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RESEARCH ARTICLE

Dissecting pleiotropic functions of the wheat Green Revolution gene *Rht-B1b* in plant morphogenesis and yield formation

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ABSTRACT

The utilization of reduced plant height genes *Rht-B1b* and *Rht-D1b*, encoding homeologous DELLA proteins, led to the wheat Green Revolution (GR). However, the specific functions of GR genes in yield determination and the underlying regulatory mechanisms remained unknown. Here, we validated that *Rht-B1b*, as a representative of GR genes, affects plant architecture and yield component traits. Upregulation of *Rht-B1b* reduced plant height, leaf size and grain weight, but increased tiller number, tiller angle, spike number per unit area, and grain number per spike. Dynamic investigations showed that *Rht-B1b* increased spike number by improving tillering initiation rather than outgrowth, and enhanced grain number by promoting floret fertility. *Rht-B1b* reduced plant height by reducing cell size in the internodes, and reduced grain size or weight by decreasing cell number in the pericarp. Transcriptome analyses uncovered that *Rht-B1b* regulates many homologs of previously reported key genes for given traits and several putative integrators for different traits. These findings specify the pleiotropic functions of *Rht-B1b* in improving yield and provide new insights into the regulatory mechanisms underlying plant morphogenesis and yield formation.

KEY WORDS: *Triticum aestivum*, Green Revolution gene, Functional dissection, Plant architecture, Yield components

INTRODUCTION

Plant height has significant effects on lodging resistance and yield potentials in crops. The introduction of the reduced height (semi-dwarf) genes *Rht-B1b* and *Rht-D1b* in the 1960s was a crucial component of the Green Revolution (GR) in wheat (*Triticum aestivum* L.) (Hedden, 2003). *Rht-B1b* and *Rht-D1* are homoeoloci on chromosome arms 4BS and 4DS, respectively, and encode truncated DELLA proteins homologous to the gibberellin-insensitive (GAI) protein in *Arabidopsis* (Chandler et al., 2002; Ikeda et al., 2001; Peng et al., 1997, 1999). DELLA proteins have been identified as key integrators of multiple regulatory pathways that modulate plant growth and development in model plants, such as *Arabidopsis* and rice (Davière and Achard, 2016; Nolan et al., 2020; Tong and Chu, 2018; Xu et al., 2014). DELLA proteins directly bind with GID1, BZR1, SPY, EIN3, JAZ1, ARF7 and D14 in the gibberellin (GA), brassinosteroid (BR), cytokinin (CTK), ethylene (ETH), jasmonate (JA), auxin (IAA) and strigolactone (SL) signaling pathways, respectively (Nolan et al., 2020; Tong and Chu, 2018), and also interact with GRF4 and NGR5, key factors for yield and nitrogen-use efficiency in rice, shaping a module to boost a sustainable GR (Li et al., 2018c; Wu et al., 2020). The GA signaling pathway has been studied extensively to explore the function of DELLA in plant growth and development. GID2 and SLY1 are F-box proteins, components of a ubiquitin E3 ligase complex in *Arabidopsis* and rice, respectively. The binding of GA with its receptor GID1 promotes the recruitment of DELLA to the $SCF^{SLY1/GID2}$ E3 ligase complex for polyubiquitylation and ubiquitinated DELLA is then degraded by the 26S proteasome (Murase et al., 2008; Shimada et al., 2008; Sun, 2011; Wu et al., 2011). However, the truncated proteins encoded by *Rht-B1b* and *Rht-D1b* cannot be degraded owing to lack of the DELLA domain that is responsible for the binding with GID1, but they can still repress the promotion effect of GA on plant height probably through binding to GA response factors, such as homologs of phytohormone-interacting factors (PIFs) and BZR1 (de Lucas et al., 2008; Feng et al., 2008; Pearce et al., 2011; Peng et al., 1999; Wu et al., 2011; Bai et al., 2012).

