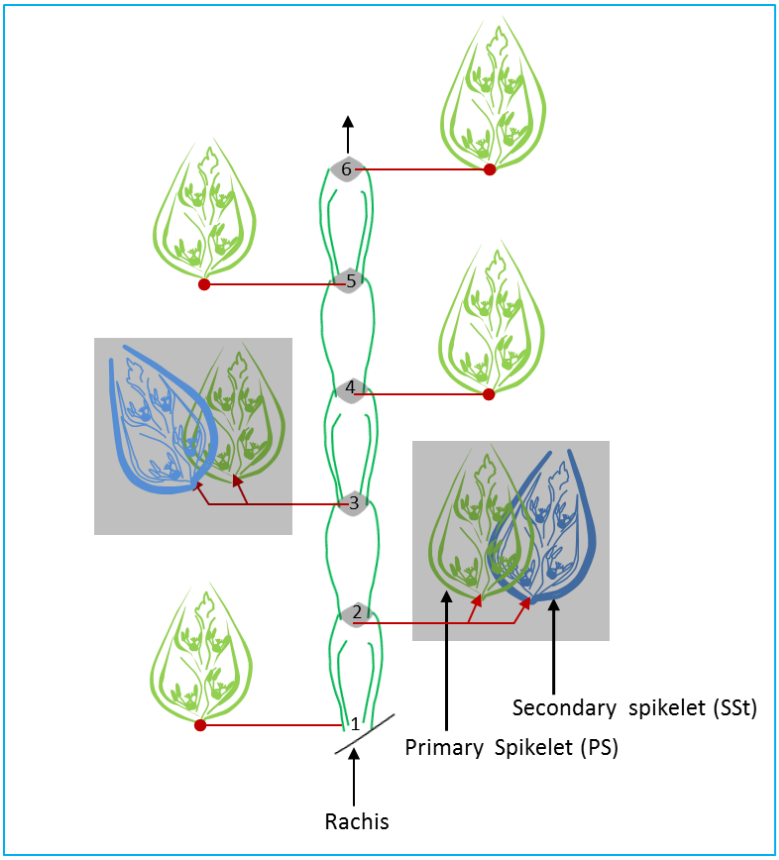


Figure 4-2. Illustration of wheat spikelet arrangement and SS formation in the BC3F3 NILs.
In a standard wheat spike, spikelets are arranged in a distichous fashion where a single spikelet is attached to each rachis node(numbered); whereas, in the case of BC3F3 NILs, the spike carries two or more spikelets sharing the same rachis node (shaded region).

4.4 Results

4.4.1 Plant growth and development after de-tillering

De-tillerd and control plants significantly differed in several traits (Table 4-1). Traits which showed significant differences between FT and DT plants includes PH, PdL, SL, SDW_H, GPS, and GNS. This also holds true for the recurrent parent Floradur and spike-branching mutant check lines. The major difference in plant height was due to the difference in PdL, which showed significant differences between the FT and DT plants. Plant height without spike and peduncle length did not show significant difference; indicating that DT plants invested more resources for peduncle and spike development. Analysis of tiller fertility (TF) in FT plants showed that about 16% of tillers at maturity were not fertile at all i.e. none of them developed any spike. Even if they are fertile, the size of the developed spikes from tillers was small.

Table 4-1. Summary of the effect of de-tillering on plant growth and development.
The values represent Mean ± SD. Significant level between FT and DT was calculated based on a two-tailed Student's *t*-test analysis: at P-value of 0.05

Trait	Treatment	BC3F3 Families					Floradur (Recurrent parent)	Spike-branching Mutant (check)
		Fam1	Fam2	Fam3	Fam4	Fam5		
TN	FT	4.63 ±0.44	4.05 ±0.76	4.31 ±0.55	3.98 ±0.37	4.52 ±0.81	4.02 ±0.39	2.95 ±0.66
	DT	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TF(%)	FT	70 ±19	87 ±4	88 ±9	90 ±10	87 ±8	90 ±13	61 ±20
	DT	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PH(cm)	FT	62.21 ±3.41 *	65.4 ±2.34 *	65.6 ±2.25 *	66.5 ±2.98 *	61.8 ±1.96 *	63.1 ±3.46 *	118.05 ±8.06 *
	DT	66.75 ±4.96	72.42 ±0.82	76.42 ±2.1	72.12 ±2.78	67.62 ±3.41	71.55 ±2.32	132.17 ±4.72
PdL (cm)	FT	25.59 ±1.5 ns	33.33 ±3.2 *	33.42 ±2.26 *	31.15 ±3.94 *	30.08 ±1.4 *	33.57 ±2.35 *	48.6 ±5.96 *
	DT	27.86 ±5.9	39.5 ±1.31	42.63 ±1.18	36.88 ±2.95	34.14 ±2.72	41.75 ±1.69	55.48 ±6.71
SL(cm)	FT	7.49 ±0.55 ns	6.71 ±0.17 *	6.52 ±0.34 *	6.46 ±0.35 *	6.96 ±0.14 *	6.78 ±0.19 *	9.14 ±0.3 *
	DT	7.74 ±0.41	7.33 ±0.19	7.26 ±0.17	7.41 ±0.25	7.53 ±0.26	7.45 ±0.33	10.24 ±0.33
PH_wPdL (cm)	FT	29.12 ±3.18 ns	25.36 ±2.29 ns	25.66 ±3.14 ns	28.89 ±3.59 ns	24.76 ±0.99 ns	22.74 ±2.15 ns	60.31 ±5.28 *
	DT	31.14 ±3.88	25.59 ±1.36	26.53 ±2.49	27.83 ±1.68	25.95 ±2.63	22.35 ±1.74	66.45 ±4.18
SDW_H (g)	FT	2.46 ±0.21 *	2.74 ±0.25 *	2.95 ±0.35 *	2.8 ±0.34 *	2.63 ±0.17 *	2.1 ±0.27 *	4.33 ±0.69 *
	DT	3.65 ±0.76	3.84 ±0.23	4.03 ±0.36	3.53 ±0.4	3 ±0.49	2.99 ±0.13	6.31 ±0.59
NPS	FT	20.38 ±2.7 ns	16.47 ±1.35 ns	17.12 ±1.89 ns	17.31 ±1.96 ns	17.69 ±1.28 ns	13.33 ±1.9 ns	24.14 ±0.75 ns
	DT	20.13 ±2.89	17.14 ±0.94	17.36 ±1.47	17.60 ±1.61	17.5 ±1.14	13.26 ±1.77	25.02 ±0.75
GNS	FT	47.13 ±3.84 *	47.71 ±4.63 *	49.21 ±5.92 *	46.14 ±5.51 *	44.07 ±4.1 *	37.93 ±3.7 *	74.93 ±18.01 *
	DT	59.06 ±11.4	69.12 ±4.8	68.5 ±5.05	62.52 ±9.35	52.43 ±13.33	52.64 ±5.39	105.19 ±14.29
addSPS	FT	7.36 ±1.41 ns	5.45 ±0.91 ns	6.52 ±1.53 ns	4.88 ±0.94 ns	6.1 ±1.45 ns		66.86 ±7.4 ns
	DT	7.33 ±1.83	4.95 ±0.36	5.19 ±1.26	4.53 ±0.84	5.1 ±1.84		68.5 ±14.87

TN,Tiller per plant; TF, Tiller Fertility %; PH, Plant height (cm); PdL, Peduncle Length (cm); SL, Spike Length (cm); PH_wPdL, Plant height without peduncle length; SDW_H, Spike Dry Weight at harvest (g); NPS, Node Number Per Spike; GNS, grain Number per spike; addSPS, additional spikelet per spike; FT, Free tillering; DT, De-tillered.

Table 4-1. Continued...

Trait	Treatment	BC3F3 Families					Floradur (Recurent parent)	Spike-braching head Mutants (check)
		Fam1	Fam2	Fam3	Fam4	Fam5		
totSPS	FT	27.74 ±3.89 ns	21.92 ±2.11 ns	23.64 ±3.01 ns	22.19 ±2.39 ns	23.79 ±2.42 ns	13.33 ±1.9 ns	91 ±7.52 ns
	DT	27.46 ±2.77	22.09 ±1.15	22.55 ±2.42	22.13 ±2.14	22.60 ±2.36	13.26 ±1.77	93.52 ±15.25
GPS	FT	1.74 ±0.41 *	2.29 ±0.23 *	2.12 ±0.18 *	2.21 ±0.21 *	1.85 ±0.18 *	2.87 ±0.28 *	0.82 ±0.19 *
	DT	2.44 ±0.23	3.13 ±0.22	3.1 ±0.26	2.88 ±0.3	2.57 ±0.26	4.01 ±0.46	1.13 ±0.06
GPS_PS	FT	2.17 ±0.38 *	2.8 ±0.31 *	2.77 ±0.2 *	2.84 ±0.25 *	2.27 ±0.19 *	-	
GPS_SSt	FT	0.87 ±0.21	1.62 ±0.15	1.63 ±0.26	1.77 ±0.21	1.57 ±0.29	-	
GPS_PS	DT	2.78 ±0.45 *	4.14 ±0.38 *	3.64 ±0.31 *	3.54 ±0.5 *	2.93 ±0.66 *	-	
GPS_SSt	DT	1.65 ±0.54	2.81 ±0.34	2.85 ±0.42	2.73 ±0.41	2.26 ±0.66	-	
TKW_PS (g)	FT	51.38 ±5.87 *	48.02 ±2.02 *	46.19 ±4.78 *	50.04 ±4.88 *	48.45 ±5.5 *	-	
TKW_SSt (g)	FT	41.2 ±5.78	37.63 ±2.28	38.37 ±2.57	40.79 ±4.61	41.08 ±3.82	-	
TKW_PS (g)	DT	58.79 ±8.27 *	45.1 ±2.64 *	47.76 ±2.89 *	45.77 ±2.73 *	49.8 ±5.16 *	-	
TKW_SSt (g)	DT	49.15 ±4.29	39.63 ±4.32	42.52 ±2.78	40.75 ±2.36	42.28 ±5.67	-	
TKW (g)	FT	48.26 ±3.01 *	42.77 ±1.29 ns	42.48 ±2.53 ns	47.17 ±3.22 *	44.98 ±4.08 ns	41.8 ±3.41 ns	44.97 ±5.37 ns
	DT	53.49 ±6.54	41.65 ±2.99	43.73 ±3.52	42.06 ±2.94	43.78 ±3.76	43.32 ±2.44	47.06 ±3.42
Chaff	FT	0.65 ±0.36 *	0.68 ±0.05 *	0.87 ±0.15 *	0.72 ±0.09 *	0.64 ±0.12 *	0.53 ±0.05 ns	1.14 ±0.35 ns
	DT	1.03 ±0.13	0.95 ±0.11	1 ±0.11	0.95 ±0.05	0.89 ±0.54	0.64 ±0.16	1.41 ±0.17
SHI	FT	0.69 ±0.06 ns	0.75 ±0.02 ns	0.70 ±0.07 ns	0.74 ±0.03 ns	0.76 ±0.05 ns	0.76 ±0.01 ns	0.79 ±0.02 ns
	DT	0.73 ±0.03	0.75 ±0.03	0.75 ±0.02	0.73 ±0.02	0.77 ±0.08	0.79 ±0.05	0.78 ±0.01
ND	FT	2.71 ±0.24 ns	2.32 ±0.24 ns	2.58 ±0.28 ns	2.53 ±0.3 ns	2.57 ±0.19 *	1.96 ±0.26 *	2.64 ±0.08*
	DT	2.59 ±0.31	2.34 ±0.15	2.38 ±0.22	2.4 ±0.22	2.32 ±0.21	1.77 ±0.17	2.45 ±0.06
HD (days)	FT	97.2 ±5.22 ns	90.55 ±1.2 ns	89.06 ±0.85 ns	90.57 ±1.23 ns	91.5 ±1.66 ns	88.79 ±0.39 ns	105.7 ±2.39 *
	DT	99.47 ±4.8	91.69 ±0.85	90.24 ±1.1	92.43 ±1.92	91.64 ±0.8	88.38 ±0.49	102.5 ±5.72

totSPS, total spikelets per spike; GPS, grain Number per spikelet; PS,Primary Spikelets; SSt, Secondary Spikelets; TKW, thousand Kernel Weight (g); SHI, Spike Harvest Index; ND, Node Density; HD, Heading Date; FT, Free tillering; DT, De-tillered.

4.4.2 Response of spike development after de-tillering

Spike traits such as SL (without awns), SDW_H, NPS, addSPS, and totSPS were analyzed. Except for SDW and SL, which showed a significant difference between FT and DT plants, no significant difference was detected for the other traits, indicating that de-tillering did not affect the number of organs formed (Table 4-1). However, de-tillering significantly affected spikelet fertility i.e. GPS. Hence, the effect of de-tillering on SDW_H was mainly attributed to the development and fertility of the spikelets. Although the spike length in the DT plants has significantly increased as compared to the FT plants, there was no additional spikelet as a result of de-tillering. Hence, ND (number of node per unit length of the spike) in the DT group was lower as compared to the FT plants, indicating that de-tillering also somehow accelerated internode development. This has contributed to higher chaff weight in DT plants. Nevertheless, there was no significant difference in SHI, which was calculated as the ratio of grain weight to SDW_H. This suggests that the accumulated biomass has not been completely converted into harvestable grain yield.

