The semi-dwarfing gene Rht-dp from dwarf Polish wheat (Triticum polonicum L.) is the “Green Revolution” gene Rht-B1b

Keywords: Dwarf Polish wheat, homologous cloning, molecular mapping, Rht-B1b, RNA-seq

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Abstract

**Background:** The wheat dwarning gene increases lodging resistance, the grain number per spike and harvest index. Dwarf Polish wheat (*Triticum polonicum* L., 2n = 4x = 28, AABB, DPW), initially collected from Tulufan, Xinjiang, China, carries a semi-dwarning gene *Rht-dp* on chromosome 4BS. However, *Rht-dp* and its dwarning mechanism are unknown.

**Results:** Homologous cloning and fine mapping revealed that *Rht-dp* is the ‘Green Revolution’ gene *Rht-B1b*. A haplotype analysis in 59 tetraploid wheat accessions showed that *Rht-B1b* was only present in *T. polonicum*. Transcriptomic analysis of two pairs of near-isogenic lines (NILs) of DPW×Tall Polish wheat (*Triticum polonicum* L., 2n = 4x = 28, AABB, TPW) revealed 41 differentially expressed genes (DEGs) as potential dwarfsm-related genes. Among them, 28 functionally annotated DEGs were classed into five sub-groups: hormone-related signalling transduction genes, transcription factor genes, cell wall structure-related genes, reactive oxygen-related genes, and nitrogen regulation-related genes.

**Conclusions:** These results indicated that *Rht-dp* is *Rht-B1b*, which regulates pathways related to hormones, reactive oxygen species, and nitrogen assimilation to modify the cell wall structure, and then limits cell wall loosening and inhibits cell elongation, thereby causing dwarfism in DPW.

**Background**

Plant height is an important agronomic trait of crops. The discovery of semi-dwarning genes in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) triggered the “Green Revolution”, as this trait not only improves lodging resistance [1], but also increases the grain number per spike and harvest index [2-3]. Increasing numbers of dwarf varieties of crops are being bred for production [4], and the dwarning mechanisms in crop plants, including rice and wheat, have been studied in detail [5-7].

In wheat, 27 dwarning genes including 32 alleles are present on chromosomes 2A, 2B, 2D, 3B, 4B, 4D, 5A, 5D, 6A, 7A, and 7B [8-16]. Twenty-two of those genes were discovered from hexaploid wheat, including *Rht1* (*Rht-B1b*), *Rht2* (*Rht-D1b*), *Rht8*, and *Rht12*. Those genes are widely utilized to breed new cultivars while only *Rht1* and *Rht2* have been cloned [6, 17-18]. As the parent of hexaploid wheat, tetraploid wheat also has many dwarning genes, for example, *Triticum durum* contains *Rht14, Rht15*, *Rht16, Rht18, Rht19*, and *Rht-R107* [19-20]; *Triticum turgidum* contains *Rht22* [21]; *Triticum aethiopicum* contains *Rht-B1f* [15]; and *Triticum polonicum* contains *Rht-B1IC12196* and *Rht-dp* [10, 14]. Additionally, *T. polonicum* has a high 1000-grain weight and accumulates high concentrations of nutrients, including zinc and iron, in grains [22]. As a valuable material for wheat genetic improvement, it has attracted the interest of breeders [22]. However, the details of its dwarning genes, *Rht-dp* and *Rht-B1IC12196*, are still unknown.

As a gibberellin (GA)-insensitive semi-dwarning gene, *Rht-dp* was found in a spontaneous dwarf accession of *T. polonicum* (dwarf polish wheat, DPW) [10], which was initially collected from Tulufan, Xingjiang, China [23]. Transcriptomic and proteomic analyses suggested that *Rht-dp* is probably involved in the phenylpropanoid pathway; it was found to reduce the contents of lignin, cellulose, and S-adenosyl-
methionine, and increase the contents of flavonoids. Lines harbouring Rht-dp show limited auxin transport and reduced extensibility of the cell wall, which ultimately limits cell expansion and causes dwarfism [24]. Although those results indicated the potential mechanism of Rht-dp, the candidate gene of Rht-dp remained unknown. Kang et al. mapped Rht-dp onto chromosome 4BS between the SSR markers Xgpw3017 and Xwmc511, and suggested that Rht-dp may be an alternative allele at the Rht-B1 locus [10]. However, a genomic alignment against the genome of Triticum aestivum 'Chinese Spring' (IWGSC RefSeq v1.0) (International Wheat Genome Sequencing Consortium, 2018) indicated that the region between Xgpw3017 and Xwmc511 did not include the Rht-B1 locus. Meanwhile, genetic analysis of the recombinant inbred line (RIL) derived from the cross of DPW and tall Polish wheat (TPW) indicated that Rht-dp should be a recessive gene [10]; while, Rht-B1b and its alleles are semi-dominant genes [6, 25-26]. Therefore, further research is needed to determine whether Rht-dp is Rht-B1b or its allele, or a new gene.