It is well known that GR genes promote yield stability by reducing plant height and concomitant reduced lodging, but how they improve yield potential remains elusive. Linkage mapping and association analyses showed that GR genes confer pleiotropic effects on yield component traits, including spike number per unit area (SN), grain number per spike (GNS) and thousand grain weight (TGW) (reviewed by Cao et al., 2020). Many near-isogenic lines (NILs) have been generated to investigate further the role of GR genes in improving yield (Allan, 1989; Börner et al., 1993; Borrell et al., 1991; Flintham et al., 1997; Keyes and Sorrells, 1989; Kowalczyk et al., 1997; Lanning et al., 2012; Miralles and Slafer, 1995; Velu et al., 2017; Wu et al., 2020). However, both genetic analyses and NIL comparisons produced inconsistent and even opposite results regarding the impacts of GR genes on yield component traits. It is almost a consensus that GR genes reduce TGW (Allan, 1989; Beharav et al., 1991; Börner et al., 1993; Cabral et al., 2018; Flintham et al., 1997; Guan et al., 2018; Keyes and Sorrells, 1989; Kowalczyk et al., 1997; Miralles and Slafer, 1995; Schulthess et al., 2017; Tian et al., 2017; Velu et al., 2017; Xu et al., 2019), but no difference in TGW caused by GR genes was detected in some genetic studies (Achilli et al., 2022; Li et al., 2018a). The majority of reports showed that GR genes increased SN or tiller number (Allan, 1989; Borrell et al., 1991; Keyes and Sorrells, 1989; Wu et al., 2020), although negative or minor effects on SN were observed in several studies (Guan et al., 2018; Li et al., 2018a; Tian et al., 2017). The effects of GR genes on GNS are highly controversial; some reports

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indicated enhanced GNS (Allan, 1989; Beharav et al., 1991; Börner et al., 1993; Borrell et al., 1991; Flintham et al., 1997; Kuchel et al., 2007; Lanning et al., 2012; Liu et al., 2014; Miralles and Slafer, 1995; Schulthess et al., 2017; Velu et al., 2017), whereas others showed that GR genes could reduce GNS (Keyes and Sorrells, 1989; Kowalczyk et al., 1997; Wu et al., 2020; Zhang et al., 2013).

Transgenic technology is one of the most widely used and reliable tools to investigate gene function. The function of GR genes in regulating plant height was initially confirmed by transformation in rice (Peng et al., 1999; Wu et al., 2011) and later confirmed in wheat (Li et al., 2012; Van De Velde et al., 2021). However, their specific roles in yield formation were not dissected. There is also no molecular evidence to address the reason why GR genes improve yield potential. Here, we pinpointed the function of GR genes in modulating plant architecture and yield traits using transgenic overexpression and knockout assays. The developmental causes for the resulting phenotypic changes were also explored by microscopy and dynamic investigations. We further dissected the underlying molecular regulatory networks through multi-omics analyses.

RESULTS

Effects of *Rht-B1b* on plant architecture

Rht-B1b and *Rht-D1b* have equivalent genetic effects on agronomic traits (Eshed and Lippman, 2019; Lanning et al., 2012; Zhang et al., 2013). To clarify the function of GR genes, we chose *Rht-B1b* to create overexpression (*Rht-B1b*-OE) and knockout (*Rht-B1b*-KO) lines. Three *Rht-B1b*-OE lines and two *Rht-B1b*-KO lines with