4.4.3 Position-based spikelet fertility in response to de-tillering

Source-sink manipulation in wheat was mainly based on induced treatments without genetically altering the source-sink relationship. In the current study, although de-tillering was an induced treatment, BC3F3 plants (whose sink-source relationship has been affected due to the introgressed *bh* allele(s)) were used. The major target was to investigate the capacity of the source in sustaining the increased sink size; and secondly, to further analyze the effect of assimilate distribution within/between the primary (PS) and secondary spikelets (SSt) sharing the same rachis node on the spike (Figure 4-2). This is because PS and SSt are attached to a similar nutrient source; which in this case is the rachis. Hence, comparison was made between PS and SSt for spikelet fertility and the grain weight. Grain number per spikelet or GPS was used as an indicator for spikelet fertility in both treatments, while TKW was used as a measure for grain quality parameter (Table 4-1). Interestingly, GPS and TKW between PS and SSt i.e. GPS-PS and GPS-SSt showed highly significant differences in both treatments i.e. FT and DT plants. GPS for the SSt i.e. GPS-SSt was always below the GPS from PS i.e. GPS-PS; indicating that the SSt are less fertile and grain development was also affected as compared to those from the PS although both of them are connected to similar assimilate source. Hence, PS are more favored as compared to SSt. However, as more nutrients are available (due to de-tillering), spikelet fertility of SSt in the DT plants i.e. GPS-SSt increased by 65% as compared to SSt in the FT plants, suggesting that spikelet

infertility in the SSt was partly affected by source related problems. Generally, DT plants showed enhanced plant growth and development including spikelet fertility due to the allocation of more nutrients to the main culm, which otherwise was used by tillers.

4.4.4 Effect of de-tillering on grain development and grain quality

Grain size is one of the determinant factors of grain yield in wheat and it is tightly controlled by genetic factors. Grain size is mainly determined by the three-dimensional configuration of the grain (i.e. grain length, width, and area) and the degree of grain filling (plumpness). Grain filling is largely accompanied by the transport of nutrients, mainly sucrose from source to the endosperm and accumulation of the storage compounds in the sink (Zuo and Li, 2014). Furthermore, grain development largely depends on the source, sink, and translocation capacity of assimilates from the source (Ma et al., 1990; Slafer and Savin, 1994; Yu et al., 2015). Besides spikelet fertility, grain weight was also analyzed between FT and DT plants (Table 4-1). The overall TKW from the whole spike did not show any significant differences between FT and DT plants. This might be due to the negative relationship between grain number and grain weight (Pinthus and Millet, 1978; Slafer and Miralles, 1993; Gambín and Borrás, 2010; Griffiths et al., 2015). Nevertheless, TKW between the PS and SSt i.e. TKW_PS and TKW_SSt were highly significant both in the FT and DT plants. This clearly shows that besides the variation in spikelet fertility between the PS and SSt, there also exists a variation in grain development even though both are connected to similar assimilate source through the shared rachis node; suggesting certain assimilate allocation and distribution criteria between the PS and SSt. Furthermore, the process seemed to be a dynamic process where the allocation and distribution process also depends on other factors like nutrient availability. Millet (1986) showed a significant positive correlation between the size of floret cavity and the grain weight in wheat (Millet, 1986a). In this regard, it is worthwhile to study the floral development between PS and SSt. Furthermore, factors within the grain itself should not be completely ignored as they also influence grain size and shape. For example, there are several correlative evidence showing the type and volume of the starch granule with the endosperm cell number and weight as well as sucrose synthase activity in wheat (Wardlaw, 1990).

4.4.5 Response of the peduncle growth after de-tillering

Peduncle is the last segment of the wheat culm. In this study, peduncle length was measured from the base of the flag leaf sheath until the base of the spike. Data analysis showed that Peduncle length in the DT plants was significantly longer as compared to FT plants (Table 4-1). This indicates

that enhanced peduncle growth in the DT plants was due to the allocation of more assimilates, which otherwise were invested on side branches. Besides its role in photosynthesis (Kong et al., 2010), wheat peduncle plays a major role in assimilate storage and transport (Gebbing, 2003) suggesting that the enhanced peduncle development in DT plants might have played the major role in spike development and fertility. Earlier research also showed that the exposed part of peduncle play role in buffering grain development during grain-filling (Kong et al., 2010) besides maintaining more water suggesting its role during drought and heat stress conditions (Wardlaw, 2002; Modarresi et al., 2010).

4.4.6 Benefits of combining *tin* and *bh* loci

Improved grain setting ability of the SSt in DT plants suggests the importance of having mono/single culm (reduced tillering) wheat ideotype. Being monoculm was also one of the traits used to define the hypothetical wheat plant ideotype by CM Donald (Donald, 1968). Although there might be a strong argument that fertile tillers can also contribute to grain yield; having monoculm wheat ideotype can also be assessed from several points of views. The first advantage of having mono/single culm wheat ideotype is linked to the very nature of tiller growth and development i.e. most tillers do not complete their life cycle (hardly produce ears). This is due to the aggressive competition among tillers for nutrients and water. Based on tillers counted from the FT plants after physiological maturity, where most tillers were dead by then, tiller fertility (TF) on average was found to be 84% (Table 4-1) indicating wasteful investment to unproductive tillers. Earlier Studies also suggested that many tillers do not survive to produce spikes (Pinthus, 1967; Masle, 1985; Davidson and Chevalier, 1990; Sharma, 1995). For example, winter wheat produces more tillers as compared to spring wheat before heading, but attain the same number of tillers with spring wheat at the end of the growing season (Pinthus, 1967); clearly indicating that only a few number of tillers complete their life cycle and contribute to the final grain yield in wheat. Furthermore, it has been suggested that tiller abortion in wheat and barley also coincides with rapid stem and spike development and elongation phases suggesting the strong connection of tiller abortion or death with critical phases of spike development (Mohamed and Marshall, 1979; Kemp and Whingwiri, 1980; Fraser et al., 1982; Davidson and Chevalier, 1990). Therefore, the key question still remained to be answered is: how many tillers (if any at all) should a wheat plant have for optimum yield?

So far, no single gene has been identified in wheat controlling tillering. However, a reduced tillering wheat mutant line (*tin* wheat mutant) has been agronomically characterized (Duggan et al., 2005a; Duggan et al., 2005b; Kebrom et al., 2012; Mitchell et al., 2013; Gaju et al., 2014; Hendriks et al., 2016) and mapped (Spielmeyer and Richards, 2004; Kuraparthy et al., 2008). Generally, it has been shown that the *tin* wheat mutant is known for the increased spike partitioning index (accumulation of spike dry matter); increased grain number per spike, higher harvest index, reduced plant height, an increased duration of the flag leaf and increased interception of photosynthetically active radiation which possibly linked with leaf architecture (Richards, 1988; Gaju et al., 2014). In the current experiment, the *tin* mutant phenotype i.e. mono/single culm phenotype was phenocopied through de-tillering i.e. the DT plants. Hence, the observed significant differences in traits between FT and DT plants (such as PdL, GPS, SL, GNS, SDW_H) clearly show the advantage of having monoculm wheat ideotype (Table 4-1). The increased spikelet fertility of SSt (GPS_SSt) after de-tillering also highlighted the benefit of combining *tin* and *bh* loci into a single genomic background for further yield trials; especially from the view of creating and testing IWPA as defined by CM Donald (Donald, 1968).

4.5 Discussion

Plants require dynamic coordination of several signal transduction pathways between the sources (plant organs capable of producing photosynthate in excess of their own needs) and sinks (plant organs that do not produce enough photosynthate to support their own growth or storage needs). Sink strength, which is the competitive ability of a sink organ to attract assimilates, is believed to be a critical yield limiting factor in wheat (Rawson, 1970; Slafer and Savin, 1994; Richards, 1996; Reynolds et al., 2000; Borrás et al., 2004; Miralles and Slafer, 2007; Foulkes et al., 2011) which is generally affected by sink size and activity (Marcelis, 1996; White et al., 2016). Hence, improving sink size and strength remained to be one of the major targets for increasing grain yield in wheat. To increase sink size, several options were suggested. The first one is improving the spike index, which is the ratio of grain mass to the total spike biomass (Dencic and Borojevic, 1992; Hucl and Graf, 1992). For this, increasing assimilate partitioning to the developing spike has been suggested as one of the strategies (Austin et al., 1980; Reynolds et al., 2007). The other suggested strategy for increasing spike biomass is to increase the relative duration of spike growth phase (Slafer et al., 1996). This is because spike length and spikelet number are positively associated with spike growth phase during which spikelet number is determined (Rawson, 1970; Fischer, 1985). Increasing fruiting efficiency of the spike, which is the number of grains set per unit of spike dry weight at anthesis (Slafer and Miralles, 1993), was also suggested as an alternative to increase spike fertility wheat(Ferrante et al., 2015; Slafer et al., 2015; Elía et al., 2016; Terrile et al., 2017). Improving the overall radiation use efficiency was also among the suggested strategies (Reynolds et al., 2000). This requires improving the photosynthetic capacity of the canopy by manipulating leaf architecture (Sakamoto et al., 2006).

During the vegetative growth phase, water soluble carbohydrates mainly fructan and sucrose are the major forms of storage carbohydrate in photosynthetically active organs of wheat (Scofield et al., 2009). These sugars are also believed to buffer grain yield as a long-term storage reserve. Although starch accumulation is believed to be rare during vegetative growth, Scofield *et.al* was able to detect starch accumulation in the flag leaf, penultimate internodes and the enclosed part of the peduncle suggesting the concomitant re-utilization of starch during peduncle growth and during the early development phases of the reproductive structures. Therefore, the availability of more assimilates in the DT plants might have played the major role in buffering all developmental programs in the peduncle as well as the spike as compared to the FT plants, where resources are relatively limiting (Table 4-1). In another study, Gebbing reported that fructans mainly accumulate

in the covered (lower) section of the peduncle while sucrose accumulates in the exposed part of the peduncle at mid grain-filling stage (Gebbing, 2003). In short, starch, fructans, and sucrose can be readily re-utilized for peduncle and spike growth. Hence, enhanced peduncle and spike growth in DT plants were due to the availability of more assimilates to buffer all developmental programs in these organs.

Although several physiological investigations were made in a source-sink relationship in wheat; as such there was no genetic manipulation to further dissect the sink-source relationship in wheat. Hence, FL-*bh*- NILs are valuable genetic materials to further dissect the source-sink relationship in wheat. Due to SS formation or spike-branching, FL-*bh*- NILs have enlarged sink (spike) size controlled by *QSS.ipk-2AS* and *QSS.ipk-2BS.* Spike-branching is associated with reduced spikelet fertility (see Table 2-16) which might be due to development related problems or simply due to competition effect. However, the improvement in spikelet fertility after de-tillering suggest that besides the generally accepted sink limitation in wheat (Rawson, 1970; Slafer and Savin, 1994; Richards, 1996; Reynolds et al., 2000; Borrás et al., 2004; Miralles and Slafer, 2007; Foulkes et al., 2011; Guo and Schnurbusch, 2015), there might also be source related problem which might be related to translocation and/or distribution problems. Although sucrose translocation from the source to the sink tissues takes place via the phloem sieve elements, it is believed to happen as bulk/mass movement (van Bel and Hafke, 2005; Ham and Lucas, 2014). Pressure gradient force created by the turgor pressure differences between source and sink ends of each sieve tube elements drives mass movement of sucrose (Ham and Lucas, 2014). However, the process by which plants control assimilate allocation to specific sink organs is largely unknown. Bulk/mass delivery of assimilates from the source to the sink organ, however, is directly proportional to the radius (thickness) of the sieve tubes and inversely proportional to the distance between source and sink (van Bel and Hafke, 2005). Furthermore, the differential priority of assimilate translocation among sink organs also related to the so-called ‘sink strength’ or ‘sink dominance’, which is a function of sink size and sink activity (Ho, 1988; Lemoine et al., 2013). Besides sink size and sink activity, the age of the sing organ has also been suggested as a determining factor for sink strength (Marcelis, 1996). Therefore, more assimilates will be delivered to the metabolically active sink tissues due to the decreased turgor pressure as a result of elevated local consumption of the imported sugars (Ham and Lucas, 2014).

Spikelet differentiation in wheat starts at the center of the spike and then proceeds to the apical and basal part of the spike (Bonnett, 1936); meaning that spikelets/ florets at the center of the

spike are most advanced (Whingwiri and Stern, 1982) and most likely able to divert more assimilates as compared to those at the apex and basal part. Florets within each spikelet also mature acropetally (bottom to top) (Bonnett, 1936); and hence, the first four florets within each spikelet are usually metabolically active (Kirby, 1974; Ghiglione et al., 2008). Thus, these florets are probably strong sink organ as well. A number of studies also indicated that other internal factors such as hormonal homoeostasis, and external factors like water and nutrient availability also affect the extent of assimilate delivery to the sink tissues (Lalonde et al., 1999; Toyota et al., 2001; Lemoine et al., 2013; Albacete et al., 2014; Lin et al., 2014; Yu et al., 2015).

Assimilate allocation to the individual spikelets on the spike appeared to be based on certain assimilate allocation criteria which might be triggered by the internal and/or external factors. This conclusion is made because of the fact that under normal circumstances (free-tillering plants), spikelets adjacent to SS are the most benefited ones in terms of resource allocation even if both of them share the same rachis node (Figure 4-2). Once the availability of more assimilate is sensed, for example after de-tillering, the resource allocation criterion has been improved for both resulting in the improvement of spikelet fertility (Table 4-1). Of course, the vascular anatomy including the vascular bundle size and distribution along the spike and within the spikelets are not well understood in wheat. Therefore, little information is available about the histology and cytology of the vascular bundle in the spike/spikelets. Nevertheless, Whingwiri and Stern (1982) studied the vascular system of the rachis of a wheat spike and suggested that the total number of vascular bundles start to decline acropetally as it goes from the peduncle to the rachis internode (Whingwiri et al., 1981). They further suggested that vascular bundles branch out in the first 4 basal spikelets while those spikelets beyond 4 are equipped with complete vascular bundles with the possibility of branching. The size of the vascular bundles from node 5 to 12 (from the base of the spike) was relatively larger than those vascular bundles assigned to node 13 and beyond; indicating the existence of vascular bundle size variations along the spike (Whingwiri et al., 1981). Thus, they proposed branching of the vascular bundles at every node of the rachis, i.e. one going to the next rachis internode while the other to an immediate spikelet. They further suggested that the vascular system between the rachis and the floret develops as the floret is initiated (Whingwiri et al., 1981; Whingwiri and Stern, 1982).

