RESEARCH PAPER

Sequence variations of the partially dominant DELLA gene Rht-B1c in wheat and their functional impacts

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Abstract

Rht-B1c, allelic to the DELLA protein-encoding gene Rht-B1a, is a natural mutation documented in common wheat (Triticum aestivum). It confers variation to a number of traits related to cell and plant morphology, seed dormancy, and photosynthesis. The present study was conducted to examine the sequence variations of Rht-B1c and their functional impacts. The results showed that Rht-B1c was partially dominant or co-dominant for plant height, and exhibited an increased dwarfing effect. At the sequence level, Rht-B1c differed from Rht-B1a by one 2 kb Veju retrotransposon insertion, three coding region single nucleotide polymorphisms (SNPs), one 197 bp insertion, and four SNPs in the 1 kb upstream sequence. Haplotype investigations, association analyses, transient expression assays, and expression profiling showed that the Veju insertion was primarily responsible for the extreme dwarfing effect. It was found that the Veju insertion changed processing of the Rht-B1c transcripts and resulted in DELLA motif primary structure disruption. Expression assays showed that Rht-B1c caused reduction of total Rht-1 transcript levels, and up-regulation of GATA-like transcription factors and genes positively regulated by these factors, suggesting that one way in which Rht-1 proteins affect plant growth and development is through GATA-like transcription factor regulation.

Key words: DELLA protein, dominance, GATA transcription factor, sequence variation, wheat.

Introduction

Wheat is a food staple for ~35% of the world population, and the continued population growth is presenting us with a formidable challenge to meet even greater demand for wheat in the near future. Nevertheless, recent data indicate that wheat yield potential is approaching a ceiling because of the lack of a complete understanding of yield formation mechanisms, and the paucity of breakthrough genes in modern cultivars. Because appropriate plant height is a key factor contributing to greater yield potential, investigations to elucidate the functional mechanisms involved in dwarfing are of substantial value to the future of agriculture.

Multiple genetic alternations can result in dwarfism. A common cause is the mutation of genes controlling biosynthesis of the plant hormones gibberellins (GAs), and genes involved in the GA signalling pathway (Peng et al., 1999; Hedden, 2003; Sun and Gubler, 2004). GAs are a group of diterpenoid carboxylic acids, and play a crucial role in the regulation of physiological processes related to plant growth and development, for example seed germination, shoot/stem elongation, cell division, and flower development (Davies, 1985; Fleet and Sun, 2005; Swain and Singh, 2005). Ross et al. (1997) classified GA-related dwarf mutants based on exogenous GA responses into GA-deficient and GA-insensitive types. GA-deficient mutants are typically ascribed to being GA biosynthesis deficient, and the GA-insensitive mutants result from mutations of positive and negative regulatory genes in the GA signalling pathway (Gomi and Matsuoka, 2003). Several negative regulators have been isolated by
characterization of the recessive (loss-of-function) slender mutants and dominant (gain-of-function) GA-unresponsive dwarf mutants (Peng et al., 1997; Ikeda et al., 2001). DELLA proteins are well-studied regulators of GA signalling, which in planta function as repressors, and are components of the GID1–DELLA–SCF\textsuperscript{GID2} complex (Sun and Gubler, 2004; Murase et al., 2008). The complex formation stimulated by GA leads to DELLA protein degradation by means of the 26S proteasome and repression release.

Since the isolation of the DELLA protein gene \textit{GAI} in \textit{Arabidopsis thaliana} (Peng et al., 1997), its orthologues have been characterized in several other plants, including \textit{d8} in maize (\textit{Zea mays}), \textit{VvGAI} in grape (\textit{Vitis vinifera}), \textit{MdRGL1} in apple (\textit{Malus×domestica} Borkh), \textit{BrRGA} in Brassica rapa, \textit{SLN1} in barley (\textit{Hordeum vulgare}), \textit{Rht} in wheat (\textit{Triticum aestivum}), and \textit{SLR1} in rice (\textit{Oryza sativa}) (Peng et al., 1999; Boss and Thomas 2002; Chandler et al., 2002; Itoh et al., 2002; Muangprom et al., 2005; Foster et al., 2007; Lawt et al., 2010). The encoded proteins share an N-terminal DELLA domain, including DELLA and VHYNP motifs, a poly(S/T) domain, and a C-terminal GRAS domain. DELLA domain defects render the proteins resistant to GA-induced degradation, and result in dwarf phenotypes.

More than 20 dwarfing or reduced height genes (\textit{Rht}) and alleles have been reported in wheat, including GA-insensitive genes (\textit{Rht}-1: \textit{Rht-Blb}, \textit{Rht-B1c}, \textit{Rht-B1d}, \textit{Rht-D1c}, etc.) and GA-sensitive genes (\textit{Rht4}, 5, 6, 7, etc.) (Konzak, 1988; Ross et al., 1997). The GA-insensitive and some GA-sensitive genes, such as \textit{Rht16} and 18, also cause coleoptile length reduction and leaf elongation, while the remaining GA-sensitive genes do not compromise early plant growth (Ellis et al., 2004). Even at the same locus, different alleles of the dwarfing gene exhibit various effects on plant height (Pearce et al., 2011). For example, of the five alleles detected at the \textit{Rht-B1} locus, \textit{Rht-B1c} (\textit{Rht3}) exhibited a three times stronger effect on plant height compared with \textit{Rht-Blb} (\textit{Rht1}) (Flintham and Gale, 1983; Flintham et al., 1997). Furthermore, \textit{Rht-B1c} resulted in increased tiller and root number, a higher photosynthetic rate, and a higher chlorophyll content (Zhang et al., 1995; Li et al., 2006). Particularly intriguing was the finding that \textit{Rht-B1c} exclusively inhibited \textit{α}-amylase production in ripening grains, and enhanced seed dormancy (Flintham and Gale, 1982; Flintham et al., 1997). However, the mechanisms underlying the functional differences of various alleles remain unclear.

