THE IMPACT OF *TEOSINTE BRANCHED1* AND *REDUCED HEIGHT* MUTATIONS IN DURUM WHEAT

by

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DEDICATION

To my mom, Sue, for her ready ear and unwavering support even on the bad days.

To my dad, Scot, for his involvement in agriculture that caught my fascination at an early age and his continued interest in the topics that I get excited about.

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ABSTRACT

Increasing the yield of wheat requires identifying new genetic combinations of alleles by crossing or by creating variation in yield limiting genes. Wheat yield is impacted by tiller number and seeds per tiller, both of which are impacted by the *Reduced height* (*Rht*) and *Teosinte branched1* (*TB1*) genes. In this study, durum plants varying for *Rht* and *TB1* alleles created by EMS mutagenesis were studied to determine the impact of each allele upon agronomic and seed traits. Both projects aimed to increase durum yield, one through an increase in tiller number; the other through the development of a plant with height between current full-height and semi-dwarf varieties that can allocate more resources to seed production. The impact of *TB1* null alleles were studied alone and together in greenhouse and field trials, along with an RNA sequencing study to determine the impact of *TB1* mutation upon global gene expression in developing meristems. *TB1* single and double null mutants produced more biomass and tillers per plant, and expression of genes in meristems varied. A screen of wheat varieties grown in Montana identified that several spring and durum wheat varieties contain different *TB1* alleles, but none contained TB1 null alleles. *Rht* experiments included field trials, coleoptile length and gibberellic acid responsiveness assays, and an *in-vitro* test to determine the impact of each *Rht* mutation upon binding to Gibberellin Interacting Domain 1 (GID1) that directly influences plant height. It was found that the previously described *Rht-B1b-E529K* allele reduced both plant height and coleoptile length while two newly characterized *Rht* mutations had lesser impacts with trends towards intermediate-height plants. The results of this research demonstrate that *Rht* alleles that alter RHT binding to GID1 and *TB1* null alleles may prove useful in increasing durum tillering and optimizing plant height for different growing conditions.

INTRODUCTION

Durum Wheat

Durum wheat, *Triticum turgidum* ssp. *durum*, is the tetraploid wheat species used to produce semolina for pasta worldwide, and several specialty bread and dessert products in certain cultures. In the U.S., durum wheat is grown mainly in four states: North Dakota, Montana, California, and Arizona (NASS 2020b).

U.S. durum wheat acres consisted of between 0.46 and 0.98 million hectares planted yearly from 2011 to 2020 (NASS 2020c). Accounting for only 2-4% of the total wheat hectares planted in those years (NASS 2020a), durum wheat acres were worth just a fraction of the overall U.S. wheat crop value. However, to those localities where durum wheat is grown, it is economically important. For example, Montana harvested 550,000 acres of durum wheat in 2019, worth about 104 million dollars (NASS 2019). Breeding program objectives include both regionally-important agronomic traits such as race-specific disease resistance and universally-desired traits such as grain yield and end-use quality traits like high seed protein content, semolina yield, gluten strength, pasta firmness, and semolina and pasta color (Clark et al. 1998, Royo et al. 2009).

When choosing a variety to grow, farmers consider several factors including yield stability, disease and insect resistance, and seed quality. Not surprisingly, yield is the number one factor since profitability is most affected by yield. Thus, it is important for breeding programs to create and release locally adapted, high yielding durum cultivars.

Yield Components

Total grain yield is impacted by genetic and environmental components. Environmental factors including nutrient and water availability, temperature throughout the growing season, competition from weeds, disease and insect pressure impact yield, but genetic factors are what determine the yield potential of a variety under ideal field conditions (Duggan et al. 2000, Destro et al. 2001).

One of the difficulties in selecting for increasing yield is that many yield-impacting traits such as productive tillers, spikelets per head, and seed size are negatively correlated (Fischer et al. 1977, Cantrell and Haro-Arias 1986). As a result, studies designed to increase yield generally focus on one or more of these factors with varying levels of success. Some methods, with a few mentioned below, have a universally beneficial effect. Other studies have shown that what impacts yield under certain environmental conditions has little effect in others (Garcia del Moral et al. 2003, Elhani et al. 2007, Moayedi et al. 2009, Destro et al. 2001).

Mutations in Yield-Affecting Genes

Selecting for mutations in genes that both reduce height and increase productive tillers is known to increase yield, as seen with the commonly used *Rht-B1b* semi-dwarfing gene which increases yield by ~8.7% in durum wheat (Mathews et al. 2006). With a reduction in height, the plant is able to redirect the energy that would have been expended on stem growth to seed production instead (Youseffian et al. 1992, Flintham et al. 1997). Although *Rht-B1b* is well studied and is effective at reducing height,

increasing productive tillers, and increasing yield in hexaploid wheat (Flintham et al. 1997, Hoogendoorn et al. 1990, Li et al. 2006, Pearce et al. 2011, Peng et al. 1999, Fick and Qualset 1979, Jobson et al. 2018, Jobson et al. 2019), there still exists opportunities for other variation in *Rht* that may have more practical applications in tetraploid wheat. The effect of *Rht-B1b* in durum is greater than in hexaploid wheat (Mathews et al. 2006), so a less dramatic height reduction allele would be useful for breeding programs. By exploring the effect of *Rht-A* mutations in durum wheat both with and without the *Rht-B1b* allele, we hope to identify an allele that would confer height intermediate between standard and semi-dwarf plant height.

Along with *Rht*-related yield increases from reduced height and increased productive tillers, other genes could also provide a yield increase through similar mechanisms. Genes that are known to affect tillering, such as *Teosinte Branched 1 (TB1)*, could be manipulated to increase the number of tillers a plant produces (Dixon et al. 2018). An increase in total tiller number, when associated with an increase in productive tiller number, can increase plant yield (Destro et al. 2001, Elhani et al. 2007).

The *Rht* and *TB1* genes are closely linked and both affect tillering, so experiments that analyze the effect of one must consider the other as well. This research consisted of two parts: a study focused on *Rht* mutations, in which the plant material was fixed for the wild type *TB1* allele that aimed to identify alleles which produced intermediate height durum plants, and a study focused on *TB1* mutations that examined their effects both in the presence and the absence of the *Rht-B1b* height-reducing mutation. Both studies include of a range of physical plant measurements and grain trait measurements, and

while one concentrates more on height and the other on tiller number, the end goal of both is to provide insight into mechanisms by which breeding programs may increase durum yield.

CHAPTER ONE

EVALUATING THE IMPACT OF RHT MUTATIONS IN DURUM WHEAT

Introduction

Incorporation of semi-dwarfing alleles of the Reduced Height (*Rht*) gene into hexaploid wheat (*Triticum aestivum*) varieties led to increased yield during the middle part of the 20th century (Hedden 2003, Pearce et al. 2011). Mutant alleles *Rht-B1b* and *Rht-D1b* have nearly identical impacts on hexaploid wheat height and grain yield. Each confers a height reduction of ~20% and yield increase of ~10% (Hoogendoorn et al. 1990, Flintham et al. 1997). Most modern hexaploid wheat varieties are semi-dwarf and contain either *Rht-B1b* or *Rht-D1b* (Worland et. al. 1998, Knopf et al. 2008). The *Rht* semi-dwarfing alleles increase seeds per plant and productive tiller number, which result in increased yield (Flintham et al. 1997, Hoogendoorn et al. 1990, Li et al. 2006, Pearce et al. 2011, Peng et al. 1999). *Rht-B1b* and *Rht-D1b* also confer a decrease in seed size and protein, along with reducing coleoptile and stem internode length (Fick and Qualset 1979, Schillinger et al. 1998, Jobson et al. in press 2020, Amram et al. 2015, Liatukas and Ruzgas 2011).

In the semi-dominant, altered function *Rht-B1b* and *Rht-D1b* alleles, a premature stop codon in the N-terminal DELLA domain prevents GID1 (GIBBERELLIN INSENSITIVE DWARF1, a receptor protein) from binding (Peng et al. 1999, Pearce et al. 2011). It is proposed that *Rht* translation reinitiates after the stop codon, which

produces N-terminally truncated DELLA proteins (Mo et al. 2018). These shorter DELLA proteins cannot detect GID1-mediated gibberellic acid (GA) signals, and plants are rendered partially GA insensitive (Gale & Marshall 1973, Peng et al. 1999, Youssefian et al. 1992). This insensitivity confers the semi-dwarf phenotype seen in *Rht-B1b* and *Rht-D1b* hexaploid wheat plants.

Genes belonging to the DELLA domain play key roles in GA signaling pathways and are present in many plant species. Characterized orthologs of wheats' *Rht-1* genes include *GAI* and *RGA* (*Arabadopsis*), *Slender1* (*SLN1* in barley; *SLR1* in rice), and *Dwarf 8* and *Dwarf 9* (maize) (Wilhem et al. 2013, Ikeda et al. 2001). Each of these acts in a similar manner to *Rht-1,* with mutant alleles causing reduced GA responsiveness and plant growth (Peng et al. 1997, Chandler et al. 2008, Ikeda et al. 2001, Winkler and Freeling 1994, Chandler et al. 2002). Other *Poeacae* orthologs include Bradi1g11090.1 (*B. distachyon*), Si039400m (foxtail millet), Sb01g010660 (sorghum), GenBank accession #JN793959 (*E. tef*), *Dwarf8* and *Dwarf9* (maize), and GenBank accession #DQ062091.1 (sugarcane) (Wilhelm et al. 2013). Of the listed orthologs, 80-95% of the protein residues are shared with *Rht-1* in Chinese Spring wheat (Wilhelm et al. 2013).

Much research has focused on the effects of *Rht-B1b* and *Rht-D1b* in hexaploid wheat, but less is known about the impact of *Rht* alleles in durum wheat. Decreasing plant height has been shown to increase yield in durum wheat as well, with the commonly used *Rht-B1b* semi-dwarfing gene increasing yield by ~8.7% in durum wheat (Mathews et al. 2006). However, this moderate yield increase comes at the cost of a severe decrease in height of over 45% (Mathews et. al. 2006). Plants that are too short present

harvesting challenges. The combine header must be lowered enough to capture the plant heads, and if the moving metal parts strike a rock there is both fire danger and possible equipment damage. Additionally, short coleoptiles are associated with poor emergence when seeds are planted deeper to reach the soil moisture, as is common in dry climates (Schillinger et al. 1998). In Montana, only one of the commonly grown durum varieties carries the *Rht-B1b* semi-dwarfing allele. This provides an opportunity for a breeding program to create varieties with a more moderate height decrease but increased yield potential over the currently grown full-height varieties.

The impacts of other mutant *Rht* alleles including *Rht-B1c, Rht-B1d, Rht-B1e, Rht-D1c, Rht-D1d, Rht-8, Rht-3* (the Tom Thumb dwarfing gene), and *Rht-10* have also been thoroughly examined in hexaploid wheat, but no *Rht-A* mutations have been found to have a height-reducing effect on plants (Flintham et al. 1997, Gale and Youssefian 1985, Pearce et al. 2011). However, due to durum's tetraploid genome, phenotypic effects in hexaploid wheat may be different in tetraploid durum wheat. *Rht* mutant alleles are dosage-dependent, so in tetraploid wheat the effect of an *Rht* mutation in one genome out of two could be stronger than the effect of a single-genome mutation and two wild type genomes. In hexaploid wheat, *Rht-A* is expressed in the stem at similar levels as *Rht-B* (Pearce et al. 2011); therefore, we proposed that *Rht-A* mutations in durum wheat may also have a height-suppressing effect. Less-severe mutations in either *Rht-A1* or *Rht-B1* could create a plant phenotype intermediate between the current *Rht-B1b* and the wild type *Rht-B1a*.

To test this hypothesis, an EMS-mutagenized population of durum wheat was screened for mutations in *Rht-A*. Lines containing missense mutations with low PROVEAN scores and reduced plant height were identified as sources for variation. These lines were crossed with both a standard-height and a semi-dwarf parent to create segregating populations that were then grown in field trials to evaluate for differences in height. Coleoptile length differences were also evaluated. Additionally, *in-vitro* testing of the alleles was conducted with a yeast-2-hybrid assay.

Along with the novel *Rht-A* mutations tested in this project, the previously identified and described *Rht-B1b-E529K* mutation was included in each of the described experiments to confirm its effects in Montana-adapted varieties. This missense mutation confers an intermediate-height plant by partially suppressing the semi-dwarf phenotype typically associated with the *Rht-B1b* allele (Mo et al. 2018). *Rht-B1b-E529K* was found to increase plant height by 21% and is associated with increases in coleoptile length, seedling shoot length, and stem internode length, all relative to *Rht-B1b* (Mo et al. 2018).

