TILLING FOR *TTBH-1* GENE AND ITS HYBRIDIZATION TO TURKISH DURUM WHEAT CULTIVARS KIZILTAN-91 AND FUATBEY-2000

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submitted by **ABDULHAMİT BATTAL** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Department of Biotechnology , Middle East Technical University** by**,**

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Assoc.Prof.Dr. Çağdaş Devrim Son _____
Head of Department, **Biotechnology**

Prof. Dr. Meral Yücel
Supervisor, **Biology Department, METU** _____

Prof. Dr. Füsun Eyidoğan
Co-Supervisor, **Education Faculty, Başkent University** _____

Examining Committee Members:

Prof. Dr. Hüseyin Avni Öktem _____
Molecular Biology and Genetics Dept., KFAU

Prof. Dr. Meral Yücel _____
Biology Dept., METU

Assoc. Prof. Dr. Remziye Yılmaz _____
Food Engineering Dept., Hacettepe University

Assoc.Prof.Dr. Çağdaş Devrim Son _____
Biology Dept., METU

Assoc.Prof.Dr. Mehmet Cengiz Baloğlu _____
Genetics and Bioengineering Dept., Kastamonu University

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name :

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ABSTRACT

TILLING FOR *TtBH-1* GENE AND ITS HYBRIDIZATION TO TURKISH DURUM WHEAT CULTIVARS KIZILTAN-91 AND FUATBEY-2000

Battal, Abdulhamit

Ph.D., Department of Biotechnology

Supervisor : Prof.Dr. Meral Yücel

Co-Supervisor : Prof.Dr. Füsun Eyidoğan

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Wheat is one of the main crops to get daily calory requirement for people. Improving wheat yield is one of the most important aims in crop research today. Spike structure genes play a crucial role for yield increase. *TtBH-A1* gene has a single nucleotide polymorphism at the conserved domain causing branched spikes producing more spikelet, grains and yield in “Miracle-Wheat”.

In this study, *TtBH-1* homoeologous (*TtBH-A1* and *TtBH-B1*) gene were screened in tetraploid Kronos Targeting Induced Local Lesions In Genomes (TILLING)

population. 80 new alleles were confirmed for *TtBH-1* gene. One mutation leading to a premature termination codon was validated for the *TtBH-B1* gene in line T4-2432 (Q14*), while a mutation in a conserved domain was validated for *TtBH-A1* gene. Glycine at the position 61 of *TtBH-A1* protein was converted to serine as a result of mutation of guanine at the position 181 of the gene to adenine in line T4-2447. Supernumerary spikelets were observed in the A-genome homozygous mutant as short branches, whereas no effects were observed in the B genome mutants. Lateral spikelet development leading to additional spikelet side of the normal spikelet was initiated during glume promordium formation according to immature inflorescence observations. T4-2447 mutants produced significantly more spikelet than Kronos . A genome and B genome mutants were combined to make double mutants. *TtBH-1* mutations was backcrossed into parental Kronos to eliminate background mutations. Additionally, T4-2447 mutant line and “Miracle-Wheat” was hybridized with Turkish domestic cultivars Kızıltan-91 and Fuatbey-2000. Yield components were evaluated for T4-2447 mutants.

Keywords: TILLING, Yield, Branched Spike, Spikelet Number, Supernumerary Spikelet.

ÖZ

TtBH-1 GENİ İÇİN TILLING VE BU GENİN TÜRK DURUM BUĞDAYI KIZILTAN-91 VE FUATBEY-2000 ÇEŞİTLERİNE HİBRİDİZASYONU

Battal, Abdulhamit
Doktora, Biyoteknoloji Bölümü
Tez Yöneticisi : Prof.Dr. Meral Yücel
Ortak Tez Yöneticisi : Prof.Dr. Füsun Eyidoğan

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Buğday insanlar için günlük kalori ihtiyacını karşılamak için kullanılan ana tahıllardan biridir. Buğday verimini artırmak günümüz tahıl araştırmalarında en önemli hedeflerden birisidir. Başak yapı genleri verim artışı için önemli bir rol oynamaktadır. *TtBH-A1* geni korunmuş bölgesinde "Miracle-Buğdayı" nda daha çok başakçık, tane ve verim üreten dallanmış başakların oluşumuna sebep olan tek nükleotid polimorfizmine sahiptir.

Bu çalışmada, *TtBH-1* geninin homologları (*TtBH*-A1 ve *TtBH*-B1 tetraploid Kronos TILLING populasyonunda taranmıştır. *TtBH-1* geni için 80 yeni allel onaylanmıştır. *TtBH-A1* geni için korunmuş bölgesinde önemli bir mutasyon doğrulanırken, B genom homologu için *TtBH-B1* geni için T4-2432 hattında stop kodona yol açan bir mutasyon (Q14*) doğrulanmıştır. *TtBH-A1* proteininin 61inci pozisyonunda ki glisin T4-2447 hattında genin 181inci pozisyonunda ki guaninin adenine mutasyonu sonucu serine çevrilmiştir. B genom mutantlarında herhangi bir etki görünmemesine karşın, ekstra başakçılar kısa dallanmalar olarak A genomu homozigot mutantlarında gözlenmiştir. Normal başakçığın yan kısmında ekstra başakçığa yol açan yan başakçık gelişimi olgunlaşmamış başak taslağı gözlemlerine göre kavuz taslağı oluşumu sırasında başlamaktadır. T4-2447 mutantları anlamlı olarak Kronostan daha fazla başakçık üretmişlerdir. A ve B genomu mutasyonları ikili mutant yapmak için birbirlerine bağlanmışlardır. *TtBH-A1* mutasyonu arka plan mutasyonlarını azaltmak için ebeveyn Kronos ile geri çaprazlanmıştır. İlave olarak, T4-2447 mutantları ve "Miracle-Buğdayı" Türk yerel çeşitlerinden Kızıltan-91 ve Fuatbey-2000 çeşitleriyle hibritlenmiştir. Verim bileşenleri T4-2447 mutantları için değerlendirilmiştir.

Anahtar Kelimeler: TILLING, Verim, Dallanmış Başak, Başakçık Sayısı, Ekstra Başakçık.

To my lovely spouse Mehtap and my source of happiness Halil Mete and Oğuz Alp

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LIST OF ABBREVIATIONS

CRISPR-Cas9	Clustered, Regularly Interspaced, Short, Palindromic Repeats
DNA	Deoxyribonucleic Acid
EMS	Ethyl methanesulfonate
FZP	*FRIZZY PANICLE*
KASP	Kompetitive Allele Specific PCR
MgCl2	Magnesium Chloride
NaOAc	Sodium Acetate
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA interference
SNP	Single Nucleotide Polymorphism,
SS	Supernumerary Spikelet
TALEN	Transcription Activator-Like Effector Nuclease
TGW	Thousand Grain Weight
TILLING	Targeting Induced Local Lesions In Genomes

CHAPTER 1

INTRODUCTION

1.1.Wheat Plant

Wheat (*Triticum ssp.*) has been very important protein and calorie sources for humanity since its first domestication, nearly 10000 years ago in the Southeast part of Anatolia (Mesopotamia) (Figure 1.1). It has been one of the most cultivated crop plants for centuries. Bread wheat (2N=6X=42, *Triticum aestivum*) and durum wheat (2N=4X=28, *Triticum turgidum*) are the mostly planted species in the world. Two types of wheat are mostly cultivated: spring wheat and winter wheat. Winter wheat needs a vernalization period between 0 $^{\circ}$C and 10 $^{\circ}$C. Vernalization period is different for each variety to induce reproductive stage and not necessary for spring wheat varieties (Chouard, 1960; Pugsley, 1971; Semenov & Halford, 2009).

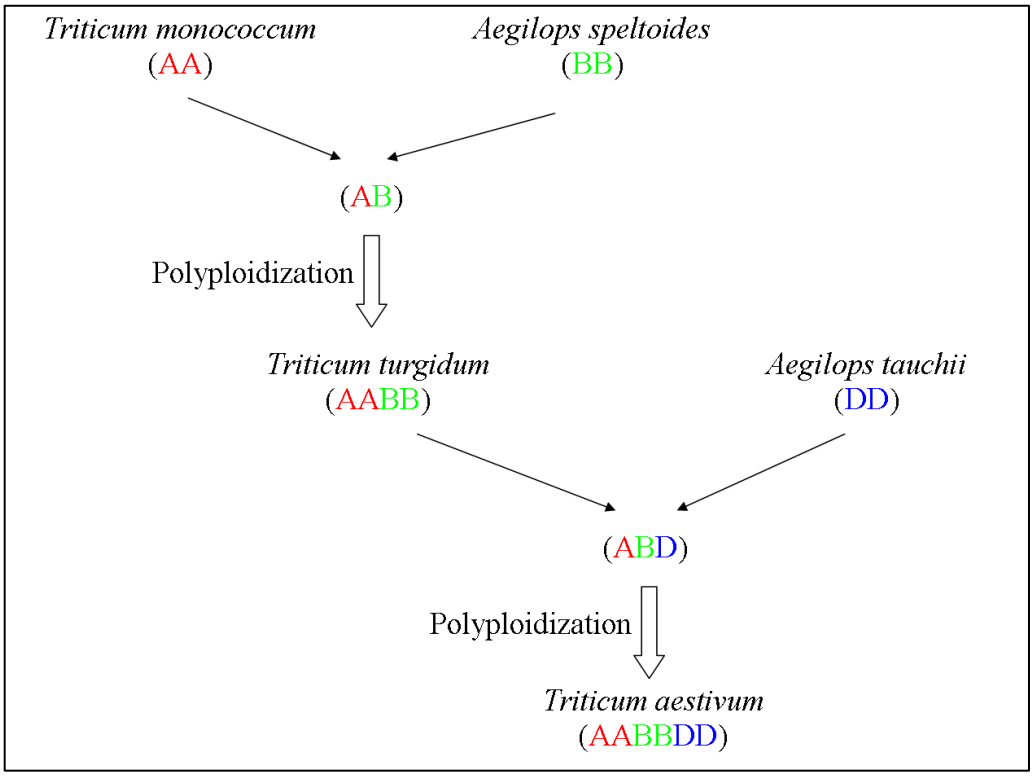


Figure 1. 1. Wheat evolution.

1.2.Wheat Development

Grain has high amount of carbohydrates (70%), protein (12%), water (12%), lipids (2%), vitamins and minerals (2%) and crude fibers (2%) (USDA).

Roots, coleoptile, leaves, tillers, stem and head are the main parts of wheat. When it is sowed, water intake begins quickly. 40% of humidity is enough for germination (Evans *et.al.*, 1975). Enzymatic activity starts after grain imbibition. Radicle (primary root node) emerges firstly as a result of germination. Secondary roots develop after third leaf. Roots main mission is intake water and nutrients for healthy growth (Baker & Gallagher, 1983; Hay & Kirby, 1991).

Coleoptile occurs after radicle development. It is a preservative sheath and a propellant force towards to surface for the first leaf. A leaf is composed of a sheath

and a leaf blade. Photosynthesis is the main task for leaves. They also help increase in strength of the stem. The last emerging leaf called flag leaf covers head (White & Edvards, 2008).

Lateral shoots developing axils or base of the main plant are called tillers (Figure 1.2). They can give heads and contribute to the yield. Stem carries head and leaves. Stem ends up with peduncle and head structures (White & Edvards, 2008).

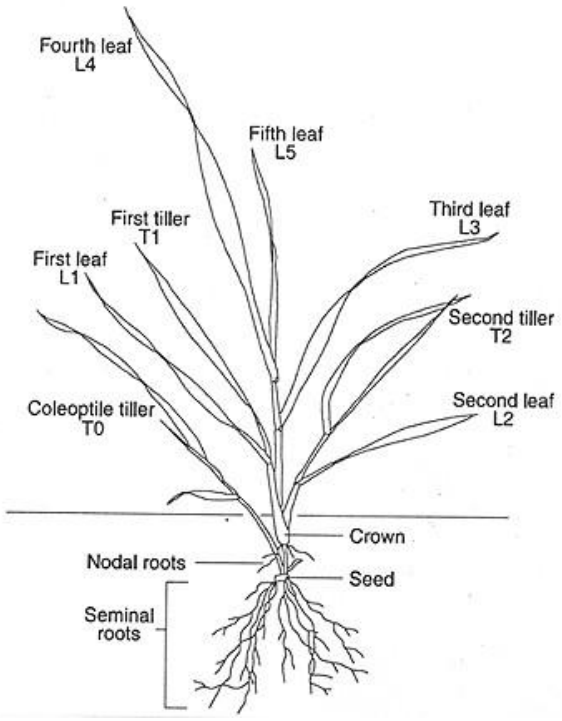


Figure 1. 2. Wheat development during tiller development stage. (NDSU)

The top of wheat plant is called head or spike or ear carrying spikelet having different number of florets according to species (Figure 1.3 A) (Allison & Daynard 1976; Kirby & Appleyard 1984; Rawson 1971; Rahman & Wilson, 1978). There are nodes and internodes in rachis the main part of spike (Figure 1.3 B). Spikelets emerge these nodes in both sides. Spikelets are connected to rachis by rachilla.

Apex embryonic part of plant is induced to form ear after fourth or fifth leaves emerged. It is 0.5 or 1.2mm long in this first stage called double ridge (Kirby, 1988; Kirby & Appleyard, 1987; Hay & Kirby, 1991). Spikelet formation begins firstly in the middle part of the small ear form as one spikelet in each node in opposite direction and continues up and down direction until terminal spikelet development (Figure 1.3 B). Terminal spikelet formation is a key factor for determination of spikelet number. When terminal spikelet formation is completed, ear has between 20 - 30 spikelet according to wheat genotype (Figure 1.3 C) (Allison & Daynard, 1976; Kirby & Appleyard, 1984). Glumes, lemmas and paleas are differentiated in this period (Barnard, 1955; Williams, 1975). Primary, secondary and central florets are formed as a result of differentiation of spikelet meristem to floral meristem (Figure 1.3 D). Each floret has three stamens and one ovary (Figure 1.3 E) (Kirby, 1988; Kirby & Appleyard, 1987; Hay & Kirby, 1991). Development of ear continues along with stem elongation. Spike emerges inside in flag leaf before anthesis (Krumm *et.al.*, 1990). Wheat is mostly self pollinated plant (Martin *et.al.*, 1967). Anthers are produced as white colour (Percival, 1921; Bennett *et.al.*, 1973). The colour changes firstly green and then yellow. Yellow anthers are ready for pollination. Stigma and style develop in the same period for anthesis taking in 3-10 days. Anthesis starts middle part of spike and continues basal and apical parts of spike (Peterson, 1965). Grains begin to enlarge their sizes after fertilization (Figure 1.3 F). Endosperm is formed until maturations. Peduncle changes yellow – brown colour after grain filling and maturity (Hanft & Wych, 1982).

Seeds are harvested after maturation. It takes nearly six months from planting to harvesting for spring wheats (Figure 1.4.). Winter wheat growth takes more time because of vernalization period between 0 and 10 °C.

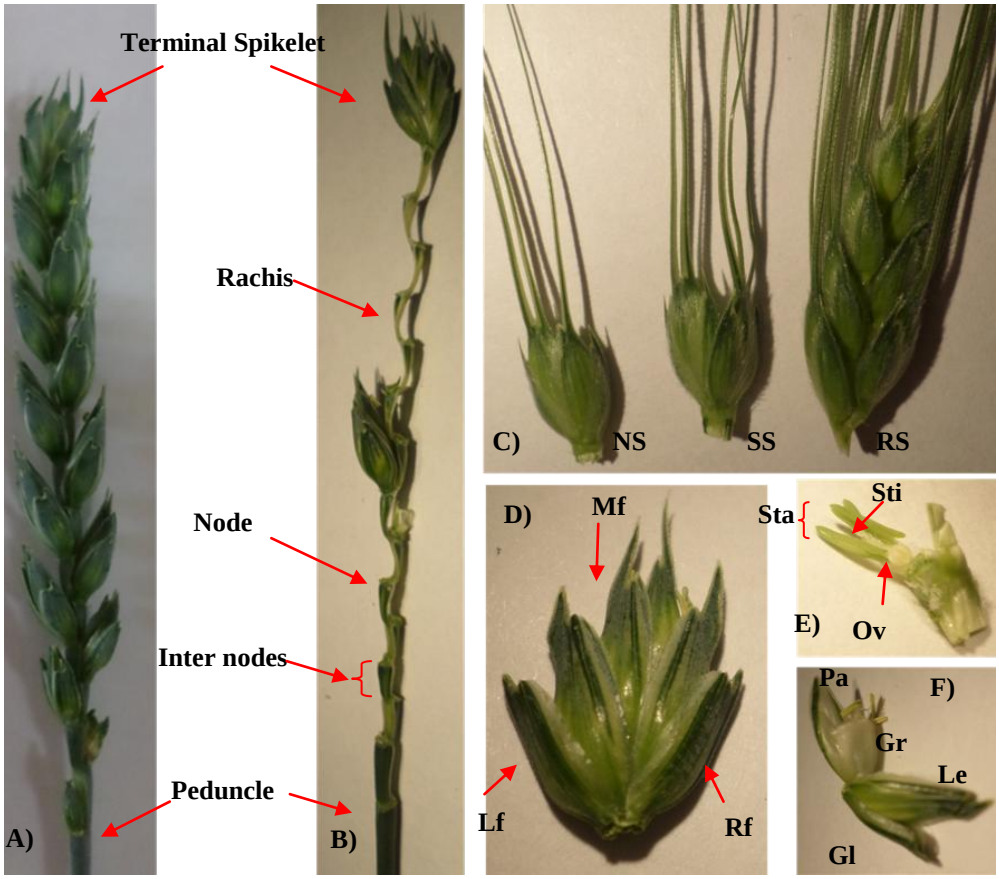


Figure 1. 3. Spike structure in wheat. A) A spike with spikelet. B) Rachis structure. C) Spikelet types. NS: Normal spikelet, SS: Supernumerary spikelet, RS: Ramified spikelet. D) A spikelet. Mf: Middle floret, Rf: Right floret, Lf: Left floret. E) A floret. Sta: Stamens, Ov: Ovary, Sti: Stigma. F) A pollinated floret. Pa: Plaea, Gr: Grain, Le: Lemma, Gl: Glume.

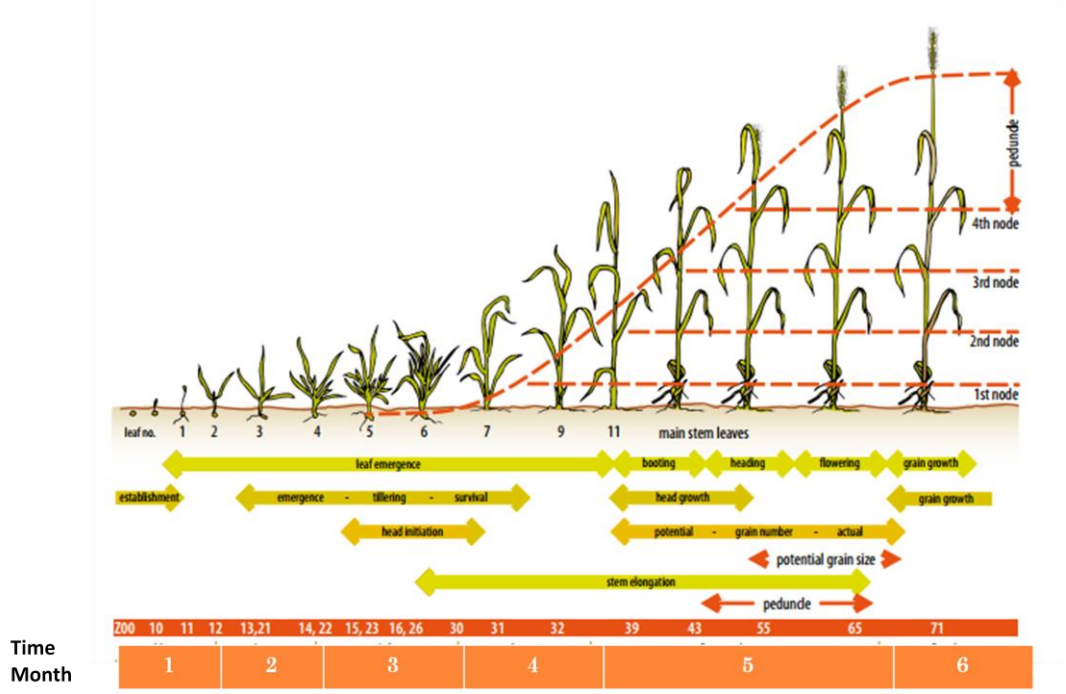


Figure 1. 4. Wheat life cycle for wheat. Adapted from (White & Edvards, 2008).

1.3.Global Wheat Production

Wheat production was about 729M (million) tonnes in the world and yield was 3289 kg/ha in 2014. China produced 126M tonnes wheat as a top country. India (94.5M tonnes), Russia Federation (59,7M tonnes), The United States of America (55,4M tonnes) and France (39M tonnes) were the other big wheat producers. Ireland had the highest yield 10014 kg/ha for wheat. Belgium (9412 kg/ha), The Netherlands (9169 kg/ha), Germany (8629 kg/ha) and New Zealand (8626 kg/ha) were the other countries having high yield. Russia Federation, China, India and Kazakhstan were the top seed producer in 2013 (Figure 1.5). (FAO)

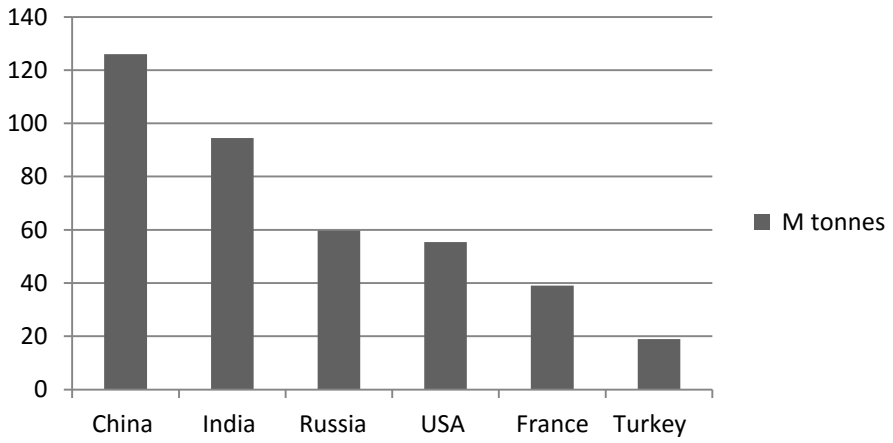


Figure 1. 5. World top wheat producers in 2014. (FAO)

1.4.Wheat Production and Trade in Turkey

Wheat was the most produced crop plant in Turkey in 2014. It was produced 19M tonnes and yield was 2429 kg/ha in the same season (Figure 1.6 and Figure 1.7) (FAO). It was reported that wheat produced 22.6M tonnes in 2015 and yield was 2890 kg/ha. Turkey needs about 22M tonnes of wheat according per year (TUIK).

If wheat trade in Turkey was evaluated between 2005 and 2015 according to FAO data, there was a sharp increase in import quantity 2007 and 2008. There were decrease in production and yield in 2007 because of highly dry season (Figure 1.6). However, import wheat quantity continued to increase after 2007. Whereas the wheat production was 21.8M tonnes, imported wheat quantity was increased up to 5M tonnes in 2011 because of demand for cultivation of higher yield potential cultivars and quality needs for flour production. 5233 tonnes of wheat was exported in the same year (Figure 1.8). There was more than 900 times difference between imported wheat quantity and exported wheat quantity. This is a major problem that must be overcome for Turkey.

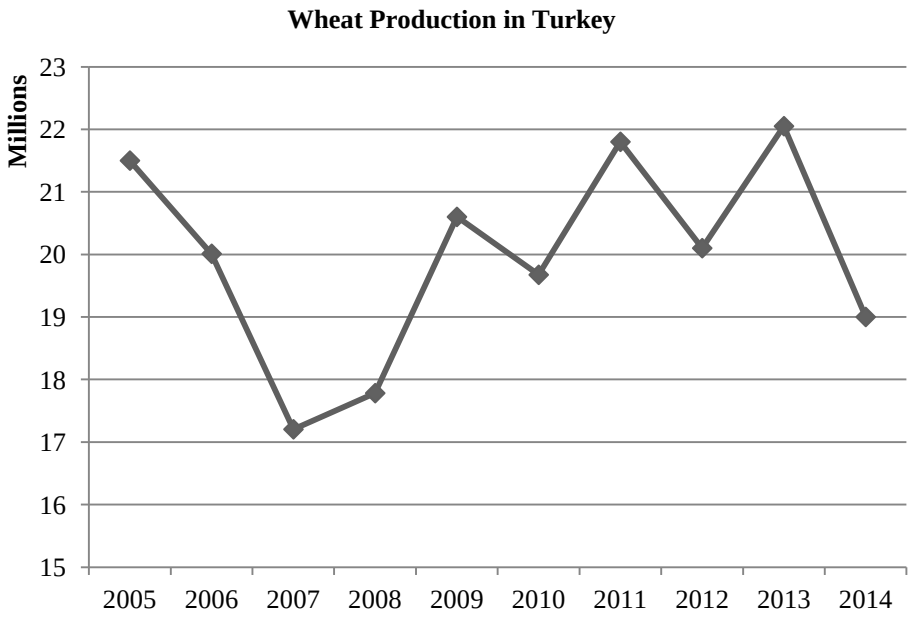


Figure 1. 6. Turkey wheat production in the last 10 years in million tonnes. (FAO)

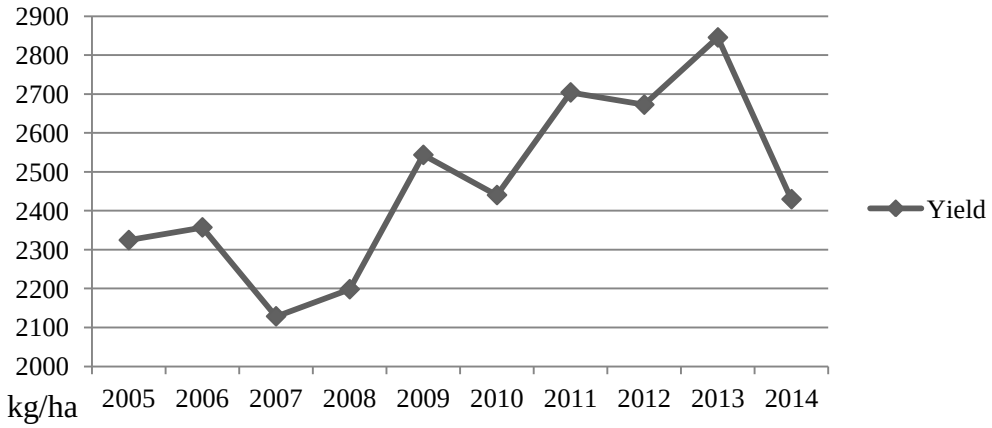


Figure 1. 7. Turkey wheat yield in last 10 years. (FAO)

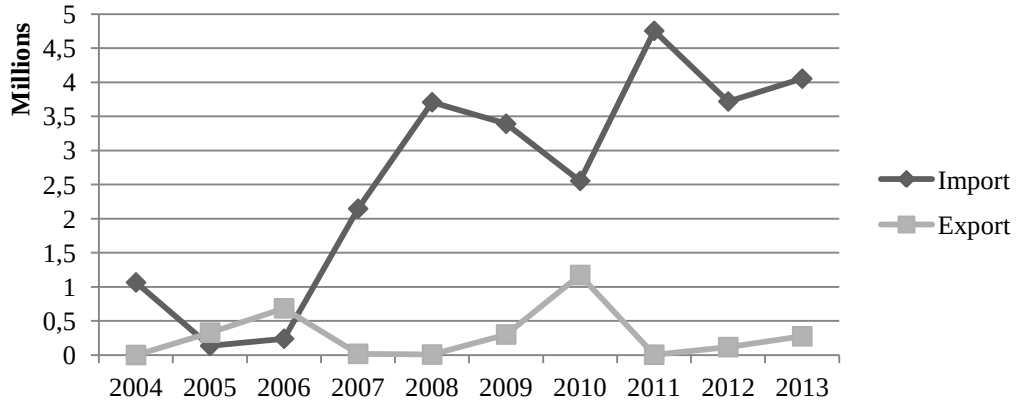


Figure 1. 8. Wheat trade quantities in Turkey between 2004-2013 years in million tonnes. (FAO)

1.5.Wheat Genetics and Biotechnology

Combination of genetics, modern biotechnology tools such as transgenics, functional genomics, -omics technologies, next generation sequencing, marker assisted breeding and classic breeding help to scientists to achieve boosting wheat yield.

