

Molecular Characterization of *Rht-1* Dwarfing Genes in Hexaploid Wheat^{1,2}[C][W][OA]

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The introduction of the *Reduced height (Rht)-B1b* and *Rht-D1b* semidwarfing genes led to impressive increases in wheat (*Triticum aestivum*) yields during the Green Revolution. The reduction in stem elongation in varieties containing these alleles is caused by a limited response to the phytohormone gibberellin (GA), resulting in improved resistance to stem lodging and yield benefits through an increase in grain number. *Rht-B1* and *Rht-D1* encode DELLA proteins, which act to repress GA-responsive growth, and their mutant alleles *Rht-B1b* and *Rht-D1b* are thought to confer dwarfism by producing more active forms of these growth repressors. While no semidwarfing alleles of *Rht-A1* have been identified, we show that this gene is expressed at comparable levels to the other homeologs and represents a potential target for producing novel dwarfing alleles. In this study, we have characterized additional dwarfing mutations in *Rht-B1* and *Rht-D1*. We show that the severe dwarfism conferred by *Rht-B1c* is caused by an intragenic insertion, which results in an in-frame 90-bp insertion in the transcript and a predicted 30-amino acid insertion within the highly conserved amino-terminal DELLA domain. In contrast, the extreme dwarfism of *Rht-D1c* is due to overexpression of the semidwarfing *Rht-D1b* allele, caused by an increase in gene copy number. We show also that the semidwarfing alleles *Rht-B1d* and *Rht-B1e* introduce premature stop codons within the amino-terminal coding region. Yeast two-hybrid assays indicate that these newly characterized mutations in *Rht-B1* and *Rht-D1* confer "GA-insensitive" dwarfism by producing DELLA proteins that do not bind the GA receptor GA INSENSITIVE DWARF1, potentially compromising their targeted degradation.

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The introduction of semidwarfing genes into rice (*Oryza sativa*) and wheat (*Triticum aestivum*) was a major factor in breeding higher yielding varieties during the Green Revolution (for review, see Hedden, 2003). The higher yields were associated with improved lodging resistance and the resulting ability to tolerate higher rates of inorganic nitrogen-based fertilizer (Gale and Youssefian, 1985). The decrease in stem stature resulted in an increase in assimilate partitioning to developing ears, enabling greater floret survival at anthesis and increased grain numbers per ear (Youssefian et al., 1992). Different *Reduced height-1 (Rht-1)* genes, and combinations, gave optimal yields depending on the height of the background variety (Flintham et al., 1997).

In the wheat and rice semidwarf mutants, reduced stature is conferred through alterations in growth mediated by the GA phytohormones. This class of plant hormones has a well-characterized role in controlling stem elongation, as illustrated by the reduced stature of GA biosynthetic and signaling mutants in many plant species (Ross, 1994). In hexaploid wheat, dwarfing has been achieved mainly through the introduction of the *Rht* alleles *Rht-B1b* and *Rht-D1b*, now found in the majority of varieties grown worldwide (Evans, 1998), which confer decreased responsiveness to GA (Gale and Marshall, 1973; Pinthus et al.,

1989). Six additional alleles at *Rht-B1* and *Rht-D1* have been identified by genetic mapping, four on chromosome 4B and two on chromosome 4D, which produce dwarfs with a broad range of plant height (Fig. 1; Gale and Youssefian, 1985; Borner et al., 1996; Flintham et al., 1997). For example, the *Rht-B1b* and *Rht-D1b* alleles produce only about a 20% reduction in height, while *Rht-B1c* and *Rht-D1c* are about half the size of the appropriate tall controls (Hoogendoorn et al., 1990; Flintham et al., 1997). The *Rht-B1* and *Rht-D1* genes were shown by Peng et al. (1999) to encode DELLA proteins, transcriptional regulators that act to repress GA signaling (for review, see Sun, 2010). Peng et al. (1999) found that *Rht-B1b* and *Rht-D1b* contain single nucleotide substitutions that introduce premature stop codons in the N-terminal coding region. They suggested that translational reinitiation could lead to the production of N-terminally truncated proteins that confer dwarfism through increased repression of GA signaling.

In many cases, environmental and developmental signals promote plant growth by increasing the levels of bioactive GAs (for review, see Yamaguchi, 2008), which promote the degradation of the DELLA proteins, thus causing the transcriptional changes that mediate the growth responses (for review, see Sun, 2010). Studies in *Arabidopsis* (*Arabidopsis thaliana*) and rice have demonstrated that targeted degradation of DELLAs is initiated by GA binding within a pocket of the GA receptor, GA INSENSITIVE DWARF1 (GID1; Ueguchi-Tanaka et al., 2005; Murase et al., 2008; Shimada et al., 2008). This leads to closing of the N-terminal lid of GID1, allowing DELLAs to bind through their conserved N-terminal DELLA/TVHYNP motifs. GID1/DELLA binding enables recognition by the GID2/SLEEPY1 F-box component of an SCF Ub E3 ligase, leading to ubiquitination of DELLAs and their subsequent 26S proteasome-mediated degradation (Fu et al.,

2002; Sasaki et al., 2003; Dill et al., 2004; Griffiths et al., 2006; Feng et al., 2008; Hirano et al., 2010). DELLA gain-of-function mutations, which result in reduced GA sensitivity and hence dwarfism, have been identified in many plant species, including rice and *Arabidopsis*. The majority of these mutations produce amino acid substitutions or deletions in the DELLA/TVHYNP motifs that affect binding to GID1 (Peng et al., 1997; Dill et al., 2001; Chandler et al., 2002; Asano et al., 2009), but not their transcriptional regulatory activity, which resides in the C-terminal GRAS domain (Silverstone et al., 1998; Chandler et al., 2002; Itoh et al., 2002; Hirano et al., 2010). The resulting reduction in GA-induced degradation of the mutant DELLAs confers dwarfism through enhanced repression of growth (Dill et al., 2001; Gubler et al., 2002; Itoh et al., 2002). The N-terminally truncated DELLA proteins that are proposed to be translated from *Rht-B1b* and *Rht-D1b* transcripts would similarly lack part of the conserved motifs that are necessary for GA-induced degradation, allowing them to accumulate and repress growth (Peng et al., 1999). However, currently, there is no biochemical evidence to support this hypothesis.

A more detailed understanding of how the *Rht-1* genes confer dwarfism could potentially allow the development of novel alleles with improved specificity for agronomic traits. For example, in semiarid environments where deep sowing is necessary to access moisture for grain germination, the shorter coleoptiles of *Rht-B1b* and *Rht-D1b* cause poor emergence (Rebetzke et al., 2007). In order to obtain further information on *Rht-1* gene structure and dwarfism, we have analyzed at the molecular level five of the six previously uncharacterized alleles at this locus. As well as discovering novel mutations, our results indicate that the severity of dwarfism in *Rht-D1c* is due to increased expression of the *Rht-D1b* allele. We also provide further evidence that the mutant RHT-1 proteins are unable to interact with the GA receptor, TaGID1, which would result in growth repression that is less responsive to GA. To determine the importance for the control of stem elongation of *Rht-A1*, for which no dwarfing alleles have been identified, we have cloned *Rht-A1a* and demonstrated that it displays a very similar expression profile to *Rht-B1a* and *Rht-D1a*.