Materials and Methods

Identification and Selection of Mutant Lines

To analyze variation caused by *Rht-A1* mutations in the presence and absence of *Rht-B1b,* an EMS (ethyl methanesulfonate) population was created in the standard height durum variety "Divide" (PI 642021). It was created by mutagenizing 10,000 seeds using the method described by Slade et al. (2005) and modified by Feiz et al. (2009). The population was then advanced through single seed descent through the M_3 generation. One thousand, nine hundred M_4 head rows were grown in the field and plants were bulk

harvested for $M_{3:5}$ seed. The head rows were measured for plant height, and the 192 shortest rows were screened for mutations in the entire coding sequence of the *Rht-A* gene. Seven missense and four silent mutations were identified, and the two lines containing missense mutations with the lowest PROVEAN scores were chosen for this project: *Rht-A1-S50F* and *Rht-A1-L358F* (Table 1). The "Kronos" TILLING mutant (UC Davis, Tsai et al. 2011, Krasileva et al. 2017) allele *Rht-B1b-E529K* was also included to analyze and compare its effect in Montana adapted lines to the observations demonstrated in Mo et al. (2018).

Table 1: Summary of *Rht-1* mutations including location and predicted effect on protein function.

Allele	DNA change (base pairs from start codon)	Original Codon	New Codon	Provean score[a]
Rht-A1-S50F	149	TCC	TTC	-2.12
Rht-A1-L358F	1072	CTC	TTC	-3.82
Rht-B1b-E529K	1585	GAG	AAG	-3.49[b]

[a]Provean (Protein Variation Effect Analyzer) score used to predict effect: A score of -2.5 for single amino acid substitutions is predicted to be deleterious 80% of the time, with higher scores having a deleterious effect less often and lower scores more often.
[b]Rht-B1b-E529K contains both a stop mutation at Q420* and a missense mutation downstream at the position shown above. The Provean score for this situation cannot be accurately calculated; the score shown is the missense mutation by itself.

Creation of BC_1F_3 Lines

Recurrent, Montana-adapted parents for backcrossing include "MTD16005" (full height durum, fixed for *Rht-B1a*) and "MT112219" (semi-dwarf durum, fixed for *Rht-B1b*). F_1 crosses were made between each of the three mutant lines and both parents, for a total of 6 F_1 crosses. The first backcross was made, then plants allowed to self for BC_1F_2 seed. DNA was extracted from the BC_1F_2 plants, then PCR was performed using

the nested approach described by Li et al. (2013) in which genome specific primers are used for initial segment amplification, then primers are used to amplify the region containing the *Rht-A1-L50F* and *Rht-A1-L358F* mutations. The primers from Mo et al. (2018) were used to detect the *Rht-B1b-E529K* mutation. PCR products were Sanger sequenced (GENEWIZ, Inc., Cambridge, MA, USA), then the sequence analyzed against reference accessions JF930277.1 (*Rht-A1*) and JF930278.1 (*Rht-B1*) (www.ncbi.nlm.nih.gov) using the DNASTAR SeqMan Pro software (DNASTAR Version 15.0.1.1, Madison, WI, USA). Lines were genotyped for *Rht-A* mutations, *Rht-B1b-E529K*, and *Rht-B1b*, and plants that were heterozygous for *Rht-A* mutations and *Rht-B1b-E529K* but fixed for *Rht-B1a* and *Rht-B1b* were kept. This allowed for selection only for the mutations of interest in future generations.

BC_1F_2 seed was grown in the 2019 field season as spaced plants, then genotyped as described above and only *Rht* homozygous plants were kept for measurement and harvest. Each single plant provided $BC_1F_{2:3}$ seed for one row in the 2020 field season.

Greenhouse Growing Conditions

All crossing occurred in the greenhouse, set at 22.2° C in the daytime and 18.3° C at night. Seeds were planted in Sunshine mix (Sungro Horticulture, Agawam, MA, USA) potting soil, 4 seeds per 20.3 cm pot. Plants were watered daily with a 100 ppm solution of 20-20-20 fertilizer using a Dosatron (Dosatron International, Inc., Clearwater, FL, USA) once plants reached the three-leaf stage. After flowering, the temperature was increased to 25° C day and 21° C night until harvest. A micronized sulfur solution (Bonide Products, Inc., Oriskany, NY, USA) at a rate of 4.38 g/l was applied to plants at

the 2-3 leaf stage (Z 12-Z13 on the Zadoks growth scale for wheat) for powdery mildew and mite prevention, and Marathon (Imidacloprid 1%, OHP, Inc., Bluffton, SC, USA) at a rate of 2.7 g per pot was applied at the same growth stage for aphid control.

Field Growing Conditions and Layout

One hundred BC_1F_2 plants from each of the six crosses were grown in 2019 under irrigated conditions at the Arthur H. Post Agronomy Farm near Bozeman, MT (latitude 45.67N, longitude 111.15W, elevation 1,455 m, soil type is Amsterdam silt loam). After testing soil, 185 kg per ha urea (46-0-0) was added. Seeds were hand planted to a depth of 2.5 cm on May 10th, 2019. At harvest maturity, single plants were cut at ground level, weighed, and grain collected using a single-plant thresher the second week of September. From May 1st to August 31st, the research station received 18.6 cm of precipitation. The highest recorded air temperature was on July 23 at 32.3 °C, while the lowest recorded air temperature was -0.56 °C on May 19 (NOAA Bozeman). Five cm irrigation water was applied using hand line sprinklers on July 1. A 1.1 l/ha application of Huskie (Pyrasulfotole 3.3%, Bromoxynil Octanoate 13.4%, Bromoxynil Heptanoate 12.9%; Bayer CropScience, Research Triangle PK, NC, USA) was applied for broadleaf weed control on June 10, and throughout the growing season weeds were rogued out by hand. The plants were grown in 3-m rows with 30-cm spacing between adjacent rows. Within each row, seeds were sown 23 cm apart with 13 plants per row. There were 100 spaced plants of each of the 6 BC_1F_2 crosses planted.

One hundred and twenty BC_1F_2-derived F_3 lines (ten mutant lines and ten wild type lines from each of the 6 crosses) were grown in two replicates of a randomized

complete block design under the above-described irrigated conditions at the Arthur H. Post Agronomy Farm in 2020. Seeds were hand planted to a depth of 2.5 cm on April 28th. At physiological maturity, single plants were cut at ground level, weighed whole, and threshed with a single-plant thresher the first week of September. From May 1 to August 31, the research center received 14.3 cm of precipitation. The highest recorded air temperature was 34.4 °C on both August 17 and 18, while the lowest recorded air temperature was -0.6 °C on May 8 and 11 (NOAA Bozeman). The plants were grown in 1.5 m rows with 30cm spacing between rows. Within a row, seeds were sown 15 cm apart with 8 plants per row. The trial was fertilized with 40 kg per ha urea (185 kg N per ha), and weed/disease control consisted of 0.06 liter per ha of Affinity Broadspec (Thifensulfuron-methyl 25%, Tribernuron-methyl 25%; FMC, Philidelphia, PA, USA), 0.58 liter per ha MCPA Ester 4 (2-ethylhexyl ester of 2-methyl-4-chlorophenoxyacetic acid 69.7%; Albaugh, LLC, Ankeny, IA, USA), and 1.2 liter per ha Discover NG (Clodinafop-propargyl 6.4%; Syngenta Crop Protection, LLC, Greensboro, NC, USA), and 0.29 liter per ha Propi-Star EC (Propiconazole 41.8%; Albaugh, LLC, Ankeny, IA, USA) applied on June 1.

The same trial was planted on May 11, 2020 at the Central Agricultural Research Center near Moccasin, MT (latitude 47.06N, longitude 109.96W, elevation 1,297 m, soil type is Danvers-Judith clay loam). This was a dryland site, and as such did not receive any additional moisture from irrigation. Seeds were planted to a depth of 2.5 cm and harvested the third week of August. From May 1 to August 31, the research center received 16.03 cm of precipitation. The highest recorded air temperature was on August

17 at 33.9 °C, while the lowest recorded air temperature was -1.1 °C on May 12 (NOAA Stanford). The plot was fertilized with 67.3 kg/ha ESN slow-release nitrogen fertilizer (44-0-0, Nutrien, Saskatoon, SK, CA). Vendetta (Octanoic acid ester of bromoxynil 31.7%, 2-Ethylhexyl ester of 2-methyl-chlorophenoxyacetic acid 34%; Wilbur-Ellis Agribusiness, Aurora, CO, USA) was applied on May 27 for broadleaf weed control. .

Plant Measurements

Plant height was measured near physiological maturity, after growth had ceased, by determining the distance from the soil surface to the terminal spikelet of the tallest tiller. Tiller number was counted at the base of these plants, regardless of whether the tiller produced a head. Flag leaf length of the tallest tiller was measured from culm to tip, and width at the widest point of the leaf. The number of productive heads included only heads that formed seed. Seed protein content was analyzed with the LECO-FP 528 (LECO Co., St. Joseph, MI, USA; AACCI Method 46-30.01) and individual seed weight was calculated by hand, using a Contador seed counter (Pfeuffer, Kitzingen, Bavaria, Germany) on a sample of 100 seeds. In 2019, single plants were measured after genotyping, while in the 2020 five representative plants per row were measured and averaged together.

GA Responsiveness Assay: Coleoptile Length

To evaluate the impact of the *Rht-1* mutations on coleoptile length and responsiveness to GA, we used the 'cigar roll' technique described by Bai et al. (2013). BC_1F_4 seeds were surface sterilized and soaked in water for 24 hours. One roll consisted of 10 seeds from a single BC_1F_4 line placed approximately 2 cm apart on 46 x 31 cm

germination paper (Anchor Paper Co., St. Paul, MN, USA). The germination paper was pre-soaked in water or in a 100 μM GA_3 solution. The seeds were sandwiched by folding over the germination paper, and then rolled to a diameter of 2 cm. The BC_1F_4 lines consisted of ten mutant and wild type lines from each of the six crosses, for a total of 120 rolls in one replication of the experiment. Two separate replications were performed. The rolls were randomized and placed upright in test tube racks, then placed in tubs which submerged the bottom two cm of the rolls in either water or 100 μM GA_3 solution. The tubs were then placed in a dark growth chamber at 18 °C. Coleoptile length, measured as the distance from the edge of the seed to the end of the coleoptile, was recorded after 10 days.

GA Responsiveness Assay: GID1 Interaction

Yeast-2-hybrid assays were used to analyze protein-protein interaction. The bait plasmid (pGBKT7) contained GID1, while the prey plasmids (pGADb) each contained one *Rht* allele. Genes were synthesized and cloned into vectors using GENEWIZ Gene Synthesis Services (GENEWIZ, Plainfield, NJ, USA). Bait and prey vectors were co-transformed into the *Saccharomyces cerevisae* strain Y2HGold using the Yeastmaker Yeast Transformation System 2 (Clontech, Mountain View, CA, USA) and the Matchmaker Gold Yeast Two-Hybrid System (Takara Bio Inc., Mountain View, CA, USA). Colonies were grown on selectable SD media lacking leucine and threonine (SD/-L-T) for 4-5 days to select for successful co-transformation. SD/-L-T broth was then inoculated with single colonies and grown overnight. Each line to be tested was brought to an internally consistent OD_{600} reading of ~0.8, then serial dilutions of 1:10, 1:100,

1:1000, and 1:10000 were prepared. Dilutions, along with full strength solution, of each line were plated on SD media lacking leucine, threonine, adenine, and histidine, (SD/-L-T-A-H) containing 100 μM GA and the same media containing no GA. Growth of each *Rht* mutant line was compared to *Rht-A1a* and *Rht-B1a* positive controls and a GID1 negative control consisting of the pGBKT7-GID1 vector co-transformed with an empty pGAD vector.

Statistical Analysis

Comparisons between the mutant and wild type sister line means were made using a one-way analysis of variance (ANOVA) in R (R Foundation for Statistical Computing, Vienna, Austria) for the 2019 spaced plant trial. After using ANOVA to check for potential genotype x environment interactions that would require further analysis, data were combined over the two locations for the 2020 trials first computing the mean over the two replications for each line. Then a mixed linear model was fitted in R using the lmer4 package in R (Bates et al., 2015) where the model included location, genotype class, lines within genotype, and location x genotype class. Individual lines within genotype class were considered random and other factors were fixed.

Data for the coleoptile length trial were analyzed as a randomized complete block design where the model included replication, genotype class and lines within genotype class using the lmer4 package in R where lines within genotype class were considered fixed. Differences in length between mutant and wild type means were determined from an ANOVA F ratio.

Results

Field 2019 Trial

The effect of durum *Rht* mutations in BC_1F_2 sister lines under field conditions was tested with a spaced, single-plant trial (Tables 2a and 2b). In the semi-dwarf *Rht-B1b* background, only the *Rht-B1b-E529K* mutation caused significant differences, reflected as an increase in plant height of 22% (16.5 cm). In the full height *Rht-B1a* background, the *Rht-B1b-E529K* mutation decreased height by 11% (10.1 cm). Both *Rht-A1-S50F* and *Rht-A1-L358F* also caused a decrease in plant height, the former by 7% (6.2 cm) and the latter by 3% (3.1 cm). *Rht-A1-L358F* also increased tillers by 17% (2.7 tillers) and protein by 5.5% (0.9%), relative to the wild type plants.