1.5.1. Wheat Genetics

Tetraploid durum wheat (AABB) was evolved from hybridization of two diploid genomes, *Triticum urartu* (AA) and *Aegilops speltoides* (BB) (Feldman & Sears, 1981). Bread wheat has hexaploid genome (2n=42, AABBDD) (Sears 1954; Okamoto 1962). It was derived from hybridization of tetraploid durum wheat (2n=28, AABB) and diploid grass genome (DD, *Aegilops tauschii*) (Figure 1.1). While durum wheat has 10 gigabase pair genome in 14 pairs of chromosomes, bread wheat has 17 gigabase pair genome in 21 pairs of chromosomes. Wheat genome is complex to study because of its huge size and 80% of repetitive sequences (Choulet *et.al,* 2010). The International Wheat Genome Sequencing Consortium (IWGSC, http://www.wheatgenome.org/) was founded as an

international consortium by farmers, plant scientists and breeders to accelerate sequencing of bread wheat for development of improved wheat varieties. Advances in next generation sequencing technology help scientist to overcome this huge and complex genome. A chromosome based draft sequence for bread wheat was released by IWGSC in 2014 (Mayer *et.al.*, 2014). High quality physical maps for 6 more chromosome arms (2BL, 2BS, 4BL, 4BS, 5DL and 5BL) have been completed in addition to current 12 chromosomes in 2015 by IWGSC. It is estimated that creating a reference sequence for bread wheat will be completed until 2018.

1.5.2. Wheat Functional Genomics and Molecular Markers

Functional genomics is interested with discovery of a specific gene function. Transgenic technology and mutagenesis are the most widely methods for this aim. Molecular markers are very useful tools for breeding programs to follow easily phenotype.

1.5.2.1.Transgenic Technology

Transgenic technology is composed of tissue culture, regeneration and genetic transformation studies. Immature inflorescence, immature embryo and mature embryo are the main sources as explants for wheat tissue culture (Battal, 2010). Transformation introducing a gene into a genome to up or down regulate its function is performed after tissue culture optimization. Biolistic or particle bombardment and *Agrobacterium* mediated methods are generally used to transform wheat tissues. The high cost of preparing transformation construct (between 8000-10000$) is one of the disadvantages of transgenic research (Borrill *et.al.*, 2015a). Whereas high regeneration efficiency has been achieved, transformation efficiency is still very low for the most of the commercial wheat cultivars. Wheat genotype is the key factor for regeneration and genetic transformation. Bobwhite, Fielder and Chinese spring are the most widely used cultivars to observe a specific gene function. Recently, it was reported that Fielder

had a high transformation efficiency by using *Agrobacterium* protocol (Ishida *et.al.*, 2015). In addition to this study, commercial durum and bread wheat were successfully transformed by *Agrobacterium* mediated transformation method without selection marker (Richardson *et.al.*, 2014). Improvements in both of transformation efficiency and completed wheat sequence will open new doors for transformation studies.

RNA interference (RNAi) method has been applied successfully to wheat functional genomics (Fu *et.al.*, 2007). A, B and D homoeologoues having the same DNA region could be silenced simultaneously using a single RNAi sequence (Travella *et.al*., 2006). Yield and biomass (Ral *et.al.*, 2012; Bednarek *et.al.*, 2012; Hong *et.al.*, 2014; Borrill *et.al.*, 2015b), amylose content (Li *et.al.*, 2005; Sestili *et.al.*, 2010; Sestili *et.al.*, 2013), gluten content (Gil-Humanes *et.al.*, 2008; Gil-Humanes *et.al.*, 2012; Tyler *et.al.*, 2015), nutrient accumulation (Waters *et.al.*, 2009; Guttieri *et.al.*, 2013), disease resistance (Bhuiyan *et.al.*, 2009; Zhang, H. *et.al.*, 2012; Panwar *et.al.*, 2013; Cheng *et.al.*, 2015; Chen *et.al.*, 2015), and stress tolerance (Du *et.al.*, 2013; Liang *et.al.*, 2014) were studied by RNAi silencing method.

Genome editing technologies have been very popular recently. Transcription Activator-Like Effector Nuclease (TALEN) (Boch *et.al.*, 2009; Moscou & Bogdanova, 2009) and Clustered, Regularly Interspaced, Short, Palindromic Repeats (CRISPR-Cas9) (Cong *et.al.*, 2013) are promising developments for wheat functional genomics (Chen and Gao, 2013). First studies for genome editing in wheat were based on practicability by using transient expression systems (Upadhyay *et.al.*, 2013; Shan *et.al.*, 2013). A single TALEN construct prepared for *TaMlo* (*Mildew Resistance Locus O*) gene was transformed to bread wheat by particle bombardment. This single construct edited all 3 homoeologous (A, B and D copies). Triple mutants having edited *TaMlo* A, B and D homoeologous conferred resistance to powdery mildew (Wang *et.al*, 2014). CRISPR/Cas9 construct edited only A genome homoeologous in the same study. Genome editing protocol by CRISPR/Cas9 system for wheat and rice was reported (Shan *et.al.*, 2014).

Improvements in sequencing, genome editing technology and transformation efficiency will open new insights for wheat functional genomics.

1.5.2.2. Mutagenesis

Mutagenesis is a powerful method for both of forward genetics or classic genetics (from phenotype to gene) and reverse genetics (from gene to phenotype). Chemical and radiation mutagenesis are the most widely used techniques to create mutant population. Ethyl methanesulfonate (EMS) is one of the mostly used chemical agent causing induced point mutations in genome.

'Targeting Induced Local Lesions (mutations) In Genomes' (TILLING) is a non-transgenic and a reverse genetic tool (Henikof *et.al.*, 2004). Rapidly generation of high-troughput allelic series for any gene, sensitivity and cost effectiveness are the other advantages of TILLING. It was firstly applied to *Arabidopsis*, a model organism (McCallum *et.al.*, 2000). TILLING has enabled valuable information for functional genomics for many organisms such as, *Arabidopsis* (McCallum *et.al.*, 2000; Martin *et.al.*, 2009), zebrafish (Wienholds *et.al.*, 2003; Winkler *et.al.*, 2011; Da Costa *et.al.*, 2014); tomato (Piron *et.al.*, 2010), *Brassica* (Stephenson *et.al.*, 2010), soybean (Cooper *et.al.*, 2008), *Brachypodium* (Dalmais *et.al.*, 2013), rice (Wu *et.al.*, 2005; Till *et.al.*, 2007a; Serrat *et.al.*, 2014), barley (Caldwell *et.al.*, 2004; Talame *et.al.*, 2008) and wheat (Slade *et.al.*, 2005a; Uauy *et.al.*, 2009; King *et.al.*, 2015).

Mutated population development, isolation and pooling of DNA and detection of mutations are important parameters for TILLING (Figure 1.9). The best mutagen causes base changes, or small insertions and deletions at a high density in the genome in TILLING studies (Till *et.al.*, 2007b). EMS has a potential to produce mostly point mutations (missense and nonsense) at a high frequency (Koornneef *et.al.*, 1982; Uauy *et.al.*, 2009). Therefore, EMS is used as a chemical mutagen agent the most of the mutagenesis studies. It was reported that more than 99% of identified mutations were Guanine>Adenine and Cytosine>Thymine transitions in

wheat (Slade *et.al.*, 2005a). Seeds are treated with EMS in different concentrations. EMS concentration must be optimized for each genotype. M1 plants are grown from M0 seeds treated with EMS. They are self fertilized to generate M2 plants. While M1 plants have heterozygous progeny, M2 plants have 1:2:1 Mendelian ratio. DNA is isolated from young leaves of M2 plants. DNA extraction method, quantification and DNA quality directly affect screening step. Isolated DNA can be pooled into 2-, 4-, 6-, 8- and 16- individual DNA into one pool. M3 seeds are stored for further mutation validation and phenotype analysis (Till *et.al.*, 2007b; Henikoff *et.al.*, 2004).

When a mutation is detected in a pool, each individual in this pool is screened to discover mutant plant. Fluorescently labelled gene specific primers are used for about 1.5 kb product after PCR. Heteroduplexes (one DNA strand from wild type and one DNA strand from mutant) are generated by adding a heating cooling step end of the PCR. COODLE (for Codons Optimized to Detect Deleterious Lesions, http://blocks.fhcrc.org/~proweb/input/) is an easy web-based tool to design primer for TILLING studies (Till *et.al.*, 2003). PCR reaction is increased up to 99°C after final extension. Heteroduplexes are formed during slowly cooling step. *Cel*1 enzyme, a member of S1 nuclease family, extracted from celery can recognize and digest mismatches on the 3'-downstream of DNA (Desai & Shankar, 2003; Oleykowski *et.al.*, 1998; Yang *et.al.*, 2000). SNPs as a result of mutagen are detected by denaturing high performance liquid chromatography (dHPLC), denaturing gradient gel electrophoresis (DGGE), single strand conformational polymorphism analysis (SSCP) and sequence analyzers (slab gel or capillary systems) (Comai & Henikoff, 2006; Weil, 2009). Li-COR® DNA gel analyzer system is used widely to identify SNP. *Cel*1 digested fragments could be analyzed by ABI3730XL sequencer (Le Signor *et.al.*, 2009). Individual mutant DNAs in the determined pool are amplified and sequenced to identify which mutant plant has the mutation. Identified mutations are evaluated according to functional results for protein such as, causing a truncation or nonsense mutation and conserved domain and DNA binding region missense mutations. PARSESNP (for Project Aligned

Related Sequences and Evaluate SNPs; http://blocks.fhcrc.org/~proweb/input/) is one of the best web tool for mutation evaluation for its functional result (Taylor & Greene, 2003). This program gives some values for SNP effect such as position specific scoring matrix (PSSM) for amino acids and SIFTS (Sorting Intolerant From Tolerant) (Ng & Henikoff, 2003). If SNP has a large positive PSSM, mutation can be resulted with dramatically changing with protein function. Therefore, PARSESNP output gives information restriction sites on the SNP points lost or gained enzymes (Kurowska *et.al.*, 2011). Other web based tools should be used for identification of mutation effect such as NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/ Structure/cdd/wrpsb.cgi). This tool gives also PSSM value about amino acid position according to their presence in the sequence. However, this program needs to eye-aid study.

M3 seeds are planted for mutation validation and phenotype observations. Mutant plants have lots of background mutations because of high density mutation. Bread wheat had the top mutation density (1 mutation/25kb), and durum wheat had 1 mutation/34kb (Slade *et.al.*, 2005b). While barley had 1 mutation/Mb (Caldwell *et.al.*, 2004), rice had 1 mutation/500Kb (Wu *et.al.*, 2005). High mutation density capacity of wheat is very useful for functional genomics. More than 65 mutations should be identified for 1.5 kb fragment for wheat TILLING population having 1500 individual. 6 or 7 new alleles should be discovered for diploid TILLING population having 1500 individual. The probability of identification of truncation mutants (premature termination codon or splice acceptor/donor site) is 95% and 85 % for bread wheat and durum wheat relatively. However, this probability is 25 % for diploids (Borrill *et.al.*, 2015a). Therefore, mutant plants are two or three times backcrossed with wild type plant to eliminate their phenotypic effects of background mutations (Dong *et.al.*, 2009a; Simmonds *et.al.*, 2016). As previously stated, durum wheat has at least two homoeologous (A and B) and bread wheat has at least three homoeologous (A, B, and D) for a gene. Linking A and B genome mutation to make double mutant for durum wheat and combining A, B, and D copy mutations to make triple mutant give stronger phenotype (Slade *et.al.*, 2005b).

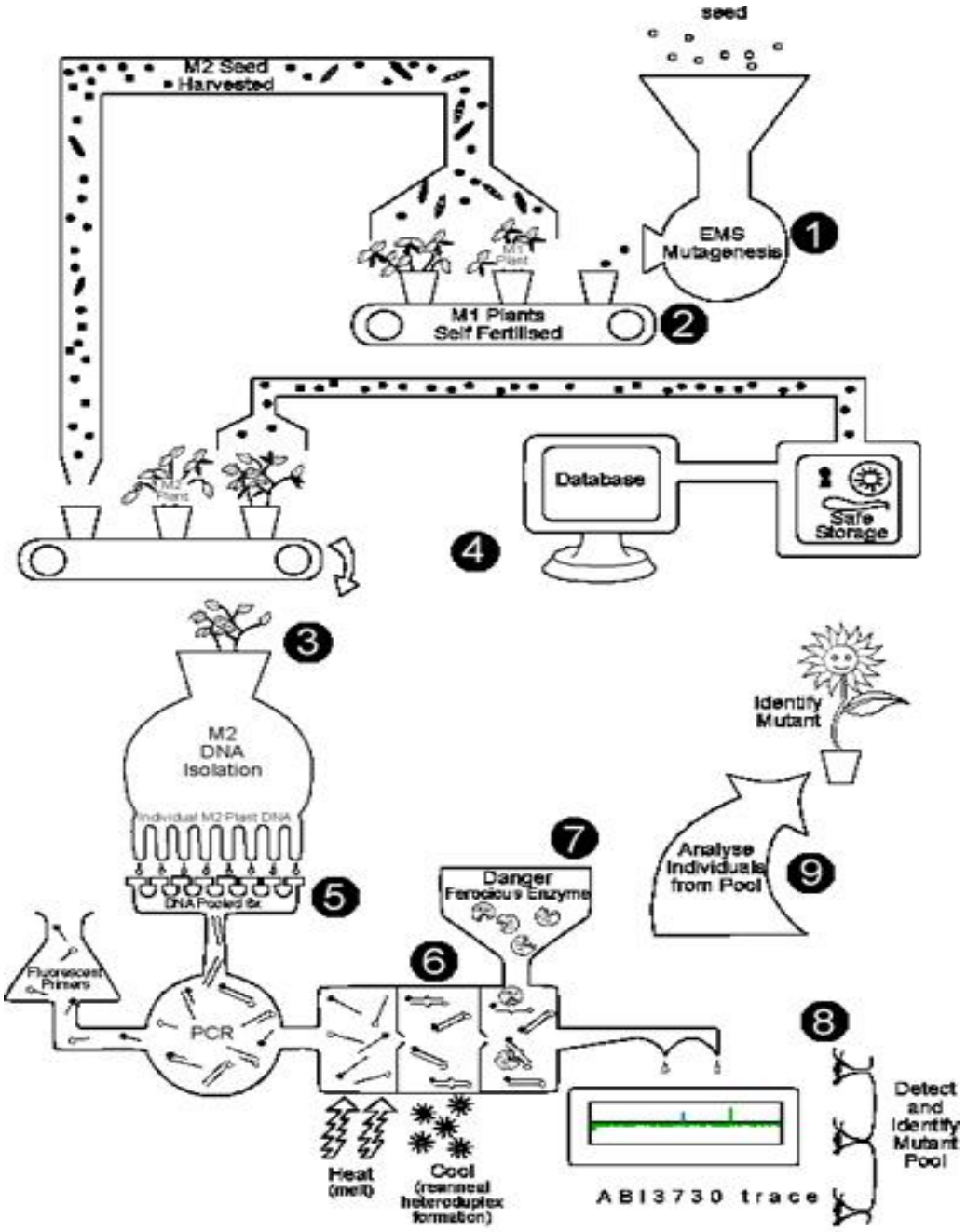


Figure 1. 9. TILLING Process. 1. EMS Mutagenesis 2. Selfing M1 plants to harvest M2 seeds, 3. DNA extraction, 4. Database and M3 seed storage, 5. Amplification, 6.Heteroduplex Formation, 7. Cleavage by *Cel*1, 8. Cleavage products, 9. Mutant identification. (Wang *et. al*., 2012)

There are many studies for wheat TILLING studies. The first report was about that 246 new alleles were discovered for *waxy* genes for three homoeologous in TILLING population. It was observed that triple mutant was nearly null phenotype (Slade *et.al.*, 2005b).

waxy gene was evaluated in another TILLING population having 2348 plant mutated by EMS. 121 mutations were discovered for A and D homoeologous. A complete waxy wheat was generated as a result of crossing of truncation mutants for A and D genome and naturally null B genome (Dong *et.al.*, 2009a).

A very useful TILLING population was generated for tetraploid and hexaploid wheat. Mutation frequency was 1 mutation/38 kb for common wheat and 1 mutation/51 kb for durum wheat. Non-polyacrylamide gel screening was compared with Li-COR method. 275 novel alleles were identified for 11 gene/genome combinations for both of population (Uauy *et.al.*, 2009).

High Resolution Melting (HRM) analysis and Mutation Surveyor® software were tested for *Starch Synthase II* (*SSII*) gene in wheat TILLING population. They were offered as an alternative method to detect mutation for three homoeologous. Combination of HRM scanning and Mutation Surveyor software analysis was too sensitive even if heterozygous state (Dong *et.al.*,2009b).

EMS induced common wheat TILLING population was studied for disease related genes such as, kinase START gene. More than 100 mutations causing premature stop and conserved domain structure and missense were observed (Fu *et.al.*, 2009).

Starch branching enzyme IIa (*SBEIIa*) was screened in a hexaploid TILLING population using high resolution melting analysis. More than 100 mutations were identified for A, B, and D homoeologous of this protein. Increase in amylose content in double mutant was reported (Botticella *et.al.*, 2011).

TILLING population having 1532 individual was generated for diploid wheat. Mutation frequency of this population was higher than other diploids (Rawat *et.al.*, 2012).

SBEII genes was also studied another durum wheat TILLING population. While increase amylase and resistant starch content was not significantly observed in single mutants, double mutants had the desired phenotype (Hazard *et.al.*, 2012).

Increase in amylose content and resistant starch content was observed in tetraploid and hexaploid wheat mutants (Slade *et.al.*, 2012).

Wheat flowering was studied in durum wheat double mutants. It was reported that homozygous double mutants (having a truncation mutation both of A and B genome) had the drastic changes in spike architecture (Chen *et.al.*, 2014).

A splice site mutation verified in tetraploid TILLING population caused increase in thousand grain weight in tetraploid and hexaploid wheat (Simmonds *et.al.*, 2016).

Advances in sequencing technology have presented public available in silico TILLING source. Two TILLING platforms have been developed for wheat by exon capture. University of California Davis wheat TILLING service (http://dubcovskylab.ucdavis.edu/wheat-tilling) re-sequenced more than 1000 mutants for Kronos durum wheat (Tsai *et.al.*, 2011). Mutations on the open reading frame were identified. They were evaluated according to their effects for protein function. Therefore, exome capture and next-generation sequencing was efficiently used for EMS-induced mutation detection for pasta wheat (Henry *et.al.*, 2014). Another wheat TILLING service has been announced by support of joint project including John Innes Centre, The Genome Analysis Centre, Rothamsted Research and University of California Davis. More than 1000 EMS mutated durum wheat cultivar Kronos and more than 600 EMS mutated bread wheat cultivar Cadenza have been exon-captured by Illimuna next generation sequencing technology. Re-sequenced mutant lines were aligned with Chinese spring cultivar sequence available in IWGSC. Identification of mutations and protein annotations were

completed for most of the mutants (King *et.al.*, 2015). These TILLING platforms present very valuable source for wheat functional genomics and breeding.

1.5.2.3.Molecular Markers

Markers are used to follow desired trait in the phenotype. They have been applied to most of the crop plants including rice, wheat, barley etc. Morphological, biochemical and molecular markers can be used for this aim. While morphological and biochemical markers can change by environmental effects, molecular markers are heritable and more stable (Kordrostami & Rahimi, 2015). Molecular markers or DNA markers the mostly used markers are based on DNA variations caused by a mutation or natural way. They help to breeders and scientists to discover new quantitative traits to improve new cultivars having higher yield and quality potential, resistance to biotic and abiotic stresses (Goutam *et.al.*, 2013; 2015). Hybridization-based markers (Restriction Fragment Length Polymorphism, RFLP (Botstein *et.al.*, 1980; Paull *et.al.*, 1995; Alvarez *et.al.*, 2013; Naz *et.al.*, 2014)), PCR-based markers (Random Amplification of Polymorphic DNA, RAPD (Williams *et.al.*, 1990; Qi *et.al.*, 1996; Shi *et.al*, 1998; Bhutta & Amjad, 2015; Mohamed *et.al.*, 2015; Qadir *et.al.*, 2015); Amplified Fragment Length Polymorphisms, AFLPs (Vos *et.al.*, 1995; Balta *et.al.*, 2014; Ejaz *et.al.*, 2015; Das *et.al.*, 2015); Cleaved Amplified Polymorphic Sequences, CAPS (Konieczny & Ausubel, 1993; Shcherban *et.al.*, 2015; Jaiswal *et.al.*, 2015; Hanif *et.al.*, 2016); Microsatellite or Simple Sequence Repeats, SSR (Litt & Luty, 1986; Wang *et.al.*, 2002; Simmonds *et.al.*, 2008; Das *et.al.*, 2015; Liu *et.al.*, 2015; Wang *et.al.*, 2015; Bansal *et.al.*, 2015; Lu *et.al.*, 2016; Islam *et.al.*, 2016)) and sequence-based markers (Single Nucleotide Polymorphism, SNP (Lander, 1996; Tyrka *et.al.*, 2015; Liu *et.al.*, 2016; Wang *et.al.*, 2016)) are the most widely used DNA marker types. These markers are mostly used for marker assisted selection (MAS), quantitative trait loci (QTL) mapping, physical mapping of gene, comparative breeding, taxonomic classification and breeding (Hayward *et.al.*, 2015).

Development of SNP based markers is recent trend as a result of next generation sequencing. Minimum assay cost, locus specificity, simplicity and high-throughput potential are some of the advantages of SNP markers (Rafalski, 2002; Schlötterer, 2004; Semagn *et.al.*, 2014). Many SNP based platforms have been developed such as, BeadXpress™, GoldenGate™, and Infinium® from Illumina (http://www.illumina.com); GeneChip™, and GenFlex™ Tag array from Affimetrix (http://www.affymetrix.com); SNaPshot™ and TaqMan™ from Applied Biosystems (http://www.appliedbiosystems.com) and competitive allele-specific PCR (called Kompetitive Allele Specific PCR, or KASP™) from KBioscence or LGC Genomics (http://www.lgcgenomics.com) are some of the these SNP based genotyping platforms.

1.6.Yield increase in wheat

Spike length, spikelet number, grain number and size are the main yield parameters. Whereas genotype has an important role on these parameters, biotic (insects, microorganisms and fungi) and abiotic stresses (drought, salinity, water deficiency, heavy metals, micronutrient deficiency and heat and cold etc.) cause drastic decrease in yield potential. Many morphological, physiological, biochemical, molecular and biotechnological studies have been performed in wheat to understand of the background of these stresses at Yücel & Öktem Plant Biotechnology Laboratory (Öktem *et.*al, 1999; Durusu, 2001; Demirbaş, 2004; Kavas *et.al.*, 2007 & 2008; Battal, 2010; Baloğlu, 2011; Battal *et.al.*, 2012; Baloğlu *et.al.*, 2012 & 2013,; Kayıhan, 2014; Öz *et.al.*, 2014).

Spike development is directly affected as a result of biotic and abiotic stresses. Spike architecture genes have a crucial role to prevent yield decrease in crops. Sink (seed) capacity is a limiting factor for wheat yield (Miralles & Slafer, 2007). Increase in grain number contributes to yield increase. Normally, spike has one spikelet per node at the opposite sides. More than one spikelet per node or extended rachilla having spikelet instead of florets was called ear abnormality. This phenotype was called supernumerary spikelet (SS) structure or branching for this

abnormality (Martinek & Bednar, 1998). Miracle wheat (*T. Turgidum convar. compositum (L.f.) Filat.*) having a highly brached spikelet phenotype has a higher yield potential than unbrached tetraploid wheats (Figure 1.10). If additional spikelet emerges side of the normal spikelet it was called right angle spikelet or true spikelet. On the other hand, additional spiklet occuring base of the normal spikelet was called parallel type spikelet. It was considered that genetic control mechanisms of this characters are different (Masubuchi, 1974).

Figure 1. 10. Branched and unbranched wheat. A) Branched Miracle wheat. B) Unbranched Kronos.

Sharman in 1944 was reported that recessive brached head (*bh*) gene controlling branching in durum wheat under normal conditions. Longer daylight or high temperature prevented formation of branched spikes.

Twin spikelet production was observed in hexaploid wheat plants nullisomic for chromosome 2A and 2D (Sears, 1954).

Koric (1966) informed that the number of seeds of branched hexaploid wheat increased to 180-200 and also thousand grain weight increased up 30-35 grams.

Rana (1969) reported that branching spikes were observed in bread wheat mutant population. The normal spikelets were changed to as a small spike appearance. Day length and temperature affected occurrence of branched spikelet. Grain number was changed between 100 and 160 in the main tiller and first few tillers. It was 70 for wild type.

Branched common wheats compared with normal cultivars to determine yield by Rawson & Ruwali in 1972. Floral initiation and terminal spikelet differentiation was later in branched wheat than normal cultivars. It was observed that branched wheat produced more than 80 spikelets and up to 128 grain in an ear. There were branched spikelets in the lower part of the ear. Also, supernumerary spikelet phenotype was present in the middle part of the ear. Rest of the ear was the normal spikelet. Most of the spikelets were not fertile. Branching structure was decreased under severe drought conditions.

Masubuchi in 1974 used five spike having additional spikelet to test branching genetic background. True spikelet, short branches and long branches (ramification) abnormalities were observed in crossed population. Frequency of these abnormalities was increased in further generations according to results. Additional spikelet and short branches were also observed when branched wheat crossed with normal cultivars. However, the frequency and quality was lower than parental branched one. Environmental factors had an important role for branching degree according to results.

It was reported that crossing of branched durum wheat with domestic cultivars resulted in yield increase (Aslam & Buhtta, 1977).

Inheritance of supernumerary spikelet in wheat was reported by Pennell & Halloran in 1983. There were two independent gene controlling supernumerary spikelet character according to this study. Inheritance was recessive for these genes. In addition to this, environmental factors could be affected branching.

Effects of vernalization and photoperiod were evaluated for two durum wheat and five common wheat having supernumerary spikelet phenotype (Pennell & Halloran, 1984). Multiple sessile spikelet (MSS) and indeterminate rachilla spikelets (IRS) types were identified. It was introduced that the use of some genotypes being more stable for supernumerary spikelet phenotype could contribute increase in grain yield per spike in breeding programs for commercial wheat.

Supernumerary spikelet formation was observed after terminal spikelet development. Lower number of florets and grains in supernumerary spikelet wheat were found than normal spikelet wheat. However, total number of grains were higher in supernumerary spikelet wheat than normal wheat. It was reported that reduction of expression of the indeterminate rachilla spikelet could cause increase in productivity of supernumerary spikelet (Kadkol & Halloran, 1988).

Chromosome location of supernumerary spikelet was found for tetraploid wheat (Klindworth *et.al.*, 1990). Short arm of chromosome 2A was determined as a major gene location for supernumerary spikelet phenotype. In addition to this, chromosome 2B had some minor gene locations according to segregation analysis. It was introduced that chromosome 2D had a location with a strong inhibitor gene for supernumerary spikelet phenotype.