Rht-B1b encodes a premature DELLA protein, which prevents GID1 from binding to its target [12]. This truncates the GA response, resulting in dwarfism. Rht-B1b originates from the native Japanese dwarf variety ‘Norin 10’ [27]. It was successfully transferred from ‘Norin 10’ to ‘Cando’ in the 1960s and widely used in durum wheat breeding [28]. Its three alleles, Rht-B1f, Rht-R107, and Rht19, were discovered from T. aethiopicum and T. durum [15, 19]. Although T. polonicum is a different species from T. aethiopicum and T. durum [29], Rht-dp, Rht-B1b and its alleles are localized on 4BS in T. polonicum, T. aethiopicum, and T. durum [10, 23, 28, 30]. Thus, we hypothesized that the candidate gene of Rht-dp may be Rht-B1b or its one of alleles.

To test this hypothesis and to understand the dwarng mechanism of Rht-dp in DPW, we firstly cloned Rht-B1 to investigate sequence differences in Rht-B1 between DPW and TPW. Secondly, we developed and applied a specific molecular marker of Rht-B1 and SSR markers on 4BS to genetically confirm the candidate region using three RILs. Thirdly, two pairs of near-isogenic line (NIL) obtained from the F7 population of DPW×TPW were conducted transcript analyses to reveal the molecular mechanism of Rht-dp; meanwhile, F1 plants and a F2 population derived from the cross of a pair of NIL were developed for further genetic analysis. Finally, we conducted a haplotype analysis of Rht-dp to reveal the natural distribution among 59 tetraploid wheat accessions.

Methods

Plant materials and growth conditions

The DPW and TPW lines were originally collected from Tulufan, Xinjiang province, China, by Prof. Chi Yen and Junliang Yang (Sichuan Agricultural University, China) in the 1980s. The F1 population of DPW×TPW and the F2 population (401 plants) derived from DPW×TPW were individually developed for trait investigation. Two RIL populations (F7 including 330 lines and F6 including 300 lines) derived from DPW×TPW, and a RIL population (F6 including 194 lines) derived from DPW×Ailanmai (AABB, 2n = 4x = 28, T. turgidum L.), were developed for gene mapping. Two pairs of NILs (D_60/T_58, and D_33/T_35, D
and T represent dwarf and tall phenotype, respectively) derived from two heterozygous F₇ lines were selected for transcript analyses. Meanwhile, F₁ plants and a F₂ population (244 plants) derived from the cross of D₆₀ and T₅₈ were developed for trait investigation. The haplotype analysis was conducted using 59 tetraploid wheat accessions (Table S1).

DPW, TPW and their F₁ plants and F₂ population were grown at the Wenjiang experimental field of Sichuan Agricultural University, Chengdu, China, in the 2011–2012 (from October 2011 to June 2012) and 2012–2013 (from October 2012 to June 2013) wheat growing seasons. The F₇ and F₈ RIL populations of DPW×TPW were grown at two experimental fields (Wenjiang and Chongzhou) of Sichuan Agricultural University (Chengdu, China) in the 2017–2018 (from October 2017 to June 2018) and 2018–2019 (from October 2018 to June 2019) wheat growing seasons, respectively. The F₆ RIL population, the F₁ plants of D₆₀×T₁₈, two pairs of NILs, and 59 tetraploid wheat accessions were grown at the Wenjiang experimental field in the 2018–2019 (from October 2018 to June 2019) wheat growing season. The F₂ population of D₆₀×T₁₈ was grown at the Wenjiang experimental field in the 2019–2020 (from October 2019 to June 2020) wheat growing season. Each line was planted with 20 plants per row. Rows were 2 m long and the spacing between rows was 30 cm.

**Phenotypic measurements and analysis**

Plant height, spike length, and stem length were measured at maturity. We selected three individual plants per line and calculated the average value. Data were analysed using SPSS software (version 18.0; SPSS, Chicago, IL, USA) Figures were drawn using SigmaPlot software (version 12.0; Systat, Point Richmond, CA, USA).

**Homologous cloning of Rht-B1**

According to the genomic sequence of T. aestivum cv. ‘Chinese Spring’ (IWGSC RefSeq v1.0), a pair of Rht-B1-specific primers (forward: 5’-CGATGCCGTC TACAACTACT-3’; reverse: 5’-CAACTCCTAGATCGGAAACTT-3’) was designed using Beacon designer software (version 7.0; Premier Biosoft International, Palo Alto, CA, USA). These primers were used to amplify the full-length Rht-B1 sequence from DPW and TPW. Each PCR reaction mixture contained 2 μl DNA, 2 μl mixture of forward and reverse primers (4 pmol/μl), 2 μl dNTP (2.5 mM/μl), 1 μl Ex-Taq polymerase (5 U/μl), 2 μl MgCl₂ (2.5 mM/μl), 2.5 μl 10× PCR buffer, and 13.5 μl ddH₂O. The PCR amplification conditions were 95°C for 5 min, 40 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 2 min), and final extension at 72°C for 10 min. Each amplified fragment was cloned into the pMD19-T vector for sequencing. Differences in Rht-B1 sequences between DPW and TPW were detected in an alignment analysis using Vector NTI software (version 11.5.1; Invitrogen, Carlsbad, CA, USA).

**Exploitation of indel marker of Rht-B1 for mapping**

According to the sequence differences in Rht-B1 between DPW and TPW, a pair of Rht-B1-specific primers (Rht-B1 Indel-F: 5’-GGCGGGAGATCGAAGTAC-3’, Rht-B1 Indel-R: 5’-GACACCGTGCACTACAAC-
3’) was designed using Beacon designer software.

