CHARACTERIZATION AND IDENTIFICATION OF NOVEL REDUCED HEIGHT (*RHT-1*) ALLELES IN WHEAT

by

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DEDICATION

To my grandpa, Reuben Swanson, who taught me: the keys to success are faith, arable land, and a good education.

To my parents, my brother, and Jake; thank you for your encouragement and support throughout this endeavor.

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ABSTRACT

Since their introduction in the 1960s, the semi-dwarfing *Reduced Height* (*Rht-1*) genes in wheat have been incorporated into the majority of modern wheat varieties. Their popularity has been driven by their positive impact on yield. The two most common semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, reduce height ~20% and increase yield ~6% compared to tall varieties. Their shorter stature makes them less susceptible to lodging under increased water and nitrogen inputs compared to tall wheat varieties. Despite their prevalence, the exact mechanism by which *Rht-B1b*/*Rht-D1b* increase yields is still unknown. Furthermore, very little research has been done to characterize their impact on bread making and end use quality. Finally, beyond *Rht-B1b* and *Rht-D1b*, there is very little allelic diversity available to wheat breeders. The objectives of this research were to investigate the impact of *Rht-B1b* on photosynthesis, characterize the impact of *Rht-B1b/Rht-D1b* on bread making and dough rheology, and to identify and test novel *Rht-1* alleles created using EMS mutagenesis. In regards to photosynthesis: we found *Rht-B1b* reduces flag leaf photosynthetic rate (18%) and chlorophyll A content (23%) compared to the tall wildtype at anthesis. In regards to end use quality: we found *Rht-B1b/Rht-D1b* decrease total grain protein content (2%) but increase gluten index (21%), bake mixing time, and bake mixing tolerance compared to the tall lines. Increased gluten index and mixing time in the semi-dwarfing lines was shown to be associated with increased high molecular weight glutenins. In regards to developing novel alleles: we identified three nonsense *Rht-1* alleles and characterized their impact on coleoptile length, gibberellin responsiveness, and DELLA/GID1 interaction. Further research will be needed to investigate their impact on agronomic traits and found that each abolished GID1 interaction in the absence but not the presence of Gibberellic acid. Overall this dissertation provides new insight on the impact of the semi-dwarfing alleles on wheat growth and development, wheat milling and baking properties and increases the available allelic diversity through the introduction of three new *Rht-1* nonsense alleles.

CHAPTER ONE:

INTRODUCTION

History and Significance of *Rht-1*

One of the defining features of the Green Revolution during the 1960s was the release and widespread cultivation of high-yielding cereal grain varieties. Since 1961, global cereal production has quadrupled from 740 million metric tons to 2.98 billon metric tons in 2017 (World Bank, 2019). However, agricultural land used for cereal production has only increased about 30% during the same time period. For wheat and rice, the greatest impact of the Green Revolution may have been the introduction and integration of high-yielding semi-dwarfing genes.

In wheat (*Triticum aestivum*), the most common semi-dwarfing alleles are *Rht-B1b* and *Rht-D1b*: they are both mutant alleles of the *Reduced Height* (*Rht-1*) gene. *Rht-B1b* and *Rht-D1b* were initially introduced in 1935 by Gonjiro Inazuka at the Iwate Prefectural Agriculture Experiment Center in Japan. Both *Rht-B1b* and *Rht-D1b* derived from the same semi-dwarf Japanese landrace. Inazuka crossed this semi-dwarf landrace to two American cultivars to create an improved semi-dwarfing variety, Norin-10 (Lumpkin, 2015). By 1949, USDA wheat breeder, O.A. Vogel had incorporated Norin-10 into his breeding program in Pullman, Washington (Reitz and Salmon, 1968). During this time, Vogel crossed Norin-10 to two high-yielding American varieties, Brevor and Baart.

In 1953, Vogel sent the Norin 10 x Brevor and Norin 10 x Baart crosses to Norman Borlaug at the International Maize and Wheat Improvement Center in Mexico

(Lumpkin, 2015). Borlaug's breeding program was focused on breeding wheat with high yield potential and resistance to lodging under increased water and nitrogen inputs (Borlaug, 1968). In 1962, Borlaug released two high-yielding semi-dwarf varieties, Pitic 62 and Penjamo 62. These varieties along with other semi-dwarf varieties were tested internationally and found to consistently increase yield (Reynolds and Borlaug, 2006). Borlaug began promoting the semi-dwarfing germplasm as part of a larger plan to build better agricultural infrastructure globally. For his efforts, Borlaug was awarded the Nobel Peace Prize in 1970, and the semi-dwarfing genes were given the title, 'The Genes of the Green Revolution' (Reynolds and Borlaug, 2006; Hedden, 2003). Since their release, the original semi-dwarfing genes have been incorporated into over 70% of modern wheat cultivars (reviewed in Evans, 1998).

Agronomic Impact of *Rht-B1b* and *Rht-D1b*

There is a copy of *Rht-1* on each of the group 4 chromosomes in wheat (Gale, *et al*. 1975; Gale and Marshall, 1975; Gale and Marshall, 1976; McVittie *et al*., 1978; Sourdille *et al*., 1998). *Rht-B1b* introduces a premature stop codon in the B genome, and *Rht-D1b* introduces a similar premature stop codon in the D genome. There is currently no Rht-A1 semi-dwarfing alleles. The position of the *Rht-B1b* and *Rht-D1b* mutations only differs by a single amino acid (Peng *et al*., 1999). Due to their proximity, *Rht-B1b* and *Rht-D1b* are functionally indiscriminate (Flintham *et al*., 1997). Both decrease height ~20% and increase yield (Hoogendoorn *et al*., 1990; Flintham *et al*., 1997). Due to their decreased height, varieties with either *Rht-B1b* or *Rht-D1b* are less susceptible to lodging

under increased water and nitrogen inputs (reviewed in Hedden, 2003). Furthermore, the impact of *Rht-B1b* and *Rht-D1b* are dosage dependent. The presence of either *Rht-B1b* or *Rht-D1b* decreases plant height by ~15%, while the presence of both *Rht-B1b* and *Rht-D1b* decreases plant height by ~40% (Flintham *et al*., 1997).

Despite their agronomic benefits, *Rht-B1b* and *Rht-D1b* are also associated with decreased coleoptile lengths. This can reduce seedling emergence, especially in arid environments (Fick and Qualset 1976; Schillinger *et al*., 1998). Another disadvantage of the semi-dwarfing alleles is their negative impact on grain protein content and seed size (Gale and Youssefian, 1985; Appleford *et al*., 2007; Casebow *et al*., 2016). Higher grain protein is more desirable for bread making (Park *et al*., 2006; Wang *et al*., 2007; Sherman *et al*., 2014). Despite decreasing coleoptile length and grain protein content, the yield increase associated with *Rht-B1b* and *Rht-D1b* has made them some of the most broadly utilized and well-studied genes in wheat breeding programs.

Rht-1 encodes a DELLA protein

Rht-B1b and *Rht-D1b* decrease plant height by reducing the ability of the plant to respond to gibberellins (GA). Gibberellins are phytohormones responsible for regulating many key events throughout plant growth and development, including seed germination and stem elongation (reviewed in Hauvermale *et al*., 2012). *Rht-1* encodes a DELLA (Asp-Glu-Leu-Leu-Ala) protein, which acts as a negative regulator of GA response (Zentella *et al*., 2007). The *Rht-1* DELLA protein is ~ 620 amino acids, with no introns.

When GA is present in the cell, it is bound by its receptor, GID1 (GA INSENSITIVE DWARF 1) (Figure 1.1). This interaction results in a GID1 conformational change so that it can easily bind to DELLA proteins (Shimada *et al*., 2008). The GA-GID1 complex is further stabilized by binding to DELLA. Once bound to GA-GID1, DELLA is recognized by GID2, an F-box protein as part of the ubiquitin E3 ligase SCF complex (Lou *et al*., 2016). DELLA is then polyubiquitinated and degraded via the 26S proteasome (Ueguchi-Tanaka *et al.*, 2005; 2007; Griffiths *et al.*, 2006). The degradation of DELLA proteins allows the plant to respond to GA. In the absence of GA, the DELLA proteins are not bound by GID1, and therefore DELLA is not degraded. DELLA proteins then continue to repress GA responses via interactions with transcription factors to regulate gene expression (reviewed in Ito *et al*., 2018; Sun, 2010).

DELLA proteins are characterized by conserved motifs. The amino terminal domain contains the DELLA and TVHYNP motifs, which are involved in the interaction between DELLA and GID1 (Murase *et al*., 2008). The carboxy terminal domain is also referred to as the GRAS (GAI, RGA, and SCARECROW) domain (Silverstone *et al.,* 1998; Ikeda *et al*., 2001; Itoh *et al*., 2002). It contains the VHIID, PFYRE, and SAW motifs, as well as two leucine heptad repeats, LHR1 and LHR2 (Bolle, 2004; reviewed in Nelson and Steber 2018). This region is critical for interaction between DELLA and GID2 (Hirano, et al., 2010; Dill et al., 2004).

Mutations in and near the conserved binding motifs can dramatically impact DELLA function, and therefore plant growth and development. The first semi-dwarfing DELLA mutations were identified in *Arabidopsis thaliana*. Researchers found that a 17

amino acid deletion near the DELLA region of the gene resulted in a semi-dominant, GA-insensitive, semi-dwarf plant (Koorneef *et al*., 1985; Peng *et al*., 1997). Since then, similar mutations near the DELLA motif resulting in semi-dwarfing phenotypes have been identified in many other crops. Mutations near the DELLA domain inhibit the interaction between DELLA and GID1. Therefore, DELLA is not degraded, and continues to repress GA responses (Pearce *et al*., 2011). There have also been studies that have identified C-terminal mutations which also result in dwarfed phenotypes by inhibiting the ability of DELLA to bind to the F-box protein (Muangprom *et al*., 2005).

Rht-B1b and *Rht-D1b* are examples of mutations near the DELLA domain, which inhibit the ability of GID1 to bind DELLA, and therefore result in a GA insensitive plant (Pearce et al., 2011). It is not surprising that a plant which cannot respond to GA has a semi-dwarfed phenotype. However, other impacts of *Rht-B1b* and *Rht-D1b* such as increased yield, resistance to certain pathogens, and modified grain composition are still a mystery.

This dissertation aims to provide further insight on the impacts of the current semi-dwarfing alleles. Chapters 2,3, and 4 all describe projects focused on characterizing the effects of the current semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*. Chapter 2 discusses the impact of *Rht-B1b* on photosynthesis and carbon and nitrogen metabolism. Chapters 3 and 4 characterize the effect of the semi-dwarfing alleles on bread making and dough rheology. Chapter 5 describes the development of novel *Rht-1* alleles; which has increased *Rht-1* allelic diversity, as well as illustrated how different regions of *Rht-1* gene impact protein function. The overall aim of the research presented is to advance our

understanding of *Rht1*, one of the most most agronomically significant genes for global wheat production.

Figure 1.1: Diagram of interaction between GA-GID1-DELLA-GID2
DELLA proteins inhibit GA responses in the absence of GA by interacting with transcription factors. In the presence of GA: GA is bound by GID1, which then binds DELLA and targets it for degradation via the SCF (Skp, Cullin, F-box) complex and the 26S proteasome (Hirano et al., 2008).

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CHAPTER TWO:

THE IMPACT OF THE WHEAT RHT-B1B SEMI-DWARFING ALLELE ON PHOTOSYNTHESIS AND SEED DEVELOPMENT UNDER FIELD CONDITIONS

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Abstract

The Reduced Height (*Rht*) genes formed the basis for the green revolution in wheat by decreasing plant height and increasing productive tillers. There are two current widely used *Rht* mutant alleles, *Rht-B1b* and *Rht-D1b*. Both reduce plant height by 20% and increase seed yield by 5–10%. They are also associated with decreased seed size and protein content. Here, we tested the degree to which *Rht-B1b* impacts flag leaf photosynthetic rates and carbon and nitrogen partitioning to the flag leaf and grain during grain fill under field conditions using near isogenic lines (NILs) that were either standard height (*Rht-B1a*) or semi-dwarf (*Rht-B1b*). The results demonstrate that at anthesis, *Rht-B1b* reduces flag leaf photosynthetic rate per unit area by 18% and chlorophyll A content by 23%. *Rht-B1b* significantly reduced grain protein beginning at 14 days post anthesis (DPA) with the greatest difference seen at 21 DPA (12%). *Rht-B1b* also significantly decreased individual seed weight beginning at 21 DPA and by 15.2% at 28 DPA. Global expression analysis using RNA extracted from developing leaves and stems demonstrated that genes associated with carbon and nitrogen metabolism are not substantially altered by *Rht-B1b*. From this study, we conclude that *Rht-B1b* reduces flag leaf photosynthetic rate at flowering while changes in grain composition begin shortly after anthesis.

Introduction

The introduction of the semi-dwarfing trait into wheat cultivars during the 1960s and 1970s was a defining characteristic of the "Green Revolution" (reviewed in Hedden, 2003). Due to the impressive yield increases associated with these genes, by the late 1990s more than 70% of wheat cultivars grown globally incorporated one of the original semi-dwarfing genes (reviewed in Evans, 1998). The genes associated with the green revolution are mutant forms of the Reduced Height-1 (*Rht*) gene which reduce plant height by decreasing the ability of the plant to respond to gibberellic acid (GA) (Allan *et al*., 1959; Allan 1970; Gale and Gregory, 1977). A single functional copy of *Rht* resides on each of the group four chromosomes of wheat (Gale, *et al*. 1975; Gale and Marshall, 1975; Gale and Marshall, 1976; Sourdille *et al*., 1998; McVittie *et al*., 1978).

The two most common mutant forms of the *Rht-1* gene are *Rht-B1b* and *Rht-D1b*. Both mutations were introduced into standard height wheat varieties via crosses with semi-dwarf wheat. Both *Rht-B1b* and *Rht-D1b* contain a premature stop codon near the N terminus of the RHT protein (Peng *et al*., 1999). There is no measurable functional difference between *Rht-B1b* and *Rht-D1b* in that both produce similar increases in wheat productivity and reductions in plant height (Flintham *et al*., 1997; Lanning *et al*., 2012). Since the 1960s many other allelic variants of *Rht-1*, as well as distinctly different genes which also reduce height, have also been discovered. However, none of these mutations have been as useful or as widely incorporated into wheat varieties as *Rht-B1b* or *Rht-D1b*.

The agronomic results seen in varieties carrying either the *Rht-B1b* or the *Rht-D1b* allele is a 15-20% reduction in plant height and increased grain yield (Hoogendoorn *et al*., 1990; Flintham *et al*., 1997). The effects of *Rht-B1b* and *Rht-D1b* are similar in winter and spring wheat (Gent *et al*., 1997). In all varieties, their advantage is reduced in drought or heat stressed environments, due to decreased seedling emergence (Flintham *et al*., 1997) caused by reduced coleoptile length. Due to their decreased height, cultivars carrying *Rht-B1b* or *Rht-D1b* are less prone to lodging, especially under high water and nitrogen conditions (reviewed in Hedden, 2003; Rebetzke *et al*., 2012; Casebow *et al*., 2016). Furthermore, different combinations of *Rht-1* alleles can be used to achieve a more diverse range of plant height and agronomic phenotypes. The presence of *Rht-B1b* or *Rht-D1b* alone reduces plant height by 14.6%, but the presence of *Rht-B1b* and *Rht-D1b* together reduces height by 41% (Flintham *et al*., 1997). In addition to being shorter than genotypes containing *Rht-B1a* and *Rht-D1a*, lines containing *Rht-B1b* or *Rht-D1b* also have smaller leaves. However, the overall biomass of *Rht-1* semi-dwarf lines is similar to standard height cultivars (Flintham *et al*., 1997).

The *Rht-1* semi-dwarfing mutations are also associated with decreased seed size and protein content (Gale and Youssefian, 1985; Appleford *et al*., 2007; Casebow *et al*., 2016). However, these studies have only reported differences in grain protein and seed size at maturity, and no studies have investigated when these differences arise during grain fill. Furthermore, very little is known regarding the impact of the semi-dwarfing alleles on seed starch. However, the semi-dwarfing alleles have been associated with decreased alpha amylase activity compared to tall varieties (Van De Velde, 2017). Alpha-

amylase is a hydrolytic enzyme which can degrade starch. Additionally, it is largely unknown how *Rht-B1b* impacts leaf protein and starch content. One study conducted in the 1970s found that semi-dwarfing lines had increased nitrogen in their stems compared to tall varieties, but that there was no difference in nitrogen translocation efficiency (Deckard, *et al*., 1977)

Despite the widespread use of *Rht-B1b* and *Rht-D1b* in modern wheat varieties, the precise mechanism by which they impact plant growth, seed development, and increase yield is not well understood. One explanation suggests that the yield increase is due to reduced stem elongation and vegetative dry matter accumulation, which leads to increased partitioning of water and nutrients to the spike resulting in increased fertile florets and harvest index (Youseffian *et al*., 1992) *Rht-B1b* and *Rht-D1b* are also associated with increased productive tillers, which also contribute to increased yield (Kertesz *et al*., 1991; Lanning *et al*., 2012, Sherman *et al*., 2014). Other studies have attributed the increased productivity to increased photosynthetic capacity associated with *Rht-B1b* and *Rht-D1b* (LeCain *et al*., 1989; Morgan *et al*., 1990; Bishop and Bugbee, 1998).

Initial studies suggested an inverse relationship between wheat plant height and photosynthetic capacity (LeCain *et al*., 1989; Morgan *et al*., 1990; Bishop and Bugbee, 1998). These studies theorized that the decreased cell size in semi-dwarf wheat varieties resulted in a higher concentration of photosynthetic machinery and an increased ratio of chlorophyll containing mesophyll cells compared to non-photosynthesizing cells, which resulted in greater photosynthetic capacity (Morgan et al. 1990). Other earlier studies

concluded that in addition to total plant height, flag leaf area was also inversely related to photosynthetic rates (Gale *et al*. 2009).

However, a more recent study indicates there is no difference in photosynthetic rates between tall and semi-dwarf wheat (Nenova *et al*., 2014). This study also investigated the impact of the semi-dwarfing genes on seedling leaf structure. Although they reported no difference in net photosynthetic rates between the tall and semi dwarf varieties, the semi-dwarf plants had increased stomatal density and leaf thickness (Nenova *et al*., 2014). Another recent study compared the photosynthetic capacity of tall plants to the "super dwarf" *Rht-B1c* mutant and again found no difference in photosynthetic rate, but that the dwarf lines had increased chlorophyll content (Dobrikova *et al*., 2017).

There have been few studies illustrating strong correlations between genetic improvements for yield, and increased photosynthetic rates (Evans, 2013). Due to the prevalence and usefulness of *Rht* dwarfing alleles, it is important to gain a better understanding of how *Rht* alleles increase plant productivity and modify photosynthetic rates. Furthermore, prior experiments investigating the effects of semi-dwarfing alleles on photosynthesis have been conducted in growth chambers which may not be representative of field conditions. It is also important to investigate how the *Rht* semi-dwarf alleles affect assimilate partitioning, and if that also plays a role in increased productivity.

The objectives of these experiments were to investigate the impact of *Rht-B1b* on: photosynthesis of plants grown under field conditions, carbon and nitrogen partitioning in major organs throughout development, and seed development throughout grain fill. From these experiments we hope to observe key differences associated with the *Rht* semi-dwarfing alleles which can later be used to help explain the mechanism by which *Rht* mutations increase plant productivity.