Branched durum wheat was compared with two common wheat having normal ears by Hucl and Fowler in 1992. They tested branched wheat yield potential. It was observed that branched wheat had less kernel than common wheats. In addition to this, yield of branched wheat decreased in drought area.

It was presented that two recessive gene and some other genes had a control for "Branched Spikes 33" in different environments (Wenye *et.al.*, 1995).

Branched head (*bh*) gene was mapped with a distance 8.5 cM $\pm$ 2.1 cM from the centromere of chromosome 2A short arm (2AS) (Klindworth *et.al.*, 1997).

Barley, a close relative to wheat, *branched-5* (*brc5*) gene was mapped on chromosome 2 using AFLP markers (Castiglioni *et.al.*, 1998).

Chromosomal locations of genes for supernumerary spikelet in bread wheat was studied by Peng and collegues in 1998. They used bread wheat "Yupi" line having branching forms and Chinese Spring monosomic series. They discovered that *bh* genes of "Yupi" were recessive according to F_1 monosomic evaluation. 2D having a recessive *bh* gene had the strongest effect for supernumerary spikelet phenotype in bread wheat. *bh* genes were hemizygous-effective and dosage-independent as a result of this study. In addition to chromosome 2D, supernumerary spikelet frequency was significantly high for 4A and 5A F_2 populations. They discovered that chromosome 4B had a *bh* gene locus. However, it was considered as a weak effective gene.

FRIZZY PANICLE (*FZP*) gene orthologue of *BRANCHED SILKLESS1* (*BD1*) in maize was cloned from rice by Komatsu and colleagues in 2003. They observed highly branched phenotype in *fzp* mutants. It was reported that *AP2/ERF* domain and acidic domain were neccessary for *FZP* function. Mutation on these domains caused severe, weak and temperature sensitive phenotype. Floral fate was maintained by normal *FZP* gene activity according to results. *FZP* worked as a transcriptional activator for the transition of spikelet meristem to floral meristem.

"FEN33" having many branched ears and higher yield potential was evaluated for histologic, morphologic and inheritance characters (Yan, 2007). It was mapped using SSR markers for brached ear. Two recessive genes located on chromosome 2A and 2D seriously affected branched spike phenotype according to F2 population

results. In addition to this two gene, some genes were also had a role for this phenotype.

Supernumerary spikelet gene on chromosome 2D was mapped using microsatellite markers (Dobrovolskaya *et.al.*, 2009). *Mrs* (*Multi row spikelet*) phenotype, a different type of supernumerary spikelet, was observed under control of a recessive gene at a single locus on chromosome 2D. It was reported that *Mrs1* gene was othologue for *bh* gene in durum wheat.

Aliyeva (2009) discovered a new type of branching in "171ACS" line as a new source for hard wheat.

Inheritance of new braching type originated by "171ACS" line (Aliyeva, 2009) was investigated in hybrid populations (Aliyeva & Aminov, 2011). They stated that this new type of branching formation controlled a single recessive gene without dosage effects.

Spike branching genes (*sb1* and *sb2*) in "FEN33" was evaluated for agronomic traits and ear characters in near isogenic line (Zhang, W. *et.al.*, 2012). It was reported that heading and anthesis time were significantly delayed as a result of *sb1* gene function. Decrease in seed number was presented as a result of *sb2* gene function.

It was observed that branched spike was only under control of a recessive gene at a single locus on distal part of chromosome 2AS in durum wheat (Haque *et.al.*, 2012). It was suggested that *bh* gene was orthologue with *MRS* gene in choromosome 2D reported by Dobrovolskaya and co-authors (2009). It was discussed that increase in sink capacity for wheat was achiavable by using branched wheat causing more spikelet.

Rachis and grain characters were investigated under different environments for spike hetero branching wheat (SHBW) in bread wheat originated from "FEN33" having branched phenotype and "Weimai" a Chineese cultivar (Zhao *et.al.*, 2012).

They reported that branched rachis were more open to environmental changes. It was stated that selecting branched spike having more grain and longer rachis could be useful for breeding programs.

Hybrid populations originated from "171ACS" line having a novel type of branching was searched for effect of D genome into this phenotype expression (Aliyeva & Aminov, 2013). They found that B and G genomes did not have a role for this novel type branching, while D genome had an inhibitor effect. A genome had for this branching phenotype gene. This novel phenotype was called as "sham ramification" similar to "vavilovoid type of branching" (Sharman, 1962) characterized by a lengthening of the rachilla with florets attached on extending spikelet rachillas (Amagai *et.al*., 2014a).

"Sham ramification" previously described as a novel type of branching was mapped in F2 populations for three durum wheat (two branched and one normal) using by microsatellite markers (Amagai *et.al.*, 2014a). It was presented that sham ramification phenotype was recessively controlled by *sham ramification1* (*shr1*) and *sham ramification2* (*shr2*) according to segregation analysis. It was stated that *shr2* gene was linked with extra glume gene and located on chromosome 2A long arm and *shr1* gene was located on chromosome 5A long arm.

It was stated that control of the *branched head* (bh^m) in monococcum located in chromosome $2A^mS$ as a recessive allele in a single locus in *Triticum monococcum* L. ($2n = 4X = A^m A^m$) (Amagai *et.al.*,2014b).

The genetic basis of supernumerary spikelet trait in bread wheat was evaluated in a recombinant inbred line population originated from breeding of a branched parent and an elite parent (Echeverry-Solarte *et.al.*, 2014). It was found that five chromosome (2D, 5B, 6A, 6B and 7B) affected to supernumerary spikelet trait. Supernumerary spikelet phenotype was observed in different expression level from two sessile spikelet per node to highly branched ears. The major contribution to character was chromosome 2D and 7B. It was stated that control of branching was

also controlled with a lot of minor genes with epistatic interactions. It was reported that supernumerary character was positively affected by grain protein content and mixogram-related characters.

It was observed that mutation in *branched head D.1* (*Bh-D.1*) gene caused additional spikelet without any chromosomal aneuploidy and rearrangements in bread wheat (Dobrovolskaya *et.al.,* 2014). It was stated that lateral meristem, formation occured instead of floral meristem in the basal part of spikelet. Additional spikelets were formed from this lateral meristems according to morphological results of mutant inflorescence.

Recombinant inbred line population generated by an elite wheat and an exotic branched wheat was investigated for new QTLs for eight characters (thousand kernel weight, kernel volume weight, grain protein content, percent of flour extraction and four mixograph-related characters) (Echeverry-Solarte *et.al.*, 2015a).

Recombinant inbred line of bread wheat originated from a supernumerary spikelet parent and an elite parent was investigated for 10 agronomic and 9 spike related characters under different environments with genome wide mapping technique (Echeverry-Solarte *et.al.*,2015b). It was stated that chromosome 2DS was very rich for spike related and agronomic characters. Especially, this chromosome had a major QTL for supernumerary spikelet characters.

Sham ramification gene was mapped in hexaploid wheat population, "171ACS" line (Amagai *et.al.*, 2015). It was found that shr^{171ACS} gene was located on chromosome 5AL as a completely linked with *extra glume* gene.

Dobrovolskaya and co-authors (2015) discovered wheat *FRIZZY PANICLE* gene homoeologous (*WFZP-A*, *WFZP-B* and *WFZP-D*) in common wheat. It was introduced that wheat *FZP* genes played an important role for supernumerary spikelet formation. It was observed that *WFZP-D* gene was the major contributor for this trait according to expression analysis.

Boden & co-authors (2015) found another spike architecture related gene *Photoperiod-1* (*Ppd-1*) gene causing paired spikelet a different type of branching in wheat.

The genetic background of branching was revealed in "Miracle-Wheat" and "Compositum-Barley" having branched ears (Poursarebani *et.al.*, 2015). It was reported that bh^t (*branched head*^{t}) locus orthologue to *compositum 2* (*com2*) gene causing branching in "Compositum-Barley" was identified in tetraploid "Miracle-Wheat". It was stated that *TtBH-A1* gene and *com2* gene were synthenic in chromosome 2 group. It was introduced that a single amino acid change (L96P) in the DNA binding site of the *AP2/ERF* conserved domain resulted with branch formation in durum wheat. Furthermore, inflorescence development of "Miracle-Wheat" and "Compositum-Barley" was investigated. It was presented that "Miracle-Wheat" produced significantly more grain number, spike dry weight at anthesis and grain yield than elite durum cultivars, however, thousand kernel weight was higher in elite durum cultivars.

Finally, branching phenotype in wheat is important for limited sink capacity. Producing more spikelets and florets can help to overcome this limitation. Functional loss of orthologues of *FRIZZY PANICLE* (*FZP*) gene caused branched ears in maize (Colombo *et.al.*, 1998; Chuck *et.al.*, 2002), in rice (Mackill *et.al.*, 1993; Komatsu *et.al.*, 2001 and 2003; Yi *et.al.*, 2005; Kato & Horibata, 2012; Bai *et.al.*, 2016), in Brachypodium (Derbyshire & Byrne, 2013; Dobrovolskaya *et.al.*, 2015), in barley (Castiglioni *et.al.*, 1998; Rossini *et.al.*, 2006) and in hexaploid wheat (Dobrovolskaya *et.al.*, 2015). Orthologues of *FZP* gene a transcription factor shared the same conserved domain *APETELA2/ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR* (*AP2/ERF*) (Accession number: cd00018) having eleven GCC DNA binding site (Chuck *et.al.*, 1998). This conserved domain specifically binds to 11 bp GCC box of ethylene response element a promoter element essential for ethylene responsiveness.

TtBH-A1 gene orthologue of *FZP* gene was cloned in branched tetraploid wheat called “Miracle-wheat”. Homoeologous of *TtBH-1* gene had also *AP2/ERF* highly conserved domain having 11 DNA binding sites between 57 and 116 amino acids (Figure 1.11). Amino acid change at the conserved domain of *TtBH-A1* gene in “Miracle-Wheat” caused branching. All branched ears having a single amino acid substitution produced more spikelet and grain yield per spike (Poursarebani *et.al.*, 2015).

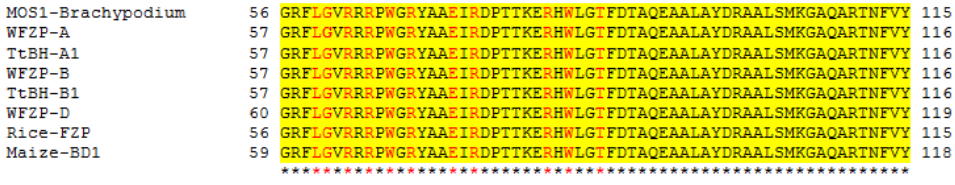


Figure 1. 11. Multiple sequence alignment of the conserved domain of *TtBH-1* orthologues. *MOS1* Brachypodium; *WFZP-A*, *WFZP-B* and *WFZP-D* homoeoloug in *T. aestivum*; *TtBH-A1* and *TtBH-B1* homoeoloug in *T.durum*; *FZP: FRIZZY PANICLE* gene in rice; *BD1*: *branched silkless1* gene in maize. Conserved domain *AP2* (*APETELA2*) was highlighted by yellow. DNA binding sites on the conserved domain was coloured by red.

1.7.Aim of the Study

Boosting crop yield is considered as an important solution to feed sharply growing world population. Spike related genes play crucial role to boost yield. Focusing these genes is one of the ways to achieve this aim. *TtBH-1* gene homoeologues are spike architecture genes in durum wheat. “Miracle-Wheat” having a SNP in *TtBH-A1* gene resulting with branched ears produced nearly two times more grain than UK durum wheat cultivars according to preliminary studies. However, the grains

were significantly smaller. Although the thousand kernel weight of “Miracle-Wheat” was lower the yield per spike was greater.

The main objective of this study was to till homoeologous of *TtBH-1* gene (*TtBH-A1* and *TtBH-B1*) in Kronos TILLING population to investigate their function on the spike architecture. In addition to this, one of the important aims was to increase spikelet number in durum wheat. Additionally, making double mutants to get more severe phenotype, backcrossing of mutants with Kronos to eliminate background mutations and to analyze yield parameters (spikelet number, seed number, thousand grain weight and size parameters) for mutants were other targets as a part of this study.

Another purpose of this study was to hybridize mutant phenotype with domestic Turkish durum wheat cultivars Kızıltan-91 and Fuatbey-2000 to observe yield parameters in next generations. Lastly, one of the aims was to hybridize Miracle wheat with Turkish domestic cultivars to investigate branching phenotype.

CHAPTER 2

MATERIALS & METHODS

2.1.Materials

2.1.1. DNA Source and Plant Materials

Tetraploid Kronos® TILLING population (Uauy *et.al.*, 2009) was used as a genetic source to find mutations for the *TtBH-1* gene. 1139 mutant DNA were organized as four DNA in one pool into three 96 well plates. They were called Plate1, Plate2 and Plate3. Mutant seeds were kindly provided by Prof. Dr. Jorge DUBCOVSKY from U.C. Davis, US. Tetraploid *Triticum turgidum durum* cultivar Kızıltan-91 (were obtained from Field Crop Central Research Institute) and cultivar Fuatbey-2000 (were obtained from Eastern Mediterranean Agricultural Research Institute) were used as domestic cultivars for breeding. Miracle wheat was also used to cross domestic cultivars.

2.1.2. Chemicals, Kits and Reagents

The chemical materials and solutions in this research were purchased from Sigma-Aldrich Corporation (St. Louis, MO, US), New England BioLabs Inc. (NEB; Ipswich, MA, US), LGC Genomics (T11W 0LY, UK) Merck KGaA (Darmstadt, Germany), Qiagen (Hilden, Germany), Thermo Fisher Scientific Inc. (MA, US) and

Clonetech Laboratories (CA, US). Chemicals, oligonucleotides, enzymes, and kits for molecular biology studies such as polymerase chain reaction (PCR), electrophoresis, nucleic acid purification and handling, KASP marker assay, enzymatic digestion, and sequencing were purchased mainly from Qiagen, Clonetech, NEB, LGC Genomics, Thermo Fisher Scientific, Sigma-Aldrich and Roche.

2.1.3. Instruments

PCR reactions were carried out G-Storm GS4 (TA11 7JH, UK) and MJ Research PTC-225 Peltier Thermal Cycler (Bio-Rad Laboratories, CA, US) machines. Sequencing was done on the ABI 3730XL sequencing platform (Applied Biosystems Inc; Foster City, CA, US) at RevGen TILLING Service (NR4 7UH, Norwich, UK) and Eurofins Genomics (Germany). Sigma 4-15-C centrifuge (Sigma GmbH; Osterode, Germany) and Heraeus Biofuge Pico (Thermo Fisher Scientific Inc., MA, US) centrifuges were used. Vortex-Genie 2 (Scientific Industries, Inc., NY, US) was used to mix reagents. Gel electrophoresis was performed using systems manufactured by Bio-Rad Laboratories Inc (CA, US). Gel documentation was performed using GelDoc-It™ Imaging System (UVP Ltd; Cambridge, UK). Qiagen TissueLyser II, TissueLyser Beads and Dispenser (Qiagen, Hilden, Germany) and GenoGrinder® 2000 (SPEXSamplePrep, NJ, US) were used to grind plant tissue. NanoPhotometer (Implen, Germany) was used to measure of DNA concentration. KASP assay was carried out using by Eppendorf Mastercycler® Pr384 Vapo.Protect® (Eppendorf AG, Hamburg, Germany). Incubations were done in Bachofer Incubator (Germany). Tecan Safire 96/384 fluorescence microplate reader (Tecan Group Ltd., Switzerland) was used to read KASP assay results. MARVIN (GTA sensorik Gmbh, Germany) was used to analyze grain yield potential. Nikon SM2645 (Nikon Corporation, Tokyo, Japan) microscope was used for immature inflorescence dissection. Some pictures were taken under Leica M205FA (Wetzlar, Germany) fluorescent stereo microscope and Leica DFC310FX Camera (Wetzlar, Germany).

2.1.4. Computer Programs and Bioinformatic Tools

Vector NTI (Life Technologies, Thermo Fisher Scientific Inc., MA, US), GeneMapper® Software (Life Technologies, Thermo Fisher Scientific Inc., MA, US), Mutation Surveyor® (SOFTGENETICS®, PA, US), KlusterCaller™ (LGC Genomics, T11W 0LY, UK) and BioEdit (Ibis Biosciences, CA, US) were programs used in this study.

NCBI web tools (blastn, blastp, CD Search etc.) (http://www.ncbi.nlm.nih.gov/), Primer3 (http://primer3.ut.ee/), PolyMarker (http://polymarker.tgac.ac.uk/), Expasy web tools (http://prosite.expasy.org/), URGI (Unité de Recherche Génomique, France) web tools (http://wheat-urgi.versailles.inra.fr/Tools), CODDLE and PARSESNP (http://blocks.fhcrc.org/~proweb/input/) (Taylor & Greene, 2003) and some internal tools of John Innes Centre were used for bioinformatics.

2.2. Experimental Strategy

This study covers five main parts (Figure 2.1).

1. TILLING for *TtBH-1* gene was the first part of this study. PCR was firstly optimized for fluorescently labeled primer. Kronos TILLING population was screened after PCR optimization. Mutations were identified and confirmed using proper tools.
2. Mutants were verified in the second part. M3 seeds for mutants were sowed in greenhouse for validation of *TtBH-A1* and *TtBH-B1* mutations. Spikes were phenotyped for branching.
3. Combining *TtBH-A1* and *TtBH-B1* mutations, backcrossing with Kronos and hybridization of domestic cultivars Kızıltan-91 and Fuatbey-2000 were performed in the third step of this research.
4. Genetic and phenotypic segregation analysis of *TtBH-A1* M4 mutants were investigated in the fourth part.
5. In the last part of this study, yield component analysis was performed for *TtBH-A1* mutants.

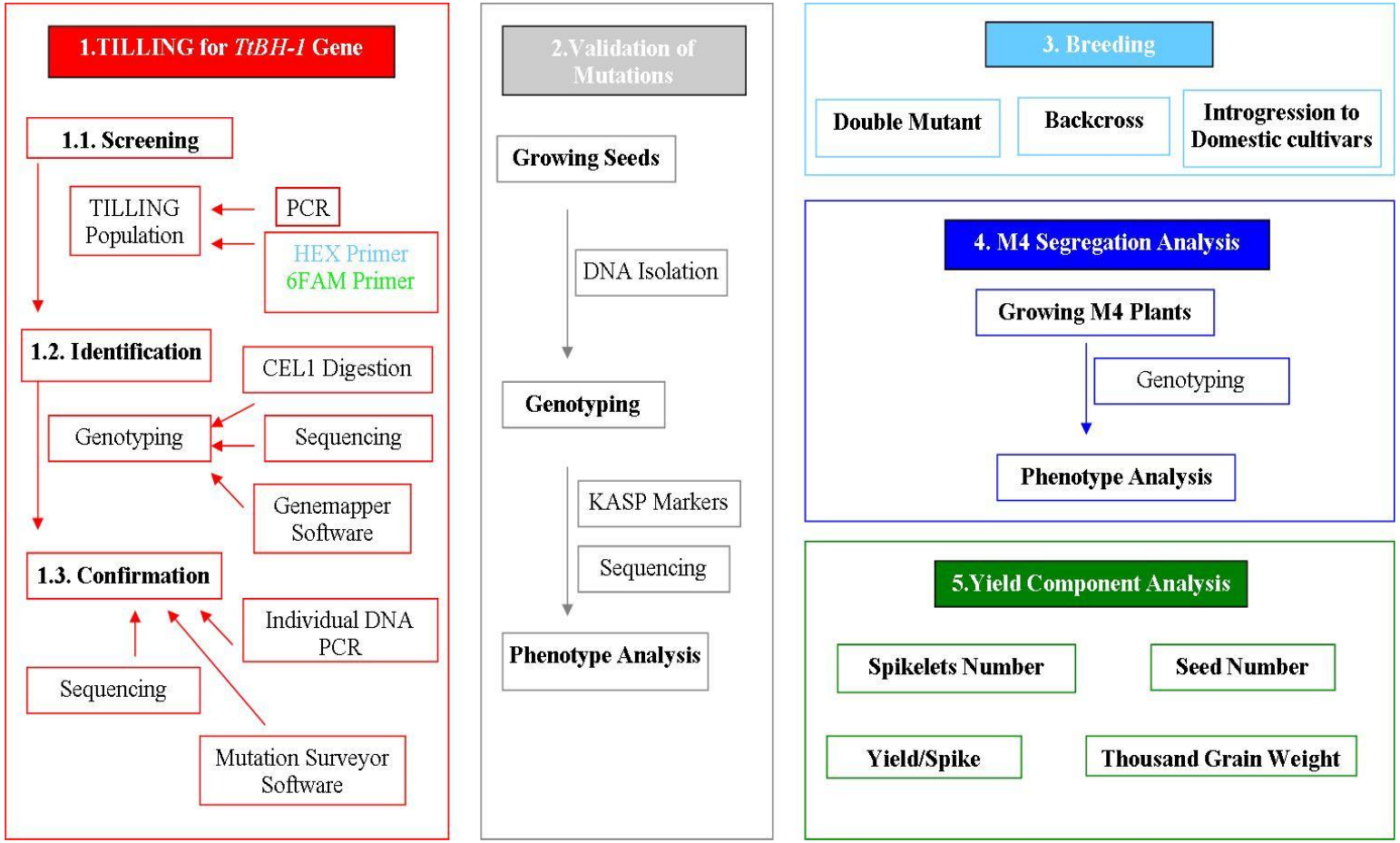


Figure 2.1. Experimental strategy. 1. TILLING for *TtBH-1* gene. 2. Validation of mutants 3. Breeding. 4. M4 Segregation analysis. 5. Yield component analysis.

2.3. Methods

2.3.1. TILLING for *TtBH-1* Gene

In this study, firstly, PCR reagents and conditions were optimized for fluorescently labeled primers to screen *TtBH-A1* and *TtBH-B1* homoeologous in Kronos TILLING population. Mutations were identified and confirmed in next steps of TILLING part.

2.3.1.1. PCR Optimization For *TtBH-1* Homoeologous Using Fluorescent Labeled Primers

Forward primers were fluorescently labeled with 6FAM fluorescent dye and reverse primers were labeled with HEX fluorescent dye to screen *TtBH-1* gene homoeologous in Kronos TILLING population in this study (Table 2.1.). They were dissolved in 0.1M TE buffer (pH 7.5) as a 100 µM. The fluorescently labeled gene specific primer stocks were stored in -20 °C and dark because of light sensitivity.

TtBH-A1 and *TtBH-B1* genes were cloned from "Miracle-Wheat" using gene specific primers. Homoeologous of *TtBH-1* gene which is orthologue to *FZP* gene having only one exon was sequenced and determined on chromosome 2AS and 2BS. Forward primer 5'GCTAGGCGGGAGCAGTAGTA3' and reverse primer 5'GTGGGCACAGCAGACCAC3' amplified 1011 bp for *TtBH-A1* gene (Figure 2.2). Forward primer 5'TCCCCTCCCCTACCCAAG3' and reverse primer 5'TGAGTACGTAAGAGGCTAAGATCG3' amplify 1218 bp for *TtBH-B1* (Figure 2.3) (Poursarebani *et.al.*, 2015).

Table 2. 1. Gene specific primers for *TtBH-1* gene.

PrimerID	**Orientation**	**Sequence**	**Tm**	**Product**
TtBH-A1	Forward	6FAM GCTAGGCGGGAGCAGTAGTA	60.82	1011
TtBH-A1	Reverse	HEX GTGGGCACAGCAGACCAC	60.98	-
TtBH-B1	Forward	6FAM TCCCCTCCCCTACCCAAG	59.21	1218
TtBH-B1	Reverse	HEX TGAGTACGTAAGAGGCTAA GATCG	59.49	-

Labeled primers were cleaned up with sodium acetate and ethanol precipitation to prevent background activity during genotyping. 100 µl of labeled primer was transferred to eppendorf tube. 10 µl 3.0 M NaOAc (pH 5.2) and 275 µl of ethanol (2.5X volume) were added to tube. Mixture was incubated at -20°C for 1 hour. It was spun at maximum speed for 15 minutes after incubation. Supernatant was removed immediately to prevent resolving. Pellet was washed with 80 % cold ethanol and dissolved with 80 µl of 0.1M TE buffer (pH 5.2). Working stock was prepared as a 2 µM concentration for labeled and unlabelled primers. Cleaned (sodium acetate and ethanol precipitated) labeled primers and un-cleaned labeled primers were tested for the best amplification. Unlabelled, labeled and mixed (labeled and unlabeled (3/2 ratio)) primers were used for primer optimization.

> *TtBH-A1* Chromosome_2AS (TILLING amplicon 1011bp)

GCTAGGCGGGAGCAGTAGTATAGTAGCCAGCCAACCTCACTTCACTTCTGCCATGAGCTCTCGCAG
CAGCAGCGGCGGCGGCGGTGCCTCCCAGATGATGGCCTTCTCGGAGCATTCGCTGCCGAAGCCGAT
CGCCGGTCACCCGCAGCCGCAGCCGTCCCCGCCGTCGTCGCCGAGCGAGCGGCCGGCGGCGCGCGG
CAGGCGGCGCGCGCAGGAGCCCGGGCGCTTCCTGGGCGTGCGCCGGCGGCCGTGGGGCCGGTACGC
GGCCGAGATACGCGACCCGACCACCAAGGAGCGGCACTGGCTCGGCACCTTCGACACGGCGCAGGA
GGCCGCCCTGGCCTACGACCGCGCCGCGCTCTCCATGAAGGGCGCGCAGGCGCGCACCAACTTCGT
CTACGCGCACGCCGCCTACAACAACTACCCGCCCTTCCTCGCGCCGTTCCACGCGCAGCACCAGCC
CGCCGCCTACGCCGCGTCCTCGGCCATGCCGTACGGCGGCCAGCAGCAGCACGCGGGCGCGGGGCC
GCCGCACATTGGCAGCTCGTACCACCACGGCCACGGCTACCACCAGCAGGGCCCGGGCGAGTGTTC
CATGCCGGTGCCCAGTGCCGCGGATCACGGCGCCAGCGGCCCGATGGACGTGCGCGGCAGCAGCGG
CCACGACTTCCTCTTCCCCAGCGCCGACGACAACTCCGGGTACCTGAGCAGCGTGGTGCCGGAGAG
CTGCCTCCGGCCCCGCGGCGGCGACCTGCAGGACGCGCGGCGCTACTCCGTGTCCGACGCCGACGC
CTACGGGCTGGGCCTCCGGGAGGACGTGGACGACCTGGCGACGATGGTGGCCGGCTTCTGGGGCGG
CGCCGACGCGCCGTACGGCGGCGGCCACGACATGGTCGCCTCGTCGCAGGGCTCGGACAACGGCTA
CTCCCCCTTCAGCTTCCTCTCCCACTGAAACGGACGCGCTGGCATTGCTCTTAGCCTCTTACGTAC
TCAGTGGTCTGCTGTGCCCAC

>*TtBH-A1* Chromosome_2AS (Protein sequence, 299a.a.)

MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPGRFLGVRRRP
WGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFH
AQHQPAAYAASSAMPYGGQQQHAGAGPPHIGSSYHHGHGYHQQGPGECSMPVPSAADHGASGPMDV
RGSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARRYSVSDADAYGLGLREDVDDLATMVA
GFWGGADAPYGGGHDMVASSQGSDNGYSPFSFLSH*

Figure 2. 1. *TtBH-A1* gene nucleotide and protein sequences. Forward and reverse primers were boxed. Open reading frame of *TtBH-A1* gene was underlined.