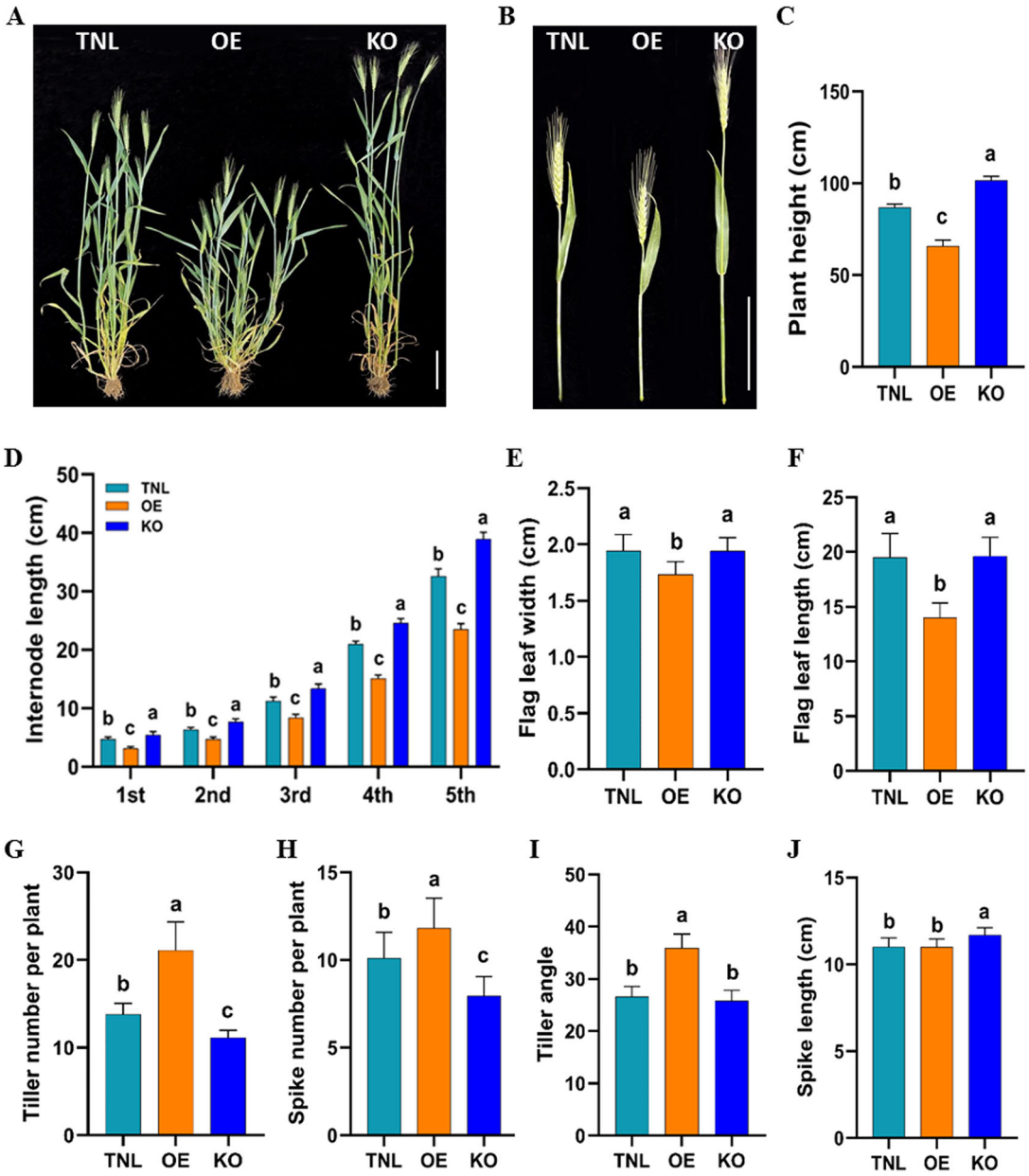

Fig. 1. Effects of *Rht-B1b* on plant architecture. (A,B) Representative images of plant architecture (A), and uppermost internodes, flag leaves and spikes (B) of *Rht-B1b* overexpression lines (OE), *Rht-B1b* knockout lines (KO) and transgenic null lines (TNL). Scale bars: 10 cm. (C-J) Statistical analyses of plant height (C), stem internode length (D), flag leaf width (E), flag leaf length (F), tiller number per plant (G), spike number per plant (H), tiller angle (I) and spike length (J) of OE, KO and TNL. Error bars represent s.d. of three biological replicates. Different letters on the bars indicate significant differences in given traits at $P<0.05$ between different lines.

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frame-shift mutation were used for subsequent analyses (Fig. S1). As expected, plant height was significantly reduced in *Rht-B1b*-OE lines and increased in *Rht-B1b*-KO lines compared with transgenic null lines (TNLs) (Fig. 1A,C). Stem internode length was also significantly reduced in *Rht-B1b*-OE lines and increased in *Rht-B1b*-KO lines (Fig. 1D). The change in uppermost internodes (peduncles) caused by *Rht-B1b* was the greatest among all stem internodes (Fig. 1B,D). Flag leaves were significantly smaller in *Rht-B1b*-OE lines than those in *Rht-B1b*-KO lines and TNLs, but there was no significant difference in flag leaf size between TNLs and *Rht-B1b*-KO lines (Fig. 1B,E,F). *Rht-B1b*-OE lines generated more tillers per plant than the TNLs, and *Rht-B1b*-KO lines had fewer tillers (Fig. 1A,G). Accordingly, overexpression of *Rht-B1b* increased earbearing tiller number per plant, i.e. spike number per plant (Fig. 1H). *Rht-B1b* overexpression also significantly increased tiller angle (Fig. 1A,I). Spikes in *Rht-B1b*-KO lines were obviously more slender than those in *Rht-B1b*-OE lines and TNLs (Fig. 1B). Although there was little difference in spike length between *Rht-B1b*-OE lines and TNLs, spike length was significantly increased in *Rht-B1b*-KO lines compared with TNLs (Fig. 1J). Overall, *Rht-B1b* had clear effects on plant height, leaf size, tiller number, tiller angle and spike length, indicating its role in modulating plant architecture.