Field 2020 Trial

To expand on the 2019 field trial, ten BC_1F_3 lines from the mutant genotype and ten from the wild type genotype within each cross were chosen to be evaluated in short-row spaced plant trials (Tables 3a and 3b). The *Rht-B1b-E529K* mutation showed significant effects in the semi-dwarf background, increasing height by 16% (10.6cm) relative to wild type, as did *Rht-A1-S50F*, which decreased height by 3% (1.9cm). In the tall background, *Rht-B1b-E529K* was associated with an 8% (6.18cm) decrease in height and a 4.6% (1.03cm) decrease in flag leaf length, while the *Rht-A1-S50F* mutation increased flag leaf width by 4.7% (0.07cm) and the *Rht-A1-L358F* mutation decreased the number of spikelets per head by 3%. (0.49 spikelets per head). There was a trend amongst all the mutations in the tall background to decrease height relative to the wild type plants.

Table 2a: The impact of *Rht* mutations on genotype class mean values for agronomic traits from 2019 Bozeman field trials.

Recurrent Parent *Rht* Allele	***Rht-1* mutant allele**		**n[a]**	**Plant Height[b]** (**cm**)	**Tillers[b]** (**No./plant**)	**Productive Heads[b]** (**No./plant**)	**Flag Leaf Length[b]** (**cm**)	**Flag Leaf Width[b]** (**cm**)
Rht-B1b	*Rht-B1b-E529K*	mut	22	90.0±2.07***	11.8±0.71	8.23±0.73	18.5±0.49	1.59±0.02
	Rht-B1b	wt	16	73.5±0.94	12.0±0.72	7.38±0.76	18.4±0.52	1.52±0.00
	Rht-A1-S50F	mut	23	61.3±0.67	15.7±0.76	7.09±0.34	17.5±0.42	1.50±0.04
	Rht-A1a	wt	22	62.2±0.75	15.3±1.00	6.55±0.39	16.9±0.39	1.43±0.03
	Rht-A1-L358F	mut	17	67.4±0.96	14.4±0.90	5.88±0.46	17.4±0.45	1.61±0.03
	Rht-A1a	wt	16	67.4±0.91	14.1±1.16	6.13±0.56	17.6±0.48	1.64±0.04
Rht-B1a	*Rht-B1b-E529K*	mut	20	85.6±1.19***	14.0±0.85	7.75±0.50	21.1±0.49	1.55±0.03
	Rht-B1b	wt	27	95.1±1.28	14.2±0.70	8.30±0.61	21.3±0.40	1.60±0.03
	Rht-A1-S50F	mut	25	87.9±1.10***	13.6±0.83	6.63±0.40	21.9±0.37	1.60±0.03
	Rht-A1a	wt	23	94.0±1.09	15.1±0.90	6.52±0.46	22.0±0.34	1.58±0.04
	Rht-A1-L358F	mut	21	93.5±0.66**	18.4±0.71*	5.88±0.46	22.2±0.42	1.70±0.03
	Rht-A1a	wt	13	96.6±0.93	15.8±0.73	6.13±0.56	22.0±0.47	1.64±0.03

[a] n represents the number of plants in each genotypic group.

[b] Values represent the average for each genotype ± the standard error.

*, **, and *** denote *P* values of <0.05, 0.01, and 0.001, respectively.

Table 2b: The impact of *Rht* mutations on genotype class mean values for seed traits from 2019 Bozeman field trials.

Recurrent parent *Rht* Allele	***Rht-1* mutant allele**		**n**[a]	**Yield** g/plant	**Grain Protein**[b] %	**Grain Weight**[b] mg/seed	**Seeds per Prod. Head**[b] No./head
Rht-B1b	*Rht-B1b-E529K*	mut	22	19.2±1.69	17.3±0.08	49.6±1.14	47.1±1.92
	Rht-B1b	wt	16	16.0±1.51	17.2±0.38	47.2±3.01	45.4±2.38
	Rht-A1-S50F	mut	23	18.9±0.78	16.0±0.09	44.9±0.82	61.5±2.86
	Rht-A1a	wt	22	16.1±1.36	15.7±0.05	44.6±0.64	54.4±2.74
	Rht-A1-L358F	mut	17	14.6±1.41	15.8±0.21	47.7±0.86	52.8±3.06
	Rht-A1a	wt	16	15.7±1.72	16.4±0.49	48.8±1.06	53.4±3.06
Rht-B1a	*Rht-B1b-E529K*	mut	20	25.6±1.71	16.3±0.34	46.4±2.38	68.0±2.01
	Rht-B1b	wt	27	25.7±1.47	16.6±0.04	46.9±1.80	63.5±2.22
	Rht-A1-S50F	mut	25	20.0±1.52	17.6±0.08	45.5±1.95	63.3±2.52
	Rht-A1a	wt	23	17.7±1.53	17.4±0.05	46.1±0.57	58.8±2.07
	Rht-A1-L358F	mut	21	24.4±1.34	17.4±0.18*	47.1±0.56	71.9±0.93
	Rht-A1a	wt	13	21.4±1.08	16.5±0.23	47.1±0.83	74.9±1.18

[a] n represents the number of plants in each genotypic class.
[b] Values represent the mean for each genotype ± the standard error.
*, **, and *** denote P values of <0.05, 0.01, and 0.001, respectively.

Table 3a: The impact of *Rht* mutations on genotype class mean values for agronomic traits from 2020 field trials averaged over two Montana locations.

Recurrent parent *Rht* Allele	***Rht-1* mutant allele**		**Plant Height[a]**	**Tillers[a]**	**Spikelets[a]**	**Productive Heads[a]**	**Flag Leaf Length[a]**	**Flag Leaf Width[a]**
			cm	**No./plant**	**No./plant**	**No./plant**	**cm**	**cm**
Rht-B1b	*Rht-B1b-E529K*	mut	78.6±1.86***	13.2±0.45	15.8±0.13	10.6±0.34	20.9±0.18	1.43±0.01
	Rht-B1b	wt	68.0±0.52	13.1±0.66	15.8±0.11	10.2±0.50	21.2±0.47	1.43±0.02
	Rht-A1-S50F	mut	57.1±0.61*	17.4±0.10	16.9±0.48	12.7±0.76	19.4±0.31	1.42±0.02
	Rht-A1a	wt	59.0±0.51	16.3±0.84	16.3±0.18	12.0±0.66	18.9±0.13	1.39±0.02
	Rht-A1-L358F	mut	65.5±0.54	15.5±0.77	15.6±0.17	11.4±0.59	20.0±1.18	1.50±0.02
	Rht-A1a	wt	65.2±0.65	14.6±0.73	15.6±0.14	10.9±0.51	18.6±0.22	1.46±0.02
Rht-B1a	*Rht-B1b-E529K*	mut	80.4±1.06**	15.1±0.96	18.8±0.22	11.5±0.73	21.9±0.20**	1.44±0.02
	Rht-B1b	wt	86.5±1.57	15.1±1.02	19.4±0.24	10.9±0.73	22.9±0.27	1.48±0.02
	Rht-A1-S50F	mut	81.6±0.96	14.7±0.83	18.1±0.18	11.0±0.64	22.8±0.32	1.56±0.02*
	Rht-A1a	wt	84.1±1.43	15.0±1.25	18.0±0.31	11.3±0.92	22.7±0.37	1.49±0.02
	Rht-A1-L358F	mut	85.9±0.58	13.6±1.01	17.8±0.14*	10.8±0.59	22.6±0.33	1.48±0.03
	Rht-A1a	wt	86.9±0.47	14.5±0.95	18.3±0.16	11.4±0.51	22.4±0.34	1.49±0.02

[a] Values represent the average for each genotype ± the standard error.
Each genotype class had 10 lines, where each line was the mean of 5 plants in two replications and two locations.
*, **, and *** denote P values of <0.05, 0.01, and 0.001, respectively.

Table 3b: The impact of *Rht* mutations on genotype class mean values for seed traits from 2020 field trials averaged over two Montana locations.

Recurrent parent *Rht* Allele	***Rht-1* mutant allele**		**Biomass[a]**	**Yield[a]**	**Grain Protein[a]**	**Grain Weight[a]**	**Seeds per Prod. Head[a]**	**Harvest Index[a]**
			g/plant	**g/plant**	**%**	**mg/seed**	**No./head**	
Rht-B1b	*Rht-B1b-E529K*	mut	29.2±1.26	26.4±1.21	13.4±0.31	45.5±1.01	54.7±1.88	0.47±0.01
	Rht-B1b	wt	29.1±1.68	28.1±1.67	12.9±0.31	43.9±0.93	64.2±4.55	0.49±0.01
	Rht-A1-S50F	mut	26.5±1.85	23.1±1.86	13.7±0.15	38.0±0.94	47.6±2.37	0.47±0.01
	Rht-A1a	wt	24.7±1.51	22.6±1.63	13.5±0.15	39.2±1.02	48.2±2.03	0.46±0.01
	Rht-A1-L358F	mut	24.1±2.54	23.1±1.97	12.8±0.22	41.0±0.88	52.8±5.10	0.49±0.01
	Rht-A1a	wt	23.5±1.67	22.7±1.62	12.7±0.22	40.6±0.86	48.9±2.31	0.49±0.01
Rht-B1a	*Rht-B1b-E529K*	mut	37.2±3.19	29.0±3.16	12.6±0.27	41.1±1.12	58.3±2.54	0.43±0.01
	Rht-B1b	wt	40.6±4.06	29.6±3.42	12.5±0.22	41.4±1.16	61.9±2.97	0.41±0.01
	Rht-A1-S50F	mut	35.2±2.10	25.6±1.62	14.0±0.29	40.5±0.69	58.3±2.67	0.42±0.01
	Rht-A1a	wt	35.1±3.39	24.5±2.44	14.0±0.32	40.6±0.72	52.7±1.96	0.41±0.01
	Rht-A1-L358F	mut	27.4±2.20	22.9±2.07	13.8±0.28	38.8±0.79	56.7±3.00	0.45±0.02
	Rht-A1a	wt	30.1±2.63	23.3±2.22	13.9±0.22	38.1±0.93	53.5±1.77	0.44±0.02

[a] Values represent the mean for each genotype ± the standard error.
Each genotype class had 10 lines, where each line was the mean of 5 plants in two replications and two locations.

GA Responsiveness Assay: Coleoptile Length

The impact of durum *Rht* mutations on coleoptile length was evaluated in the presence and absence of GA (Table 4). Length was compared between the mutant and wild type in each cross, and between the GA and water treatments. This allowed us to determine both the effects of the mutations on coleoptile length and on the impact of GA on coleoptile length. The *Rht-B1b-E529K* mutation significantly affected coleoptile length in both the semi- dwarf and full height backgrounds, reducing coleoptile length by 20% (18.44mm) ($P<0.001$) without GA present and 28% (29.23mm) ($P<0.001$) with GA in the full height background. Coleoptile length in the semi-dwarf background was increased by 23% (16.71mm) ($P<0.01$) in water and 25% (20.12mm) ($P<0.01$) in the GA

Table 4: The impact of *Rht* mutations on coleoptile length and GA responsiveness.

Recurrent parent *Rht* allele	***Rht-1* mutant allele**		**n**[a]	**Coleoptile length in water (mm)**[b]	**Coleoptile length in GA (mm)**[b]	**GA/ Water**[c]
Rht-B1b	*Rht-B1b-E529K*	mut	10	88.0±13.42**	99.4±14.56**	1.13*
	Rht-B1b	wt	10	71.3±4.91	79.3±8.04	1.11
	Rht-A1-S50F	mut	10	64.7±7.29	65.4±7.92	1.01
	Rht-A1a	wt	10	63.1±4.76	60.4±6.00	0.96
	Rht-A1-L358F	mut	10	62.4±5.05	58.9±4.37	0.94
	Rht-A1a	wt	10	60.5±6.36	58.3±5.13	0.96
Rht-B1a	*Rht-B1b-E529K*	mut	10	74.6±7.67***	74.9±8.62***	1.00*
	Rht-B1a	wt	10	93.1±11.96	104.1±7.14	1.11
	Rht-A1-S50F	mut	10	107.7±2.20	114.3±2.81	1.06
	Rht-A1a	wt	10	107.9±2.40	113.4±3.19	1.05
	Rht-A1-L358F	mut	10	104.9±2.34	109.5±2.38	1.04*
	Rht-A1a	wt	10	106.7±2.64	115.7±2.17	1.08

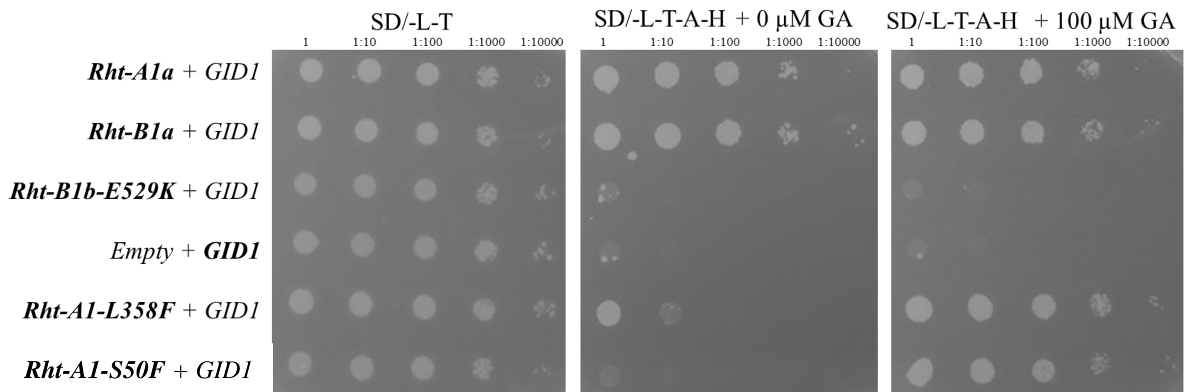
[a] n represents the number of values in each genotype after averaging two replicates together
[b] Values represent the average for each genotype ± the standard error
[c]Difference between GA and water treatments, expressed as a ratio.
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

solution. There were no significant differences between the sister lines containing *Rht-A* mutations. When comparing the length of the coleoptile in GA to that in water for the same genotype, the presence of GA increased coleoptile length by 11% for the wild-type line in the *Rht-B1b-E529K* mutation in both backgrounds and increased length by 8% in the wild type line in the full-height background *Rht-A1-L358F* pair.

