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Evaluating the impact of *Rht* hypomorphic mutations in durum wheat

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Abstract

Increasing the yield of wheat (*Triticum* spp.) requires identifying new allelic combinations by crossing or by creating useful variation in yield limiting genes. Wheat yield is impacted by many factors, including tiller number and seeds per tiller, both of which are impacted by the *Reduced height* (*Rht*) gene. Durum wheat [*T. turgidum* L. subsp. *durum* (Desf.) van Slageren] varieties are either standard height, wild type for *Rht* (*Rht-B1a*), or are semidwarf and carry the *Rht-B1b* allele. *Rht-B1b* increases productive tillers but can result in plants too short for easy harvest in the northern United States and shorter coleoptiles that reduce dry soil germination. In this study, durum plants varying for *Rht* alleles created by ethyl methanesulfonate (EMS) mutagenesis were studied to determine the impact of each allele upon agronomic and seed traits. The projects' goal is to increase durum wheat yield through the development of a plant with height intermediate between current full-height and semidwarf varieties. Experiments included field trials, coleoptile length and gibberellic acid (GA) responsiveness assays, and an in vitro test to determine the impact of each *Rht* mutation upon binding to Gibberellin Insensitive Dwarf1 (GID1). It was found that the *Rht-B1b-E529K* allele conferred plant height and coleoptile length intermediate between *Rht-B1b* and *Rht-B1a* containing plants, while two *Rht-A1* alleles had lesser impacts with trends toward intermediate-height plants. The results of this research demonstrate that hypomorphic *Rht* alleles that alter *Rht* binding to GID1 may prove useful in optimizing durum wheat height to increase yield across different growing conditions.

1 | INTRODUCTION

Durum wheat [*T. turgidum* L. subsp. *durum* (Desf.) van Slageren] is the tetraploid wheat species whose seeds are milled into semolina for pasta production. In the United States, durum wheat is grown mainly in four states: North Dakota, Montana, California, and Arizona (National Agricultural Statistics Service, 2020b). Durum wheat production ranged between 0.5 to 1 million ha in the United States from 2011 to 2020 (National Agricultural Statistics Service, 2020c). While durum wheat accounts for only 2–4% of the total wheat hectares planted (National Agricultural Statistics Service, 2020a), it is regionally economically important. For example, Montana harvested 220,000 ha of durum wheat in

Abbreviations: EMS, ethyl methanesulfonate; GA, gibberellic acid; GID, GIBBERELLIN INSENSITIVE DWARF1; PCR, polymerase chain reaction.

2019, worth about US$104 million (National Agricultural Statistics Service, 2019).

Durum wheat breeding program objectives include developing varieties with regionally important agronomic traits (e.g., race-specific disease resistance) and universally desired traits (e.g., high grain yield) (Clarke et al., 1998; Royo et al., 2009). Additional selection criteria that are important in durum wheat breeding programs include end-use quality traits like high seed protein content, semolina yield, gluten strength, pasta firmness, and yellow semolina and pasta color (Troccoli et al., 2000). One of the difficulties in selecting for increased yield is that many yield-impacting traits such as productive tillers, spikelets per head, and seed size are negatively correlated (Cantrell & Haro-Arias, 1986; Fischer et al., 1977). As a result, many studies designed to increase wheat yield by impacting a single yield component trait fail because of decreases in other yield components. Other studies have shown that factors impacting yield in some environments were ineffective in others (Destro et al., 2001; Elhani et al., 2007; Garcia del Moral et al., 2003; Moayedi et al., 2009).

Incorporation of semidwarfing alleles of the *Reduced height* (*Rht*) gene into hexaploid wheat (*Triticum aestivum* L.) varieties increased yield during the middle of the 20th century (Hedden, 2003; Pearce et al., 2011). The naturally occurring mutant alleles, *Rht-B1b* and *Rht-D1b*, each reduce hexaploid wheat height by of ∼20% and increase yield ∼10% (Flintham et al., 1997; Hoogendoorn et al., 1990; Jobson et al., 2018). Most modern hexaploid wheat varieties are semidwarf and contain either *Rht-B1b* or *Rht-D1b* (Knopf et al., 2008; Worland et. al. et al., 1998). The *Rht* semidwarfing alleles increase seeds per plant and productive tiller number, thereby increasing yield (Flintham et al., 1997; Hoogendoorn et al., 1990; Li et al., 2006; Pearce et al., 2011; Peng et al., 1999). However, *Rht-B1b* and *Rht-D1b* also decrease seed size and protein, along with reducing coleoptile and stem internode length (Amram et al., 2015; Fick & Qualset, 1976; Jobson et al., 2018; Liatukas & Ruzgas, 2011; Schillinger et al., 1998). *Rht-B1b* was also observed to decrease flag leaf photosynthetic rates at flowering (Jobson et al., 2019).

In the semidominant, altered function *Rht-B1b* and *Rht-D1b* alleles, a premature stop codon in the N-terminal DELLA domain prevents GIBBERELLIN INSENSITIVE DWARF1 (GID1; a receptor protein) from binding (Pearce et al., 2011; Peng et al., 1999). *Rht* translation reinitiates after the stop codon, which produces N-terminally truncated DELLA proteins that create the semidwarfing phenotype (Van De Velde et al., 2021). 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 creates the semidwarf phenotype seen in *Rht-B1b* and *Rht-D1b* hexaploid wheat plants.