> *TtBH-A1* Chromosome_2AS (TILLING amplicon 1011bp)

GCTAGGCGGGAGCAGTAGTATAGTAGCCAGCCAACCTCACTTCACTTCTGCCATGAGCTCTCGCAG
CAGCAGCGGCGGCGGCGGTGCCTCCCAGATGATGGCCTTCTCGGAGCATTCGCTGCCGAAGCCGAT
CGCCGGTCACCCGCAGCCGCAGCCGTCCCCGCCGTCGTCGCCGAGCGAGCGGCCGGCGGCGCGCGG
CAGGCGGCGCGCGCAGGAGCCCGGGCGCTTCCTGGGCGTGCGCCGGCGGCCGTGGGGCCGGTACGC
GGCCGAGATACGCGACCCGACCACCAAGGAGCGGCACTGGCTCGGCACCTTCGACACGGCGCAGGA
GGCCGCCCTGGCCTACGACCGCGCCGCGCTCTCCATGAAGGGCGCGCAGGCGCGCACCAACTTCGT
CTACGCGCACGCCGCCTACAACAACTACCCGCCCTTCCTCGCGCCGTTCCACGCGCAGCACCAGCC
CGCCGCCTACGCCGCGTCCTCGGCCATGCCGTACGGCGGCCAGCAGCAGCACGCGGGCGCGGGGCC
GCCGCACATTGGCAGCTCGTACCACCACGGCCACGGCTACCACCAGCAGGGCCCGGGCGAGTGTTC
CATGCCGGTGCCCAGTGCCGCGGATCACGGCGCCAGCGGCCCGATGGACGTGCGCGGCAGCAGCGG
CCACGACTTCCTCTTCCCCAGCGCCGACGACAACTCCGGGTACCTGAGCAGCGTGGTGCCGGAGAG
CTGCCTCCGGCCCCGCGGCGGCGACCTGCAGGACGCGCGGCGCTACTCCGTGTCCGACGCCGACGC
CTACGGGCTGGGCCTCCGGGAGGACGTGGACGACCTGGCGACGATGGTGGCCGGCTTCTGGGGCGG
CGCCGACGCGCCGTACGGCGGCGGCCACGACATGGTCGCCTCGTCGCAGGGCTCGGACAACGGCTA
CTCCCCCTTCAGCTTCCTCTCCCACTGAAACGGACGCGCTGGCATTGCTCTTAGCCTCTTACGTAC
TCAGTGGTCTGCTGTGCCCAC

>*TtBH-A1* Chromosome_2AS (Protein sequence, 299a.a.)

MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPGRFLGVRRRP
WGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFH
AQHQPAAYAASSAMPYGGQQQHAGAGPPHIGSSYHHGHGYHQQGPGECSMPVPSAADHGASGPMDV
RGSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARRYSVSDADAYGLGLREDVDDLATMVA
GFWGGADAPYGGGHDMVASSQGSDNGYSPFSFLSH*

Figure 2. 2. *TtBH-B1* gene nucleotide and protein sequences. Forward and reverse primers were boxed. Open reading frame of *TtBH-B1* gene was underlined.

Home-made *Taq*-DNA Polymerase and commercial Takara Ex-*Taq* DNA polymerase were tested for fine PCR amplification for *TtBH-1* homoeologous. $MgCl_2$ was optimized testing as a final concentration 2.5 mM, 3.0 mM, and 3.5 mM in reaction. Betaine was used as 1.0 M final concentration in PCR reaction. dNTP mix was used 0.8 mM as a final concentration. Wild type Kronos DNA, mutant T4-2235 DNA and some tetraploid DNA sources were used as a template for PCR optimization. All parameters were optimized for *TtBH-A1* and *TtBH-B1* genes.

PCR master mix was prepared and dispersed to PCR tubes and 96-well plates in an equal volume according to reaction.

Touch-down step was added to PCR procedure and optimized. Gradient PCR was carried out between 55 - 60 °C to find the best annealing temperature. PCR cycles were tested as 35 and 45 cycles. All parameters were separately optimized for *TtBH-A1* and *TtBH-B1* genes. Amplified PCR products were run in 1 % agarose gel electrophoresis at 100V for 30 min.

PCR products were digested with restriction enzyme *BsrI* to correct amplified genes. The *BsrI* restriction enzyme recognized and cleaved ACTGG(1/-1)^ sites. 65 °C was the best incubation temperature for its activity with appropriate buffer. 5 µl of amplified PCR products, 0.6 µl of enzyme, 1 µl of NEBuffer 3.1 and 3.4 µl of water were incubated at 65 °C at least 3 hour or overnight for complete digestion. Reaction was finalized with 80 °C for 10 min to inactivate enzyme. Digested DNA and 5 µl of undigested PCR products were run to 3 % agarose gel electrophoresis at 100 V for 40 min. 2-Log DNA ladder (0.1- 10kb) (NEB) was used to check amplifications.

2.3.1.2. Screening of Kronos TILLING Population for *TtBH-A1* and *TtBH-B1* Genes

Plate1, Plate2 and Plate3 belonged to Kronos TILLING population were screened with mixed primers (3/2 ratio for labeled/unlabeled primers) after PCR optimization. Some wells were checked for true amplification and *BsrI* enzyme

digestion to continue further steps. Amplified plates were sent to REVGEN Tilling Service for *Cel*1 digestion and genotyping. TILLING steps were performed adapted from Le Signor & co-authors (2009).

Some wells were checked with E-gel to see amplification specificity and strength of *TtBH-A1* and *TtBH-B1* genes. Clean, specific and strength bands for genes were important for *Cel*1 digestion and further sequencing. It was extracted from celery juice. 5 µl of PCR products of *TtBH-1* homoeologous was used with 1 µl of *Cel*1 enzyme (1:9 diluted) randomly cleaving mismatches in DNA and 2 µl of 10X *Cel*1 enzyme buffer (Table 2.2). Volume was completed to 20 µl with water. *Cel*1 master mix was prepared and dispersed to each well using multichannel pipettes. Plates were sealed and spun after *Cel*1 mix added. Reaction was incubated at 45 °C for 15 minutes and stopped by adding 5 µl of 150 mM EDTA. Isopropanol clean up step was carried out after Cel1 digestion step. 100 µl of 75 % isopropanol was added to *Cel*1 cleaved PCR products. They were spun at 5000 rpm for 30 minutes after 15 minutes at room temperature in dark incubation. Plates were inverted 20 seconds at 300 rpm. 10 µl of ROX/Hi-Di Formamide mix (0.1 µl of ROX 1kb ladder and 10 µl of Hi-Di Formamide) were immediately added to isopropanol washed samples after second wash. Plates were vortexed, spun down and stored at 4 °C overnight or 30 minutes at 4 °C by vortexing frequently. They were incubated 95 °C for 3 minutes before genotyping. Samples were run on ABI3730XL sequencer for genotyping.

Table 2. 2 *Cel*1 reaction mix.

Reagent	**Volume (µl)**
PCR Product	5
Water	12
*Cel*1 buffer (10X)	2
*Cel*1 enzyme (1:9)	1
Total	20

2.3.1.3.Identification *TtBH-A1* and *TtBH-B1* Gene Mutations

GENEMAPPER® software was used to analyze ABI3730XL results to identify mutations for *TtBH-A1* and *TtBH-B1* genes. This software found cleaved sites on DNA in the each well for *TtBH-1* homoeologous. "Rox1kb Advanced" was used as analysis method and "Rox1kbA1000less50and75" was size standard in this study. This size standard removed the first and last sections of the fragment (50 and 75bp respectively), and so removed noise. 6FAM labeled forward direction gave blue colour and HEX labeled reverse direction gave green colour. ROX dye was red colour as a reference dye. Tables were prepared showing well number, blue size, green size and product size for *TtBH-A1* and *TtBH-B1* genes.

2.3.1.4.Confirmation of *TtBH-A1* and *TtBH-B1* Gene Mutations

Identified DNA pools were amplified individually using *TtBH-A1* and *TtBH-B1* gene specific primers and sequenced to confirm which line has the mutation in the gene. *SAP*/*EXO* (*Shrimp alkaline phosphatase*/*Exonuclease1*) clean up was performed before sequencing (Stemke-Hale *et.al.*, 2008). 8 µl of PCR product, 0.8 µl of SAP and 0.4 µl of *Exo*1 were incubated for 30 minutes at 37 °C and then 10 minutes at 80 °C. Individual mutant line sequence results of *TtBH-A1* and *TtBH-B1* genes were evaluated with using Mutation Surveyor® software, Vector NTI software and some web based bioinformatics tools (NCBI Blast, PARSESNP (Project Aligned Related Sequences and Evaluate SNPs), Bioedit and Prosite Translate etc.). *TtBH-A1* and *TtBH-B1m*utations were evaluated according to their effect (missense, nonsense or silent), genotype (homozygous or heterozygous) and place (conserved domain region, DNA binding site etc.).

To find a premature stop codon was the first aim in Kronos TILLING population for these branching genes to determine its function. Glutamine (CAA to TAA and CAG to TAG), arginine (CGA to TGA) and tryptophan (TGG to TGA and TGG to TAG) were the main targets for search a premature stop codon in this study. If a premature stop codon was not found, an amino acid change at the conserved domain

or DNA binding site or highly conserved place was searched for *TtBH-A1* and *TtBH-B1* genes. NCBI protein BLAST tool was used for conserved domain search. Position Specific Scoring Matrix (PSSM) scores were evaluated for confirmed mutations.

PARSESNP (http://blocks.fhcrc.org/~proweb/input/) web based tool analyzing SNPs in gene using conserved blocks was used to see mutation effect. It gives a SIFT and PSSM scores for variations in blocks (Taylor & Greene, 2003).

The mutation frequency (rate) was evaluated according to the following formula (Till *et.al.*, 2007a):

$$\text{Mutation Rate} = \frac{\text{The total surveyed DNA length (TILLED fragment length} \times \text{individuals in population) x Screened homoeoloug number}}{\text{Confirmed mutation number}}$$

Mutation reports for *TtBH-A1* and *TtBH-B1* mutant lines were prepared showing mutant ID, mutation position, mutation genotype, mutation effect and PSSM scores.

2.3.2. Validation of *TtBH-A1* and *TtBH-B1* Gene Mutations

All *TtBH-A1* mutants having missense mutation and important *TtBH-B1* mutants were kindly provided by Professor Jorge Dubcovsky (U.C. Davis, USA). Available seeds were planted in summer greenhouse and DNA isolation was carried out for *TtBH-1* homoeologous. KASP markers were designed and tested for genotyping. All mutants were sequenced for validation.

2.3.2.1. Sowing of *TtBH-A1* and *TtBH-B1* Gene Mutant Lines

Kronos, *TtBH-A1* and *TtBH-B1* mutants were surface sterilized with 70 % ethanol and put into in petri dishes with wet paper for 1 week at room temperature. They were transferred to soil after germination. Each individual was labeled as follow "T4-Mutant Id – individual number". For example, "T4-177-1" was T4-177 was mutant ID and 1 was the first individual number of the first plant.

2.3.2.2.DNA Isolation From *TtBH-A1* and *TtBH-B1* Mutants and Kronos

DNA isolation was performed in two or three leaf stage adapted from Pallotta & co-authors (2003). Kronos, *TtBH-A1* and *TtBH-B1* mutant plant DNAs were organized 96-well plates. Fresh leaf tissues of Kronos, *TtBH-A1* and *TtBH-B1* mutant plants about 2.5 cm long were put into 1.2 ml round shape collection tubes. 1-3 mm of tungsten beads were dispersed to collection tubes. 500 µl of DNA extraction buffer incubated at 65 °C was added to tubes. Grinding parameters were 2 minutes at 160 strokes (20Hz) in Spex GenoGrinder 2000 machine to get powdered tissue. Samples were incubated at 65 °C for at least 1 hour. Plates were put into fridge for cooling to room temperature. 250 µl of 6M ammonium acetate was added to each well. Plates were incubated at fridge for 25 minutes before centrifuge for 15 minutes at 5000 rpm to remove proteins and plant tissue. 600 µl of supernatant was transferred to new collection tubes including 360 µl of isopropanol for DNA precipitation. Reaction was carefully pipetted and incubated for 5 minutes. Centrifuge step was repeated for 15 minutes at 5000 rpm to pellet down DNA. Plates were dried on paper towel at maximum 1 minute to prevent DNA loss after carefully supernatant removal. 500 µl 70 % ethanol was used for washing DNA pellet. Plates were incubated at 65 °C for 30 minutes to evaporate removal 70 % of ethanol after centrifuge step 15 minutes at 5000 rpm. DNA pellets were dissolved in 100 µl of double distilled sterile water, vortexed and incubated at 65 °C for 15 minutes. Expected concentration was nearly 100 ng/µl. Isolated DNAs were used for sequencing and marker assay.

2.3.2.3.Genotyping of *TtBH-A1* and *TtBH-B1* Mutant Plants

Mutations for *TtBH-A1* and *TtBH-B1* mutant plants were validated by genotyping after DNA isolation. KASP markers and sequencing were used for this aim.

2.3.2.3.1. KASP Markers for Mutants

Kompetitive Allele Specific PCR genotyping system (KASP^{TM}) is genotyping tool to detect a single nucleotide polymorphism (SNP) or insertion/deletion in the genome (Semagn *et.al.*, 2014). Allele specific fluorescent labeled primers and common primer were used to amplify Kronos, *TtBH-A1* and *TtBH-B1* mutant plants. HEX (excitation 535 nm and emission 556 nm) and FAM (excitation 485 nm and emission 520 nm) were fluorophores to distinguish genotypes. ROX (Excitation 575 nm and emission 610 nm) was used for normalization as a reference dye to prevent signal differences between wells. KASP reaction results for Kronos, *TtBH-A1* and *TtBH-B1* mutant plants were transferred to computer reading by FRET (Fluorescence Resonance Energy Transfer) plate reader. KlusterCaller^{TM} was used to view and evaluate KASP results for Kronos, *TtBH-A1* and *TtBH-B1* mutant plants. Homozygous FAM alleles were close to X-axis (mutant *TtBH-A1* and *TtBH-B1* genotype) and homozygous HEX alleles were close to Y-axis (Kronos and wild type genotype of *TtBH-A1* and *TtBH-B1*). Heterozygous alleles were between X and Y axis for *TtBH-A1* and *TtBH-B1* mutant plant.

PolyMarker program (Ramirez-Gonzalez *et.al.*, 2015) and Primer3 web tool were used to design labeled primers and common primers for KASP assay for *TtBH-A1* and *TtBH-B1* mutations.

Primer mix was prepared for each mutation using 12 µl of FAM labeled primer, 12 µl of HEX labeled primer, 30 µl of common primer and 46 µl of water (Table 2.3). KASP reaction mix was prepared using 2 µl of DNA (1:10 diluted), 2 µl of KASP mix and 0.056 µl of primer mix solution (Table 2.4). KASP reaction was carried out in 384-well micro-plates. PCR was carried out provider instructions.

Table 2. 3. KASP marker primer mix.

Reagents	**Volume (µl)**
FAM Labeled Primer	12
HEX Labeled Primer	12
Common Primer	30
Water	46

Table 2. 4. KASP reaction mix.

Reagents	**Volume (µl)**
DNA	2
KASP mix	2
Primer mix	0.056

2.3.2.3.2. Direct Sequencing of *TtBH-1* Mutants

PCR was carried out using *TtBH-A1* and *TtBH-B1* gene specific primers and PCR products were digested with *Bsr*I enzyme. Sequencing reaction was performed using BigDye Terminator Kit (Rosenblum *et.al.*, 1997) for forward and reverse direction after *EXO*/*SAP* clean up as previously described. Samples were sent to Eurofins Genomics (Germany) company for sequencing.

Sequence results were analyzed by using Vector NTI and BioEdit tools. Mutation allele type (wild, heterozygous and homozygous) was determined for *TtBH-A1* and *TtBH-B1* mutant plants.

2.3.2.4.Phenotype Analysis of *TtBH-1* Mutants

Phenotypes of *TtBH-1* mutants were analyzed according to spike structure. They were screened for spike architecture after heading stage. Spikelets number was counted for mutants.

2.3.3. Breeding Strategy

Breeding strategy was designed to make double mutants combining important mutations on *TtBH-A1* and *TtBH-B1* genes in one plant and to clear background mutations.

Mutants were prepared for crossing after heading stage (Figure 2.4, A). Firstly, spikelets were discarded on the lower and upper part of the spike because of delayed development (Figure 2.4, B). Generally, there were 3 florets (two laterals and one central) in a spikelet (Figure 2.4, E-F). Central floret was discarded because of late development (Figure 2.4, D). Upper parts of glumes, paleas and lemmas were cut carefully not to disturb female organs for each floret (Figure 2.4, C). After that, florets were emasculated removing all stamens (Figure 2.4, D). Each floret had 3 stamens in greenish colour. Emasculated spikes were labeled and covered with crossing bags to prevent pollen contamination (Figure 2.4, G). Pollination was carried out according to breeding strategy which was backcrossing or making double mutant when stigma was ready to get pollen. Pollinated spikes were relabeled and bagged.

2.3.3.1.Making Double Mutants for *TtBH-1* Gene and Backcrossing

There were two homeoelogous of *TtBH-1* gene. Important mutations according to phenotype and genotype analysis for *TtBH-A1* and *TtBH-B1* were linked to see more strength phenotype.

TtBH-A1 and *TtBH-B1* mutants had lots of background mutations as a result of EMS mutation. They were backcrossed with parental Kronos to reduce background mutations.

Figure 2. 3. Crossing process. A) A spike at booting stage. B) Removal of spikelets at the top and bottom part of the spike. C) Prepared spike for emasculation. D) Emasculated spike. E) Green stamens and ovary. F) Yellow stamens and feathery ovarium and G) Bagged spikes after crossing.

2.3.3.2.Hybridization of *TtBH-A1* Mutant Gene Into Domestic Cultivars

TtBH-A1 mutant gene and Miracle wheat were crossed with domestic Turkish durum wheat cultivars Kızıltan-91 and Fuatbey-2000 in winter greenhouse at METU. 15 seeds for each Kızıltan-91, Fuatbey-2000, Miracle and *TtBH-A1* mutants were germinated in petri dishes after surface sterilization with 70 % of ethanol. Germinated seedlings were transferred to soil. Miracle wheat and Kızıltan-91 needed vernalization for at least 4 weeks. *TtBH-A1* mutant gene was hybridized with Kızıltan-91 and Fuatbey-2000. Additionally, these domestic cultivars were crossed with Miracle wheat.

2.3.4. Segregation and Phenotyping of M_4 Generation of T4-2447 Mutants

TtBH-A1 M4 mutant seeds showing supernumerary spikelet phenotype were planted in winter greenhouse in John Innes Centre to observe genotype and phenotype segregation. Seeds were sowed and DNA was isolated from mutants as previously described. Genotyping was carried out by sequencing. Genotype analysis was performed according to sequencing results.

2.3.4.1.Spike Development in "Miracle-Wheat"

Miracle spike development was investigated at different developmental stages. Apex, immature inflorescences, spikelets and spike structures were observed under light microscope.

2.3.4.2. Phenotyping of M4 Generation of T4-2447 Mutants

Immature inflorescences in the first three tillers of *TtBH-1* mutants and Kronos were dissected under stereo microscope aid of forceps and scalpel (Figure 2.5). Spike architecture was observed for branching and supernumerary spikelet at earlier development stages under light microscope. Spikelet number, supernumerary spikelet number and total spikelet number were counted for each tiller.

Figure 2. 4. Immature inflorescence isolation under stereo microscope.

2.3.5. Yield Component Analysis for M4 Generation of T4-2447 Mutants

Yield analysis was carried out for T4-2447 M4 generation mutants and Kronos. Spikelet number, seed number, yield per spike, thousand grain weight (TGW), grain width and length and area were evaluated using MARVIN seed analyzer.

2.4. Statistical Analysis

Chi-square tested was calculated using Microsoft Excel program. Statistical analyses were performed using one way analysis of variance (ANOVA) program.

CHAPTER 3

RESULTS & DISCUSSION

Results of 3.1 TILLING for *TtBH-1* gene, 3.2 Validation of *TtBH-A1* and *TtBH-B1* Mutants and 3.4 Segregation and Phenotyping of M4 Generation of T4-2447 Mutants were published as a part of "The Genetic Basis of Composite Spike Form in Barley and 'Miracle-Wheat'" article in Genetics journal (2015 Sep;201(1):155-65. doi: 10.1534/genetics.115.176628. Epub 2015 Jul 7).

3.1.TILLING for *TtBH-1* Gene

TtBH-A1 and *TtBH-B1* genes were secreened, identified and confirmed after PCR optimization for fluorescently labeled primers.

3.1.1. PCR Optimization For *TtBH-1* Homoeologous Using Fluorescent Labeled Primers

The optimum reagent volumes and final concentrations to amplify *TtBH-A1* gene and *TtBH-B1* gene were determined. While commercially Takara *Ex-Taq* DNA polymerase amplified *TtBH-B1* gene, Home-made *Taq* DNA polymerase was used to amplify *TtBH-A1* gene (Figure 3.1). Home-made *Taq* DNA polymerase did not work nice for *TtBH-B1* gene.

The restriction enzyme *BsrI* was used to establish the correct amplicon. *TtBH-A1* gene had two restriction sites at 305 bp and 606 bp for *BsrI* enzyme. If *BsrI* cut the correct amplicon, three bands 301bp, 305 bp and 405 bp were visualized on the agarose electrophoresis gel (Figure 3.2). *TtBH-B1* gene had one cut site at 502 bp for *BsrI*. There were two fragments 502 bp and 716 bp on the gel after digestion. (Figure 3.3)

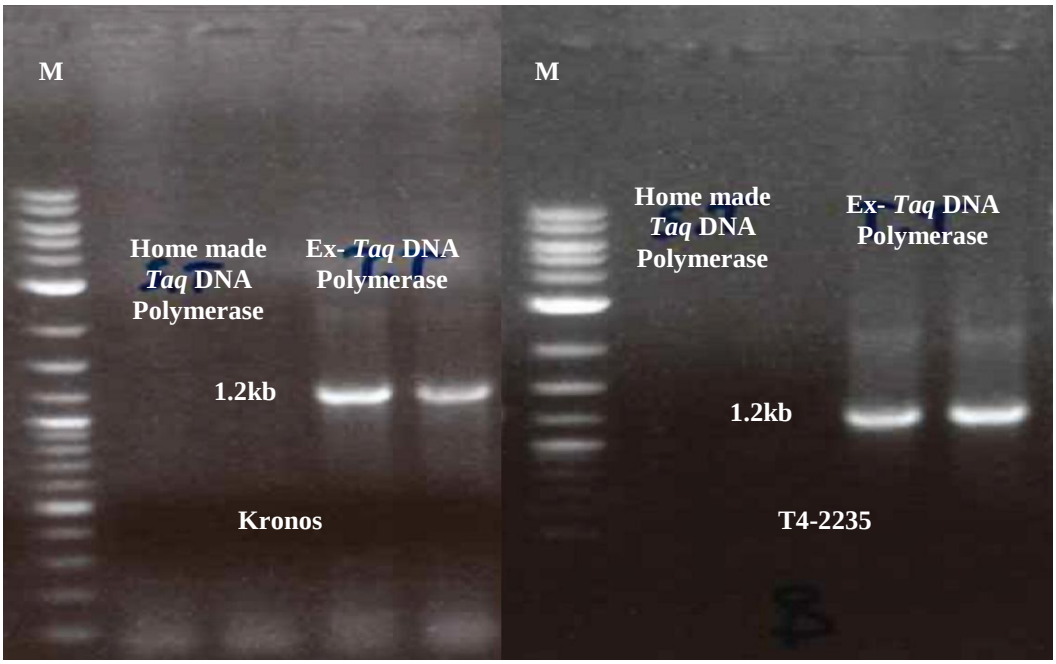
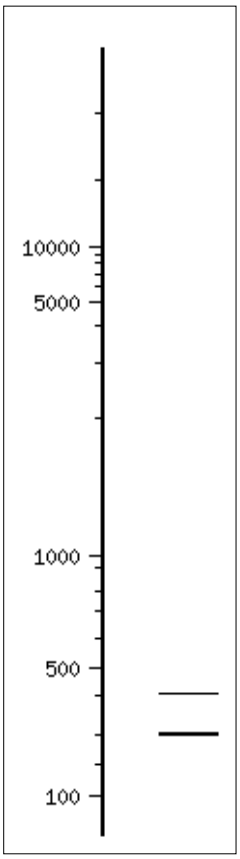
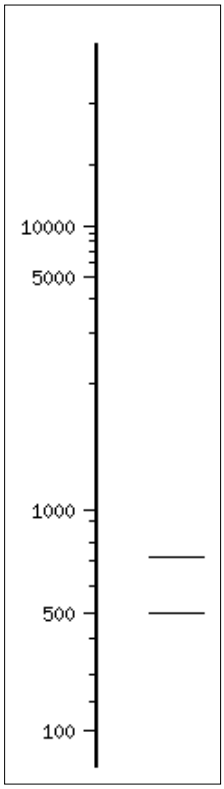


Figure 3. 1. Amplification of *TtBH-B1* gene. Home-made *Taq* DNA polymerase and commercial Takara *Ex-Taq* DNA polymerase were compared using Kronos wild type DNA and 2235 mutant line DNA for *TtBH-B1* gene. M: Marker. 2-Log DNA ladder (0.1- 10kb).



#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-BsrI	1-305	305
2	BsrI-BsrI	306-606	301
3	BsrI-(RightEnd)	607-1011	405

Figure 3. 2. Pseudogel image and fragment lengths of *TtBH-A1* gene after *BsrI* digestion.



#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-BsrI	1-502	502
2	BsrI-(RightEnd)	503-1218	716

Figure 3. 3. Pseudogel image and fragment lengths of *TtBH-B1* gene after *BsrI* digestion.

3.0 mM and 3.5 mM of $MgCl_2$ gave more strength bands relative to 2.5 mM concentration. 3.0 mM of $MgCl_2$ was used for further PCR reactions (Figure 3.4). Betaine was added as a final concentration 1M. It helps amplification of GC rich DNA sequences (Henke *et.al.*, 1997).

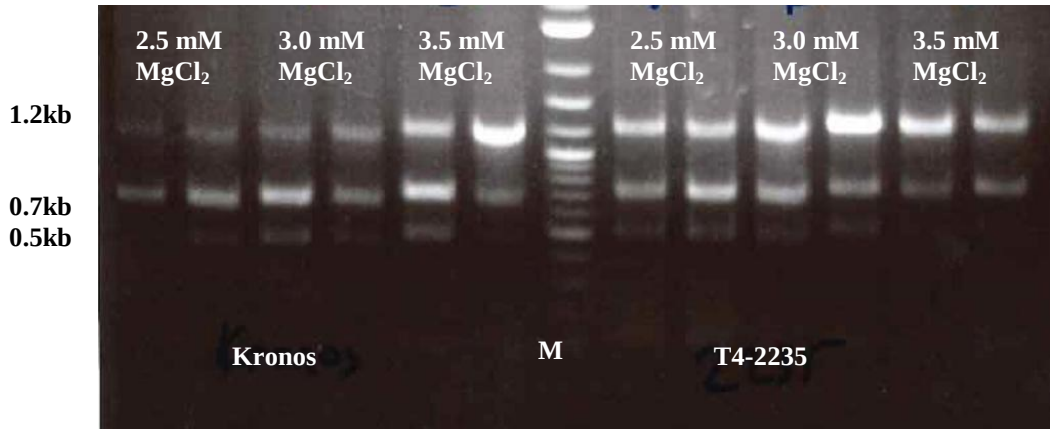


Figure 3. 4. Optimization of $MgCl_2$ concentration for *TtBH-1* gene. *TtBH-B1* gene tested for different $MgCl_2$ concentration using Kronos and T4-2235 mutant line. There were 1.2 kb undigested product, 700 bp and 500 bp digested ones. M: Marker. 2-Log DNA ladder (0.1- 10kb).