The present study served to elucidate why the \textit{Rht-B1c} allele has multiple effects on plant growth, and the underlying factors contributing to increased phenotypic expression in reducing plant height. The \textit{Rht-B1c} genomic sequence covering both the promoter and coding regions was isolated, and it was shown that the sequence insertion in the promoter and some single nucleotide polymorphisms (SNPs) represent evolution’s imprint on the B genome, and the retrotransposon insertion in the coding region leads not only to \textit{Rht-B1c} transcript change and DELLA motif primary structure disruption, but also to modifications of the \textit{Rht-1} gene expression level and responses to GA.

Materials and methods

Plant materials

DS3 and DY3 lines were provided courtesy of Professor Zhao Yinhui of Jiangsu Academy of Agricultural Sciences (JASS). They were developed through crossing common wheat \textit{S} and \textit{Y} with the \textit{Rht-Blc} donor Tom Thumb, and backcrossing for 23 generations using \textit{S} and \textit{Y} as recurrent parents. Segregating \textit{F}_2 populations were developed by selling \textit{BC}_2\textit{F}_1 plants.

One hundred and twenty-six lines from the core common wheat germplasm collection were obtained from the Chinese Academy of Agricultural Sciences (Supplementary Table S1 available at JXB online). Other plant materials are listed in Supplementary Table S2.

Growth conditions and morphological evaluation

\textbf{Plant height measurement} The \textit{BC}_2\textit{F}_1 families and parentals were planted in a field on the JASS campus in 2003. The 126 common wheat lines were planted in a field at the Jiangpu Experimental Station (JP) in 2008, 2009, and 2011 for plant height evaluation. The trials were performed in a randomized complete block design, each with two replicates. Each plot of the trials had two 1.5 m long rows spaced 0.5 m apart. Thirty seeds were sown in each row. At the two-leaf stage, 10 evenly distributed plants were retained in each row for further growth. Field management followed common wheat production practices. The main culm height of 10 plants per plot was measured at maturity.

\textbf{Leaf dimension and chlorophyll content measurements} Plants used for leaf dimension measurements were grown in a field at JP. Each line was grown in 15 1.5-m long rows spaced 27 cm apart. At flowering stage, the length and width of the flag leaf, and the first, second, and third leaf below the flag leaf on the main culm were measured. Ten plants were sampled from each line. Leaf area was estimated using the following formula: area=leaf length\times leaf width×0.835 (Miralles and Slafer, 1991).

The plants for relative chlorophyll content measurement were grown in two locations, one in a field at JP and one in a field on the Nanjing Agricultural University campus. SPAD readings, a measure of relative leaf chlorophyll content (Kariya et al., 1982), were recorded using a portable Minolta SPAD-502 chlorophyll meter (Spectrum Technologies, Inc., IL, USA) from 9 to 10 o’clock on sunny days. Ten plants at flowering stage were sampled from each line. Five readings were obtained from the proximal to distal end of a fully expanded leaf blade adjacent to the main leaf vein. Mean values were calculated and used as the data entry value.

\textbf{Epidermal cell observation} Samples were collected from plants at flowering stage. Tissue coating and microscopic observations were conducted following the procedures described in Wu et al. (2011). Photographs were taken using an Eclipse 80i microscope (Nikon Japan) at \times100 magnification. The length and width of 20 cells for each tissue were measured with an eyepiece graticule at \times200 magnification.

Genomic DNA isolation and amplification

Genomic DNA was extracted from young wheat leaves according to the procedures described by Ma and Sorrells (1995). Bacterial artificial chromosome (BAC) clones carrying \textit{Rht-1} genes were obtained by screening a \textit{T. aestivum} cv. Wangshuibai (2n=6x=42) BAC library representing three haploid genome equivalents using forward primer 5’-CAGGACGGAGCGCAAGAAAGC-3’, and reverse primer 5’-AAGGGGCTTAGGAGGAGTTTTACG-3’, which amplified all three \textit{Rht-1} genes. Positive BAC clones were fingerprinted with restriction enzymes \textit{BamH}I and \textit{XhoI} that produced fingerprints distinguishing the three \textit{Rht-1} genes based on published sequences (Peng et al., 1999; Febrer et al., 2009). \textit{Rht-A1} and \textit{Rht-D1} promoter regions were obtained by subcloning the
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BACs and end sequencing. *Rht-B1b* and *Rht-D1b* diagnostic assays followed Ellis *et al.* (2002), and *Rht* detection was conducted by surveying with the linked microsatellite marker xgw261 (Schmidt *et al.*, 2004).

KOD-FX DNA polymerase was employed for PCR amplification (Toyobo, Shanghai, China). Target bands were excised from agarose gels and, following purification, were ligated into the pTA-2 vector (Toyobo) for transformation into competent DH5α cells.

**RT–PCR and cDNA cloning**

RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol, and quantified with Ultraspec 2100 pro (GE Healthcare Biosciences). RNA samples were treated with DNase I (Fermentas) for genomic DNA removal. The RNA sample for 3′-rapid amplification of cDNA ends (RACE) was reverse-transcribed with ThermoScript reverse transcriptase (Invitrogen). RACE PCR was conducted using KOD-FX DNA polymerase (Toyobo) following the procedures described in the SMARTTM RACE cDNA Amplification manual (Clontech). The gene-specific primer was BF (Ellis *et al.*, 2002), and the nested primer was 5′-ATGAAGCGGACTACCCAAGAGGAC-3′. RNA for reverse transcription–PCR (RT–PCR) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen). *Rht-A1, Rht-B1*, and *Rht-D1* primers are provided in Supplementary Fig. S2 at JXB online, which were designed based on 3′-untranslated region (UTR) sequences (Supplementary Fig. S2). *Rht-B1c*S primers were 5′-TGTCGGGTCGCGGCCTGC-3′ and 5′-TCTTCTGTCCACCAAGGAAAGGC-3′. RT–PCR primers for other genes amplified in this study are listed in Supplementary Table S3, and were designed according to expressed sequence tags (ESTs; in the NCBI database) from wheat homologues of the corresponding genes. The wheat α-tubulin gene was amplified as a control to quantify expression levels using primers 5′-ATCTCCACTTCCAGTTCG-3′ and 5′-TCATCGCCTTCATACCGTC-3′.