**Exploitation of SSR markers on 4BS for mapping**

According to the genomic sequence of 4BS of *T. aestivum* cv. ‘Chinese Spring’ (IWGSC RefSeq v1.0) ([http://plants.ensembl.org/](http://plants.ensembl.org/)), microsatellites were predicted using the MicroSAellite identification tool ([https://webblast.ipk-gatersleben.de/misa/](https://webblast.ipk-gatersleben.de/misa/)) [31-32]. Beacon designer software was used to design SSR markers (Table S2).

**Genotyping and genetic mapping**

Genomic DNA was extracted from DPW, TPW, Ailanmai and the mapping populations RIL$_6$ (DPW×Ailanmai), RIL$_7$ and RIL$_8$ (DPW×TPW) using a plant genomic DNA kit (TIANGEN BIOTECH, Beijing, China). Each PCR reaction mixture contained 1 μl DNA, 2 μl mixture of forward and reverse primers (4 pmol/μl), 1.5 μl dNTP (2.5 mM/μl), 0.5 μl Taq polymerase (5 U/μl), 1.5 μl MgCl$_2$ (2.5 mM/μl), 2 μl 10× PCR buffer, and 11.5 μl ddH$_2$O. The PCR amplification conditions were 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, with final extension at 72 °C for 7 min. The PCR products were separated on 8% polyacrylamide gels. The polymorphic bands between the parents were used to genotype individual line of the mapping populations.

The *Rht-B1 Indel* marker and 15 polymorphic SSR markers were first used for genetic mapping of *Rht-dp* in the F$_7$ RIL population. Then, *Rht-B1Indel* and its four flanking SSR markers (Xgpw2994.1, Xgpw3128.1, Xgpw3427.1, and Xgpw4800.1) were further used to confirm the candidate region in the F$_8$ RIL and F$_6$ RIL populations. The F$_7$ RIL population was hybridized on the wheat 55K SNP array by CapitalBio Technology (Beijing, China) (unpublished data).

Linkage analysis was performed using the JoinMap software (version 4.0; Kyazma BV, Wageningen, Netherlands) with a logarithm of odds (LOD) threshold of 3.0. The Kosambi mapping function was used to convert the recombination frequencies into genetic distances (cM) [33].

**Haplotype analysis of Rht-B1 in 59 tetraploid wheat accessions**

Genomic DNA was extracted from each tetraploid wheat accession using a plant genomic DNA kit (TIANGEN BIOTECH, Beijing, China), and PCR amplification was performed as described in the section “Homologous cloning of *Rht-B1*. The amino acid sequence was deduced using ExPASy software ([http://web.expasy.org/ translate/](http://web.expasy.org/translate/)). All sequences were aligned using Vector NTI software (Invitrogen). A phylogenetic tree was constructed using the neighbour-joining algorithm in MEGA5 ([https://www.megasoftware.net/](https://www.megasoftware.net/)).

**Expression analysis of *Rht-B1b***

Tissues at the three growth stages (jointing, booting, and grain filling stages) were collected, including roots, basal stems, leaf sheaths, leaf blades, young leaves, lower leaf blades, first and second internodes,
flag leaves, and spikes. The collected tissues were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted using a Plant RNA Kit (Omega Bio-Tek, American). cDNA was synthesized using the M-MLV First Strand cDNA Synthesis kit (Invitrogen).

Quantitative real-time PCR (qPCR) was performed on the CFX-96 system as described by Wang et al. using a pair of \textit{Rht-B1b}-specific primers (forward: 5′-GGCGGGAGATCGAAGTAC-3′; reverse: 5′-GACACCGTGCACTACAAC-3′) [34]. To normalize gene expression levels, the \textit{Actin} gene was used as the reference gene [34]. Relative expression levels were calculated according to the \(2^{ΔΔCt}\) method using the CFX Manager (version 3.1; Bio-Rad, Hercules, CA, USA).

\textbf{Transcript analysis of two pairs of NILs}

\textit{Sample collection}

At the booting stage, the first internode was collected individually from two pairs of NILs, and then snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

\textit{RNA isolation, quantification, and qualification}

Total RNA was isolated as described above, and RNA degradation and contamination were monitored on 1\% agarose gels. The RNA purity was checked using a NanoPhotometer® spectrophotometer (Implen GmbH, Munich, Germany). The RNA concentration was measured using the Qubit® RNA Assay Kit with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The RNA integrity was assessed using the RNA Nano 6000 Assay Kit with a Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA).

\textit{Library preparation and sequencing}

mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5x) (New England Biolabs, Ipswich, MA, USA). First-strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, the NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments 250~300 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, CA, USA). Then, 3 µl USER Enzyme (New England Biolabs) was reacted with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system) and the library quality was assessed using the Agilent Bioanalyzer 2100 system.

Index-coded samples were clustered using the cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. After cluster
generation, the prepared libraries were sequenced on the Illumina Hiseq platform.

**Quality control**

Raw data (raw reads) of in fastq format were first processed using in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N, and low-quality reads from the raw data. All the downstream analyses were conducted using clean, high-quality data.

**Read mapping to reference genome**

The Chinese Spring (IWGSC RefSeq v1.0) reference genome and gene model annotation files were downloaded from the genome website (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v1.0). The D genome sequences were excluded from the reference before mapping the processed reads of the tetraploid lines (A and B genomes). An index of the Chinese Spring reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12.