Core Ideas

- *Rht* hypomorphic alleles result in intermediate plant height.
- *Rht-A* hypomorphic mutations reduce plant height.
- Intermediate-height plants carrying hypomorphic *Rht* alleles have longer coleoptiles.

Genes belonging to the DELLA domain family 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* [*Arabidopsis thaliana* (L.) Heynh.], *Slender1* [*SLN1* in barley (*Hordeum vulgare* L.)]; *SLR1* in rice (*Oriza sativa* L.), and *Dwarf 8* and *Dwarf 9* in maize (*Zea mays* L.) (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 altered plant growth (Peng et al., 1997; Chandler et al., 2008; Ikeda et al., 2001; Winkler & Freeling, 1994; Chandler et al., 2002). Of the listed orthologs, 80–95% of protein residues are shared with *Rht-1* (Wilhelm et al., 2013).

Much of the wheat *Rht*-related research has focused on the effects of *Rht-B1b* and *Rht-D1b* in hexaploid wheat, and less is known about the impact of *Rht* alleles in durum wheat. Incorporation of *Rht-B1b* into durum wheat increased grain yield ∼9% compared with the wild-type allele, while decreasing height by 45% (Mathews et al., 2006). The large height reduction can render semidwarf durum wheat varieties too short for mechanical harvest (Addisu et al., 2009). In addition, *Rht-B1b* reduces coleoptile length (Pandey et al., 2015). Short coleoptiles are associated with poor emergence when seeds are planted deeper to reach soil moisture as is common in dry climates (Schillinger et al., 1998). In Montana, only one of the commonly grown durum wheat varieties carries *Rht-B1b*. This provides an opportunity to create *Rht* variants in durum wheat that confer plant height intermediate between standard height and *Rht-B1b* semidwarfs but maintain increased yield potential.

The impacts of other mutant *Rht* alleles, including *Rht-B1c, Rht-B1d, Rht-B1e, Rht-D1c, Rht-D1d, Rht-8, Rht-3*, and *Rht-10*, were examined in hexaploid wheat, but no *Rht-A1* mutations have been found that reduce plant height (Flintham et al., 1997; Gale & Youssefian, 1985; Pearce et al., 2011). The phenotypic effects of *Rht-B1b* in durum wheat are more pronounced than in hexaploid wheat since *Rht-B1b* semidwarf hexaploid wheat still contains two wild-type *Rht* genes. In hexaploid wheat, *Rht-A* is expressed in stems at similar levels as *Rht-B* (Pearce et al., 2011); therefore, we hypothesized that *Rht-A* mutations in durum wheat would also have a height-suppressing effect. Hypomorphic

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 of a missense amino acid change: 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 Q64* 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 alone.

alleles of *Rht-A1* or *Rht-B1* could create a plant phenotype intermediate between *Rht-B1b* and *Rht-B1a*. A previously described hypomorphic mutation, *Rht-B1b-E529K*, increased plant height by 21% relative to *Rht-Blb* by partially suppressing the semidwarf phenotype associated with *Rht-B1b* (Mo et al., 2018).

Our goal here was to determine the impact of two *Rht-A1* missense alleles and the Rht-*B1b-E529K* allele on plant height, agronomic traits, coleoptile length and in vitro binding to GID1. The results demonstrate that the hypomorphic *Rht-A1* and *Rht-B1* alleles confer plant height and coleoptile length intermediate between that of durum wheat carrying only *Rht-1* wild-type alleles or *Rht-B1b*.

2 | MATERIALS AND METHODS

2.1 | Identification and selection of *Rht-A1* alleles

An ethyl methanesulfonate (EMS) population was created in the standard height durum wheat 'Divide' (Elias & Manthey, 2007) (PI 642021) by mutagenizing 10,000 seeds using the method described by Slade et al. (2005) and modified by Feiz et al. (2009), which involved soaking the seeds in a solution of 0.65% EMS for 16 h followed by washing them in cold tap water for 5 h and then planting in the greenhouse. The population was advanced by 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 $M_{4:5}$ seed. Plant height was measured on each head row and the 192 shortest rows were screened for mutations in *Rht-A* by direct sequencing of the *Rht-A* coding sequence after polymerase chain reaction (PCR) amplification as in Li et al. (2013). Seven missense and four silent mutations were identified, and the two lines containing *Rht-A1* missense mutations with the lowest protein variation analyzer (PROVEAN) scores were chosen for this project: *Rht-A1-S50F* and *Rht-A1-L358F* (Table 1). The durum wheat 'Kronos' TILLING mutant (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 with those reported by Mo et al. (2018).

2.2 | Creation of BC_1F_3 lines

Montana-adapted parents for backcrossing include 'Lustre' (standard height durum wheat, fixed for *Rht-B1a*) (Hogg et al., 2021) and line MT112219 (semidwarf durum wheat, fixed for *Rht-B1b*). The F_1 crosses were made between each of the three *Rht*-allele-containing lines and both recurrent parents. The first backcross was made, then plants allowed to self to produce BC_1F_2 seed. DNA was extracted from individual BC_1F_2 plants and PCR was performed using the nested approach of 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. The PCR products were Sanger sequenced (GENEWIZ, Inc.), then the sequence was compared 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). Plants 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.