Semi-quantitative RT–PCR was performed in a 25 μl total reaction volume. The PCR profile was as follows: 94 °C for 3 min; 20-30 cycles at 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s; and a final 5 min extension at 72 °C. A 6 μl aliquot from the PCR product of each reaction was electrophoresed on a 2% agarose gel, and viewed under UV light after standard staining with ethidium bromide.

Quantitative RT–PCR amplification was performed using the StepOne™ Real-time PCR instrument (Applied Biosystems) in a 20 μl volume containing 10 μl of SYBR-Green PCR Mastermix (Toyobo), and 10 pmol of each primer. Reactions were conducted in triplicate. Cycle threshold values for each target gene were normalized based on values obtained in corresponding reactions for the wheat α-tubulin gene. Relative expression was estimated employing the 2^ΔΔCT^ method (Livak and Schmittgen, 2001).

**Transient assays**

The 2.1 kb and 2.3 kb *Rht-B1a* and *Rht-B1c* upstream sequences were amplified using KOD-FX DNA polymerase (Toyobo) with the forward primer containing a PstI restriction site, and the reverse primer containing a SmaI restriction site. The restricted amplicons were used to substitute for the *Cauliflower mosaic virus* (CaMV) 35S promoter in the CaMV35SS-GUS–NOS sequence of a pUC19-based expression vector. Following ligation and transformation into DH5α competent cells, the constructs were confirmed by sequencing.

**Transient expression and microscopy**

The PDS-1000 particle delivery system (Bio-Rad) was used to introduce the fusion constructs through 1.1 mm tungsten particles into epidermal cells of onion (*Allium cepa*) bulb scales or maize (*Zea mays*) shoot tips placed on half-strength MS medium agar plates following the manufacturer’s recommended procedure. Maize shoot tips were from seedlings 2–3 d following germination at 25 °C. Bombardment parameters were 1100 psi of pressure, 85 mm from the macrocarrier to the samples, and 28 in of mercury vacuum. After bombardment, tissues were incubated in the dark for 12 h at 25 °C, and subsequently stained in X-glue solution for 4 h following Jefferson (1987). β-Glucuronidase (GUS) expression was detected under light microscopy (Nikon ECLIPSE 80i).

**GA treatment**

Seeds were germinated in Petri dishes at room temperature. One-week-old seedlings grown under 16h of light per day were sprayed with 100 μM GA₃, and were harvested 1 h later for RNA extraction. Samples without GA₃ spraying were harvested as controls.

**Sequence analysis**

Positive clones were sequenced at Takara Bio, Inc., Dalian, China. Basic sequence analysis was conducted with Macvector 10.0 (Accelrys, Oxford, USA). The transcription start site (TSS) was predicted with the plant promoter prediction program TSSP (http://www.softberry.com). Putative cis-acting elements were identified using the PLACE database of Higo *et al.* (1999).

**Statistical analysis**

Comparison of quantitative data between samples was subjected to two-tailed and unpaired Student’s t-test.

**Accession numbers**

The sequence data of *Rht-A1a, Rht-B1a, Rht-B1c*, and *Rht-D1a* were deposited in NCBI (http://www.ncbi.nlm.nih.gov) under accession nos KC767924–KC767927.

**Results**

**Effects of Rht-B1c on plant growth and development**

*Rht-1* genes are key regulators of the GA signalling pathway, which has multiple effects on plant growth and development. Therefore, the *Rht-B1c* near-isogenic lines D-Sumai 3 (DS3) and D-Yangmai 3 (DY3) were compared with their recurrent parents Sumai 3 (S3) and Yangmai 3 (Y3) for plant height, leaf morphology, epidermal cell length and width, and chlorenchyma content.

DS3 and DY3 were dwarfed at the early seedling stage (Fig. 1A, B). At maturity, S3 was ~15–20 cm taller than Y3, but DS3 and DY3 were similar in plant height and exhibited an ~50% reduction relative to S3 and Y3 (Fig. 1C–E), compared with a 10–20% height reduction by *Rht-B1b* and *Rht-D1b* (Finlathom *et al.*, 1997). In the BC₂₃F₁₂ families, the plants could be distinctly classified into three groups (a, b, and c; Supplementary Table S4 at JXB online) based on plant height. Groups a and c were similar to S3/Y3 and DS3/DY3, respectively, in plant height, and represented the parental genotypes. Group b exhibited plant height values intermediate to the parental values, suggesting they were heterozygous at *Rht-B1*. Group b with the Y3 background showed a mean plant height close to the mid-parental values, and the dominant effect mean was nearly zero (Supplementary Table S4), suggesting that *Rht-B1c* in this background was co-dominant in conditioning plant height. However, the *Rht-B1c* dominant effects in the three BC₂₃F₁₂ families with the S3 background were all
negative, with a mean of –5.5 (Supplementary Table S4), indicating partial dominance for reduced height. Consistent with these results, Flintham et al. (1997) reported that the Rht-B1c dominant effects in three different genetic backgrounds varied from –4.4 to –8.5, with a mean of –6.1. However, Rht-B1b and Rht-D1b were either co-dominant or partially recessive for reduced height (Gale et al., 1989; Keyes and Sorrells, 1989; Flintham et al., 1997; Flintham and Gale, 1998).

Rht-B1c introduction resulted in significantly shorter and wider leaves at all leaf positions \((P < 0.0001)\). The effects increased as the position approached the spike in the S3 background plants (Fig. 2A); however, in Y3 background individuals, effects on the flag leaf morphology were not as prominent as on the second upper leaves (Fig. 2B). The length and width changes consequently resulted in leaf area variation. DS3 and DY3 flag leaf area was reduced by 8.7% and 4.9%, respectively; both were significantly different \((P < 0.0001)\) from the taller counterparts, namely S3 and Y3. Moreover, applying SPAD units as measurements, it was found that the relative chlorophyll contents in the flag leaf and the leaf directly below the flag leaf were significantly higher in DS3 and DY3 than in S3 and Y3, with \(P < 0.0001\) (Fig. 3). This is consistent with darker green leaves observed in plants with Rht-B1c.