Firstly, unlabeled primers and labeled primers (after clean up step) were diluted to 2 µM stock solutions. Final concentration was 0.8 µM after mixing of 3/2 (Labelled/Unlabelled) ratio. The initial PCR was unsuccessful (Figure 3.5). A possible explanation could be ethanol because of utilization of clean labeled primers. The reaction could be affected by ethanol. Another reason could be primer secondary structure, hairpin and high probability of primer dimer, especially for *TtBH-A1* gene. It was observed that using only labeled primers and mix with unlabeled ones gave less strength bands than only unlabeled ones because of labeled primer having a big fluorescent dye. Also, binding to DNA strand took more time and so the annealing time was longer than normal PCR protocols. After that,

uncleaned 100 µm labeled primers were tried unlabeled 100 µm unlabelled primers 3/2 ratio. 50 % forward mix and 50 % reverse mix were used to prepare primer master mix. It was added 0.1 µl to PCR reaction mix as final concentration 0.8 µM (Figure 3.6).

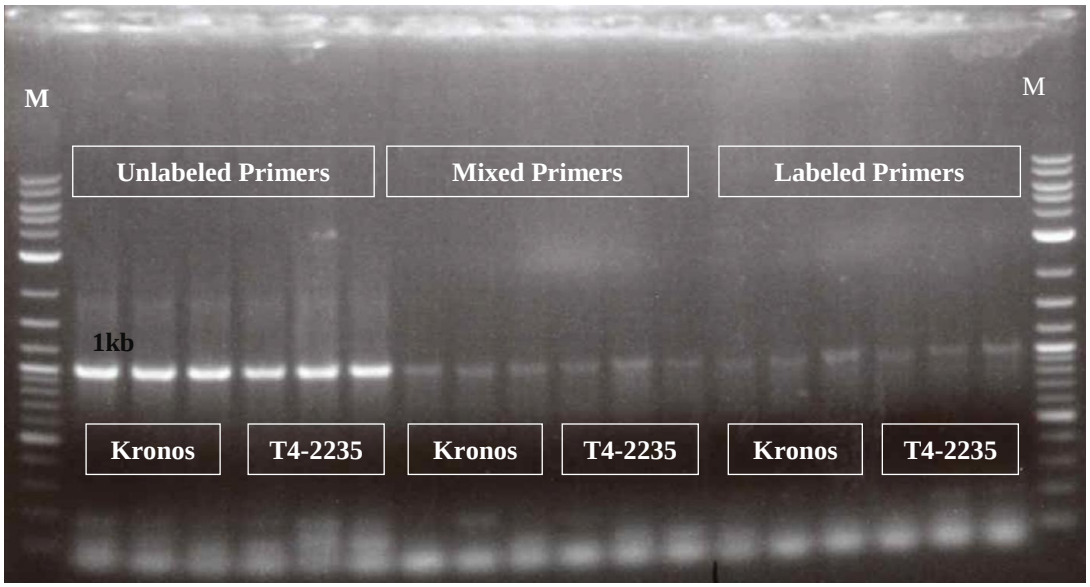


Figure 3. 5. PCR primer optimization for *TtBH-A1* gene using 2 µM stock labeled primers. Unlabeled primers, cleaned labeled and mix of them were prepared from 2 µM stock and used as 0.8 µM final concentration to amplify *TtBH-A1* gene from Kronos and 2235 mutant line. M: Marker. 2-Log DNA ladder (0.1- 10kb).

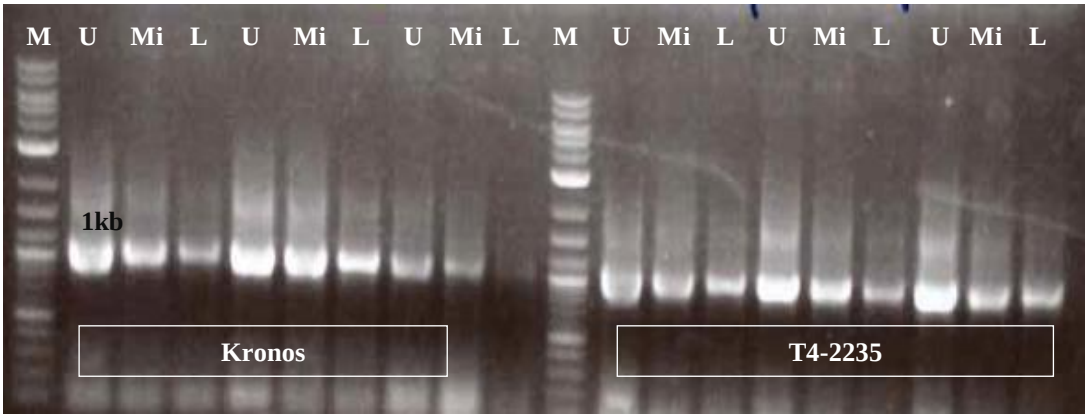


Figure 3. 6. PCR primer optimization for *TtBH-A1* gene using 100 µM stock labeled primers. 100 µM stock of primers were directly used as final concentration 0,8 µM to amplify *TtBH-A1* gene from Kronos and 2235 mutant line without a clean-up step. M: Marker, U: Only Unlabeled primers, L: Only Labelled primers and Mi: Mixed primers 3/2 Labeled/Unlabeled ratio. 2-Log DNA ladder (0.1- 10kb).

Gradient PCR was used from 55 to 60 °C to find the best annealing temperature. 58 °C for *TtBH-A1* (Figure 3.7) and 57 °C for *TtBH-B1* (Figure 3.8) gene were found the best annealing temperatures. The PCR cycle number was used 35 cycles for testing unlabelled primers. Cycle number was increased to 45 cycles to gain more strength bands for further analysis (Figure 3.7).

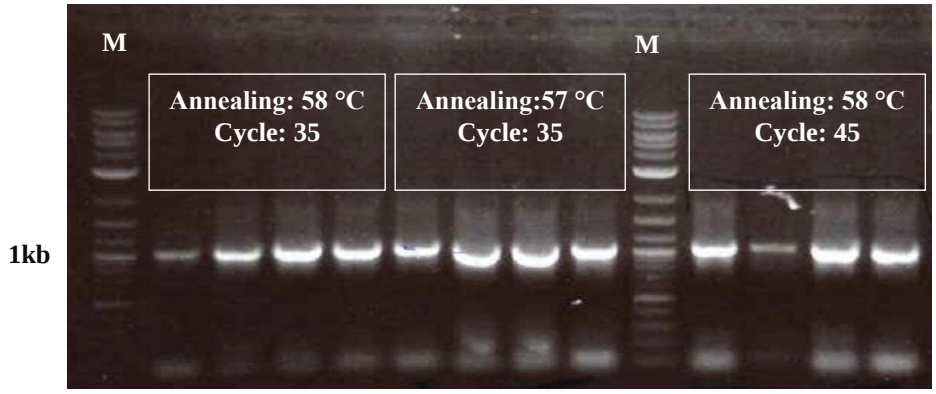


Figure 3. 7. Optimization of annealing temperature and cycle number for *TtBH-A1* gene. 2-Log DNA ladder (0.1- 10kb).

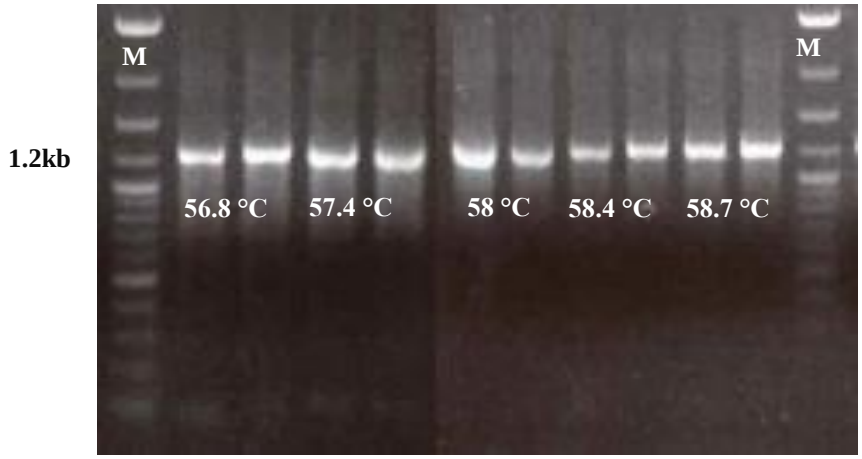


Figure 3. 8. Optimization of annealing temperature for *TtBH-B1* gene. 2-Log DNA ladder (0.1- 10kb).

Touch down step was optimized as starting from 65 °C for *TtBH-A1* gene and starting from 63 °C for *TtBH-B1* gene decreasing -0.7 °C/cycle.

Optimized PCR reagents (Table 3.1) and conditions Table 3.2 for *TtBH-A1* and Table 3.3 for *TtBH-B1* were used for further steps. Heteroduplex formation (formation of one strand from wild type and one strand from mutant line from amplified PCR products) was used only TILLING screening step.

Table 3. 1. Optimized PCR reagents.

Reagent	**Stock Conc.**	**Volume (µl)**	**Final Conc.**
Pooled DNA	~30 ng/µl	5.0	~150 ng
Water		0.3	
PCR Reaction Buffer	5X	2.5	1X
Betaine	5 M	2.5	1 M
$MgCl_2$	25 mM	1.5	3 mM
dNTP Mix	10 mM	1.0	0.8 mM
Primer Master Mix	100 µM	0.1	0.8 µM
Taq DNA Polymerase	5 U/µl	0.1	0.5 U
TOTAL		13.0	

Table 3. 2. Optimized PCR conditions for *TtBH-A1* gene.

1.	95˚C for 3 minutes	Denaturation
2.	95˚C for 20 seconds	Touch Down Step
3.	From 65˚C -0,7˚C/cycle 30 seconds	
4.	72˚C for 1:20 minutes	
5.	GO TO 2 for 9 times	
6.	98˚C for 15 seconds	Denaturation
7.	58˚C for for 45 seconds	Annealing
8.	72˚C for 1:20 minutes	Extension
9.	GO TO 6 for 44 times	
10.	72˚C for 10 minutes	Final Extension
11.	99˚C for 10 minutes	Heteroduplex Formation (ONLY TILLING)
12.	70˚C for 20 seconds, -0.3˚C/cycle	
13.	GO TO 12 for 69 times	
14.	12˚C for ever	Storage
15.	End	

Table 3. 3. Optimized PCR conditions for *TtBH-B1* gene.

1.	95˚C for 3 minutes	Denaturation
2.	95˚C for 20 seconds	Touch Down Step
3.	From 63˚C -0.7°C/Cycle for 30 seconds	
4.	72˚C for 1:40 minutes	
5.	GO TO 2 for 9 times	
6.	98˚C for 15 seconds	Denaturation
7.	57˚C for 45 seconds	Annealing
8.	72˚C for 1:40 minutes	Extension
9.	GO TO 6 for 44 times	
10.	72˚C for 10 minutes	Final Extension
11.	99˚C for 10 minutes	Heteroduplex Formation (ONLY TILLING)
12.	70˚C for 20s, -0.3˚C per cycle	
13.	GO TO 12 for 69 times	
14.	12˚C for ever	Storage
15.	End	

3.1.2. Screening of Kronos TILLING Population For *TtBH-A1* and *TtBH-B1* Genes

Mutant DNA pools were screened for *TtBH-A1* and *TtBH-B1* genes after PCR optimization. One row of PCR products of each plate was digested with *BsrI* enzyme to test PCR efficiency. (Figure 3.9 and Figure 3. 10).

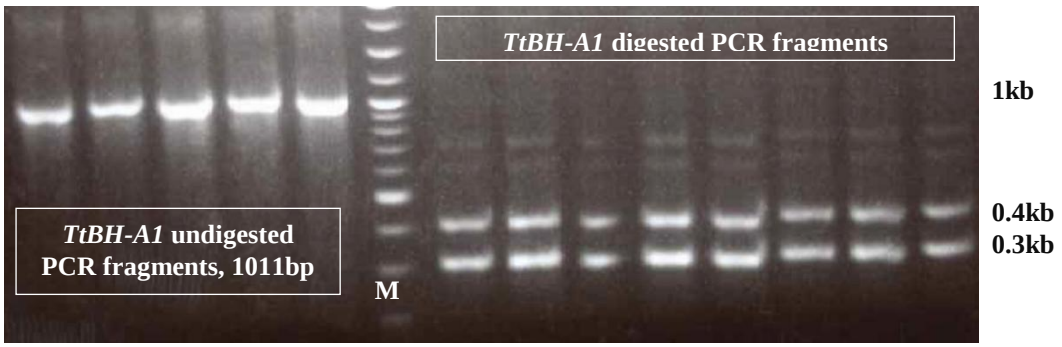


Figure 3. 9. *Bsr*I digestion of Plate2 before *Cel*1 treatment for *TtBH-A1* gene. M: Marker. 2-Log DNA ladder (0.1- 10kb). *TtBH-A1* gene had two *BsrI* digestion sites. 301 bp and 305 bp fragments were nearly the same size. They were together on agarose gel as a one band at 300 bp region. The other band was 400 bp region. It was observed that nearly 600 bp and 700 bp bands were on the gel in addition to expected bands, if the samples were not completely digested. Undigested samples were loaded to gel as a control.

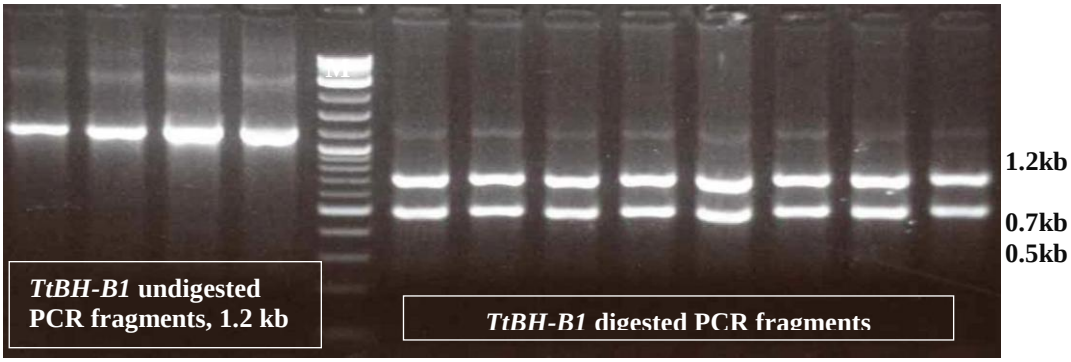


Figure 3. 10. *Bsr*I digestion of Plate2 before *Cel*1 traetment for *TtBH-B1* gene. M: Marker. 2-Log DNA ladder (0.1- 10kb). *TtBH-B1* gene had one *BsrI* digestion site at 502 bp. There were two bands on the gel nearly 500 bp and 700 bp. Undigested samples were loaded to gel as a control.

Plates corrected by *BsrI* digestion for *TtBH-A1* gene and *TtBH-B1* gene were sent to RevGen TILLING Service for the *Cel*1 digestion. PCR products were run using E-gel to see enough fragment strength before *Cel*1 digestion. E-gel was a check point before further steps. Smear PCR products and too much primer dimers could be problem working with *Cel*1. *Cel*1 enzyme recognized and randomly cut the mismatch pairing on both of the strands of *TtBH-1* homoeologous. Sequencing by ABI3730XL was the last step of screening.

3.1.3. Identification of *TtBH-A1* and *TtBH-B1* Mutations

ABI3730XL sequencer results were analyzed by using GENEMAPPER® software. If there was a mismatch on the heteroduplex strand of *TtBH-1* homoeologous, two peaks were observed one for blue color (forward direction) and one for green color (reverse direction). Also, the sum of the blue and green peak sizes was close to product size with $\pm$ 50 bp, if *Cel*1 worked correctly (Figure 3.11). Generally, there was noisy activity on nearly first 150 – 200 bp region in the sequence results. So, it was very difficult to determine this region mismatches. Successive peaks were evaluated as an artefact. They were discarded.

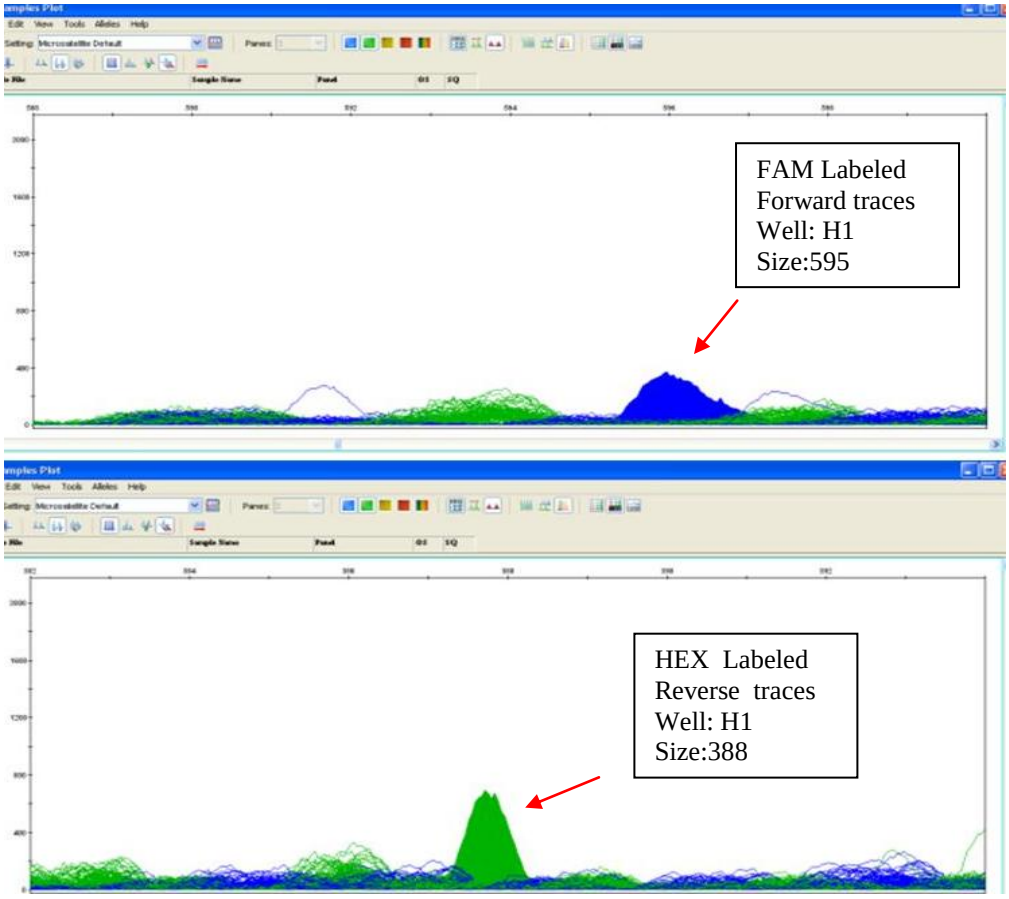


Figure 3. 11. Plate1 GENEMAPPER® Software result for *TtBH-A1* gene. There were three blue peaks (forward direction) out of the motif. Selected peak belonged to H1 well and its size was 595. Green peak size was 388 and sum of them were equal to product size (983bp).

Table 3.4 (*TtBH-A1* gene) and Table 3.5 (*TtBH-B1* gene) were prapared for GENEMAPPER® software results. Firstly, GENEMAPPER software results for *TtBH-A1* gene were evaluated. Totally, 12 mismatches for the Plate1, 15 mismatches for the Plate2 and 14 mismatches for the Plate3 were discovered. Totally, 41 DNA pools had mismatch pairs according to analysis (Table 3.4). Product size for *TtBH-A1* was 1011 bp. The minimum product size was 977 bp in Plate1 well F3. The maximum product size was 995 bp in Plate2 well G7.

The sum of size of blue and green peaks for one well was equal or close to product size 1218 bp for *TtBH-B1* gene, if *Cel*1 worked correctly. 20 mismatches for Plate1, 14 mismatches for Plate2 and 12 mismatches for Plate3 were determined according to *Cel*1 digestion results. In summary, 46 mismatches were found for *TtBH-B1* gene (Table 3. 5). The minimum product size was 1193 bp in Plate1 well F6. The maximum product size was 1209 bp in Plate2 well H6.

Table 3. 4. GENEMAPPER analysis of *TtBH-A1* gene.

			SIZE		
Plate ID	**No**	**Well ID**	**Blue Peak**	**Green Peak**	**Sum**
Plate 1	1	H1	595	388	983
	2	A2	580	404	984
	3	B3	704	281	985
	4	F3	507	470	977
	5	H3	670	314	984
	6	C4	754	232	986
	7	G4	591	393	984
	8	B6	203	780	983
	9	F8	891	97	988
	10	A11	516	468	984
	11	B12	597	385	982
	12	E12	486	496	982
Plate 2	13	E1	167	827	994
	14	E2	97	896	993
	15	G2	216	778	994

Table 3.4 (cont'd)

	16	D3	425	568	993
	17	G3	161	833	994
	18	Bl4	733	254	987
	19	C4	471	514	985
	20	G7	85	910	995
	21	F8	160	834	994
	22	D9	508	480	988
	23	G10	514	471	985
	24	B11	659	326	985
	25	C11	880	107	987
	26	D11	523	463	986
	27	G11	975	10	985
Plate 3	28	E1	631	352	983
	29	F1	287	702	989
	30	G1	412	571	983
	31	B2	215	777	992
	32	F2	789	203	992
	33	C3	679	304	983
	34	E3	584	399	983
	35	D6	563	420	983
	36	F6	226	765	991
	37	C8	465	520	985
	38	B10	752	231	983
	39	D11	884	102	987
	40	E11	664	318	982
	41	C12	146	844	990

Table 3. 5. GENEMAPPER analysis of *TtBH-B1* gene.

Plate ID	No	Well ID	SIZE		
			Blue Peak	Green Peak	Sum
Plate1	1	F2	612	588	1200
	2	C3	708	493	1201
	3	C4	989	206	1195
	4	F4	964	230	1194
	5	F4	402	798	1200
	6	H4	281	920	1201
	7	G5	516	685	1201
	8	C6	865	329	1194
	9	C6	680	521	1201
	10	F6	884	309	1193
	11	F6	680	521	1201
	12	A8	667	533	1200
	13	B8	931	262	1193
	14	B9	547	654	1201
	15	D10	964	231	1195
	16	E10	978	216	1194
	17	E12	401	798	1199
	18	E12	510	690	1200
	19	E12	727	474	1201
	20	E12	851	343	1194
Plate2	21	C1	541	659	1200
	22	H1	860	334	1194
	23	G3	748	448	1196
	24	A4	770	426	1196
	25	E4	477	725	1202
	26	B5	526	675	1201
	27	C5	682	519	1201
	28	H6	1116	93	1209
	29	A7	454	746	1200
	30	B7	780	416	1196
	31	D7	635	565	1200
	32	E8	653	547	1200
	33	F11	655	545	1200
	34	H11	708	490	1198
Plate3	35	F1	698	500	1198
	36	F2	745	452	1197

Table (3.5 cont'd)

	37	B3	614	588	1202
	38	B4	403	800	1203
	39	H5	257	943	1200
	40	C6	285	915	1200
	41	H7	630	570	1200
	42	E9	560	636	1194
	43	F9	511	690	1201
	44	C10	288	912	1200
	45	F11	510	690	1200
	46	F11	727	474	1201

3.1.4. Confirmation of *TtBH-A1* and *TtBH-B1* Mutations

Individual mutant lines for *TtBH-A1* (Figure 3. 12) and *TtBH-B1* (Figure 3. 13) were amplified and sequenced to confirm which line had the mutation. Sequence results were evaluated using Mutation Surveyor® software an in silico tool which is very sensitive and accurate and some bioinformatics tools such as Vector NTI, BioEdit, NCBI Blast, PARSESNP and Prosite etc. Table 3.6 (*TtBH-A1*) and Table 3.7 (*TtBH-B1*) were prepared mutations showing Mutant ID, mutation place, mutation type (heterozygous or homozygous), amino acid change and Position Specific Scoring Matrix (PSSM) from NCBI and PARSESNP and SIFT scores..

15 homozygous and 25 heterozygous mutations were confirmed for *TtBH-A1* gene in this TILLING study. While 28 mutations were missense, 12 were silent mutation in totally 40 new alleles for *TtBH-A1* gene. 6 mutations were identified at the conserved domain, 2 silent and 4 missense mutations. 4 mutations (T4-929, T4-2151, T4-2261 and T4-2447) were at the conserved domain region (Table 3.6).

The strongest mutation was T4-2447 mutant. Glycine (**G**GC) was changed to serine (**A**GC) at the 61th amino acid because of heterozygous mutation on the 181 bp guanine to adenin (Figure 3. 14). It had the highest PSSM score (0.94) according to NCBI Conserved Domain Search. PSSM is used to understand how often amino

acid substitution occurs for each position in a protein multiple sequence alignment. This muation was at the DNA binding site and highly conserved. It was expected that protein function might be affected by this mutation. Also, PSSM score was 27.9 and SIFT score was zero according to PARSESNP result.

T4-2151 mutant line having homozygous mutation (A102T) on conserved domain had also high PSSM score, 0.78 (Poursarebani *et.al.*, 2015). There was no PSSM score result for T4-2151 line according to PARSESNP result.

T4-929 (G57A, homozygous) and T4-2261 (G57W, homozygous) lines had the same amino acid mutation at the position 57 which was the start of conserved domain.

These results showed that mutant line T4-2447 had very important mutation for *TtBH-A1* gene. (Figure 3.15 and Table 3.6)

The orthologues of *TtBH-1* gene were conserved in crop plants (Komatsu *et.al.*, 2001 and 2003; Chuck *et.al.*, 2002; Derbyshire & Byrne, 2013; Dobrovolskaya *et.al.*, 2015; Poursarebani *et.al.*, 2015). *TtBH-A1* and *TtBH-B1* genes had this *AP2/ERF* highly conserved domain having 11 DNA binding sites between 57 and 116 amino acids. Mutation on this conserved domain caused severe branched phenotype in other crops. This conserved domain was the nice target beside of truncation mutations. Mutation on *AP2/ERF* conserved domain caused branching phenotype in rice (Komatsu *et.al.*, 2003 and Yi *et.al.*, 2005) and bread wheat (Dobrovolskaya *et.al.*, 2015).

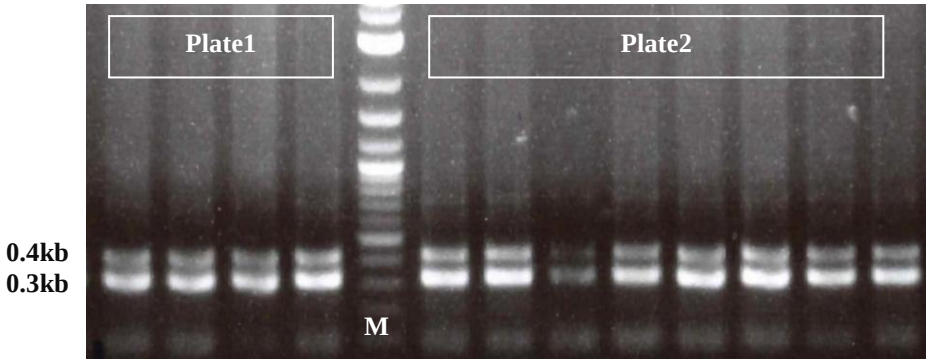


Figure 3. 12. *Bsr*I digested Plate1 and Plate2 individuals for *TtBH-A1* gene. M: Marker. 2-Log DNA ladder (0.1- 10kb).

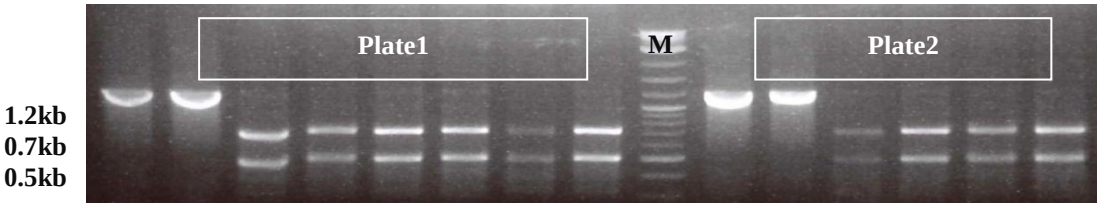


Figure 3. 13. Undigested and *Bsr*I digested Plate1 and Plate2 individuals for *TtBH-B1* gene. M: Marker. 2-Log DNA ladder (0.1- 10kb).