Materials and Methods

Plant Material

The near-isogenic lines (NILs) used here were previously described (Lanning *et al*., 2012). The standard height hard red spring variety Fortuna (CI 13596), was used as the recurrent parent line. Fortuna carries the tall, wildtype form of the gene, *Rht-B1a*. The donor semi-dwarf hard red spring parent 'Hi-Line' (PI 549275) was used to introduce the *Rht-B1b* allele. Backcrosses to Fortuna continued to the BC_4 generation, and their genotype was confirmed as described by Ellis *et al*. (2002). Subsequent generations beyond BC_5 were allowed to self-pollinate and plants homozygous for the *Rht-B1b* allele were selected for comparison with standard height Fortuna. For this project we excluded the *Rht-D1b* allele; previous studies have shown *Rht-D1b* to be functionally indistinguishable from *Rht-B1b* (Flintham *et al*., 1997; Lanning *et al*., 2012.)

Growing Conditions

Plants were grown under non-limiting irrigated conditions at the Arthur H. Post Field Research Center near Bozeman, MT (latitude 45.67N, longitude 111.00W, elevation 1455 m, soil type is Amsterdam silt loam). Seeds were planted to a depth of 3.5 cm on April 20th, 2016, and harvested the final week of August. From April 20th to September 1st, the research center received 15.7cm of precipitation. The highest recorded air temperatures were on July 22 and July 23 at 35.6°C, the lowest recorded air temperature was -2.8°C on May 11, 2016 (https://www.ncdc.noaa.gov). Additionally, irrigation was applied using hand line sprinklers one week pre-and post-anthesis with 5 cm of water applied each time. Throughout the growing season, weeds were rogued out by hand, and the plants were covered by nets to prevent herbivory. The plants were grown in 2.9 m rows with 30 cm spacing between each row. Within a row, seeds were sown 15 cm apart with 19 plants per row. There were 20 rows total, alternating *Rht-B1a* and *Rht-B1b* for a total of 10 rows for each isoline. The entire field was surrounded by four rows of barley to minimize edge effects.

Plant Sampling

Within each row of 19 plants, five plants were designated and labeled as sampling plants to extract tissue throughout the growing season. another unique group of five plants were designated and labeled for non-invasive measurements. These plants were randomly selected within each row excluding the plants on the end of each row to minimize edge effects. The five plants selected for tissue collection will be referred to as the “sampling population,” the five plants selected for non-invasive measurements, such

as photosynthesis, height, tiller number, and yield, will be referred to as the "non-sampling" population. This was done to ensure that these measurements were not affected by removing or damaging any part of the plant during the growing season. The first five primary heads on each plant to flower from both populations were tagged at heading and used for measurements and tissue collections. This was done to assure that collections and measurements were done on heads of similar maturity. The majority of heads from both lines anthesed on July 6, 2016. If specific heads emerged or anthesed later than the primary heads, it was noted, and collections and measurements were altered to account for this difference.

From the "sampling population" one head and respective flag leaf was collected from each plant at anthesis, as well as at 7, 14, 21, and 28 days post anthesis (DPA), and the same plants were used for each collection. The samples from the five plants collectively within one row were considered one biological replicate. All tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C. The leaf tissue from these collections was used for: chlorophyll quantification and metabolomic profiling. The grain tissue from these collections was used for: metabolomic profiling and measuring grain protein and starch. For this study, anthesis was defined as the point at which half the primary heads of a plant began extruding anthers. Physiological maturity was denoted as when the primary heads were half brown.

Additionally, one plant from each row was used for starch and protein sampling over a diurnal period. This plant was also randomly selected within each row, excluding the plants on the ends of the row. The first five heads on these plants were also tagged

during heading. At 14 DPA, one flag leaf and its respective head from each plant was removed throughout the photoperiod: 30 min before sunrise, 30 min post sunrise, mid-day, and 30 min before sunset (twilight). These time points were chosen to represent the plant's physiological response after being in total darkness, limited exposure to light, full light, and reduced light. For these experiments, the single plant per row is considered one biological replicate. This plant was not included in any further measurements.

Finally, the plants used for RNA sequencing were grown in unique rows from the rest of the experiment. At 14 DPA leaves and stems were collected from three rows of Fortuna, *Rht-B1b*, and *Rht-D1b*. Within each row, tissue samples were collected from three individual plants and the composite of these three plants from one row was considered one biological replicate.

Agronomic Measurements

Plant height was measured from the non-sampling population at maturity. It was measured as the distance from the soil surface to the top of the head (excluding the awns) and reported for each plant as the average height of the three tallest tagged tillers. The average from the five plants "non-sampling" plants within the row was considered one biological replicate.

Tiller number at anthesis was measured as the total number of tillers per plant. Measurements were taken on the five "non-sampling" plants within each row, and one biological replicate represents the average of the measurements taken from the five plants per row. Productive tiller number at maturity only counted tillers which set seed.

Similarly, each row was considered one biological replicate, and is representative of the average of the measurements taken from five "non-sampling" plants within that row.

Flag leaf length and width were measured on one of the five tagged heads from each of the non-sampling population plants at 14 DPA. Leaf length was measured from the base of the leaf to the tip, leaf width recorded the maximum width of each flag leaf. Each row was considered a biological replicate and represented the average of the measurements from the five non-sampling plants within that row.

Above ground biomass and grain yield were also recorded considering each row as one biological replicate which represented the average of the measurements taken from the non-sampling population within that row. Above ground biomass was measured as the total mass of the plant cut at the soil level and adjusted to a 10% moisture basis. Then, the grain was threshed and weighed and adjusted to a 10% moisture basis for calculations. Harvest index was calculated as grain weight divided by total biomass.

Photosynthetic Measurements

Photosynthetic measurements were collected at anthesis and 14 DPA from flag leaves on one of the "non-sampling" plants within each row and the measurement from one plant is considered one biological replicate. The measurements were taken at these points in development to illustrate photosynthetic capacity at the beginning and midway through grain fill. The measurements were taken between 13:00-15:00 in direct sunlight. The max air temperature at anthesis and 14 DPA was 23.3°C and 30°C respectively. Data were collected using a CI-340 (CID Bio-Science, Camas, WA) photosynthesis meter according to the methods described in Smidansky *et al.* (2007) and adapted by Oiestad *et*

al. (2016). This did not include any period of dark adaptation. The leaf area measured was set at 6.5 cm^2, with a 0.61 min^{-1} flow rate in an open system. Photosynthetic chamber light intensity was held constant using the light attachment module set at 2000 µE m^{-2} s^{-1} PAR. The light attachment module used red/blue LED lights; the blue peak at 470nm, and the red peak at 660nm. Measurements were collected under ambient CO_2 conditions where the CID photosynthesis meter was calibrated to 404 µL L^{-1} CO_2 (http://co2now.org, 14 July 2016 and 28 July 2016). Measurements were recorded every 60 seconds. Evapotranspiration and stomatal conductance were also calculated using this apparatus. Initially, seven measurements were taken on a single leaf to allow the machine to stabilize, then a single measurement was reported per plant to ensure all measurements were taken under similar environmental conditions.

Chlorophyll Measurements

The tissue for chlorophyll quantification was collected from the "sampling" population within each row at anthesis. One flag leaf from each of the five plants per row was removed and immediately frozen in liquid nitrogen. Later, the five flag leaves from each row were combined and ground into a coarse powder using a mortar and pestle. One biological replicate represents the composite tissue from the five plants within a row. A subsample of the powder was ground into a fine powder using three, three-millimeter diameter glass beads in a two ml tube by beating for 20 seconds in a bead beater (Biospec Products, Bartlesville, OK). Chlorophyll was quantified using the methods described in Ni *et al.* (2009) with the quantities adapted for a smaller amount of fresh tissue. To extract the chlorophyll, 0.5 ml of 80% acetone was added to 20mg of plant tissue powder

and agitated. Samples were centrifuged for five minutes and the supernatant was transferred into a new tube, and the process repeated two more times. Total chlorophyll was quantified using a Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA). The reaction was kept in the dark to prevent the degradation of the chlorophyll. The absorbance was measured at 663 and 646 nm. Chlorophyll was quantified using the following equations from Ni *et al*. (2009), where V is the volume of extract (ml) and W is the mass of the leaf tissue (mg).

Chlorophyll a (μg/g) = $[12.7 \times A663 - 2.69 \times A646] \times V/1000 \times W$

Chlorophyll b (μg/g) = $[22.9 \times A646 - 4.86 \times A663] \times V/1000 \times W$

Chlorophyll a & b (μg/g) = $[20.20 \times A646 + 8.02 \times A663] \times V/1000 \times W$

<u>Grain Weight, Number of Grains per Head, Grain Protein, and Starch Content</u>

Protein and starch content was measured on grain harvested from the “sampling population” within each row at 7, 14, 21, and 28 DPA. At each collection timepoint, one tagged head from each plant within the “sampling population” was harvested and total head fresh weight, individual grain fresh weight, and the number of grains per head was recorded. The samples were then immediately frozen in liquid nitrogen. Later, the grain samples from all five plants within a row were bulked so that one biological replicate represented a composite of all plants within that row. All samples were ground using a mortar and pestle, lyophilized for 24 hours in a VirTis BenchTop Freeze Dryer (SP Industries Inc, Warminster, PA) and then allowed to re-equilibrate to ambient moisture (10%). They were then finely ground using a bead beater, as described above. Protein content was quantified using 0.1 g of dry powder on a 10% moisture basis on the LECO –

FP 528 (LECO Co., St. Joseph, MI) combustion method nitrogen analyzer using N to protein conversion ratios of 6.25 for seed and 5.7 for leaf tissue (AACC Method 46-30, 1995).

Starch was extracted and quantified as described by Smith and Zeeman (2006) and adapted by Schlosser *et al*. (2014). One ml of 80% ethanol was added to 10mg each plant sample in a two-ml tube and the samples were incubated at 80°C for three minutes while mixing at 1400 rpm. After three minutes, the samples were centrifuged at 13,000 g for five minutes and the supernatant was discarded. This process was repeated two additional times and the samples were dried for one hour in a speed-vac concentrator (Fisher Scientific, Waltham, MA). The pellets were re-suspended in 100 mM sodium acetate (pH 4.8). The starch was digested as described by Schlosser *et al*. (2014) using 0.05 U α-amylase and 0.15 U amyloglucosidase mg^{-1} dry weight. Starch was quantified as described by Rösti et al. (2006) using a standard curve based on known amounts of purified wheat seed starch.

Flag Leaf Protein and Starch Content

Flag leaf protein and starch were quantified using the same methods as described above at 14 DPA. However, the flag leaf tissue was collected over a diurnal cycle (30 min pre-sunrise, 30 min post-sunrise, mid-day, and 30 minutes pre-sunset), rather than 7, 14, 21, 28 days DPA. Furthermore, measurements for flag leaf protein and starch were all collected from a single plant per row, rather than the five plants of the “sampling population.” The first five heads of the plant to be sampled were tagged during heading, and 14 DPA each head and respective flag leaf were removed throughout the diurnal

cycle. Once collected, the tissue was immediately frozen, lyophilized, and ground as described above.

Expression Analysis

Tissue was collected for expression analysis from stems between the first and second internode as well as 14 DPA flag leaves at mid-morning. A single leaf and internode was sampled from three distinct *Rht-B1a* and *Rht-B1b* plants from different rows, for a total of three biological replicates for each line per tissue type. Total RNA was extracted and sequenced as described in Oiestad *et al*. (2016) using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and quantified with a Bioanalyzer (Agilent Technologies, Santa Clara, California). One mg of RNA was used to create a cDNA library using TruSeq RNA-SEQ library kits (Illumina Inc., San Diego, California). The sequence data were analyzed using ArrayStar (DNASTAR, Madison, Wisconsin), with the parameters set as: match setting at 100% for a minimum 50 bp, and all other settings left as default. The resultant data were presented as reads per kilobase of transcript for million mapped reads (RPKM) (Mortazavi *et al*., 2008) and normalized to *Act-2*, an actin like protein which was similarly expressed in both genotypes, and previously reported as a reliably expressed gene for normalization (Tenea *et al*., 2011). The data was analyzed globally against the available rice genome. Specific wheat genes of interest were also analyzed. Genes central to either carbon or nitrogen metabolism were identified based on expression profiling done in rice by Hirose (2006) and adapted in Schlosser (2014) to identify the most prevalent form of the gene in green tissue during development. Wheat

genome orthologs were identified using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and used for expression analysis.

Leaf and Grain Metabolites

Tissue for metabolite analysis was collected from the from flag leaves at anthesis and 14 DPA seeds from the "sampling population." The leaf and grain tissue from each individual row was bulked together to create four or five biological replicates for each genotype for each tissue. Metabolites were extracted as described by Schmidt *et al*. (2011) and adapted by Oiestad *et al*. (2016). The tissue was frozen in liquid nitrogen and ground to a fine powder as described above, then 350 µl of methanol (75°C) was added to 20 mg of each sample and incubated at 60°C for ten minutes. After the samples were vortexed, they were incubated in a sonicating water bath for ten minutes. After ten minutes, 350 µl of chloroform was added to each sample and vortexed. Finally, 300 µl of ddH_2O was added and the samples were vortexed, then centrifuged at 13,000 g for five minutes. The polar fraction was transferred to a GC-MS glass vial in a volume dependent manner (150 mL per 30 mg FW) and dried in a speed-vac concentrator. Samples were analyzed on an Agilent 6890 gas chromatograph (Agilent Technologies). Data acquisition, metabolite identification and normalization were performed as in Fiehn *et al*. (2008).

Statistical Analysis

Statistical analysis to compare the difference between the *Rht-B1a* and *Rht-B1b* allelic groups was done using a two-tailed, paired sample *t* test. A paired t test was used

because the two genotypes were paired in adjacent rows. The number of replications (rows) and subsamples varied for each experiment.

Results

Agronomic Measurements

As expected, there was a significant height reduction (P <0.001) between *Rht-B1b* and *Rht-B1a* under the field conditions used. The *Rht-B1b* NIL had a 23% reduction in height (Table 2.1). No difference was observed in productive tiller numbers between *Rht-B1b* and the *Rht-B1a* in this study in which plants were space planted 15 cm apart. Plants carrying *Rht-B1b* had a decreased biomass (by 16.4%) and increased harvest index (17.2 %). We also observed a significant (P<0.0001) decrease in flag leaf length in the *Rht-B1b* line compared to *Rht-B1a*, 12.6 cm and 14.7 cm respectively. There was also a significant (P < 0.05) decrease (12%) in flag leaf width.

Photosynthetic Capacity

Photosynthetic rates were measured in the field at anthesis and at 14 DPA. At both time points, photosynthetic rates trended down in the *Rht-B1b* NIL (Table 2.2), and were significantly decreased (17.8 %, P<0.01) at anthesis. Evapotranspiration and stomatal conductance were also decreased to a similar degree (13.7 and 20.2%, respectively P<0.05) at anthesis and trended lower at 14 DPA in the *Rht-B1b* NIL. Similarly, *Rht-B1b* flag leaf chlorophyll trended lower relative to *Rht-B1a* (Figure 2.1).

The difference between *Rht-B1b* and *Rht-B1a* was pronounced in chlorophyll A, the primary molecule responsible for photosynthesis, (23% reduction in *Rht-B1b).*

Leaf Protein and Starch Content throughout Photoperiod

Flag leaf protein and starch content were recorded throughout the photoperiod during anthesis (Figure 2.2 at 30 min prior to and after sunrise, mid-day (14:00) and 30 min pre-sunset. For all time points, leaf protein content trended upwards in the *Rht-B1b* NIL, with a significant difference (1.67%, $P<0.05$) observed at twilight. While total protein as predicted by N content was different, there was no difference in the abundance of the major photosynthetic proteins as quantified by SDS PAGE (results not shown) between *Rht-B1a* and *Rht-B1b*.

Flag leaf starch content was also elevated in *Rht-B1b* compared to *Rht-B1a*. The greatest difference in leaf starch content between the two NILs was observed in flag leaves collected at mid-day. At that point, there was 18.3 µg starch/mg dry weight in *Rht-B1b* (mutant) and 9.4 µg starch/mg dry weight in the *Rht-B1a* ($P<0.05$) (Figure 2.2B). The *Rht-B1b* NIL had the greatest increase in starch between the post sunrise and mid-day measurements increasing from 6.24 to 18.31 µg starch/mg dry weight, and the *Rht-B1a* variety had its greatest increase of starch production between the mid-day to twilight measurements, increasing from 9.37 to 22 µg starch/mg dry weight.

Starch and Protein Content of Grain throughout Development

The impact of *Rht-B1b* upon seed starch and protein content during development was measured to assess when differences first develop. Developing seeds were isolated

by harvesting heads from primary tillers at 7, 14, 21, and 28 DPA. Starch and protein content in developing seeds through maturity at 28 DPA is summarized in Table 2.3. There were no significant differences in seed starch content between the *Rht-B1a* and *Rht-B1b* NILs throughout development. However, protein content was reduced in *Rht-B1b* relative to *Rht-B1a* NILs throughout development. The difference in seed protein content was statistically significant beginning at 14 DPA when protein content was measured as 13.1% in *Rht-B1a* and 12.0 % in *Rht-B1b* (P<0.01). This observed difference between the isolines was consistent through maturity. At 28 DPA the *Rht-B1b* line had a 1.83% decrease in protein content compared to *Rht-B1a* (P<0.05).

Seed Number and Individual Kernel Weight throughout Development

Three aspects of grain fill were measured, these being the average seed per head, total weight of the head, and individual seed fresh weight (Figure 2.3). Beginning at 7 DPA, the *Rht-B1b* NIL had an increased seed number per head compared to the *Rht-B1a*, (38.8 versus 29.4). Initially, total head weight was also increased in the *Rht-B1b* NIL compared to *Rht-B1a*. However, by 28 DPA both NILs had an average head weight of 2.2g. The individual kernel weight was consistently greater in *Rht-B1a* beginning at 14 DPA. The final individual grain fresh weight for *Rht-B1a* and *Rht-B1b* NILs were 61.7 mg and 52.3 mg/seed, respectively.

Effect of Rht-B1b Mutation on Metabolite Production

Total methanol soluble metabolites were extracted from flag leaf tissue harvested at anthesis and seeds at 14 DPA at 10:00. 127 compounds were identified in the leaf, and

126 compounds were identified in the grain. The metabolites which were identified were grouped into four different categories: fatty acid, sugar and sugar alcohols, amino acid derivatives, and amino acids. There were no major differences observed among these four groups between *Rht-B1b* and *Rht-B1a*, suggesting *Rht-B1b* does not have a widespread global effect on plant metabolism.

Expression Analysis of Leaf and Stem RNA

RNA sequencing data was analyzed globally as well as for the expression of genes involved in photosynthesis, carbon metabolism, and nitrogen metabolism (Table 2.4). The presence of *Rht-B1b* did not cause significant global gene expression changes in leaf tissues. However, it did impact gene expression in stem tissue. In regard to photosynthesis, the RuBisCo small subunit was significantly increased in *Rht-B1b* stem tissue ($P<0.01$). Genes associated with carbon metabolism in stems were also generally upregulated in *Rht-B1b*, including starch synthase ($P<0.04$). Genes involved in nitrogen metabolism were less effected, though glyeraldehyde-3-phosphate dehydrogenase was increased significantly in *Rht-B1b* ($P<0.04$). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE124940 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124940).

Discussion

Incorporation of height reducing (*Rht*) semi-dwarf genes *Rht-B1b* and *Rht-D1b* into wheat cultivars has led to dramatic increases in grain yield (Flintham *et al*., 1997).