Interestingly, Zee and O'Brien (1971) also indicated that vascular tissues, where the sterile glumes, lemma, palea and caryopsis are attached to the rachis and rachilla, are surrounded by transfer cells suggesting the role of transfer cells and assimilate translocation in wheat spikelets and floral

structures (Zee and O'brien, 1971). Transfer cells, which are involved in solute transfer are absent from leaf, leaf sheath, internode, rachis, rachilla, sterile glumes, palea, lemma, and pericarp; except at the nodes where sterile glumes, palea, lemma, and pericarp are attached (Zee and O'brien, 1971).

By studying the vascular system of in wheat spikelet, Hanif and Langer (1972) suggested that florets closer to the rachis node, i.e. the basal three florets, were directly supplied by the principal vascular bundles of the rachilla, while the distal florets were subject to sub-vascularization (Hanif and Langer, 1972). This suggests that florets in each spikelet have no equal chance of accessing assimilates from the source. Contrary to this, Bremner and Rawson (1978) did basal grain removal experiment and suggested that the vascular system in the spikelet is adequate; but suggested that the limitation to the distal florets is associated with the maintenance of sufficient assimilates (Bremner and Rawson, 1978). So what determines the assimilate partitioning to each floret in each spikelet?

By studying, old and modern wheat cultivars, under irrigation and high fertility, Fischer and HillerisLambers (1978) concluded that, unlike in some of the old bread wheat and triticale cultivars, grain weight in modern wheat is source limited with a significant environmental effect (Fischer and Hillerislambers, 1978). Similarly, Blade and Baker (1991) studied grain weight in response to source-sink manipulation (through plot thinning at the six-leaf stage or at heading, spikelet and flag leaf removal at anthesis or 8 or 18 days after anthesis) using eight spring wheat cultivars differing in kernel weight and found a significant interaction between cultivar and spikelet removal treatment suggesting that some of the cultivars are sink limited (Blade and Baker, 1991). BY modifying, the source-sink relationship, Slafer and Savin also studied grain mass at different positions within the spike in wheat (Slafer and Savin, 1994). Using two field-grown cultivars, de-tillering at anthesis and spikelet removal after ten days of anthesis, they suggested that grain development under two different environments was largely insensitive to the increase in assimilate availability and concluded that grain yield in wheat is either sink-limited or co-limited by both source and sink but never source-limited. Contrary to this, Cruz-Aguado et al. (1999) modified the source-sink relation of three wheat cultivars through spikelet and flag leaf removal and suggested that grain filling in wheat is source limited (Cruz-Aguado et al., 1999). Therefore, the lack of such inconsistent results highlights the effect of genotypic variations and/or environmental factors in controlling assimilate distribution in wheat spike and/or spikelets (Fischer and Hillerislambers, 1978; Yu et al., 2015; Asseng et al., 2016; Marti et al., 2016). Taking

all together, there is no concrete justification for the cause of floral abortion in wheat, i.e. whether it is due to weak structural connectivity (related to the vascular bundle and sub-vascularization), or it is due to source limitation, or sink limitation or some other unknown factor(s).

Taking into account the carbon assimilation from the source (leaves), sucrose translocation via a phloem network and carbon accumulation in a sink organs, Seki *et.al* proposed a mathematical model for phloem sucrose transport in rice panicle (Seki et al., 2015). In rice panicle, large vascular bundle containing multiple sieve tubes (the cells of the phloem that conduct sugars, amino acids, hormones and other organic materials throughout the plant (Ham and Lucas, 2014), is assigned to each primary branches of the rachis; thus, the number of large vascular bundles and primary branches are the same (Seki et al., 2015). However, it was not clear as to how each of these sieve tubes in the large vascular bundles behaves during panicle branching i.e. whether each of these sieve tubes bifurcates into daughter tubes during panicle branching. For this, they compared the vascular system of plants and animals i.e. the pipe and aorta models (Figure 4-3R, S, T). The comparison was made based on simulation as well as actual data generated from a rice variety. In the pipe model, they have assumed that each sieve tube was considered as an independent (Figure 4-3 S; meaning that there is no bifurcation or exchange of solutes between them at any site. Because the radius of each sieve tube is the same in the pipe model, a shorter distance between the spikelet and source should result in faster grain growth; although it was not to the expectation (Seki et al., 2015). Whereas in the aorta model, which was adopted from blood vessel system in the animal kingdom, a single tube bifurcates into multiple daughter tubes at each branching points (junction) following Murray's law; which states that the radius of the mother tube is equal to the sum of the radii of the two daughter tubes (Figure 4-3 R, T). The two daughter tubes, however, can have different radii based on the 'radius allocation rule' which of course result in asymmetric branching of the tubes. They concluded that the 'radius allocation rule' determines the efficiency of sucrose transport in the aorta model as main sieve tube becomes thinner and thinner after each bifurcation steps, especially during panicle branching which reduces the efficiency of sucrose transport towards the tips of the branches. Hence, they suggested that there exists a unique 'radii allocation rule' that maximizes grain yield in rice panicle. It is unique, because when the allocation of the radius to the main branch is maximum, the apical grains develop sufficiently while basal grain in the secondary branches becomes smaller (due to smaller radii allocated to them i.e. limited sucrose transport); and when two daughter

radii receive the same size, the basal grain on the secondary branch grew well while the upper grain on primary branches do not mature properly as the tubes get thinner and thinner as it gets farther. They also suggested the existence of competition among grains for sucrose in both pipe and aorta models. In the case of pipe model, the competitions are mainly distance-dependent; meaning that shorter distance between the spikelet and source (the leaf) result in faster grain growth although it is not the case. In the aorta model, there exists a complex position (distance and unique allocation parameter) dependent sucrose competition. The grain yield predicted by the aorta model was higher than the pipe model, and thus, the aorta model was suggested as a working model for sieve tube structure in rice panicle (Seki et al., 2015).

Little is known about the phloem system in wheat florets, especially to the distal florets which are always aborting. Based on the nature of floret and grain size distribution in each of the wheat spikelet, the aorta model of phloem sucrose transport is proposed for wheat. The model is further supported by the data generated from SS and spikelets of BC3F3 plants in this study as well as literature review from other studies. Diagrammatic representation of the proposed aorta model in wheat spike/spikelet is shown in Figure 4-3.

As it has been already suggested earlier that distance and position based assimilate competition is one of the characteristics of the aorta model of phloem sucrose translocation in rice panicle (Seki et al., 2015). This also fits for wheat due to the very nature of wheat floret development in the each spikelet. The two florets, which are most proximal to the rachis or assimilate source, are always fertile i.e. Floret 1 and Floret 2 (Figure 4-3) while the fate of more distal florets (Floret 5 and beyond) are subject to abortion (Kirby, 1974; Sibony and Pinthus, 1988; Gonzalez-Navarro et al., 2015; Guo and Schnurbusch, 2015) which seemed to be competition effect. As florets develop from bottom to top; F1 and F2 are relatively older, more stable, and have larger carpel size (Kirby, 1974; Guo et al., 2015b; Xie et al., 2015), suggesting that these florets are more capable of diverting assimilates as compared to other florets whose fate still depends on the ability to compete with Floret 1 and Floret 2 in addition to other, yet unknown factors controlling their fate. The fate of Floret 3 and 4 depends on their position on the spike whether it is from basal, central or apical part of the spike. Floret 3 is often fertile in the central spikelets while the fate of Floret 4 is uncertain (Langer and Hanif, 1973; Ferrante et al., 2013; Guo and Schnurbusch, 2015).

Floret removal (Floret 3 and beyond) from each spikelet resulted in further increase in weight of the two basal grains which might be due to assimilate re-allocation (Pinthus and Millet, 1978). Similarly, the removal of the basal florets (Floret 1 or Floret 2) from the central spikelets before

anthesis compensated the grain setting rate as well as grain weight of the distal florets in the central spikelets (Rawson and Evans, 1970). This clearly suggests that there exists a gradient of sink size strength (dominance) among the florets which resulted in gradient based variation of floret development and fertility in wheat.

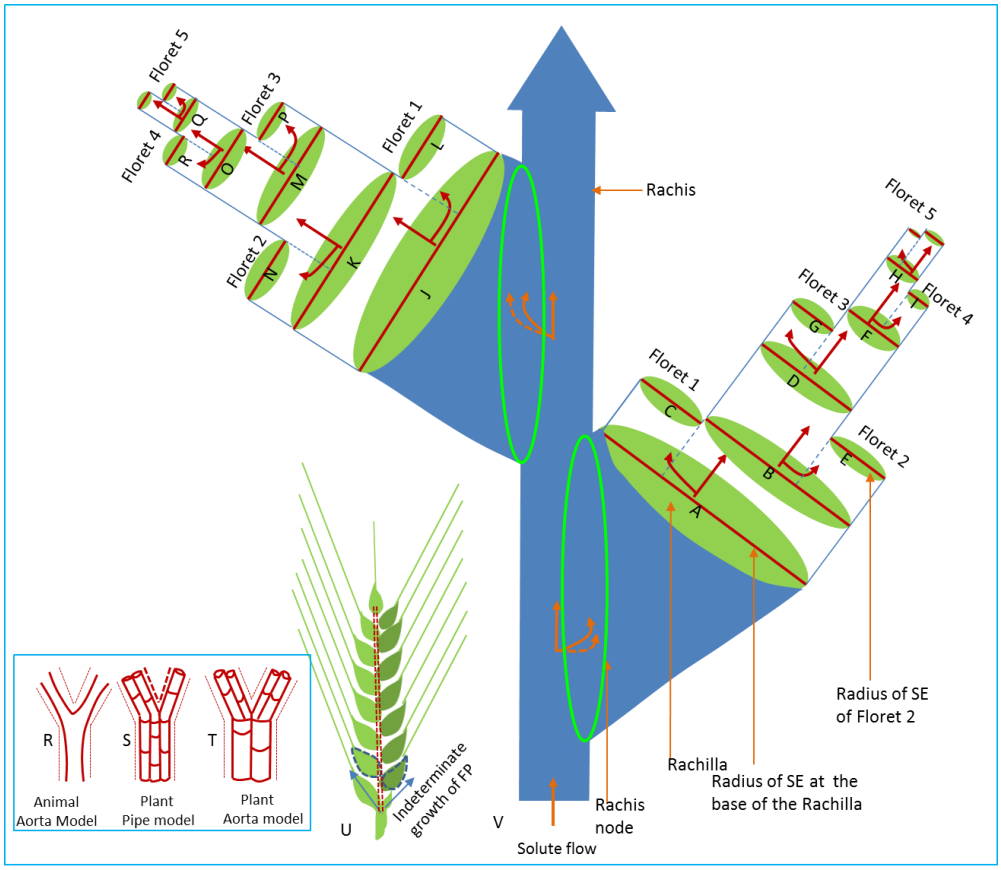


Figure 4-3. Proposed aorta model of sucrose phloem network in wheat spike and spikelet.
The inset figures (R, S, T) show simplified vascular networks from the animal vascular system (R) and plant xylem (S and T) (McCulloh et al., 2003). In animal cardiovascular system (R) a single continuous tube bifurcates or branches into daughter tubes. In a plant pipe model (S), tubes are not continuous but divided into conduits that run in parallel within each branch. In plant 'aorta model' (T), the number of conduits increases as the branch number increases due to bifurcations of each conduits. Based on the proposed aorta model of phloem network, assumption was made in such a way that multiple sieve tubes are approximated as a single large tube (T) in the rachis of the spike. At each rachis node point; the tube bifurcates into two daughter tubes in such a way that one of the daughter tubes feeds the immediate floret (i.e. Floret 1) and the other tube follows the rachilla and further bifurcates into two daughter tubes at the next node point, where one of the daughter tubes feeds Floret 2 and other tube follows to the rachilla until the next node point where the tube further bifurcates following the same fashion. During the bifurcation of the tube at each node point, a relationship has been assumed following Murray's law which states that the cubic radius of the mother tube is the sum of cubic radii of the two daughter tubes (Murray, 1926; Seki et al., 2015). This means that neither of the daughter tubes is thicker than its mother tube. However, the two daughter tubes can assume different radii giving them characteristics of asymmetric branching which can be decided by the 'radius allocation rule'. Of course the 'radius allocation rule' is not known in wheat. Thus, 'A', which is the radius of the mother tube diverted from the rachis to the rachilla of the first spikelet (V), bifurcates into daughter tubes 'B' and 'C' where 'C' feeding floret 1. Tube 'B' further extends following the rachilla until the next rachilla node where it bifurcates further into two daughter tubes: 'D' and 'E', where E feeds Floret 2 while D further extends following the path of the rachilla until the next rachilla node. Bifurcation of each tube continues indefinitely following the same rule as rachilla grows indeterminately (U). Thus, the pattern of bifurcation of mother tube will follow this pattern: A=B+C; B=D+E; D=F+G; and F= H+I and so forth. Hence, size of the the SE diminishes as bifurcation continues which possibly affects the survival of distal florets, i.e. Floret 4 and 5. Bend solid and broken arrow indicates spikelets sharing same rachis node i.e. the PS and SSt which are attached to similar assimilate source through the rachis node. SE, Sieve element; FP, Floret Primordia.