RESULTS

Identification and Sequence Analysis of the *Rht-A1* Gene

Although the existence of the homeologous DELLA gene, *Rht-A1*, is indicated (Peng et al., 1999; Febrer et al., 2009), its contribution to GA-dependent growth is currently unclear. In order to address this, a full-length putative *Rht-A1a* sequence was amplified by PCR using cDNA derived from seedling leaf tissues (var Cadenza). Across the predicted coding region, the amino acid sequence is approximately 96% identical to

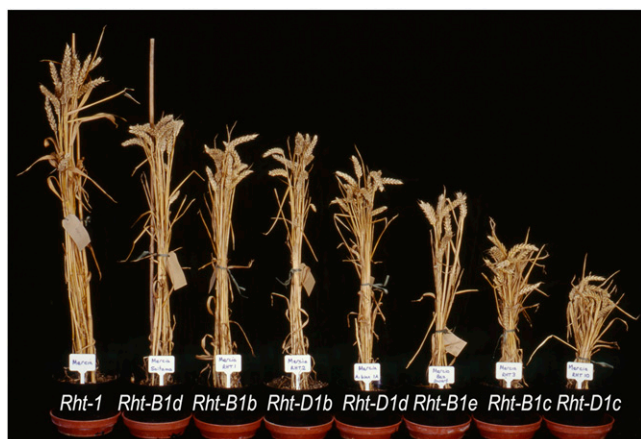


Figure 1. Phenotypes of *Rht-B1* and *Rht-D1* dwarfing alleles in NILs. Wheat NILs (var Mercia) were grown to maturity. The photograph is from the John Innes Centre archives (produced by Tony Worland). [See online article for color version of this figure.]

corresponding RHT-B1A and RHT-D1A sequences (Fig. 2A). Furthermore, nucleotide positions 289 to 769 of the coding region are identical to an *Rht-A1* sequence amplified from a Chinese Spring bacterial artificial chromosome library (Febrer et al., 2009), making it likely that this represents the *Rht-A1* gene. To confirm the chromosomal location of this gene, specific PCR primers were designed to each *Rht-1* homeolog and used to amplify genomic DNA (gDNA) from chromosome 4 aneuploid wheat lines (Brewer et al., 1969). Whereas all three homeologs were amplified to a comparable level using the wild-type Chinese Spring gDNA template, the putative *Rht-A1* sequence was not amplified from the template lacking chromosome 4A (N4AT4D; Supplemental Fig. S1), consistent

with it representing the *Rht-A1* gene on chromosome 4A.

The predicted RHT-A1 amino acid sequence contains all known motifs that are conserved in functional DELLA proteins. These include the DELLA, LEXLE, and TVHYNP motifs within the N-terminal regulatory domain (Fig. 2A), which are responsible for binding to GID1 to initiate GA-mediated degradation (Murase et al., 2008; Shimada et al., 2008), and a highly conserved C-terminal GRAS domain that is important for functionality (Peng et al., 1997; Silverstone et al., 1998; Hirano et al., 2010). The conservation in these regions, and the expression profile of *Rht-A1* (see below), strongly suggest that *Rht-A1*, in addition to *Rht-B1* and *Rht-D1*, encodes a functional DELLA protein.

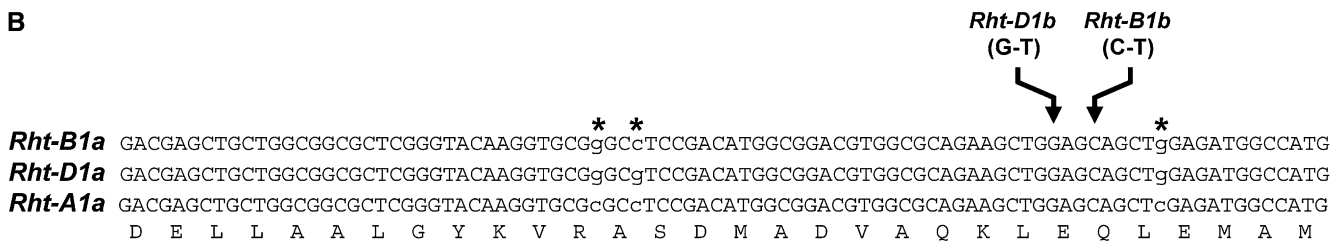
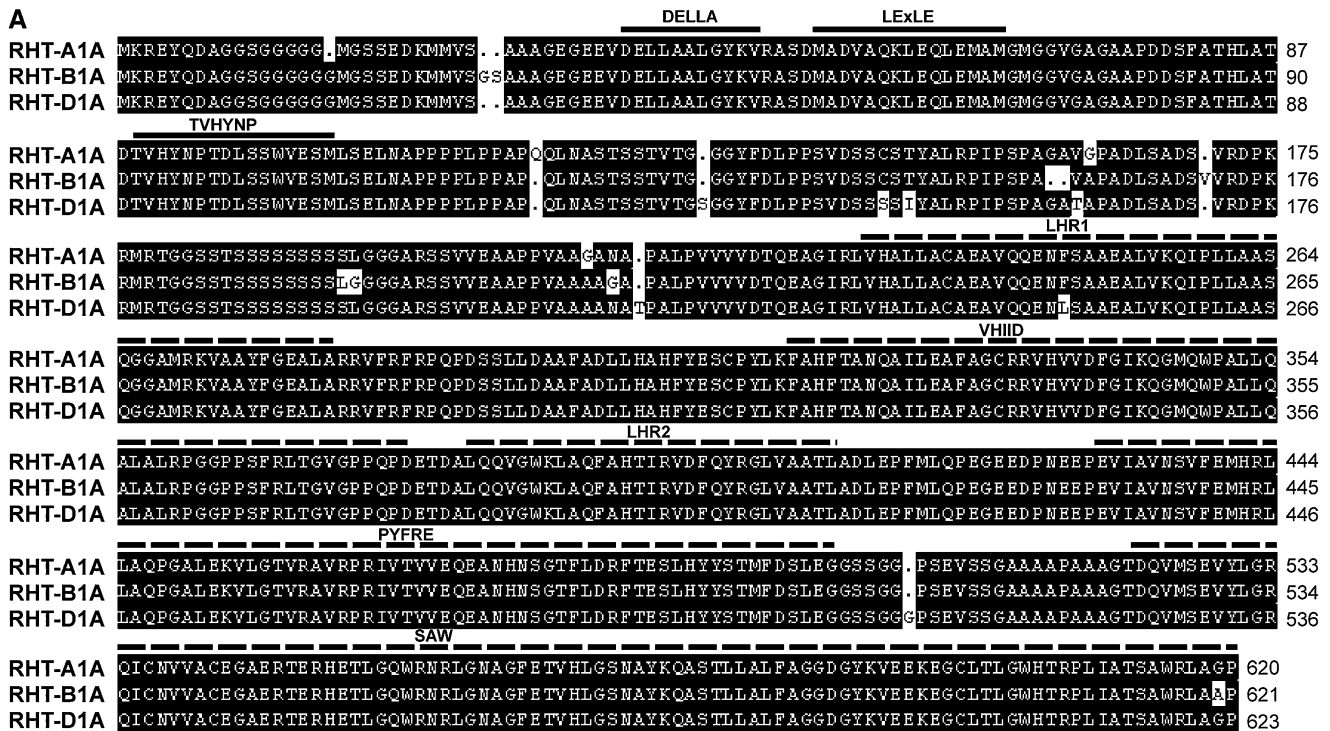


Figure 2. Predicted RHT-1 sequences. A, Amino acid sequence alignment of RHT-A1A, RHT-B1A, and RHT-D1A proteins. The amino acid sequences are predicted from nucleotide sequences amplified from var Cadenza. Gaps introduced to improve the sequence alignment are indicated by dots. Conserved N-terminal regulatory motifs (DELLA, LEXLE, and TVHYNP) are indicated by thick solid lines above the sequences. The C-terminal (LHR1, VHIID, LHR2, PFYRE, and SAW) functional domains are indicated by thick dashed lines above the sequences. B, Comparison of the nucleotide sequences of the *Rht-1* homeologs across the conserved N-terminal coding region. SNPs between the *Rht-1* homeologs are indicated by lowercase letters and asterisks above the sequences. The *Rht-B1b* and *Rht-D1b* point mutations are shown, and their positions are indicated by arrows above the sequences. The predicted amino acid sequences of RHT-A1A, RHT-B1A, and RHT-D1A are identical in this region.