Rht-B1c effects on cell morphology were examined through microscopic observation and two-dimensional measurement of the epidermal cells in the peduncle, flag leaf sheath, and blade. The results showed that the epidermal cells of all observed tissues had reduced length and increased width (Fig. 4A). Peduncle epidermal cell morphology for the two Rht-B1 genotypes is provided in Fig. 4B.

Rht-B1c differs from Rht-B1a by fragment insertions and SNPs in promoters and coding regions

The Rht-B1c allele effects on plant growth and development were distinct from those of Rht-B1b; therefore, it was of interest to determine the sequence characteristics responsible for the observed differences. The full coding Rht-1 sequences were obtained by PCR using a forward primer \((5'-CGGAAC\, CGAGGCAAGCAAAAGC-3')\) designed based on one EST sequence (gi55686840) that showed the best homology to the Rht-B1a 5' region (Peng et al., 1997), and a reverse primer \((5'-AAGGGGGCTTAGGAGGAGTTTTACG-3')\) designed based on the 3'-UTRs from a group of Rht-1 3'-RACE products (data not shown). S3 genomic DNA amplification generated three products established by fingerprinting with restriction enzymes BamHI and XhoI. The Rht-B1a-like product exhibited a 67 bp 5'-UTR and 147 bp 3'-UTR (data not shown). This sequence was used to design one Rht-B-specific reverse primer \((5'-GCTTCTCCTTCTTGAAGCGTTACG-3')\) ending 116 bp downstream from the stop codon. S3 and DS3 genomic DNA amplification using the Rht-B-specific reverse primer, and the Rht-B1-specific forward primer BF (Ellis et al., 2002), which initiated 24 bp upstream from the start codon, produced an expected 2006 bp

Fig. 1. Phenotypes of Rht-B1c near-isogenic lines. (A and C) Phenotypes of S3 (left) and DS3 (right). (B and D) Phenotypes of Y3 (left) and DY3 (right). (E) Plant height (cm) of the three genotypic groups in S3 and Y3 backgrounds, calculated with pooled data from BC2F2 populations. Error bars indicate the standard deviation. The sample sizes of the two populations were 80 (S3) and 163 (Y3) plants, and the three groups were significantly different from each other in plant height, with \(P < 0.0001\), and statistically fit to a 1:2:1 genotypic ratio (B1a/B1a:B1a/B1c:B1c/B1c). Genotypic classification was verified by a \(F_{2:3}\) progeny test. (This figure is available in colour at JXB online.)

Fig. 2. Percentage leaf length reduction and leaf width increase in (A) DS3 and (B) DY3 relative to S3 and Y3. Numbers 1–4 refers to the flag leaf, and the first, second, and third leaf below the flag leaf.

Fig. 3. Chlorophyll content of flag leaf and the leaf directly below the flag leaf in S3, Y3, DS3, and DY3. The data are mean values from 10 plants. The vertical line indicates the standard error.
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Rht-B1a sequence in S3, and a 4032 bp sequence in DS3. The DS3 sequence differed from Rht-B1a by a 2026 bp insertion 151 bp downstream from the start codon, two SNPs preceding the indel, and one SNP downstream from the indel (Fig. 5A). These sequence variations were verified by repeating the PCRs.

Association of the 4kb fragment with plant height was determined by surveying the DS3-derived BC3F12 families using one primer (5’-GCCCTCTTTGGCCTACATTTTCTCAT-3’) located within the insertion, and one primer (5’-GCTCCAGCTTCTGGCCACGT-3’) located downstream from the indel. PCR revealed three genotypes, exhibiting co-segregation with variation in plant height (Fig. 5B). A similar result was obtained in the DY3-derived BC3F12 families. Assuming that the wheat genome size is equivalent to 17 Gb, only ~2 kb of donor DNA will be maintained in a plant derived from 23 backcrossed generations. Consequently, the variable sequence region revealed in the near-isogenic lines should correspond to the introduced Rht-B1c.

The Rht-B1c 2kb insertion was characterized by a 5bp (AGGTG) target site duplication, a 503 bp long direct terminal repeat (LTR), and a 1015 bp internal domain flanked by LTRs (Fig. 5A). The LTR showed TG–CA inverted terminal repeats. This type of sequence organization was similar to the terminal-repeat retrotransposon in miniature (TRIM) family termed Veju, characterized in wheat (SanMiguel et al., 2002).

Differences between Rht-B1a and Rht-B1c in the sequence upstream from the coding region were examined by isolating a 2135 bp and a 2332 bp upstream sequence, respectively, from S3 and DS3 via two rounds of PCR-based genome walking (O’Malley et al., 2007). Both fragments were confirmed by genomic DNA PCR amplification using gene-specific primers (5’-AATTCGAACTGCACATACGTATGAAGATG-3’ and 5’-CGCCGCCGCCGACCCAGA-3’) flanking the fragments. Relative to the S3 fragment, seven SNPs, and one 197 bp sequence insertion upstream from –591 in the DS3 fragment were detected (Fig. 5A). This 197 bp indel also co-segregated with the height phenotypes in the BC3F2 populations (data not shown). All Rht-B1c sequence variations were confirmed in ‘Tom Thumb’, the donor parent.

Rht-B1 is more distant from Rht-A1 and Rht-D1 than Rht-A1 is from Rht-D1

The Rht-1 gene upstream sequence variations were examined by cloning Rht-A1a and Rht-D1a sequences corresponding to the Rht-B1 upstream sequence via subcloning of BACs carrying the respective genes. The respective Rht-A1a and Rht-D1a sequences, 3298 bp and 4331 bp, were structurally unique from Rht-B1c by an 868 bp insertion and a 1902 bp insertion, respectively, 728 bp upstream from the 197 bp indel, besides a number of small indels (Supplementary Fig. S1 at JXB online). Regardless of the large indels, Rht-B1 exhibited 89.0% and 92.8% similarity to Rht-A1 and Rht-D1, respectively, and Rht-A1 and Rht-D1 were 90.3% similar. In terms of the 197 bp indel, Rht-A1 and Rht-D1 remained consistent in showing the highest similarity. A comparable relationship was observed in the coding regions.