<u>GA Responsiveness Assay: GID1 Interaction</u>

Durum *Rht* mutations and their interaction with the GID1 receptor was tested in vitro with a yeast-2-hybrid assay (Figure 1). The positive controls *Rht-A1a* and *Rht-B1a* showed strong interaction on both media types, while the GID1/empty vector negative control showed no interaction. *Rht-B1b-E529K* also showed no interaction on either media, indicating that the addition of the *E529K* mutation to *Rht-B1b* does not alter its effect in vitro. The missense mutations had a varied effect. *Rht-A1-S50F* showed no

Figure 1: Yeast-2-hybrid assay of durum EMS-derived *Rht* mutations to characterize the interaction between DELLA and GID1 in the presence or absence of 100 μM GA.

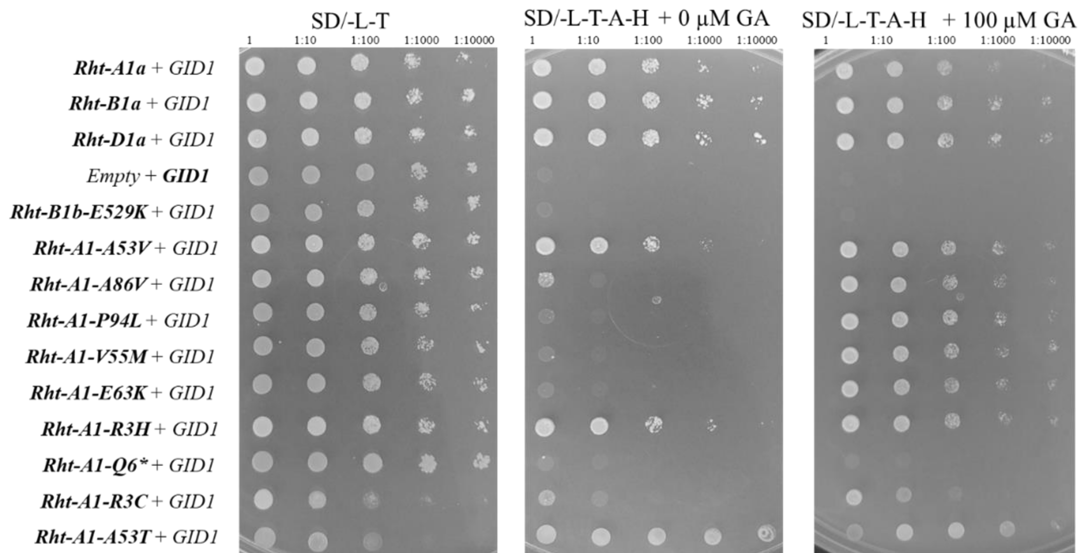


Serial dilutions on SD/-L-T (control) media, SD/-L-T-A-H media, and SD/-L-T-A-H media + 100 μM GA are shown. *Rht-A1a* and *Rht-B1a* are positive controls, and GID1 is a negative control with an empty pGADT7 vector to ensure no random activation is occurring. Plates are representative of three independent replications.

interaction without GA, but restored interaction near the level of wild type when GA was present. *Rht-A1-L358F* demonstrated a weaker interaction than wild type in the absence of GA, and a strong interaction similar to wild type when GA was present.

The same interaction assay was performed on a series of *Rht-A1* EMS mutations identified in hexaploid wheat (Figure 2). Positive controls *Rht-A1a*, *Rht-B1a*, and *Rht-D1a* showed strong interaction with GID1 in both the presence and absence of GA, while negative controls *Rht-B1b-E529K* and GID1/empty vector did not. *Rht-A* mutations varied in their effect. *Rht-A1-A53V*, *Rht-A1-R3H*, and *Rht-A1-A53T* each showed interaction close to that of the positive controls when no GA is present, while *Rht-A1-A86V* and *Rht-A1-R3C* showed interaction only at the strongest concentration. *Rht-A1-*

Figure 2: Yeast-2-hybrid assay of EMS-derived *Rht* mutations found in hexaploid wheat indicating the interaction between DELLA and GID1 in the presence or absence of 100 µM GA.



Serial dilutions on SD/-L-T (control) media, SD/-L-T-A-H media, and SD/-L-T-A-H media + 100 µM GA are shown. *Rht-A1a*, *Rht-B1a, and Rht-D1a* are positive controls, *Rht-B1b-E529K* is a negative control, and GID1 is a negative control with an empty pGADT7 vector to ensure no random activation is occurring. Plates are representative of three independent replications.

P94L, *Rht-A1-V55M*, and *Rht-A1-E63K* did not grow on the no-GA plate. The addition of GA restored the interaction of *Rht-A1-A53V*, *Rht-A1-A86V*, *Rht-A1-P94L*, *Rht-A1-V55M*, *Rht-A1-E63K*, *Rht-A1-R3H*, and *Rht-A1-A53T* to the same level as the wild-type positive controls. *Rht-A1-Q6** mimicked the negative controls, with no growth on either plate. Both *Rht-A1-A53T* and *Rht-A1-R3C* had reduced growth on the control plate, but in comparing the interaction plates still showed enough growth to interpret.

Discussion

Two *Rht-A* missense mutations and the previously reported *Rht-B1b-E529K* allele were tested to see impacts on various plant physical traits and grain characteristics. Sister lines were created using each of the mutations in two different Montana-adapted parent lines, for a total of 6 mutant genotypes to be compared to their corresponding wild type sister line (Table 1).

In both the BC_1F_2 (Table 2a) and BC_1F_3 (Table 3a) generations, we observed the height-increasing effect of *Rht-B1b-E529K* when crossed to a semi-dwarf parent, as previously reported (Mo et al. 2018). The effect was greater in 2019, with a 22% increase versus a 16% increase in 2020. We also found that the *Rht-B1b-E529K* decreased plant height when crossed to a full-height parent. Again, the effect was more pronounced in the 2019 trial with an 11% reduction compared to an 8% reduction in 2020. Both the semi-dwarf and the full-height crosses to *Rht-B1b-E529K* resulted in an intermediate-height durum plant, between current semi-dwarf and full height varieties.

When comparing the *Rht-A* mutations to their semi-dwarf sister lines, neither *Rht-A1-S50F* nor *Rht-A1-L358F* provided the desired height increase. Both *Rht-A* missense mutations showed significant height reduction compared to their wild type sister lines in 2019 but that trend was not statistically significant in 2020. One possible reason for the differing results between years may be that plants overall were taller in 2019 than 2020, potentially due to more moisture in 2019. Another possibility is that the plots were located in slightly different locations within the farm each year and may have had underlying soil differences that influenced plant growth. None of the mutations tested here contributed to an increased grain yield (Tables 2b and 3b).

Although no height-reducing *Rht-A* alleles have been identified in hexaploid wheat (Flintham et al. 1997, Gale and Youssefian 1985, Pearce et al. 2011), this data suggests that when integrated into durum varieties with the wild type *Rht-B1a* allele, *Rht-A* missense mutations could bring about shorter plants. *Rht-B1b-E529K* was shown to be useful for creating intermediate height durum plants both when combined with *Rht-B1b*, as shown in (Mo et al. 2018), and with *Rht-B1a*.

Along with field trials to test the agronomic impacts of the mutations, two GA-responsiveness assays were also conducted. The interaction between GID1, a known receptor of wild type *Rht* when GA is present, and various *Rht-A1* mutations was analyzed in-vitro with yeast-2-hybrid assays. Pearce et al. (2012) showed the *Rht-A1a, Rht-B1b,* and *Rht-D1b* all have DELLA/GID1 interactions, while there is no interaction with *Rht-B1b*, either in the presence or absence of GA. In this experiment, the co-transformed yeast was spotted onto three types of media to view the interactions in the

presence of GA, in the absence of GA, and on a non-selective media as a control. All three mutations in the durum field trials were included (Figure 1), as well as a second assay that examined 9 *Rht-A* mutations found in hexaploid wheat (Figure 2). We observed no growth on either plate for *Rht-B1b-E529K* when examining the durum mutations, and since Pearce et al. 2012 observed that *Rht-B1b* did not grow, it is shown that the in-vitro effect of *Rht-B1b* is not altered by the addition of the *E529K* mutation. Therefore, it was included in the hexaploid mutation assay as a negative control.

Of the mutations tested, only one was a nonsense mutation. *Rht-A1-Q6** did not grow on either plate, showing the same pattern of no interaction when a stop mutation is present that was demonstrated by Pearce et al. (2012) and with the *Rht-B1b-E529K* mutation in this study, but disagrees with Jobson et al. (2020, in press) who found that GA restored the function of three stop mutations.

The missense mutations varied in effect. *Rht-A1-S50F, Rht-A1-P94L*, *Rht-A1-V55M*, and *Rht-A1-E63K* had no interaction without GA, but their function was fully restored when GA was present. *Rht-A1-L358F* and *Rht-A1-A86V* interacted slightly on the no-GA plates, while *Rht-A1-A53V* and *Rht-A1-R3C* showed intermediate interaction, midway between the positive and negative controls. With each of those four, the addition of GA restored full function. *Rht-A1-A53T'*s growth appeared similar on both plates but reduced on the control plate which leads to a less reliable interpretation of its results. Alleles that grew on both plates similar to wild type alleles are expected to exhibit plant growth similar to wild type plants, whereas alleles that differed when GA was or was not present are expected to produce plants that are intermediate in height.

Rht-B1b and *Rht-D1b* reduce coleoptile length compared to wild type *Rht* alleles (Fick and Qualset 1979, Schillinger et al. 1998, Jobson et al. in press 2020, Amram et al. 2015, Liatukas and Ruzgas 2011), and the *Rht-B1b-E529K* mutation partially repressed that length reduction compared to *Rht-B1b* (Mo et al. 2018). We observed a coleoptile length increase compared to *Rht-B1b*, which agrees with the results of the durum experiment described above, and a length decrease compared to *Rht-B1a* (Table 4). These results mirror the effect seen in field-grown plants of the same genotypes, as observed in previous studies correlating height of plants to coleoptile length (Fick and Qualset 1979, Schillinger et al. 1998, Liatukas and Ruzgas 2011, Jobson et al. in press 2020). The *Rht-B1b-E529K* mutation's effect on coleoptile length was increased when GA was present, 28% versus 20% reduction in the *Rht-B1a* cross and 25% versus 23% increase in the *Rht-B1b* cross. This mutation-induced increased GA-sensitivity response was not observed in the other crosses examined, nor was a difference in coleoptile length detected between sister lines, which supports Jobson et al. (in press 2020)'s findings that mutations other than *Rht-B1b* and *Rht-D1b* did not impact coleoptile growth or GA responsiveness.

The wild type sister line in the *Rht-A1-L358F, Rht-B1a* cross did show a GA response, with an increased coleoptile length of 8% when GA was present. This supports previous studies (Fick and Qualset 1979, Schillinger et al. 1998, Liatukas and Ruzgas 2011, Jobson et al. in press 2020) showing that wild type *Rht-B1a* plants are GA-responsive and grow longer coleoptiles when exposed to GA. Since all of the genotype groups had coleoptiles long enough to emerge from soil if planted at 2.5 cm, it cannot be

determined if the increases/decreases observed in this experiment would be of agronomic relevance or not.

Field experiment results showed that height was unaffected by *Rht-A* mutations when *Rht-B1b* was present, but when combined with *Rht-B1a* the mutations did provide a height decrease. Coleoptile data revealed a similar pattern. The yeast-2-hybrid results indicated that both *Rht-A* mutations would confer intermediate interaction with GID1, which may produce plants intermediate in height. These results indicate that by adding *Rht-A* mutations to full-height durum varieties, plant height can be reduced, while the same technique in semi-dwarf varieties is not likely to show the desired intermediate height plants.