2.3 | Field trials

One hundred BC_1F_2 plants from each of the six crosses were grown in 2019 under irrigation at the Arthur H. Post Agronomy Farm near Bozeman, MT (45.67° N, 111.15° W, 1, 455 m asl) in an Amsterdam silt loam (Fine-silty, mixed, superactive, frigid Typic Haplustolls) with pH 6.5. Soil tests were conducted and 185 kg urea ha^{-1} (46-0-0) was added to ensure sufficient available N. The plants were grown in 3-m rows with 30-cm spacing between adjacent rows. Within each

row, seeds were sown by hand to a depth of 2.5 cm on 10 May 2019. Seeds were planted 23 cm apart with 13 plants per row. Plants were 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 the 2020 field trial.

From 1 May to 31 August, the research station received 18.6 cm of precipitation. The highest recorded air temperature was 32.3 ˚C on 23 July, while the lowest recorded air temperature was −0.56 ˚C on 19 May (https://www.ncdc.noaa.gov/cdo-web/datasets/GHCND/stations/GHCND:USC00241047/detail). Five centimeters irrigation water was applied using hand line sprinklers on 1 July. A 1.1 L ha^{-1} application of Huskie (pyrasulfotole 3.3%, bromoxynil octanoate 13.4%, bromoxynil heptanoate 12.9%; Bayer CropScience) was applied for broadleaf weed control on 10 June, and throughout the growing season, weeds were rogued out by hand. At harvest maturity, single plants were cut at ground level, weighed, and grain collected using a single-plant thresher the second week of September.

In 2020, 120 $BC_1F_{2:3}$ lines (10 lines in each of the two *Rht* allelic groups for each of the six 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. A plot was a single 1.5-m row with 30-cm spacing between rows. Within a row, seeds were planted 15 cm apart with eight plants per row. Seeds were hand planted to a depth of 2.5 cm on 28 April. From 1 May to 31 August, the research center received 14.3 cm of precipitation. The highest recorded air temperature was 34 ˚C on both 17 and 18 August, while the lowest recorded air temperature was −0.6 ˚C on 8 and 11 May (NOAA Bozeman). The trial was fertilized with 185 kg urea ha^{-1} , and weed and disease control consisted of 0.06 L ha^{-1} of Affinity Broadspec (thifensulfuron-methyl 25%, tribernuron-methyl 25%; FMC Corp.), 0.58 L ha^{-1} MCPA Ester 4 (2-ethylhexyl ester of 2-methyl-4-chlorophenoxyacetic acid 69.7%; Albaugh, LLC), and 1.2 L ha^{-1} Discover NG (Clodinafop-propargyl 6.4%; Syngenta Crop Protection, LLC), and 0.29 L ha^{-1} Propi-Star EC (Propiconazole 41.8%; Albaugh, LLC) applied on 1 June.

The same trial was planted on 11 May 2020 at the Central Agricultural Research Center near Moccasin, MT (1,297 m asl) in a mix of soil types: Danvers clay loam (fine, smectitic, frigid Vertic Argiustolls) and Judith clay loam (fine-loamy, carbonatic, frigid Typic Calciustolls). This was a dryland site and, as such, did not receive any additional moisture from irrigation. Seeds were planted as in Bozeman. From 1 May to 31 August, the research center received 19.5 cm of precipitation. The highest recorded air temperature was on 18 August at 36 ˚C, while the lowest recorded air temperature was −1 ˚C on 12 May (https://agresearch.montana.edu/carc/weather/index.html). The plot area was fertilized with 67.3 kg ha^{-1} ESN slow-release nitrogen fertilizer (44-0-0, Nutrien). Vendetta (Octanoic acid ester of bromoxynil 31.7%, 2-Ethylhexyl ester of 2-methyl-chlorophenoxyacetic acid 34%; Wilbur-Ellis Agribusiness) was applied on 27 May for broadleaf weed control.

2.4 | Plant measurements

Plant height was measured at harvest maturity as 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. The number of productive heads included only heads that formed seed. Number of spikelets per head was determined as the number of fertile spikelets per head. Likewise, number of seeds per head was the number of seeds per productive head. Flag leaf length of the tallest tiller was measured from culm to tip and flag leaf width at the widest point of the leaf. These plant measurements were taken from single plants in 2019 and from five representative plants per plot in the 2020 trials. At physiological maturity, five representative plants from each plot were cut at ground level. The plants were bundled and weighed and then threshed with a single-plant thresher. Straw weight was determined as the difference between biomass yield (grain plus straw) and grain weight. The biomass, straw, and grain weights were adjusted to 10% moisture and harvest index was computed. Seed protein content was analyzed with the LECO-FP 528 (LECO Co.) (AACC, 2009) and individual seed weight was calculated by hand by weighing a sample of 100 seeds counted using a Contador seed counter (Pfeuffer GmbH).

2.5 | GA responsiveness assay: Coleoptile length

To evaluate the impact of the *Rht-1* mutations on coleoptile length and responsiveness to GA, the 'cigar roll' technique described by Bai et al. (2012) was used. The BC_1F_4 seeds were surface sterilized and soaked in water for 24 h. One roll consisted of 10 seeds from a single $BC_1F_{2:4}$ line placed ~2 cm apart on 46- by 31-cm germination paper (Anchor Paper Co.). The germination paper was presoaked 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. There were 10 lines for each of the two *Rht* allelic groups $BC_1F_{2:4}$ 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 that submerged the bottom 2 cm of the rolls in either water or 100-μM GA_3. 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 d.