Furthermore, using FL-*by*-NILs (at BC3F3-generation), position-based spikelet fertility and grain weight was compared to test the hypothesis of aorta-based phloem sucrose transport in wheat spike/spikelet. The pipe model seemed to be less likely because the assumption of the pipe model is that each conducting sieve tubes, which neither bifurcates nor tapers at any site (Figure 4-3S), are assumed to connect each spikelet to the source (leaf) i.e. each spikelet/floret have their own pipe that connects them to the source. Because competition for sucrose in the pipe model is distance-dependent (Seki et al., 2015); PS and SSt, which are attached to the same rachis node (Figure 4-2), should have benefited equally (should have similar spikelet fertility index). However, SSt has lower spikelet fertility (GPS_SSt) as compared to the spikelet (GPS_PS) under both FT and DT plants (Table 4-1). This indicates that there exists a unique, yet unknown 'radii allocation rule' between these types of spikelets to maximize spikelet fertility (Figure 4-3V). Hence, the pipe model is less likely to fit in wheat.

The second reason why the pipe model is less likely in wheat is the very nature of basal spikelets on the spike. Although the basal spikelets are closer to the source (leaf) as compared to those in the center or apical portion of the spike, according to the pipe model they should have benefited better than those spikelets in the center and/or apical part of the spike. However, this is generally not the case in wheat; because the fertility of the basal spikelets is generally inferior to those at the central of the spike (Kirby, 1974; Whingwiri et al., 1981; Guo et al., 2015a; Guo and Schnurbusch, 2015). Therefore, the pipe model is less likely to fit in wheat again.

If we consider the aorta model, however, where the main vascular system branches out and gets thinner and thinner as it gets farther and farther (Figure 4-3V); it is the most likely model fitting in a wheat spikelet. This is because it fits well with the general trend of floret abortion in a wheat spikelet. Floret primordia abortion starts from the most distal part of each spikelet where bifurcation of the main vascular system becomes thinner and thinner (following Murray's law) carrying less amount of sucrose that might not be enough for sustaining all the distal florets (Figure 4-3V). As a result, cell divisions and metabolism in the distal florets will cease (Ghiglione et al., 2008) resulting in the interruption of normal floral development which of course led to floret abortion or degeneration. A study also indicated that due to increased demand for carbohydrates (glucose and fructose) by the developing spike, a decrease in glucose and fructose has been detected after terminal spikelet stage (Ghiglione et al., 2008), suggesting an elevated demand for carbohydrates during spike development stages. Interestingly, the authors performed sucrose feeding experiment through the flag leaf during the floret degeneration period and were able to

rescue more florets per spike than otherwise degenerate. Of course, floret abortion or degeneration also varies along the spike. Relatively, more florets from the central part of the spike, where spikelet differentiation first started, reach anthesis and set more grains, suggesting that vascular system connectivity and bifurcation also follow the same pattern.

The aorta model can also be justified from SSt of the DT plants (Table 4-1). Due to the availability of more assimilates (as a result of de-tillering), SSt showed enhanced spikelet fertility (grain setting rate) as compared to SSt in the FT plants. This means that the more assimilates are available, the more florets are to be rescued before the level of assimilates drops off to the level that cannot sustain floret development anymore (Figure 4-3V).

Tiller removal experiment based on 12 hexaploid spring wheat cultivars delayed the floral degeneration process and increased the number of florets that can set grain which otherwise will be aborted (Guo and Schnurbusch, 2015). Data from basal grain removal experiment by Bremner and Rawson also suggested that abortion of distal florets is due to assimilate related problem (Bremner and Rawson, 1978). These suggest that there is clear position and age-based competition among florets for sucrose in wheat. Hence, the current position-based analysis of spikelet fertility and grain weight suggests that the aorta model is the most likely working model to further study floral development and the identification of factors driving floral degeneration in wheat. This requires further studies, especially, when and how the vascular bundles are developed and connected besides the size and distribution of vascular bundle in each floret. Furthermore, studying the exact role and connectivity of the transfer cells at the attachment point of the glume, lemma, palea, and pericarp are also equally important.

5 Outlook

Regardless of the slow yield increment in wheat, the demand is expected to rise as world population rises. To cope up such a rising demand, wheat yield needs to be increased. Although wheat produces florets indeterminately, early degeneration of the florets has been a major challenge for increasing yield directly by increasing floret fertility. Furthermore, there is no clear understanding of the cause for floret abortion (degeneration) in wheat. Hence, increasing grain yield by increasing fertile floret is not an easy task. Therefore, other options need to be sought. One of these is increasing spikelet number per spike. For this, we have now the alleles controlling spikelet number with a pleiotropic effect in spikelet fertility. However, a detailed understanding of genetic interaction network is still unknown to further understand the molecular genetic basis of spike architecture in wheat. Nevertheless, the spike-branching alleles can give new opportunity for increasing grain number per spike. Although spike-branching is negatively correlated with spikelet fertility, it can be improved by partitioning more assimilates to the developing florets. One way of achieving this is by combining *bh* allele(s) with the *tin* wheat mutant where more assimilates can be diverted to the developing inflorescence which otherwise would be invested on tillers unnecessarily. Because of the involvement of other unknown internal as well as external (environmental) factors, spike-branching is a plastic phenotype in terms of phenotypic penetrance and expressivity. By creating and further studying double knockout mutants (by simultaneous silencing *BH* homoeo-alleles) using tools such as CRISPR-Cas9 might give a better understanding of the phenotypic penetrance and expressivity, as well as the complex regulatory network of inflorescence architecture in wheat. Furthermore, the source-sink relation in wheat is also a key factor for unlocking grain yield limitations in wheat. However, available information concerning the source-sink relationship in wheat is largely inconsistent as to whether yield limitation in wheat is source or sink driven. Hence, the developed FL-*bh*-NILs provide further opportunity to understand and genetically dissect source-sink relationship in wheat. Furthermore, combing *bh* allele(s) with *tin* locus may pave a way for gene pyramiding towards creating the ideal wheat plant architecture.

6 Summary

Although the gene underlying the *bh^t-A1* locus, *TtBH-A1,* has already been cloned, spike-branching is a quantitatively inherited trait. Hence, other loci are believed to be involved in modifying the phenotypic penetrance and expressivity of spike-branching. Besides the large magnitude of phenotypic plasticity, QTL mapping identified three SS QTL located on chromosome 1A (*QSS.ipk-1AS*), 2A (*QSS.ipk-2AS*), and 2B (*QSS.ipk-2BS*). From these three QTL, *QSS.ipk-2AS* and *QSS.ipk-2BS* were found to be major and medium effect loci, respectively, controlling the spike-branching phenotype in all environments. *QSS.ipk-2AS* and *QSS.ipk-2BS* are linked to CAPS markers derived from *TtBH-A1* and *TtBH-B1*, respectively, suggesting that *QSS.ipk-2AS* and *QSS.ipk-2BS* are homoeoloci. This has been further confirmed by expression analysis of *TtBH-A1* and *TtBH-B1* at glume, floret and terminal spikelet stages of spike development, where *TtBH-A1* was found to be the most abundantly expressed allele in all stages followed by *TtBH-B1.* Furthermore, CDS analysis of *TtBH-B1* from 49 different wheat accessions/varieties revealed that besides carrying the mutant allele *bh^t-A1*, all branching mutant accessions were found to carry a non-synonymous mutation at *bh^t-B1* that is frequently linked with the strong branching phenotype. This suggests that both homoeo-alleles, *bh^t-A1*and *bh^t-B1*, are mutated in these accessions. Besides the identified loci, expressivity of spike-branching or SS formation is also affected by environmental factors including plant competitions and other yet unknown factors. Mapping result also revealed that besides controlling spike-branching, *QSS.ipk-2AS* and *QSS.ipk-2*BS were also found to affect spikelet fertility (grain number per spikelet) in wheat.

To further characterize the effect of spike-branching, FL-*bh*-NILs were developed using standard tetraploid wheat cv. Floradur as a recurrent parent. Genotyping of 67 FL-*bh*-NILs at BC4F2 plants revealed that all of the NILs carried the *bh^t-A1*allele from the donor (TRI 19165) segment. Based on RpGR and size of the introgressed segment, 20 best NILs with introgressed fragment size ranging from 2.3 to 37 cM were selected for further analysis. Phenotypic analysis at BC3F2 plants showed that FL-*bh*-NILs produced 3 to 5 additional spikelets per spike leading to 12 to 23.6 % change in spike dry weight at harvest. This indicates that besides carrying more spikelets per spike, FL-*bh*-NILs are valuable genetic materials for studying the source-sink relationship in wheat.

Source-sink manipulation through de-tillering of FL-*bh*-NILs plants at BC3F3 lead to about 65% improvement in SS fertility after de-tillering, suggesting that reduced spikelet fertility during spike-branching is partly connected with assimilate availability and distribution. The differential spikelet

fertility between PS and SSt, both of which are connected to similar assimilate source through the shared node, suggests that there might be a limited, and yet unknown assimilate allocation criteria in the spikelets and florets of wheat. Nevertheless, assimilate allocation in wheat spikelet/floret might follow the 'Aorta Model' where the main vascular bundle (assimilate source) bifurcates following Murray's law at each node point of each spikelet and floret. Hence, source capacity and size of the vascular bundle or allocation criteria might be the major factors controlling the amount of assimilate allocated to specific sink organ affecting spikelet and floret fertility in wheat.

7 Curriculum Vitae

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1. Doctoral student at Martin Luther University of Halle-Wittenberg and, Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Germany: **November 2012 to present**
2. PGD (Post Graduate Diploma) in Computer Sciencece from HILCOe School of Computer Science & Technology, Ethiopia: **January 2007-2009**
3. MSc, in Plant Biotechnology from Wageningen University, The Netherlands: **Septemeber 2004-2006**
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Other trainings

1. IPK BARLOMICS summer school on topics Epigenetics, Phenomics and Genome engineering using TALENS and RGNS: IPK, Gatersleben Germany: **14-17 September 2015**
2. Histological and ultrastructural analysis of plant tissue using Light, Scanning and Transmission Electron Microscopy: IPK, Gatersleben Germany: **13-17 April 2015**
3. Practical Course in Fluorescence and Confocal Microscopy in Plant Science: IPK, Gatersleben Germany: **9-13 May 2014**
4. Contemporary approaches to genetic resources conservation and uses: Wageningen University and Research, Centre for Development Innovation, The Netherlands: **12- 30 April 2010**
5. Basic molecular markers and data analysis for enhancing the efficiency of mutation induction in crop improvement: In collaboration with the regional (AFRA) and International Atomic Energy Agency (IAEA), Eldoret, Kenya: **4-8 October 2010**

6. Physical Protection and Security management of radioactive sources: National Nuclear Security Administration (NNSA), Addis Ababa, Ethiopia: **17-19 November 2010**
7. Policy seminar on why does famine persist? Poverty Reduction and Food security strategies in Eastern Africa: Organisation for Social Science Research in Eastern and Southern Africa (OSSREA), Addis Ababa, Ethiopia: **20 April – 1 May 2009**
8. Mutation breeding for market-oriented traits in African crops: Addis Ababa, Ethiopia: **18-22 June 2007**
9. Mutation methodology and results of experiments on Genetic improvement of neglected African crops: In collaboration with the regional (AFRA) and International Atomic Energy Agency (IAEA), Duala, Cameroon: **24-28 June 2002**
10. Radiation protection and safety of radiation sources: Addis Ababa, Ethiopia: **13-17 May 2002**
11. Design and analysis of agricultural experiments: Addis Ababa, Ethiopia: **29 May-3 June 2001**
12. Scientific paper writing: Addis Ababa, Ethiopia: **12-15 November 2001**
13. Planning, monitoring, and evaluation of research proposals: Addis Ababa, Ethiopia: **24-29 September 2001**

Employment

1. Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), DFG HEISENBERG-Research Group Plant Architecture, Germany: **November 2012 - July 2016, position held: E13 TV_L**
2. Rothamsted Research, Department of Plant Biology & Crop Science, Harpenden United Kingdom: **May 2011 – May 2012, position held: Post-doctoral Research**
3. Ethiopian Institute of Agricultural Research, Debre Zeit Agricultural Research Centre: **October 2006 - May 2011, Position held: Research Associate**
4. Ethiopian Institute of Agricultural Research, Debre Zeit Agricultural Research Centre: **March 2001 – August 2004, Position held: Junior Researcher**

Awards

1. Post-doctoral Research fellowship award (May 2011 to May 2012), Rothamsted International, United Kingdom.
2. Meritorious national group award for the development of superior tef variety called “Quncho”, the ministry of science and technology, 2012, Ethiopia.