CHAPTER TWO

INVESTIGATING THE IMPACT OF DURUM *TB1* NULL MUTATIONS

Introduction

Increasing the number of tillers per plant, and presumably spikes per plant, can increase yield (Naseer et al. 2016). However, simply increasing total tillers is not sufficient. Late increases in tillering can siphon away limited resources that otherwise would contribute to grain yield, resulting in a plant with few productive tillers and lower grain yield (Elhani et al. 2006 and Motzo et al. 2002). It is necessary, therefore, to increase the number of productive tillers that contribute to yield. An allele identified at a chromosome 6B QTL was associated with increased early tiller number and led to an increase in productive tiller number (PTL) and yield in favorable environments (Naseer et al. 2016). This allele has been identified in several popular Montana-grown hard red spring wheat varieties, such as Vida (PI 642366) and Reeder (PI 613586) (Naseer et al. 2016), indicating that manipulation of tiller number is a factor already being utilized in wheat breeding programs. Demonstrating specific genes' effects on tillering is an approach that can be used to modify genetic yield potential.

One such gene that affects tillering is *Teosinte Branched1* (*TB1*). First identified in maize (*Zea mays*) as one of the main genes that differentiates modern maize from its progenitor, *TB1* expression in maize decreases the branching architecture specific to teosinte (Doebley 1995). High levels of *TB1* expressed in maize, compared to that in teosinte, is associated with the modern single-stalked plant with few to no upper branches

(Doebley et al. 1997). Homologs of *TB1* have been identified in multiple species: *OsTB1* in rice (*Oryza sativa*) (Sakamoto and Matsuoka 2004), *BRC1* in *Arabidopsis* (Aguilar-Martinez et al. 2007), *SbTB1* in sorghum (*Sorghum bicolor*) (Kebron et al. 2006), *PvTB1* in switchgrass (*Panicum virgatum*) (Xu et al. 2016), and *TaTB1/TB1* in bread wheat (Lewis et al. 2008, Liu et al. 2018).

As a class II member of the transcriptional regulator family known as the TCP family (Cubas et al. 1999), *TB1* and its homologs all have conserved function as negative regulators that suppress lateral branching, growth of axillary buds, and internode growth of branches (Aguilar-Martinez et al. 2007, Doebley et al. 1995 and Doebley et al. 1997, and Takeda et al. 2003). However, the effect of *TB1* on plant architecture varies across species.

In maize, *TB1* prevents outgrowth of buds at lower nodes and promotes development of ears at upper nodes, while mutant *TB1* plants develop more tillers and male inflorescences (Cubas et al. 1999). Mutant *TB1* alleles in maize were also associated with increased node length and height of overall plant (Hubbard et al. 2002) and an increase in root biomass that keeps the ratio of above-ground to below-ground growth proportionately similar across mutant and wild-type genotypes (Gaudin et al. 2014), along with reduced expression of genes in growth and development regulating hormone pathways (Dong et al. 2019).

The gene most closely related to *TB1*, *BRC1*, restricts bud outgrowth in *Arabidopsis* and other dicots (Aguilar-Martinez et al. 2007). Overexpression of *OsTB1* suppresses outgrowth by limiting tiller development to the primordial stage in rice

(Sakamoto and Matsuoka 2004), while overexpression of *PvTB1* in switchgrass inhibited tiller emergence and development, along with inducing changes in stem height and diameter, and biomass yield (Xu et al. 2014). Repressed *TB1* expression in sorghum was shown to induce axillary bud growth (Kebrom et al. 2006).

Hexaploid wheat containing *TB1* mutant alleles demonstrated to have reduced *in vitro* binding to the Flowering Time 1 protein were taller than wild type plants (Dixon et al. 2020). Altered bread wheat inflorescence architecture in the form of "paired spikelets" was associated with increased D genome expression of *TB1* (Dixon et al. 2018). Lewis et al. (2008) showed that overexpression of *TB1* in wheat results in fewer tillers, reduced axillary bud outgrowth, shorter plants, and increased inflorescence axillary outgrowth. Additionally, overexpression of the tae-miR156 regulator resulted in reduced expression of *TaTB1* and enhanced hexaploid wheat tillering (Liu et al. 2018).

These studies provide the basis for our research. Overexpression of *TB1* is associated with decreased tillering (Xu et al. 2014, Sakamoto and Matsuoka 2004), while wild-type *TB1* (and orthologs) plants exhibit apical dominance with few main branches (Aguilar-Martinez et al. 2007, Doebley et al. 1995, Doebley et al. 1997, and Takeda et al. 2003) and mutant *TB1* plants reveal a bushier architecture consisting of more tillers (Cubas et al. 1999, Xu et al. 2014, Kebrom et al. 2006, and Liu et al. 2018) and increased height (Dixon et al. 2020). We hypothesized that tiller number can be increased and utilized as a method to increase total biomass and/or grain yield in durum and other wheat relative species by suppressing *TB1* expression by selecting for *TB1* null alleles.

To analyze the effects of *TB1* null mutations in durum, an EMS mutagenized durum wheat population was screened for stop codon mutations. Three lines containing *TB1* stop mutations were chosen for crossing into wild type durum, then greenhouse and field trials were conducted on the populations created from those crosses to evaluate phenotypic differences including plant and root biomass, height, and tillering. Additionally, an expression study using RNAseq was conducted to determine the effect the mutations had on the expression of genes involved in seed dormancy and germination in developing meristems. A screen of commonly grown Montana winter, spring, and durum wheat was also conducted to determine whether commonly grown wheat varieties vary in *TB1* alleles. A study on root biomass was conducted to determine if above-ground phenotypic differences were reflected below ground as well.

Materials and Methods

Plant Material

The wheat TILLING population database (UC Davis, Tsai et al. 2011, Krasileva et al. 2017) was screened using online tools for *TB1* null mutations in the *Triticum turgidum* variety "Kronos" (D03-21) and three lines containing *TB1* stop mutations were chosen for this project: *TB1-A1-W339**, *TB1-B1-W341**, and *TB1-B1-Q142** (see Table 5). M_4 seed of those lines was obtained and M_4 plants grown in fall 2018. F_1 crosses between the *TB1-A1-W339** line and both *TB1-B1* mutant lines were made and the resulting F_1 seeds were planted, grown, and allowed to self-pollinate. DNA was extracted from the F_2 plants, then PCR was performed using primers shown in Table 6. Conditions for PCR were 40 cycles of 30 s denaturation at 96 °C, 30 s annealing at 65 °C,

and 60 s extension at 72° C, for both primer sets. PCR products were Sanger sequenced (GENEWIZ, Inc., Cambridge, MA, USA), then analyzed using the DNASTAR SeqMan Pro software (DNASTAR Version 15.0.1.1, Madison, WI, USA) against the reference genes from Liu et al. (2017). Only homozygous mutant and homozygous wild type plants were kept for advancement. In this manner, four genotypes of interest for each cross were created: AABB, aabb, AAbb, and aaBB for both *TB1-A1-W339** x *TB1-B1-W341** and *TB1-A1-W339** x *TB1-B1-Q142**. F_2 homozygotes were grown and allowed to self-pollinate to produce seed of F_2-derived F_3 lines for planting in the field during spring 2019. The number of F_2-derived F_3 lines varied from 9 to 19 in each of the four genotype classes for each cross.

Table 5: Summary of *TB1* mutations.

Allele	**DNA change (base pairs from start codon)**	**Original Codon**	**New Codon**
TB1-A-W339Stop	1016	TGG	TAG
TB1-B-W341Stop	1023	TGG	TGA
TB1-B-Q142Stop	424	CAG	TAG

Table 6: Amplification and sequencing primers used to detect *TB1* mutations.

Mutation Detected	**Primer Sequence[a]**	**Primer Type**	**PCR Product Size**
*TB1-A1-W339**	5’ GATCGACAACCAGCCGCCA 3’	Forward	862 bp
	3’ GGGCTGCGAGTTGGGAAA 5’	Reverse	
	5’ GACAAGGAGTCGAGGACG 3’	Sequencing	
*TB1-B1-W341**	5’ GATCGACAACCAGTCGCCG 3’	Forward	859 bp
	3’ GGGCTGGGAGTTGGGAAA 5’	Reverse	
	5’ GACAAGGAGTCGAGGACG 3’	Sequencing	
*TB1-B1-Q142**	5’ GATCGACAACCAGTCGCCG 3’	Forward/ Sequencing	859 bp
	3’ GGGCTGGGAGTTGGGAAA 5’	Reverse	

[a]Primers developed from sequence alignment from Liu et al. 2018. (Genbank accession numbers TraesCS4A02G271300 and TraesCS4B02G042700).

Greenhouse Growing Conditions

Seeds were planted in Sunshine mix (Sungro Horticulture, Agawam, MA, USA) potting soil, 4 seeds per 20.3 cm pot. Plants were watered daily with a 100 ppm solution of 20-20-20 fertilizer applied through a Dosatron (Dosatron International, Inc., Clearwater, FL, USA) once plants reached the three-leaf stage. The temperature was set at 22.2 °C in the daytime and 18.3 °C at night. After flowering, the temperature was increased to 25 °C day and 21 °C until harvest. A micronized sulfur solution (Bonide Products, Inc., Oriskany, NY, USA) at a rate of 4.38 g/l was applied to plants at the 2-3 leaf stage (Z 12-Z13 on the Zadoks growth scale for wheat) for powdery mildew and mite prevention, and Marathon (Imidacloprid 1%, OHP, Inc., Bluffton, SC, USA) at a rate of 2.7 g per pot was applied at the same growth stage for aphid control.

Initial Greenhouse Trial

A single randomized replicate of the F_2 lines (102 entries) were grown in the greenhouse under the above-described conditions to produce seed for field trials. These plants were also evaluated for genotypic differences to confirm that field trials were warranted.

Field Experiment

The F_2-derived F_3 lines (102 entries) were grown in two replications of a randomized complete block design at the Arthur H. Post Agronomy Farm near Bozeman, MT (latitude 45.67N, longitude 111.15W, elevation 1,455 m, soil type is Amsterdam silt loam). The plants were grown in 2-m rows with 30 cm spacing between each row. Within a row, seeds were sown 15 cm apart with 13 plants per row. Seeds were hand

planted to a depth of 2/5 cm on May 9, 2019. After testing soil, 185 kg/ha urea (46-0-0) was added. From May 1st to August 31st, the research station received 18.62 cm of precipitation. The highest recorded air temperature was on July 23 at 32.3 °C, the lowest recorded air temperature was -4.18 °C on May 1. Five cm irrigation water was applied using hand line sprinklers on July 1. A 1.1 l/ha application of Huskie (Pyrasulfotole 3.3%, Bromoxynil Octanoate 13.4%, Bromoxynil Heptanoate 12.9%; Bayer CropScience, Research Triangle PK, NC, USA) was applied for broadleaf weed control on June 10, and throughout the growing season weeds were rogued out by hand.

F_2-derived F_4 seed harvested from the 2019 trial was used for similar field trials in 2020 at the Arthur H. Post Agronomy Farm and at the Central Agricultural Research Center (CARC) near Moccasin, MT (latitude 47.06 N, longitude 109.96W, elevation 1,297 m, soil type Danvers-Judith clay loam). Experimental design at both locations was a randomized complete block design with two replications with 93 entries per replication. Plots were 1.5-m rows with 30-cm spacing between rows. Within rows, seeds were sown 15 cm apart with eight plants per row. Plots were hand-planted to a depth of 2.5 cm on April 25, 2020 at the Bozeman location and on May 11, 2020 at the Moccasin location.

The Moccasin plot was fertilized with 67.3 kg/ha ESN slow-release nitrogen fertilizer (44-0-0, Nutrien, Saskatoon, SK, CA) and in Bozeman with 403.5 kg/ha urea (46-0-0). The Bozeman trial received 5 cm irrigation water on July 2, while the Moccasin location received no added irrigation. From May 1 to August 31, the Post farm received 14.3 cm of precipitation. The highest recorded air temperatures were on August 17 and 18 at 34.4 °C, the lowest recorded air temperature was -0.6 °C on May 8 and 11.

The CARC received 16.03 cm of precipitation. The highest recorded air temperature was on August 17 at 33.9 °C, the lowest recorded air temperature was -1.1 °C on May 12 (NOAA Stanford). Herbicides applied to the Bozeman crop were 0.06 liter per ha of Affinity Broadspec (Thifensulfuron-methyl 25%, Tribernuron-methyl 25%; FMC, Philidelphia, PA, USA), 0.58 liter per ha MCPA Ester 4 (2-ethylhexyl ester of 2-methyl-4-chlorophenoxyacetic acid 69.7%; Albaugh, LLC, Ankeny, IA, USA), 1.2 liter per ha Discover NG (Clodinafop-propargyl 6.4%; Syngenta Crop Protection, LLC, Greensboro, NC, USA), and 0.29 liter per ha Propi-Star EC (Propiconazole 41.8%; Albaugh, LLC, Ankeny, IA, USA) applied on June 1. In Moccasin, Vendetta (Octanoic acid ester of bromoxynil 31.7%, 2-Ethylhexyl ester of 2-methyl-chlorophenoxyacetic acid 34%; Wilbur-Ellis Agribusiness, Aurora, CO, USA) was applied on May 27 for weed control.