All Rht-B1c sequence variations, except for SNP 43, SNP 75, and the Veju insertion, are present in Rht-1 genes on A, B, and G genomes

Unique Rht-B1c sequence variations relative to Rht-B1 were investigated by cloning Rht-1 sequences up to 1625 bp...
upstream from 848 (Fig. 5A) on A, B, S, and G genomes of the Triticeae tribe by pairing the Rht-B1-specific primer 5’-TTACCTCTTGTGATTCCCACCG-3’ or the degenerate primer 5’-CAGAACCGTGTGTCGCATTAGCTA-3’. The 22 examined accessions representing different Triticeae species or subspecies and their genome compositions are listed in Supplementary Table S2 at JXB online. Nucleotide C at SNP 43 and nucleotide A at SNP 75, as well as the Veju insertion, did not occur in any of these accessions. The other SNPs in the corresponding regions were detected in Rht-1 genes in at least one of the examined genomes (Supplementary Table S2). The Rht-B1c 197 bp indel was found in Rht-1 genes of all but the B genomes of tetraploid and hexaploid species, thus probably representing an ancient form of the Rht-B1 sequences, even though there are a few SNPs, and small indels among the orthologues (data not shown). It was concluded that SNP 43, SNP 75, and the Veju insertion were the candidates resulting in the mutation from Rht-B1a to Rht-B1c.

**Rht-B1c promoter activity and Rht-1 gene cis-element variations**

A TSS was identified 163 bp upstream from the Rht-B1 gene start codon through plant promoter prediction with TSSP (Fig. 5A). This was confirmed by aligning the sequence with the full-length cDNA (AK332917) obtained through the CAP-trapper method of Kawaura et al. (2009). A putative TATA box was present 36 bp upstream from the TSS. Similar sequence features were found in all three Rht-1 genes at the two sites, with the exception of a few indels.

The effects of the Rht-B1c upstream sequence variations on promoter activity were investigated through transient expression assays. The transient expression constructs were prepared by fusing 2.1 kb Rht-B1a and 2.3 kb Rht-B1c upstream sequences to the GUS reporter gene (Fig. 6A). Initially, following bombardment of the constructs into onion epidermal cells, no GUS activity was detected. However, when the onion epidermal cells were replaced with young maize shoot tips for bombardment, comparable GUS activity was detected for all constructs (Fig. 6B), implying that Rht-B1c and Rht-B1a promoters do not differ substantially in promoter activity despite the sequence variations. Quantitative PCR analysis (results shown below) showed that upstream sequence polymorphisms of all Rht-1 genes exhibited no effects on basic promoter activity.

PLACE was used to identify cis-elements in the Rht-1 gene regions starting at 728 bp upstream from the 197 bp indel. Results showed that 84% of putative upstream regulatory elements of Rht-B1a, which has the shortest target sequence, were found in the other three genes, suggesting substantial conservation in expression regulation. However, putative cis-element evolution was also evident based on predictions. Rht-B1c lost the CACGTG motif predicted in Rht-B1a, and gained eight motifs due to the SNPs and 197 bp indel. Three of these gained motifs, including CELLCYCLESC, P1BS, and S1FBOXSORPS1L21, were also not detected in Rht-A1a and Rht-D1a (Supplementary Table S5 at JXB online). With a comparable target sequence
length (1593 bp and 1616 bp, respectively), *Rht-A1a* and *Rht-D1a* were distinguished by a total of 25 putative cis-elements (Supplementary Table S5). Consequently, the results suggested that although the basic *Rht-1* gene functions in wheat are potentially redundant, their roles in growth and development might have differentiated.

The 197 bp indel and coding region SNPs did not contribute to the major dwarfing effect

The influences of the 197 bp promoter region insertion, the *Veju* insertion, and the three coding region SNPs on plant height of 126 wheat cultivars in China’s wheat germplasm pool core collection were investigated. The corresponding regions were examined by PCR amplification using *Rht-B1* specific primers 5'-TTACCTCTTGTGATTCCCACCG-3' and 5'-TGGCGGTGAAGTGGGCGAAC-3'. One hundred and sixteen lines produced reproducible products identical in size to the corresponding S3 *Rht-B1a* sequence, indicating that the samples did not possess the 197 bp and *Veju* insertions. These results were confirmed by sequencing products of 22 of these cultivars. The remaining 10 lines generated products longer than the S3 *Rht-B1a* sequence. Sequencing revealed that seven of these lines possessed the 197 bp insertion and were identical to *Rht-B1c* at the three SNP sites, and three had a 160 bp insertion upstream from -356 and were identical to *Rht-B1a* at these SNP sites. Consequently, the 126 cultivars were classified into three haplotypes (Fig. 7; Supplementary Table S1 at JXB online). SNP43 showed a gain of the *Hae*III restriction recognition site in *Rht-B1c*, and SNP75 detected in *Rht-B1c* exhibited a loss of the *Bcc*I restriction recognition site in *Rht-B1a*. Therefore, it was possible to examine their presence in wheat cultivars by restriction enzyme digestion of the 123 bp PCR products beginning with -24 to 99. The 94 cultivars without sequence data all produced PCR products digestible with *Bcc*I but not *Hae*III, and were therefore assigned to haplotype III.

Based on 3 year field trial data, plant height of the seven cultivars representing haplotype I (i.e. *Rht-B1c*) exhibited a 90.5 cm average. However, one cultivar of this type, Caizhuang, was >150 cm in height (Supplementary Table S1 at JXB online). It does not possess a known dwarfing gene. The other six cultivars, all with a plant height <100 cm, carried *Rht8*, *Rht-D1b*, or dwarfing quantitative trait loci (QTLs) based on the literature, sequencing, or diagnostic assays.

This suggests that instead of the 197 bp insertion and three SNPs, the *Veju* insertion is most probably responsible for the extreme *Rht-B1c* dwarfing capacity. Haplotype II plant height ranged from 80.5 cm to 126.4 cm, with an average of 110.2 cm. Although haplotype III and haplotype II differed in the absence of the 160 bp insertion, they exhibited a similar average plant height (Fig. 7), suggesting that this insertion had no effect on dwarfing.