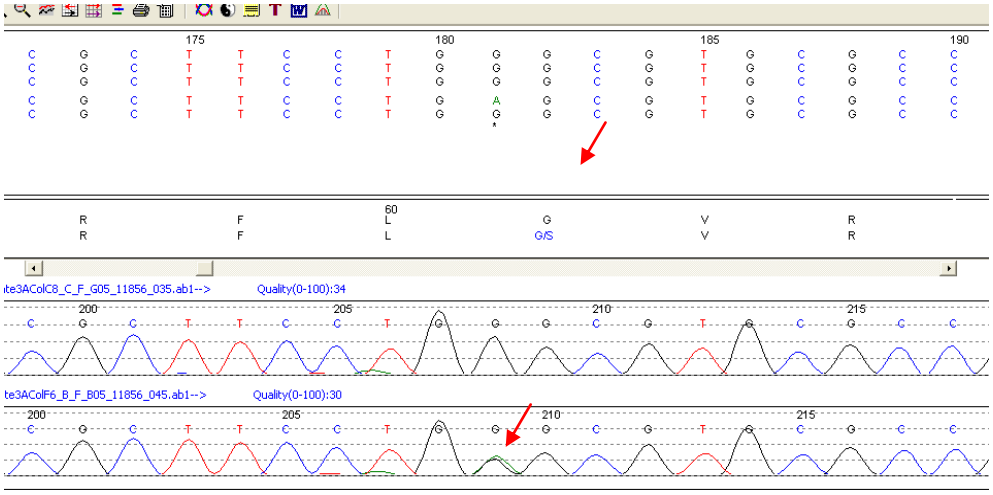


Figure 3. 14. Chromatogram for the T4-2447 line having the strongest mutation for *TtBH-A1* gene. T4-2447 mutant had a change (guanine to adenine at 181bp) at the gene sequence. This change resulted with amino acid change (glycine to serine at 61th position) in protein. This amino acid is at DNA binding site and highly conserved.

```
KRONOS_TtBH-A1      MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPGRFL 60
T4-2447_TtBH-A1     MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPGRFL 60
T4-2151_TtBH-A1     MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPGRFL 60
T4-929_TtBH-A1      MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPARFL 60
T4-2261_TtBH-A1     MSSRSSSGGGGASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAARGRRRAQEPWRFL 60
                    ************************************************************ ***

KRONOS_TtBH-A1      GVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAA 120
T4-2447_TtBH-A1     SVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAA 120
T4-2151_TtBH-A1     GVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRATLSMKGAQARTNFVYAHAA 120
T4-929_TtBH-A1      GVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRATLSMKGAQARTNFVYAHAA 120
T4-2261_TtBH-A1     GVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRATLSMKGAQARTNFVYAHAA 120
                    .************************************************:**************
```

Figure 3. 15. Multiple sequence alignment of *TtBH-A1* gene conserved domain missense mutations and Kronos. Conserved domain was highlighted by yellow, mutation points were highlighted by blue and DNA binding sites were labeled by red.

Table 3. 6. Confirmed *TtBH-A1* gene mutations. Mutant ID showed for individual mutant line. Place presented mutation position in the gene. Amino acid change was showed using abbreviations of amino acids. Abbreviations of amino acids were Appendix A. "=" symbol represented as a silent mutation. PSSM value was obtained from NCBI. Conserved domain missense mutations were bolded.

No	Mutant ID	Place	Genotype	Amino acid change	PSSM (NCBI)	PSSM (Parsesnp)	SIFT
1	T4-177	G562A	Homozygous	A188T			
2	T4-179	G546A	Homozygous	M182I			
3	T4-265	G670A	Heterozygous	E224K			
4	T4-305	C481T	Heterozygous	H161Y			
5	T4-317	G637A	Heterozygous	D213N			
6	T4-338	C809T	Heterozygous	A270V			
7	T4-341	C722T	Heterozygous	S241F			
8	T4-342	C345T	Heterozygous	V115=			
9	T4-369	G557A	Heterozygous	S186N			
10	T4-429	C167T	Heterozygous	P56L			
11	T4-700	C483T	Homozygous	H161=			
12	T4-779	G564A	Heterozygous	A188=			
13	T4-854	C119T	Heterozygous	S40L			
14	T4-913	C50T	Heterozygous	A17V			
15	**T4-929**	**G170A**	**Homozygous**	**G57A**			
16	T4-1014	C113T	Heterozygous	P38L			
17	T4-1060	C699T	Heterozygous	D233=			
18	T4-2024	C112T	Homozygous	P38S			
19	T4-2048	G472A	Homozygous	G158R			
20	T4-2103	G480A	Homozygous	P160=			
21	T4-2123	C625T	Homozygous	P209S			
22	T4-2128	C848T	Homozygous	S283L			
23	T4-2130	G488A	Heterozygous	G163D			
24	**T4-2151**	**G304A**	**Homozygous**	**A102T**	**0.78**		
25	T4-2227	G599A	Heterozygous	G200S			
26	T4-2236	G246A	Homozygous	R82=			
27	T4-2240	C174T	Heterozygous	R58=			
28	T4-2240	C378T	Homozygous	P126=			

Table 3.6 (cont'd)

29	**T4- 2261**	**G169T**	**Homozygous**	**G57W**			
30	T4-2281	G759A	Heterozygous	R253=			
31	T4-2306	G647A	Heterozygous	G216E			
32	T4-2315	G550A	Heterozygous	V184M			
33	T4-2438	C529T	Homozygous	P177S			
34	**T4-2447**	**G181A**	**Heterozygous**	**G61S**	**0.94**	**27.9**	**0.00**
35	T4-2530	C429T	Heterozygous	S143=			
36	T4-2622	C722T	Heterozygous	S241F			
37	T4-2673	G855A	Heterozygous	Q285=			
38	T4-2676	C632T	Heterozygous	A211V			
39	T4-2705	C50T	Heterozygous	A17V			
40	T4-2706	G99A	Homozygous	P33=			

40 mutations were confirmed for *TtBH-B1* gene in this TILLING study, while 46 mismatches were identified for this gene. One premature truncation was found at the 14th amino acid for T4-2432 mutant (Figure 3.16). This mutation was heterozygous. The glutamine (**C**AG) was converted to a premature stop codon (**T**AG) (Q14X), the protein lost its function (Poursarebani *et.al.,* 2015). 17 mutations were silent. There were 23 missense mutations at the protein coding sequence. 6 mutations (T4-598, T4-841, T4-1096, T4-1164, T4-1323, and T4-2302) were on the conserved domain region.

T4-1164 (A95V) and T4-598 (A102T) mutant lines had the highest PSSM scores 1.00 and 0.78 in order (Table 3.7). PSSM score was 17.8 and SIFT score was zero according to PARSESNP result for T4-1164 line. T4-2432 having premature stop codon and T4-1164 were nice targets for *TtBH-B1* gene. (Figure 3.17 and Table 3.7) Premature stop codons caused by a mutation in the *WFZP-D* gene resulted with branching phenotype in bread wheat (Dobrovolskaya *et.al.*, 2015).

Mutation rate for *TtBH-1* gene was calculated as one mutation per 26 kb. Very high GC content of tilled fragment (74.4 %) could be one of the possible reasons for high mutation rate for *TtBH-1* gene. Because, GC residues were the main targets for

EMS mutagen (Slade *et.al.*, 2005b). Moreover, GENEMAPPER® and Mutation Surveyor® programs were used in this study. They were more sensitive and accurate from previously used tools. One mutation per 51 kb mutation frequency for 50% of GC content fragment was reported for the same TILLING population (Uauy *et.al.*, 2009). Slade and co-authors (2005b) showed one mutation per 34 kb mutation rate for tetraploid wheat. One mutation per 24 kb mutation rate was reported for hexaploid wheat in the same study. The similar mutation rate was also introduced for *starch synthase II* gene homoeologous in bread wheat (Dong *et.al.*, 2009b).

Homozygous mutation and heterozygous mutation rates were 36.25 % and 63.75 % respectively. The most of the mutations were transitions, 56.25 % C to T and 42.5 % G to A as expected from EMS alkylation. Only one mutation was confirmed as G to T 1.25 %. These results were similar to previos reports (Dong *et.al.*, 2009a and 2009b; Greene *et.al.*, 2003).

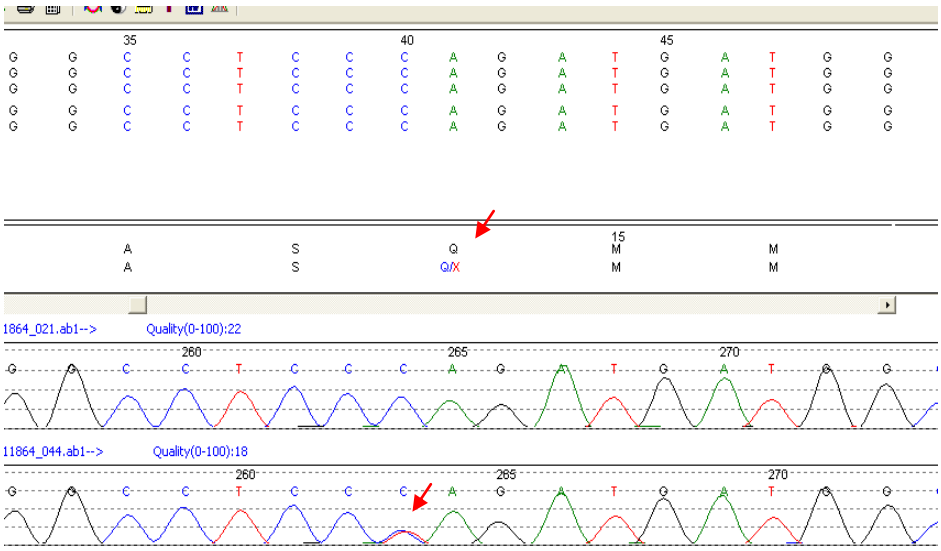


Figure 3. 16. Chromatogram for T4-2432 line for *TtBH-B1* gene. Glutamine (CAG) at the 14th amino acid in the protein was converted to stop (X) codon (TAG).

```
T4-598_TtBH-B1      MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPTTK 80
T4-841_TtBH-B1      MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPTTK 80
T4-1096_TtBH-B1     MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPITK 80
T4-1164_TtBH-B1     MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPTTK 80
T4-1323_TtBH-B1     MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYVAEIRDPTTK 80
T4-2302_TtBH-B1     MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPTTK 80
KRONOS_TtBH-B1      MSSRSSSGGGQASQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPGRFLGVRRRPWGRYAAEIRDPTTK 80
T4-2432_TtBH-B1     MSSRSSSGGGQASX---------------------------------------------------------------------- 14
                    ************

T4-598_TtBH-B1      ERHWLGTFDTAQEAALAYDRATLSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-841_TtBH-B1      ERHWLGTFDTAQEAALAYDCAALSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-1096_TtBH-B1     ERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-1164_TtBH-B1     ERHWLGTFDTAQEAVLAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-1323_TtBH-B1     ERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-2302_TtBH-B1     ERHWLGTFDTAQEAALAYDRAAFSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
KRONOS_TtBH-B1      ERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYAHAAYNNYPPFLAPFHAQQQPAAYASSTMPYGGQQRAGAAAPHI 160
T4-2432_TtBH-B1     ---------------------------------------------------------------------------------
```

Figure 3. 17. Multiple sequence alignment of *TtBH-B1* gene truncation, missense mutations on conserved domain and Kronos. Conserved domain was highlighted by yellow, mutation points were highlighted by blue and DNA binding sites were labeled by red.

Table 3. 7. Confirmed *TtBH-B1* gene mutations. Mutant ID showed for individual mutant line. Place presented mutation position in the gene. Amino acid change was showed using abbreviations of amino acids. Abbreviations of amino acids were Appendix A. "=" symbol represented as a silent mutation. PSSM value was obtained from NCBI. Conserved domain missense mutations and truncation mutation were bolded.

No	Mutant ID	Place	Genotype	Amino acid change	PSSM (NCBI)	PSSM (Parsesnp)	SIFT
1	T4-229	C372T	Heterozygous	Y124=			
2	T4-275	C470T	Homozygous	A157V			
3	T4-342	C760T	Heterozygous	L254W			
4	T4-358	G159A	Heterozygous	A53=			
5	T4-364	C735T	Homozygous	D245=			
6	T4-373	C35T	Heterozygous	A12V			
7	**T4-409**	**G273A**	**Heterozygous**	**A91=**	**0.65**		
8	T4-437	C441T	Homozygous	Y147=			
9	T4-438	G634A	Heterozygous	A212T			
10	T4-457	C112T	Homozygous	P38S			
11	T4-458	C654T	Homozygous	Y218=			
12	T4-548	C462T	Homozygous	G154=			
13	T4-549	C428T	Homozygous	S143F			
14	T4-554	C702T	Homozygous	D234=			
15	**T4-598**	**G304A**	**Heterozygous**	**A102T**	**0.78**		
16	T4-2709	C144T	Homozygous	R48=			
17	**T4-841**	**C298T**	**Heterozygous**	**R100C**	**0.22**		
18	T4-872	C628T	Heterozygous	P210S			
19	T4-1017	G513A	Homozygous	Q171=			
20	**T4-1096**	**C233T**	**Heterozygous**	**T78I**	**0.09**		
21	**T4-1164**	**C284T**	**Heterozygous**	**A95V**	**1.00**	**17.8**	**0.00**
22	T4-1171	G443A	Homozygous	G148D			
23	T4-1302	G58A	Heterozygous	E20K			
24	**T4-1323**	**C212T**	**Heterozygous**	**A71V**	**0.17**		
25	T4-1332	C546T	Heterozygous	S182=			
26	T4-1360	C396T	Heterozygous	H132=			
27	T4-2020	C413T	Homozygous	A138V			

Table 3.7 (cont'd)

28	T4-2141	C416T	Homozygous	A139V			
29	T4-2152	G471A	Heterozygous	A157=			
30	T4-2234	G460A	Heterozygous	G154S			
31	T4-2234	G677A	Heterozygous	S226N			
32	T4-2281	C510T	Heterozygous	H170=			
33	T4-2299	C373T	Heterozygous	P125S			
34	**T4-2302**	**C307T**	**Heterozygous**	**L103F**	**0.26**		
35	T4-2333	G159A	Heterozygous	A53=			
36	T4-2336	G131A	Heterozygous	R44Q			
37	T4-2724	G11A	Homozygous	R4H			
38	**T4-2432**	**C40T**	**Heterozygous**	**Q14X**	**STOP**		
39	T4-2512	G390A	Heterozygous	P130=			
40	T4-2629	G42A	Heterozygous	Q14=			

3.2.Validation of *TtBH-A1* and *TtBH-B1* Mutants

All *TtBH-A1* gene mutant plants having missense mutation and *TtBH-B1* gene mutants having truncation and missense mutations on conserved domain region seeds (Table 3.8) and Kronos wild type seeds were sowed in summer greenhouse. However, T4-2432 seeds having a truncation for *TtBH-B1* gene were not available for summer greenhouse. T4-2432 was planted during winter greenhouse season. Genotyping was carried out after DNA isolation of *TtBH-1* mutants.

3.2.1. Genotyping of *TtBH-A1* and *TtBH-B1* Mutants

Firstly, designed KASP markers were tested for genotyping. After that, all mutants were sequenced to validate mutations and to develop a breeding strategy.

Table 3. 8. Planted seeds for *TtBH-1* homoeologous in summer greenhouse.

	Gene	Mutation	Mutant ID	Seeds No	Generation
1	*TtBH-A1*	A188T	T4-177	15	M3
2	*TtBH-A1*	S241F	T4-341	15	M2
3	*TtBH-A1*	S186N	T4-369	15	M3
4	*TtBH-B1*	A102T	T4-598	15	M3
5	*TtBH-B1*	R100C	T4-841	10	M4
6	*TtBH-A1*	A17V	T4-913	20	M3
7	*TtBH-A1*	G57A	T4-929	15	M3
8	*TtBH-A1*	P38L	T4-1014	15	M3
9	*TtBH-B1*	A95V	T4-1164	15	M3
10	*TtBH-A1*	P38S	T4-2024	15	M3
11	*TtBH-A1*	G158R	T4-2048	15	M3
12	*TtBH-A1*	S283L	T4-2128	15	M3
13	*TtBH-A1*	G163D	T4-2130	15	M3
14	*TtBH-A1*	A102T	T4-2151	15	M3
15	*TtBH-A1*	G200S	T4-2227	15	M3
16	*TtBH-A1*	G57W	T4-2261	20	M4
17	*TtBH-A1*	G216E	T4-2306	15	M3
18	*TtBH-A1*	V184M	T4-2315	15	M3
19	*TtBH-A1*	P177S	T4-2438	15	M3
20	*TtBH-A1*	G61S	T4-2447	15	M3
21	*TtBH-A1*	S241F	T4-2622	15	M3
22	*TtBH-A1*	A211V	T4-2676	20	M4
23	*TtBH-A1*	A17V	T4-2705	15	M4

3.2.1.1.KASP Markers and Sequencing of *TtBH-A1* and *TtBH-B1* Mutants

KASP markers were used to validate mutant plants. T4-2151 line had a homozygous mutation (A102T, PSSM: 0.78) on conserved domain. It was observed that all plants had mutant genotype for T4-2151. T4-2622 line had a heterozygous mutation (S241F). Segregation was observed for this mutation. T4-2447 having the strongest mutation for *TtBH-A1* gene (G61S, PSSM: 0.96) did not work with designed KASP markers. (Figure 3.18)

Homozygous FAM alleles (mutant type genotype for *TtBH-1* homoeologous) were close to X-axis and homozygous HEX alleles (wild genotype) were close to Y-axis. Heterozygous mutant plants were between X-axis and Y-axis.

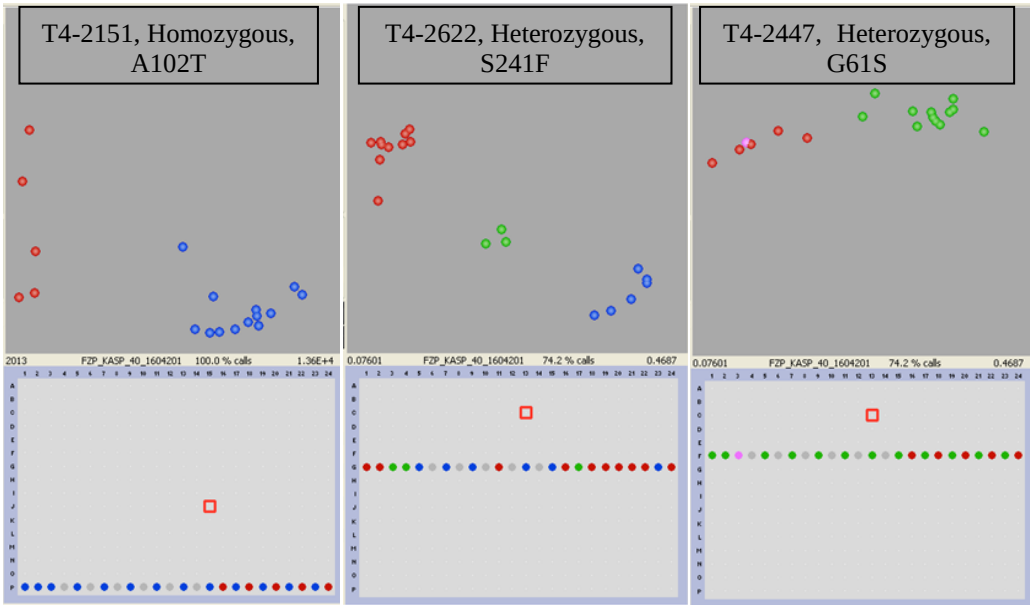


Figure 3. 18. KlusterCaller™ results for some *TtBH-A1* gene mutants. T4-2151 had homozygous mutation. T4-2622 had heterozygous mutation. Segregation was observed for T4-2622. T4-2447 had heterozygous mutation. KASP markers did not work for T4-2447. Red balls: Wild type genotype, Blue balls: Mutant genotype, Green balls: Heterozygous genotype.

Table 3.9 presented verified KASP markers for given mutant lines. However, the most of the mutant lines including T4-2247 line having the most important mutation for *TtBH-A1* gene did not work with KASP markers. All mutant plants were sequenced for genotyping and verification of KASP results.

There was only one homozygous plant genotype for G61S mutation at *TtBH-A1* gene according to sequence results. T4-2447-2, T4-2447-8 and T4-2447-11 plants were wild type and rest of the plants was heterozygous (Table 3.10). All T4-2151 plants were homozygous as expected. T4-598-3 was homozygous and T4-598-7 was heterozygous genotype for *TtBH-B1* gene. There was no mutant genotype for T4-841 and T4-1164 mutant lines. Available genotypes were used for crossing to combine A and B homoeologous.

Table 3. 9. KASP results for genotyping.

Mutant ID	**Mutation**	**Genotype**	**KASP**
T4-177	G562A	Homozygous	OK
T4-1014	C113T	Heterozygous	OK
T4-2048	G472A	Homozygous	OK
T4-2151	G304A	Homozygous	OK
T4-2227	G599A	Heterozygous	OK
T4-2306	G647A	Heterozygous	OK
T4-2315	G550A	Heterozygous	OK
T4-2438	C529T	Homozygous	OK
T4-2622	C722T	Heterozygous	OK
T4-2676	C632T	Heterozygous	OK
T4-2705	C50T	Heterozygous	OK
T4-598-B	G304A	Heterozygous	OK

Table 3. 10. T4-2447 mutant genotype and phenotype results. SS: Supernumerary Spikelet. (Poursarebani *et.al.*, 2015)

Plant ID	Mutation	Genotype	Phenotype
T4-2447-1	G181A	Heterozygous	
T4-2447-2	G181A	Wild type	
T4-2447-3	G181A	Heterozygous	
T4-2447-4	G181A	Heterozygous	
T4-2447-5	G181A	Heterozygous	
T4-2447-6	G181A	Heterozygous	
T4-2447-7	**G181A**	**Homozygous**	**SS phenotype**
T4-2447-8	G181A	Wild type	
T4-2447-9	G181A	Heterozygous	
T4-2447-10	G181A	Heterozygous	
T4-2447-11	G181A	Wild type	
T4-2447-12	G181A	Heterozygous	
T4-2447-13	G181A	Heterozygous	

3.2.2. Phenotype Analysis for *TtBH-1* Gene

Mutant plants were phenotyped after mutation validation to see phenotype. Supernumerary spikelets were observed on spike of T4-2447-7 plant which was the strongest mutation for *TtBH-A1* gene (Table 3.10). Extra spikelets emerged side of normal spikelets and connected to rachis (Figure 3.19). This mutation was on DNA binding site, glycine was changed to serine at 61st position of protein. The PSSM for this amino acid was 0.94. T4-2447 plant was homozygous mutant. There were 9 heterozygous and 3 wild type mutant plants for this line. However, we could not observe any extra spikelets for these 12 plants (Table 3.10). It was reported that orthologues of *TtBH-1* gene had recessive inheritance (Pennel & Halloran, 1983; Wenye *et.al.*, 1995; Dobrovolskaya *et.al.*, 2009 and 2015; Haque *et.al.*, 2012). Dobrovolskaya and co-authors (2015) reported similar results for *WFZP-A* gene in hexaploid wheat orthologue to *TtBH-A1* gene in tetraploid wheat. Moreover, similar phenotype was observed as a result of mutation in this gene orthologue in other crops (Colombo *et.al.*, 1998; Chuck *et.al.*, 2002; Mackill *et.al.*, 1993; Komatsu

et.al., 2001 and 2003; Yi *et.al.*, 2005; Kato & Horibata, 2011; Bai *et.al.*, 2016; Derbyshire & Byrne, 2013; Castiglioni *et.al.*, 1998; Rossini *et.al.*, 2006)

T4-2447-7 plant had totally twelve spikes. Seven of them had supernumerary spikelets, 58.3%. One spike with 5 extra spikelets, two spikes with 4 extra spikelets, one spike with 2 extra spikelets and three spikes with 1 extra spikelet were observed on T4-2447-7 plant. Most of these spikes were used for crossing.

Also, other planted mutants for *TtBH-A1* gene were screened for phenotype. However, any branching and supernumerary spikelet phenotype was not observed, even if conserved domain mutants.

T4-2432 mutant line having a premature stop mutation for *TtBH-B1* gene was screened for phenotype. There were 9 homozygous, 2 heterozygous and 3 wild plants for this mutation. Any supernumerary spikelet or branching was not observed for *TtBH-B1* gene mutants. T4-598 line having a mutation on conserved domain (A102T, PSSM: 0.78) did not have a mutant phenotype. T4-2432 line had a premature stop codon (Q14X). However, any plant belong to this line did not have different phenotype from wild type (Table 3.11). *TtBH-B1* gene did not have any contribution to branching phenotype in durum wheat according to these results. It was reported that *WFZP-B* gene in hexaploid wheat was nearly silent according to A and D genome homoeologous (Dobrovolskaya *et.al.*, 2015). In addition to this, *TtBH-B1* gene did not affected “Miracle-Wheat” phenotype (Poursarebani *et.al.*, 2015).

Table 3. 11. T4-2432 mutant genotype results. (Poursarebani *et.al.*, 2015)

Mutant ID	Mutation	Genotype
T4-2432-1	C40T	Homozygous
T4-2432-2	C40T	Wild type
T4-2432-3	C40T	Heterozygous
T4-2432-4	C40T	Homozygous
T4-2432-6	C40T	Homozygous
T4-2432-8	C40T	Wild type
T4-2432-9	C40T	Homozygous
T4-2432-10	C40T	Homozygous
T4-2432-11	C40T	Homozygous
T4-2432-12	C40T	Homozygous
T4-2432-13	C40T	Homozygous
T4-2432-14	C40T	Wild type
T4-2432-15	C40T	Homozygous
T4-2432-16	C40T	Heterozygous

Figure 3. 19. Observed extra spikelets on T4-2447-7 plant. Extra spikelets were labeled with red arrow.

3.3.Breeding Strategy

3.3.1. Creating Double Mutants and Backcross with Kronos

Conserved domain mutations for *TtBH-A1* (T4-2447, T4-929 and T4-2151) were crossed with *TtBH-B1* premature stop codon (T4-2432) and conserved domain mutation (T4-598) to make double mutants. Totally, 56 seeds were produced as result of combining T4-2432 and T4-2447 lines. Combining two homoeologous caused more severe phenotype than its alone effect (Slade *et.al.*, 2005b; Wang *et.al.*, 2012; Botticella *et.al.*, 2011; Hazard *et.al.*, 2012; Chen *et.al.*, 2014). Combination of *WFZP-A* and *WFZP*-D genes in bread wheat caused more severe branching phenotype in bread wheat (Dobrovolskaya *et.al.*, 2015).

Kronos wild type was crossed with *TtBH-1* gene mutants to eliminate background mutations. Crossing summary was on Table 3.12. Developed grains were also observed on extra spikelets on T4-2447-7 plant crossed with Kronos (Figure 3. 20).

Any phenotype was not observed both of backcrosses and double mutants because of recessive inheritance of *TtBH-A1* gene in F1 seeds. Some of the crosses were backcrossed with parent Kronos wild type to continue background mutation clean.

Figure 3. 20. Developed grains after crossing on extra spikelets. Extra spikelets were labeled with red arrows.

Table 3. 12. *TtBH-1* gene crossing summary.