Expression Analysis of the *Rht-1* and GA Biosynthetic Genes during Wheat Stem Elongation

Differences in the expression profiles of the three *Rht-1* homeologs during stem elongation could, in part, explain the phenotypic diversity observed in the *Rht-1* mutants and the absence of identified semidwarfing alleles of *Rht-A1* that are analogous to *Rht-B1b* or *Rht-D1b*. Transcript levels for the three homeologs were monitored in different regions of an elongating stem (var Cadenza) at a stage prior to the main period of peduncle expansion (Supplemental Fig. S2) using quantitative real-time reverse transcription (qRT)-PCR with homeolog-specific primers (Fig. 3A). The results indicated that *Rht-A1* is expressed with a similar profile to *Rht-B1* and *Rht-D1* in all regions of the stem that were analyzed. All three homeologs are expressed at a slightly higher level in the peduncle and lower section of the P-1 internode, the two tissues that are undergoing cell expansion, but otherwise the expression is similar in other stem tissues. A slight increase in the levels of expression of the *Rht-1* homeologs is observed in the ear compared with most regions of the stem. In contrast, on the basis of RNA-blot hybridization, Appleford et al. (2006) reported considerable tissue specificity in the expression of the GA-biosynthesis genes, *TaGA20ox1* and *TaGA3ox2*. We confirmed this by qRT-PCR, which showed highest levels of expression of *TaGA20ox1* in the ear and peduncle node (Fig. 3B), while *TaGA3ox2* is most highly expressed in the peduncle and lower part of the P-1 internode (Fig. 3C).

In order to localize the expression domains of *Rht-A1*, *TaGA20ox1*, and *TaGA3ox2* more precisely, promoter-GUS transcriptional fusion constructs were generated using approximately 1.5 kb of sequence upstream of the predicted initiating ATG. Multiple transgenic wheat lines were produced for each construct showing consistent expression patterns in at least five independent T1 lines (data not shown). GUS activity was determined in the elongating wheat stems of the *pRht-A1::GUS*, *pGA20ox1::GUS*, and *pGA3ox2::GUS* lines at an equivalent developmental stage to that used for the qRT-PCR study described above. GUS activity in the *pRht-A1::GUS* lines was observed at similar levels in the nodes and P-1 internode region (Fig. 3D), whereas the *pGA20ox1::GUS* and *pGA3ox2::GUS* lines displayed GUS activity predominantly in the P-1 and peduncle nodal tissues (Fig. 3, E and F), with lower levels detectable in the P-1 internode. In contrast to the *pGA20ox1::GUS* line, GUS activity in the *pGA3ox2::GUS* line extended from the P-1 node up into the lower internode (Fig. 3F). These differences in expression profiles of *GA3ox2* and *GA20ox1* are supported by our qRT-PCR analysis (Fig. 3C) and the earlier study by Appleford et al. (2006). However, the level of GUS activity observed in the lower internode of the *pGA3ox2::GUS* lines is not entirely consistent with the high transcript levels of *GA3ox2* observed by qRT-PCR. This observation potentially indicates the

requirement for additional regulatory elements within the *GA3ox2* gene that are necessary to direct the appropriate expression within this tissue. The expression profiles for *TaGA20ox1*, *TaGA3ox2*, and *Rht-A1* inferred from the promoter-GUS reporter lines, which in most cases were consistent with the qRT-PCR data, clearly illustrate differences in the regulation of GA biosynthetic and DELLA genes during wheat stem elongation.

Characterization of *Rht-B1* and *Rht-D1* Mutations

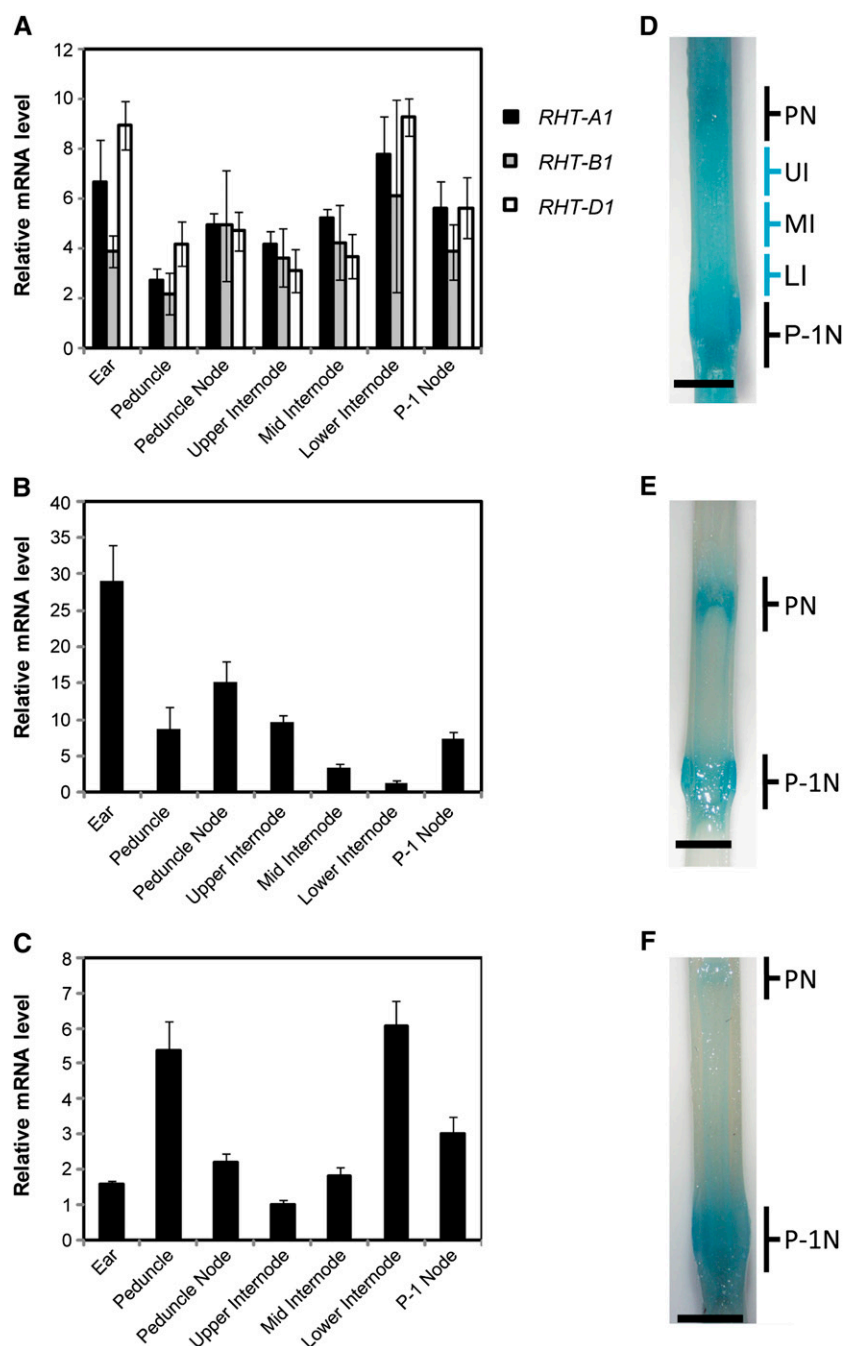
In addition to *Rht-B1b* and *Rht-D1b*, six further dwarfing alleles of *Rht-B1* or *Rht-D1*, conferring different degrees of dwarfism, have been identified by genetic mapping (Fig. 1) and reviewed by Borner et al. (1996). The phenotypes range from a mild decrease in plant stature in *Rht-B1d* to moderate in *Rht-D1d* and *Rht-B1e* to more severe in *Rht-B1c* and *Rht-D1c* (Fig. 1). While the molecular basis of the dwarfing mutations in *Rht-B1b* and *Rht-D1b* has been determined (Peng et al., 1999), there is currently no sequence information regarding the six further *Rht-1* dwarfing alleles. Characterization of these different alleles at the molecular level should aid understanding of how different mutations at homeologous loci induce such variation in plant height. Such information would also allow the design and use of allele-specific PCR markers, permitting simple genotyping at these loci.