**Quantification of gene expression levels**

HTSeq v0.6.1 was used to count the number of reads mapped to each gene. The mean fragments per kilobase of transcript per million mapped reads (FPKM) value for each gene was calculated based on the length of the gene and the number of reads mapped to it [35].

**Differential expression analysis**

Read counts were adjusted by the edgeR program package through one scaling normalized factor. Analysis of differential gene expression between two pairs of NILs (D33/T35 and D60/T58) was performed using the DESeq R package. The P-values were adjusted using the Benjamini and Hochberg method. A corrected P-value of 0.005 and log2 (fold change) of 1 were set as the thresholds for significantly different gene expression.

**QPCR for validation**

Two differentially expressed genes Auxin-repressed protein (ARP) and *L-ascorbate oxidase homolog* (*ASCO*) from RNA-Seq were verified by qPCR, and their gene-specific primers sequences were **ARP** (forward: 5′-ATTAAGCAGTCGCCG TCGAT-3′; reverse: 5′-TCGCTGTAAAGCCAG TCGTA-3′) and **ASCO** (forward: 5′-AATGGCAATAGGTTCACAGTAGA-3′; reverse: 5′-CTTCACGAGGAACGAGTG AGG-3′), respectively.

**Results**

**Phenotype of plants harbouring Rht-dp**
The average heights of DPW and TPW were 91.52 ± 2.97 cm and 189.88 ± 1.72 cm, respectively. No significant difference of plant heights between F$_1$ plants (179.12 ± 3.65 cm) and TPW was observed (Fig. S1). The plant heights of F$_2$ plants ranged from 65 to 185 cm. According to the frequency distribution of plant height, F$_2$ plants were separated into two groups of dwarf and tall phenotypes at 110 cm (Fig. 1A). The dwarf and tall phenotype groups included 107 and 294 plants, respectively, consistent with the expected Mendelian segregation ratio of 1:3 ($X^2$=0.606, $p$>0.05). These results validate that Rht-dp should be a major recessive gene. However, the separated threshold of plant height with 110 cm was significant larger than the plant height of DPW with 91.52 ± 2.97 cm, which implied that the effect of Rht-dp on reducing plant height might be partially covered by one or more non-allelic loci.

To fine-map Rht-dp, two RIL populations including 330 F$_7$ and 300 F$_8$ plants were constructed. The plant heights of F$_7$ and F$_8$ plants ranged from 65 to 165 cm (Fig. 1B) and from 65 to 170 cm (Fig. 1C), respectively. For F$_7$ population, the average heights of dwarf and tall phenotypes were 84.07 ± 1.97 cm and 133.75 ± 2.01 cm, respectively. Compared with the tall phenotype, the lines harbouring Rht-dp showed a reduction in plant height of up to 37.14%. The reduced plant height was because of the shortened first internode (by 14.83%), second internode (by 7.15%), and basal internode (by 1.46%), but the length of the spike was not affected (Fig. 1D). These results indicate that Rht-dp reduces plant height mainly by restricting elongation of the first and second internodes at the booting stage.

To validate the candidate region of Rht-dp in a different genetic background, an F$_6$ RIL population including 194 lines derived from DPW×Ailanmai was constructed. The average height of Ailanmai was 100.98 ± 0.37 cm. Ailanmai has a recessive dwarng gene Rht22, which has an additive effect with Rht-dp. The RIL population was grouped into dwarf and tall phenotypes with heights ranging from 20 to 60 cm and from 120 to 160 cm, respectively (Fig. 1E).

**Differences in sequence of Rht-B1 between DPW and TPW**

To test the hypothesis that the candidate gene of Rht-dp is Rht-B1b or one of its alleles, the sequences of Rht-B1 were cloned from DPW and TPW. Sequence analysis showed that Rht-B1 of DPW is Rht-B1b, with a single nucleotide change from C to T at the nucleotide position 190 when compared with Rht-B1a (Fig. 2A) that results in a premature termination codon at amino acid position 64 (Fig. 2B). Although Rht-B1 of TPW did not have this single nucleotide change from C to T at nucleotide position 190, it had a three-nucleotide deletion at nucleotide position 386–388 when compared with Rht-B1a (Fig. 2A), resulting in a serine (S) deletion at amino acid position 129 (Fig. 2B). These results imply that the candidate gene of Rht-dp might be Rht-B1b. An Rht-B1 Indel marker was developed from the three-nucleotide deletion of Rht-B1 in TPW for further analysis.

**Mapping of Rht-dp**

To confirm that the candidate gene of Rht-dp is Rht-B1b, the Rht-B1Indel marker was first used to determine whether Rht-B1 was tightly linked with Rht-dp. Genetic mapping analyses confirmed that the
Rht-B1Indel marker completely co-segregated with Rht-dp in three RIL populations (Fig. 3).

To further confirm that Rht-B1b is located in the candidate region of Rht-dp, 190 pairs of SSR markers were exploited according to the genome reference of 4BS (Table S2). Fifteen pairs of SSR markers exhibited polymorphism between DPW and TPW, and were linked with Rht-dp in the F7 RIL population. Of them, two SSR markers, Xgpw2994.1 and Xgpw3128.1, were tightly linked with Rht-dp with a genetic distance of 0.6 cM (Fig. 3A). Xgpw2994.1 and Xgpw3128.1 were confirmed as tightly linked markers flanking Rht-dp in the F6 (Fig. 3B) and F8 (Fig. 3C) RIL populations.