	Cross ID	T4-2447	T4-2151	T4-929	
Kronos	HB1	19			**Grain number**
	HB2	14			
	HB3		11		
	HB4	4			
	S14-36	18			
	S14-38	15			
	S14-48		13		
	S14-49		10		
	S14-50		13		
	S14-42			6	
	Total	**70**	**47**	**6**	
T4-598	HB5			10	
	HB7		14		
	S14-37	4			
	NA1	19			
	Total	**23**	**14**	**10**	
T4-2432	W14-66	14			
	W14-94	13			
	W14-102	12			
	W14-59	17			
	Total	**56**			

3.3.2. Hybridization of *TtBH-A1* Gene Into Turkish Domestic Cultivars

Triticum turgidum durum cultivars Kızıltan-91 and Fuatbey-2000 were Turkish durum wheat cultivars. *TtBH-A1* mutation in T4-2447 line was introgressed to these cultivars. These two Turkish cultivars were also crossed with "Miracle-Wheat" having branched spikes. F1 seeds were harvested for these crosses (Table 3.13 and Figure 3.21). They were expected that all of them heterozygous for this trait. Further analyses will be performed in self pollinated plants after two times backcrossed with parents. Aslam & Buhtta (1977) investigated crossing of domestic durum cultivar with branched durum wheat caused increase in yield.

Table 3. 13. Hybridization of T4-2447 mutants and "Miracle-Wheat" with Turkish domestic cultivars.

	Cross ID	T4-2447	"Miracle-Wheat"	
Fuatbey-2000	METU-S-15-1		5	**Grain number**
	METU-S-15-3	8		
	METU-S-15-4	7		
	METU-S-15-5	12		
	METU-S-15-6	3		
	Total	**30**	**5**	
Kızıltan-91	METU-S-15-7	7		
	METU-S-15-8		7	
	METU-S-15-9	5		
	METU-S-15-11	7		
	Total	**19**	**7**	

Figure 3. 21. F1 seeds for crossing of Turkish domestic durum wheat cultivars with Miracle and T4-2447 mutant.

3.4. Segregation and Phenotyping of M4 Generation of T4-2447 Mutants

There were 9 heterozygous plants and one homozygous plant for T4-2447 line in M3 plants. Moreover, 10 seeds for each heterozygous M4 plants and 20 seeds for homozygous plant (T4-2447-7 plant; 10 from branched ear and 10 from unbranched ear) were planted.

1:2:1 Segregation (16 Wild type, 49 Heterozygous and 20 Homozygous) was observed for heterozygous plants in M3 generation for T4-2447 (χ^2=2.37, *P*=0.306). *TtBH-A1* gene was recessive as it was expected. Recessive control of supernumerary spikelet trait was reported in previous studies (Pennel & Halloran, 1983; Wenye *et.al.*, 1995; Dobrovolskaya *et.al.*, 2009 and 2015; Haque *et.al.*, 2012).

3.4.1. Spike Development in "Miracle-Wheat"

Miracle spike had ramified and supernumerary spikelet beside of normal spikelet (Figure 3. 22, A and Figure 3.23, F). Ramified spikelets were mostly between peduncle and middle part of spike. Supernumerary spikelets were observed in different numbers after branched spikelets (Figure 3. 22, B and C). Spike had normal spikelets close to terminal spikelet. Terminal spikelet was not developed completely in spike and ramified spikelets (Figure 3. 22, A). Miracle wheat spike development was observed under light microscope to investigate branching. Spike development was similar during double ridge stage (Figure 3. 23, A). Ectopic spikelet formation was observed instead of outer glume formation (Figure 3. 23, B). Ectopic spikelet was changed ramified or supernumerary spikelet in Miracle wheat. Floret development (Figure 3. 23, C) and awn development (Figure 3. 23, D) were observed. Ramified spikelets had different number of spikelet between 4 and 7 ending with a terminal spikelet not completely developed (Figure 3. 23, E). Terminal spikelet development is a key for spike development. Spikelet formation is stopped when terminal spikelet is completely developed. Spikelet meristem was converted to floral meristem for floral organ development. Delayed terminal

spikelet development could be possible reason for longer spikelet meristem activity. Therefore, spikelet development continued in “Miracle-Wheat” longer than unbranched wheat. These findings were similar to Poursarebani & co-authors (2015) report investigating branched phenotype development and Rawson & Ruwali (1972) report. Similar branching formation was also published in other crops (Chuck *et.al.*, 2002; Komatsu *et.al.*, 2003).

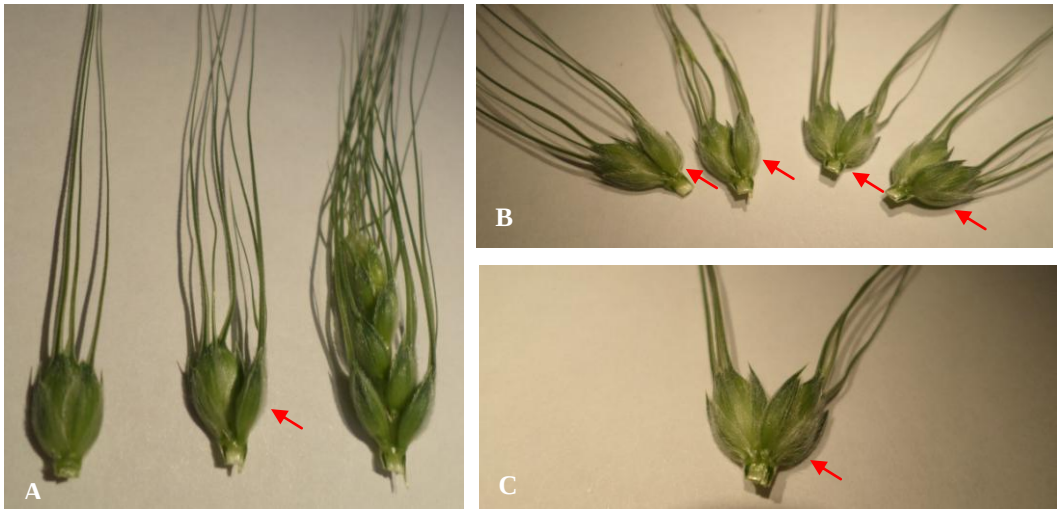


Figure 3. 22.Spikelet types in “Miracle-Wheat”. Extra spikelet was labeled with red arrow. (A) Normal Spikelet (Left), Supernumerary Spikelet (Middle) and Ramified Spikelet (Right); (B) and (C) Supernumerary spikelets. TS: Terminal spikelet.

Figure 3. 23. Spike development in “Miracle-Wheat”. Ectopic spikelets were labeled with red asterix (*). A) Double ridge stage, B) Initiaion of ectopic spikelet, C) Development of florets, D) Awn development, E) A branched spikelet and F) Branched spike. TS: Terminal spikelet.

3.4.2. Phenotyping of M4 Generation of T4-2447 Mutants

The first three tillers of T4-2447 M4 generation plants were screened for extra spikelet and branching. While ramified spikelet phenotype was not observed, supernumerary spikelet phenotype was observed in homozygous mutants for *TtBH-A1* gene. 3:1 phenotypic ratio (66 Wild type and 19 Mutant) was observed as expected (χ^2=0,317, *P*=0,573). 19 homozygous plants had at least one extra spikelet connected to rachis and side of main spikelet. Lateral spikelet development was observed instead of floral meristem activity after glume promordia initiation. In other words, spikelet meristem was still active. This phenotype was the same with "Miracle-Wheat" supernumerary spikelet phenotype (having only one additional spikelet) observed between ramified spikelet and normal spikelet. Extra spikelet was mostly observed lower part of spike instead of outer glume of main spikelet (Figure 3. 24, Figure 3. 25, Figure 3.26 and Figure 3.27). Inflorescence development observations were reliable with previously reported study about heterochronic floral development in diploid, tetraploid and hexaploid wheats (Shitsukawa *et.al.*, 2009). The similar additional spikelet phenotype was reported in hexaploid wheat (Echeverry-Solarte *et.al.*, 2014).

In addition to M4 generation of heterozygous T4-2447 mutants, 17 mutant M4 plants from T4-2447-7 line having supernumerary spikelet phenotype were screened. Plant ID was labeled with "ex" for originating from supernumerary spike. All M4 plants for T4-2447-7 line had supernumerary spikelet.

Spikelet number, supernumerary spikelet number and total spikelet number was counted for T4-2447 M4 generation. Homozygous plants data was presented in Table 3.14 (Poursarebani *et.al.*, 2015). Supernumerary spikelet structure was mostly observed on Tiller2 and Tiller3. Total spikelet number with 6 extra spikelets was counted as 22 the maximum number on Tiller2 of 2447-6-10 plant. There was 37.5 % increase in spikelet number for this spike. The extra spikelet was not observed on any tiller of 2447-9-9 plant although it was homozygous.

Also, any supernumerary spikelet was not observed for wild type and heterozygous *TtBH-A1* mutants and heterozygous double mutants. Immature inflorescences were dissected to observe earlier spike development for these plants and Kronos wild type.

Figure 3. 24. Mutant phenotype for *TtBH-A1* gene. Extra spikelet was labeled with red arrow.

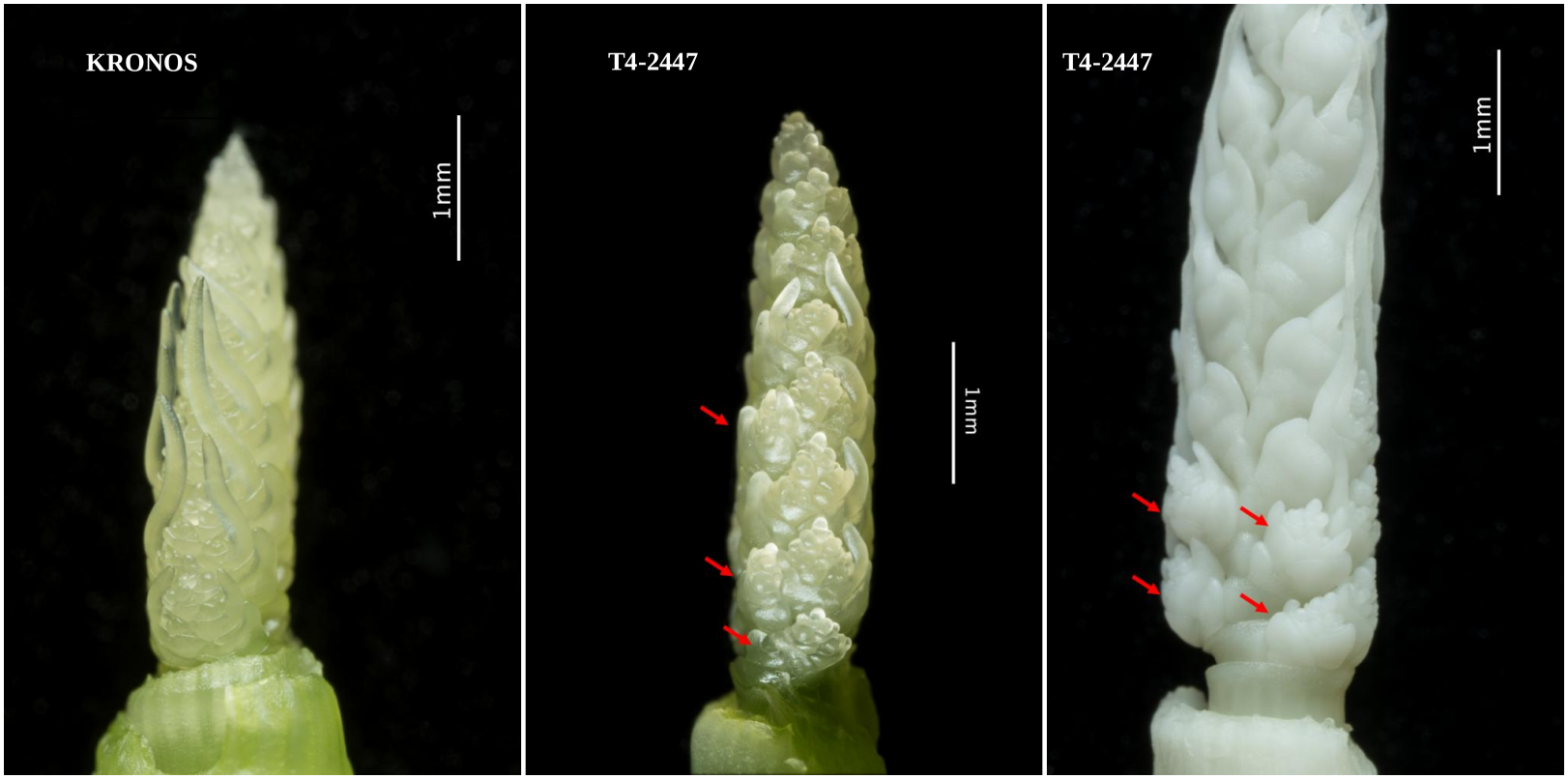


Figure 3. 25. Immature inflorescences for Kronos wild type, *TtBH-A1* mutants (T4-2447). Extra spikelet was labeled with red arrows.

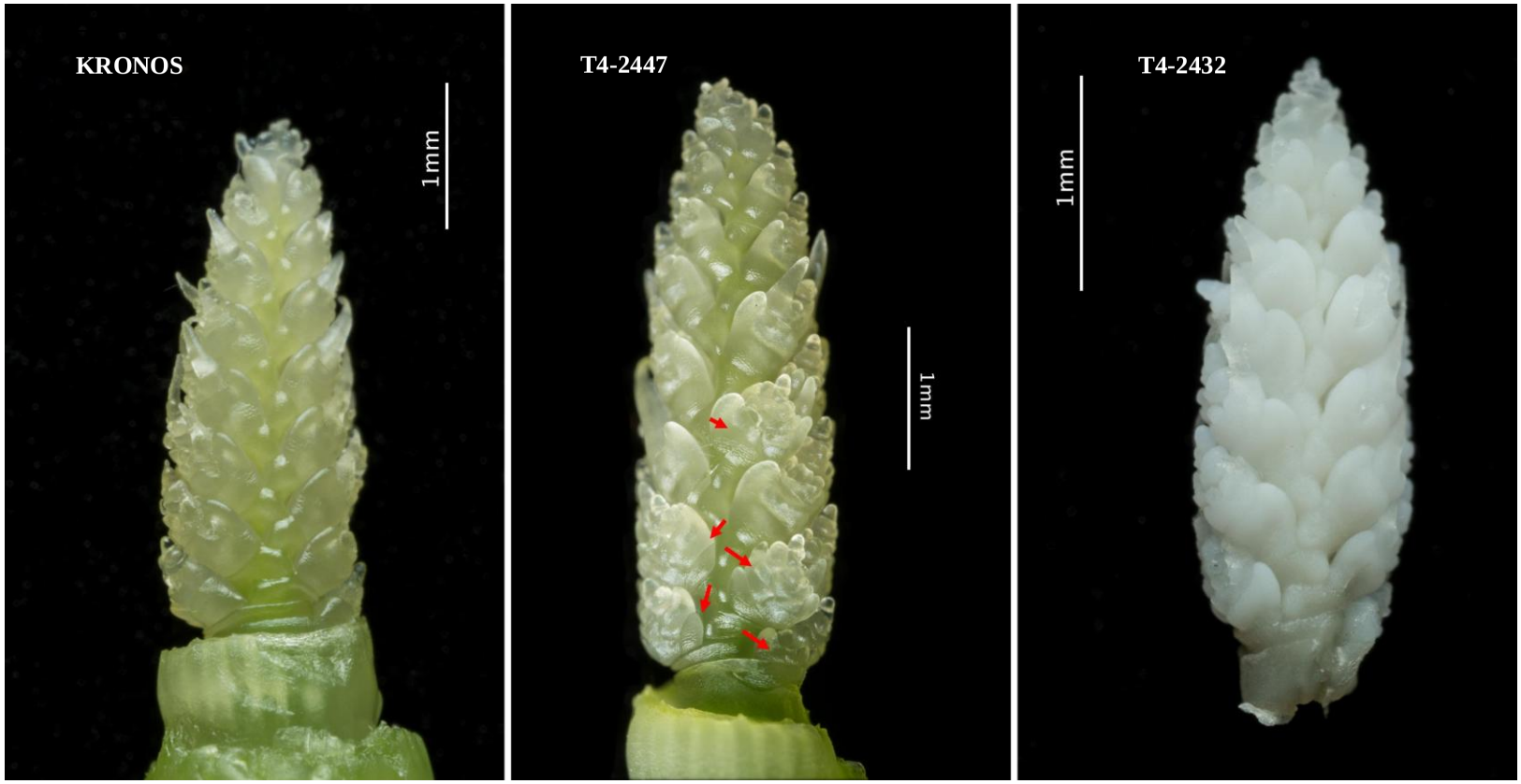


Figure 3. 26. Immature inflorescences for Kronos wild type, *TtBH-A1* mutant (T4-2447) and *TtBH-B1* mutant (T4-2432). Extra spikelet was labeled with red arrows.

Figure 3. 27. Maturing spikes for Kronos and T4-2447 mutants. (Poursarebani *et.al.*, 2015) Extra spikelet was labeled with red arrows.

Table 3. 14. Spikelet numbers for the first three tillers of T4-2447 M4 generation homozygous plants. (Poursarebani *et.al.*, 2015) S No: Spikelet number; SS No: Supernumerary Spikelet number; Position: Supernumerary spikelet position; Total: Total number of all spikelet; ex: Originated from having an extra spikelet.

	Tiller 1			Tiller 2			Tiller 3		
Plant ID	**S No**	**SS No**	**Total**	**S No**	**SS No**	**Total**	**S No**	**SS No**	**Total**
2447-1-2	18	1	19	15	1	16	15	2	17
2447-1-5	15	0	15	15	2	17	14	0	14
2447-1-10	17	0	17	15	1	16	16	1	17
2447-3-2	18	2	20	17	3	20	17	4	21
2447-3-4	16	0	16	16	2	18	15	0	15
2447-3-5	16	1	17	16	3	19	13	0	13
2447-4-2	15	0	15	16	1	17	14	1	15
2447-5-8	ND	ND	ND	17	1	18	16	0	16
2447-5-9	14	0	14	14	0	14	14	1	15
2447-6-4	14	0	14	16	1	17	13	2	15
2447-6-7	17	0	17	15	1	16	16	1	17
2447-6-8	17	0	17	17	1	18	16	1	17
2447-6-10	16	1	17	16	6	22	14	2	16
2447-9-3	14	0	14	16	0	16	17	1	18
2447-9-9	15	0	15	15	0	15	14	0	14
2447-10-2	17	0	17	14	3	17	15	2	17
2447-10-7	16	1	17	15	4	19	16	4	20
2447-12-3	18	1	19	15	2	17	16	2	18
2447-12-5	16	0	16	16	1	17	14	1	15
2447-13-1	15	0	15	15	4	19	13	0	13
2447-7-ex-1	15	0	15	14	1	15	14	1	15
2447-7-ex-2	15	1	16	15	5	20	15	2	17
2447-7-ex-3	15	0	15	15	2	17	15	2	17
2447-7-ex-4	15	0	15	15	3	18	13	0	13
2447-7-ex-5	15	0	15	17	3	20	15	2	17
2447-7-ex-6	17	1	18	16	3	19	15	2	17
2447-7-ex-7	13	0	13	18	2	20	17	4	21
2447-7-ex-8	17	0	17	15	2	17	16	1	17
2447-7-ex-9	16	0	16	ND	ND	ND	14	1	15
2447-7-ex-10	17	0	17	16	0	16	15	2	17

Table 3.14 (cont'd)

2447-7-1	15	1	16	16	1	17	14	0	14
2447-7-2	15	0	15	16	1	17	15	2	17
2447-7-3	16	0	16	15	4	19	17	3	20
2447-7-4	17	1	18	15	1	16	15	1	16
2447-7-5	15	0	15	16	1	17	14	0	14
2447-7-6	17	0	17	16	3	19	17	3	20
2447-7-7	16	0	16	15	2	17	14	0	14
2447-7-9	16	1	17	17	2	19	15	2	17

3.5.Yield Component Analysis for M4 Generation of T4-2447 Mutants

Yield analysis was carried out for T4-2447 M4 generation *TtBH-A1* mutants. Spikelet number, seed number, yield per spike, thousand grain numbers, grain width and length and area were measured for yield. These parameters were the main components of the yield.

Spikelet numbers on Tiller1, Tiller2 and Tiller3 were counted for mutants (**WT**: Wild type for *TtBH-A1*; **Het**: Heterozygous for *TtBH-A1*; **M**: Homozygous for *TtBH-A1* without supernumerary spikelet; **M(SS)**: Homozygous for *TtBH-A1* including supernumerary spikelet). In addition to mutants, Kronos wild type spikelet number was counted to make a comparison. T4-2447 mutants having supernumerary spikelet produced significantly more spikelet from wild type, heterozygous and Kronos genotype. M and M(SS) had significantly more spikelet than Het and WT on Tiller1 ($p<0.001$). Also, Kronos produced significantly more spikelet than WT on Tiller1 ($p<0.006$). M(SS) was significantly the highest spikelet producer on Tiller2 and Tiller3. In addition to this, M(SS) produced significantly more spikelet than Kronos on Tiller2 ($p<0.001$). M and Het significantly had more spikelet than WT genotype on Tiller2 and Tiller3. It was clear that *TtBH-A1* gene had direct effect on spikelet number in durum wheat. (Table 3. 15 and Figure 3. 28)

Supernumerary spikelet number was counted for mutants. Only M plants produced supernumerary spikelet on Tiller1, Tiller2 and Tiller3. Tiller2 had significantly more supernumerary spikelet than Tiller1($p<0.00$) and Tiller 3 ($p<0.046$) (Table 3. 16 and Figure 3. 29).

It was observed that mutant phenotype (M and M(SS)) significantly had more spikelets than WT and Het plants according to spikelet number per spike. They shared the same mutation background except *TtBH-A1* gene. Spikelet number was significantly higher than Kronos ($p<0.026$). (Table 3. 17 and Figure 3. 30)

Table 3. 15. Spikelet number for T4-2447 mutants.

Genotype	**Total Spikelets T1**	**Total Spikelets T2**	**Total Spikelets T3**
WT	13,32 ± 2,36 c	13,80 ± 1,56 d	13,04 ± 1,67 d
Het	14,67 ± 1,74 b	14,57 ± 1,54 c	14,02 ± 1,73 c
M	15,84 ± 1,21 a	15,62 ± 0,92 b	14,95 ± 1,21 b
M (SS)	16,16 ± 1,52 a	17,59 ± 1,69 a	16,34 ± 2,13 a
Kronos	15,60 ± 0,97 a,b	15,60 ± 0,70 b	15,90 ± 1,45 a

WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*; M (SS): Homozygous for *TtBH-A1* including supernumerary spikelet; T1: Tiller1; T2: Tiller2; and T3: Tiller3. Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test.

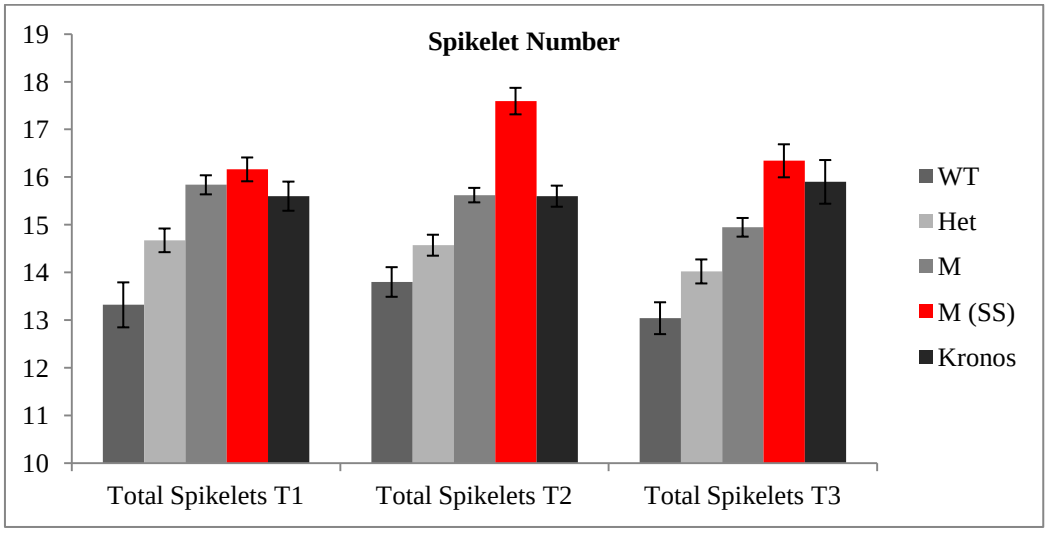


Figure 3. 28. Spikelet number for T4-2447 mutants. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*; M (SS): Homozygous for *TtBH-A1* including supernumerary spikelet; T1: Tiller1; T2: Tiller2; and T3: Tiller3.

Table 3. 16. Supernumerary Spikelet (SS) number for T4-2447 mutants.

Genotype	SS Number T1	SS Number T2	SS Number T3
WT	0,00	0,00	0,00
Het	0,00	0,00	0,00
M	0,32 ± 0,53 c	2,00 ± 1,41 a	1,39 ± 1,18 b
Kronos	0,00	0,00	0,00

WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*; SS: Supernumerary Spikelet; T1: Tiller1; T2: Tiller2; and T3: Tiller3. Means denoted by different letters in a row are significantly different at $P < 0.05$ according to One-Way ANOVA test.

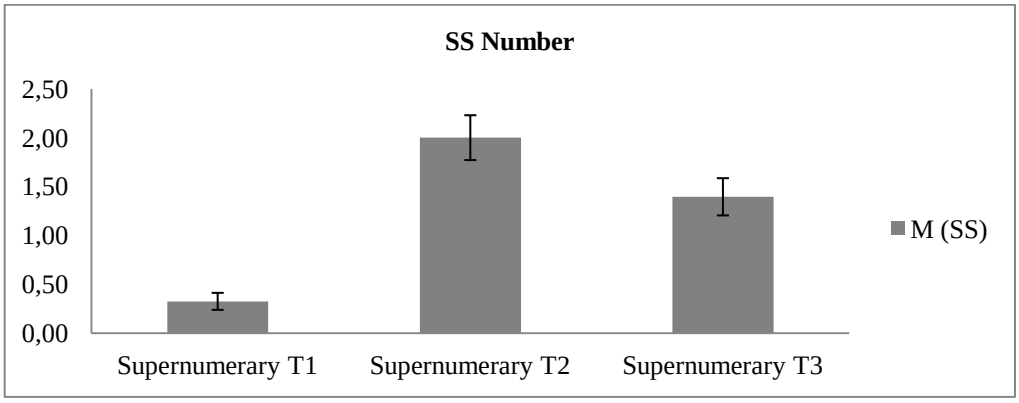


Figure 3. 29. Supernumerary Spikelet (SS) number for T4-2447 mutants. T1: Tiller1; T2: Tiller2; and T3: Tiller3.

Table 3. 17. Spikelet number/spike for T4-2447 mutants and Kronos.

Genotype	Spikelet Number/Spike
WT	13,39 ± 1,48 d
Het	14,43 ± 1,33 c
M	15,47 ± 0,76 b
M (SS)	16,69 ± 1,30 a
Kronos	15,70 ± 0,73 b

WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*; M (SS): Homozygous for *TtBH-A1* including supernumerary spikelet. Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test.