The high GC content within the *Rht-1* genes (approximately 71% over their coding regions) made their amplification from gDNA or cDNA templates difficult. However, homeolog-specific amplification of the entire coding sequences of *Rht-B1* and *Rht-D1* was achieved by amplifying overlapping 5' and 3' fragments.

Rht-B1c

The *Rht-B1* coding region was amplified using cDNA prepared from independent lines carrying the *Rht-B1c* allele, including the variety Tom Thumb (the original source of the dwarfing gene) and *Rht-B1c* near-isogenic lines (NILs) in Maris Huntsman, Maringá, and Nainari 60 (Gale and Marshall, 1973; Gale and Yousefian, 1985). Strikingly, we found that the *Rht-B1c* transcript carries a 90-bp in-frame insertion within the region encoding the conserved N-terminal DELLA domain (Supplemental Fig. S3). In addition, the *Rht-B1c* allele also contains two single nucleotide polymorphisms (SNPs) upstream of the insertion that result in amino acid substitutions (Fig. 4A; G15R and M25I substitutions). In the C-terminal GRAS domain, there were two silent mutations (codons Ala-271 [GCA to GCG] and Ala-617 [GCA to GCC]). Interestingly, a much larger insertion was present in gDNA from all lines containing *Rht-B1c* that were examined, although attempts to amplify across the full insert to determine its size were unsuccessful. However, PCR and sequencing indicated the

Figure 3. Expression analysis of the three *Rht-1* homeologs and two GA biosynthesis genes in the upper expanding tissues of the developing wheat stem. A to C, Relative expression levels of *Rht-A1*, *Rht-B1*, and *Rht-D1* (A), *TaGA20ox1* (B), and *TaGA3ox2* (C) in different regions of the extending wheat stem at 7 weeks post germination. The P-1 internode was divided into three equal sections for tissue collection. The mRNA levels of these genes were determined by qRT-PCR from three biological replicates for each sample type, normalized against *UBIQUITIN*. Results are plotted as the ratio to the lowest detectable level \pm se. D to F, Localization of GUS activity under the control of the *Rht-A1* (D), *TaGA20ox1* (E), and *TaGA3ox2* (F) promoters in an elongating wheat stem at the same stage of development as analyzed by qRT-PCR. The positions of the peduncle node (PN) and the P-1 node (P-1N) are indicated. The positions of the upper (UI), middle (MI), and lower (LI) internodes are indicated in D. Bars = 5 mm.



presence of at least a 508-bp fragment that included the 90-bp insertion found in the transcript (Supplemental Fig. S3). Furthermore, the larger insertion contains potential splice sites at its 5' end and at the start of the 90-bp insertion (predicted using <http://genes.mit.edu/GENSCAN.html>). This suggested that most of the insertion in *Rht-B1c* is subsequently excised during transcript processing to give an mRNA containing a 90-bp in-frame insertion and a protein with a 30-amino acid insertion within the DELLA domain (Fig. 4A). Allele-specific PCR-based markers were designed to detect the presence of the insertion in

Rht-B1c, allowing it to be distinguished from other *Rht-B1* alleles (Supplemental Fig. S4A).

In order to determine whether mutations in *Rht-B1* affected *Rht-1* gene expression, the relative levels of the homeologous *Rht-1* transcripts were compared in *Rht-B1a* (*Rht-1*), *Rht-B1b*, and *Rht-B1c* seedlings by qRT-PCR. There were no significant differences in the levels of expression of *Rht-A1* and *Rht-D1* in the two *Rht-B1* mutants that were analyzed (Fig. 4, C and E). Similarly, expression of *Rht-B1* was largely unaltered in the *Rht-B1b* mutant compared with the *Rht-1* control (Fig. 4D), but its expression was significantly reduced