Based on the gene annotation of wheat 4BS from 29.94 to 31.29 Mbp, flanked by Xgpw2994.1 and Xgpw3128.1, there were five potential genes: TraesCS4B01G042700 (encodes a teosinte branched 1 protein), TraesCS4B01G042800 (encodes an uncharacterized protein), TraesCS4B01G042900 (a RING finger protein), TraesCS4B01G043000 (EamA-like transporter family), and TraesCS4B01G043100 (Rht-B1 encodes a DELLA protein) (Fig. 3D). Except of Rht-B1, sequence difference of other four genes (primers listed in Table S4) between DPW and TPW was not found. These results implied that the candidate gene of Rht-dp should be Rht-B1b.

**Characterization of Rht-dp in F1 plants and F2 population derived from the cross of a pair of NIL**

Although the current data supported that the candidate gene of Rht-dp should be Rht-B1b, there was a discrepancy that Rht-dp is a recessive dwarfing gene resulted from the genetic analysis of F1 and F2 of DPW×TPW, but Rht-B1b is a semi-dominant dwarfing gene [6]. Additionally, the threshold of plant height separated dwarf and tall plants in F2 population was larger than the plant height of DPW. The information promoted us to do a hypothesis that the effect of Rht-dp on reducing plant height was probably influenced by one or more non-allelic loci derived from TPW. To test this hypothesis, a QTL analysis was performed on F7 RIL population using the wheat 55K SNP array. Except of a major-locus on 4BS (Rht-dp) derived from DPW caused dwarfism, a micro-locus on 5A derived from TPW heightened plant was detected (unpublished data). To further confirm the information of Rht-dp, we measured the plant height of F1 plants and F2 population derived from the cross of a pair of NIL (D_60 and T_58). The average heights of D_60 and T_58 were 93.52 ± 1.83 cm and 159.67 ± 2.72 cm, respectively; the average plant height of F1 was 123.23 ± 2.55 cm. Compared with T_58, F1 plants harbouring Rht-dp showed a reduction on plant height up to 22.82%. The plant heights of F2 plants ranged from 65 to 155 cm. According to the frequency distribution of plant height, F2 plants were separated into two groups of dwarf and tall phenotypes at 95 cm (Fig. 4A). The dwarf and tall phenotype groups included 62 and 182 lines, respectively, consistent with the expected Mendelian segregation ratio of 1:3 (X²=0.021, p>0.05). Meanwhile, the separated threshold of plant height with 95 cm was similar to the plant height of D_60 with 93.52 ± 1.83 cm. In addition, the Rht-B1 Indel marker completely co-segregated with Rht-dp in this F2 populations (Fig. 4B). Thus, these results indicated that the dwarfing gene of Rht-dp is a single semi-dominant gene, and confirmed that the candidate gene is Rht-B1b.

**Expression patterns of Rht-B1b in DPW**
To confirm that $Rht-B1b$ reduces plant height via its effects on elongation of the first and second internodes at the booting stage, the transcriptional patterns of $Rht-B1b$ were investigated in different DPW tissues at the jointing, booting, and grain-filling stages. $Rht-B1b$ was mainly expressed in the first and second internodes at the booting stage, and at dramatically higher levels in those tissues than in other tissues at the jointing, booting, and grain-filling stages (Fig. 5).

**Allelic variations of Rht-B1 in tetraploid wheat accessions**

Since $Rht-B1b$ is the candidate gene of $Rht-dp$ in DPW, the haplotypes of $Rht-B1b$ in 59 tetraploid wheat accessions were analysed. Among them, five accessions were dwarf phenotypes including two $T. turgidum$ (AS313 and AS2239), two $T. polonicum$ [AS304 (DPW) and IC12196], and one $T. durum$ (ZH2237). The 59 sequences cloned from the 59 tetraploid wheat accessions were grouped into eight types. $Rht-B1b$ was only obtained from DPW and IC12196; and $Rht-B1t$ and $Rht-B1u$ were only obtained from $T. turgidum$. subsp. dicoccon (PI191781) and $T. turgidum$. subsp. Turanicum (PI184543), respectively. Of them, five novel types (named $Rht-B1q–B1u$, respectively) were identified by comparison with $Rht-B1a$ (Fig. 6B). $Rht-B1q$ contained an S deletion at position 129 (S129); $Rht-B1r$ carried a mutation at position 30 (A30S) and an S deletion at position 129 (S129); $Rht-B1s$ contained a mutation at position 363 (P363S). $Rht-B1t$ had two mutations at positions 15 (G15R) and 363 (P363S). $Rht-B1u$ also had two mutations at positions 136 (Y136D) and 363 (P363S) (Fig. 6B).

Among these variations, $Rht-B1q$ had the highest frequency (43.9%). The frequencies of $Rht-B1a$, $Rht-B1b$, $Rht-B1h$, $Rht-B1r$, $Rht-B1s$, $Rht-B1t$, and $Rht-B1u$ were 15.3%, 3.4%, 13.6%, 13.6%, 6.8%, 1.7%, and 1.7%, respectively.