The *Veju* insertion changes processing and translated products of *Rht-B1c* transcripts

Since *Rht-B1c* has one 2026 bp insertion and three SNPs in the region corresponding to the *Rht-B1a* open reading frame (ORF), *Rht-B1c* transcripts were isolated by amplifying the cDNA beginning at the start codon by 3'-RACE. PCR generated two reproducible products. One product was 363 bp in length, and was designated as Rht-B1cS; the other, 2370 bp in length, was designated Rht-B1cL. Alignment of Rht-B1cS with *Rht-B1a* cDNA and *Rht-B1c* genomic DNA sequences showed that Rht-B1cS resulted from premature polyadenylation of the *Rht-B1c* transcripts, and it included a 150 bp 5' region identical to *Rht-B1a*, and 186 bp of the 5' *Veju* sequence. The sequences flanking the polyadenylation site contained multiple conserved polyadenylation signal elements (Fig. 8A). Rht-B1cL did not possess a stop codon. In eukaryotes, mRNA lacking a stop codon might be degraded via a non-stop decay pathway (Frischmeyer et al., 2002; van Hoof et al., 2002), which, if also applicable in plants, might explain why Rht-B1cS abundance was 40 times less than that of Rht-B1cL at the mRNA level (Fig. 8B).

Rht-B1cL contains a 1956 bp ORF and a 414 bp 3'-UTR. Comparison with the *Rht-B1c* genomic DNA sequence provided evidence that this transcript resulted from removal of the 1936 bp from 149 to 2084 (Fig. 5). Relative to *Rht-B1a*, the Rht-B1cL ORF retained a 90 bp 3' sequence of the *Veju* insertion, which subsequently resulted in a predicted 30 residue
addition following K49, residing within the DELLA motif (Fig. 9). The SNP downstream from the Veju insertion was identified as a nonsense mutation, but the two SNPs before the Veju insertion result in G to R and M to I changes, both of which reside in non-conserved regions (Fig. 9). With the exception of these variations, the predicted Rht-B1cL translation product was identical to Rht-B1a.

**Rht-B1c reduced Rht-1 gene expression levels but not the transcriptional response to GA treatment**

Since Rht-B1cL was the major Rht-B1c transcript in cells, its abundance was examined to determine Rht-B1c mRNA levels in the following experiments. Semi-quantitative RT–PCR assays indicated that Rht-B1 was primarily expressed in stems and leaves, and lower expression levels were found in roots and milky stage spikes (Fig. 10A). Overall, the Rht-B1 expression level was consistently lower in DS3 than in S3, irrespective of growth stage (Fig. 10A, B). Seedling treatment with 100 μM GA3 resulted in notable Rht-B1a and Rht-B1c up-regulation, but the Rht-B1c transcription levels remained lower (Fig. 10B). However, the transcription level of Rht-B1c could be higher taking into consideration the Rht-B1cS transcript.

**Rht-B1c up regulated genes involved in cell elongation, chlorophyll biosynthesis, and chloroplast development**

As Rht-B1c plant lines exhibited shorter cells and higher chlorophyll content (Fig. 3), S3 and DS3 seedling leaves were evaluated to determine if Rht-B1c regulated cell elongation and chlorophyll biosynthesis through GATA-like transcription factors, similar to what was reported in *A. thaliana* (Richter et al., 2010; Hudson et al., 2011). Quantitative RT–PCR for GATA22, the wheat GATA-like transcription factor gene, showed that DS3 expressed almost three times more of this gene relative to S3 (Fig. 11A). GA3 application decreased its expression in both lines; nevertheless, the expression level...
remained notably higher in DS3 than in S3. At the tillering stage, DS3 still maintained a higher GATA22 expression level (Fig. 11B). The expression of four wheat genes homologous to HEMA1, GUN4, CAO1, and PorB was also examined, and they all had higher expression levels in DS3 (Fig. 11B). These four genes were positively regulated for chlorophyll biosynthesis by GATA transcription factors in A. thaliana (Hudson et al., 2011).

Chiang et al. (2012) demonstrated that GATA transcription factors modulated chloroplast biogenesis. Therefore, to determine if Rht-B1c affected chloroplast development and photosynthesis, the expression of rbcS and CAB genes was measured for chlorophyll alb binding. rbcS showed an almost 6-fold increase in the expression level, and the CAB genes also displayed a significantly higher level in DS3 (Fig. 11B).

**Discussion**

Sequence characteristics of Rht-B1c and its homologues

Pearce et al. (2011) and Wu et al. (2011) reported Rht-B1c cDNA cloning. Wu et al. (2011) also isolated this gene’s coding region genomic DNA. In the present study, using homology-based cloning and genome walking strategies, the full genomic DNA sequence of Rht-B1c and Rht-B1a, including both the coding region and the upstream 2 kb sequence, was cloned. In agreement with Wu et al. (2011), the present results revealed that in the coding region, Rht-B1c differed from Rht-B1a by one 2026 bp insertion 151 bp downstream from the start codon. The retrotransposon was determined to be different from Veju_S and Veju_L elements (SanMiguel et al., 2002; Sabot et al., 2005). Furthermore, it was found that the Rht-B1c coding region carried three SNPs, with SNP43 and SNP75 preceding the insertion, and SNP723 following the insertion. This was consistent with the findings of Pearce et al. (2011). However, the SNP A417 reported by Pearce et al. (2011) was not confirmed. SNP723 does not cause a codon change. In the 2 kb upstream region, Rht-B1c was characterized by one 197 bp insertion preceding −591, and seven SNPs (Fig. 5A). Genetic analyses showed that the insertions co-segregated with plant height in segregating populations.