Plant Measurements

Greenhouse and field measurements were conducted similarly. Plants were measured near physiological maturity, after growth had ceased. Height of plant was determined by measuring from the soil surface to the terminal spikelet of the tallest tiller. Tiller number was counted at the base of the plant, regardless of whether the tiller produced a head. Spikelets per head was counted on the tallest tiller only. Flag leaf length was measured from stem to tip, and width at the widest point of the leaf. Number of productive heads included only heads that formed seed. Biomass was measured at harvest by weighing the whole plant after cutting at the base and adjusted for moisture content and seed weight after harvesting seed. Seed protein and moisture were measured using a Foss Infratec 1241 Machine (Foss Analytics, Eden Prairie, MN, USA; AACCI

Method 39-11.01). Individual seed weight was measured with a Single Kernel Characterization System 4100 (Perten, Springfield, IL, USA; AACCI Method 55-31.01).

Field data was measured on five consecutive plants in the middle of each row, then averaged for one data point per row. The five plants were tagged prior to measurements to ensure consistency, then harvested together. Greenhouse plants were measured individually, for one data point per plant.

RNA Sequencing Expression Analysis

F_4 seed of each genotype (AABB, aabb, AAbb, and aaBB) from the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross population was planted in the greenhouse in 20.3 cm pots, with 15 seeds per pot. Three different lines from each genotype were planted in individual pots for three biological replicates, for a total of 45 seeds per genotype, repeated three times for three technical replicates. Developing spikes (Dixon et al., 2018) at 3 weeks post planting were used for analyses. Plants were removed from soil and rinsed, then ~1 cm of tiller tissue was excised. A composite sample of 45 mg tissue was collected and immediately frozen in liquid N. RNA extraction was completed with a Qiagen RNeasy Plant Mini Kit and the protocol listed therein (Qiagen Catalog #74904, 4th edition, April 2006). RNA samples were analyzed using GENEWIZ Next Generation Sequencing service (GENEWIZ, South Plainfield, NJ, USA).

Variety Screen

Fifteen winter wheat varieties, 15 spring wheat varieties, and 10 durum varieties along with controls of Chinese spring wheat and one representative line of each of the

four genotypes of interest (Kronos AABB, aabb, AAbb, and BBaa) were planted in the greenhouse (Table 7), then DNA extracted at the two-leaf stage. Each line was genotyped with primers designed to capture the whole *TB1* gene (see Table 8 for primer sequences and product size). PCR conditions were as follows: initialization 96°C for 5 min., then 40 cycles of 96°C for 30 sec, 30 sec at the annealing temperature shown in Table 8, 1 minute at 72 °C, and a 7 min. final extension at 72 °C. PCR products were Sanger sequenced by GENEWIZ (GENEWIZ, Inc., Cambridge, MA, USA), then the sequence was analyzed for differing alleles using the DNASTAR SeqMan Pro software (DNASTAR Version 15.0.1.1, Madison, WI, USA) against the reference genes from Liu et al. (2017).

Table 7: List of wheat varieties screened for *TB1* alleles.

Variety/Name	Type	Identifier
Alum	Spring	WA8166
Brennan	Spring	Syngenta 2009
Chinese Spring	Spring	CI 14108
Choteau	Spring	PI 633974
Corbin	Spring	WestBred, LLC 2006
Duclair	Spring	PI 660981
Egan	Spring	PI 671855
Lanning	Spring	PI 676978
McNeal	Spring	PI 574642
NS Presser CL	Spring	Northern Seed LLC 2016
Reeder	Spring	PI 613586
SY Ingmar	Spring	Syngenta 2015
SY Soren	Spring	Syngenta 2011
SY Valda	Spring	Syngenta 2015
Vida	Spring	PI 642366
WB Gunnison	Spring	WestBred, LLC 2011
Bearpaw	Winter	PI 665228
Bobcat	Winter	PI 693235
Decade	Winter	PI 660291
Flathead	Winter	PI 693237
FourOSix	Winter	PI 689753

Judee	Winter	PI 665227
Loma	Winter	PI 680576
MT1435	Winter	MSU experimental line
Northern	Winter	PI 676026
Rampart	Winter	PI 593889
Ray	Winter	PI 689754
StandClear CLP	Winter	PI 693236
SY Clearstone 2CL	Winter	Syngenta 2012
Warhorse	Winter	PI 670157
Yellowstone	Winter	PI 643428
Alzada	Durum	WestBred, LLC 2004
CDC-Precision	Durum	CFIA 7832, 2015
CDC-Vivid	Durum	CFIA #7220, 2012
Divide	Durum	PI 64021
Joppa	Durum	PI 673106
Mountrail	Durum	PI 607540
MT112219	Durum	MSU experimental line
MTD16005	Durum	MSU experimental line
ND-Riveland	Durum	PI 687796
Tioga	Durum	PI 660664

Table 8: Primers used for *TB1* mutation screen.

Name[a]	**Primer Sequence**	**Product Size bp**	**Annealing Temp. °C**
Tb-A-FLW TB1-ABD-RLW	5’ CGAGCCCCATGGACTTA 3’ 3’ CTTGTACCGTACGCATGTC 5’	1064	59
Tb-B-FLW TB1-ABD-RLW	5’ CATTCCTCCCCTGCATTT 3’ 3’ CTTGTACCGTACGCATGTC 5’	981	59
Tb-D-FLW TB1-ABD-RLW	5’ GGTGCAATGGCTCCTCG 3’ 3’ CTTGTACCGTACGCATGTC 5’	634	59
TB1-A Frag 2 For TB1-A Frag 2 Rev	5’ GCACATCTGATCATAGCTCCT 3’ 3’ GGGCTGCGAGTTGGGAAA 5’	1191	66
TB1-B Frag 2 For Tb-B1-RevLW	5’ CATGCCCTTTCCACCAC 3’ 3’ GAGGAGCCATTGCACCGTC 5’	1033	61
TB1-D Frag 2 For TB1-D Frag 2 Rev	5’ AGACGGGTGGTCTCAGGGT 3’ 3’ GGGCTGCGAGTTGGTAAA 5’	1094	65

Gene IDs used for developing primers for *TB1-A1, TB1-B1, and TB1-D1* are TraesCS4A02G271300, TraesCS4B02G042700, and TraesCS4D02G040100, respectively.

Root Analysis

F_4 seed of each genotype (AABB, aabb, AAbb, and aaBB) from the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross population was planted in the greenhouse in 20.3 cm pots filled with ceramic granules (PROFILE Products LLC, Buffalo Grove, IL, USA), with eight seeds per pot, thinned to four spaced plants per pot when plants were at the two-three leaf stage. Three different lines from each genotype were planted in individual pots as biological replicates, repeated three times as technical replicates. This whole 9-pot experiment was duplicated three times, for two data collection time points: one set one month after planting and two sets two months after planting. Pots were placed randomly into blocks by time point. Plants were gently removed from the ceramic granules, then rinsed in water to remove any granules clinging to the roots. Total roots in each pot were compiled in a paper bag for drying, with the same being done with the above-ground biomass. Tiller counts were also taken.

Statistical Analysis

In the original greenhouse trial, data were analyzed with one-way ANOVA and Tukey's test to compare all genotypes groups to each other using the agricolae package (de Mendiburu 2020) for R (R Foundation for Statistical Computing, Vienna, Austria). Based on the information gained from that experiment, we decided to compare each mutant genotype to a control, the double wild type group, in the field trials. The mean for each line over both replicates in each field trial environment was computed, then data was combined over all three environments before a mixed linear model was fitted in R using the lmer4 package in R (Bates et al., 2015) where the model included location,

genotype class, lines within genotype class, and location x genotype class interaction. Lines within genotype class were considered random and other factors were fixed. When genotype x environment interaction was present, each location was analyzed separately. Dunnett's test for comparing groups to a control was performed using the DescTools package in R (Signorell et al. 2020).

Root data was analyzed using a one-way ANOVA treating each date separately. Pairwise comparisons among genotype means were conducted using the emmeans package (Lenth 2020) for R data analysis software (R Foundation for Statistical Computing, Vienna, Austria). RNA data was first normalized to Actin using the method in (Oistad et al. 2016), then analyzed with two-tailed t-tests to detect differences in fold change for expression of genes in mutant genotypes versus the *TB1* wild type sample.

Results

Initial Greenhouse Trial

To test the impact of *TB1* mutations on plant growth under controlled conditions, F_2 *TB1* homozygous plants were grown in the greenhouse. Tillers and spikelets were counted at plant maturity. Total tillers, productive tillers, spikelets per head, and productive spikelets per plant were all compared across genotypes for each cross (Tables 9a and 9b). There were no statistically significant differences at the $P<0.05$ level among genotypes for either cross, however, the trend for both was towards increased total and productive tillers when one or more *TB1* null mutations were present.

Table 9a: Tiller, spikelet, and productive head comparisons of genotype class means from the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-W341** cross population in greenhouse trial.

Genotype	**n**	**Total Tillers (mean ± SE, No./plant)**	**Productive Tillers (mean ± SE, No./plant)**	**Spikelets/ Head (mean ± SE)**
TB1-A1, TB1-B1	15	6.27 ± 0.54[a]	2.93 ± 0.21[a]	14.49 ± 0.29[a]
*TB1-A1, TB1-B1- W341**	18	7.00 ± 0.49[a]	2.72 ± 0.19[a]	13.66 ± 0.26[a]
TB1-A1-W339, TB1-B1*	7	6.71 ± 0.79[a]	2.57 ± 0.31[a]	13.18 ± 0.42[a]
TB1-A1-W339, TB1-B1-W341**	9	7.89 ± 0.69[a]	3.00 ± 0.27[a]	14.12 ± 0.37[a]

[a] Numbers with different superscript letters differ at P<0.05.

Table 9b: Tiller, spikelet, and productive head comparisons of genotype class means from the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross population in greenhouse trial.

Genotype	**n**	**Total Tillers (mean ± SE, No./plant)**	**Productive Tillers (mean ± SE, No./plant)**	**Spikelets/ Head (mean ± SE)**
TB1-A1, TB1-B1	13	5.38 ± 0.39 [a]	2.92 ± 0.19 [a]	13.83 ± 0.28 [a]
*TB1-A1, TB1-B1- Q142**	12	6.42 ± 0.41 [a]	2.83 ± 0.20 [a]	13.19 ± 0.29 [a]
TB1-A1-W339, TB1-B1*	13	6.54 ± 0.39 [a]	2.92 ± 0.19 [a]	13.27 ± 0.28 [a]
TB1-A1-W339, TB1-B1-Q142**	12	6.17 ± 0.41 [a]	3.42 ± 0.20 [a]	13.07 ± 0.29 [a]

[a] Numbers with different superscript letters differ at P<0.05.

Field Trials

To test the impact of *TB1* null mutations on plant growth under field conditions, F_2:F_3 homozygous plants were grown in 2019 at one location and F_2:F_4 lines derived from that trial were grown in 2020 at two locations. Only tiller number in the *Tb1-A1-W339*/Tb1-B1a, Tb1-A1a/Tb1-B1-Q142** cross differed significantly (P<0.01) by environment, so only it was examined individually by location (Table 10). The double mutant genotype had 27 % more tillers than the wild type, and 18-21% more than the mutant groups in 2019, but no differences were found in either of the 2020 locations.

Table 10: Tiller number comparisons of genotype class means from the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross population grown in three environments.

Genotype	**n**	**Bozeman 2019 Tillers/plant**	**Bozeman 2020 Tillers/plant**	**Moccasin 2020 Tillers/plant**
TB1-A1, TB1-B1	11	16.2±0.58	15.9±0.71	11.3±0.67
*TB1-A1, TB1-B1-Q142**	10	17.6±0.56	16.4±0.53	11.4±0.50
TB1-A1-W339, TB1-B1*	11	17.3±0.90	15.5±0.52	12.4±0.64
TB1-A1-W339, TB1-B1-Q142**	10	21.0±0.80***	16.5±0.85	12.5±0.46

n denotes the number of lines in each genotype averaged across two replications.
Values represent the average for each genotype ± the standard error.
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

Finding no other GxE interactions, analysis was continued by averaging the two replications in an environment together, then combining the three environments. As shown in Tables 11a and 11b, the double mutant *TB1-A1-W339*, TB1-B1-Q142** had reduced spikelets and reduced flag leaf length, while *TB1-A1-W339*, TB1-B1* reduced only flag leaf length. Number of tillers when both mutations were present versus neither differed at P<0.05, while productive heads and plant height differed near P=0.07.