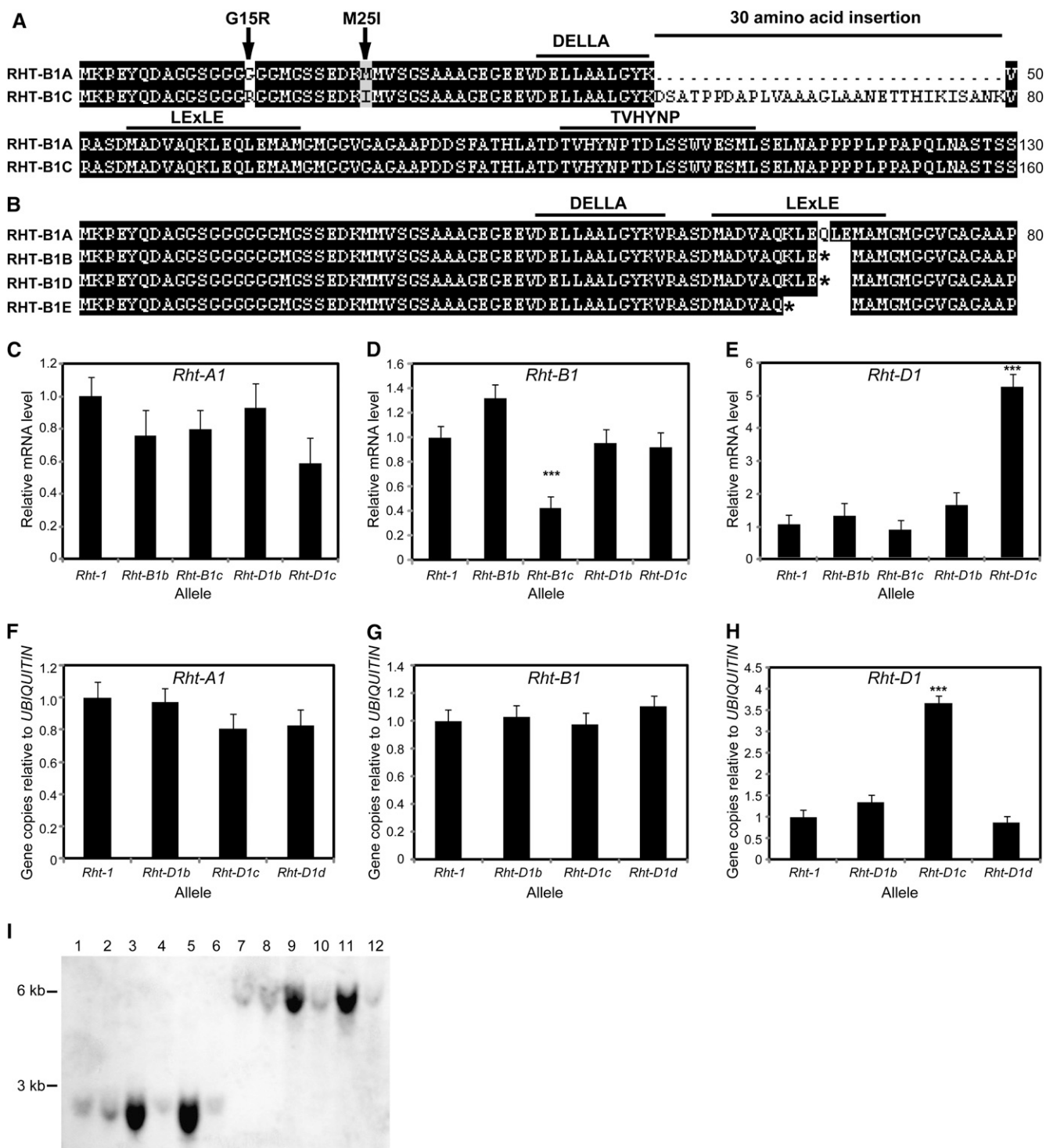


Figure 4. *Rht-1* allele characterization. A, Amino acid alignment of RHT-B1A and RHT-B1C. The translated RHT-B1C protein contains a 30-amino acid insertion close to the DELLA motif and two upstream amino acid substitutions (indicated with arrows above the sequences; G15R and M25I). Conserved N-terminal regulatory (DELLA, LExLE, and TVHYNP) domains are indicated by lines above the sequences. B, Amino acid alignment of the N-terminal domains of *Rht-B1* alleles. *Rht-B1d* carries the same point mutation as *Rht-B1b*, while *Rht-B1e* expresses a point mutation that introduces a premature stop codon three amino acids farther upstream. C to E, mRNA levels for *Rht-1* in 2-week-old seedlings of wheat NILs in the Mercia variety, determined by qRT-PCR using primers designed to amplify all three homeologs. Values shown are means of three biological replicates, normalized against *UBIQUITIN*. Results are plotted as the ratio to the level in the *Rht-1* control \pm SE. *** Significantly different from the *Rht-1* control ($P < 0.01$). F to H, Gene copy number of the *Rht-1* homeologues in the *Rht-D1* allelic series (NILs in the Mercia variety)

in both the Mercia and Maris Huntsman *Rht-B1c* NILs (Fig. 4D; Supplemental Fig. S5B). The reduction in *Rht-B1* transcript levels in *Rht-B1c* is potentially due to a lower splicing efficiency during mRNA processing.

Rht-B1d and *Rht-B1e*

Rht-B1d is derived from the Japanese variety Saitama 27 (Worland and Petrovic, 1988). A recent study by Pestsova et al. (2008) using a PCR-based assay indicated the presence of the *Rht-B1b* point mutation in the *Rht-B1d* allele. We confirmed this result by amplifying and sequencing the *Rht-B1* gene from genomic DNA extracted from *Rht-B1d* seedlings (Fig. 4B). Surprisingly, no further mutations were found in the coding region of this gene. Plants carrying the *Rht-B1d* mutation are less severely dwarfed than those carrying *Rht-B1b* alone (Fig. 1; Worland and Petrovic, 1988), which suggests that another, undetected, mutation outside of the *Rht-B1* coding region is affecting plant height.

Another source of GA-insensitive dwarfism that is used commercially has its origins in the Russian variety Bezostaya, which carries the *Rht-B1e* allele (Worland, 1986; Borner et al., 1996). PCR-based assays indicated that *Rht-B1e* is distinct from *Rht-B1b* (Pestsova et al., 2008). To characterize this allele, the *Rht-B1* gene from two *Rht-B1e* semidwarf lines, Krasnodar dwarf and Bezostaya dwarf, was amplified and sequenced, revealing a novel mutation introducing a stop codon three codons upstream of the *Rht-B1b* point mutation (Fig. 4B). An A-to-T nucleotide substitution changes the Lys-61 codon (AAG) to a translational stop codon (TAG). This is in an almost identical position to both the *Rht-B1b* and *Rht-D1b* mutations, where similar point mutations result in the conversion of *Rht-B1* codon 64 and *Rht-D1* codon 61 to stop codons (Fig. 4B; Peng et al., 1999). No further mutations in the *Rht-B1e* coding sequence were detected. We have designed PCR-based markers that detect the presence of this mutation and distinguish it from both the *Rht-B1a* and *Rht-B1b* sequences (Supplemental Fig. S4B). Based on the similarity in nature and its position within the coding region, it is likely that the *Rht-B1e* mutation confers its GA-insensitive semidwarf phenotype in a manner analogous to *Rht-B1b*, potentially through the production of an N-terminally truncated protein product that represses GA signaling, as hypothesized by Peng et al. (1999).

Rht-D1c and *Rht-D1d*

The extreme dwarfing allele *Rht-D1c* is derived from the Chinese variety Ai-bian 1, which is also the source of another *Rht-D1* allele, *Rht-D1d* (Borner et al., 1996). This latter allele arose spontaneously from an *Rht-D1c* population and was distinguished by an increase in height compared with the *Rht-D1c* plants (Fig. 1). We used genomic DNA extracted from Ai-bian 1, *Rht-D1c*, and *Rht-D1d* as templates to amplify the *Rht-D1* coding region using homeolog-specific primers. Sequence analysis revealed that all of these lines carry the *Rht-D1b* mutation, as suggested by screening using PCR markers (Pestsova et al., 2008). Since no further mutations were detected, the sequences of *Rht-D1b*, *Rht-D1c*, and *Rht-D1d* are identical across the coding region. However, the far more severe dwarf phenotype of *Rht-D1c* lines compared with *Rht-D1b* suggests that it may carry an additional mutation outside the coding region that alters its expression level. This was investigated by comparing the transcript abundance for the three *Rht-1* homeologs in seedlings of *Rht-D1a*, *Rht-D1b*, *Rht-D1c*, and *Rht-D1d* by qRT-PCR using specific primers. There were no significant differences in the levels of expression of *Rht-A1* and *Rht-B1* in the *Rht-D1* mutants compared with *Rht-D1a* (Fig. 4, C and D). However, while *Rht-D1* was expressed at similar levels to *Rht-1* in *Rht-D1b* (Fig. 4E), its expression level in *Rht-D1c* was about 4-fold higher (Fig. 4E). We then investigated whether the increased *Rht-D1* expression in *Rht-D1c* is due to the presence of extra copies of *Rht-D1* by performing real-time qPCR on gDNA isolated from *Rht-D1a* (tall), *Rht-D1b*, *Rht-D1c*, and *Rht-D1d* lines. The copy number of *Rht-A1* and *Rht-B1* was found to be very similar in all these lines (Fig. 4, F and G). However, while the *Rht-D1* copy number in *Rht-D1b* and *Rht-D1d* was nearly identical to that in the *Rht-D1a* control (Fig. 4H), it was about 4-fold higher in *Rht-D1c* (Fig. 4H), providing a good correlation between gene copy number and expression level. To confirm this increase in *Rht-D1* copy number in *Rht-D1c*, we hybridized a *Rht-D1*-specific DNA probe to a Southern blot containing *EcoRI*- or *DraI*-digested gDNA isolated from an *Rht-D1* allelic series. The probe (D1-2855) was generated by amplifying a DNA fragment between 2,855 and 3,097 bp downstream of the *Rht-D1*-initiating ATG. Hybridization of D1-2855 to gDNA fragments from *Rht-1*, *Rht-D1b*, and *Rht-D1d* (Mercia NIL and Aibian 1A progenitor) produced similar band intensities (Fig. 4I), suggesting

Figure 4. (Continued.)

determined by qPCR of gDNA template. The relative gDNA copy number was normalized against *UBIQUITIN*. Results are plotted as the ratio to the level in the *Rht-1* control. Values shown are means of three biological replicates \pm se. *** Significantly different from the *Rht-1* control ($P < 0.01$). I, Hybridization of D1-2855 to a Southern blot containing gDNA isolated from wheat lines containing different *Rht-D1* alleles: *Rht-D1a* Mercia (*Rht-1*) control, lanes 1 and 7; *Rht-D1b* Mercia NIL, lanes 2 and 8; *Rht-D1c* Mercia NIL, lanes 3 and 9; *Rht-D1d* Mercia NIL, lanes 4 and 10; *Rht-D1c* Ai-bian 1, lanes 5 and 11; *Rht-D1d* Ai-bian 1a, lanes 6 and 12, digested with *DraI* (lanes 1–6) or *EcoRI* (lanes 7–12).

equal gene copy numbers in these lines. In contrast, the intensity of signal for the equivalent gDNA fragments from *Rht-D1c* (observed in both Mercia NIL and Aibian 1 progenitor) was highly elevated (Fig. 4I), supporting an increase in Rht-D1 copy number. Taken together, these findings strongly suggest that the severe dwarfing caused by the *Rht-D1c* allele results from the presence of multiple gene copies of *Rht-D1b*, producing elevated transcript levels and potentially higher amounts of the GA-insensitive DELLA repressor. While *Rht-D1d* is potentially derived from *Rht-D1c* (Borner et al., 1991), it does not appear to have an increase in *Rht-D1* copy number compared with the *Rht-D1a* control (or *Rht-D1b*; Fig. 4H). It is likely, therefore, that the substantial increase in height of *Rht-D1d* compared with *Rht-D1c* (Borner et al., 1991, 1996) is due to this reduction in the number of gene copies of *Rht-D1b*.