2.6 | 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. The *Rht* coding sequences for the tested alleles were synthesized and cloned into vectors using GENEWIZ Gene Synthesis Services (GENEWIZ). Bait and prey vectors were co-transformed into the *Saccharomyces cerevisae* strain Y2HGold using the Yeastmaker Yeast Transformation System 2 (Clontech) and the Matchmaker Gold Yeast Two-Hybrid System (Takara Bio Inc.). Colonies were grown on selectable SD media lacking leucine and threonine (SD/-L-T) for 4–5 d to select for successful co-transformation. The 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, 1:1,000, and 1:10,000 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 tested *Rht* allele was compared with *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.

2.7 | Statistical analysis

Comparisons between the *Rht-1* allelic class means for each cross were made using a one-way analysis of variance (ANOVA) in R (R Foundation for Statistical Computing) for the 2019 spaced-plant trial. Data for the 2020 trials were combined over locations for each cross. The model was as follows:

Response = location + replication within location + genotype class + lines within genotype class + location × genotype class + location × lines within genotype class + random error. Individual lines within genotype class and their interaction with location were considered random and other factors were fixed using the lmer4 package in R (Bates et al., 2015).

Data for the coleoptile length trial were analyzed as a randomized complete block design for each cross. The model included replication, genotype class, and lines within genotype class using the lmer4 package in R, where lines within genotype class were considered random. Differences in length between *Rht* allelic group means were determined from an ANOVA *F* ratio.

3 | RESULTS

3.1 | 2019 Field trial

The effect of durum wheat *Rht* mutations in BC_1F_2 sister lines under field conditions was tested with a spaced, single-plant trial (Tables 2 and 3). In the semidwarf *Rht-B1b* background, only the *Rht-B1b-E529K* mutation caused any allelic differences ($P < .001$). The *Rht-B1b-E529K* allelic group was 17 cm taller than *Rht-B1b*-containing genotypes. In the standard height *Rht-B1a* background, the *Rht-B1b-E529K* allele decreased height by 10 cm ($P < .001$) (11%) relative to *Rht-B1a*. Both *Rht-A1-S50T* and *Rht-A1-L358F* also decreased plant height, the former by 6 cm ($P < .001$) (7%) and the latter by 3 cm (3%) ($P < .001$). *Rht-A1-L358F* also increased tillers by 2.7 tillers per plant ($P < .05$) (17%) and grain protein by 0.9% ($P < .05$) (Table 3), relative to *Rht-A1a* in the *Rht-B1a* background. Differences between allelic class means were not detected for the other traits.

3.2 | 2020 Field trial

To expand on the 2019 field trial, 10 $BC_1F_{2:3}$ lines from the *Rht* EMS allele genotype and 10 from the native *Rht* genotype within each cross were chosen to be evaluated in space-planted, replicated trials at two locations (Tables 4 and 5). Although location effects were detected for most traits for individual crosses, location × genotype effects were infrequent ($P < .05$). Therefore, mean values are presented averaged over locations. Important interactions are described below. The *Rht-B1b-E529K* mutation showed significant effects ($P < .001$) in the semidwarf (*Rht-B1b*) background, increasing height by 11 cm (16%) relative to *Rht-B1b*. The *Rht-A1-S50F* mean, was 2 cm (3%) shorter ($P < .05$) than *Rht-B1b* alone. In the standard height (*Rht-B1a)* background, the *Rht-B1b-E529K* mutation reduced plant height 6 cm (8%) ($P < .01$). The plant height reduction from the *Rht-B1b-E529K* mutation was significant ($P < .05$) in both locations but more so at Bozeman than Moccasin, which gave rise to the significant ($P < .05$) genotype × location interaction. The *Rht-B1b-E529K* allele decreased flag leaf length by 1 cm (4.6%) ($P < .01$) compared with *Rht-B1a* (Table 4). The *Rht-A1-S50F* mutation increased flag leaf width by 0.1 cm (4.7%) ($P < .05$) in the *Rht-B1a* background, but the *Rht-A1-L358F* mutation decreased flag leaf width in the *Rht-B1b* background. Genotype class means were in opposite directions for the *Rht-B1b-E529K* vs. *Rht-B1b* class means but neither difference was significant ($P < .05$). The *Rht-A1-L358F* mutant in the standard height background gave narrower leaves than the *Rht-A1a* counterpart at Moccasin (1.36 vs 1.46 mm, $P < .05$) but not for the Bozeman location (1.58 vs 1.54 mm). This led to