Among the insertions and SNPs, the Veju insertion was the only one not detected in the Rht-B1 genes of all 126 surveyed common wheat cultivars, and in the Rht-1 orthologues present in the A, B, S, and G genomes of the Triticeae species, the close relatives of wheat. Therefore, unlike most identified DELLA mutants resulting from deletion (gai), premature termination (rga-1, Rht-B1b, Rht-B1d, Rht-B1e, Rht-D1b, and shr1), or amino acid substitutions (rga-2, sln1d, and Brrga1-d) (Peng et al., 1997, 1999; Silverstone et al., 1998; Pearce et al., 2011; Ikeda et al., 2001; Chandler et al., 2002; Muangprom et al., 2005), the Veju insertion clearly emerged as the mutation responsible for the origin of the Rht-B1c allele from Rht-B1.
SNPs C$_{43}$ and A$_{75}$ in *Rht-B1c* caused a codon change, but an association with dwarfing was not observed. Furthermore, the substituted amino acids reside in a non-conserved region, which is not implicated in the DELLAs–GID1 interaction (Murase *et al.*, 2008), suggesting no crucial functional relationship. SNPs C$_{43}$ and A$_{75}$ were identified in seven of the 126 cultivars with SNP G$_{723}$ and the 197 bp upstream insertion (haplotype I in Fig. 7). Sequences homologous to the 197 bp insertion and SNPs T$_{131}$ and G$_{273}$ were resolved in *Rht-B1* orthologues in *S* genome carriers *Aegilops speltoides* and *A. longissima*, and *G* genome carriers *T. timopheevii* and *T. araraticum*. Because the *S* genome was viewed as the ancestor of *B* and *G* genomes (Wang *et al.*, 1997), these results suggested that *Rht-B1c* originated from the *Veju* insertion into an ancient *Rht-B1* haplotype that experienced point mutations from G$_{43}$ to C$_{43}$ and G$_{75}$ to A$_{75}$. All *Rht* orthologues detected in *A* and *D* genomes possessed the 197 bp insertion, providing additional support for its ancestral origin. The 197 bp insertion, together with the seven SNPs detected in the *Rht-B1c* upstream region, appeared to exhibit negligible effects on basic promoter activity. In common wheat, haplotype III (Figure 7) was the most common haplotype, differing from the *S* genome orthologs by the G$_{43}$ to C$_{43}$ mutation in the coding region and the loss of 197 bp fragment. All detected tetraploid species and hexaploid sub-species of wheat examined in our study belonged to this haplotype. Haplotype II, the most rare haplotype, might have originated from haplotype III by insertion of the 160 bp fragment.

Compared with the DNA coding region, the upstream regions of *Rht-1* genes in various genomes were much more diverse, characterized by a number of indels and SNPs. Generally speaking, *Rht-A1* and *Rht-D1* were more similar relative to *Rht-B1*. Sequence variations in the upstream regions of these genes caused loss or gain of the predicted *cis*-elements. In *A. thaliana*, five DELLA protein genes have been reported with overlapping but differentiated functions (Dill and Sun, 2001; Tyler *et al.*, 2004). Gallego-Bartolomé *et al.* (2010) suggested that this functional diversification largely relied on changes in gene expression patterns rather than on the capacity of the protein to interact with different regulatory partners. It would be interesting to ascertain if upstream sequence variations in *Rht-1* genes caused functional diversification. The polyploid nature of wheat could surely allow such modifications.

**The influence of Veju insertion on Rht-B1c expression and function**

Two *Rht-B1c* transcripts were identified, termed Rht-B1cS and Rht-B1cL. Rht-B1cL is identical to the *Rht-B1c* cDNA reported by Pearce *et al.* (2011) and Wu *et al.* (2011). The transcript encodes a protein with 30 extra residues within the N-terminal DELLA motif, due to retention of the 90 bp 3’ *Veju* sequence in mature mRNA. Disruption of the DELLA motif could cause failure of its interaction with the GA receptor GID1, which is required for GA–GID1–DELLA complex formation underlying GA signal transduction (Murase *et al.*, 2008). Wu *et al.* (2011) found that rice plants transformed with *Rht-B1c* cDNA were much shorter than those transformed with *Rht-B1b*.

*Rht-B1cS* contained only the 336 bp 5’ region initiating at the start codon. The multiple conserved polyadenylation signal elements surrounding the putative Rht-B1cS polyadenylation site and the presence of two *Rht-B1c* transcripts in cells suggested that Rht-B1cS resulted from alternative polyadenylation during RNA processing. Real-time RT–PCR analysis revealed that Rht-B1cL accounted for ~97.5% of the *Rht-B1c* transcripts in seedling leaves. However, this proportion might actually be much less since Rht-B1cS probably has a short lifetime due to the absence of a stop codon. The mRNA without a stop codon in *Saccharomyces cerevisiae* would trigger a non-stop decay pathway (van Hoof *et al.*, 2002; Frischmeyer *et al.*, 2002, Vasudevan *et al.*, 2002). Indeed, *Rht-B1c* transcripts in DS3 were substantially less than *Rht-B1a* transcripts in S3, regardless of tissue types. If *Rht-B1a* in S3 and *Rht-B1c* in DS3 are assumed to have comparable expression levels, generation of short-lived Rht-B1cS would result in less Rht-B1cL. The truncated polypeptide translated from Rht-B1cS possessed a truncated DELLA motif, and therefore probably does not serve a major role in GA signalling.

Since the aberrant Rht-B1c protein cannot interact with GID1 (Pearce *et al.*, 2011; Wu *et al.*, 2011), it would not be degraded through the GA-stimulated GID1–DELLA–SCF$^{GID2}$ complex, and consequently would accumulate in cells. It is proposed that this could in turn down-regulate *Rht-1* gene expression through a feedback mechanism. When external GA$_3$ is applied, DELLA protein degradation encoded by *Rht-A1a* and *Rht-D1a* could provide the induction signal for *Rht-1* gene expression. However, this induction was limited compared with S3 due to the aberrant Rht-B1c protein.

**Plant morphogenesis affected by Rht-B1c**

*Rht-1* genes were first known for their role in determining plant height. *Rht-1* mutants varied in their genetic and physiological effects on plant growth and development. In contrast to the co-dominant or partially recessive *Rht-B1b* and *Rht-D1b*, *Rht-B1c* was co-dominant or partially dominant for reduced height, as shown in this study and others (Uddin and Marshall, 1989; Flintham *et al.*, 1997; Jošt *et al.*, 2011). This was in disagreement with the report of Wu *et al.* (2011), in which *Rht-B1c* was claimed to be completely dominant. Different from *Rht-B1b* and *Rht-D1b* that reduced cell length in vegetative organs and had no affect on cell width or cell division (Miralles *et al.*, 1998), *Rht-B1c* notably reduced cell length, and substantially increased cell width.