Mutant forms of the Reduced Height-1 (*Rht*) gene reduce plant height by decreasing the ability of the plant to respond to gibberellic acid (GA) (Allan *et al*., 1959; Allan 1970; Gale and Gregory, 1977) However, the underlying causal factor for the increased productivity, and the effects on plant growth and development is has not yet been fully characterized. To understand the effects of the semi-dwarfing alleles on plant growth and development more completely, we investigated the effect of *Rht-B1b* on photosynthesis as well as carbon and nitrogen partitioning during grain fill under field conditions.

Our results show the *Rht-B1b* NIL had reduced plant height, increased harvest index and decreased grain protein compared to *Rht-B1a*, NIL (Table 1). These results agree with Lanning *et al*. (2012) who used the same Fortuna derived NILs as this study. These results are consistent with previous studies which reported 20-25% height reductions (Hoogendoorn, *et al*., 1990; Butler *et al*., 2005; Mathews *et al*., 2006; Lanning *et al*., 2012). Previous experiments have also described an increase in productive tillers, leading to increased yields in *Rht-B1b* genotypes (Kertesz *et al*., 1991; Lanning, *et al*., 2012; Sherman *et al*., 2014). Lanning *et al.* (2012) reported a 9% grain yield advantage for *Rht-B1b* (mutant) over the *Rht-B1a* for these NILs from solid seeded conditions. We did not detect a difference in grain yield. This may have been because our trial was grown in spaced planted conditions, which would have reduced resource competition for both NILs.

The effects of *Reduced Height* genes on photosynthesis have been inconclusive. Morgan *et al*. (1990) reported increased photosynthetic rates, increased soluble protein, chlorophyll, and RuBisCo content in semi-dwarf wheat when compared to near isogenic

tall lines. It has also been previously thought that there was an inverse relationship with photosynthesis and wheat plant height (LeCain *et al*., 1989; Bishop and Bugbee, 1998). However, when Nenova *et al*. (2014) compared photosynthetic rates of near isogenic lines, they found no significant difference between the semi-dwarf and tall lines. None of these studies investigated the photosynthetic capacity of the *Rht-B1b* lines compared to tall *Rht-B1a* under field conditions and using near isogenic lines. Our results indicate that photosynthesis per unit area is decreased in the *Rht-B1b* line compared to *Rht-1a* (Table 2.2). We observed a decrease in both photosynthetic rate and chlorophyll (Table 2.2 and Figure 2.1) content in the *Rht-B1b* NIL. However, these results do not take into account the total photosynthetic capacity of the plant canopy, and our experiment did not measure the total leaf area per plant. Additionally, our experiment did not observe increased tiller number, which have been previously associated with *Rht-B1b*. Plants with relatively more tillers would likely have a greater net photosynthetic capacity compared to plants with fewer tillers. From the experiments we conducted, we can conclude that when grown under space planted, irrigated field conditions, *Rht-B1b* decreases photosynthetic rate per unit area compared to the tall *Rht-B1a* during early grain development.

Although the photosynthetic rate and chlorophyll content of *Rht-B1b* was decreased, we observed an increase in both leaf starch and protein concentration for both genotypes during the grain fill period. Previous studies have documented carbohydrate accumulation in leaves to decrease the expression of photosynthetic genes, and therefore photosynthesis (Paul *et al*., 2001). It is possible that since the *Rht-B1b* line had increased leaf starch content, this was inhibiting the photosynthetic capacity. Furthermore, research

has suggested that inactivation of sucrose symporters may also reduce photosynthesis, and would increase the amount of starch in the leaf (reviewed in Sukhov, 2016).

While the grain starch and protein content have been previously characterized at maturity, the leaf starch and protein content throughout a diurnal period during anthesis was unknown. It appears that presence of the *Rht-B1b* allele resulted in starch accumulating in flag leaves earlier in the day relative to *Rht-B1a* (Figure 2.2). However, by the end of the day, there was no significant difference between *Rht-B1b* and *Rht-B1a*. We observed no significant difference in protein content of flag leaves throughout the diurnal period.

Rht-B1b has been previously associated with decreased grain protein content, (Gale and Youssefian, 1985; Lanning, 2012; Sherman, 2014). However, it is unknown at what point during grain development the differences in protein arise. Lanning et al (2012) showed *Rht-B1b* had mature seed protein 1.1% lower than the *Rht-B1a*. Our results agree with this finding and show that there is a significant reduction in grain protein content beginning 14 DPA and continuing through maturity (Table 2.3). We did not observe significant differences in seed starch content between *Rht-B1b* and *Rht-B1a*. It has also been previously determined that varieties containing *Rht-B1b* have a greater number of seeds per spike, but that the seeds are smaller (reviewed in Gale and Youssefian, 1985), and have decreased protein content at maturity (Lanning *et al*., 2012). Our results support these findings as well as indicate that those differences are present beginning at 14 DPA, and that the differences increase as plants mature (Figure 2.3).

We also investigated the effect of *Rht-B1b* on leaf and seed metabolites and global gene expression, which had both been previously uncharacterized. We detected no significant changes in leaf or seed metabolites, indicating there was likely no significant difference in carbohydrates due to differences in photosynthesis or carbon metabolism. Furthermore, RNA sequencing analysis showed no difference in leaf photosynthetic gene expression between *Rht-B1b* and *Rht-B1a* (Table 2.4). However, there were significant differences in expression detected in stem tissue. Genes involved in photosynthesis and carbon metabolism were upregulated in the *Rht-B1b* stem tissue. Specifically, *agp2*, a gene encoding the ADP-glucose pyrophosphorylase large subunit was upregulated in *Rht-B1b*. Upregulation of *agp2* has been associated with increased starch content (Zhang *et al*., 2016). This may partially explain the increased starch content in the *Rht-B1b* lines. We also found the Rubisco small subunit was significantly increased in the stem tissue of the *Rht-B1b* line. Global expression analysis did not indicate any large groups of differentially expressed genes (data not included), indicating *Rht-B1b* likely has little effect on global gene regulation.

It is clear that the presence of the *Rht-B1b* semi-dwarfing alleles has dramatic effects on wheat plant growth and development, and that those effects begin during early development and continue throughout development. At anthesis *Rht-B1b* NIL plants had decreased photosynthesis and chlorophyll content in flag leaves. *Rht-B1b* NIL plants also had reduced grain protein content and size as early as 14 DPA. However, despite these changes in plant growth and development, we did not detect any significant changes in global gene expression due to the presence of the semi-dwarfing allele.

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Table 2.1: Comparison of plant height, flag leaf dimensions, tiller number, and biomass between *Rht-B1a* (wildtype) and *Rht-B1b* (mutant) near isogenic lines.
*, *** denote significance between genotypes (t-test) at *P* value < 0.05, 0.001 respectively
Values reported as the mean ± standard error
‡ PTN, Productive Tiller Number
For height and tiller measurements, n represents the average of three plants measured per row; for the remaining measurements, n represents the number of rows where five plants were measured and averaged from an individual row; plant height measurements were measured at physiological maturity.

	N	***Rht-B1a* (wildtype)**	***Rht-B1b* (mutant)**
Plant height (cm)	13	95.0 ± 1.35	73.1 ± 0.19***
Tiller no. at Anthesis	13	24.0 ± 1.98	23.7 ± 1.71
PTN‡ at Maturity	13	20.0 ± 0.60	19.1 ± 0.60
Flag leaf length (cm)	10	14.7 ± 0.37	12.6 ± 0.31***
Flag leaf width (mm)	10	13.9 ± 0.28	12.5 ± 0.21*
Biomass (g/plant)	10	53.0 ± 2.24	44.3 ± 1.83***
Harvest index	10	0.29 ± 0.01	0.34 ± 0.01***
Grain yield (g/plant)	10	15.4 ± 0.74	15.4 ± 0.68

Table 2.2: Photosynthetic rates of *Rht-B1b* (mutant) and *Rht-B1a* (wildtype) NILs measured as carbon exchange in flag leaves at two stages of development.

*, ** denote significance between genotypes at P value < 0.05 and 0.01, respectively;
Values reported as the mean ± standard error
N represents the measurements taken from one flag leaf per row; all measurements were collected on flag leaves in direct sunlight.

	N	Photosynthetic Rate (µmol $m^{-2}s^{-1}$)	Evapotranspiration (mmol $m^{-2}s^{-1}$)	Stomatal Conductance (mmol $m^{-2}s^{-1}$)
Anthesis				
Rht-B1a (wildtype)	10	23.7 ± 0.84	5.12 ± 0.22	160.0 ± 9.56
Rht-B1b (mutant)	10	19.5 ± 1.09**	4.42 ± 0.11*	127.6 ± 9.41*
14 DPA				
Rht-B1a (wildtype)	6-9	10.3 ± 0.79	4.08 ± 0.24	63.0 ± 5.98
Rht-B1b (mutant	5-8	8.3 ± 1.38	3.18 ± 0.43	57.0 ± 2.88

Table 2.3: Starch and protein content in grain throughout development for *Rht-B1a* (wildtype) and *Rht-B1b* (mutant) near isogenic lines.
*, ** denote significance between genotypes at P value < 0.05, 0.01 respectively;
Values reported as the mean ± standard error
N = 5, where n represents the number of rows where each row was a composite of five sampling plants.

	N	7 DPA	14 DPA	21 DPA	28 DPA
Percentage Starch					
Rht-B1a (wt)	5	36.3 ± 1.93	50.1 ± 0.74	68.6 ± 3.73	66.6 ± 3.00
Rht-B1b (mt)	5	32.9 ± 0.97	50.6 ± 3.95	64.8 ± 1.99	66.3 ± 1.58
Percentage Protein					
Rht-B1a (wt)	5	10.0 ± 0.32	13.1 ± 0.25	12.4 ± 0.29	13.3 ± 0.23
Rht-B1b (mt)	5	10.1 ± 0.33	12.1 ± 0.10**	11.0 ± 0.16**	12.0 ± 0.33*

Table 2.4: Gene expression values in leaves and stems of genes involved in photosynthesis and assimilate partitioning. Data represents the average ± the standard error and is reported as the reads per kilobase million (RPKM), n=3, *P*-value represents a two-tailed paired t test, expression normalized to actin (Tenea *et al.,* 2011).

		Stems				Leaves			
Name	**GenBank Accession Number**	***Rht-B1a* Average RPKM**	***Rht-B1b* Average RPKM**	***P* value**	***Rht-B1b/ Rht-B1a***	***Rht-B1a* Average RPKM**	***Rht-B1b* Average RPKM**	***P* value**	***Rht-B1b/ Rht-B1a***
Carbon Metabolism									
Granule-bound starch synthase (*GBSSII*)	AF109395	1146 ± 109	1978 ± 283	0.09	1.7	1457 ± 259	1465.5 ± 190.5	0.98	1.0
Starch branching enzyme (*Sbe2*)	AF286319	230 ± 17	345 ± 41	0.10	1.5	329.0 ± 66.6	318.7 ± 33.7	0.92	0.9
Sucrose transporter (*SUT1D*)	AF408845	974 ± 131	887 ± 50	0.64	0.9	13194.8 ± 1224.2	16627.5 ± 870.6	0.14	1.3
ADP-glucose pyrophosphorylase large subunit (*agp2*)	AJ563452	1657 ± 202	3785 ± 613	0.05	2.3	365.6 ± 37.8	304.0 ± 26.8	0.34	0.8
starch synthase isoform IV	AY044844	378 ± 27	589 ± 50	0.04	1.6	880.2 ± 94.3	1065.9 ± 104.1	0.34	1.2
ADP-glucose pyrophosphorylase small subunit	AY727927	1236 ± 197	1599 ± 86	0.24	1.3	1535.9 ± 402.1	897.3 ± 185.0	0.30	0.6
Starch Branching Enzyme (*sbeiia*)	HE591389	2112 ± 259	2405.9 ± 269.2	0.56	1.1	2508.7 ± 492.2	2798.4 ± 315.4	0.71	1.1
Sucrose Transporter (*SUT2D*)	KJ812205	559 ± 27	579.6 ± 12.8	0.60	1.0	725.1 ± 54.6	686.3 ± 46.2	0.68	0.9
RuBisCo Small Subunit (*rbcS*)	AB042066	85754 ± 1267	125966.0 ± 5180.2	0.00	1.5	1166680.2 ± 289990.1	1250289.4 ± 150391.2	0.84	1.1
Nitrogen Metabolism									
Ferrodoxin dependent glutamate synthase (*NADH-GOGAT-3B*)	KC960544	2818.5 ± 83.0	2083.5 ± 380.9	0.20	0.7	434.9 ± 17.0	424.8 ± 25.2	0.80	0.9
Glyeraldehyde-3-phosphate dehydrogenase (*GAPC8*)	KR029493	65648.5 ± 8387.6	101553.1 ± 4195.1	0.04	1.6	39053.0 ± 2791.0	40016.2 ± 3241.7	0.86	1.0
Rht Genes									
Rht-D1	AJ242531	1311.9 ± 60.7	1739.5 ± 123.0	0.06	1.3	707.8 ± 81.2	808.1 ± 18.4	0.38	1.1
Rht-B1	FR668586	1823.0 ± 40.7	4512.2 ± 165.2	0.00	2.5	1116.2 ± 146.4	2407.4 ± 247.5	0.02	2.2
Housekeeping Gene									
Actin	AB181991	1432.5 ± 0.0	1432.5 ± 0.0			1432.5 ± 0.0	1432.5 ± 0.0		

Figure 2.1: Relative amounts of chlorophyll in flag leaf tissue at anthesis for *Rht-B1a* (wildtype) and *Rht-B1b* (mutant) near isogenic lines. * denotes significance at *P* value < 0.05 in comparisons of *Rht-B1a* vs *Rht-B1b,* N = 4, where n represents the composite of five sampling plants per row, error bars represent the standard error

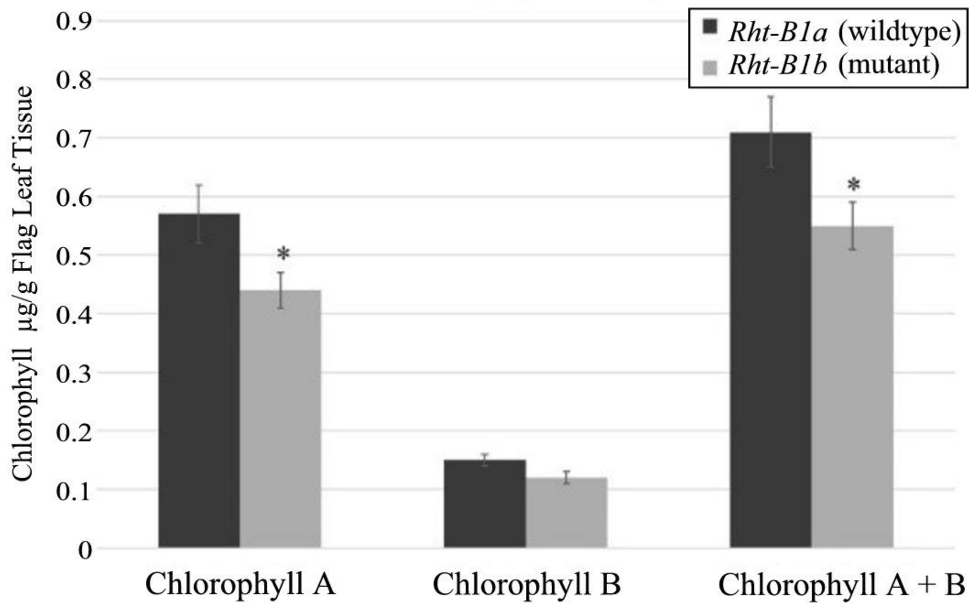


Figure 2.2: Abundance of protein and starch quantified in flag leaves over a diurnal period at anthesis for *Rht-B1a* (wildtype) and *Rht-B1b* (mutant) near isogenic lines. **(A)** Leaf protein abundance throughout photoperiod. **(B)** Leaf starch throughout diurnal period. * denotes significance at $P < 0.05$ in comparisons of *Rht-B1a* vs *Rht-B1b*, for **(A)** N = 4, where n represents the composite of five flag leaf samples taken per row; error bars represent the standard error; For **(B)** N= 5, where n represents the composite of five flag leaf samples taken per row error bars represent the standard error.

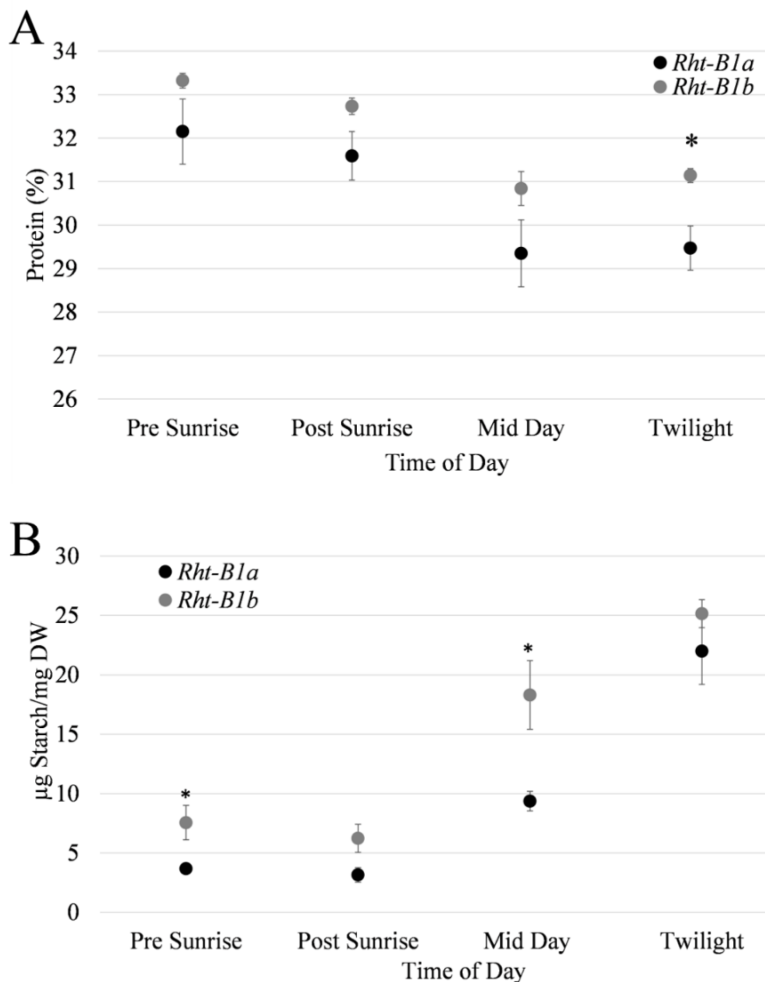
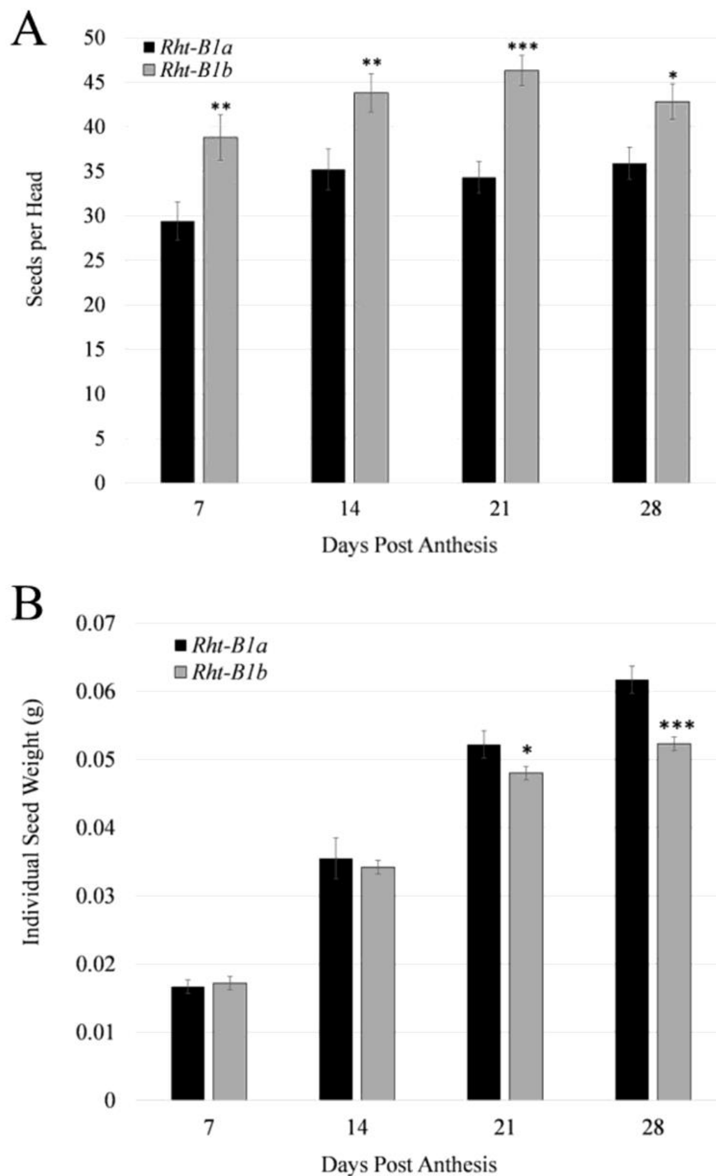


Figure 2.3. Comparison of grain number and size throughout grain fill for *Rht-B1a* (wildtype) and *Rht-B1b* (mutant) near isogenic lines. **(A)** Number of seeds per head throughout grain fill. **(B)** Individual seed mass over grain fill. *, **, *** denotes *P* value at 0.05, 0.01, 0.001 respectively; N =10, where n represents tissue collected from one plant per row; error bars represent the standard error.