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

Recurrent parent *Rht* allele	*Rht-1* allele	*n* [a]	Plant height[b]	Tillers[b]	Productive heads[b]	Flag leaf length[b]	Flag leaf width[b]
			cm	No. plant^{-1}		cm	
Rht-B1b	*Rht-B1b-E529K*	22	90.0 ± 2.07***	11.8 ± 0.71	8.23 ± 0.73	18.5 ± 0.49	1.59 ± 0.02
	Rht-B1b	16	73.5 ± 0.94	12.0 ± 0.72	7.38 ± 0.76	18.4 ± 0.52	1.52 ± 0.00
	Rht-A1-S50F	23	61.3 ± 0.67	15.7 ± 0.76	7.09 ± 0.34	17.5 ± 0.42	1.50 ± 0.04
	Rht-A1a	22	62.2 ± 0.75	15.3 ± 1.00	6.55 ± 0.39	16.9 ± 0.39	1.43 ± 0.03
	Rht-A1-L358F	17	67.4 ± 0.96	14.4 ± 0.90	5.88 ± 0.46	17.4 ± 0.45	1.61 ± 0.03
	Rht-A1a	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*	20	85.6 ± 1.19***	14.0 ± 0.85	7.75 ± 0.50	21.1 ± 0.49	1.55 ± 0.03
	Rht-B1a	27	95.1 ± 1.28	14.2 ± 0.70	8.30 ± 0.61	21.3 ± 0.40	1.60 ± 0.03
	Rht-A1-S50F	25	87.9 ± 1.10***	13.6 ± 0.83	6.63 ± 0.40	21.9 ± 0.37	1.60 ± 0.03
	Rht-A1a	23	94.0 ± 1.09	15.1 ± 0.90	6.52 ± 0.46	22.0 ± 0.34	1.58 ± 0.04
	Rht-A1-L358F	21	93.5 ± 0.66**	18.4 ± 0.71*	5.88 ± 0.46	22.2 ± 0.42	1.70 ± 0.03
	Rht-A1a	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.
*Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level.

TABLE 3 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]	Grain yield	Grain protein[b]	Grain weight[b]	Seeds per productive head[b]
			g plant^{-1}	g kg^{-1}	mg seed^{-1}	No. head^{-1}
Rht-B1b	*Rht-B1b-E529K*	22	19.2 ± 1.69	173 ± 0.8	49.6 ± 1.14	47.1 ± 1.92
	Rht-B1b	16	16.0 ± 1.51	172 ± 3.8	47.2 ± 3.01	45.4 ± 2.38
	Rht-A1-S50F	23	18.9 ± 0.78	160 ± 0.9	44.9 ± 0.82	61.5 ± 2.86
	Rht-A1a	22	16.1 ± 1.36	157 ± 0.5	44.6 ± 0.64	54.4 ± 2.74
	Rht-A1-L358F	17	14.6 ± 1.41	158 ± 2.1	47.7 ± 0.86	52.8 ± 3.06
–	*Rht-A1a*	16	15.7 ± 1.72	164 ± 4.9	48.8 ± 1.06	53.4 ± 3.06
Rht-B1a	*Rht-B1b-E529K*	20	25.6 ± 1.71	163 ± 3.4	46.4 ± 2.38	68.0 ± 2.01
	Rht-B1a	27	25.7 ± 1.47	166 ± 0.4	46.9 ± 1.80	63.5 ± 2.22
	Rht-A1-S50F	25	20.0 ± 1.52	176 ± 0.8	45.5 ± 1.95	63.3 ± 2.52
	Rht-A1a	23	17.7 ± 1.53	174 ± 0.5	46.1 ± 0.57	58.8 ± 2.07
	Rht-A1-L358F	21	24.4 ± 1.34	174 ± 1.8*	47.1 ± 0.56	71.9 ± 0.93
–	*Rht-A1a*	13	21.4 ± 1.08	165 ± 2.3	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.
*Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level.

the two significant genotype × location interactions for leaf width. Spikelets per head did not differ among allele class means except where *Rht-A1-L358F* had 0.5 (3%) ($P < .05$) fewer spikelets per head than *Rht-A1a* in the *Rht-B1a* background. The *Rht-B1b*-E529K vs. *Rht-B1b* in the semidwarf background had equal means for spikelet number averaged over locations but showed interaction with location. Trends were opposite for Bozeman (15.6 vs. 16.0 spikelets per head) and Moccasin (16.0 vs. 15.5, $P < .05$). The only other significant difference in allelic class means was for productive tillers, where the *Rht-B1b-E529K* allele increased productive tillers by 1.2 tillers per plant ($P < .05$).

TABLE 4 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	Tillers	Productive heads	Spikelets	Flag leaf length	Flag leaf width
		cm	No. plant^{-1}			cm	
Rht-B1b	*Rht-B1b-E529K*	79.6 ± 1.86***	13.2 ± 0.45	10.6 ± 0.34	15.8 ± 0.13	20.9 ± 0.18	1.43 ± 0.01
	Rht-B1b	67.7 ± 0.52	13.1 ± 0.66	10.2 ± 0.50	15.8 ± 0.11	21.2 ± 0.47	1.43 ± 0.02
	Genotype × location	NS[a]	NS	NS	**	NS	*
	Rht-A1-S50F	56.9 ± 0.61*	17.4 ± 0.10	12.7 ± 0.76	16.9 ± 0.48	19.4 ± 0.31	1.42 ± 0.02
	Rht-A1a	59.1 ± 0.51	16.3 ± 0.84	12.0 ± 0.66	16.3 ± 0.18	18.9 ± 0.13	1.39 ± 0.02
	Genotype × location	NS	NS	NS	NS	NS	NS
	Rht-A1-L358F	65.5 ± 0.54	15.5 ± 0.77	11.4 ± 0.59	15.6 ± 0.17	18.9 ± 1.18	1.44 ± 0.02*
	Rht-A1a	65.2 ± 0.65	14.6 ± 0.73	10.9 ± 0.51	15.6 ± 0.14	19.8 ± 0.22	1.52 ± 0.02
–	Genotype × location	NS	NS	NS	NS	NS	NS
Rht-B1a	*Rht-B1b-E529K*	80.0 ± 1.06**	15.4 ± 0.96	11.8 ± 0.73*	18.8 ± 0.22	21.9 ± 0.20**	1.44 ± 0.02
	Rht-B1a	87.0 ± 1.57	14.8 ± 1.02	10.6 ± 0.73	19.4 ± 0.24	22.9 ± 0.27	1.48 ± 0.02
	Genotype × location	*	NS	NS	NS	NS	NS
	Rht-A1-S50F	81.0 ± 0.96**	14.7 ± 0.83	10.8 ± 0.64	18.1 ± 0.18	22.8 ± 0.32	1.57 ± 0.02*
	Rht-A1a	84.7 ± 1.43	15.0 ± 1.25	11.4 ± 0.92	18.0 ± 0.31	22.7 ± 0.37	1.48 ± 0.02
	Genotype × location	NS	**	*	NS	NS	NS
	Rht-A1-L358F	85.9 ± 0.58	13.6 ± 1.01	10.4 ± 0.59	17.8 ± 0.14*	22.6 ± 0.33	1.47 ± 0.03
	Rht-A1a	86.9 ± 0.47	14.5 ± 0.95	10.8 ± 0.51	18.3 ± 0.16	22.4 ± 0.34	1.50 ± 0.02
–	Genotype × location	NS	NS	NS	NS	NS	**