Experience and skills

- Wet lab, both in DNA and RNA technologies (isolation, primer design, PCR, qPCR)
- Plant molecular biology techniques used in genetics and genomics
    - ✓ Molecular marker development and analysis
    - ✓ Linkage map construction and QTL mapping in complex (polyploid) plant genome
    - ✓ Comparative genomics and identification of candidate gene (cloning of the QTL)
    - ✓ Forward and reverse genetic tools such as TILLING
    - ✓ Marker assisted selection and development of mapping population in complex (polyploid) plant genome

- ✓ DNA sequence analysis using bioinformatics software such as Geneious and Blast2GO
- ✓ Understanding of next-generation sequence data processing and analysis
- ✓ Plant transformation and tissue culture

- Working under field and greenhouse conditions, phenotyping and data analysis using:
  - ✓ Statistical software such as Excel, SPSS, and GenStat
  - ✓ QTL and linkage mapping software like Joinmap, WinQTLCart, QTLNetwork, and GenStat
- Computer skills
  - ✓ Computer programming languages, web design, Microsoft office applications and troubleshooting

Publications

1) Ahmad M. Alqudah; Ravi Koppolu1; **Gizaw M. Wolde**; Andreas Graner and Thorsten Schnurbusch (2016). The genetic architecture of barley plant stature, Front Genet 7, 117.
2) Poursarebani, N., T. Seidensticker, R. Koppolu, C. Trautewig, P. Gawroński, F. Bini, G. Govind, T. Rutten, S. Sakuma, A. Tagiri, **Gizaw M. WOLDE**, H.M. Youssef, A. Battal, S. Ciannamea, T. Fusca, T. Nussbaumer, C. Pozzi, A. Börner, U. Lundqvist, T. Komatsuda, S. Salvi, R. Tuberosa, C. Uauy, N. Sreenivasulu, L. Rossini and T. Schnurbusch (2015). The genetic basis of composite spike form in barley and 'Miracle-Wheat'. Genetics 201: 155-165
3) Solomon Chanyalew, Kebebew Assefa and **Gizaw M. Wolde** (2012)**.** Phenotypic and Molecular Diversity in Tef. Proceeding of the second international tef workshop
4) Kebebew Assefa, Solomon Chanyalew and **Gizaw M. Wolde** (2012). Conventional and Molecular Breeding of tef. Proceeding of the second international tef workshop
5) Assefa, K., A. Sherif, G. Belay**, Gizaw M. Wolde**, H. Tefera and M. Sorrells. 2010. *Quncho*: The First Most Popular Tef Variety in Ethiopia. International Journal of Agricultural Sustainability 9, no. 1 (2011): 25-34.
6) Belay, G., A. Zemede, K.Assefa, **Gizaw M. Wolde** and H.Tefera. (2009) Seed size effect on grain weight and agronomic performance of tef [*Eragrostis tef* (Zucc.) Trotter]. African J. of Agricultural Research
7) Belay, G., H. Tefera, A. Getachew, K. Assefa & **Gizaw M. Wolde** (2007). Highly client-oriented breeding in Ethiopian tef [*Eragrostis tef* (Zucc.) Trotter]. African J. of Agricultural Research.
8) Belay, G., H. Tefera, B. Tadesse, **Gizaw M. Wolde**, D. Jarra and T. Tadesse. (2006) Participatory variety selection in the Ethiopian cereal tef [*Eragrostis tef* (Zucc.) Trotter]. Experimental Agriculture 42:91-101.

Posters

1) **Gizaw M.Wolde** and Thorsten Schnurbusch (2013).
   Inflorescence architecture as a tool for floret fertility and resource use efficiency in wheat- 9th Plant science student conference (PSSC 2013), Martin Luther University of Halle-Wittenberg, Germany.
2) **Gizaw M.Wolde**, Martin Mascher and Thorsten Schnurbusch (2015).
   TtBH-B1 is involved in the determination of spike architecture of tetraploid wheat- Institute's day, Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.
3) **Gizaw M.Wolde**, Martin Mascher and Thorsten Schnurbusch (2016).
   The 'Miracle Wheat' Homoeo-Allele, *bh^{t}-B1*, Affects Spike-Branching in Tetraploid Wheat- Plant and Animal Genome XXIV, San Diego, USA.

Declaration

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Date _____ Signature of the Applicant_____

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9 Supplementary materials

Table 9-1. Sequence variation in the upstream of *TtBH-B1* ORF.

The comparison was made based on sequence generated from four non-branching tetraploid wheat varieties (Floradur, Bellaroi,Tamaroi, and Wollaroi) and three branching (TRI 19165, TRI 27966, and TRI 7282) accessions. The Chinse spring is included for comparison. Haplotype group for each of these varieties and accessions is shown in Supplementary Table 11-1.

Accession/variety		-559	-545	-520	-482	-469	-426 to -413	-392	-374	-362	-330 to -314	-311	-308	-269	-137	-54
CS	…	A…	C…	T…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	A…
TRI 19165[P1]	…	A…	T…	C…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	G…
TRI 27966	…	A…	T…	C…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	G…
TRI 7282	…	A…	C…	T…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	A…
Floradur [P3]	…	A…	T…	C…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	G…
Bellaroi [P2]	…	G…	T…	T…	A…	C…	del…	T…	C…	A…	del…	T…	A…	G…	T…	A…
Tamaroi	…	A…	C…	T…	G…	A…	in…	C…	T…	G…	in…	C…	G…	A…	A…	A…
Wollaroi	…	G…	T…	T…	A…	C…	del…	T…	C…	A…	del…	T…	A…	G…	T…	A…

P1, P2 were parents of the mapping population while P3 was used as the recurrent parent for FL-*bh*-NILs. The number shows the position of the nucleotide from the start codon. in, insertion; del deletion

Table 9-2. List of accession/varieties based on SNP found in the coding and 3'UTR of *TtBH-B1*

Haplotype	Name	Country	Type	Spike	Branching Intensity	1	C144T		C267T		A491G (Y164C)		A619T (F207I)		T785C (L262S)	924		G994A		1145	1146	1147
Hap I-YIL-G-del	Auradur	Aut	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	Elsadur	Aus	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	Langdon	-	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	Logidur	AUS	Winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	Tamaroi	AUS	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	Td24	GER	Winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 11066	UZB	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 13541	ITA	Winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 1669	ALB	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 19273	TUR	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 3023	ALB	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 3504	POR	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 3720	ESP	Winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 4292	TUR	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 4522	CHN	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 7056	FRA	Winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 758	TUR	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
	TRI 9547	ARM	winter	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	-	-	-
Hap I-YIL-G-in	TRI 2230	USA	spring	W	-	ATG…	C	…	C	…	A	…	A	…	T	…TGA	…	G	…	T	A	G
Hap III-YIS-G-del	PI225308	IRAN	spring	M	Medium to high	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	PI438971	KAZ	Spring	M	low to medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 1781	GER	Winter	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 1782	GER	Winter	M	medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 18959	FRA	Spring	M	medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 19165[P1]	-	Winter	M	strong	ATG…	C	…	T	…	A	…	A	…	c	…TGA	…	G	…	-	-	-

Table 10-2 Continued ...

Haplotype	Name	Country	Type	Spike	Branching Intensity	1	C144T		C267T		A491G (Y164C)		A619T (F207I)		T785C (L262S)	924		G994A		1145	1146	1147
	TRI 19292	FRA	Winter	M	Strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
Hap III-YIS-G-del	TRI 27966	-	spring	M	medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 28396	ITA	Winter	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 3261	ESP	spring	M	Strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 3411	SU	spring	M	Strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4045	EUR	spring	M	Medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4270	ITA	spring	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4341	EUR	spring	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4461	EUR	Winter	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4653	AUS	winter	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 5283	CHN	spring	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 5911	IRAN	spring	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 984	EUR	spring	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	CITr 13712	USA	spring	M	medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	CITr 13713	USA	spring	M	medium	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 9548	ARM	winter	M	strong	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	TRI 4886	UK	Winter	W	-	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	-	-	-
	Floradur [P3]	AUS	Spring	W	-	ATG…	C	…	T	…	A	…	A	…	c	…TGA	…	G	…	-	-	-
Hap IV-YIS-G-in	TRI 7021	POL	Winter	W	-	ATG…	C	…	T	…	A	…	A	…	C	…TGA	…	G	…	T	A	G
Hap V-CFS-G-in	Kalka	AUs	spring	W	-	ATG…	T	…	C	…	G	…	T	…	c	…TGA	…	A	…	T	A	G
	Bellaroi [P2]	Aut	Spring	W	-	ATG…	T	…	C	…	G	…	T	…	c	…TGA	…	A	…	T	A	G
	TRI 17236	TUR	spring	W	-	ATG…	T	…	C	…	G	…	T	…	c	…TGA	…	A	…	T	A	G
	TRI 9546	ARM	winter	W	-	ATG…	T	…	C	…	G	…	T	…	c	…TGA	…	A	…	T	A	G
	Wollaroi	AUS	spring	W	-	ATG…	T	…	C	…	G	…	T	…	c	…TGA	…	A	…	T	A	G

P1, P2 were parents of the mapping population while P3 was used as the recurrent parent for FL-*bh*-NILs. The number shows the position of the nucleotide from the start codon.

Table 9-3. Wheat microsatellite markers used for background selection of the BC2F1 generation. S, Short arm; L, long arm; C, Centromere; bP, base pair

Marker	Chromosome	Location	Allele size (bP)		Difference(bP)
			Floradur	TRI 19165	
Xgwm0691	1A	S	150	161	11
Xgwm1148	1A	L	180	197	17
Xgwm0905	1A	S	257	243	14
Xgwm1050	1B	S	211	197	14
Xgwm0268	1B	L	204	217	13
Xgwm0018	1B	S	184	191	7
Xgwm0659	1B	L	176	253	77
Xgwm0448	2A	C	244	229	15
Xgwm0294	2A	L	100	85	15
Xgwm1036	2A	L	270	242	28
Xgwm0636	2A	S	88	94	6
Xgwm0425	2A	S	75	60	15
Xgwm0726	2A	S	75	50	25
Xgwm1198	2A	S	158	121	37
Xgwm1027	2B	L	122	135	14
Xgwm1128	2B	S	162	150	12
Xgwm0619	2B	L	153	142	11
Xgwm0429	2B	S	215	221	6
Xgwm0720	3A	C	129	152	23
Xgwm0005	3A	C	173	160	13
Xgwm0247	3B	L	140	155	15
Xgwm0493	3B	S	201	214	13
Xgwm0896	3B	L	167	182	15

Table 10-3 continued ...

Marker	Chromosome	Location	Allele size (bP)		Difference(bP)
			Floradur	TRI 19165	
Xgwm0610	4A	L	168	153	15
Xgwm0894	4A	L	143	169	26
Xgwm0937	4A	L	179	205	26
Xgwm1091	4A	S	235	249	14
Xgwm1093	4A	S	176	161	15
Xgwm0959	4A	L	247	261	14
Xgwm0857	4B	S	180	190	10
Xgwm0251	4B	L	90	104	14
Xgwm0856	4B	S	102	116	14
Xgwm0930	4B	L	193	180	13
Xgwm0995	5A	L	160	141	19
Xgwm1236	5A	L	146	122	24
Xgwm1089	6A	L	140	150	10
Xgwm1150	6A	L	199	177	22
Xgwm0427	6A	L	212	191	21
Xgwm1233	6B	S	152	164	12
Xgwm0680	6B	C	135	123	12
Xgwm0219	6B	L	154	188	34
Xgwm1061	7A	L	177	166	11
Xgwm1207	7A	L	238	295	57
Xgwm0282	7A	L	193	205	12
Xgwm0951	7B	S	159	136	23
Xgwm0577	7B	L	130	188	58
Xgwm1085	7B	C	135	157	22

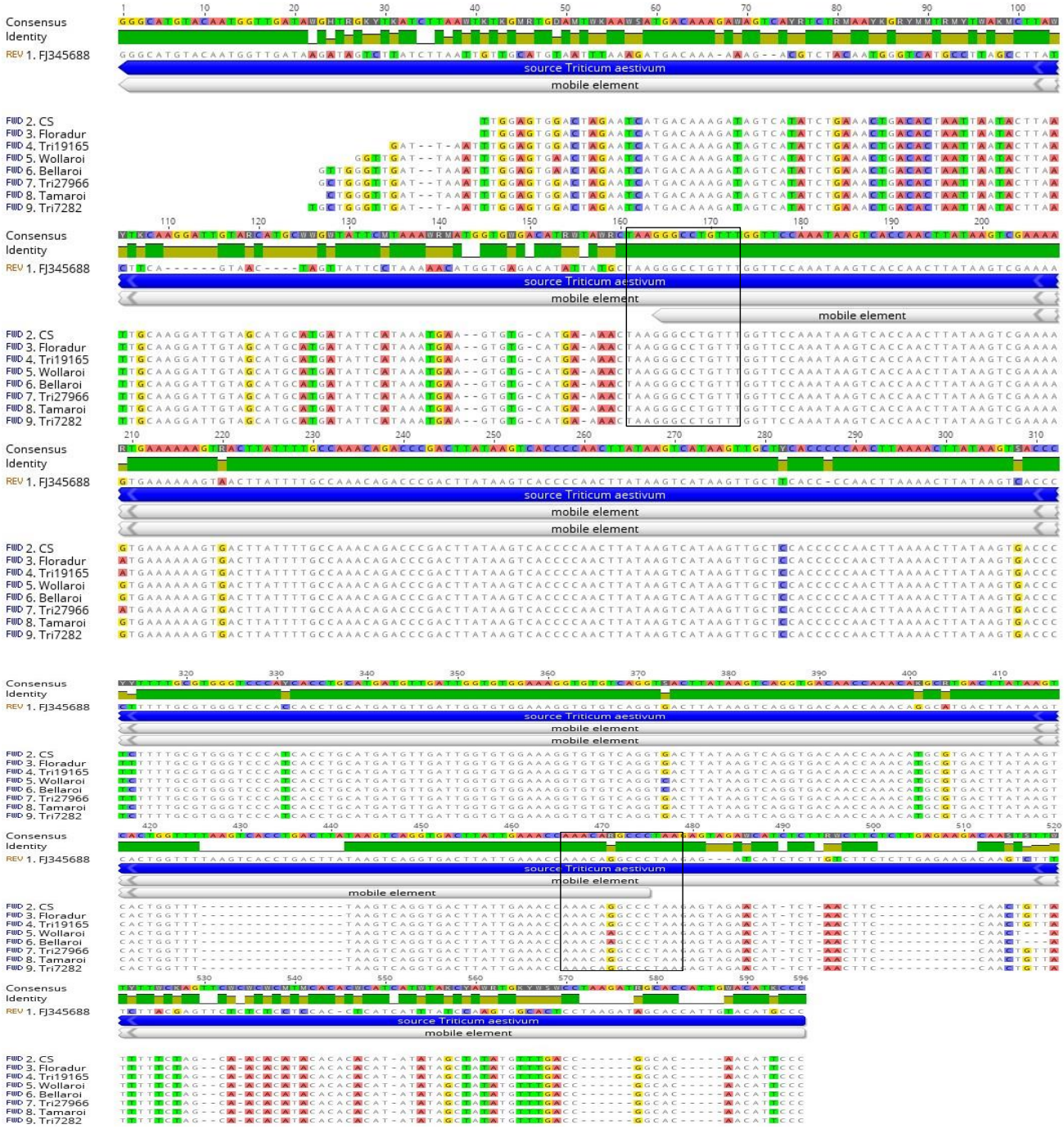


Figure 9-1. Identification of Miniature Inverted-repeat Transposable Element (MITE) in tetraploid wheat.