Contributions of *Rht-B1b* to grain yield and yield component traits

To ascertain how GR genes improve yield potential, we investigated the effects of *Rht-B1b* on yield-contributing traits SN, GNS and TGW. *Rht-B1b*-OE lines had significantly increased total tiller number per m^2 and SN (herein referring to spike number per m^2) compared with TNLs, and both traits were reduced in *Rht-B1b*-KO lines (Fig. 2A,B). Nevertheless, the earbearing tillering rate was reduced in *Rht-B1b*-OE lines and enhanced in *Rht-B1b*-KO lines relative to that in TNLs (Fig. 2C). Thus, *Rht-B1b* improved SN by enhancing tillering capacity. GNS was significantly increased in *Rht-B1b*-OE lines and decreased in *Rht-B1b*-KO lines compared with TNLs (Fig. 2D). However, *Rht-B1b*-KO lines had significantly higher spikelet number per spike than TNLs, and *Rht-B1b* overexpression led to a very slight decrease in floret and spikelet number per spike, but this was not statistically significant (Fig. 2E,F). Obviously, *Rht-B1b* increased GNS by enhancing floret fertility.

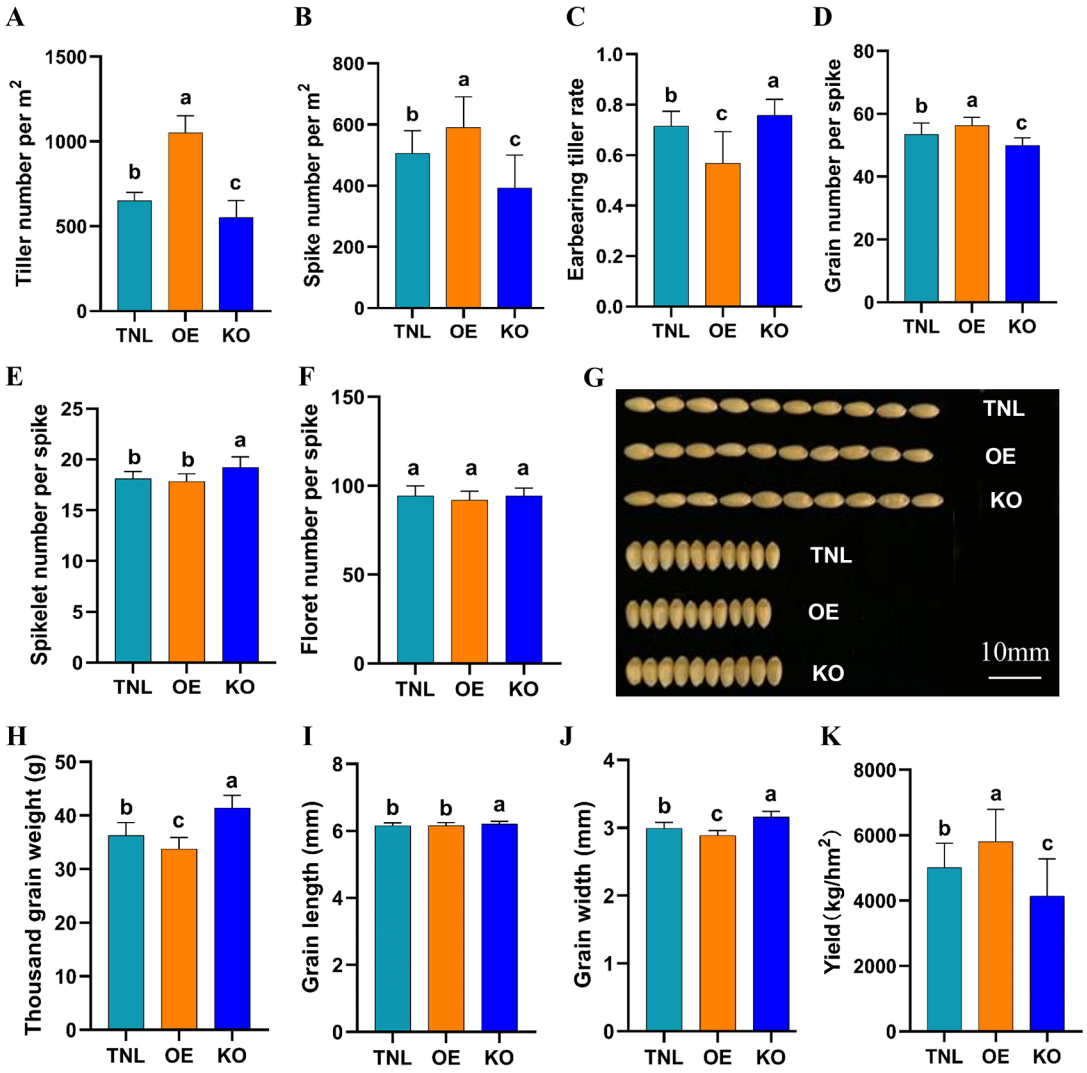

Fig. 2. Impacts of *Rht-B1b* on yield-contributing traits. (A-F,H-K) Statistical analyses for tiller number per m^2 (A), spike number per m^2 (B), earbearing tillering rate (C), grain number per spike (D), spikelet number per spike (E), floret number per spike (F), thousand grain weight (H), grain length (I), grain width (J) and yield (K) of *Rht-B1b* overexpression lines (OE), *Rht-B1b* knockout lines (KO) and transgenic null lines (TNLs). Error bars indicate s.d. of three biological replications. Different letters represent significant differences (P<0.05) in target traits between different lines. (G) Grain morphology of OE, KO and TNL.

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TGW was lower in *Rht-B1b*-OE lines and higher in *Rht-B1b*-KO lines than in TNLs (Fig. 2H). Considering that grain size is a major determinant of grain weight, we investigated grain length (GL) and grain width (GW) (Fig. 2G). GL was significantly increased in *Rht-B1b*-KO lines compared with TNLs (Fig. 2I). Likewise, grain width was less in *Rht-B1b*-OE lines and greater in *Rht-B1b*-KO lines (Fig. 2J). Thus, we conclude that *Rht-B1b* reduces TGW largely by modulation of grain size. Most importantly, grain yield was significantly enhanced in *Rht-B1b*-OE lines and reduced in *Rht-B1b*-KO lines compared with TNLs (Fig. 2K), indicating that *Rht-B1b* has an overall positive effect on yield-contributing traits. Enhanced SN and GNS were apparently sufficient to compensate for the reduction in grain weight.