Note. Values represent the average for each genotype ± the standard error. $N = 20$ values in each genotype group, each value representing the average of five plants each in two replicates and in two locations.

*Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level.

[a]NS, genotype × location not significant with P value > .05.

We did detect genotype × location interaction for the *Rht-A1-S50F* mutant and its alternate allele in both the semidwarf and standard-height backgrounds for biomass, straw yield, and grain yield (Table 5). This was primarily because the Bozeman location had greater differences, and the differences between allele class means were in opposite directions in some cases for the two locations. In the semidwarf background, differences between *Rht-A1-S50F* and *Rht-A1a* classes were 63.8 vs. 55.3 g plant^{-1} ($P < .05$) at Bozeman and 36.1 vs. 39.7 g plant^{-1} at Moccasin for biomass; 33.5 vs. 27.8 g plant^{-1} ($P < .05$) at Bozeman and 20.3 vs. 21.2 g plant^{-1} at Moccasin for straw yield and 30.3 vs. 27.5 g plant^{-1} at Bozeman and 15.7 vs. 18.5 g plant^{-1} at Moccasin. Similarly, in the standard-height background, those allele class mean differences were 81.2 vs. 71.5 g plant^{-1} at Bozeman and 48.1 vs. 39.5 g plant^{-1} at Moccasin for biomass; 40.6 vs. 47.4 g plant^{-1} at Bozeman and 28.7 vs. 23.9 g plant^{-1} at Moccasin for straw yield; and 31.1 vs. 34.1 g plant^{-1} at Bozeman and 19.4 vs. 15.6 g plant^{-1} at Moccasin for grain yield

3.3 | GA Responsiveness assay: Coleoptile length

The *Rht-B1b-E529K* mutation significantly affected coleoptile length in both the semidwarf and standard-height backgrounds (Table 6). Coleoptile length was reduced by 18 mm (20%) ($P < .001$) without GA present and 29 mm (28%) ($P < .001$) with GA in the standard-height background. However, in the semidwarf background, coleoptile length was increased by 17 mm (23%) ($P < .01$) in water and 20 mm (25.3%) ($P < .01$) in the presence of GA.

There were no significant differences between the sister lines segregating for *Rht-A* mutations.

In the semidwarf background, coleoptile length increased in GA compared with water in the *Rht-B1bE529K* allelic group ($P < .08$) and *Rht-B1b* ($P < .05$) classes. The two *Rht-A* EMS allelic groups were unchanged between GA and water in the presence of *Rht-B1b*. Coleoptile length increased in GA compared with water for the *Rht-B1a* group from each of the