Alignment of 'MITE Tourist-2' (accession no: FJ345688 from cultivar Xiaoyan 54) with MITE Tourist-2 sequence generated from four tetraploid wheat elite varieties (Floradur, Wollaroi, Bellaroi and Tamaroi), three landraces (TRI 19165, TRI 27966, TRI7282) and Chinese spring(CS). The conserved terminal inverted repeats (TIR) and direct target site duplication (TSD) for the nested 'MITE: Islay Tourist' are boxed. Rev, Reverse, FWD, Forward.

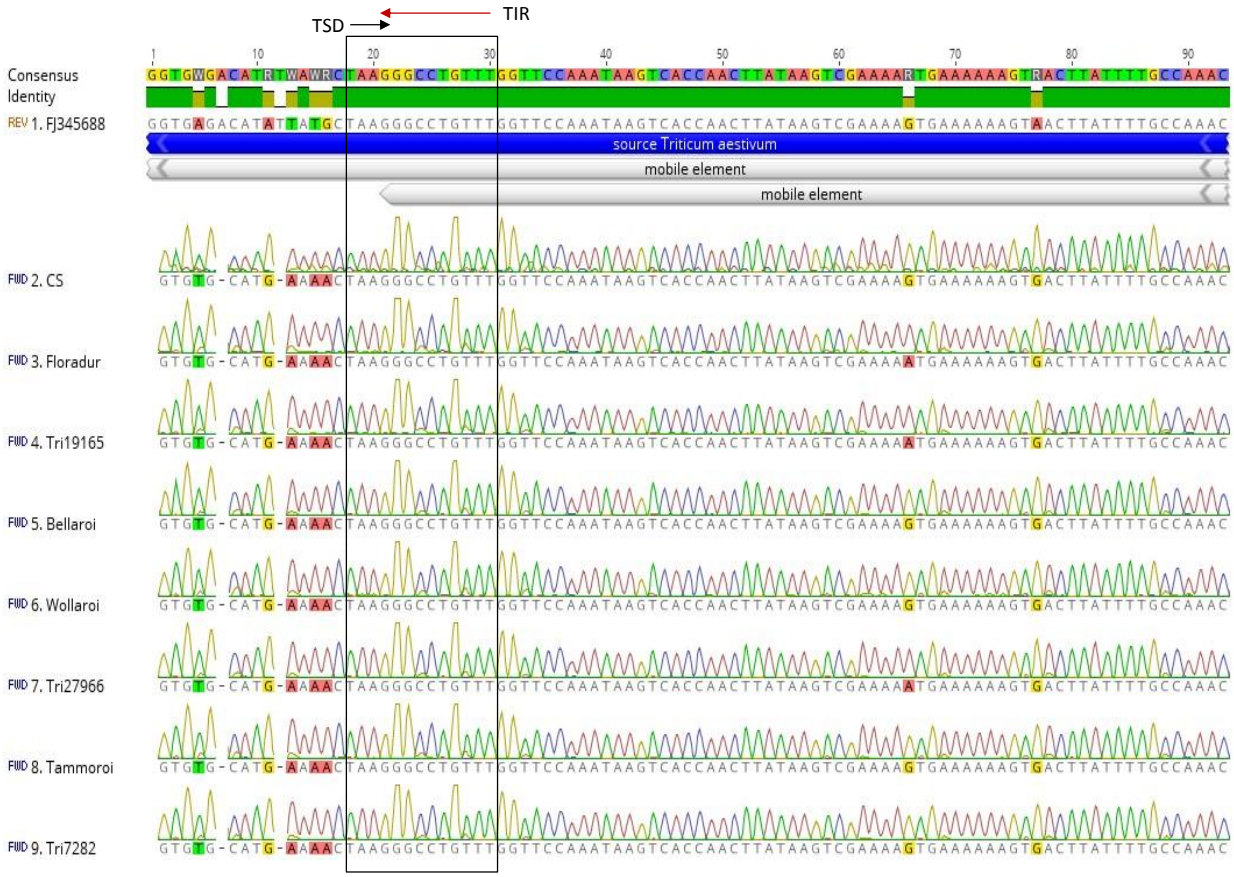


Figure 9-2. Sequence chromatogram of the nested 'MITE: Islay Tourist' from seven tetraploid wheat and the Chinse spring.
'MITE Tourist-2'(accession no: FJ345688 from cultivar Xiaoyan 54) has been used for comparison. The conserved terminal inverted repeats (TIR) and direct target site duplication (TSD) from 'MITE: Islay Tourist' are boxed. Rev, Reverse; FWD, Forward.

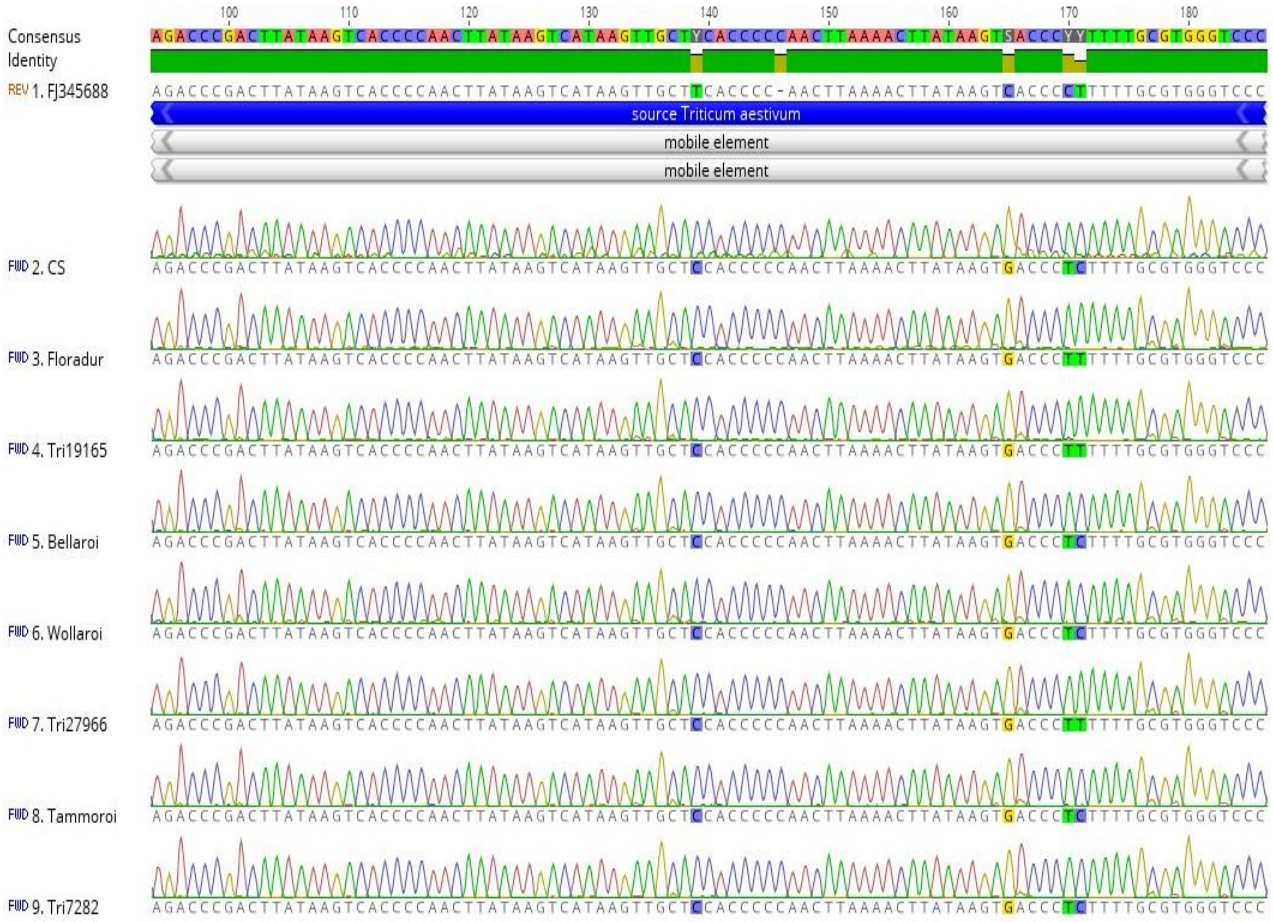


Figure 10-2 continued ...

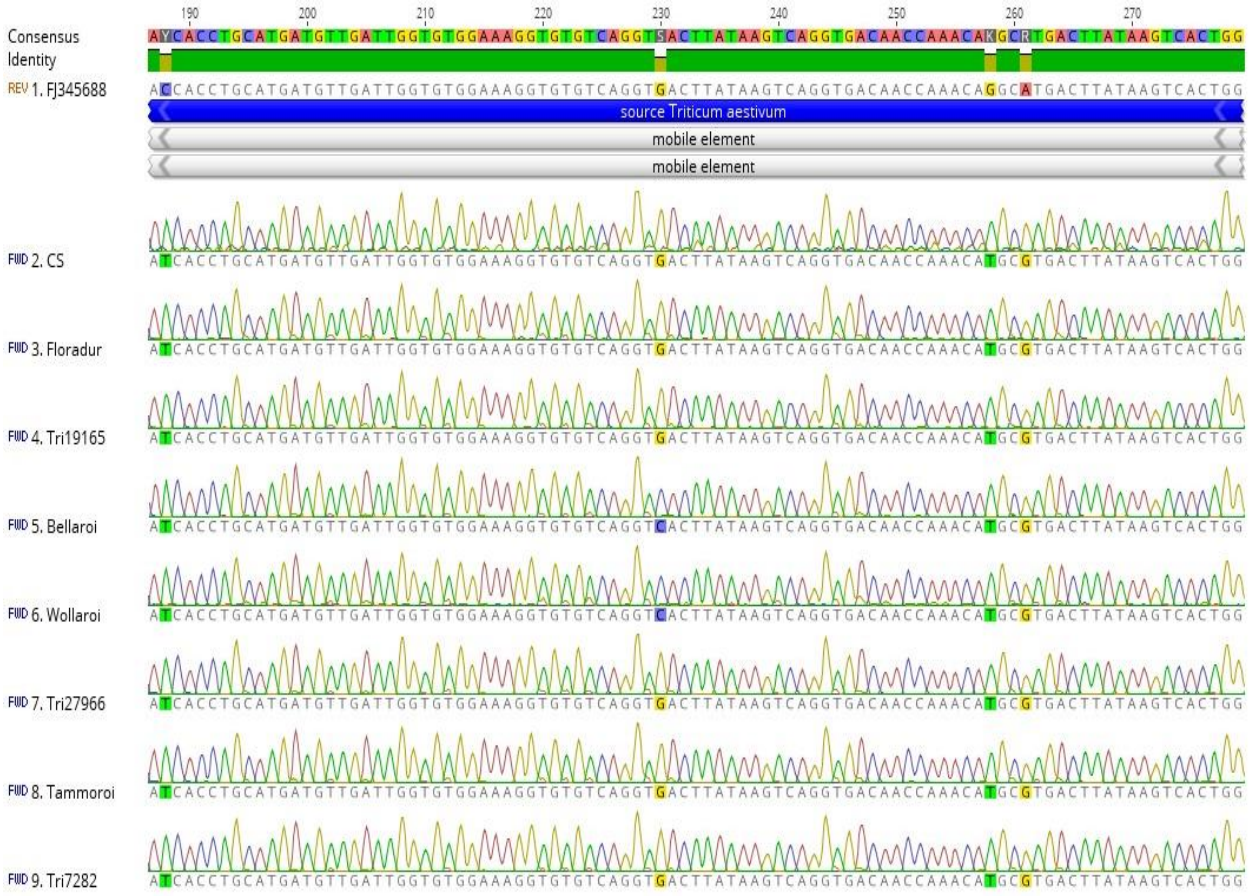


Figure 10-2 continued ...