Dwarfing Mutations in RHT-B1 Abolish the Interaction with TaGID1 in Yeast Two-Hybrid Assays

Binding of bioactive GAs within a pocket in the GID1 protein induces a conformational change that permits interaction between the DELLA and GID1 proteins (Murase et al., 2008; Shimada et al., 2008). This interaction with GID1 involves several conserved amino acids within the DELLA N-terminal region. We hypothesize that disruption of these conserved motifs in mutant RHT-1 proteins would prevent their interaction with GID1 and allow the proteins to accumulate, despite adequate endogenous GA levels. In the case of *Rht-1* mutations that introduce premature stop codons, it has been suggested that translational reinitiation occurs, producing an N-terminally truncated protein product that lacks the GID1 interaction motifs (Peng et al., 1999; Willige et al., 2007). However, an alternative explanation is that the reduced GA sensitivity results from expression of the short N-terminal peptide, which could sequester GID1 and thereby reduce its effective concentration. In an attempt to distinguish between these possibilities and to examine the impact of the 30-amino acid insertion in RHT-B1C, we used yeast two-hybrid assays to assess the interaction between the predicted RHT-B1B and RHT-B1C mutant proteins and GID1. A GID1 bait construct was prepared by amplifying a *TaGID1* sequence from wheat cDNA. The *TaGID1* sequence is 95% identical to the barley (*Hordeum vulgare*) GA receptor, GSE1 (Chandler et al., 2008), strongly suggesting that it encodes a functional GA receptor. The prey constructs containing the domains indicated in Figure 5A were cotransformed into yeast with the *TaGID1* bait, and the interactions were assessed in the absence or presence of bioactive GA₃. The full-length RHT-B1A interacted strongly with *TaGID1* in yeast even in the absence of GA, and this interaction was enhanced by the presence of GA₃ (Fig. 5B). This observation provides support for *TaGID1* being a functional GA receptor that binds RHT-B1A, potentially targeting it for degradation via a pathway

that appears conserved in both dicots and monocots (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Willige et al., 2007). RHT-B1C, which contains the 30-amino acid insertion within the DELLA domain, failed to interact with *TaGID1* in the absence or presence of GA (Fig. 5B). The predicted N- and C-terminally truncated RHT-B1B protein products also did not interact with *TaGID1* in the yeast two-hybrid assays, irrespective of whether GA₃ was included (Fig. 5B).

DISCUSSION

The major contribution to global wheat production from the introduction of the *Rht-B1b* and *Rht-D1b* semidwarfing genes in the 1960s is well documented (Evans, 1998). However, it was not until 1999 that the mutant genes were characterized at the molecular level (Peng et al., 1999), and the biochemical basis for the resulting semidwarf phenotype has still not been demonstrated. In addition to *Rht-B1b* and *Rht-D1b*, a number of other dwarfing alleles at the *Rht-B1* and *Rht-D1* loci have been described, several having applications in agriculture (Borner et al., 1996). We have characterized three further dwarfing *Rht-B1* alleles and two at the *Rht-D1* locus and provide evidence to support the proposed biochemical basis for the phenotype in these and the *Rht-B1b* and *Rht-D1b* mutants. No dwarfing allele has been identified in *Rht-A1*, and it was unclear whether this gene was expressed. Our results here indicate that *Rht-A1* is expressed at similar levels to the other *Rht-1* homeologs. Furthermore, the nucleotide sequence in the N-terminal coding region encompassing the premature stop codons in *Rht-B1b*, *Rht-B1e*, and *Rht-D1b* is identical in all three genes (Fig. 2B), which should allow the introduction of similar stop codons into *Rht-A1*. The similarity in phenotype between the *Rht-B1b*, *Rht-D1d*, and *Rht-B1e* mutants suggests that mutations introducing a premature stop within at least three codons (codons 61, 62, and 64; Fig. 2B) will confer GA insensitivity. The absence of additional *Rht-B1* and *Rht-D1* alleles with novel mutations at these positions illustrates the rarity of these mutational events. Therefore, it is possible that corresponding mutations in the *Rht-A1* gene will also confer GA insensitivity but have yet to occur or be selected. However, it is also conceivable that these mutations do not confer an obvious phenotype due to changes in the functional role of *Rht-A1* compared with the other *Rht-1* homeologs. To distinguish between these two scenarios, it will be important to introduce the equivalent *Rht-A1* mutations using targeted mutagenesis-based approaches.

The genetic lesions of all previously characterized DELLA gain-of-function mutants are either nucleotide substitutions or in-frame deletions (Peng et al., 1997, 1999; Boss and Thomas, 2002; Chandler et al., 2002; Muangprom et al., 2005; Asano et al., 2009). Here, we demonstrate that *Rht-B1c* contains a novel type of DELLA mutation, a genomic insertion that results in

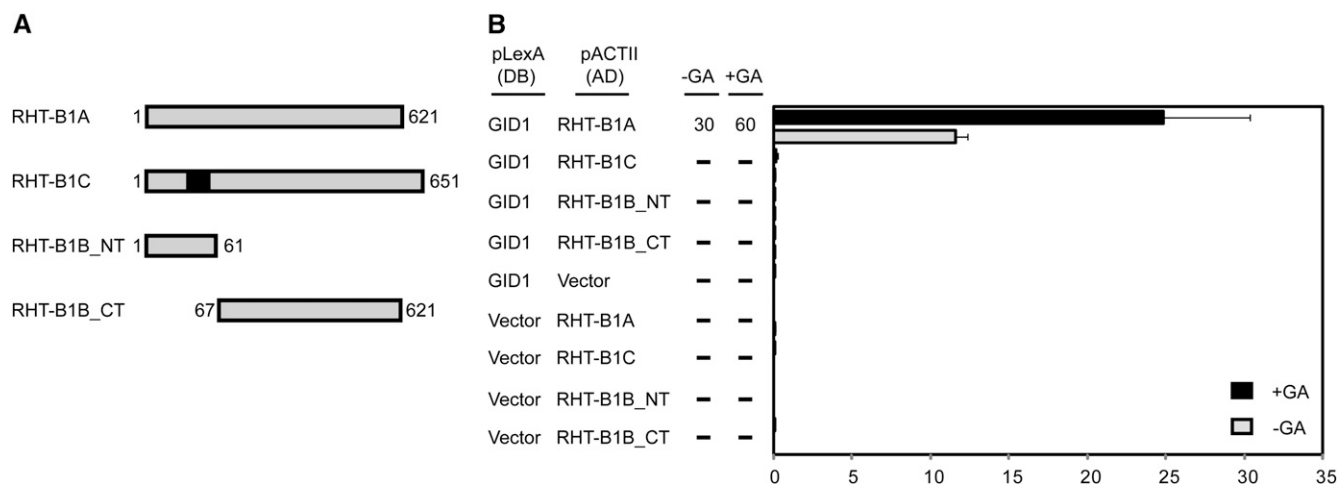


Figure 5. Interaction between wild-type and mutant forms of RHT-B1 and TaGID1 in yeast two-hybrid assays. A, Schematic diagram showing full-length and mutant forms of RHT-B1, which were expressed as LexA activation domain (AD) fusions in yeast. Numbers indicate the amino acid positions within RHT-B1. The black box in the RHT-B1C schematic diagram indicates the position of the 30-amino acid insertion. The full-length TaGID1 was expressed as a LexA DNA-binding domain (DB) fusion protein in yeast. B, Interactions between activation domain and DNA-binding domain protein fusions were determined in cotransformed L40 yeast cells by scoring growth on His⁻ medium containing 3-aminotriazole (3-AT; 0, 2, 5, 10, 30, and 60 mM). Data presented are maximum concentrations of 3-AT at which growth was observed; dashes signify no growth on 2 mM 3-AT. Quantitative values for each interaction were determined by measuring β -Gal activity in the presence and absence of 100 μ M GA₃. Values are means of three biological replicates \pm SE.