TABLE 5 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 yield	Straw yield	Grain yield	Harvest index	Grain protein	Seed weight	Seeds per productive head
		g plant^{-1}				g kg^{-1}	mg seed^{-1}	No. head^{-1}
Rht-B1b	*Rht-B1b-E529K*	55.6 ± 1.5	29.4 ± 1.26	26.3 ± 1.21	0.48 ± 0.01	134 ± 3.1*	45.5 ± 1.01	54.7 ± 1.88*
	Rht-B1b	56.9 ± 2.4	28.9 ± 1.68	27.9 ± 1.67	0.49 ± 0.01	129 ± 3.1	43.9 ± 0.93	64.2 ± 4.55
	Genotype × location	NS[a]	NS	NS	NS	NS	NS	–
	Rht-A1-S50F	50.0 ± 2.2	26.9 ± 1.85	23.0 ± 1.86	0.45 ± 0.01	137 ± 1.5	38.0 ± 0.94	47.6 ± 2.37
	Rht-A1a	47.5 ± 3.1	25.4 ± 1.51	23.0 ± 1.63	0.48 ± 0.01	135 ± 1.5	39.2 ± 1.02	48.2 ± 2.03
	Genotype × location	*	*	*	NS	NS	NS	–
	Rht-A1-L358F	45.9 ± 5.0	23.4 ± 2.54	22.6 ± 1.97	0.48 ± 0.01	128 ± 2.2	41.0 ± 0.88	52.8 ± 5.10
	Rht-A1a	47.0 ± 1.5	24.3 ± 1.67	22.6 ± 1.62	0.48 ± 0.01	127 ± 2.2	40.6 ± 0.86	48.9 ± 2.31
–	Genotype × location	NS	NS	NS	NS	NS	NS	–
Rht-B1a	*Rht-B1b-E529K*	66.7 ± 5.2	37.4 ± 3.19	29.3 ± 3.16	0.43 ± 0.01	126 ± 2.7	41.1 ± 1.12	58.3 ± 2.54
	Rht-B1a	69.5 ± 5.3	40.3 ± 4.06	29.2 ± 3.42	0.41 ± 0.01	125 ± 2.2	41.4 ± 1.16	61.9 ± 2.97
	Genotype × location	NS	NS	NS	NS	NS	NS	–
	Rht-A1-S50F	59.9 ± 3.0	34.7 ± 2.10	25.3 ± 1.62	0.42 ± 0.01	140 ± 2.9	40.5 ± 0.69	58.3 ± 2.67
	Rht-A1a	60.5 ± 2.8	35.7 ± 3.39	24.8 ± 2.44	0.41 ± 0.01	140 ± 3.2	40.6 ± 0.72	52.7 ± 1.96
	Genotype × location	*	*	*	NS	NS	NS	–
	Rht-A1-L358F	51.3 ± 2.9	27.4 ± 2.20	22.9 ± 2.07	0.44 ± 0.02	138 ± 2.8	38.8 ± 0.79	56.7 ± 3.00
	Rht-A1a	53.3 ± 2.5	30.1 ± 2.6	23.3 ± 2.22	0.42 ± 0.02	139 ± 2.2	38.1 ± 0.93	53.5 ± 1.77
–	Genotype × location	NS	NS	NS	NS	NS	NS	–

Note. Values represent the average for each genotype ± the standard error. $N = 20$ values in each genotype group, each value representing the average of five plants each in two replicates and in two locations.

*Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level.

[a]NS, genotype × location not significant with P value > .05.

TABLE 6 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[b]	Coleoptile length in GA[b]	GA/water[c]
			mm		
Rht-B1b	*Rht-B1b-E529K*	10	88.0 ± 4.24**	99.4 ± 4.60**	1.13
	Rht-B1b	10	71.3 ± 1.55	79.3 ± 2.54	1.11**
	Rht-A1-S50F	10	64.7 ± 2.31	65.4 ± 2.50	1.01
	Rht-A1a	10	63.1 ± 1.50	60.4 ± 1.90	0.96
	Rht-A1-L358F	10	62.4 ± 1.60	58.9 ± 1.38	0.94
	Rht-A1a	10	60.5 ± 2.01	58.3 ± 1.62	0.96
Rht-B1a	*Rht-B1b-E529K*	10	74.6 ± 2.43***	74.9 ± 2.73***	1.00
	Rht-A1a	10	93.1 ± 3.78	104.1 ± 2.26	1.11*
	Rht-A1-S50F	10	107.7 ± 2.20	114.3 ± 2.84	1.06
	Rht-A1a	10	107.9 ± 2.40	113.4 ± 3.19	1.05
	Rht-A1-L358F	10	104.9 ± 2.34	109.5 ± 2.38	1.04
	Rht-A1a	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.

*Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level.

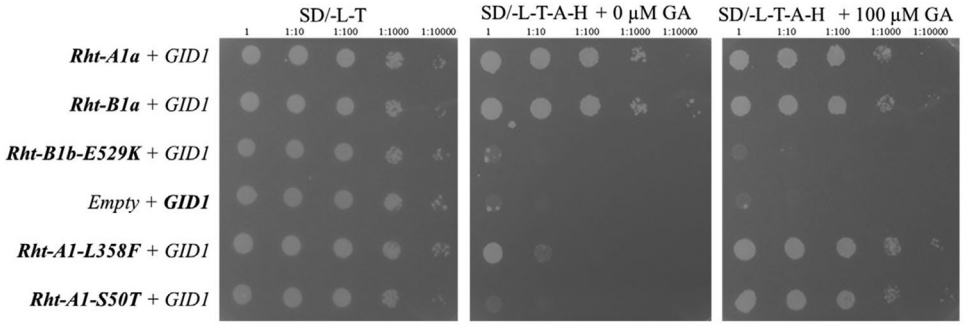

FIGURE 1 Yeast-2-hybrid assay of durum wheat ethyl methanesulfonate-derived *Rht* mutations to characterize the interaction between DELLA and GID1 in the presence or absence of 100-µM GA. Serial dilutions on SD/-L-T (control) media, SD/-L-T-A-H media, and SD/-L-T-A-H media + 100-µM GA are shown. *Rht-A1a* 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

three crosses but only reached significance at the 5% level for two of the three groups. The *Rht-B1bE529K* allelic group had nearly identical values between water and GA, while the two *Rht-A* mutants trended higher in GA than in water in the *Rht-B1a* background.

3.4 | GA Responsiveness assay: *Rht*–GID1 interaction

Durum wheat *Rht-A1* 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 varied effects. *Rht-A1-S50F* showed no interaction without GA but restored interactions 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 like *Rht-A1a* when GA was present.