Micromorphological investigations of the effect of *Rht-B1b* on plant architecture and yield-contributing traits

Histochemical staining showed that cell length and width in the internodes were significantly reduced in the *Rht-B1b*-OE lines and increased in the *Rht-B1b*-KO lines compared with TNLs, suggesting that *Rht-B1b* functions as a repressor of cell growth and causes a concomitant reduction in plant height (Fig. 3A). Considering that *Rht-B1b* reduced grain size, we also investigated the cell size of the grain pericarp. Electron microscopy showed that *Rht-B1b*-OE and *Rht-B1b*-KO lines were not different from TNLs in this respect (Fig. 3B). As expected, *Rht-B1b* had little effect on cell number per area in the grain pericarp (Fig. 3B). *Rht-B1b* is a negative regulator of grain size (Fig. 2G,I,J). Further investigation showed that the enhanced expression of *Rht-B1b* reduced the area of grain pericarp and caused a concomitant decrease in cell number (Fig. 3B). *Rht-B1b* reduces plant height by diminishing cell size in the internodes, and reduces grain size by decreasing cell number in the pericarp, suggesting that it modulates plant height and grain size through different pathways.

Given the large effects of *Rht-B1b* on tiller number, we investigated tiller initiation and outgrowth at the seedling stage. *Rht-B1b*-OE lines had more tiller buds than the TNLs, and the *Rht-B1b*-KO lines had fewer (Fig. 3C). Conversely, enhanced expression of *Rht-B1b* repressed tiller outgrowth, accounting for a lower rate of earbearing tillers in *Rht-B1b*-OE lines (Fig. 2C). We also investigated developing spikes and observed that there was little difference in spikelet number among the *Rht-B1b*-OE and *Rht-B1b*-KO lines and TNLs at the early stage of spike development (Fig. 3D). However, there were smaller spikelets at the top of the spikes in *Rht-B1b*-OE lines owing to retarded growth at the late stage of spike development, explaining why *Rht-B1b* reduced spikelet and floret number per spike (Fig. 3D). The *Rht-B1b*-OE lines had fewer underdeveloped sterile florets than the TNLs and the *Rht-B1b*-KO lines (Fig. 3E). We also compared pollen fertility in the normal florets and observed little difference in pollen staining among the three groups (Fig. 3F). Thus, *Rht-B1b* enhances floret fertility by improving floral development.