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CHAPTER THREE:

THE IMPACT OF THE RHT-B1B, RHT-D1B, AND RHT-8 WHEAT SEMI-DWARFING GENES ON FLOUR MILLING, BAKING, AND MICRONUTRIENTS

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: E.M. Jobson
Contributions: Designed the study, conducted experiments, analyzed data, interpreted results, wrote first draft of manuscript

Co-Author: M.J. Giroux
Contributions: Designed the study, provided guidance for analysis and interpretation of results, edited manuscript

Co-Author: J.M. Martin
Contributions: Designed the study, provided guidance for analysis and interpretation of results, edited manuscript

Co-Author: T.M. Scheider
Contributions: Assisted with data collection

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Abstract

The introduction of the semi-dwarfing *Reduced Height (Rht*) genes in wheat led to dramatic yield increases. The two most common forms of the gene, *Rht-B1b* and *Rht-D1b* have been widely incorporated into wheat varieties. However, despite their agronomic benefits and prevalence in modern wheat cultivars, much less is known regarding their impact on end use quality and nutrition. For this study, we compared near isogenic lines (NILs) carrying either *Rht-B1b*, *Rht-D1b*, *Rht-8* or no semi-dwarfing alleles. The trials were grown under rainfed and irrigated field conditions. Our agronomic results agreed with previous studies; we observed a 25% height reduction, 13% yield increase, and a 2% decrease in grain protein content in the semi-dwarf NILs. However, despite the decreased protein content, the *Rht-B1b/Rht-D1b* NILs had increased bake mixing time (4.6 minutes), but reduced loaf volume (7%). We also observed that although the semi-dwarfing alleles decreased kernel weight by 15%, they were associated with a 2% increase in flour yield. Flours prepared from the semi-dwarf NILs had decreased Zinc, Iron, and Manganese while having increased levels of Potassium and Calcium. These findings demonstrate that while *Rht-B1b/Rht-D1b* negatively impact kernel size and protein content they positively impact flour yield and dough mixing strength.

Introduction

High agronomic yield under locally adapted conditions and at least acceptable end-use qualities are two of the most important characteristics for a successful wheat variety. The single most dramatic yield increase in modern wheat breeding history occurred in the 1960s and 1970s during the "Green Revolution" (reviewed in Hedden, 2003). During this time, Norman Borlaug popularized semi-dwarfing alleles which significantly reduced plant height and decreased lodging while increasing the number of fertile tillers per plant. These semi-dwarfing alleles are mutant forms of the *Reduced Height-1* (*Rht-1*) gene.

There are functional copies of the *Rht-1* gene on all the group 4 chromosomes in wheat (Gale, *et al*. 1975; Gale and Marshall, 1975; Gale and Marshall, 1976; McVittie *et al*., 1978; Sourdille *et al*., 1998). One mutant allele is found on the B genome (*Rht-B1b*), and another is on the D genome (*Rht-D1b*). Both *Rht-B1b* and *Rht-D1b* introduce a premature stop codon near the N terminus of the RHT protein (Peng et al., 1999). The resultant protein decreases plant height via inhibiting the ability of the plant to respond to gibberellic acid (GA) (Allan *et al*., 1959). Since their introduction during the Green Revolution, *Rht-B1b* and *Rht-D1b* have been widely incorporated into modern wheat cultivars (reviewed in Evans 1998). *Rht-B1b* and *Rht-D1b* are functionally similar (Flintham et al., 1997). Both decrease height by ~20% and increase grain yield (Flintham *et al*., 1997). Despite their wide incorporation into wheat breeding programs and agronomic benefits, much less is known regarding their effect on end use quality.

For this study we investigated near isogenic lines varying in plant height developed in a hard-red spring wheat. Hard red spring wheat is grown in moisture-limited environments. The lack of available water usually limits grain yield but gives high protein grain. High grain protein is desirable for bread making (Park et al., 2006; Wang et al., 2007; Sherman et al., 2014). High grain protein is useful for bread making because the proteins allow the dough to stretch and capture the CO_2 produced from yeast during the dough proofing. Therefore, flour protein is positively related to loaf volume (Finney, 1948; Tipples and Kilborn, 1974; Faergestad et al., 2000). Other characteristics used to measure the bread making quality of hard red spring wheat include: the time for the dough to reach maximal resistance, the amount of water needed for the dough to reach maximal resistance, and the time the dough remains near maximal resistance (tolerance). High water absorption, a longer time to reach maximal resistance, and good tolerance are all desirable traits which are also associated with increased loaf volume (Cressey, et al., 1987; Branlard et al., 1991). Hagberg falling number is another test used as a measurement of end use quality. It is a measurement of alpha amylase activity. Low falling numbers are associated with excessive alpha amylase which are associated with negative effects on bread crumb texture and crust color (Chamberlain, 1981).

Despite the prevalence of the *Rht-1* semi dwarfing alleles in modern wheat cultivars, there has not been much work done to investigate their effect on end-use quality. The most well documented effect of *Rht-B1b* and *Rht-D1b* on end use quality is their negative effect on grain protein content and kernel size (Flintham et al., 1997; Gooding et al., 1999; Mann et al., 2009; Lanning et al., 2012). Sherman et al., (2014)

compared end use quality traits using a recombinant inbred line population segregating for *Rht-B1a* (standard height) and *Rht-D1b* (semi-dwarf). They found that the semi dwarf allele (*Rht-B1b*) was associated with decreased flour yield and flour protein, increased mixing tolerance and bake mixing time, and decreased loaf volume. A separate study which investigated the impact of the semi-dwarfing alleles on falling number found that lines carrying *Rht-B1b* and *Rht-D1b* increase Hagberg falling number (Gooding et al., 1999; Casebow et al., 2016).

There has also been increasing research regarding the effect of the semi dwarfing alleles on the micronutrient content of wheat. Initial studies showed an inverse relationship between yield and micronutrient concentration (Oury et al., 2006). Later, a study using NILs of the semi-dwarfing alleles showed that despite their increased yield, lines with *Rht-B1b* or *Rht-D1b* did not increase uptake of Copper, Iron, Magnesium, or Zinc compared to the lower yielding tall cultivars (Gooding et al., 2012). Recently, a study quantified micronutrients in grain and found that *Rht-B1b* and *Rht-D1b* decreased grain Zinc, Iron, Magnesium, and Manganese relative to tall cultivars (Velu, et al., 2017).

This study investigated the impact of the semi-dwarfing alleles on end use quality using NILs developed in a hard-red spring wheat cultivar. Lines carried either *Rht-B1b*, *Rht-D1b, Rht-8* or no semi-dwarfing alleles. *Rht-8* is another distinct gene which has been identified to decrease plant height, but does not affect the GA pathway (Korzun et al., 1998; Rebetzke and Richards 2000). It was used in this study as a control to illustrate that differences observed were due to *Rht-1* mutations, and not simply the effect of a semi-dwarfed plant architecture. *Rht-8* is the primary alternative to *Rht-B1b* and *Rht-D1b*

and is popular in hot and dry environments. Rather than impacting the gibberellin biosynthetic pathway, *Rht-8* reduces stem elongation by altering sensitivity to brassinosteroids (Gasperini et al., 2012). It is located on the short arm of chromosome 2D (Korzun et al., 1998). Lanning et al., (2012) found that *Rht-8* reduced plant height, but less so than *Rht-B1b* and *Rht-D1b*. They also determined that *Rht-8* reduced grain protein content less than *Rht-B1b* and *Rht-D1b*. Otherwise, very little is known regarding the impact of *Rht-8* on end use quality traits.

It is important to fully understand the impact of the semi-dwarfing alleles on the end use quality of wheat since *Rht-B1b* and *Rht-D1b* have become so prevalent in wheat breeding programs across the world. This study provides a comprehensive investigation of the impact of the semi-dwarfing alleles on bread making and end use quality of wheat.

Materials and Methods

Plant Material

The near isogenic lines (NILs) for this study were developed in the hard red spring wheat, 'Fortuna' (CI 13596) as previously described in Lanning et al. (2012). Fortuna is a standard height cultivar which carries no semi-dwarfing alleles. 'Hi-Line' (PI 549275) introduced the *Rht-B1b* allele*, Rht-D1b* was introduced from 'McNeal' (PI 574642), and the donor parent for *Rht-8* was 'Mara' (PI 244854). Each line was backcrossed to the BC_4 by using Fortuna as the recurrent parent. *Rht-B1b* and *Rht-D1b* genotypes were confirmed in the BC_4 generation as described by Ellis et al. (2002); *Rht-8* genotypes were confirmed as described by Ellis et al. (2005).

Field Design and Conditions

The three NILs plus the Fortuna recurrent parent were grown in a randomized complete block design with five replications. Plots were 4 rows wide and 3 m long with 30 cm between rows. Seeding rate was 3.3g per meter of row, and the seeding rate was approximately 65.6 plants per meter. The same experiment was planted in two separate fields with one receiving no added irrigation water and the other receiving 10.2 cm of irrigation. There was 44 cm of precipitation throughout the growing season. All trials were conducted over the 2017 growing season near Bozeman, MT at the Arthur H. Post Field Research Center (latitude 45.67N, longitude 111.00W, elevation 1455m, soil type is Amsterdam silt loam). The soil fertility for the irrigated field was: N (0-121.92cm): 186.06 kg/ha, PK 33-407ppm, and S(0-60.96cm): 60.54kg/ha. The fertility profile for the rain fed field was: N(0-121.92cm): 165.80 kg/ha, PKS 10-219ppm, and S(0-60.96cm): 35.83 kg/ha. Weeds were rogued out by hand throughout the growing season. Plants were harvested using a combine at the end of the season.

End Use Quality Analysis

AACC approved methods were used for quality analysis. For each test, there was a total of ten biological replicates for each genotype: five from the irrigated plots, and five from the rainfed plots. After samples were harvested, they were cleaned and dockages were removed using a Forster Cyclone Grain Scourer, 1930, size 6 (Forster Manufacturing CO, Wichita, KS) and a Dockage Test Machine XT7 2014 (Carter International, Minneapolis, MN). Whole seeds were tested for moisture and protein using a Foss Infratec 1241 Machine (Foss Analytics, Eden Prairie, MN) (AACCI Method 39-

11.01). Kernel hardness and kernel weight were assessed using a Single Kernel Characterization System 4100 (Perten, Springfield, IL) (AACCI Method 55-31.01). Kernel length and width were measured using digital calipers and the measurement for each plot represented the average of five individual kernels. Test weight was measured through the Foss system and through AACCI Method 55-10.01. Samples were tempered to 14.5% moisture for milling (AACCI Method 26-10.02). A Quadrumat Jr II Mill (C. W. Brabender Instruments Inc., Hackensack, NJ) was used to mill samples for white flour and bread tests (AACCI Method 26-50.01). Milled samples were sifted using a 149-micron USA Standard Testing Sieve (Seedburo Equipment CO., Chicago, IL) and a Ro-Tap Rx-29 shaker (W. S. Tyler, Mentor, OH) to remove shorts and further purify flour. Whole wheat flour milling for falling number and ash tests were milled using Laboratory Mill 3303 (Perten) and Laboratory Mill 3100 (Perten), respectively. Alpha-amylase activity was measured using the Falling Number 1000 system (Perten) (AACCI Method 56-81.03). Ash content was measured after baking samples in an IsoTemp Muffle Furnace (Fisher Scientific, Pittsburg, PA) (AACCI Method 08-01.01). Gluten index was characterized using a Glutomatic System (Perten) (AACCI Method 38-12.02). Dough mixing properties were measured using Mixographs (National Manufacturing CO., Lincoln, NE) following AACCI Method 54-40.02 using a 10g bowl. Mixing tolerance was scored by an expert baker after examining the farinograph, and assigned a numerical value 1-8 (weak to strong). Baking and bake mixing properties were done according to the straight-dough bread-making method for 100g samples and mixed on a Finney Special mixer (National Manufacturing CO, Lincoln, NE). Baking pan dimensions were

approximately 14.29 x 7.94 cm on the top side and 12.86 x 6.35cm on the bottom outside with a depth of 5.72 cm (AACCI Method 10-10.03). Water absorption was measured as the amount of water needed for optimum dough consistency as judged by an expert baker. Loaf volume was measured by Rapeseed Displacement (AACCI Method 10-05.01). Ash content of whole wheat and white flour was measured by heating three grams of each sample at 580°C in a muffle furnace for 18 hours, weighing the remaining residue, and accounting for 14.5% moisture (AACC Method 08-01.01). Micronutrient analysis was performed on whole wheat and white flours. Five grams of flour was sent to the University of Georgia Agricultural & Environmental Services Laboratory for analysis. Samples were digested in a CEM Mars 6 Microwave (CEM Corporation, Matthews, NC) according to EPA Method 3052 (USEPA, 1995), and analyzed according to EPA Method 200.8 (Creed et al., 1994) using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) (Spectro Arcos FHS16, Germany).

Statistical Analysis

Each response variable was analyzed via analysis of variance using a model for a randomized complete block combined over environments. All factors were considered fixed. Comparisons among the NIL means were performed using Least Significant Difference (LSD) with the ‘Agricolae’ R package (De Mendiburu and Simon, 2015).

Results

The *Rht* isolines and the Fortuna recurrent parent were grown in two environments where the environments were differentiated by added irrigation water.

Many traits were impacted by the environment. For example the irrigated trial gave taller plants (88.3 vs 82.6 cm) higher grain yield (6144 vs v 4097 kg ha^{-1}) but lower grain protein (14.1 vs 14.7%) than the rainfed trial. The analysis of variance showed that the genotype x environment interaction was generally not significant ($P<0.05$), meaning that although the environment means may have differed the relative difference between genotypes remained the same between environments so genotype means are presented averaged over the two environments.

We observed the expected ~27% height reduction in the *Rht-B1b/Rht-D1b* lines compared to the tall isoline, as well as the characteristic yield increase of ~13% (Table I). As previously reported in Lanning et al. (2012), *Rht-8* was intermediate between *Rht-B1b/Rht-D1b* and Fortuna in both height reduction and yield increase.

The inverse relationship between plant height and kernel weight has previously been reported (reviewed in Gent and Kiyomoto, 1996). We observed *Rht-B1b* and *Rht-D1b* reduced single kernel weight (16% and 13%, respectively) and kernel diameter (10% and 8%) compared to Fortuna (Table 2). The same pattern was observed for kernel length where Fortuna had longer and wider kernels than did *Rht-B1b* and *Rht-D1b*. However, we did not observe a significant increase in test weight between both semi-dwarf varieties and Fortuna, which agreed with previous findings (Allen et al., 1986). In addition to decreasing seed size, the *Rht-B1b* and *Rht-D1b* NILs also decreased grain protein compared to Fortuna with the *Rht-8* NIL being intermediate between Fortuna and *Rht-B1b* and *Rht-D1b* (Figure 1). These results agree with previous studies which characterized the negative association between the semi-dwarfing alleles and grain

protein content (Lanning et al., 2012.) There were no significant differences in grain hardness between the isolines.

Rht-B1b, *Rht-D1b*, and *Rht-8* all increased flour yield and decreased flour protein content compared to Fortuna ($P<0.05$) (Table 3). *Rht-B1b* and *Rht-D1b* also significantly ($P<0.05$) increased falling number compared to Fortuna. Falling numbers for *Rht-B1b* and *Rht-D1b* were 314.8 and 313.5 seconds, compared to 299.1 seconds for Fortuna (Table 3). Alpha amylase and flour ash did not vary among the four genotypes. Both *Rht-D1b* and *Rht-D1b* NILs had longer mix times, greater mixing tolerance, absorbed less water during baking and had lower loaf volumes with higher crumb grain scores than the Fortuna recurrent parent (Table 4, Figure 2). The *Rht-8* NIL had values intermediate between the *Rht-B1b* and *Rht-D1b* and Fortuna for all these traits. Differences in micronutrient content in whole wheat flour were observed among the four genotypes for all measured nutrients (Table 5). The *Rht-B1b* and *Rht-D1b* NILs had lower values than Fortuna for P, S, Cu, Fe and Zn but greater values than Fortuna for Ca and K. The *Rht-8* NIL did not differ from Fortuna except for Mn, where it exceeded the other three genotypes.

Discussion

The introduction of the semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*, into wheat varieties was associated with the most dramatic yield increases in modern plant breeding history (reviewed in Hedden, 2003). However, despite their prevalence, much less is known about the impact of the semi-dwarfing alleles on end use quality. Lack of water

often limits the yield potential of hard red spring wheat. But lower grain yields are usually accompanied by higher grain protein which commands a premium price. In this study, we investigated the impact of the semi-dwarfing alleles on agronomic, milling and baking and mineral composition in the grain in near isogenic lines created in a hard red spring wheat cultivar grown in irrigated and rainfed conditions.

We observed the expected height reduction and yield increase previously associated with *Rht-B1b* and *Rht-D1b* (Hoogendoorn *et al*., 1990; Flintham *et al*., 1997). We also found *Rht-8* was intermediate for plant height and grain yield between *Rht-B1b/D1b* and Fortuna, consistent with Lanning et al. (2012). Our measurements of kernel characteristics also agreed with previous findings. We observed a decrease in grain protein content, from 15.4% in Fortuna, to 13.5% in *Rht-B1b* and 13.8% in *Rht-D1b*, which agrees with the previously described relationship between yield and grain protein content (Simmonds, 1994) However, presence of the semi-dwarfing alleles also decreased kernel weight, kernel diameter, and kernel length.