4 | DISCUSSION

Two *Rht-A1* missense alleles created via EMS mutagenesis and the previously reported *Rht-B1b-E529K* allele (Table 1) were demonstrated to impact several plant growth traits. In both the BC_1F_2 (Table 2) and BC_1F_3 (Table 4) generations, we observed the previously reported height-increasing effect of *Rht-B1b-E529K* when crossed to a semidwarf parent (Mo et al., 2018). The effect was greater in 2019, with a 22% increase vs. a 16% increase in 2020 and may reflect differences in growing conditions between the two years. We also found that the *Rht-B1b-E529K* decreased plant height when crossed to a standard-height parent. Again, the effect was more pronounced in the 2019 (Table 2) trial, with an 11% reduction compared with an 8% reduction in 2020 (Table 4). Both the semidwarf and the standard-height crosses to *Rht-B1b-E529K* gave an intermediate-height phenotype between current semidwarf and standard-height varieties.

When comparing the *Rht-A1* EMS alleles to *Rht-A1a* in the presence of *Rht-B1b*, neither *Rht-A1-S50T* nor *Rht-A1-L358F* changed plant height in the 2019 trial (Table 2); however, the *Rht-A1-S50T* group was significantly shorter ($P < .05$) than the *Rht-A1a* group in the 2020 trial (Table 4). When the *Rht-A1* missense mutations were crossed to the standard-height parent, the *Rht-A1* EMS alleles were significantly shorter ($P < .01$) relative to *Rht-A1a* in 2019. Only the *Rht-A1-S50T* mutation showed significant ($P < .05$) height reduction in the 2020 trial. One possible reason for the differing results between years may be that plants overall were taller in 2019 than 2020 because of more favorable rainfall, and the differences between class means were accentuated. None of the mutations tested here contributed to consistent changes in agronomic and seed traits (Tables 2–5). Observing differences in yield traits will require full-density field trials and isolines created in multiple backgrounds.

Although no height-reducing *Rht-A* alleles have been identified in hexaploid wheat (Flintham et al., 1997; Gale & Youssefian, 1985; Pearce et al., 2011), our results indicate that the specific *Rht-A* missense mutations presented here would result in reduced-height plants in the absence of *Rht-B1b* or *Rht-D1b*. *Rht-B1b-E529K* was shown to be useful for creating intermediate-height durum wheat plants when used in place of *Rht-B1b* as shown in (Mo et al., 2018).

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 two *Rht-A1* EMS missense alleles was analyzed in vitro with yeast-2-hybrid assays. Pearce et al. (2011) 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 (Figure 1). 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 nonselective media as a control. We observed no growth on either plate for *Rht-B1b-E529K* when examining the durum wheat mutations, and since Pearce et al. (2011) 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.

Rht-B1b and *Rht-D1b* reduce coleoptile length compared with wild-type *Rht* alleles (Fick & Qualset, 1976; Schillinger et al., 1998; Jobson et al., 2020; Amram et al., 2015; Liatukas & Ruzgas, 2011), and the *Rht-B1b-E529K* mutation partially repressed coleoptile length reduction compared with *Rht-B1b* (Mo et al., 2018). We observed a coleoptile length increase from *Rht-B1b-E529K* compared with *Rht-B1b*, as in Mo et al. (2018), and a length decrease compared with *Rht-B1a* (Table 6). These results mirror the effect seen in field-grown plants of the same genotypes, as observed in previous studies correlating plant height to coleoptile length (Fick & Qualset 1976; Schillinger et al., 1998; Liatukas & Ruzgas, 2011; Jobson et al., 2020). The *Rht-B1b-E529K* mutation's effect on coleoptile length was increased when GA was present: 28 vs. 20% reduction in the *Rht-B1a* cross and 25 vs. 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. (2020) findings that mutations other than *Rht-B1b* and *Rht-D1b* did not impact coleoptile growth or GA responsiveness.

The *Rht-A1a* group 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 & Qualset, 1976; Schillinger et al., 1998; Liatukas & Ruzgas, 2011; Jobson et al., 2020), showing that wild-type *Rht-1* plants are GA responsive and grow longer coleoptiles when exposed to GA. Since all the genotypic groups had coleoptiles long enough to emerge from soil if planted at 2.5 cm, it cannot be determined if the increases and decreases observed in this experiment would be of agronomic relevance or not.

Field experiment results showed that plant height was unaffected by *Rht-A* mutations when *Rht-B1b* was present, but when combined with *Rht-B1a*, the *Rht-A-S50F* mutation decreased plant height in both years. Coleoptile data revealed a similar pattern. The yeast-2-hybrid results indicated that both *Rht-A* EMS alleles would confer intermediate interaction with GID1, which may produce shorter plants. These results indicate that by adding *Rht-A* mutations to standard-height durum wheat varieties, plant height can be reduced without reducing coleoptile length. Intermediate height alone is also likely to prove useful in certain environments and would likely be accompanied by smaller reductions in seed size and protein content than are conferred by *Rht-B1b*.

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AUTHOR CONTRIBUTIONS

McKenna M. Brown: Investigation; Supervision; Writing-original draft. John M. Martin: Conceptualization; Methodology; Supervision; Writing-original draft; Writing-review & editing. Emma M. Jobson: Investigation; Methodology. Andrew C. Hogg: Investigation; Methodology. Patrick M. Carr: Investigation; Methodology; Supervision; Writing-review & editing. Michael J. Giroux: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing-review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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