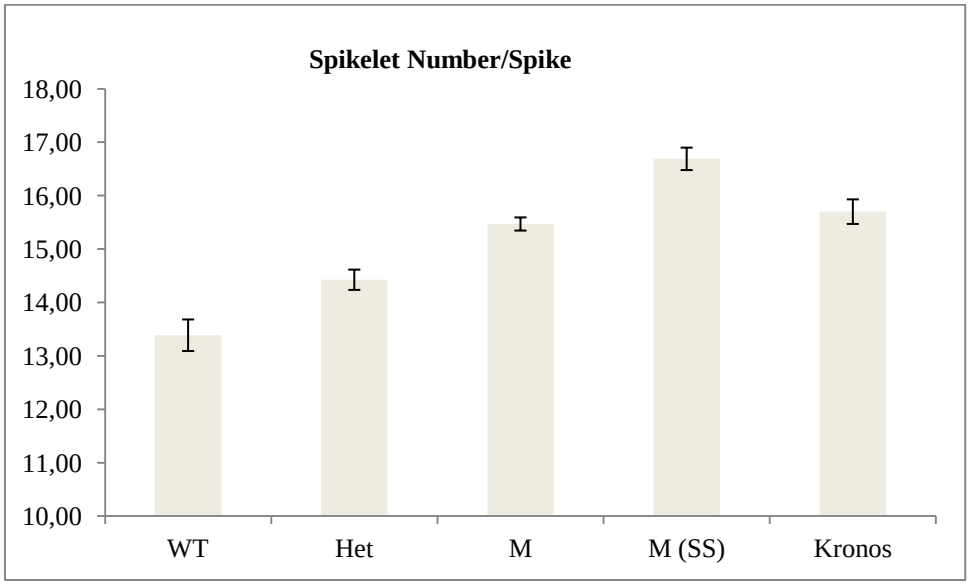


Figure 3. 30. Spikelet number/Spike for T4-2447 mutants and Kronos. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1* M (SS): Homozygous for *TtBH-A1* including supernumerary spikelet.

Seeds/Spike, Yield/Spike and TGW were also another important parameters to get useful information about yield potential. Kronos had significantly higher scores for Seeds/Spike and Yield /Spike than T4-2447 mutants (Table 3. 18 and Figure 3. 31 and Figure 3. 32). However, there was no significantly difference for TGW scores. (Table 3. 18 and Figure 3. 33)

Table 3. 18. Seeds/Spike, Yield/Spike and TGW parameters for T4-2447 mutants and Kronos.

Genotype	Seeds/Spike	Yield/Spike (g)	TGW(g)
WT	25,58 ± 5,69 b	1,57 ± 0,40 b	60,92 ± 5,28
Het	26,90 ± 5,53 b	1,64 ± 0,37 b	60,62 ± 3,59
M	25,69 ± 5,69 b	1,54 ± 0,39 b	59,77 ± 6,41
Kronos	32,74 ± 5,76 a	1,95 ± 0,40 a	59,32 ± 2,22

WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*. Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test.

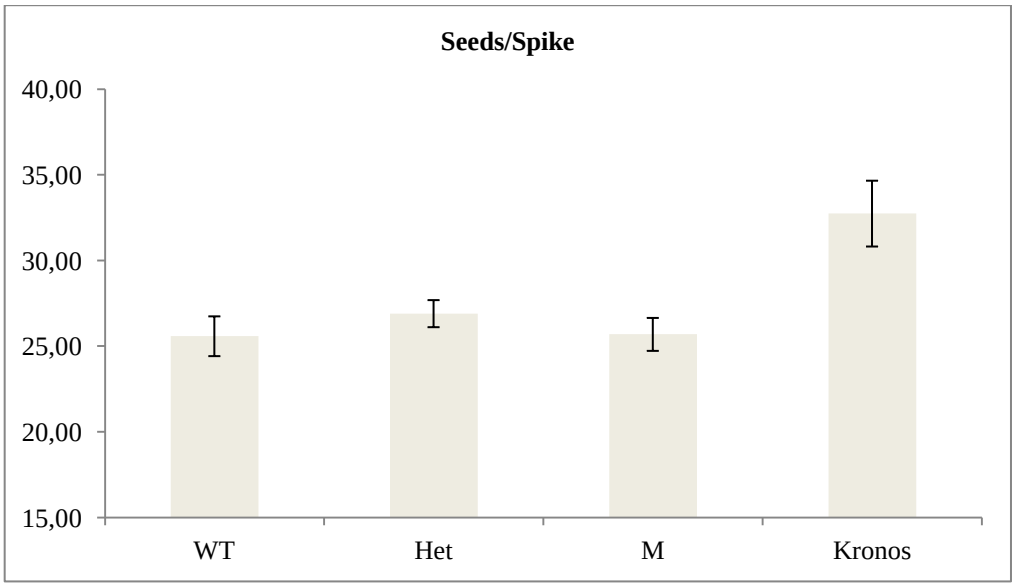


Figure 3. 31. Seeds/Spike for T4-2447 mutants. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

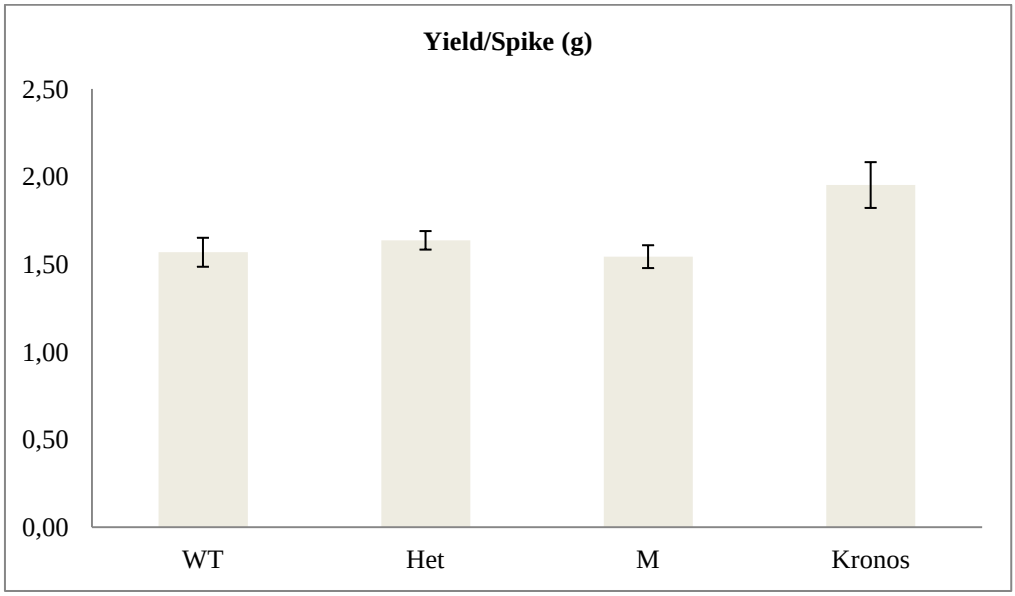


Figure 3. 32. Yield/Spike for T4-2447 mutants. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

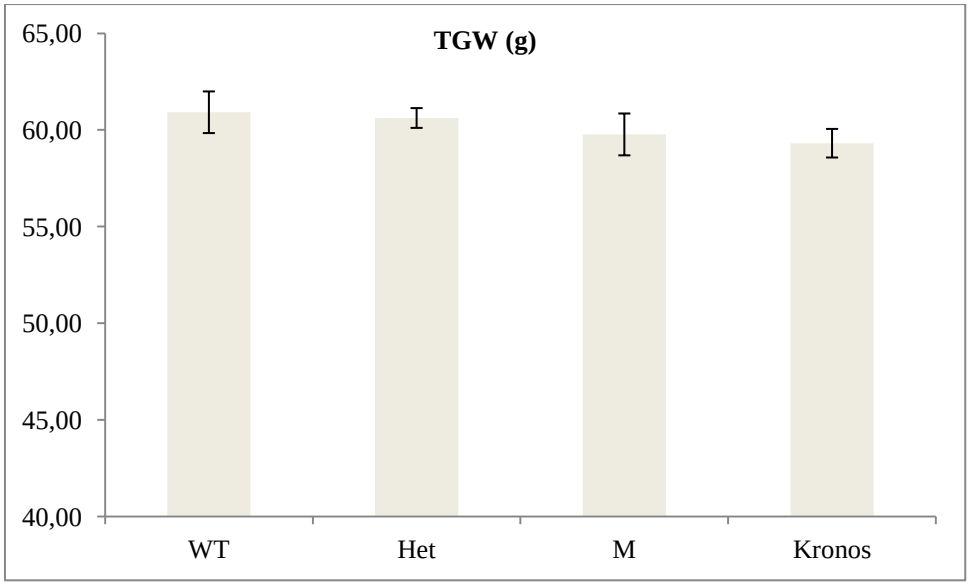


Figure 3. 33. Thousand grain weight (TGW) for T4-2447 mutants. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

Seed width and length and area were also important parameters for yield (Table 3. 19). Kronos seeds were significantly longer than all T4-2447 mutants (Figure 3. 34). However, WT and Het mutants were significantly wider than Kronos (Figure 3. 35). It was observed that area was not significantly difference between seeds (Figure 3. 36).

Table 3. 19. Area, width and length parameters for T4-2447 mutants and Kronos.

Genotype	**Length (mm)**	**Width (mm)**	**Area (mm^2)**
WT	7,12 b ± 0,18	3,86 a ± 0,14	21,38 ± 1,18
Het	7,12 b ± 0,13	3,86 a ± 0,09	21,36 ± 0,81
M	7,11 b ± 0,17	3,85 ab ± 0,12	21,27 ± 0,99
Kronos	7,30 a ± 0,11	3,77 b ± 0,06	21,29 ± 0,43

WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*. Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test.

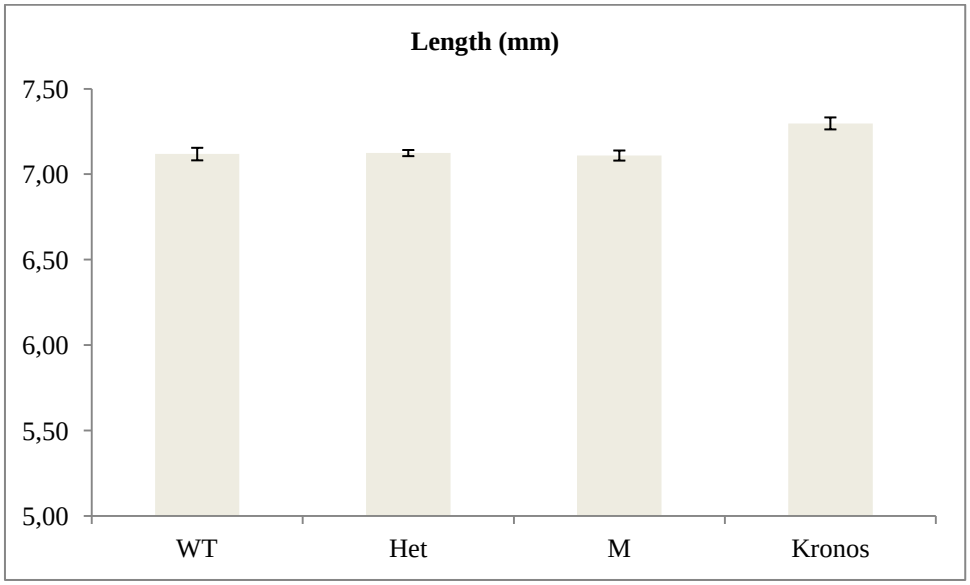


Figure 3. 34. Seed length for T4-2447 mutants and Kronos. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

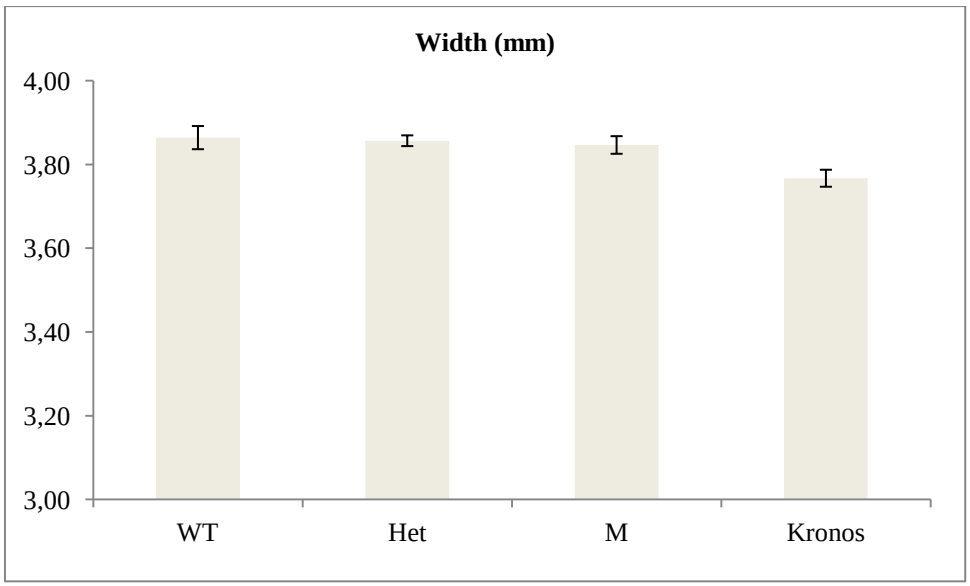


Figure 3. 35. Seed width for T4-2447 mutants and Kronos. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

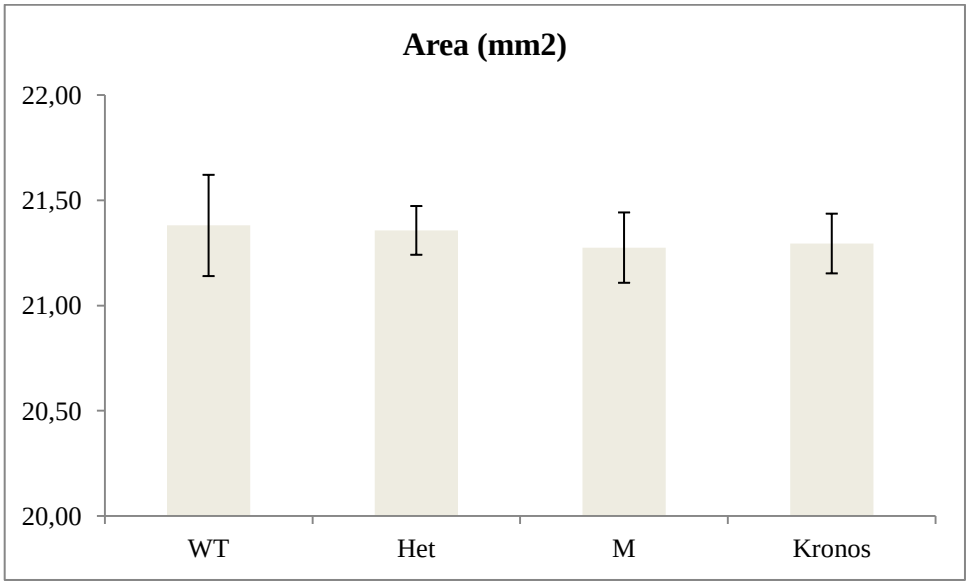


Figure 3. 36. Seed area for T4-2447 mutants and Kronos. WT: Wild type for *TtBH-A1*; Het: Heterozygous for *TtBH-A1*; M: Homozygous for *TtBH-A1*.

As a result, T4-2447 mutant plants having a SNP at the DNA binding site of *AP2/ERF* highly conserved domain produced significantly more spikelets. Kronos had more Seeds/Spike and Yield/Spike than mutants. However, TGW and area parameters were not significantly different in all plants. T4-2447 mutants had some background mutations. These results were informative for further studies. Yield analyses should be repeated after two times backcrossing with Kronos.

CHAPTER 4

CONCLUSION

Firstly, TILLING was performed for *TtBH-A1* and *TtBH-B1* genes in mutagenized Kronos TILLING population in this study. 41 mismatches for *TtBH-A1* gene and 46 mismatches for *TtBH-B1* gene were identified using GENEMAPPER® software after PCR optimization using fluorescently labeled primers.

80 new alleles were confirmed for *TtBH-1* gene. Mutation rate was calculated as one mutation per 26 kb in this study. This rate was one of the highest mutation rates for wheat TILLING populations. Using highly sensitive tools (Genemapper and Mutation Surveyor softwares) and high GC content of gene were possible reasons for this high mutation frequency. 98.75 % of mutation was guanine to adenine transition as expected because of EMS alkylation.

40 new mutations were confirmed for A genome copy. 37.5 % of mutations was homozygous for *TtBH-A1* gene as expected, rest was heterozygous. Missense and silent mutations were found for *TtBH-A1* gene 28 and 12 respectively. 4 of missense mutations were on conserved domain region that was very important for proper function of gene. The strongest mutation according to position specific scoring matrix (PSSM) and SIFT scores was determined in T4-2447 line having a mutation at the 181 bp of gene guanine was converted to adenine leading to glycine to serine change. This mutation was at highly conserved and DNA binding site of the gene.

40 mutations were confirmed for B genome copy. While 35 % of mutation was homozygous as expected, 65 % was heterozygous. 23 of mutations were missense and one of them was truncation at the 14 position of amino acid sequence, glutamine was converted to premature stop codon. T4-2432 had this mutation as a heterozygous. Moreover, 6 more mutations were confirmed at the conserved domain of *TtBH-B1* gene.

Mutant lines were validated using KASP markers and sequencing after DNA isolation. KASP markers were designed and tested for mutant plants. However, designed KASP markers did not work for most of them.

Mutant plants were screened for supernumerary phenotype after sequencing. Lateral spikelet development leading to supernumerary spikelet was observed on ear of the T4-2447 having the strongest mutation for *TtBH-A1* gene. There was only one homozygous plant (Plant ID: T4-2447-7) for this line. 7 of 12 ears of T4-2447-7 plant had the at least one additional spikelet on the side of the normal spikelet. The maximum additional spikelet number was 5 in a spike. Any supernumerary spikelet phenotype was not observed in rest of the other *TtBH-A1* mutant plants. Moreover, any spike and spikelet abnormalities were not noted for T4-2432 having a premature stop codon for *TtBH-B1* gene and other B genome mutants.

T4-2447 mutant having supernumerary phenotype was crossed with B genome mutants to make double mutants. Additionally, T4-2447 mutants were backcrossed with parental Kronos to eliminate other mutations to see real effect of *TtBH-A1* mutation. Moreover, T4-2447 mutant was crossed with domestic cultivars (Kızıltan-91 and Fuatbey-2000) to observe yield parameters after two times backcrossing. Therefore, tetraploid “Miracle-Wheat” having highly branched ears was crossed with Turkish domestic cultivars. F1 seeds for these crosses were harvested.

Lateral spikelet development was observed only one plant of M3 generation. Therefore, M4 seeds obtained from heterozygous M3 plants for T4-2447 line were planted to see inflorescence development and yield performance. 1:2:1 genetic

segregation (χ^2=2.37, *P*=0.306) and 3:1 phenotype segregation (χ^2=0.317, *P*=0.573) were observed as it was expected. While 20 plants had the mutation genotype, 19 plants had the supernumerary spikelet phenotype. Lateral spikelet development began during glume primordial initiation. First three tillers of T4-2447 mutants and Kronos were counted for spikelet number, supernumerary spikelet number and total spikelet number. The maximum supernumerary spikelet number was 6 on a spike and total spikelet number was 22. 37.5 % increase in spikelet number was observed in this spike. Additionally, inflorescence development of "Miracle-Wheat" was investigated under light microscope at different stages. Ramified (highly branched) spikelet, supernumerary spikelet and normal spikelet were observed in "Miracle-Wheat" inflorescences. However, terminal spikelet development was very weak both of ramified spikelet and main spike.

Spikelet number, grain number, yield per spike, thousand grain weight, seed length and width and area was evaluated for supernumerary spikes and normal spikes. T4-2447 mutants having supernumerary spikelet produced significantly more spikelet than wild type Kronos. Second tillers produced significantly more additional spikelet than first and third tiller in supernumerary spikes. Kronos had the higher scores for Seeds/Spike and Yield/Spike parameters than *TtBH-A1* mutants. However, thousand grain weight value was not different in compared plants. While T4-2447 mutants had longer seeds, Kronos seeds were wider than mutants. However, seed area was not different.

Finally, it was clear that *TtBH-A1* gene orthologue of *FZP* gene in rice had a role in spike architecture causing lateral spikelet development in durum wheat. T4-2447 mutants having the conserved domain mutation (G61S) produced significantly more spikelet as an additional spikelet on the side of normal spikelet. However, ramified spikelet phenotype or completely branching was not observed in mutants. T4-2447 mutants and "Miracle-Wheat" were hybridized with Turkish domestic cultivars Kızıltan-91 and Fuatbey-2000.

In future, backcrossed mutants and hybridized domestic cultivars will be evaluated for yield parameters in greenhouse and field after finished parental backcrossing. Marker will be developed for *TtBH-A1* mutation. Additionally, crossing of domestic cultivars and "Miracle-Wheat" will be used to investigate ramified spikelet phenotype.

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APPENDIX A

ABBREVIATIONS OF AMINO ACIDS

Table A. 1.List of amino acids, single letter codes (SLC) and DNA codons.

Amino Acid	SLC	DNA codons
Methionine	M	ATG
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Threonine	T	ACT, ACC, ACA, ACG
Phenylalanine	F	TTT, TTC
Isoleucine	I	ATT, ATC, ATA
Glycine	G	GGT, GGC, GGA, GGG
Alanine	A	GCT, GCC, GCA, GCG
Valine	V	GTT, GTC, GTA, GTG
Proline	P	CCT, CCC, CCA, CCG
Cysteine	C	TGT, TGC
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Stop codons	Stop	TAA, TAG, TGA

CURRICULUM VITAE

Name, Family name	:	Abdulhamit BATTAL
Birth	:	September 14, 1982, Bayburt, Turkey
Citizenship	:	Republic of Turkey
Address	:	Middle East Technical University Department of Biological Sciences, 06800 Çankaya Ankara Turkey
Phone	:	+90 312 210 5156
Mobile	:	+90 506 574 3252
e-mail 1	:	abattal@metu.edu.tr
e-mail 2	:	hamitbattal@gmail.com

EDUCATION:

2016	PhD	METU, Biotechnology Dept.
2010	MSc	METU, Biotechnology Dept.
2005	BS	Dokuz Eylül University, Biology Education

EXPERIENCES

2007 – 2016	Research Assistant, Scientific Human Resources Development Program , METU - YYU
2007 – 2016	Teaching Assistant, Department of Biological Sciences, METU, Ankara
2013 – 2014	Visitor Scientist, Crop Genetics Department, John Innes Centre, Norwich, United Kingdom

2005 – 2006 Microbiology Research Laboratory, Yüzüncü Yıl University, Van

PUBLICATIONS

1. Poursarebani, N., Seidensticker, T., Koppolu, R., Trautewig, C., Gawroński, P., Bini, F., Wolde, G. M., Youssef H.M., **Battal, A**., …. &Schnurbusch, T. (2015). The genetic basis of composite spike form in barley and 'Miracle-Wheat'. *Genetics*, *201*(1), 155-165.
2. Baloglu, M. C., **Battal, A**., Aydin, G., Eroglu, A., Oz, M. T., Kavas, M., ... & Yucel, M. (2013). Vector Construction Strategies for Transformation of Wheat Plant. *J Anim Plant Sci*, *23*(3), 906-912.
3. **Battal A.** (2010) Optimization of Mature Embryo Based Regeneration and Genetic Transformation of Turkish Wheat Cultivars. M.Sc. Thesis in Biotechnology.
4. Bildirici, İ., Şener, A., Atalan, E., **Battal, A.**, & Genç, H. (2009). Synthesis and antibacterial activity of 4-benzoyl-1-(4-carboxy-phenyl)-5-phenyl-1H-pyrazole-3-carboxylic acid and derivatives. *Medicinal chemistry research*, *18*(5), 327-340.
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Presentations in an International Conference:

1. **Battal A.**, Simmonds J. & Uauy C. "Mutation on FZP (FRIZZY PANICLE) leads to increased Spikelet number in Durum Wheat" Annual Meeting in Prague 2015, 2015. **(Poster)**
2. **Battal A.**, **Baloglu MC**, Kavas M, Yücel M, Öktem HA. (2012) Particle bombardment transformation of some Turkish wheat cultivars with TaNAC69-1 and TtNAMB2 genes, 15th European Congress on

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7. Baloglu M.C., Kalemtaş G., Eroğlu A., **Battal A.**, Öktem H.A., Yücel M. "Cloning of Wheat (*Triticum aestivum*) *TaNAC69-1* Gene and Transformation of Wheat Inflorescence by Particle Bombardment" International Symposium on Biotechnology: Development and Trends, Ankara, Turkey 2009. (**Oral**).

Presentations in a National Conference:

1. **Battal A.**, Simmonds J., Uauy C., Öktem H.A. & Yucel M. "Bazı Makarnalık Buğday Çeşitlerinin Başakçık Sayısını Artıran Mutant *TtBH-A1*

Geni ile Islah Edilmesi" (In Turkish), 18th National Biotechnology Congress, Konya, Turkey, 2015. **(Oral)**

2. A. Eroğlu, M.C. Baloglu , G.Kalemtaş, C.Kayıhan, F.Ayşin, **Battal A.**, H.A.Öktem, M.Yücel. "Cloning of NAC Type Transcription Factors to Monocot Expression Vectors" (In Turkish), 20th National Biology Congress, Denizli, Turkey, 2010. (**Oral**)
3. **Battal A.**, Yücel M., Öktem H.A. "Optimization of Mature Embryo Based Regeneration and Genetic Transformation of Some Turkish Cultivars" (In Turkish), 16th Biotechnology Congress, Antalya, Turkey, 2009. (**Oral**)
4. M.C. Baloglu, G.Kalemtaş, A.Eroğlu, **Battal A.**, F.Ayşin, C.Kayıhan, H.A.Öktem, M.Yücel "Isolation and Characterization of NAC69-1 and *NAM-B2* Genes From Wheat" (In Turkish), 16th Biotechnology Congress, Antalya, Turkey, 2009. (**Oral**)
5. M.C. Baloglu, G.Kalemtaş, A.Eroğlu, **Battal A.**, H.A.Öktem, M.Yücel. "Cloning of NAC69-1 and NAM-B2 Genes to Dicot Binary Vectors and Transform to *Agrobacterium"* (In Turkish) 16th Biotechnology Congress, Antalya, Turkey, 2009. (**Oral**)
6. F. Ayşin, C. Kayıhan, M.C. Baloglu, G. Kalemtaş, A. Eroğlu, **Battal A.**, H. A. Öktem, M. Yücel. "*TaNAC69-1* RNAi Vector Construction for Wheat" (In Turkish), 16th Biotechnology Congress, Antalya, Turkey, 2009. (**Poster**)
7. S. Okay, M.C. Baloglu, A. Eroğlu, **Battal A.**, G. Özcengiz, H.A. Öktem, M. Yücel. "Transformation of Chitinase A Gene Isolated *Serratia marcescens* to Tobacco" (In Turkish), 16th Biotechnology Congress, Antalya, Turkey, 2009. (**Poster**)

Scholarships and Awards:

1. **Research Assistant**, (2007 – 2016) Scientific Human Resources Development Program, METU – YYU.
2. **Visiting Ph.D. Student Scholarship** (12 Months, 2013 -2014) provided by TUBİTAK 2214/A Program, Ankara, Turkey. (Present)
3. Ph.D. Program **Honor Student** of the Department of Biotechnology in the Academic Year 2010-2011, METU, Graduate School of Natural and Applied Sciences, Ankara, Turkey.

Membership:

2007 – Present	Turkish Biotechnology Association
2015 – 2016	The Society of Experimental Biology (SEB)
2009 – 2010	Federation of European Biochemical Societies (FEBS)

Projects:

1. ODTÜ BAP-08-11-DPT2011K121010 "TILLING For *TtBH-1* Gene And Its Hybridization To Turkish Durum Wheat Cultivars Kiziltan-91 And Fuatbey-2000" Ph.D. Thesis, 2011- 2016
2. TUBITAK 108O786 "Development of Abiotic Stress Resistant Transgenic Wheat Cultivars Using NAC Type Transcription Factors and Investigation of Gene Expression Profiles of Transgenics Under Abiotic Stresses Using Microarrays", 2009-2011
3. ODTÜ BAP-08-11-DPT2002K120510 "Optimization of Mature Embryo Based Regeneration and Genetic Transformation of Turkish Wheat Cultivars" M.Sc. Thesis Project, 2007-2010

Research Interests:

Functional Genomics, Transgenics, TILLING, Yield Increase in Wheat