**Dwarfism-related DEGs induced by DELLA mutant Rht-B1b**

To understand the molecular networks of $Rht-B1b$, the DEGs induced by the DELLA mutation $Rht-B1b$ in the first internode of two pairs of NILs were investigated. A total of 41 DEGs was obtained, 30 of which were successfully functionally annotated (Table S3). Twenty-eight DEGs were further classed into five sub-groups; hormone-related signalling transduction genes, transcription factor genes, cell wall structure-related genes, reactive oxygen-related genes, and nitrogen regulation-related genes (Table 1). Among the hormone-related signal transduction genes, two brassinolide (BR) signal-related genes serine carboxypeptidase II-3 ($SCP$) and cytochrome $P450$ 710A1 ($CYP450$) were down-regulated; and genes encoding salicylic acid (SA)-binding protein 2 and ARP were up-regulated in the dwarf phenotype. The only down-regulated transcription factor gene was $MybAS2$. Fifteen DEGs were grouped into cell wall structure-related genes (seven pectin-related genes and eight xylan acetylation-related genes). In the dwarf phenotype, five pectin-related genes [encoding a pectate lyase 15 (PEL15), three subtilisin-like protease (SBT1.7), and an alpha-galactosidase (α-Gal)] involved in pectin modification were down-regulated; while all eight xylan acetylation-related genes, including three $GDSL$ esterase/lipase genes, two $ESKIMO$ genes, $IRX15-L$, ALTERED XYLOGLUCAN 4-like ($AXY-L$), and an uncharacterized acetyltransferase gene were up-regulated. For the reactive oxygen-related genes, plant cysteine oxidase 2 ($PCO2$) and ASCO were down-regulated; and genes encoding germin-like protein 5-1 (GLP) and blue copper protein (BCP)
were up-regulated in the dwarf phenotype. For nitrogen assimilation-related genes, two phosphoenolpyruvate carboxylase kinase 2 (PPCK2) genes and early nodulin (ENOD) were down-regulated; and asparagine synthetase (APS) was up-regulated in the dwarf phenotype. We verified the expression of ARP and ASCO in the first and second internodes at the booting stage (Fig. S2).

Discussion

The GA-insensitive dwarfing gene Rht-B1b is the predominant source of the semi-dwarf growth habit of wheat plants grown in parts of Northern Europe [36], the Mid and Lower Yangtze Valley Autumn-sown Spring Wheat Region in China [37], and the Great Plains Hard Winter Wheat Region in the USA [38]. Because Rht-B1b significantly decreases plant height to reduce plant lodging and increase wheat yield [37, 39], it has been introduced into tetraploid wheat T. durum for dwarf breeding [28]. However, it is well known that the progenitor of T. polonicum is not Norin 10 or T. durum. Additionally, DPW was originally collected from Tulufan, Xingjiang, China [23]. Thus, the dwarfing gene Rht-dp of T. polonicum cannot be derived from Norin 10 or T. durum. However, our results show that the candidate gene Rht-dp of DPW is Rht-B1b. This conclusion is supported by the following evidences: (1) Rht-dp is a single semi-dominant dwarfing gene, as is Rht-B1b [6]. (2) Rht-dp and Rht-B1b reduce plant height mainly via reducing the length of the first and second internodes (Fig. 1D), and their effects on reducing plant height were similarity about 22% [18, 39]. (3) The sequence of Rht-B1 of DPW is the same as that of Rht-B1b (Fig. 2). (4) Mapping work revealed that the candidate region of Rht-dp was between SSR markers Xgpw2994.1 and Xgpw3128.1 (Fig. 3B-D). This region contains five potential genes including Rht-B1 (Fig. 3E); except of Rht-B1, other four genes have not sequence difference between DPW and TPW. The Rht-B1 Indel marker developed based on the sequence difference of Rht-B1 between DPW and TPW is completely co-segregated with Rht-dp in three RIL populations (Fig. 3). In our haplotype analysis, Rht-B1b was only obtained from T. polonicum (Fig. 6A), implying that it might be originated from this species.

In wheat, Rht-B1b encodes a DELLla mutant protein resembling the SLRL1 protein. Its accumulation represses GA-regulated growth and developmental responses and causes the typical semi-dwarf phenotype [6, 40]. DELLla not only regulates the expression of downstream genes but also interacts with DNA-binding transcription factors. Our transcript analysis identified 28 DEGs regulated by the DELLla mutant Rht-B1b involved in the processes of nitrogen assimilation, oxidation-reduction, modification of the cell wall components and structures, and hormone-related signal transduction (Table 1). However, this list of DEGs only slightly overlaps with those identified in previous studies, suggesting that the effects of DELLla on transcription depend on the species, organ, and developmental context [41-44]. Since our results indicated that Rht-B1b is mainly expressed in the first and second internodes (Fig. 5) to dramatically reduce their lengths at the booting stage in DPW (Fig. 1D), we explored the molecular network of Rht-B1b by conducting a transcript analysis of the first and second internodes at the booting stage.
The control of plant growth and development by DELLA is dependent on GA-regulated growth and developmental responses [44-46]. However, we did not find genes involved in GA metabolism among the DEGs in this study. Instead, the DEGs identified in this study included auxin-, SA- and BR-related genes (Table 1). These results suggested that GA interacts with these hormones [46]. DELLA can directly trigger the expression of auxin- and BR-related genes to affect plant growth [47-48]. For example, the expressions of SCP and CYP450 (both grouped into BR-related genes) were dramatically down-regulated by the DELLA mutation Rht-B1b to potentially cause dwarfism in DPW (Table 1), because the expression of SCP positively affects plant growth [49]. Auxin represses the expression of ARP genes [50-51]. In a previous study, overexpression of an ARP of Brassica rapa caused a reduction in vegetative growth [50]. Auxin also modulates the expression of ASCO, which encodes a crucial enzyme that produces oxidative molecules, including H\textsubscript{2}O\textsubscript{2} [52]. Overexpression of an ASCO in cotton enhanced the accumulation of H\textsubscript{2}O\textsubscript{2} and promoted cell elongation, whereas suppression of an ASCO in tobacco and Arabidopsis inhibited stem cell growth [53]. Our results show that the DELLA mutation Rht-B1b resulted in dramatically up-regulated ARP and down-regulated ASCO in DPW (Table 1). Auxin-induced growth inhibition is accompanied by decreased levels of reactive oxygen species [54]. Thus, the accumulation of the DELLA mutant protein regulated via auxin-mediated signal transduction may reduce the contents of reactive oxygen species such as H\textsubscript{2}O\textsubscript{2} [41], thereby limiting cell expansion to cause dwarfism in DPW.