Although they were associated with decreased grain size, the semi-dwarfing alleles had a positive effect on flour yield, increasing from 70.9% in Fortuna to 72.1% in *Rht-B1b/Rht-D1b*. This is not consistent with Sherman et al. (2014) who found that presence of *Rht-D1b* significantly decreased flour yield 0.5%. This discrepancy may have been due to slight differences in milling methods. Additionally, the Sherman et al. (2014) did not use near isogenic lines; there may have been other factors influencing flour yield beyond the semi-dwarfing alleles.

We also observed decreased flour protein for the semi-dwarfing lines compared to the recurrent parent Fortuna consistent with Sherman et al. (2014). Our observation of increased falling number values for the semi-dwarfing lines compared to the recurrent parent Fortuna is consistent with the inverse relationship between plant height and falling number value described in Casebow et al. (2016) and Van de Velde et al. (2017). However, we did not see a significant decrease in alpha amylase activity between tall and semi-dwarf varieties which we may have expected with an increased falling number. More research will be needed to further understand the impact of the semi-dwarfing alleles on alpha amylase activity and dormancy.

Interestingly, despite the decreased grain and flour protein content, the semi-dwarfing alleles had a positive effect on mixing time (mixograph and bake mixing) and mixing tolerance (Figure 2). This may be due to the semi-dwarfing alleles impacting the abundance of different types of storage proteins present in the dough. Previous studies have shown that an increase of gliadins compared to glutenins decreases dough stability, loaf volume, and gluten index (Barak et al., 2013; Dhaka and Khatkar, 2014). Perhaps the semi-dwarfing alleles have less total protein, but a lower ratio of gliadins/glutenins compared to tall lines, therefore increasing dough strength.

Despite increased dough strength, the semi-dwarfing alleles did not increase loaf volume. This agrees with Shutton et al. (1991) which found an inverse relationship between grain size and loaf volume. From the baking and mixograph tests, we also observed that the semi-dwarfing alleles decreased water absorption. This agrees with previous findings which described a positive relationship between flour protein and water

absorption (Park *et al*., 2006). The semi-dwarfing alleles also had a positive impact on crumb grain score. Crumb grain score is not strongly correlated with total flour protein (Park et al., 2006), but previous studies have shown a relationship between starch granule size and the structure and appearance of the crumb grain (Park *et al*., 2004; Park *et al*., 2005). Further studies are needed to describe the impact of the semi-dwarfing alleles on starch granule morphology and its relationship with the crumb grain score.

Our analysis of micronutrient content was consistent with the findings in Velu et al. (2017). We saw the relative abundance of Copper, Iron, Manganese, Sulfur, and Magnesium was decreased in the semi-dwarf lines compared to Fortuna. However, we also noted that lines with *Rht-B1b/Rht-D1b* had increased amounts of Calcium and Potassium. It is important to understand the impact of the semi-dwarfing alleles on the nutritive quality of the grain since they have been so widely incorporated into breeding programs. It is relevant because recently more breeding programs are focused on developing cultivars to have increased nutritional value, particularly increased Iron and Zinc (reviewed in Velu et al., 2014; Goudia et al., 2015; Yu et al., 2017).

Conclusions

This study presents a comprehensive analysis of the impact of the semi-dwarfing alleles on end use quality traits. We have found that although *Rht-B1b/Rht-D1b* decrease grain and flour protein content, they positively impact flour yield and dough mixing strength. Furthermore, our results agreed with previous findings that *Rht-B1b/Rht-D1b* decrease the relative abundance of certain micronutrients. However, we also found that

they also have a positive impact on Calcium and Potassium in the grain. Further studies will be needed to investigate how the semi-dwarfing alleles reduce protein content without reducing dough strength, as well as why they are associated with decreased micronutrient content, and what impact they have on alpha amylase activity and dormancy.

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Table 3.1: Comparison of plant height and yield between tall and semi-dwarf near isogenic lines.
Values represent the mean of combined rainfed and irrigated plots ± standard error.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
LSD Calculated using Fisher's test of least significance difference using R agricolae package.
$n = 10$, where n represents one plot in either rainfed or irrigated conditions
Height refers to the distance from the soil to the top of the head, not including awns.

	Height (cm)	**Yield (kg/ha)**
Fortuna	102.9 ± 1.3[a]	4924.5 ± 334.6[b]
Rht-8	89.5 ± 1.2[b]	5160.3 ± 393.4[b]
Rht-B1b	75.4 ± 0.8[c]	5563.7 ± 381.1[a]
Rht-D1b	73.8 ± 1.3[d]	5601 ± 386.9[a]
LSD	1.53	250.27

Table 3.2: Kernel characteristics of tall and semi-dwarf wheat.
Values represent the mean of combined rainfed and irrigated plots ± standard error.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
LSD Calculated using Fisher's test of least significance difference using R agricolae package.
$n = 10$, where n represents one plot in either rainfed or irrigated conditions.

	Kernel Weight (mg)	**Kernel Diameter (mm)**	**Kernel Length (mm)**	**Grain Protein (%)**	**Kernel Hardness**
Fortuna	35.5 ± 0.62^{a}	2.8 ± 0.02^{a}	6.7 ± 0.10^{a}	15.4 ± 0.12^{a}	68.9 ± 1.52^{a}
Rht-8	34.5 ± 0.47^{a}	2.8 ± 0.02^{a}	6.5 ± 0.05^{b}	14.9 ± 0.18^{b}	69.9 ± 1.15^{a}
Rht-B1b	29.8 ± 0.34^{b}	2.5 ± 0.01^{c}	6.3 ± 0.08^{bc}	13.5 ± 0.05^{d}	70.8 ± 1.65^{a}
Rht-D1b	30.7 ± 0.54^{b}	2.6 ± 0.02^{b}	6.2 ± 0.06^{c}	13.8 ± 0.12^{c}	68.5 ± 1.68^{a}
LSD	1.11	0.04	0.18	0.17	3.98

Table 3.3: The impact of semi-dwarfing alleles on flour characteristics.
Values represent the mean of combined rainfed and irrigated plots ± standard error.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
LSD Calculated using Fisher's test of least significance difference using R agricolae package.
$n = 10$, where n represents one plot in either rainfed or irrigated conditions.

	Flour Yield (%)	**Flour Protein (%)**	**Falling Number (sec)**	**Alpha Amylase (Ceralpha Units)**	**White Flour Ash (%)**	**Whole Wheat Flour Ash (%)**
Fortuna	70.9 ± 0.33^{b}	15.0 ± 0.13^{a}	299.1 ± 4.16^{c}	0.04 ± 0.004^{a}	0.45 ± 0.01^{a}	1.51 ± 0.03^{a}
Rht-8	71.6 ± 1.06^{a}	14.6 ± 0.19^{b}	311.8 ± 8.94^{bc}	0.04 ± 0.004^{a}	0.44 ± 0.01^{a}	1.51 ± 0.02^{a}
Rht-B1b	72.1 ± 0.80^{a}	13.2 ± 0.05^{d}	314.8 ± 3.87^{a}	0.03 ± 0.003^{a}	0.44 ± 0.01^{a}	1.52 ± 0.03^{a}
Rht-D1b	72.1 ± 1.10^{a}	13.5 ± 0.11^{c}	313.5 ± 4.11^{ab}	0.04 ± 0.002^{a}	0.45 ± 0.01^{a}	1.51 ± 0.03^{a}
LSD	0.55	0.16	10.32	0.0084	0.012	0.04

Table 3.4: Comparison of bread making parameters between tall and semi-dwarf near isogenic lines of wheat**.**
Values represent the mean of combined rainfed and irrigated plots ± standard error.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
LSD Calculated using Fisher's test of least significance difference using R agricolae package.
$n = 10$, where n represents one plot in either rainfed or irrigated conditions.
Mixograph mixing time and mixing tolerance were measured from the mixograph tests using flour-water dough.
Bake Mixing time, loaf volume, water absorption, and crumb grain score were measured from the Bake Tests using full bread formulation dough.

	Mixograph Mixing Time (min)	**Bake Mixing Time (min)**	**Loaf Volume (cm^3)**	**Water Absorption (%)**	**Crumb Grain Score**	**Mixing Tolerance**
Fortuna (wt)	2.8 ± 0.22^{b}	4.3 ± 0.18^{c}	1208.0 ± 17.3^{a}	83.9 ± 0.55^{a}	5.3 ± 0.30^{b}	1.1 ± 0.21^{b}
Rht-8	3.1 ± 0.07^{b}	5.5 ± 0.33^{b}	1178.5 ± 11.9^{a}	83.8 ± 0.45^{a}	5.9 ± 0.28^{ab}	1.8 ± 0.20^{b}
Rht-B1b	4.2 ± 0.11^{a}	9.4 ± 0.50^{a}	1115.5 ± 9.5^{b}	82.3 ± 0.45^{b}	6.5 ± 0.22^{a}	2.2 ± 0.42^{a}
Rht-D1b	4.1 ± 0.15^{a}	8.4 ± 0.56^{a}	1142.0 ± 16.3^{b}	82.2 ± 0.63^{b}	6.4 ± 0.27^{a}	2.3 ± 0.67^{a}
LSD	0.40	1.03	36.1	1.53	0.86	0.50

Table 3.5: Comparison of micronutrient abundance in whole wheat flour milled from tall and semi-dwarf wheat cultivars. Values represent the mean of combined rainfed and irrigated plots ± standard error.
Values calculated as the weight of element/dry sample weight and reported as either a percentage or parts per million (ppm).
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
LSD Calculated using Fisher's test of least significance difference using R agricolae package.
$n = 10$, where n represents one plot in either rainfed or irrigated condition.

	Ca	**K**	**Mg**	**P**	**S**	**Cu**	**Fe**	**Mn**	**Zn**
			(%)				**(ppm)**		
Fortuna	0.045± 0.004^{b}	0.37± 0.013^{c}	0.156± 0.004^{ab}	0.31± 0.011^{a}	0.201± 0.003^{a}	4.61± 0.15^{a}	38.20± 1.21^{a}	48.49± 1.90^{b}	29.49± 2.19^{a}
Rht-8	0.048± 0.002^{ab}	0.37± 0.008^{c}	0.159± 0.002^{a}	0.32± 0.007^{a}	0.197± 0.002^{a}	4.42± 0.13^{ab}	38.13± 0.77^{a}	52.76± 1.50^{a}	28.76± 2.19^{a}
Rht-B1b	0.050± 0.003^{a}	0.41± 0.009^{a}	0.15± 0.003^{bc}	0.30± 0.011^{b}	0.184± 0.002^{b}	4.24± 0.19^{bc}	34.89± 0.89^{b}	47.27± 1.25^{b}	26.42± 2.21^{b}
Rht-D1b	0.050± 0.001^{a}	0.39± 0.007^{b}	0.145± 0.002^{c}	0.29± 0.008^{b}	0.184± 0.002^{b}	4.01± 0.16^{c}	33.81± 0.69^{b}	46.07± 1.53^{b}	27.01± 1.89^{b}
LSD	0.003	0.015	0.0058	0.012	0.006	0.30	1.53	2.76	1.62

Figure 3.1: Semi-dwarfing alleles decrease grain protein content as well as kernel size.
Values represent the mean averaged over 5 replications for each genotype for rainfed and irrigated conditions. Means from irrigated plots are represented in grey and means from rainfed plots are in black.

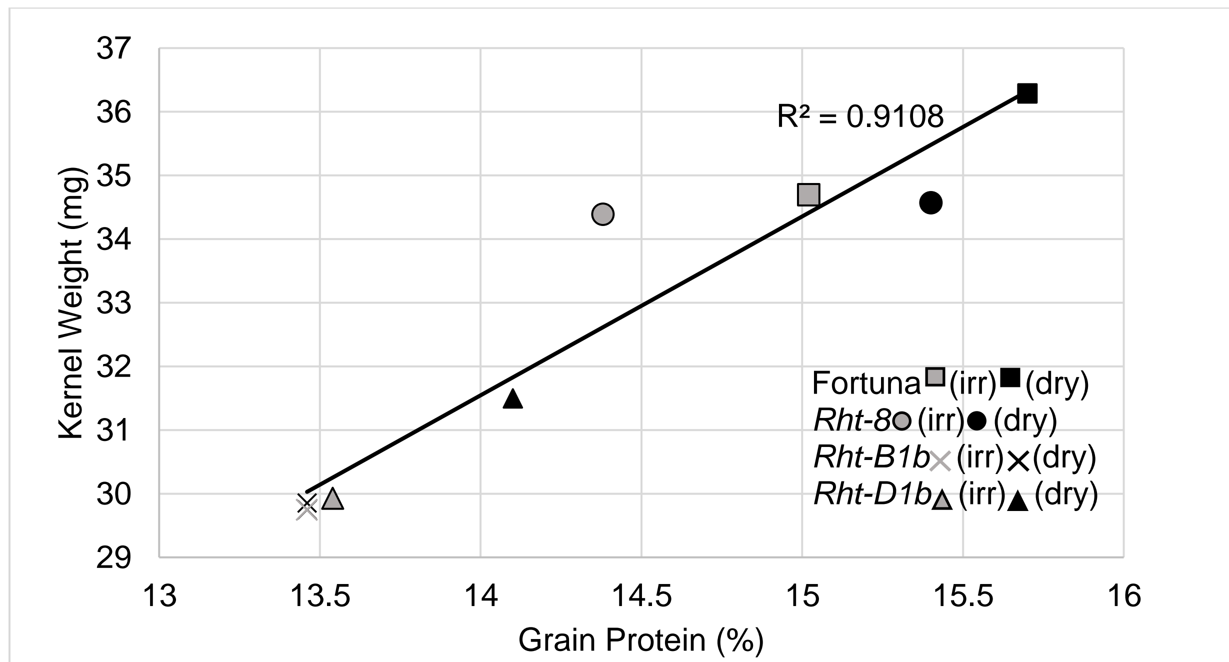

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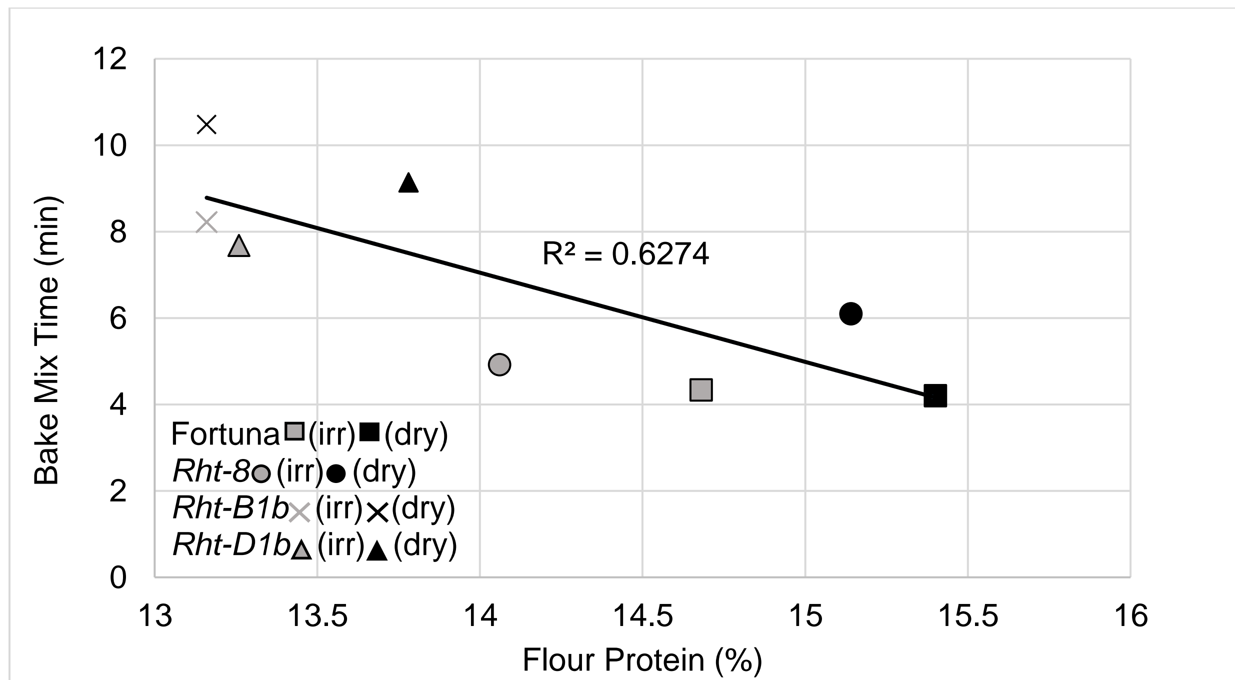
Figure 3.2: Semi-dwarfing alleles decrease flour protein content, while increasing dough mixing time. Values represent the mean averaged over 5 replications for each genotype for rainfed and irrigated conditions. Means from irrigated plots are represented in grey and means from rainfed plots are in black.



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CHAPTER FOUR:

RHT-1 SEMI-DWARFING ALLELES ALTER DOUGH RHEOLOGY BY MODIFYING GLUTEN COMPOSITION

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

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Contributions: Designed the study, conducted experiments, analyzed data, interpreted results, wrote first draft of manuscript

Co-Author: M.J. Giroux
Contributions: Designed the study, provided guidance for analysis and interpretation of results, edited manuscript

Co-Author: J.M. Martin
Contributions: Designed the study, provided guidance for analysis and interpretation of results, edited manuscript

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Contributions: Collected and analyzed HPLC protein molecular weight distribution data

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Abstract

Grain protein and starch abundance and composition are quantitative traits and play key roles in dough rheology. The dominant acting semi-dwarfing alleles of the *Reduced height* (*Rht-1*) gene present in most modern wheat varieties increase productive tillers and yield but also reduce seed size and protein content. The two most prevalent semi-dwarfing alleles are *Rht-B1b* and *Rht-D1b* which increase tillering and reduce plant height by making the plant less responsive to gibberellic acid (GA) while *Rht-8* also decreases plant height but by a GA independent pathway. Despite their negative impact on grain protein content *Rht-B1b*, *Rht-D1b*, and *Rht-8* increase dough mixing time and tolerance. This study used near isogenic lines that were either tall or were semi-dwarf lines that carried *Rht-B1b, Rht-D1b,* or *Rht-8* to investigate how each semi-dwarfing allele impacts dough mixing properties. None of the three tested semi-dwarfing alleles impacted starch swelling power or starch granule size. Each reduced flour protein content compared to the tall variety with the largest decreases in *Rht-B1b* (1.8%) and *Rht-D1b* (1.5%). We observed that the semi-dwarfing lines increased the gluten index (21.5%) compared to *Rht-1a*. Using SE-HPLC we determined that the semi-dwarfing lines had an increased relative abundance of high molecular weight glutenins compared to the tall variety (2%). This study indicates that the *Rht-1* semi-dwarfing alleles increase dough mixing time and tolerance by increasing the relative abundance of high molecular weight glutenins yielding stronger dough. This study serves as an example of how a gene primarily developed for agronomic purposes can also have significant impacts on dough rheology and bread making.

Introduction

Wheat (*Triticum aestivum*) is one of the most important crops for human consumption, and accounts for 20% of the total calories consumed worldwide (FAOSTAT, 2011). Its popularity is driven by its ability to grow well within a wide range of environments, as well as the products derived from wheat dough. Specifically, the extensibility and elasticity unique to wheat dough which allows gas bubbles; to be trapped during the baking process has contributed to the global success of wheat. Both extensibility and elasticity play important roles in dough development. Breadmaking doughs are typically stronger and more elastic compared to pastry doughs which have lower protein content and greater extensibility. Together, extensibility and elasticity are referred to as dough viscoelasticity (Shewry et al., 2002).