Transcriptome dissection of the molecular mechanisms underlying the effect of *Rht-B1b* on plant architecture and grain yield

Transcriptome analysis, which detects differentially expressed genes (DEGs) in key spatial-temporal windows, is an effective way to uncover regulatory pathways or networks underpinning genes of interest (Wang et al., 2009). To mine genes downstream of *Rht-B1b*, we compared transcriptome profiles between the *Rht-B1b*-OE lines and TNLs. Considering that plant height and grain yield are the traits most affected by the GR genes, we focused on dissection of the regulatory mechanisms controlling stem elongation, tillering, spike development and grain weight using RNA sequencing (RNA-Seq) assays.

Plant height

As the jointing and heading stages (from Feekes 6 to 10.5) are the most determinant spatiotemporal windows for plant height, the transcriptome of stems at Feekes 7 (second internode visible) and uppermost internodes at Feekes 10.1 (spike visible) were used to mine key genes downstream of *Rht-B1b*. In total, 36/30 and 599/411 DEGs were upregulated or downregulated by *Rht-B1b* overexpression at Feekes 7 and Feekes 10.1, respectively (Tables S1 and S2). Very few common DEGs were detected between these two groups of RNA-Seq data, indicating strong spatiotemporal specificity of the regulatory mechanism underlying *Rht-B1b* modulation of stem development. Several DEGs were homologs of *TCP14*, *OsMADS57*, *GID1c*, *OsPIL1*, *BRI1*, *OsYABBY4*, *HOX12*, *OsDWARF* and *OsDWARF10*, each of which have been reported to regulate plant height (Fig. 4A; Tables S1 and S2). *TCP14* in *Arabidopsis* increases internode length by promoting cell division in response to auxin (Ferrero et al., 2021; Kieffer et al., 2011) and its homologs were downregulated by *Rht-B1b* at Feekes 10.1 (Fig. 4A; Table S2). *OsMADS57* promotes plant height by repressing GA 2-oxidase genes (Chu et al., 2019). *Rht-B1b* overexpression downregulated a *MADS57*-like gene at Feekes 10.1 (Fig. 4A; Table S2). HOX12 in rice negatively regulates plant height (Gao et al., 2016) and one of its homologs is upregulated by *Rht-B1b* (Fig. 4A; Table S2). Three *GA 2-oxidase* (*GA2ox*) genes were upregulated by *Rht-B1b* (Fig. 4A; Table S2). The GA-GID1 module induces DELLA degradation in model plants (Murase et al., 2008; Shimada et al., 2008). A homolog of *GID1c* was differentially expressed between *Rht-B1b*-OE lines and TNLs (Fig. 4A; Table S2). These results indicate that *Rht-B1b* could modulate plant height through a feedback regulatory loop of GA homeostasis in stem internodes. In addition, *Rht-B1b* overexpression downregulated a homolog of *OsPIL1*, a key regulator of internode elongation (Fig. 4A; Table S2) (Todaka et al., 2012). *BRI1* functions as a receptor of BR to promote plant height (Montoya et al., 2002), and its homologs were downregulated by *Rht-B1b* overexpression (Fig. 4A; Table S2). *OsYABBY4* is a negative regulator of plant height (Yang et al., 2016), but one of its homologs was downregulated by *Rht-B1b* overexpression at Feekes 7 (Fig. 4A; Table S2). Likewise, *DWARF10* and *OsDWARF* are positive regulators of plant height (Arite et al., 2007; Hong et al., 2002), but their homologs were upregulated by *Rht-B1b* overexpression (Fig. 4A; Table S2). The contrasting expression patterns of such genes showed that they had been subjected to functional differentiation between wheat and rice. In addition to GA, other hormone-related DEGs included *9-cis-epoxycarotenoid dioxygenase* (NCED) genes for ABA synthesis (Tan et al., 1997) and *abscisic acid 8'-hydroxylase* (*CYP707A*) genes for ABA degradation (Yang and Choi, 2006), ABA receptor *PYL4*, *PP2C* and *ABF* genes for ABA signaling (Choi et al., 2000; Hao et al., 2011), ethylene receptor *OsERF3* (Wuriyanghan et al., 2009) and *ACC oxidase* genes (Qin et al., 2007) for ETH, *SAUR* genes (Zhou et al., 2013) for auxin, and *OsCKX11-like* genes (Zhang et al., 2021) for cytokinin (Fig. 4A; Table S2). A group of DEGs possibly involved in cell division and growth was also identified, including genes encoding CDC48c and expansins (Fig. 4A; Table S2) (Choi et al., 2003; Rancour et al., 2002). Based on the putative functions of DEGs, we propose a simplified regulatory model underpinning *Rht-B1b* reducing plant height (Fig. 4B).