It has become clear that *Rht-1* genes encode GA signalling proteins critical for GA-regulated growth and development. Using GA-deficient and altered GA-response *A. thaliana* mutants, Cowling and Harberd (1999) demonstrated that GA regulated cell elongation in hypocotyls grown under light and dark conditions. GAI and RGA signalling elements, both of which are DELLA proteins, were integral in these processes (Alabadi *et al.*, 2004). DELLAAs block the transcriptional activity of phytochrome-interacting factors (PIF)
by binding the DNA recognition domains in PIFs (de Lucas et al., 2008; Feng et al., 2008). PIF factors, including PIF3, PIF4, and PIF5, positively regulate hypocotyl elongation, and are antagonistically regulated by light and GA (Ni et al., 1998; Kim et al., 2003; de Lucas et al., 2008; Feng et al., 2008; Kunihiro et al., 2011). Based on the GA signalling 'relief of repression' regulatory model, degradation failure in the aberrant Rht-B1c protein could result in a constitutive block of PIF-like transcription factor activity in wheat, subsequently resulting in shorter cells and dwarfing. Richter et al. (2010) identified two homologous GATA-type transcription factors, GNC and GNL, in A. thaliana that functioned downstream from DELLA and PIF regulators in germination, greening, elongation growth, and flowering time regulation. Their results indicated that DELLA proteins provided positive regulation of GATA-type transcription factors by inhibiting PIFs that are GNC repressors. In agreement with these observations, it was shown here that GATA22 expression in Rht-B1a and Rht-B1c lines was significantly reduced by GA application. Nevertheless, relative to the Rht-B1a lines, the GATA22 expression in the Rht-B1c line consistently maintained a markedly higher expression level, even following GA application, suggesting that PIF-like regulators could remain partially inhibited even after destabilization of the normal Rht-A1a and Rht-D1a proteins by GA treatment. The observations regarding the GATA22 expression levels resulting from GA treatment are analogous to the heterozygous state of Rht-B1c in terms of Rht cellular protein composition, which underlies the partial dominance nature of this gene. This was also consistent with the observation that increased accumulation of the mutated Rht-D1 protein exhibited stronger dwarfing effects (Li et al., 2012).

**Rht-1 gene participation in chlorophyll biosynthesis and regulation of chloroplast biogenesis**

Leaf is the main photosynthetic organ, and efficient photosynthesis is critical for photosynthetic formation and yield. A number of experiments suggested that GA-mediated signalling participates in chlorophyll biosynthesis and chloroplast biogenesis regulatory processes. For example, both DS3 and DY3 wheat had higher chlorophyll content; a semi-dwarf near-isogenic line with Rht-B1b and Rht-D1b had significantly higher leaf photosynthetic rates, and chlorophyll and Rubisco concentrations compared with the tall wheat (Morgan et al. 1990, Feng et al. 2008) and Cheminant et al. (2011) demonstrated that DELLA proteins played a critical role in regulating chlorophyll and carotenoid biosynthetic pathways by PIF interactions. Several authors indicated that PIF factors were inhibitory to chlorophyll biosynthesis (Kim et al., 2003; Feng et al., 2008; Moon et al., 2008; Shin et al., 2009; Stephenson et al., 2009; Cheminant et al., 2011). In this process, GATA-type transcription factors were involved and functioned downstream from PIFs (Richter et al., 2010; Hudson et al., 2011). Thus, it was not surprising to find that Rht-B1c participated in the regulation of chloroplast biogenesis, in addition to chlorophyll biosynthesis modulation, as shown in the distinct rbcS and CAB expression patterns between Rht-B1a and Rht-B1c lines, and reported by Jiang et al. (2012). In A. thaliana, this regulatory process also required GATA-type regulators (Chiang et al., 2012) and PIFs (Shin et al., 2009). In the pifQ A. thaliana mutant grown under dark conditions, both chlorophyll biosynthetic and photosynthetic genes were highly overexpressed (Shin et al., 2009). In agreement with these findings, in GA-deficient/sensitive mutants, such as gal-3, the expression of both chlorophyll biosynthetic and photosynthetic genes was de-repressed (Alabadi et al., 2004; Cheminant et al., 2011).

Crop dwarfing was a critical factor leading to the first ‘Green revolution’. It is related to a comprehensive suite of traits, including ideal plant type, harvest index, photosynthetic efficiency, and assimilate partition, among other attributes, which are all determining factors in increasing yield potential. Pearce et al. (2011), Wu et al. (2011), and the present study clearly demonstrated that Rht-B1c, as a Rht-B1a mutant unique among other known DELLA protein gene mutants, affects many aspects of growth and development. Characterization of its structure and regulation of growth- and development-related genes will be extremely valuable in designing new crop improvement strategies. However, this is still not sufficient, since in monocots, that include most food staple cereal crops, only one DELLA protein gene locus per genome has been identified, the mechanisms underlying the GA-mediated regulation and cross-talk of GA signalling with other regulatory pathways and environmental cues, particularly the coordinative control of various physiological functions, remains poorly understood. Therefore, isolation and characterization of genes downstream from the DELLA regulator, and elucidation of their interplay with DELLA protein genes, is our next challenge.

**Supplementary data**

Supplementary data are available at JXB online

**Figure S1.** Upstream structures of Rht-1 genes.

**Figure S2.** 3’ UTR sequence alignment of Rht-1 genes.

**Table S1.** Rht-B1 haplotypes and plant height for 126 wheat cultivars.

**Table S2.** Rht-1 sequence variations in Triticeae tribe species.

**Table S3.** Primer sequences used in RT-PCR of downstream genes.

**Table S4.** Plant height of the three Rht-B1 genotypes in the individual BC3F1.5 families and the estimated dominant effect.

**Table S5.** Distinct cis-elements predicted for wheat Rht-1 genes.

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