Grain storage proteins are the major determinant of the viscoelasticity of dough (Shewry et al., 2002). Wheat grain protein accounts for approximately 8 - 18% of grain dry weight (Shewry et al., 2009). The major class of storage protein in wheat grain is gluten (Shewry, et al., 2002). Gluten is composed of hundreds of different protein subunits which form a complex matrix in dough (Wrigley and Bietz, 1988: reviewed in Weiser 2007). Gluten can be separated into its two most prevalent fractions based on their solubility; the alcohol soluble gliadins, and the alcohol insoluble glutenins. Gliadins are considered less elastic than glutenins and are primarily responsible for the extensibility of the dough. The glutenins are responsible for dough elasticity and cohesion which determines dough strength (Weiser, 2007). Furthermore, insoluble

polymeric proteins are positively correlated with bake mixing time, whereas soluble polymeric proteins are negatively correlated with bake mixing time (Park et al., 2006).

Glutenins are further characterized by their molecular weight. Low molecular weight glutenins account for approximately 20% of gluten proteins (Weiser et al., 2001). High molecular weight glutenins account for 10% of gluten proteins and have a molecular weight ranging from 67 – 83 kDa (Wieser, 2007). Of gluten component proteins, high molecular weight glutenins have the greatest impact on dough strength (Wrigley et al., 2006; Weiser et al., 2001). High molecular weight glutenins are largely controlled by loci found on the group 1 chromosomes, *Glu-A1*, *Glu-B1*, and *Glu-D1* (Payne, 1987). Recent studies have shown that these genes, as well as those associated with gliadins, are regulated by both *cis* and *trans* loci (Plessis et al., 2013). These transcription factors have been shown to impact the quantity and composition of glutenins and gliadins (Plessus et al., 2013).

In addition to grain protein, starch is also important in dough rheology. Starch accounts for 65-73% of the dry weight of flour (Pomeranz, 1988). It plays a key role in water absorption and interacts directly with the gluten matrix (Sandstedt el al., 1961, 1955; Petrofsky et al., 1995). Furthermore, the rate of starch gelatinization directly impacts dough expansion during baking (Kusunose et al., 1999).

Starch is composed of large A-type and small B-type granules. The A-type granules have a diameter greater than 10 µm and are more disk shaped than spherical; B-type granules have a diameter smaller than 10 µm and are spherical (Soulaka et al., 1985; Vermeylen et al., 2005; Kim and Huber, 2008). Although A-type granules account for

greater than 70% of total starch weight, 90% of granules are B-type (Bechtel et al., 1990; Raeker et al., 1998; Peng et al., 1999). The surface of these granules has a direct impact on dough rheology (Sipes, 1993) and the ratio of A- and B-type granules impacts bread making (Park et al., 2005).

Grain protein and starch content are quantitative traits controlled by many genes and influenced by the environment. One gene that has large impacts on protein content is *Reduced Height* (*Rht-1*). There is a *Rht-1* gene on each of the group 4 chromosomes (Gale et al., 1975; Gale and Marshall, 1975, 1976; McVittie et al., 1978; Sourdille et al., 1998). Dominant acting mutant forms of *Rht-1* reduce plant height and increase yield. The two most prevalent semi-dwarfing mutations are found in the B and D genome, *Rht-B1b* and *Rht-D1b*, respectively. Both alleles contain a premature stop codon near the RHT protein N terminus (Peng et al., 1999). The resultant truncated protein partially inhibits the plant's ability to respond to gibberellic acid (GA) (Allan, et al., 1959). The agronomic result is a 20% height reduction, increased productive tillers, and a 10% increase in grain yield (Flintham et al., 1997). *Rht-B1b* and *Rht-D1b* are also associated with decreased kernel size and grain protein content (Flintham et al., 1997; Gooding, et al., 1999; Lanning et al., 2012; Mann et al., 2009). *Rht-8* is another gene which reduces plant height but does not interfere with the plant's ability to perceive gibberellic acid (Korzun, Order, Ganal, Worland, & Law, 1998; Rebetzke & Rich ards, 2000). *Rht-8* reduces plant height approximately 6.5% by impacting the plant's ability to respond to brassinosteroids (Lanning et al., 2012; Gasperini et al., 2012). *Rht-8* was included as part

of this study to ensure that the impact of *Rht-B1b/Rht-D1b* was due to the *Rht-1* mutations, and not semi-dwarfed plant architecture.

Limited work has been done to investigate the impact of the *Rht-1* semi-dwarfing alleles on bread making and end use quality. Sherman et al. (2014) associated *Rht-D1b* with decreased flour yield, protein, and loaf volume, but increased mixing tolerance and bake mixing time. We used near isogenic lines carrying either *Rht-B1b, Rht-D1b*, or no semi-dwarfing mutation to evaluate the impact of the semi-dwarfing alleles on end use quality (Jobson et al., 2018). We also observed a decrease in flour protein content (1.8% *Rht-B1b*, 1.5% *Rht-D1b*), but an increase in mixograph mixing time (1.8 minutes) and tolerance when compared to *Rht-1a*.

The purpose of this study was to investigate the impact of the *Rht-1* semi-dwarfing alleles on dough strength and grain composition in order to better understand how they increase dough strength despite reducing flour protein content. For this study we used near isogenic lines developed in a tall hard red spring wheat cultivar. Lines either carried *Rht-B1b*, *Rht-D1b*, no semi-dwarfing gene (*Rht-1a*), or *Rht-8*.

The *Rht-1* semi-dwarfing alleles have been incorporated into most modern wheat cultivars. Therefore, it is important to not only understand their agronomic impact, but also their impact on end use quality and bread making. This study provides new insight into the impact of the semi-dwarfing alleles on grain protein and starch composition in relation to bread making quality. The semi-dwarfing alleles are some of the most broadly used genes in wheat breeding programs. Although there has been extensive research regarding their impact on plant growth and development, there is very limited research

regarding their impact on bread making and end use quality. This study provides a comprehensive analysis of the impact of the semi-dwarfing alleles on starch and protein in relation to bread making; and illustrates how genes which significantly impact agronomic traits also influence product quality.

Materials and Methods

Plant Material

This study used near isogenic lines (NILs) which carried either no semi-dwarfing alleles, *Rht-B1b, Rht-D1b,* or *Rht-8*. The NILs were developed in the standard height, hard red spring wheat, "Fortuna" (CI 13596) as described by Lanning et al. (2012). "Hi-Line" (PI 549275) was the donor parent for the *Rht-B1b* allele, "McNeal" (PI 574642) served as the donor of the *Rht-D1b* allele, and "Mara" (PI 244854) was the donor of the *Rht-8* allele. All lines were backcrossed to Fortuna as the recurrent parent to the BC_4 generation. The genotype of each line was confirmed in the BC_4 generation using the markers described by Ellis et al., (2002; 2005).

Field Design and Conditions

Trials for this study were grown as described in Jobson et al., (2018) in 2017 at the Arthur H. Post Field Research Center near Bozeman, MT (latitude 45.6 N, longitude 111.00 W, elevation 1,455 m, soil type: Amsterdam silt loam). Plants were grown under both irrigated and rainfed conditions. The trials received 13.8 cm of precipitation throughout the growing season with the irrigated field receiving an additional 10.2 cm of water with half supplied one week prior to and half one-week post heading.

The NILs (*Rht-B1b, Rht-D1b, Rht-8*, and *Rht-1a*) were grown in a randomized complete block design with five replications in both the irrigated and rainfed trials. The plots were 3 m long and 4 rows wide, with 30 cm between rows. Seeds were planted at a rate of 3.3 g per m of row.

Milling

Milling and quality analysis were done according to AACC approved methods (American Association of Cereal Chemists, 2000). Samples were cleaned using a Forster Cyclone Grain Scourer, 1930, size 6 (Forster Manufacturing CO, Wichita, KS, USA). Dockages were removed using a Dockage Test Machine XT7 2014 (Carter International, Minneapolis, MN, USA). Samples were tempered to 14.5 % moisture (AACC Method 26-10.02) and milled into straight grade white flour and bran fractions using a Quadrumat Jr II Mill (C.W. Brabender Instruments Inc., Hackensack, NJ, USA). The milled samples were further cleaned using a 149 μm USA Standard Testing Sieve (Seedburo Equipment CO., Chicago, IL, USA) and a Ro-Tap RX-29 shaker (W.S. Tyler, Mentor, OH, USA).

Flour and Dough Rheology

Flour protein and moisture content were measured using a Foss Infratec 1241 Machine (Foss Analytics, Eden Prairie, MN, USA; AACC Method 39-11.01). Whole wheat flour for flour swelling power analysis and starch swelling power was milled using a Laboratory Mill 3303 (Perten, Springfield, IL, USA). Starch was extracted and purified from 300mg of whole wheat flour as described by Hogg *et al*. (2016). Flour swelling power was measured according to AACC Method 56-21.01. Gluten Index was measured

using the Glutomatic System (Perten, Springfield, IL, USA; AACC Method 38-12.02). The pasting property of flour was measured using a Perten Rapid Visco Analyser 4500 (Perten; AACC Method 76-21.02).

Starch Granule Visualization

Measurements of starch granules were done using images captured using a Zeiss Supra 55 VP field emission gun-scanning electron microscope, (Carl Zeiss Microscopy, Peabody, MA, USA). Three starch samples purified from flour from each genotype grown under irrigated conditions were imaged. Each sample was imaged 5 times. Measurements of A- and B-type granules were determined by measuring the maximal diameter of three A- and three B-type granules from each image; totaling 15 measurements for each biological replicate.

Protein Molecular Weight Distribution

Flour from 3 replications of each genotype grown under irrigated conditions was sent to Dr. Jae-Bom Ohm at the Wheat Quality Laboratory at North Dakota State University for protein molecular weight distribution analysis. Protein molecular weight distribution (MWD) parameters were measured using size exclusion high performance liquid chromatography (SE-HPLC) as described by Gupta et al. (1993) and Ohm et al. (2009). The extractable and unextractable protein fractions were obtained from 10mg (14% moisture) of flour using a sodium phosphate/ sodium dodecyl sulfate (SDS) solution (0.5% SDS and 0.5 M sodium phosphate, pH 6.9). The SDS extractable protein fraction was then solubilized in 1 ml of a buffer solution, and vortexed for 5 minutes at 2,000 rpm using a vortex mixer (Pulsing Vortex Mixer; Fisher Scientific, Hampton NH).

The extractable protein fraction was then separated by centrifugation at 20,000 g (Eppendorf Centrifuge 5424, Hamburg, Germany) and filtered through a 0.45 μm polyvinylidene difluoride syringe filter. The unextractable protein fraction was solubilized by sonicating the residue in 1 ml of the buffer solution for 30 sec (Sonic Dismembrator 100; Fisher Scientific). The unextractable protein fraction was then separated using centrifugation and filtration as described for the extractable fraction. Immediately after filtration both the SDS extractable and unextractable protein fractions were heated at 80 °C for 2 min to prevent protein hydrolysis. Ten μl of each fraction was then injected individually for SE-HPLC fractionation.

Size exclusion HPLC was done using a liquid chromatograph (Agilent 1100; Agilent Technologies, Santa Clara, CA, USA) loaded with a size exclusion narrow bore column (300 × 4.6 mm, Yarra 3 um SEC SEC-4000; Phenomenex, Torrance, CA, USA) and a guard cartridge (BIOSEP SEC S4000; Phenomenex). The SE-HPLC system was run at a flow rate of 0.5 ml/min using an isocratic mobile phase of 50% acetonitrile and 0.1% (v/v) trifluoroacetic acid aqueous solution. Absorbance data were attained at 214 nm by a photodiode array detector (Agilent 1200; Agilent Technologies, Santa Clara, CA, USA). UV absorbance data were analyzed by in-house programs coded using MATLAB software (MathWorks, Natick, MA, USA) as described by Ohm et al. (2009). Size exclusion HPLC profiles were divided into four fractions (F) as follows, F1: 3.5– 4.7 min, F2: 4.7–5.2 min, F3: 5.2-5.8 and F4: 5.8-7.4 min. Size exclusion HPLC fractions (F1–4) were reported to be composed primarily of polymeric proteins for F1 and F2, gliadins for F3, and albumin and globulins for F4 (Larroque, Gianibelli, Batey, &

MacRitchie, 1997; Malalgoda, Ohm, Meinhardt, & Simsek, 2018). The protein molecular weight distribution parameters were derived from UV absorbance data from the four fractions.

RNA Sequencing

Grain used for expression analysis was collected and immediately frozen using liquid nitrogen at 21 days past anthesis. The frozen grain was ground using a mortar and pestle, and total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA) (Oiestad et al., 2016). The extracted RNA was quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). ArrayStar (DNASTAR, Madison, WI, USA) was used to analyze the sequence data. The parameters were match setting at 90% for a minimum of 100 bp. The data are reported as reads per kilobase of transcript for million mapped reads (RPKM) (Mortazavi et al., 2008). The data was initially analyzed globally using the most recent wheat genome sequence (Appels, et al., 2018). We then performed a targeted expression analysis focused on genes previously identified to be involved in grain starch (Cao et al., 2012) and protein synthesis (Kawaura et al., 2005). The data were normalized to *Act-2*. *Act-2* encodes an actin-like protein and has been previously shown to be a reliably expressed gene for RNAseq dataset normalization (Tenea et al., 2011).

Statistical Analysis

Response variables where data were obtained on all replications were analyzed using an analysis of variance model for a randomized complete block design which combined both rainfed and irrigated environments. All factors were considered fixed. A

model for a completely randomized design was used for protein molecular weight distribution and starch granule size variables where data were collected on a subset of the replications. The Least Significant Difference (LSD) value to compare differences between genotypes was calculated following a significant F ratio (P<0.05) using the "Agricolae" R package (De Mendiburu and Simon, 2015). The P value presented for the expression analysis was calculated using a two-tailed, independent sample t-test. This value represents any variance in expression between the wildtype and *Rht-B1b* lines.

Results

Flour and Dough Rheology

We observed the expected reduction in flour protein in the semi-dwarf NILs compared to the tall *Rht-1a* NIL. *Rht-B1b* and *Rht-D1b* decreased flour protein content by 1.8% and 1.5% compared to *Rht-1a* (P<0.05; Table 1). *Rht-B1b* and *Rht-D1b* increased gluten index 29.7% and 28.4% compared to *Rht-1a* ($P<0.05$; Table 1). *Rht-8* had intermediate flour protein content and gluten index between the semi-dwarf and tall NILs. *Rht-B1b* and *Rht-D1b* had no measurable impact compared to *Rht-1a* on starch swelling power (Table 1), flour swelling power, or starch granule size (data not shown).

The semi-dwarfing alleles impacted flour the pasting properties as measured by RVA. Peak viscosity value was increased 20% in the semi-dwarf lines compared to *Rht-1a*. The total setback value was also increased in *Rht-B1b* (26%) and *Rht-D1b* (22%) compared to *Rht-1a*.

Protein Molecular Weight Distribution

SE-HPLC results are divided by those proteins which were SDS-Extractable, and those which were not. The first two fractions in each group represent the polymeric glutenin proteins (UP1, UP2, EP1, EP2). Specifically, UP1 and UP2 are associated with the large molecular weight glutenins. We observed an increase in UP1 and UP2 in *Rht-B1b* (UP1:14.92%, UP2:4.39%) and *Rht-D1b* (UP1:14.65%, UP2:4.33%) compared to *Rht-1a* (UP1:12.73%, UP2:3.80%) ($P<0.05$; Table 2). The third fraction is associated with the unextractable and extractable gliadin subunits (UP3 and EP3). The EP3 value was decreased in the semi-dwarfing lines (*Rht-B1b*:32.7%, *Rht-D1b*:33.42%) compared to the tall variety (35.84%) ($P<0.05$; Table 2). There was no measurable difference between genotypes for the unextractable gliadins. The fourth fraction (UP4 and EP4) is associated with the albumins and globulins, we did not detect any significant differences between genotypes for this fraction.

RNA Sequencing

RNA sequencing data was initially analyzed globally. We also performed a targeted analysis focused on genes associated with seed storage proteins (Table 3). We found no statistically significant differences in storage protein gene expression between *Rht-B1b* and *Rht-1a*.

Discussion

Rht-B1b and *Rht-D1b* have a significant impact on many aspects of wheat plant growth and development. Because they relatively reliably decrease plant height and

increase yield, they are now present in most modern wheat varieties. However, despite their agronomic importance, there is limited research regarding their impact on protein and starch in relation to bread making. Since the ability of wheat flour to be baked into bread is one of the primary reasons for the global success of wheat, it is important to understand how yield genes, such as *Rht-1*, impact end use quality and bread making.

A previous study (Jobson et al., 2018) showed that *Rht-B1b* and *Rht-D1b* increase dough mixing time (4.6 minutes) while reducing flour protein. In this study we evaluated the impact of *Rht-B1b* and *Rht-D1b* on dough viscoelasticity, starch properties, and storage protein composition to understand how *Rht-B1b* and *Rht-D1b* impact dough mixing properties. *Rht-B1b* and *Rht-D1b* did not impact flour or starch swelling power, or starch granule size. Our previous study also showed *Rht-B1b* and *Rht-D1b* had no impact on alpha amylase activity (Jobson et al., 2018). Based on these results, it is unlikely that the semi-dwarfing alleles significantly impact starch content or composition.

There were measurable differences in pasting viscosity between *Rht-B1b/Rht-D1b* and *Rht-1a*. Semi-dwarf NILs flour had increased final viscosity as well as peak time compared to the tall NIL flour. This may be explained by previous findings which describe an inverse relationship between flour protein content and final viscosity and total setback (Lee, 2016; Katyal et al., 2018).

Despite having a lower flour protein, *Rht-B1b* and *Rht-D1b* increased gluten index 21.5% compared to *Rht-1a*. This agrees with our previous study which illustrated that despite decreasing total protein content, the *Rht-B1b* and *Rht-D1b* semi-dwarfing alleles increase dough strength (Jobson et al., 2018). This may be explained by a difference in

the abundance of major storage proteins. Barak et al. (2013) and Dhaka et al. (2014) found that an increased ratio of gliadins to glutenins decreased gluten index and dough stability. Barak et al. (2013) added fractionated glutenins and gliadins to fortified flour in increments of 2, 4, 6, 8, and 10%. The addition of gliadins decreased the stability and mixing time of the dough, while the addition of glutenins increased the dough stability and mixing time. A 2% addition of glutenins resulted in a 100% increase in the dough stability. Based on SE-HPLC data, *Rht-B1b/Rht-D1b* have a positive impact on glutenin quantity, specifically the high molecular weight polymers. Previous SE-HPLC studies have shown that these high molecular weight protein polymers have a significant impact on increasing dough strength, which may partially explain the increased dough strength associated with the semi-dwarfing alleles (Tsilo et al., 2010; Dachke-vitch and Autran 1989; Singh et al., 1990b; Bangur, et al., 1997; Park et al., 2006).

For almost all traits, *Rht-8* was intermediary between *Rht-B1b/Rht-D1b* and *Rht-1a*. This may be due to its intermediate grain protein content. However, further research is needed to understand the mechanism behind the impact of *Rht-8* on grain composition and end use quality.