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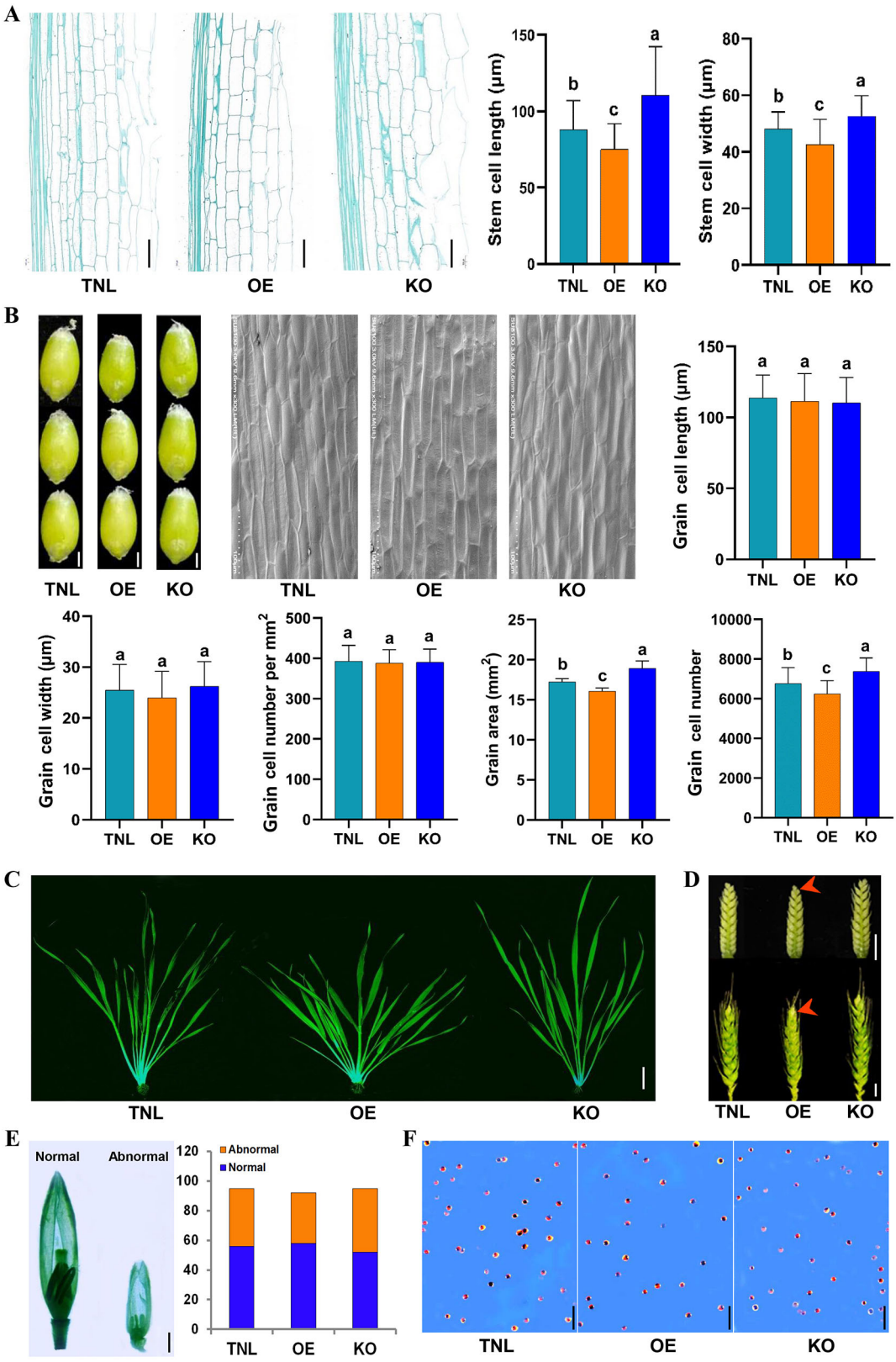