the introduction of 30 amino acids between the DELLA and LEXLE motifs, which are required for binding to the GID1 N-terminal extension (Murase et al., 2008). We were unable to amplify across the whole genomic insertion, but we identified a highly conserved 508-bp fragment that has been shown to be a repeated flanking unit of a 2,031-bp terminal repeat retrotransposon in miniature at the equivalent location in Tom Thumb (Wu et al., 2011). These tandem repeats contain splice sites such that most of the insertion is removed during RNA processing, leaving an in-frame 90-bp insertion that, as demonstrated in yeast two-hybrid assays, completely abolishes the interaction of RHT-B1C with TaGID1. It is likely, therefore, that the RHT-B1C protein acts as a repressor of GA signaling that would not be polyubiquitinated and degraded by the 26S proteasome, even in the presence of GA. In addition to the DNA insertion, *Rht-B1c* also contains two SNPs upstream of the insertion that result in two amino acid substitutions (Fig. 4; G15R and M25I). While it is conceivable that these may also compromise the interaction with GID1, this appears unlikely, given that this region is poorly conserved in DELLA proteins. Furthermore, these substitutions are not found in the regions implicated in the DELLA-GID1 association (Murase et al., 2008). It is likely that they reflect SNPs present in the original donor.

In contrast to *Rht-B1c*, the severely dwarfing *Rht-D1c* allele harbored no DNA insertions but contained instead the same base substitution present in the mildly dwarfing *Rht-D1b* allele. We could detect no other mutations within its coding sequence. The se-

verity of the dwarf phenotype in *Rht-D1c* may be due to its elevated level of expression (Fig. 4E) compared with other *Rht-1* alleles. This may be explained by a higher copy number for *Rht-D1c* (Fig. 4H), although mutations in its promoter cannot be discounted, despite none being detected within 3,686 bp of sequence upstream of the predicted start codon (data not shown). The recent partial sequencing of chromosome 3B has illustrated the high degree of transposon-mediated gene amplification in wheat (Choulet et al., 2010). It is likely that this process, potentially involving CACTA transposon-mediated gene capture and amplification at the same locus, is responsible for the increased copy number for *Rht-D1c*. The semidwarf *Rht-D1d* mutant is described as being a spontaneous mutant derived from *Rht-D1c* (Borner et al., 1991), although it does not contain the same increase in *Rht-D1* gene copy number and may have lost these additional copies through a transposon excision-mediated process. However, the similarity in phenotype to *Rht-D1b* and its identical *Rht-D1* copy number also raises the possibility that it is not a novel allele but is derived from an *Rht-D1b* contaminant within the *Rht-D1c* population or through out-crossing with *Rht-D1b*.

We have found that, in common with *Rht-B1b* and *Rht-D1b*, the previously uncharacterized *Rht-B1d*, *Rht-B1e*, *Rht-D1d*, and, as discussed above, *Rht-D1c* alleles all contain nucleotide substitutions that create premature stop codons in the N-terminal coding regions. In the cases of *Rht-B1d*, *Rht-D1c*, and *Rht-D1d*, the mutations are identical to those found in the respective *Rht-B1b* and *Rht-D1b* mutants. However, the introduction

of an alternative stop codon in the *Rht-B1e* mutant extends the genetic location within which these semi-dwarfing DELLA gain-of-function mutations can occur. Peng et al. (1999) proposed two explanations for the GA insensitivity that results from these mutations: the expression of a short N-terminal product could block GA signaling through its interaction with TaGID1 via the remaining DELLA domain, or, alternatively, translational reinitiation could result in the production of an N-terminally truncated product that constitutively represses GA signaling due to its inability to interact with TaGID1. Our findings that both truncations completely abolish the interaction with TaGID1 in yeast assays allow the first proposal to be discounted and provide support for the second. Mutant DELLA genes that are predicted to express C-terminal truncations including the complete GID1 interaction domain have been described in Arabidopsis, rice, and barley (Peng et al., 1997; Ikeda et al., 2001; Chandler et al., 2002). The findings that all of these mutations result in loss of function rather than gain of function would further suggest that the production of an N-terminal peptide is not the cause of dwarfism in the *Rht-B1b*, *Rht-D1b*, and *Rht-B1e* mutants. It will be necessary to confirm the effects of these mutations on the stability of endogenous RHT-1. However, our attempts at raising polyclonal antibodies that recognize the endogenous RHT-1 proteins in wild-type or mutant backgrounds have proved unsuccessful.

In contrast to other DELLA gain-of-function mutations, in which deletion or substitution of amino acids critical for GID1 binding causes severe dwarfism (Peng et al., 1997, 1999; Chandler et al., 2002; Murase et al., 2008; Asano et al., 2009), the *Rht-B1b*, *Rht-B1e*, and *Rht-D1b* mutations result in only mild height reductions. It is possible that a low efficiency of translational reinitiation in the *Rht-1* mutants would result in reduced levels of GA-insensitive DELLA protein being produced. This is consistent with the phenotype of *Rht-D1c*, which indicates that increased expression of the *Rht-D1b* allele is capable of enhancing the severity of dwarfism. Indeed, a gene-dose effect was observed for the Arabidopsis DELLA gene *GAI* and its gain-of-function mutant allele *gai* expressed in tobacco (*Nicotiana tabacum*), in which the degree of dwarfism was related to the level of expression (King et al., 2001; Hynes et al., 2003).

Comparison of the spatial expression profiles of *Rht-1* with those of the GA biosynthetic enzymes *TaGA20ox1* and *TaGA3ox2* within the expanding P-1 internode indicated substantial differences. In agreement with the observations of Appleford et al. (2006), expression of the biosynthetic genes is strongest in growing tissues, with *TaGA20ox1* expression located most strongly at the nodes, presumably within the intercalary meristem, and that of *TaGA3ox2* in the meristem and elongation zones. In contrast, *Rht-1* was expressed throughout the internode. These observations suggest that growth of the internode is regulated by GA production rather than response. However,

although our results for the biosynthetic genes are broadly in line with those of Kaneko et al. (2003), who, on the basis of reporter gene analysis in rice, showed expression of *OsGA20ox2* and *OsGA3ox2* in the meristem and elongation zones of the fifth internode, the same expression pattern was also found for the DELLA gene *SLR1*. It is possible that differences in the developmental stages of the plants analyzed in these studies are responsible for the observed disparities between them.