Conclusions

This study provides a comprehensive analysis of the impact of the *Rht-B1b* and *Rht-D1b* semi-dwarfing alleles on flour protein and starch content in relation to dough rheology. We found that despite decreasing total flour protein content, *Rht-B1b* and *Rht-D1b* increase dough strength compared to the tall NIL by altering the composition of

gluten component storage proteins. We observed that *Rht-B1b* and *Rht-D1b* increase the relative abundance of glutenins compared to gliadins, which has previously been shown to increase dough strength. Further studies will be needed to determine how the semi-dwarfing alleles alter gluten storage protein composition.

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Table 4.1: Impact of *Rht-1* semi-dwarfing alleles on flour traits.
[a] Starch swelling power reported as grams of water absorbed/grams of flour.
[b] RVA: Rapid Visco Analyser.
[c] Final Viscosity measured in Rapid Visco Units.
N.S. no significant difference between groups.
Values represent the mean of combined rainfed and irrigated plots ± standard error.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).
$n = 10$, where n represents one plot in either rainfed or irrigated conditions.
Flour protein previously reported in Jobson et al. (2018).

	Flour Protein (%)	**Gluten Index (%)**	**Starch Swelling Power (g/g)[a]**	**RVA[b] Final Viscosity[c]**	**RVA Peak Time (min)**
Rht-1a	15.0 ± 0.13[a]	74 ± 0.03[c]	23.4 ± 3.31[a]	159.5 ± 6.8[b]	15.8 ± 0.09[c]
Rht-8	14.6 ± 0.19[b]	82 ± 0.03[b]	20.2 ± 2.42[a]	168.1 ± 11.2[b]	15.9 ± 0.13[bc]
Rht-B1b	13.2 ± 0.05[d]	96 ± 0.03[a]	21.3 ± 5.42[a]	199.1 ± 35.7[a]	16.0 ± 0.09[ab]
Rht-D1b	13.5 ± 0.11[c]	95 ± 0.02[a]	21.9 ± 2.94[a]	195.2 ± 13.7[a]	16.0 ± 0.19[a]
LSD (0.05)	0.16	2.36	*N.S.*	16.9	0.08

Table 4.2: Protein molecular weight distribution of wheat grain storage proteins.
Values represent the mean of irrigated plots ± standard deviation, n=3.
Means followed by different letters within the same column are statistically different ($P \leq 0.05$).

EP: SDS extractable proteins, higher content of low molecular weight subunits.
UP: SDS Unextractable proteins, higher content of high molecular weight subunits.

EP1, EP2, UP1, and UP2 primarily represent the polymeric proteins, EP3/UP3 represent the gliadins, and EP4/UP4 represent albumins and globulins (Larroque et al., 1997; Malalgoda et al., 2018).

	Area (%)		
SDS Extractable	**Mean**	**Min**	**Max**
EP1			
Rht-1a	15.63 ± 0.08^{a}	15.54	15.71
Rht-8	14.96 ± 0.26^{b}	14.70	15.23
Rht-B1b	15.13 ± 0.34^{ab}	14.80	15.48
Rht-D1b	15.18 ± 0.38^{ab}	14.78	15.53
LSD (0.05)	0.54		
EP2			
Rht-1a	7.60 ± 0.25^{a}	7.44	7.90
Rht-8	7.46 ± 0.17^{a}	7.28	7.61
Rht-B1b	7.49 ± 0.21^{a}	7.33	7.73
Rht-D1b	7.45 ± 0.17^{a}	7.34	7.65
LSD (0.05)	0.38		
EP3			
Rht-1a	35.84 ± 0.35^{a}	35.52	36.21
Rht-8	35.18 ± 0.12^{b}	35.07	35.30
Rht-B1b	32.70 ± 0.18^{c}	32.52	32.87
Rht-D1b	33.42 ± 0.20^{d}	33.2	33.57
LSD (0.05)	0.42		
EP4			
Rht-1a	16.34 ± 0.65^{a}	15.73	17.03
Rht-8	16.86 ± 0.68^{a}	16.08	17.36
Rht-B1b	17.04 ± 0.48^{a}	16.72	17.59

Rht-D1b	17.00 ± 0.54^{a}	16.38	17.39
LSD (0.05)	1.12		
SDS Unextractable			
UP1			
Rht-1a	12.73 ± 0.64^{c}	12.18	13.43
Rht-8	13.54 ± 0.68^{bc}	13.15	14.33
Rht-B1b	14.92 ± 0.48^{a}	14.45	15.41
Rht-D1b	14.65 ± 0.59^{ab}	14.14	15.3
LSD (0.05)	1.14		
UP2			
Rht-1a	3.80 ± 0.07^{c}	3.75	3.88
Rht-8	4.06 ± 0.06^{b}	4.01	4.12
Rht-B1b	4.39 ± 0.14^{a}	4.26	4.54
Rht-D1b	4.33 ± 0.07^{a}	4.26	4.39
LSD (0.05)	0.17		
UP3			
Rht-1a	5.21 ± 0.57^{a}	4.93	5.47
Rht-8	5.04 ± 0.11^{a}	4.95	5.16
Rht-B1b	5.26 ± 0.32^{a}	5.06	5.63
Rht-D1b	5.02 ± 0.10^{a}	4.91	5.09
LSD (0.05)	0.42		
UP4			
Rht-1a	3.07 ± 0.04^{b}	2.81	2.89
Rht-8	2.95 ± 0.09^{ab}	2.8	2.96
Rht-B1b	2.91 ± 0.11^{a}	2.95	3.17
Rht-D1b	2.84 ± 0.13^{ab}	2.87	3.10
LSD (0.05)	0.19		

Table 4.3. Expression of wheat protein genes in developing grains 21 days past anthesis. Data is reported as the average reads per kilobase million (RPKM), n=3 individual plants grown under irrigated conditions. *P*-value represents a two tailed independent *t*-test, all expression values were normalized to actin (Tenea *et al*., 2011).

	Protein Type	**Accession #**	***Rht-B1b* Average RPKM**	***Rht-1a* Average RPKM**	***P*-value**	***Rht-B1b/ Rht-1a***
alpha gliadin	alpha gliadin	EF165552	555	694	0.08	0.80
	alpha gliadin	EF165553	44539	56612	0.25	0.79
	alpha gliadin	U51306	12353	22366	0.28	0.55
alpha/beta gliadin	alpha/beta gliadin	K03076	6783	12623	0.31	0.54
	alpha/beta gliadin	M11075	52764	58102	0.54	0.91
	alpha/beta gliadin	M11076	42756	39818	0.77	1.07
gamma gliadin	gamma gliadin	AF234644	28111	24593	0.66	1.14
	gamma gliadin	AF234646	51361	51404	1.00	1.00
	gamma gliadin	AF234647	12266	11022	0.71	1.11
	gamma gliadin	EF151018	22878	29082	0.62	0.79
	gamma gliadin	M13713	13109	14546	0.42	0.90
	gamma gliadin	M16060	10877	20196	0.48	0.54
	gamma gliadin	M16064	79750	69377	0.68	1.15
omega gliadin	omega gliadin	AF280605	6708	9290	0.36	0.72
high molecular weight glutenin	HMW x-type: 1Bx14	AY367771	195	172	0.74	1.14
	HMW x-type Bx7	DQ119142	16179	15119	0.82	1.07
	HMW x-type 1Ax2	M22208	4959	4829	0.92	1.03
	HMW x-type 1Dx5	X12928	9380	8377	0.70	1.12
	HMW y-type 1By16	EF540765	8471	6789	0.61	1.25
	HMW y-type 1Dy	X03041	9250	10509	0.17	0.88
Low molecular weight glutenin	LMW A3	AY146588	60	52	0.62	1.14
	LMW D3	AB164415	78159	56871	0.36	1.37
	LMW D3	DQ357053	8681	8935	0.85	0.97
	LMW D3	DQ357055	18816	17525	0.88	1.07
	LMW D3	DQ357056	2597	6628	0.43	0.39
	House Keeping	AB181991	353	353		

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CHAPTER FIVE:

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF NOVEL RHT-1 ALLELES IN HARD RED SPRING WHEAT

Contribution of Authors and Co-Authors

Manuscript in Chapter 5

Author: E.M. Jobson
Contributions: Designed the study, conducted experiments, analyzed data, interpreted results, wrote first draft of manuscript

Co-Author: M.J. Giroux
Contributions: Designed the study, provided guidance for analysis and interpretation of results, edited manuscript

Co-Author: J.M. Martin
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Co-Author: A.C. Hogg
Contributions: Discussed project directions and helped with line development
Co-Author: R. Sharrock
Contributions: Design and guidance for yeast 2 hybrid assays

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Abstract

The introduction of the semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b,* into wheat varieties significantly increased wheat yields beginning in the 1960s. Despite their agronomic advantage, *Rht-B1b* and *Rht-D1b* are also associated with decreased grain protein content and decreased coleoptile length. Furthermore, there is limited allelic diversity available to wheat breeders beyond *Rht-B1b* and *Rht-D1b*. We identified 16 EMS induced *Rht-1* alleles, including a nonsense mutation in each *Rht-1* gene: *Rht-A-Q555stop*, *Rht-B-Q420stop*, and *Rht-D-W559stop*. We evaluated the nonsense alleles by measuring their impact on coleoptile length, gibberellic acid (GA) sensitivity, and DELLA/GID1 interaction. *Rht-A-Q555stop* reduced coleoptile length 16 % compared to *Rht-1a* (wildtype), and repressed coleoptile GA perception similarly to *Rht-B1b* and *Rht-D1b*. *Rht-B-Q420stop* and *Rht-D-W559stop* did not impact coleoptile length. However, *Rht-B-Q420stop* reduced the ability of germinating seeds to perceive GA, but not as much as *Rht-A555stop*, *Rht-B1b*, or *Rht-D1b*. We also tested whether the novel mutations impacted the interaction between DELLA/GID1 in the presence and absence of GA. We found that *Rht-A-Q555stop, Rht-B-Q420stop* and *Rht-D-W559stop* inhibited DELLA/GID1 interaction in the absence of GA, but the interaction was restored in the presence of GA. The identification of *Rht-A-Q555stop, Rht-B-Q420stop*, and *Rht-D-W559stop* increases the allelic diversity of *Rht-1.* This study also indicates that the RHT carboxyl terminus is important for DELLA/GID1 interactions.

Introduction

Gibberellic acid (GA) is part of a family of plant hormones which regulate seed germination, stem elongation, leaf expansion, and flower development. They also play a key role in regulating plant developmental processes. (Hauvermale *et al.*, 2012). GA responses are triggered when GA is perceived by its receptor, GA-INSENSITIVE DWARF 1, GID1, a protein with similarities to hormone sensitive lipases (Ueguchi-Tanaka *et al.*, 2005). Since GA is essential to plant growth and development, it must be regulated precisely. DELLA (Asp-Glu-Leu-Leu-Ala) family proteins negatively regulate GA responses in the absence of GA (Zentella *et al.*, 2007).

In the presence of GA, DELLA proteins are bound by GID1, and polyubiquitinated via the E3 ubiquitin ligase complex and degraded by the 26S proteasome (Ueguchi-Tanaka *et al.*, 2005; 2007; Griffiths *et al.*, 2006). Once DELLA is degraded, the plant responds to the GA signal. In the absence of GA, the DELLA proteins are not degraded and repress GA responses through interactions with transcriptionfactors (reviewed in Ito et al., 2018). In addition to regulating responses associated with gibberellic acid, such as floral initiation and vegetative growth; DELLA proteins have also been shown to interact with proteins which regulate plant responses to abscisic acid (Zentella *et al.*, 2007).

DELLA proteins are characterized by an N terminal DELLA domain and a C-terminal GRAS (GAI, RGA, and SCARECROW) domain (Silverstone *et al.,* 1998; Ikeda *et al*., 2001; Itoh *et al*., 2002). The C-terminal domain contains two leucine heptad repeat motifs, LHR1 and LHR2, as well as the VHIID, PFYRE, and SAW motifs (Bolle, 2004;

reviewed in Nelson and Steber 2018). These domains are critical to bind DELLA proteins to an F-box protein (GID2) as part of the ubiquitin ligase complex for degradation (Hirano *et al*., 2010). The N terminal domain is characterized by the DELLA, TVHYNP, and poly S/T/V motifs. The DELLA motif is involved in the interaction between GID1 and DELLA (Murase *et al*., 2008).

Mutations in and near the DELLA motif have the potential to dramatically impact plant growth and development. In *Arabidopsis thaliana* a 17 amino acid deletion near the DELLA motif results in a GA-insensitive, semi-dominant, semi-dwarf phenotype (Koorneef *et al*., 1985; Peng *et al*., 1997). In wheat (*Triticum aestivum*), the *Reduced Height* (*Rht-1*) genes encode DELLA proteins. There is a single functional copy of *Rht-1* on each of the group four chromosomes (Gale *et al*., 1975; Gale and Marshall, 1975,1976; McVittie *et al*., 1978; Sourdille *et al*., 1998). The two most common semi-dwarfing alleles utilized in modern wheat varieties are *Rht-B1b* and *Rht-D1b*. Both introduce a premature stop codon in the RHT-1 N terminus, near the DELLA motif (Peng *et al*., 1999) which blocks RHT-1 binding with its receptor, GID1 (Pearce *et al*., 2011). Since it cannot be bound by GID1, the DELLA protein is not degraded and continues to repress GA responses, even in the presence of GA. Therefore, *Rht-B1b* and *Rht-D1b* reduce plant height by inhibiting the ability of the plant to respond to GA (Allan, 1970; Gale and Gregory, 1977).

Rht-B1b and *Rht-D1b* are functionally indistinguishable (Flintam *et al*., 1997b; Lanning *et al*., 2012). Both decrease plant height ~20% and increase grain yield compared to standard height wheat varieties (Hoogendoorn *et al*., 1990; Flintham *et al*.,

1997b). The phenotypic impact of the semi-dwarfing alleles is dependent on dosage. Individually, *Rht-B1b* or *Rht-D1b* reduce plant height 20%, however, when a hexaploid wheat plant carries both *Rht-B1b* and *Rht-D1b* plant height is reduced 41% (Flintham *et al*., 1997a). This allows plant breeders to use semi-dwarfing alleles in combination in order to achieve greater diversity of agronomic phenotypes.

Despite their positive influence on yield, *Rht-B1b* and *Rht-D1b* are also associated with decreased seed size and grain protein content (Lanning *et al*., 2012; Casebow *et al*., 2016). High grain protein is desirable since since increased grain protein is associated with increased bread loaf volume (Park *et al*., 2006; Wang *et al*., 2007; Sherman *et al*., 2014). *Rht-B1b* and *Rht-D1b* also reduce coleoptile length, which is associated with decreased emergence in arid and semi-arid climates where seed must be planted deeper (Fick and Qualset 1976; Schillinger *et al*., 1998). Finally, there is limited *Rht-1* allelic diversity available to wheat breeders. There is a need to discover novel *Rht-1* semi-dwarfing mutations which are well suited for a wide variety of environments and end use products.

The purpose of this project was to identify, and test novel *Rht-1* alleles created using EMS mutagenesis. After identifying novel alleles, we tested the newly identified nonsense mutations for their impact on coleoptile growth, GA responsiveness, and DELLA/GID1 interaction.

Ideal *Rht-1* semi-dwarfing mutations mutations, or combinations of mutations, would result in plants with height intermediate between the current semi-dwarfs and tall varieties. They would have longer coleoptiles to increase emergence in soils that are dry

on the surface, and have increased protein content and seed size compared to the current semi-dwarfing alleles. The mutations would be selected to be best suited for specific environments or agronomic management strategies. Table 5.1 illustrates the potential for novel mutations individually as well as in combination.

This project increases the available allelic diversity for *Rht-1* and illustrates how mutations throughout the gene impact plant growth as well as the molecular function of DELLA proteins.

Materials and Methods

Creation of EMS Population

An EMS mutagenized M_1 population was created as described by Feiz *et al*. (2009). The population was developed in Fortuna (CI 13596), a tall hard red spring wheat variety that carries no semi-dwarfing alleles (*Rht-1a*). Approximately 5,000 Fortuna seeds were soaked in 1 % EMS for 18 hours at room temperature. After 18 hours, the seeds were rinsed for 4 hours in running cold tap water. The M_1 population was grown and a single head from 356 fertile plants was harvested and advanced to the $M_{1:3}$ generation.

Screening for Novel *Rht-1* Alleles

DNA was extracted from a composite of leaf tissue from three to five $M_{1:3}$ plants at the four-leaf stage for each of the 356 EMS lines. Amplification of *Rht-A1*, *Rht-B1*, and *Rht-D1* was done using the nested PCR approach described by Li et al. (2013) which utilizes genome specific primers for initial amplification followed by non-genome

specific *Rht-1* primers for secondary amplification. Following amplification of individual *Rht-1* segments, segments were sequenced by Sanger sequencing (GENEWIZ, Inc., Cambridge, MA).

Creation of F2 Population

We identified 16 EMS induced *Rht-1* mutations. Each mutation was crossed to the non-mutagenized Fortuna parent to create F_1s. The F_1s were advanced to create an F_2 population.

SIFT scores

The impact of each new *Rht-1* EMS mutation was estimated using the SIFT (sorting intolerant from tolerant) prediction software (Ng and Henikoff, 2003). SIFT was used to estimate whether the EMS induced single nucleotide polymorphisms via mutagenesis would have a neutral or deleterious impact on RHT-1 function.

GA Responsiveness Assays and Coleoptile Length

We evaluated the impact of the *Rht-1* mutations on coleoptile length and responsiveness to gibberellic acid, following the ‘cigar roll’ methodology described by Bai *et al*. (2013). We genotyped 64 F_2 plants from each *Rht-1* segregating population to identify those that were homozygous for the presence or absence of the EMS induced *Rht-1* mutation. $F_{2:3}$ seeds from *Rht-1* mutant and wildtype plants for each novel allele were collected. The seeds were then surface sterilized and imbibed in water for 24 hrs. After 24 hrs 10 seeds from each genotype were placed approximately 2 cm apart on germination paper (25 cm x 38 cm; 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 between two sheets of germination paper, and then rolled to a diameter of 2 cm. There were 5 rolls of mutant and wildtype seeds for each novel allele, as well as 5 rolls of *Rht-B1b, Rht-D1b*, and *Rht-1a* (wildtype). The rolls were randomized in each rack. The racks were placed in tubs which either contained water or 100 μM GA_3. The rolls were arranged so the bottom 2 cm of the roll was submerged in either water or 100 μM GA_3. The racks were then placed in a dark growth chamber at 18 °C. After 10 days coleoptile length was recorded. Coleoptile length was measured as the distance from the edge of the seed to the end of the coleoptile where the first leaf emerged.

Yeast 2 Hybrid Assay

Yeast transformations and assays were done on the nonsense alleles using the Yeastmaker Yeast Transformation System 2 (Clontech, Mountain View, CA) and the Matchmaker Gold Yeast Two-Hybrid System (Takara Bio Inc., Mountain View, CA). Full length copies of *Rht-A1a*, *Rht-B1a*, and *Rht-D1a* were synthesized and cloned into the pGAD vector using GeneWiz Gene Synthesis Services (GeneWiz, Plainfield NJ). A full-length copy of GID1 was also synthesized and cloned into the pGBK vector. The mutations: *Rht-B1b*, *Rht-A-Q555stop, Rht-B-Q420stop,* and *Rht-D-W559stop* were all introduced to the full-length sequence of *Rht-1* using the GeneWiz Site Directed Mutagenesis service. The pGAD-Rht-1 mutant and wildtype proteins were co-transformed with pGBK-GID1 into the Y2HGold yeast strain using the Yeastmaker Yeast Transformation System 2. pGBK-GID1 was also co-transformed with an empty pGAD vector to be included as a negative control. The colonies were grown on SD

medium lacking both leucine and threonine to select successful co-transformations. A single colony was selected from each interaction and grown in liquid media overnight and then quantified at OD 600 nm. Once concentrations were standardized across all reactions, the solutions were serially diluted (1, 1:10, 1:1000, 1:10,000, 1:100,000) and 5 μl was plated on quadruple drop out media for Adenine, Histidine, Leucine, and Threonine. The drop-out media either had no additional gibberellic acid added, or 100 μM of gibberellic acid (GA_3). The plates were incubated at 30°C and growth of colonies was measured five days after plating.