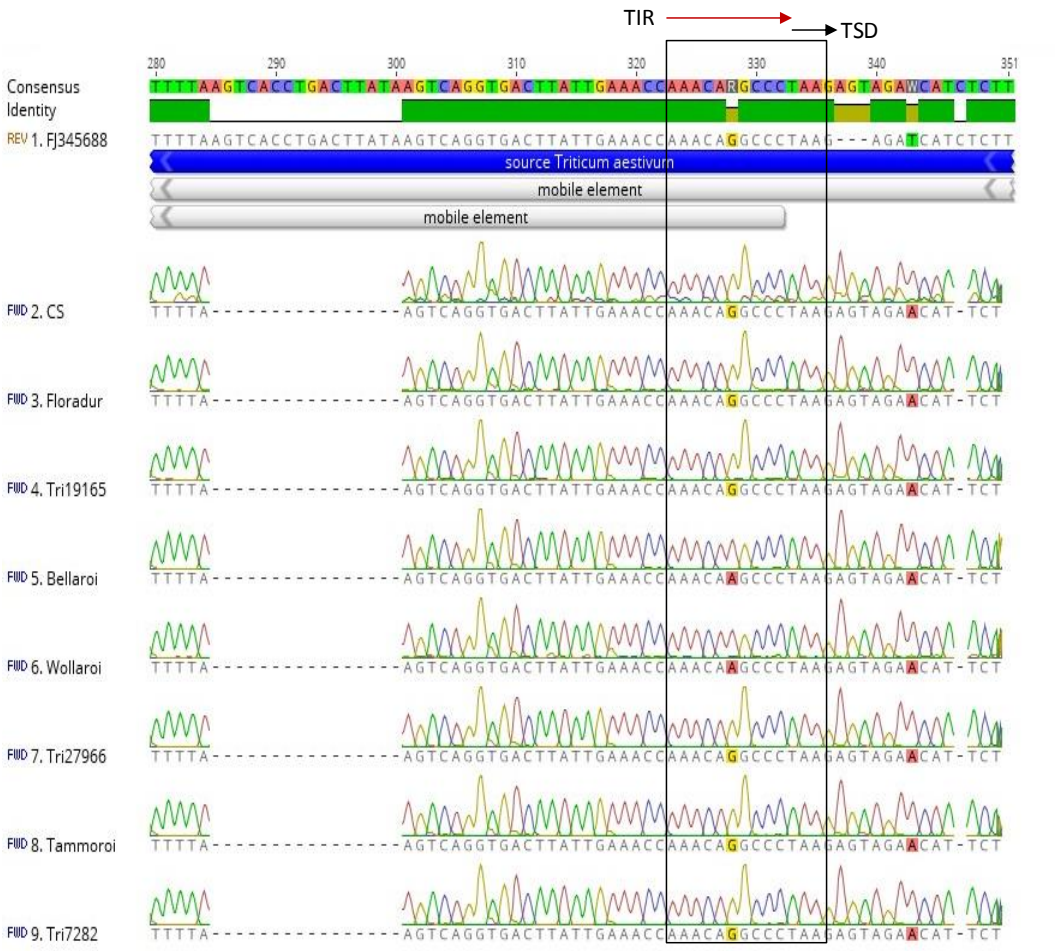


Figure 10-2 continued ...

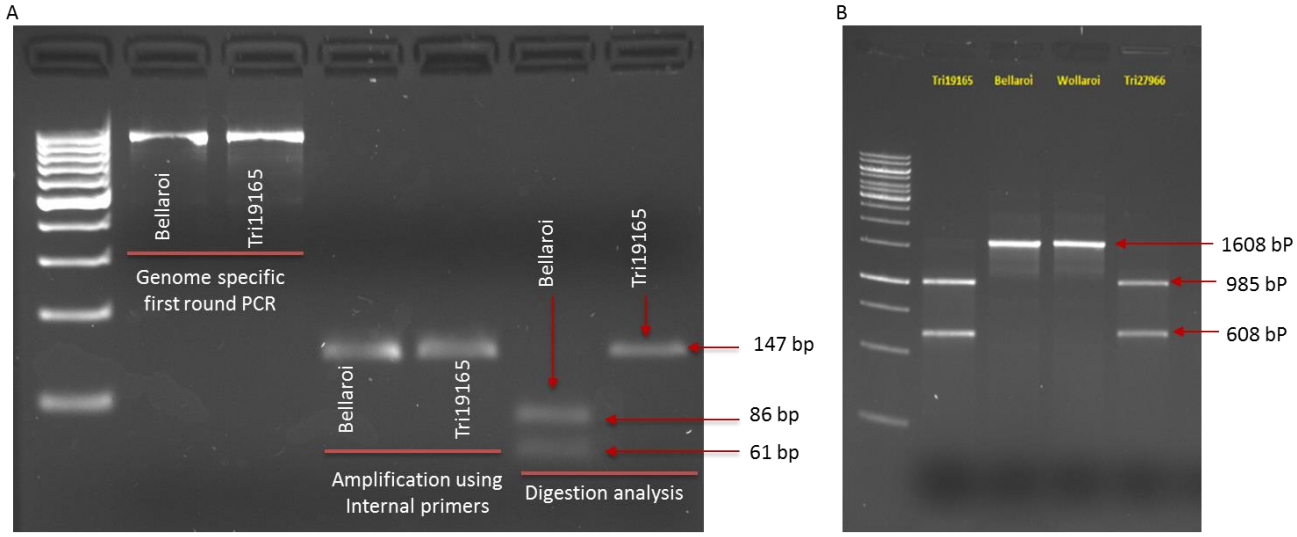


Figure 9-3. Allele-specific CAPS markers for *TtBH-A1* and *TtBH-B1*

(A) Diagnostic CAPS markers developed from *TtBH-A1* based on SNP T287C which gave tetraploid wheat the branching head phenotype. Enzyme *BstNI* was used for the restriction analysis. The marker can be used as a diagnostic marker to differentiate the standard and branching spike form in tetraploid wheat. (B) CAPS marker for *TtBH-B1*. The marker was developed based on SNP located at position -54 (G/A, see supplementary Table 11-1) to differentiate the two parental alleles. Enzyme *XhoI* was used for the restriction analysis. All the RILs were genotyped using both markers. The result has been summarized in the text.

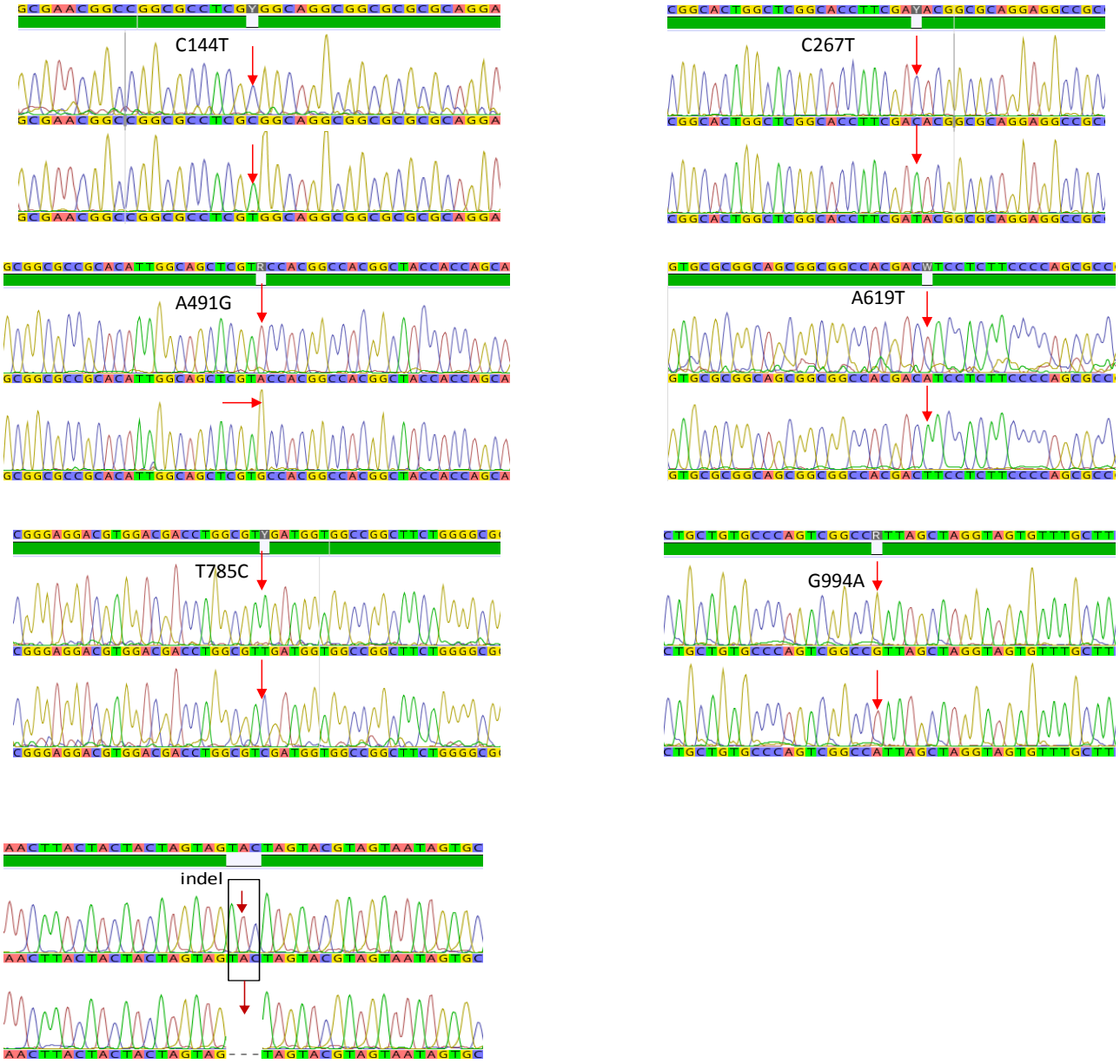


Figure 9-4. Chromatogram showing all SNP found in the CDS and 3'UTR of *TtBH-B1*

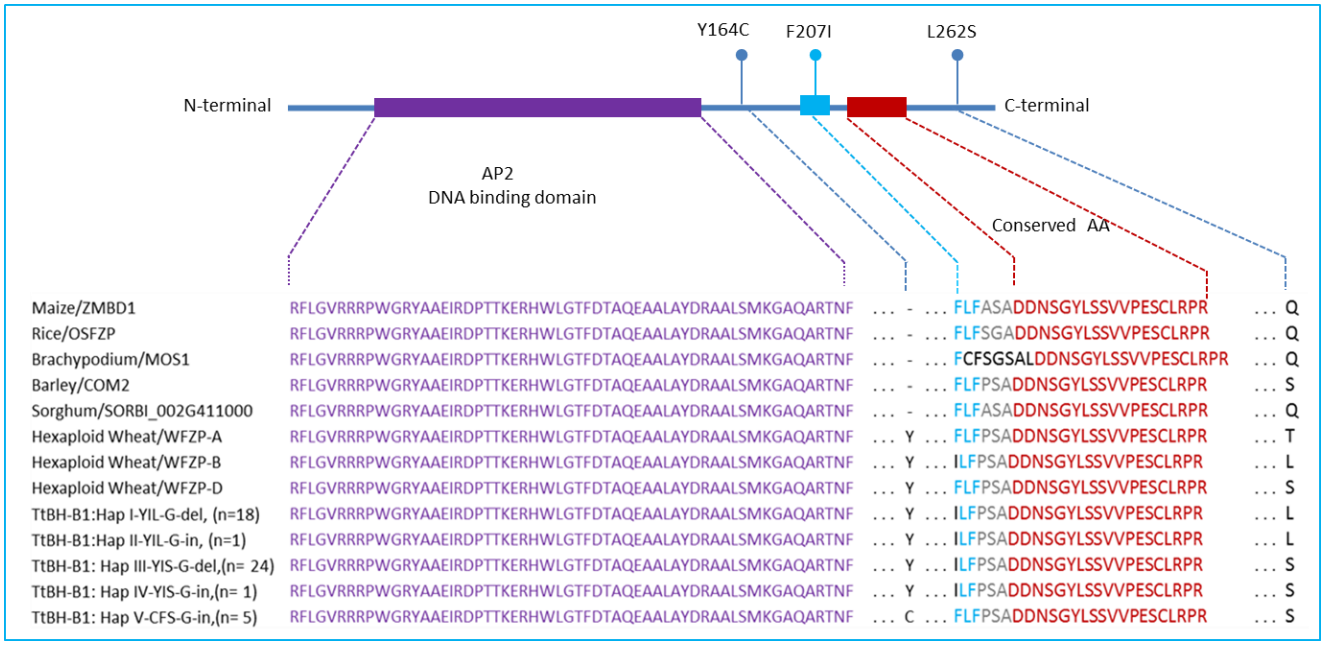


Figure 9-5. Comparison of TtBH homologous proteins from grasses.
The highly conserved AP2/ERF DNA-binding domain and highly conserved AA residues at the C-terminus are shown in purple and red boxes respectively. The three AA substitutions are indicated on the top. AA sequence from ZmBD1 (AAO21119), OsFZP (AB103120), MOS1 (KQK14788), SORBI_002G411000 (KXG36907), WFZP-A (AJA71267) WFZP-B (AJA71268), and WFZP-D (AJA71269) were downloaded from NCBI. The five haplotype and the number of accessions constituting each haplotype are shown in the parenthesis.