In the *Tb1-A1-W339*/Tb1-B1a, Tb1-A1a/Tb1-B1-W341** cross (Tables 12a and 12b), the double mutant had an increase in tillers per plant and reduced spikelets and flag leaf length. The *TB1-A1-W339*, TB1-B1* genotype also had reduced flag leaf length. Productive heads trended upwards (P=0.05) when both mutations were present.

RNA Sequencing Expression Analysis

Total RNA was extracted from a composite sample of each of the 4 TB1 genotypes in the *Tb1-A1-W339*/Tb1-B1a, Tb1-A1a/Tb1-B1-Q142** cross to assess the expression of a range of genes including *TB1* and genes associated with the GA (gibberellic acid) signaling, JA (jasmonic acid) signaling, ABA (abscissic acid)

Table 11a: Comparison of agronomic trait measurements taken on each of the four genotypes produced in the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross, from field trials combined across all three environments.

TB1 Genotype	**n**	**Plant Height cm**	**Tillers No./plant**	**Spikelets No./head**	**Productive Heads No./plant**	**Flag Leaf Length cm**	**Flag Leaf Width cm**
TB1-A1, TB1-B1	11	62.2±0.98	14.5±0.54	14.9±0.12	11.4±0.54	18.3±0.25	1.45±0.02
*TB1-A1, TB1-B1- Q142**	10	62.5±0.82	15.2±0.58	14.6±0.13	12.2±0.58	17.9±0.25	1.43±0.02
TB1-A1-W339, TB1-B1*	11	64.7±0.93	15.1±0.53	14.8±0.10	12.2±0.55	17.3±0.24**	1.44±0.01
TB1-A1-W339, TB1-B1-Q142**	10	65.1±1.01	16.7±0.76*	14.4±0.15*	13.4±0.71	16.9±0.25***	1.41±0.02

n denotes the number of ID's in each genotype averaged across two reps, in each environment.
Values represent the average for each genotype ± the standard error.
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

Table 11b: Comparison of seed trait measurements taken on each of the four genotypes produced in the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-Q142** cross, from field trials combined across all three environments.

TB1 Genotype	**n**	**Biomass g/plant**	**Yield g/plant**	**Grain Protein %**	**Seed Weight mg/seed**	**Seeds/Prod. Head No./head**	**Harvest Index**
TB1-A1, TB1-B1	11	20.4±1.11	19.9±1.39	14.3±0.11	42.5±0.88	60.6±3.10	0.48±0.02
*TB1-A1, TB1-B1- Q142**	10	21.3±0.96	20.7±1.25	14.4±0.16	44.3±0.77	56.1±2.64	0.49±0.02
TB1-A1-W339, TB1-B1*	11	20.8±0.95	20.5±1.30	14.4±0.18	42.7±0.68	56.0±2.35	0.49±0.02
TB1-A1-W339, TB1-B1-Q142**	10	22.6±1.45	20.5±1.29	14.2±0.28	41.9±1.24	51.7±2.62	0.48±0.02

n denotes the number of ID's in each genotype averaged across two reps, in each environment
Values represent the average for each genotype ± the standard error
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

Table 12a: Comparison of agronomic trait measurements taken on each of the four genotypes produced in the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-W341** cross, from field trials combined across all three environments.

TB1 Genotype	**n**	**Plant Height cm**	**Tillers No./plant**	**Spikelets No./head**	**Productive Heads No./plant**	**Flag Leaf Length cm**	**Flag Leaf Width cm**
TB1-A1, TB1-B1	16	69.7±0.83	14.9±0.42	15.0±0.11	12.0±0.42	20.2±0.22	1.48±0.01
*TB1-A1, TB1-B1- W341**	18	69.8±0.78	15.9±0.48	14.9±0.88	12.6±0.48	20.2±0.19	1.48±0.01
TB1-A1-W339, TB1-B1*	8	69.3±1.17	15.8±0.66	14.6±0.19	12.5±0.68	19.4±0.32**	1.53±0.02
TB1-A1-W339, TB1-B1-W341**	9	68.4±1.16	16.7±0.74*	14.5±0.13*	13.4±0.67	19.4±0.31***	1.47±0.02

n denotes the number of ID's in each genotype averaged across two reps, in each environment
Values represent the average for each genotype ± the standard error
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

Table 12b: Comparison of seed trait measurements taken on each of the four genotypes produced in the *Tb1-A1-W339*/Tb1-B1a*, *Tb1-A1a/Tb1-B1-W341** cross, from field trials combined across all three environments.

TB1 Genotype	**n**	**Biomass** g/plant	**Yield** g/plant	**Grain Protein** %	**Seed Weight** mg/seed	**Seeds/Prod. Head** No./head	**Harvest Index**
TB1-A1, TB1-B1	16	27.2±0.79	23.8±0.94	14.7±0.14	49.4±0.98	56.9±2.13	0.46±0.01
*TB1-A1, TB1-B1- W341**	18	29.8±0.96	23.5±0.96	14.8±0.18	50.0±0.86	55.0±2.26	0.44±0.01
TB1-A1-W339, TB1-B1*	8	27.3±1.08	23.8±1.11	15.2±0.16	49.5±1.24	55.1±2.89	0.46±0.02
TB1-A1-W339, TB1-B1-W341**	9	31.4±2.28	21.9±1.25	15.2±0.23	51.1±0.89	51.5±2.86	0.42±0.02

n denotes the number of ID's in each genotype averaged across two reps, in each environment
Values represent the average for each genotype ± the standard error
*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

biosynthesis, and ABA signaling pathways. These particular pathways were chosen due to their effect on plant growth and development. Table 13 shows significant fold change of the RPKM (mean reads per kilobase of transcript, per million mapped reads) of each mutant *TB1* sample versus the wild type *TB1*. All results were normalized to the housekeeping gene Actin. *TB1-A* and *TB1-B* were both expressed at low levels in all 4 genotypes, with *TB1-A* consistently higher. In the double mutant *TB1-A1-W339*, TB1-B1-Q142*, TB1-B* expression was decreased by 41%, while *TB1-A* expression was increased by 26%. In *TB1-A1-W339*, TB1-B1*, expression of both *TB1* genes was upregulated by around 55%. In *TB1-A, TB1-B1-Q142*,* both were downregulated, *TB1-A* by 43% and *TB1-B* by 61%.

The JA signaling pathway contained the most genes with significantly altered level of expression of the plant growth and development-regulating genes analyzed. Downregulated in that pathway were a WRKY gene, TRITD7Bv1G060720, decreased by 55% and a JAV1 gene, TRITD7Av1G252390, decreased by 74% in the double mutant sample. In the *TB1-A* mutant sample, a Jasmonyl-isoleucine synthetase gene TRITD1Bv1G222110 decreased by 23%.

Upregulated genes include two more WRKY genes: TRITD2Bv1G231460 increased by 241% in the *TB1-A* mutant, and TRITD7Bv1G060710 increased by 86% in the *TB1-B* mutant. In the ABA signaling pathway, TRITD4Av1G055360, a PYL5 gene was upregulated 47% in the *TB1-A* mutant, and a ZHD15 gene, TRITD4Bv1G015270, was downregulated 43% in the *TB1-B* mutant.

Table 13: List of genes analyzed using total RNA sequencing, with results including fold change from wild type displayed for each *TB1* mutant genotype.

		TB1-A1-W339*, TB1-B1-Q142*		***TB1-A1-W339*, TB1-B1***		***TB1-A1, TB1-B1-Q142****		***TB1-A1, TB1-B1***
Protein	**Gene**	**RPKM[a]**	**fold change**	**RPKM[a]**	**fold change**	**RPKM[a]**	**fold change**	**RPKM[a]**
Actin	TRITD4Av1G220530	144.0±0.00	1.00	144.0±0.00	1.00	144.0±0.00	1.00	144.0±0.00
TB1-A	TRITD4Av1G194310	1.41±0.12	1.26 *	1.72±0.68	1.54	0.64±0.06	0.57 **	1.12±0.06
TB1-B	TRITD4Bv1G012050	0.55±0.12	0.59	1.44±0.42	1.55	0.36±0.11	0.39 *	0.92±0.16
Gibberellin Signaling Pathway								
Rht-A[b]	TRITD4Av1G194130	94.65±12.13	0.96	101.95±12.42	1.03	83.73±1.47	0.85 *	99.07±3.79
Rht-B[b]	TRITD4Bv1G012280	122.78±12.84	1.01	137.82±17.31	1.14	107.06±5.86	0.88	121.00±12.92
IDD-like	TRITD3Av1G032110	12.17±1.07	0.87	14.81±1.87	1.06	10.70±0.17	0.77 *	13.91±1.02
BZR/BE S	TRITD2Av1G065030	30.54±5.12	1.14	29.80±2.09	1.11	23.52±0.65	0.88 *	26.74±0.85
Jasmonic Acid Signaling Pathway								
Jasmonyl-isoleucine synthetase	TRITD1Bv1G222110	4.70±0.38	0.78 *	4.68±0.27	0.77 *	5.69±0.36	0.94	6.05±0.20
Jasmonyl-isoleucine synthetase	TRITD3Av1G058120	2.11±0.25	1.65 *	2.59±0.73	2.03	1.18±0.11	0.92	1.28±0.02
COI1	TRITD3Bv1G206960	51.90±4.23	1.02	57.12±9.24	1.12	42.90±0.46	0.84	51.10±7.26
JAV1	TRITD7Av1G252390	0.12±0.02	0.26 *	0.58±0.29	1.21	0.87±0.45	1.81	0.48±0.08
WRKY	TRITD2Av1G267740	6.16±1.46	2.07	10.36±2.64	3.47 *	3.59±0.71	1.20	2.98±0.19
WRKY	TRITD2Bv1G231460	8.75±1.79	2.15 *	13.89±1.60	3.41 **	6.05±1.77	1.48	4.07±0.74
WRKY	TRITD3Bv1G082570	1.77±0.49	1.18	3.17±1.65	2.13	5.82±1.83	3.90	1.49±0.66
WRKY	TRITD6Av1G052760	38.51±5.11	1.13	51.45±19.95	1.51	69.57±12.38	2.04	34.12±11.04
WRKY	TRITD7Bv1G060710	0.32±0.03	0.90	0.75±0.32	2.09	0.67±0.01	1.86 **	0.36±0.05
WRKY	TRITD7Bv1G060720	0.35±0.04	0.45 **	0.69±0.30	0.91	0.67±0.08	0.88	0.76±0.04
JAZ	TRITD4Av1G184160	0.68±0.11	1.53	1.16±0.63	2.61	0.78±0.13	1.76	0.45±0.08
JAZ	TRITD4Bv1G007870	12.81±1.59	1.07	12.39±1.32	1.04	11.65±0.90	0.98	11.93±1.05
JAZ	TRITD0Uv1G028260	10.95±1.14	1.07	10.61±1.57	1.04	10.16±0.97	0.99	10.23±0.93
JAZ	TRITD4Bv1G172710	0.48±0.03	1.21	0.66±0.33	1.66	1.38±0.30	3.46	0.40±0.24

JAZ	TRITD6Av1G037770	26.33±0.62	1.12 *	24.57±0.69	1.05	21.51±1.57	0.92	23.44±0.54
Rht-A[b]	TRITD4Av1G194130	94.65±12.13	0.96	101.95±12.42	1.03	83.73±1.47	0.85 *	99.07±3.79
Rht-B[b]	TRITD4Bv1G012280	122.78±12.84	1.01	137.82±17.31	1.14	107.06±5.86	0.88	121.00±12.92
EIN3/EIL1	TRITD5Av1G159810	3.70±0.25	0.62 *	5.92±1.05	0.99	5.19±0.50	0.87	5.99±0.49
EIN3/EIL1	TRITD5Bv1G150070	4.42±0.72	0.67	6.27±1.18	0.95	6.39±0.86	0.97	6.61±0.76
ORA59/ ERF-like	TRITD4Bv1G001490	0.21±0.06	0.74	0.31±0.16	1.11	0.02±0.02	0.06	0.28±0.11
PDF 2.1-like	TRITD4Bv1G184330	22.28±3.68	0.94	17.70±1.62	0.75 *	18.09±2.37	0.76 *	23.71±0.97
PDF 2.1-like	TRITD6Bv1G028180	1.34±0.56	1.78	0.93±0.47	1.23	2.21±0.50	2.94	0.75±0.23
MYC-like	TRITD3Bv1G076400	12.47±1.13	0.84	16.83±2.51	1.13	18.95±3.24	1.27	14.89±0.23
MYC-like	TRITD3Bv1G148410	8.58±1.43	1.00	20.80±8.80	2.42	23.08±5.28	2.68	8.60±3.43
MYC-like	TRITD5Bv1G166430	3.30±1.08	1.15	1.61±0.28	0.56 *	3.33±1.49	1.16	2.86±0.26
MYC-like	TRITD5Bv1G166420	2.42±0.77	1.00	1.30±0.23	0.54	2.73±1.21	1.13	2.41±0.35
TPL	TRITD3Bv1G086210	44.56±6.45	0.67 *	65.89±8.18	0.98	55.05±5.01	0.82	66.96±4.10
TPL	TRITD7Av1G146530	59.46±13.74	0.76	76.61±8.01	0.98	66.94±1.26	0.86 *	77.84±2.98
TPL	TRITD7Bv1G106940	43.97±9.70	0.78	49.28±5.08	0.88	47.38±3.85	0.84	56.18±1.43
HDA19-like	TRITD6Av1G081960	51.80±3.33	0.80	51.41±2.49	0.80 *	53.68±6.41	0.83	64.38±4.95
HDA19-like	TRITD7Av1G198070	17.17±1.07	0.75 *	19.41±2.46	0.84 *	19.85±1.59	0.86	23.04±1.61
ABA Biosynthesis Pathway								
NCED/ TaNCED	TRITD2Bv1G125530	0.05±0.01	0.87	0.14±0.02	2.74 *	0.11±0.04	2.07	0.05±0.03
vp15	TRITD5Av1G193780	0.08±0.06	0.77	0.28±0.08	2.87 *	0.31±0.17	3.08	0.10±0.03
ABA Signaling Pathway								
PYR1-like	TRITD4Bv1G118420	20.44±3.23	1.46	21.36±1.34	1.53 *	13.70±1.90	0.98	13.97±1.66
PYL5	TRITD4Av1G055360	8.47±1.92	1.50	8.29±0.44	1.47 **	4.50±0.43	0.80	5.64±0.36
TEM1	TRITD1Av1G218480	1.38±0.33	1.31	3.31±1.29	3.15	2.49±0.53	2.36	1.05±0.04
ZHD15	TRITD4Bv1G015270	5.57±1.58	0.93	5.58±0.55	0.93	3.41±0.40	0.57 **	6.02±0.16