Statistical Analysis

Comparisons between wildtype and mutant alleles were analyzed using an independent sample *t*-test.

Results

We identified 16 EMS induced mutations distributed across the *Rht-1* A, B, and D genome genes (Table 5.2). These mutations included a nonsense mutation in each genome: *Rht-A-Q555stop, Rht-B-Q420stop,* and *Rht-D-W559stop* (Figure 5.1). *Rht-A-Q555stop* and *Rht-D-W559stop* are both positioned near the C terminus, while *Rht-B-Q420-stop* is more centrally located in the gene.

We initially evaluated the impact of all 16 mutations based on their SIFT (sorting intolerant from tolerant) scores (Table 5.2). The SIFT scores ranged from 0 to 0.62, with values closer to zero indicating that the mutation likely had a deleterious impact on the RHT-1 protein structure and function.

We evaluated the impact of 13 novel alleles on GA responsiveness and coleoptile growth. Three mutations were excluded due to low viability. Coleoptile measurements of the novel alleles were taken on $F_{2:3}$ seeds germinated in the presence (100 µM) or absence (0 µM) of GA_3 (Table 5.3). This allowed us to determine if the mutations had an impact on coleoptile length, as well as if the mutations interfered with the seedlings ability to perceive GA. We observed the previously described impact of *Rht-B1b* and *Rht-D1b* on coleoptile length and GA perception. *Rht-B1b* and *Rht-D1b* decreased coleoptile length by 43% compared to *Rht-1a* (*P<0.001* when germinated in water. There was almost no difference in *Rht-B1b* and *Rht-D1b* coleoptile length from seeds which were germinated in GA_3 versus water. However, *Rht-1a* (*wildtype*) coleoptiles were 14% longer when the seeds were germinated in the presence of GA. We found the *Rht-A-Q555stop* mutant reduced coleoptile length 12% compared to its sister wildtype line ($P<0.05$) when germinated in the presence of GA and 19% compared to *Rht-1a* ($P<0.01$). *Rht-B-Q420stop* and *Rht-D-Q559stop* did not have any measurable impact on coleoptile length. However, the *Rht-B-Q420stop* mutant did reduce GA perception compared to its sister wildtype line. The wildtype *Rht-B-Q420stop* coleoptiles were 14% longer when germinated in GA, the *Rht-B-Q420stop* mutant coleoptiles were only 5% longer when germinated in GA. Both wildtype and mutant *Rht-D-W559stop* coleoptiles were approximately 11% longer when germinated in the presence of GA than when germinated in water.

The novel missense alleles also had a range of effects on GA responsiveness and coleoptile growth. None of the novel alleles reduced coleoptile growth as dramatically as

the current semi-dwarfing alleles. *Rht-D-L500F* had the shortest coleoptile measurement of the novel alleles at 9.02 cm in water and 9.54 cm in GA.

We also analyzed the impact of the three stop mutations (*Rht-A-Q555stop*, *Rht-B-Q420stop*, *Rht-D-W559stop*) on DELLA/GID1 protein-protein binding using a yeast 2 hybrid assay (Figure 5.2). The current semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b* both introduce a premature stop codon, so we were initially interested in how stop codons in different regions of the gene may impact plant growth and protein function. The assay was done either in the absence or presence of added GA (100 μM). *Rht-A1a, Rht-B1a,* and *Rht-D1a* were included as positive controls and *Rht-B1b* and GID1 without DELLA were included as negative controls. As previously reported by Pearce *et al*. (2012), there was DELLA/GID1 interaction for *Rht-A1a, Rht-B1a*, and *Rht-D1a* (Figure 2). We observed no DELLA/GID1 interaction for *Rht-B1b* in the presence or absence of GA. None of the novel *Rht-1* alleles had DELLA/GID1 interactions in the absence of GA. However, the interaction was restored for *Rht-A-Q559stop*, *Rht-B420stop* and *Rht-D559stop* in the presence of GA. The interactions of the novel nonsense alleles appeared weaker than the *Rht-A1a, Rht-B1a*, and *Rht-D1a* DELLA/GID1 interaction.

Discussion

We created an EMS population using the standard height hard red spring variety Fortuna and identified 16 novel *Rht-1* alleles, including 3 nonsense mutations, one in each genome. We initially used the SIFT prediction software to estimate the impact of each mutation (Table 5.2). We also screened $F_{2:3}$ mutant seeds to investigate the impact

of the nonsense mutations on coleoptile length and GA responsiveness (Table 5.2). We observed the expected impact of *Rht-B1b* and *Rht-D1b* which reduced coleoptile length 43% compared to *Rht-1a* (Amram *et al*., 2015; Liatukas and Ruzgas 2010). Furthermore, coleoptile length of *Rht-B1b* and *Rht-D1b* was not impacted by the presence of GA, a previously reported (Amram *et al*., 2015).

Of the nonsense alleles, *Rht-A-Q555stop* had the greatest impact on coleoptile length. *Rht-A-Q555stop* coleoptiles were 13% shorter than their sister wildtype line coleoptiles, and 16% shorter than *Rht-1a*. Furthermore, *Rht-A-Q555stop* coleoptiles were only 3% longer when germinated in GA rather than water. This is much less than their sister wildtype line in which coleoptile length was increased 8% when germinated in water (Table 2). This indicates that *Rht-A-Q555stop* reduces coleoptile length, as well as reduces GA sensitivity but not as much as *Rht-B1b* or *Rht-D1b*.

Rht-B-Q420stop and *Rht-D-W559stop* did not impact coleoptile length. However, *Rht-B-Q420stop* did influence GA sensitivity. Mutant coleoptiles of *Rht-B-Q420stop* were 5% longer in the presence of GA. The coleoptiles of the sister wildtype line of *Rht-B-Q420stop* were 14% longer when germinated in GA compared to water (Table 2). This indicates that *Rht-B-Q420stop* reduces GA sensitivity, but not as severely as *Rht-A-Q555stop, Rht-B1b*, or *Rht-D1b*. The coleoptiles from both the mutant and wildtype *Rht-D-W559stop* lines were 11% longer when germinated in GA. This suggests that *Rht-D-W559stop* does not impact coleoptile length or GA sensitivity.

Of the missense alleles, *Rht-D-L500F* had the shortest coleoptile length, however there was no significant difference between the mutant and its sister wildtype line. In

general, the GA responsiveness and coleoptile measurements from the missense alleles were highly variable and it may be useful to repeat this test on a select number of alleles after developing near isogenic lines.

It may be useful to have semi-dwarfing alleles which do not impact coleoptile length and are sensitive to exogenous GA. This could be beneficial in arid environments where the current semi-dwarfing alleles are associated with decreased emergence. For example, *Rht-8* is a semi-dwarfing gene which is sensitive to GA and is well adapted to dry Mediterranean climates (Law and Worland, 1986; Korzun *et al*., 1998). It reduces plant height but does not impact coleoptile length, therefore making it better suited for dry climates. (Rebetzke *et al*., 1999; Ellis *et al.,* 2004)

We also investigated the impact of the stop mutations on the interaction between DELLA and GID1. Interestingly, *Rht-A-Q559stop*, *Rht-B-Q420stop* and *Rht-D-W559stop* blocked DELLA/GID1 interaction in the absence of GA, but interactions were restored in the presence of GA (Figure 2). DELLA/GID1 interaction is GA dependent, however, similar *in vitro* studies done in yeast have shown that wildtype DELLA can bind GID1 in the absence of GA, but the interaction is weakened (Pearce et al., 2011; Shinozaki et al., 2018). Other studies have illustrated that the GA dependent DELLA/GID1 interaction is stabilized by the GRAS domain (Hirano et al., 2010). *Rht-A-Q559stop*, *Rht-B-Q420stop* and *Rht-D-W559stop* may have inhibited DELLA/GID1 interaction in the absence of GA by destabilizing the weak protein binding. However, when GA was present, the destabilizing effect of the nonsense alleles was not enough to completely inhibit the

DELLA/GID1 interaction. The nonsense alleles DELLA/GID1 interaction in the presence of GA was weakened compared to *Rht-A1a*, *Rht-B1a*, and *Rht-D1a*.

The identification of these novel alleles increases *Rht-1* allelic diversity. The novel alleles could be used in breeding programs individually, as well as in combination. This could allow breeders to achieve a wider range of plant heights to develop ideal genotypes for specific environments and end use products. This project also illustrates the impact of mutations throughout the gene on the interaction between DELLA and GID1. Despite the prevalence of the semi-dwarfing alleles, the role and function of DELLA proteins is an ongoing research topic, especially the manipulation of DELLA proteins in relation to crop development.

Further research will be necessary to fully characterize the agronomic impact of the novel nonsense alleles, as well as to characterize the missense alleles. Due to the negative impact of the current semi-dwarfing alleles on grain protein content, it would be useful to identify a novel *Rht-1* allele which reduces height and increases tillering and yield but does not negatively affect grain protein content.

Table 5.1: Predicted agronomic impact of novel mutations for specific environments and agronomic management strategies.
[a] Expected yield estimates are based off of data reported in Lanning et al., (2012) in which yield trials were conducted with *Rht-B1b* and *Rht-D1b* isolines.
[b] Variants predicted to have partial function and moderate impact on plant height

Genotype	**Predicted Height Reduction**	**Grain Yield Increase[a]**	**Decrease in Grain Protein**	**Ideal Wheat Production Environment**
WT (*Rht-Ala, B1a, D1a*)	Standard Height Tall			Dry, normal fertilizer input
Rht-B1b	6”	10%	7%	Irrigated or dryland, mid-level fertilizer
Rht-D1b	6”	10%	7%	Irrigated or dryland, mid-level fertilizer
Single Allele Variant[b]	1”	1.7%	1.2%	Irrigated or dryland Mid to low-level fertilizer
Double Allele Variant	3”	5%	3.5%	Irrigated or dryland, mid to high-level fertilizer
Triple Allele Variant	4-5”	6.7-8.3%	4.7-5.8%	Irrigated or dryland, mid to high-level fertilizer

Table 5.2: List of novel *Rht-1* mutations and their predicted impact on protein structure and function.
[a] SIFT; sorting intolerant from tolerant, more deleterious mutations have values closer to 0 with values less than 0.05 predicted to be deleterious to protein function.

Genome	Amino acid #	Original amino acid	New amino acid	SIFT[a]
Rht-A	9	G	D	0.62
Rht-A	129	T	I	0.11
Rht-A	228	A	V	0.07
Rht-A	543	G	R	0
Rht-A	**555**	**Q**	**Stop**	**0**
Rht-B	205	E	G	0.34
Rht-B	**420**	**Q**	**Stop**	**0**
Rht-B	584	A	T	0.03
Rht-B	595	E	K	0
Rht-D	279	G	D	0
Rht-D	290	R	C	0.14
Rht-D	500	L	F	0
Rht-D	546	G	R	0
Rht-D	**559**	**W**	**Stop**	**0**
Rht-D	574	G	S	0
Rht-D	596	V	M	0
Rht-B1b	64	Q	Stop	0
Rht-D1b	61	Q	Stop	0

Figure 5.1: Location of novel mutations throughout *Rht-1* created by Ethyl methanesulfonate in the hard red spring wheat variety Fortuna
The newly identified stop mutations are indicated, gray regions represent DELLA and other conserved motifs. *Rht-B1b* and *Rht-D1b* are current standard dominant semi-dwarfing alleles. Total length of protein: *Rht-A1a*: 620 amino acids, *Rht-B1a*: 621 amino acids, *Rht-D1a*: 623 amino acids

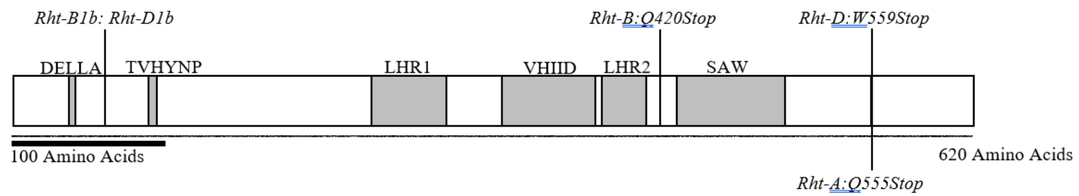

Table 5.3: Coleoptile lengths and gibberellic acid (GA_3) responsiveness assays of novel *Rht-1* alleles.
Values represent the average for each genotype ± the standard deviation
[a] n represents a single F_2-derived F_3 line.
*, **, and *** denote *P* values < 0.05, 0.01, and 0.001, respectively in comparisons between *Rht* mutant and wild type sister lines. Significance for GA/water determined using *P* values calculated from comparisons of water versus GA

Rht-1 Allele		n[a]	Coleoptile length in Water (cm)	Coleoptile length in GA (cm)	GA/water
Rht-1a		5	10.97 ± 1.00	12.48 ± 0.75	1.14**
Rht-B1b		5	7.13 ± 0.60***	7.04 ± 0.40***	0.99
Rht-A-A228V	mut	5	10.46 ± 0.55	11.29 ± 0.82	1.08*
Rht-A-A228V	wt	5	11.17 ± 0.71	12.18 ± 0.64*	1.09**
Rht-A-Q555stop	**mut**	**5**	**10.92 0.77**	**11.23 ± 0.49**	**1.03**
Rht-A-Q555stop	**wt**	**5**	**11.84 ± 1.29**	**12.76 ± 1.36****	**1.08**
Rht-A-G9D	mut	8	9.86 ± 0.39	10.66 ± 0.93	1.08**
Rht-A-G9D	wt	8	9.79 ± 0.57	10.57 ± 1.25	1.08
Rht-B-E595K	mut	7	11.51 ± 1.08	12.99 ± 1.23	1.13**
Rht-B-E595K	wt	7	12.00 ± 1.23	13.45 ± 0.90	1.12**
Rht-B-A584T	mut	9	10.27 ± 1.52	10.67 ± 1.14	1.04
Rht-B-A584T	wt	9	11.69 ± 1.87*	12.07 ± 1.01**	1.03
Rht-B-E205G	mut	5	12.37 ± 1.63	13.06 ± 0.76	1.06
Rht-B-E205G	wt	5	12.62 ± 1.82	13.67 ± 1.39	1.08
Rht-B-Q420stop	**mut**	**5**	**12.21 ± 0.50**	**12.83 ± 0.80**	**1.05**
Rht-B-Q420stop	**wt**	**5**	**11.75 1.62**	**13.36 ± 0.91**	**1.14**
Rht-D-L500F	mut	8	9.02 ± 1.18	9.54 ± 1.53	1.06
Rht-D-L500F	wt	8	9.96 ± 1.21	10.20 ± 1.95	1.02
Rht-D-G279D	mut	7	9.99 ± 1.16	10.16 ± 0.96	1.02
Rht-D-G279D	wt	7	10.99 ± 0.94*	10.94 ± 1.72	1
Rht-D-G574S	mut	6	9.86 ± 1.04	9.53 ± 1.21	0.97
Rht-D-G574S	wt	6	9.55 0.98	10.03 ± 1.27	1.05
Rht-D-V596M	mut	6	10.20 ± 1.10	11.16 ± 2.03	1.09
Rht-D-V596M	wt	6	11.46 ± 1.03*	9.95 ± 1.12	0.88**
Rht-D-G546R	mut	7	9.54 ± 1.78	11.19 ± 1.37	1.17*
Rht-D-G546R	wt	7	11.48 ± 1.59*	10.45 ± 1.41	0.91
Rht-D-W559stop	**mut**	**5**	**12.92 ± 1.02**	**14.35 ± 1.41**	**1.11**
Rht-D-W559stop	**wt**	**5**	**12.54 ± 1.66**	**14.03 ± 0.77**	**1.11**

Figure 5.2: Yeast 2 hybrid assay of novel nonsense Rht-1 alleles characterizing the DELLA/GID1 interaction in the presence or absence of 100 µM GA.
Interaction of *Rht-1* wildtypes (*Rht-A1a, Rht-B1a, Rht-D1a*) and *Rht-1* mutants (*Rht-B1b, Rht-A-Q555stop, Rht-B-Q420stop, Rht-D-W559stop*) with receptor GID1. The growth was done on SD/-Leu-Trp (control) and SD/-Leu-Trp-Ade-His supplemented either with or without 100µM GA_3.

SD/-L-T
SD/-L-T-A-H 0µM GA_3
SD/-L-T-A-H 100µM GA_3
1 1:10 1:100 1:1000 1:100000
Rht-A1a
Rht-B1a
Rht-D1a
Rht-B1b
Rht-A-Q555stop
Rht-B-Q420stop
Rht-D-W559stop
GID1

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CHAPTER SIX:

CONCLUSIONS

Since their introduction in the 1950s and 1960s, the *Reduced Height* (*Rht-1*) genes have played a key role in the development of modern wheat varieties. This has largely been due to the positive agronomic traits associated with the semi-dwarfing alleles, *Rht-B1b* and *Rht-D1b*. Despite their widespread popularity, the exact mechanism by which *Rht-B1b* and *Rht-D1b* increase yield is still unknown. Furthermore, very little research has been done regarding their impact on bread making and end use quality. Finally, beyond *Rht-B1b/Rht-D1b* there is very little allelic diversity for *Rht-1*.

The research presented in this dissertation has added to the existing knowledge surrounding the semi-dwarfing genes by: investigating the impact of *Rht-B1b* on photosynthesis and carbon and nitrogen metabolism, characterizing the impact of the semi-dwarfing alleles on bread making and dough rheology, and finally by increasing the available allelic diversity using non-GMO EMS mutagenesis.

We found that *Rht-B1b* decreased photsynthesis and flag leaf chlorophyll content at anthesis when grown under field conditions. Previous work had shown that *Rht-B1b* was associated with decreased grain protein content; we determined that this difference can be detected as early 14 days past anthesis. Despite these differences, we were unable to detect any impact of *Rht-B1b* on the expression of genes associated with carbon and nitrogren metabolism during stem elongation.

We determined that although the semi-dwarfing alleles have been primarily utilized for their agronomic effects, they also impact traits associated with bread making

and end use quality. Despite *Rht-B1b/Rht-D1b* negatively impacting grain and flour protein content, they significantly increase dough mixing time, tolerance, and the gluten index. This is likely due to *Rht-B1b* and *Rht-D1b* increasing the relative abundance of high molecular weight glutenins compared to the tall wildtype.

Lastly, this dissertation describes the development, identification, and characterization of novel *Rht-1* alleles using EMS mutagenesis; specifically, the characterization of three novel non-sense alleles, *Rht-A-Q555stop, Rht-B-Q420stop, Rht-D-W559stop.* We evaluated each mutations impact on coleoptile elongation, GA responsiveness, and DELLA/GID1 interaction. Further research will be needed to describe the agronomic impact of these novel mutations.

This research is relevant to better understand some of the most widely incorporated genes in wheat breeding programs worldwide. It is an illustration of how a yield gene can also impact end use quality. Finally, it increases the available allelic diversity of *Rht-1* so that wheat breeders may be able to better develop an ideal genotype for unique environments or end use products.

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