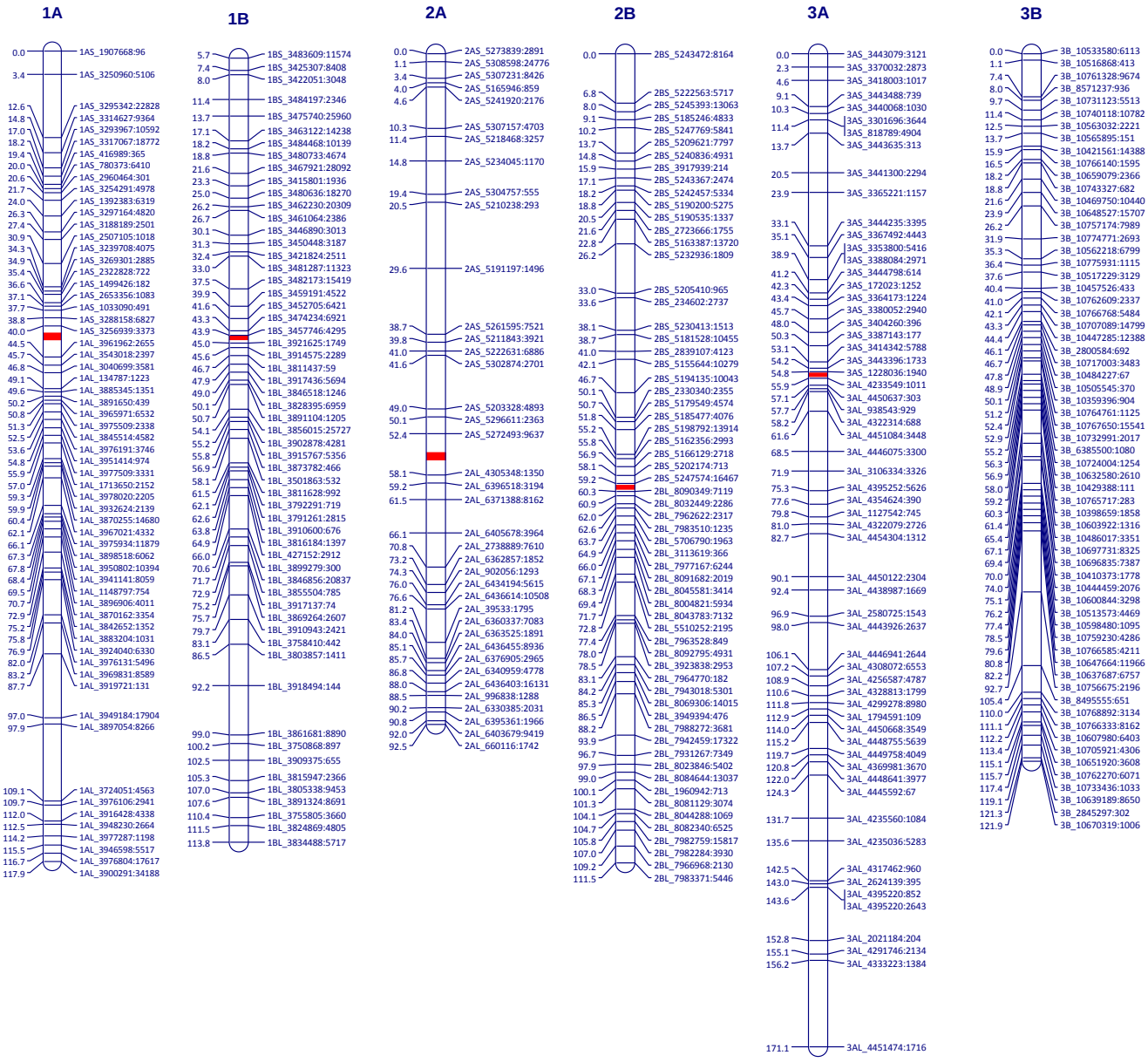
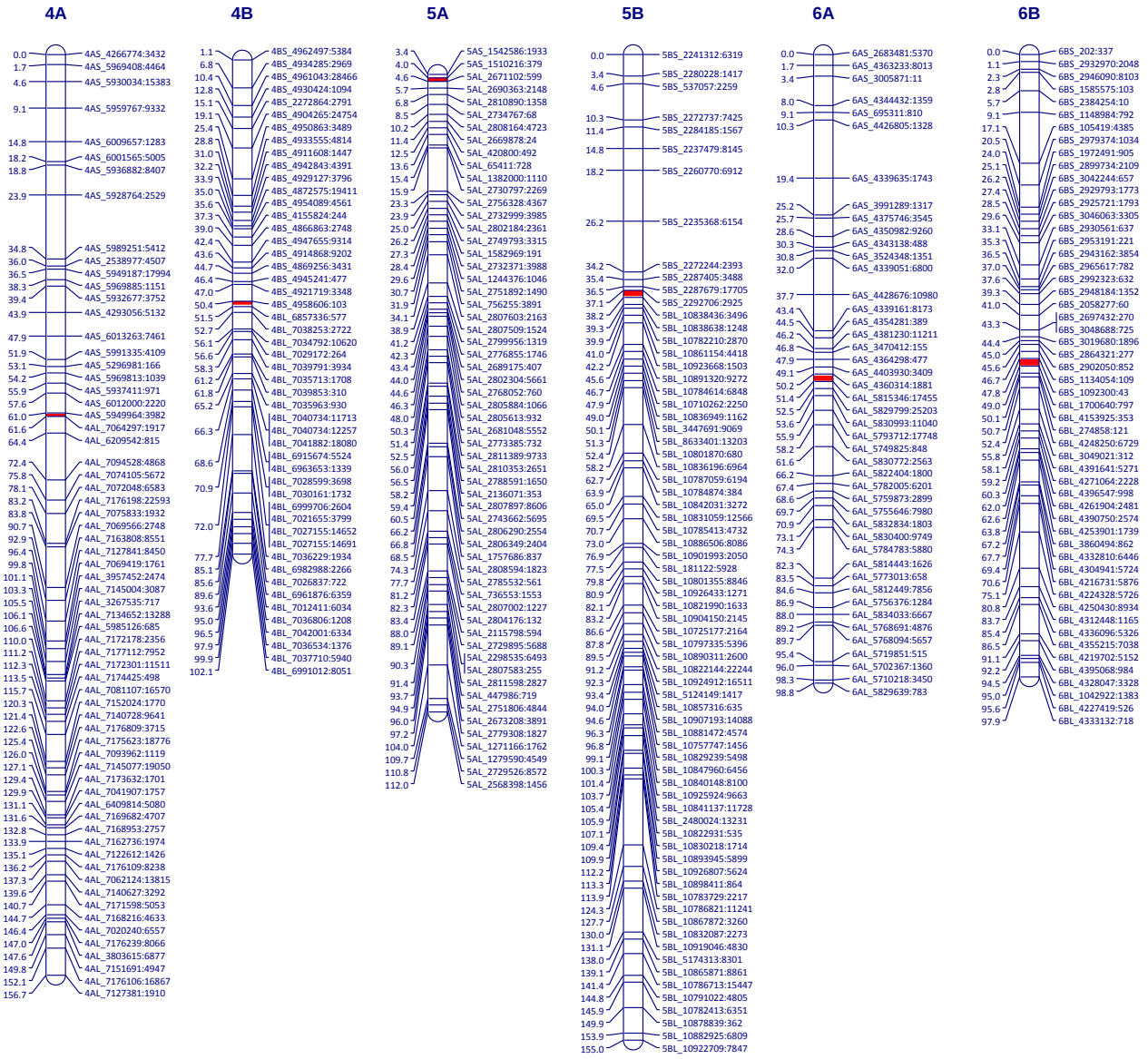
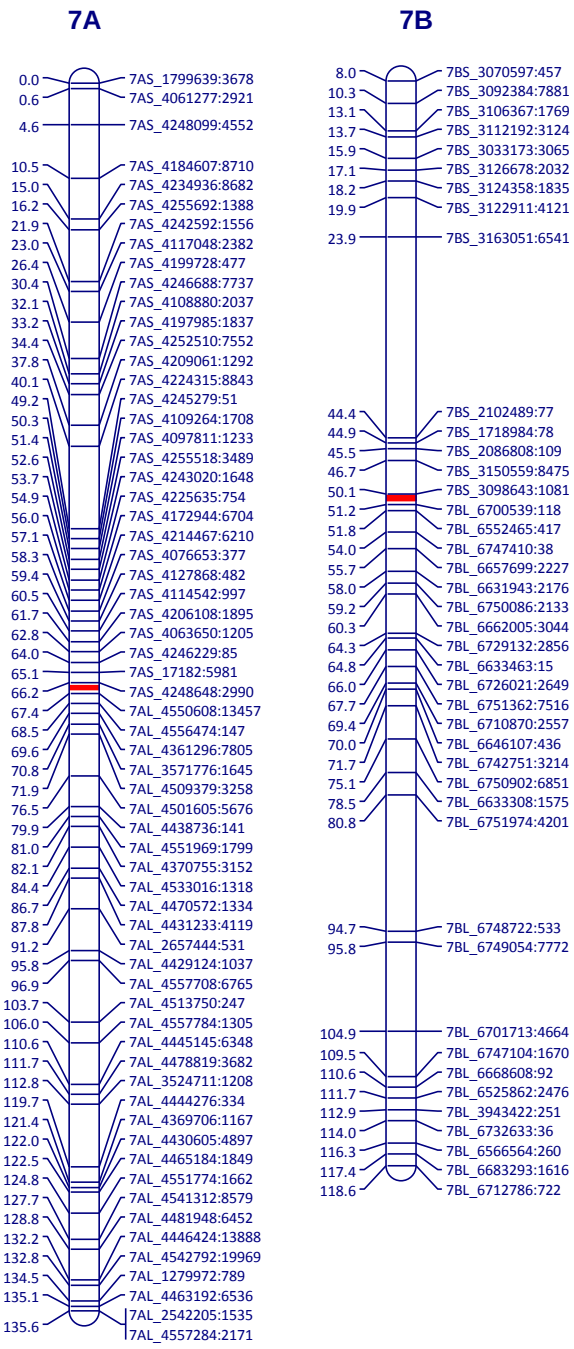


Figure 9-6. Linkage map of Markers used to genotype FL-*bh*-NILS (BC4F2 plants)



Supplementary Figure 10-6 continued...



Supplementary Figure 10-6 continued...

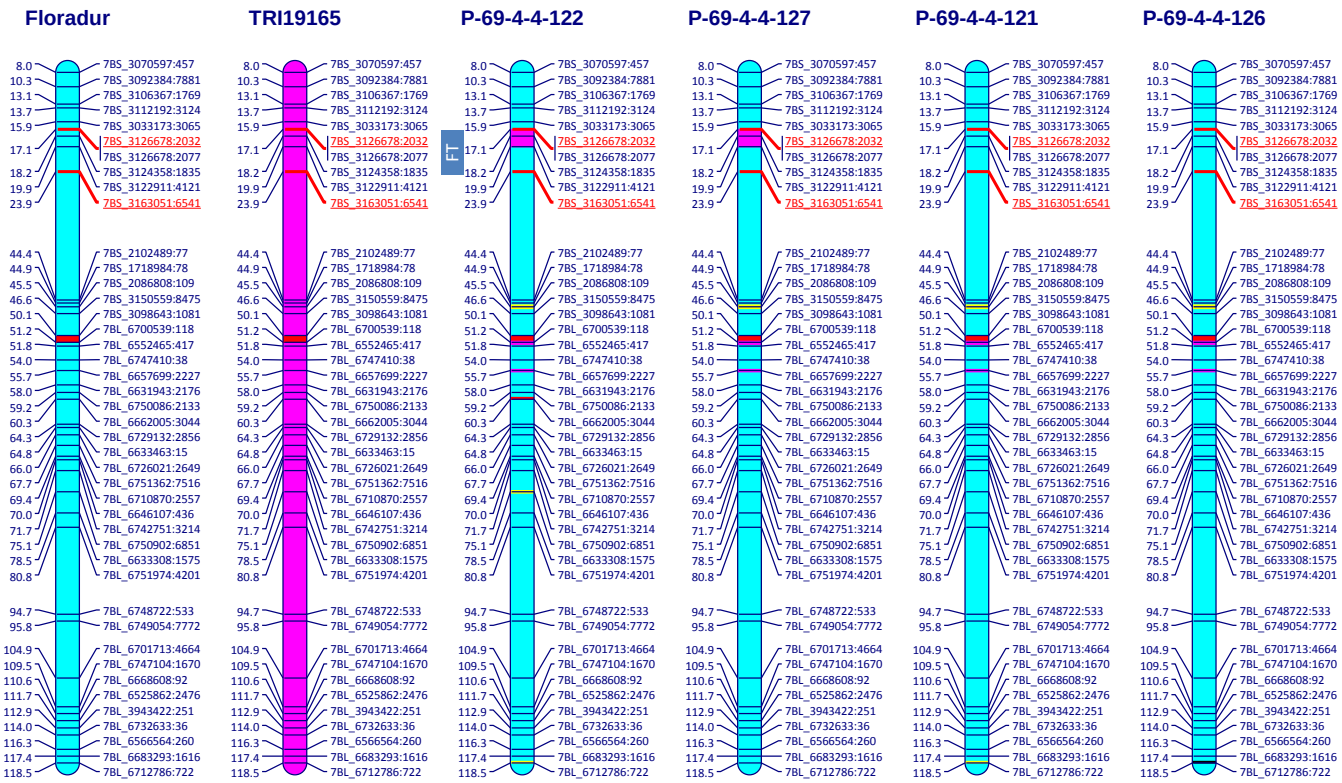


Figure 9-7. FL-*bh*-NILs from Fam1 plants carrying donor segment flanking the FT locus.
Markers flanking *QHD.ipk-7BS* (FT), which was mapped as a major effect QTL for heading date, are underlined. P-69-4-4-122 and P-69-4-4-127 carry the donor fragment from TRI 19165 while P-69-4-4-121 and P-69-4-4-126 carry allele from recurrent parent Floradur.