In rice, over-expression of an early nodulin gene resulted in improved nitrogen-use efficiency and increased nitrogen assimilation [55]. In C\textsubscript{3} plants, nitrogen assimilation is positively correlated with phosphoenolpyruvate carboxylase (PEPC) phosphorylation [56-57], which is catalysed by phosphoenolpyruvate carboxylase kinase (PPCK). The extent of phosphorylation is largely determined by PPCK activity, which is controlled by the level of PPCK transcripts [56, 58-59]. A reduction in PEPC activity leads to serious stunting of growth [60]. Our results showed that the DELLA mutation Rht-B1b led to significant down-regulation of early nodulin and two PPCKs in DPW (Table 1). Thus, decreased nitrogen assimilation and PPCK activity may decrease the activity of PEPC [43, 59] to cause dwarfism in DPW.

The hemicellulose xylan and pectins are two abundant polysaccharides in the plant cell wall [61]. Their modifications, such as methylesterification and acetylation, have been proposed to influence cell wall architecture and function, causing various plant growth phenotypes [61-64]. Our results showed that the DELLA mutation Rht-B1b led to significant down-regulation of the expression of several pectin-related genes, including PEL, three SBTs, and α-Gal (Table 1). Decreases in the transcript levels of these genes may lead to the repression of pectin degradation and the accumulation of de-esterified pectin [63], enhanced pectin methylesterase activity to stiffen the cell wall [65], and reduced adherence of pectin to the cell wall [66]. Thus, the DELLA mutation Rht-B1b may result in modifications of pectin that limit cell wall loosening and inhibit cell elongation, thereby causing dwarfism in DPW.

Many studies have reported that either excess or inadequate acetylation of xylan disrupts the cell wall structure, thereby causing dwarfism in plants. Our results show that the DELLA mutation Rht-B1b up-regulated eight xylan acetylation-related genes, including three GDSL esterase/lipase genes, two ESKIMO genes, IRX15-L, AXY-L, and an uncharacterized acetyltransferase gene (Table 1). ESKIMO and AXY-L are
xylan acetyltransferases, and IRX-L is involved in synthesis of the xylan backbone [61, 69-70]. A specific interaction between acetyltransferases and xylan backbone biosynthetic enzymes may repress acetylation of adjacent residues [70]. Therefore, even though the transcript levels of ESKIMO, IRX15-L, AXY-L and IRX-L were up-regulated (Table 1), the acetylation of xylan may be decreased. GDSL esterase/lipase is a xylan deacetylation enzyme [64]. The DELLA mutation Rht-B1b resulted in up-regulated expression of GDSL esterase/lipase, leading to enhanced xylan deacetylation. Therefore, the DELLA mutation Rht-B1b may reduce acetylation of xylan to limit cell wall loosening and inhibit cell elongation, causing dwarfism in DPW.

A model summarizing how the DELLA mutation Rht-dp causes dwarfism in DPW is proposed (Fig. 7). Whether the DELLA mutation Rht-B1b regulates the pathway of hormones, reactive oxygen species, and nitrogen assimilation, it ultimately affects the cell wall structure to limit cell wall loosening and inhibit cell elongation, thereby causing dwarfism in DPW.

Conclusion

In summary, our results indicated that the semi-dwarfing gene Rht-dp is the “Green Revolution” gene Rht-B1b. It regulates pathways related to hormones, reactive oxygen species, and nitrogen assimilation to modify the cell wall structure, and then limits cell wall loosening and inhibits cell elongation, thereby causing dwarfism in DPW.

Abbreviations

BR, brassinolide; cM, centimorgan; DEGs, differentially expressed genes; DPW, dwarf polish wheat; FPKM, fragments per kilobase of transcript per million mapped reads; GA, gibberellin; LOD, logarithm of odds; NIL, near-isogenic line; qPCR, Quantitative real-time PCR; RIL, recombinant inbred line; RNA-seq, RNA sequencing; SA, salicylic acid; TPW, tall polish wheat.

Declarations

Ethics approval and consent to participate

The DPW and TPW lines were originally collected from Tulufan, Xinjiang province, China, by Prof. Chi Yen and Junliang Yang (Sichuan Agricultural University, China) in the 1980s. No permission was necessary to collect this sample. Professor Chi Yen undertook the formal identification of the sample. The voucher specimen and the seed are deposited in the Triticeae Research Institute, Sichuan Agricultural University, Chengdu, Sichuan, China. Collection of the dwarf Polish wheat complied with the institutional, national and international guidenlines.