Yields from current wheat cultivars containing *Rht-B1b* or *Rht-D1b* alleles may be approaching their limits (Slafer et al., 2001), while a changing climate may reduce the usefulness of these genes in the future. These issues, combined with potential restrictions on the use of growth retardants and chemical fertilizer, highlight the need to develop novel dwarfing alleles. Our genetic and biochemical characterization of dwarfing *Rht-B1* and *Rht-D1* alleles and the finding that *Rht-A1* is expressed have provided further insights and opportunities for generating such genes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wheat (*Triticum aestivum*) NILs for different alleles of *Rht-B1* and *Rht-D1* in the varieties Maris Huntsman, Mercia, and Bezostaya were provided by Dr. J. Flintham (John Innes Centre). Wheat chromosome 4 aneuploid lines N4AT4B, N4DT4B, and CSDT4B- α were provided by Dr. M. Ambrose from the Biotechnology and Biological Sciences Research Council small grain cereals collection in the Germplasm Resources Unit, John Innes Centre. BC7 NILs for *Rht-B1c* in the varieties Maringá and Nainari 60 were described by Hoogendoorn et al. (1988), and grains were provided by the Australian Winter Cereal Collection. Seed for Ai-bian 1 and Ai-bian 1a were provided by Dr. Andreas Börner (Institute of Plant Genetics and Crop Plant Research, Germany). Stem expression analysis studies and transformations were carried out in the spring wheat cv Cadenza, which carries wild-type alleles of *Rht-B1* and *Rht-D1*. Plants were grown as described previously (Appleford et al., 2006).

Gene Amplification and Sequencing

Rht-B1 or *Rht-D1* mutant alleles were amplified from either gDNA or cDNA templates. Genomic DNA was extracted using a cetyl-trimethyl-ammonium bromide extraction buffer. RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the SuperScript First Strand Synthesis System (Invitrogen). *Rht-B1* and *Rht-D1* genes were amplified as two overlapping fragments using two sets of primers, listed in Supplemental Table S1. Amplified fragments were cloned into pGEM-T Easy vector (Promega) and sequenced. The presence of an insertion larger than the expected 90 bp in the *Rht-B1* gene of Maringá *Rht-B1c* was first revealed by the sizes of fragments amplified using forward primers from the beginning of the gene (e.g. Rht3F5) and reverse primers from within the 90 extra nucleotides (e.g. Rht3R5). The sizes and sequences of this and other products amplified between gene and insert sequences were all consistent with a single 508-bp insertion.

Plasmid Constructs

Promoter::GUS expression constructs were created for *Rht-A1*, *TaGA20ox1-A*, and *TaGA3ox2-3* by amplifying 1,529, 1,555, and 1,566 bp upstream of the predicted initiating ATG, respectively, by PCR. The amplified fragments were cloned in frame into the pPTGUS vector upstream of the *uidA* gene to create plasmids *pRht-A1::GUS*, *pGA20ox1-A::GUS*, and *pGA3ox2-3::GUS*. For the yeast two-hybrid assays, bait proteins were cloned as LexA binding domain fusion proteins in the pLexA-NLS expression vector and prey proteins as Gal4

activation domain fusions in the pACT expression vector. In each assay, the full-length *GID1* was used as the bait protein fusion. *RHT-B1* prey constructs were produced in pACTII by amplifying the necessary coding regions from gDNA of *Rht-1* or *Rht-B1c* (var Mercia).

Wheat Transformation and Histochemical Analysis of GUS Activity

Promoter::GUS reporter lines were produced by bombardment of wheat embryos with the plasmids *pRHT-A1::GUS*, *pGA20ox1-A::GUS*, and *pGA3ox2-3::GUS* (as described by Sparks and Jones, 2009). Expression analysis was performed on plants of the T1 generation. Plant tissues were incubated at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl- β -glucuronidase solution containing 1% Triton. Tissues were destained in 70% ethanol and examined using a light microscope.

Real-Time qRT-PCR

Gene expression analysis in the developing stem was carried out in seven separate regions from a 7-week-old plant: the ear, peduncle, peduncular node, P-1 internode split into three equal sections, and P-1 node (as described by Appleford et al., 2006). Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described previously (Rieu et al., 2008). Expression data were normalized against the *UBIQUITIN* control gene using the GeNorm procedure described by Vandesompele et al. (2002).

Four-day-old seedlings of wheat NILs (var Mercia and Maris Huntsman) differing in their *Rht-1* alleles on chromosomes 4B and 4D were used to analyze expression of the homeologous *Rht-1* genes. qRT-PCR was carried out using a DNA Engine Opticon2 Continuous Fluorescence Detector (MJ Research). RNA was extracted and cDNA was synthesized as described above. Reactions were carried out using SYBR Green Jumpstart *Taq* Ready Mix (Sigma-Aldrich). The average threshold cycle (CT) value for each gene was calculated from duplicate samples for each experiment. Data were analyzed using Opticon Monitor analysis software version 2.02 (MJ Research). For each target gene, expression was calculated relative to that of the reference gene, *UBIQUITIN* (Van Riet et al., 2006), using the Δ CT method (Pfaffl et al., 2002) and corrected for primer efficiencies.

The primers for each target gene and the *UBIQUITIN* endogenous control gene are listed in Supplemental Table S1. The *UBIQUITIN* endogenous control gene (accession no. AY297059) has been described previously (Li et al., 2008). Wheat *Rht-D1* Mercia NILs were used to determine *Rht-D1* gene dosage in an *Rht-D1* allelic series. gDNA was extracted using cetyl-trimethyl-ammonium bromide DNA extraction buffer. qPCR was performed with the three *Rht-1* homeolog-specific assays as described above but using gDNA (2 ng μ L⁻¹) as template. For each target gene, the amplification signal was normalized to *UBIQUITIN* as described above.

DNA Gel-Blot Hybridizations

Twenty micrograms of gDNA isolated from each line of the *Rht-D1* allelic series was digested with *DraI* or *EcoRI*. Gel-blot hybridizations were performed as described previously using a digoxigenin-11-dUTP-labeled probe and chemiluminescence detection (Vaughan et al., 2006). The *Rht-D1*-specific probe of 243 bp (D1-2855) was amplified using primers *Rht-D1_SF1* and *Rht-D1_SR2* from wheat gDNA (var Mercia).

Rht-B1c and *Rht-B1e* PCR Markers

To screen for the presence of the *Rht-B1c* and *Rht-B1e* alleles, two PCR markers were developed. For *Rht-B1c*, the primer pair *Rht-B1c-F1/Rht-B1c-R1* was used to amplify a 256-bp product only from lines carrying the *Rht-B1c* allele by PCR. For *Rht-B1e*, two primer sets were used: forward primer BF was used with reverse primer WR3 to amplify wild-type sequences, while PCR using BF with reverse primer MR3 amplified a 228-bp fragment only in lines carrying the *Rht-B1e* allele.

Yeast Two-Hybrid Assay

Saccharomyces cerevisiae strain L40 was used for all yeast two-hybrid assays (Vojtek et al., 1993). Yeast cells were cotransformed with binding domain and activation domain plasmid expression constructs, and transformants were

selected on –Leu–Trp medium (Q-biogene). The ability to drive the expression of the *HIS3* or *LacZ* reporter genes was tested as described previously (Dill et al., 2004) in the presence or absence of 100 μ M GA₃.

The accession numbers for the sequences described in this article are JF930277 (*Rht-A1a*), JF930278 (*Rht-B1a*), JF930279 (*Rht-B1c*), JF930280 (*Rht-B1e*), JF930281 (*Rht-D1b*), JF930282 (*TaGA20OX1-A*), and JF930283 (*TaGA3OX2-3*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Homeolog-specific qRT-PCR to validate the chromosomal location of *Rht-A1*.

Supplemental Figure S2. Wheat tissues sampled for gene expression analysis.

Supplemental Figure S3. Comparison of the nucleotide sequences of *Rht-B1a* and *Rht-B1c* alleles.

Supplemental Figure S4. PCR-based assays to detect *Rht-B1c* and *Rht-B1e* mutations.

Supplemental Figure S5. Expression analysis of the *Rht-1* homeologs in wild-type wheat and the *Rht-B1b* and *Rht-B1c* mutants.

Supplemental Table S1. Oligonucleotide primers used for standard PCR and qRT-PCR.

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