[a]Reads per kilobase of transcript, per million mapped reads, normalized to Actin (TRITD4Av1G220530). Mean plus and minus the standard error of three technical replicates

[b]Rht genes are part of both the gibberellin and jasmonic acid signaling pathways.

*, **, and *** denote P values of <0.05, 0.01, and 0.001 respectively.

Variety Screen

Forty wheat varieties were analyzed for mutations in *TB1*, along with controls containing known mutations. For specific varieties of spring and durum wheat and the mutations found, see Table 14. One silent mutation present in two varieties and one missense mutation predicted to have a neutral effect in one variety were found in the A genome of spring wheat. Two more spring wheat varieties were found to have the *TB-B1b* allele, consisting of two silent mutations and three missense mutations with predicted neutral effects, previously reported by Dixon et al. (2018). Also in the B genome, five durum varieties were found to have a missense mutation predicted to have a

Table 14: List of *TB1* mutations in Montana grown wheat.

Allele	**DNA change (bp from start codon)**	**Original Codon**	**New Codon**	**Provean Score[a]**	**Identified In:**
TB1-A1-A104A	312	GCT	GCC	NA	Alum, WB Gunnison
TB1-A1-A314P	940	GCT	CCT	-0.952	SY Valda
TB1-B1-S184G	550	AGC	GGC	-2.071	Riveland, Joppa, Tioga, Precision, Mountrail
TB1-B1-A271V[b]	812	GCA	GTA	-0.061	McNeal, Egan
TB1-B1-L340L[b]	912	TTG	TTA	NA	McNeal, Egan
TB1-B1-G66G[b]	198	GAG	GAA	NA	McNeal, Egan
TB1-B1-S103R[b]	309	AGC	AGG	-0.291	McNeal, Egan
TB1-B1-K221N[b]	663	AAA	AAT	-1.112	McNeal, Egan
TB1-D1-D110Y[c]	328	GAC	TAC	-7.939	Duclair, Vida, Choteau, NS Presser

[a]Provean (Protein Variation Effect Analyzer) score used to predict effect: A score of -2.5 for single amino acid substitutions is predicted to be deleterious 80% of the time, with higher scores having a deleterious effect less often and lower scores more often.
[b]These five mutations make up the *TB-B1b* allele from Dixon et al. 2018
[c]*TB1-D1-D110Y* from Dixon et al. 2018

borderline deleterious effect. Lastly, four spring wheat varieties were found to contain a predicted deleterious missense mutation in the D genome, also identified previously by Dixon et al. (2018).

Root Analysis

Root biomass, aboveground biomass, and tillers per pot were analyzed at one-month and two months post planting (Tables 15a and 15b). The number of lines analyzed per genotype group differed due to excluding pots that were affected by disease or mortality. At both time points, there were no detectable differences among genotypes in either root or aboveground biomass. One month after planting, plants with *TB1-A1-W339**, either with the wild type *TB1-B1* or in combination with the mutant *TB1-B1-Q142** allele, had 44% and 68% more tillers, respectively, than the *TB1-A1, TB1-B1* wild type. Two months after planting, only the double mutant *TB1-A1-W339*, TB1-B1-Q142** showed more tillers than wild type, at a 25% increase.

Table 15a: Root biomass, tillers, and aboveground biomass comparisons by genotype on a per plant basis, one month after planting.

Genotype	**n**	**Root Biomass (mean ± SE, g/plant)**	**Tillers (mean ± SE, #/plant)**	**Biomass (mean ± SE, g/plant)**
TB1-A1, TB1-B1	8	1.18 ± 0.23 [a]	12.0 ± 1.03 [a]	2.04 ± 0.30 [a]
*TB1-A1, TB1-B1-Q142**	9	1.19 ± 0.22 [a]	12.3 ± 0.97 [a]	2.17 ± 0.28 [a]
TB1-A1-W339, TB1-B1*	7	1.84 ± 0.25 [a]	17.3 ± 1.10 [b]	2.91 ± 0.32 [a]
TB1-A1-W339, TB1-B1-Q142**	8	1.78 ± 0.23 [a]	20.2 ± 1.03 [b]	2.95 ± 0.30 [a]

[a] Superscript numbers indicate groups with statistical differences at the P<0.05 level. Genotypes with the same letter mean there is no statistical difference between them.
n denotes the number of replicates (pots) measured for each genotype

Table 15b: Root biomass, tillers, and aboveground biomass comparisons by genotype on a per plant basis, two months after planting.

Genotype	**n**	**Root Biomass (mean ± SE, g/plant)**	**Tillers (mean ± SE, #/plant)**	**Biomass (mean ± SE, g/plant)**
TB1-A1, TB1-B1	14	4.69 ± 0.35 [a]	17.4 ± 0.98 [a]	18.9 ± 0.83 [a]
*TB1-A1, TB1-B1-Q142**	12	3.69 ± 0.38 [a]	16.1 ± 1.02 [a]	19.3 ± 0.90 [a]
TB1-A1-W339, TB1-B1*	13	4.07 ± 0.36 [a]	19.7 ± 1.02 [ab]	21.1 ± 0.85 [a]
TB1-A1-W339, TB1-B1-Q142**	17	3.84 ± 0.31 [a]	21.7 ± 0.92 [b]	19.6 ± 0.74 [a]

[a] Superscript numbers indicate groups with statistical differences at the P<0.05 level. Genotypes with the same letter mean there is no statistical difference between them.
n denotes the number of replicates (pots) measured for each genotype

Discussion

The initial greenhouse trial revealed trends of increases in number of total tillers and productive heads when *TB1* mutations were present, either alone or in combination (Tables 9a and 9b). Therefore, we proceeded with field trials on F_3 progenies. In the first year, we found that in the *Tb1-A1-W339*/Tb1-B1a, Tb1-A1a/Tb1-B1-Q142** cross, either mutation provided a tiller number increase of around 20%, while the mutations together added nearly 30% more tillers (Table 10). This agrees with previous research (Cubas et al. 1999, Xu et al. 2014, Kebrom et al. 2006, and Liu et al. 2018) indicating that mutations in *TB1* cause a bushier, more branched plant architecture in a variety of species. However, the current research was the first demonstration that *TB1* null mutations could be used to increase tiller number in wheat.

Continuing field trials in two locations for the second year, it was possible to examine GxE interactions. We found that only tiller number in the *Tb1-A1-W339*/Tb1-B1a, Tb1-A1a/Tb1-B1-Q142** cross interacted significantly with environment. This

interaction was further examined. The double mutant class produced more tillers per plant in the 2019 environment, but not in either of the 2020 environments (Table 10).

The remaining response variables were analyzed across environments. Spikelet numbers were reduced in the double mutant *TB1-A1-W339*, TB1-B1-Q142**, while flag leaves were shorter in both it and the *TB1-A1-W339*, TB1-B1* single mutant (Tables 11a and 11b). The reduction in spikelet number is in contrast to the findings of Dixon et al. (2018), who observed that paired spikelets were present when *TB1* was overexpressed. As expected, based on greenhouse trials, mutant genotypes had a trend of increased tillers and productive heads. There was also a trend of increased height with *TB1* null mutations, which agrees with Dixon et al. (2020) and Hubbard et al. (2002) who both found that *TB1* mutations resulted in taller plants.

In the other cross, the double mutant *TB1-A1-W339*, TB1-B1-W341** had increased tillers along with reduced spikelets per head, and it, along with the single mutant *TB1-A1-W339*, TB1-B1*, had reduced flag leaf length. All mutations alone displayed a trend towards increased biomass, more dramatically so when combined.

Gene expression analysis revealed few trends. A list of GA, JA, and ABA signaling pathway genes was analyzed along with *TB1-A* and *TB1-B*. Expression in the double mutant genotype was increased for *TB1-A*, which does not fit the expectations based on Dixon et al. (2018)'s findings that increased expression of *TB1-A* decreased tiller number. In this project, we used stop mutations that were found to increase tiller number. We were interested to see if the coding sequence change had any effect on RNA expression levels of *TB1* and other genes associated with growth regulating hormones.

TB1-B was reduced in the double mutant which did agree with Dixon et al. (2018). In the single mutant genotypes, *TB1-A* and *TB1-B* were both upregulated in the *TB1-A* mutant, and both downregulated in the *TB1-B* mutant. In the plant growth regulatory genes, expression varied considerably. There were no trends of a group of genes or even a single gene that were consistently up- or downregulated whenever mutations were present. Our results disagree with those of Dong et al. (2019) who found that in *TB1* mutant plants, all but two ABA biosynthesis and signaling genes were significantly downregulated, and all JA biosynthesis and signaling genes were significantly downregulated.

To determine if *TB1* mutant genotypes already exist amongst Montana top grown wheat varieties, a screen was conducted in which the entire *TB1* gene was sequenced and examined for mutations in 40 different varieties. In all, 9 different mutations were identified. The allele known as *TB1-B1b*, which consists of a suite of five mutations, was identified in McNeal and Egan spring wheat. This allele was previously identified by Dixon et al. (2018). Also identified by Dixon et al. (2018) research was the *TB1-D1-D110Y* mutation, which we found in four spring wheat varieties commonly grown in Montana. We also identified three mutations in durum wheat that were identified in our examination of previous literature. This indicates that there is natural allelic variation in both hexaploid and tetraploid wheat.

Along with examining the effects of *TB1* mutations on field plant characteristics and on gene expression, a belowground study on roots was conducted. Aboveground biomass, root biomass, and tiller counts were taken on the experimental plants. At one

month post-planting, the *TB1-A1-W339** allele, either with the wild type *TB1-B1* or in combination with the mutant *TB1-B1-Q142** allele, was associated with a large increase in tillering, while in the other cross there were only increased tillers when both mutations were present. By two months post-planting, only the double mutant had increased tillers. We did not see any statistically significant differences in above- or belowground biomass, which contradicts the findings of Gaudin et al. (2014), who found that in maize plants with a *TB1* mutation, increased tillering was strongly correlated with an increase in root biomass proportional to the increase in plant biomass. One factor that complicated this experiment was the tendency of the pots to hold water, which led to the development of fungal infections in the growth media and rotted roots. Overall, the plants did not appear healthy and robust compared to either the previous greenhouse trial or any of the field experiments. A different method of growing plants for root examinations may yield different and more representative results.

Each of the *TB1* experiments was intended to detect differences in tiller number and other agronomic characteristics due to *TB1* allelic differences. Although the presence of *TB1* mutations appeared to increase tiller number and plant height in the 2019 field trials, once two more environments/years were added, those results were less noticeable. Root biomass was not shown to be affected by *TB1* mutations, but that experiment may yield different results under more favorable growing conditions. Analyzing gene expression with RNA sequencing revealed that some genes which regulate plant growth hormones are upregulated, some downregulated, and many are unaffected. When taken together, these results indicate that *TB1* mutations in semi-dwarf

durum did not have the intended impact of increasing grain yield through increased tiller number when grown under spaced planted conditions. However, the trends do support further research on *TB1* mutations' impacts on wheat, including the potential to increase biomass of forage-type winter wheat and to examine the impacts in hexaploid spring wheat as well. The screen of allelic variation in *TB1* in currently growth Montana varieties show that natural variation does exist and may be a contributor to these varieties' success.

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