Consent for publication

Not applicable.
Availability of data and materials

All data generated or analyzed during this study were included in this article and the supplementary files.

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Competing interest

The authors declare that they have no conflicts of interest.

Authors’ contributions

SC, XX, JL, YW and YZ designed the research and wrote the manuscript; SC, QY, XZ and XX performed the experiments; SC, XF, JZ, LS, HK, HZ and YW performed the data analysis and revised the manuscript.

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Table

Table 1 Dwarfism-related DEGs induced by DELLA mutant Rht-dp
| Gene ID            | Description                                           | Fold change of transcript |
|-------------------|-------------------------------------------------------|---------------------------|
|                   | D_60/T_58    | D_33/T_35    |
| Hormone-related signaling transduction genes |
| *TraesCS2B01G157100* | Serine carboxypeptidase II-3                           | -32 | -20 |
| *TraesCS3B01G167400* | Cytochrome P450 710A1                                 | -25 | -18 |
| *TraesCS2B01G471800* | Salicylic acid-binding protein 2                      | 28  | 39  |
| *TraesCS4B01G070300* | Auxin-repressed 125 kDa protein                       | 12  | 15  |
| Transcription factor |
| *TraesCS1B01G055200* | Myb-related protein MYBAS2                            | -13 | -26 |
| Cell wall structure-related genes |
| *Pectin-related genes* |
| *TraesCS2A01G016500* | Pectate lyase 15                                       | -17 | -29 |
| *TraesCS4A01G237500* | Subtilisin-like protease SBT17                        | -20 | -22 |
| *TraesCS4B01G077600* | Subtilisin-like protease SBT17                        | -29 | -17 |
| *TraesCS6A01G339400* | Subtilisin-like protease SBT17                        | -14 | -12 |
| *TraesCS6B01G332900* | Alpha-galactosidase                                   | -16 | -11 |
| *TraesCS1B01G249000* | (1-3,1-4)-beta-D-glucanase                            | 21  | 28  |
| *TraesCS2A01G341400* | Sugar transport protein 5                             | 11  | 14  |
| *Xylan acetylation-related genes* |
| *TraesCS3A01G258100* | GDSL esterase/lipase                                 | 15  | 11  |
| *TraesCS3B01G290800* | GDSL esterase/lipase                                 | 13  | 10  |
| *TraesCS7B01G250700* | GDSL esterase/lipase                                 | 13  | 23  |
| *TraesCS4A01G110000* | ESKIMO 1                                             | 14  | 10  |
| *TraesCS4B01G194100* | ESKIMO 1                                             | 17  | 10  |
| *TraesCS6A01G131900* | IRX15-like                                           | 13  | 11  |
| *TraesCS7A01G191700* | ALTERED XYLOGLUCAN 4-like                            | 17  | 11  |
| *TraesCSU01G204900*  | Uncharacterized acetyltransferase                     | 28  | 16  |
| Reactive oxygen-related genes |
| *TraesCS5A01G025200* | Plant cysteine oxidase 2                              | -11 | -15 |
**L-ascorbate oxidase homolog**  
TraesCS7A01G459400  
**Germin-like protein 5-1**  
TraesCS3A01G165500  
**Blue copper protein**  
TraesCS6A01G315800  

**Nitrogen regulation-related genes**

**Phosphoenolpyruvate carboxylase kinase 2**  
TraesCS6A01G375800  
**Phosphoenolpyruvate carboxylase kinase 2**  
TraesCS6B01G413500  
**Early nodulin-93**  
TraesCS7A01G091800  
**Asparagine synthetase**  
TraesCS3B01G385400

**Figures**

**Figure 1**
Phenotypic characterization. A: Frequency distribution of plant heights in the F2 population from DPW×TPW; B: frequency distribution of plant heights in the DPW×TPW F7 population; C: frequency distribution of plant heights in the DPW×Ailanmai F6 population; D: plant height, the lengths of spike and each internode of DPW×TPW NILs F7 at the maturate stage; E: frequency distribution of plant heights in the DPW×TPW F8 population.

Figure 2

Sequences of Rht-B1 in DPW and TPW. A: nucleotide mutations of Rht-B1 in DPW and TPW; B: amino acid mutations of Rht-B1 in DPW and TPW.
Figure 3

Fine mapping of Rht-dp. A: mapping of Rht-dp in the DPW×TPW RILs F7; B: mapping of Rht-dp in DPW×Ailanmai RILs F6; C: mapping of Rht-dp in DPW×TPW RILs F8; D: candidate genes between SSR markers Xgpw29941 and Xgpw31281.
Figure 4

Genetic analysis of the F2 population from D_60×T_58. A: Frequency distribution of plant heights in the F2 population from D_60×T_58; B: mapping of Rht-dp in the F2 population from D_60×T_58.
Figure 5

Expression patterns of Rht-dp in various wheat tissues at jointing, booting, and grain filling stages.
Figure 6

Haplotype analysis of Rht-B1 in 59 tetraploid wheat accessions. A: phylogenetic relationship of Rht-B1 alleles; B: nonsynonymous mutations in Rht-B1.
Figure 7

Molecular network model of the DELLA mutation Rht-dp in DPW.

**Supplementary Files**

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- Fig.S1.tif