Fig. 3. Micromorphological investigation of the effects of *Rht-B1b* on plant height, grain size, tillering and spike development. (A) Comparisons of the cell size of stem internodes among *Rht-B1b* overexpression lines (OE), *Rht-B1b* knockout lines (KO) and transgenic null lines (TNL). Scale bars: 80 μm. (B) Comparisons of the cell size and number of seed coats among OE, KO and TNL. The images show developing grains and their coat cells. Scale bars: 1 mm. (C) Comparisons of tiller initiation and outgrowths among OE, KO and TNL during the tillering phase. Scale bar: 2 cm (D) Comparisons of young spikes among OE and KO and TNL. Red arrowheads show underdeveloped spikelets. Scale bars: 1 cm. (E) Phenotypic display and score of normal and abnormal florets per spike in OE, KO and TNL. Scale bar: 1 mm. (F) Pollen fertility assessment by iodine-potassium iodide (I_2-KI) staining. The stained pollens in blue are fertile. Scale bars: 200 μm. In A,B, the error bars represent s.d. of three biological replicates. Different letters represent significant differences (P<0.05) in the target trait between different lines.

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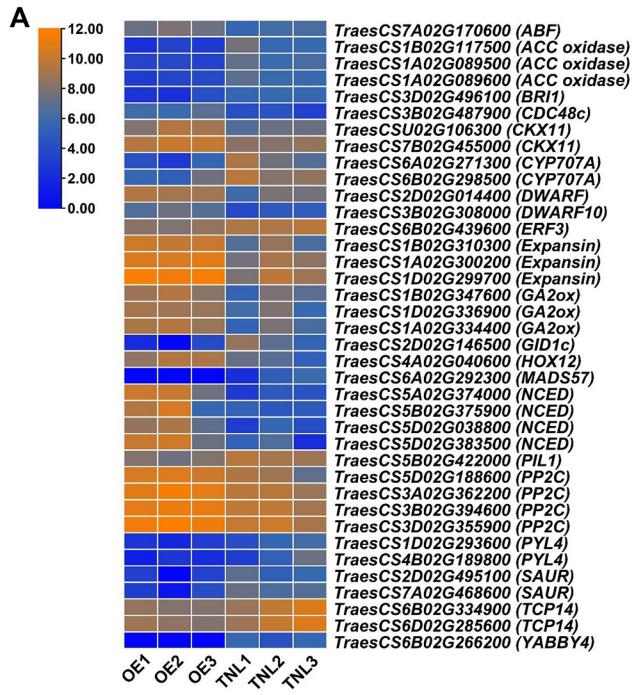

Fig. 4. Differentially expressed genes between *Rht-B1b* overexpressing lines (OE1-3) and their transgenic null lines (TNL1-3) in stem internodes based on transcriptome analysis. (A) Putative key downstream genes of *Rht-B1b*. The names of homologous genes are shown in brackets. These genes are differentially expressed between *Rht-B1b*-OE lines and TNLs according to transciriptome analysis and their homologs may be involved in modulating plant height. (B) A simplified model of the mechanism underpinning *Rht-B1b* modulation of plant height (B). Black letters in the boxes show non-differentially expressed genes; orange and blue letters show genes upregulated and downregulated by *Rht-B1b*, respectively. ABA, abscisic acid; ETH, ethylene; GA, gibberellin. Arrows show promotion of gene expression; lines with blunt ends show repression of gene expression; bold line represents direct binding. Latin prefixes of species in gene names are omitted.

Tiller initiation, outgrowth and angle

A total of 661/214 DEGs in tillers at the tillering stage (Feekes 3) were upregulated or downregulated by *Rht-B1b* overexpression (Table S3). Some homologs of *OsHOX1*, *OsHOX6*, *OsHOX12*, *OsHOX14*, *TB1*, *D2* and *D14* were differentially expressed between *Rht-B1b*-OE lines and TNLs. *OsHOX6* overexpression enhances tiller number in rice (Rahmawati et al., 2019) and its homologs were upregulated by *Rht-B1b* overexpression (Fig. 5A; Table S3). Enhanced expression of *OsHOX14* results in a dwarf phenotype (Shao et al., 2018) and *OsHOX1* has been identified as a positive regulator of tiller angle by affecting shoot gravitropism (Hu et al., 2020). *Rht-B1b* overexpression led to upregulation of the homologs of *OsHOX1* and *OsHOX14*, suggesting that these genes might mediate the function of *Rht-B1b* in repression of tiller outgrowth and increase in tiller inclination (Fig. 5A; Table S3). As such, HOX family genes as downstream targets of *Rht-B1b* play key roles in wheat tillering. Although *TaTB1* genes in chromosomes 4A, 4B and 4D repress tillering (Dixon et al., 2018) we detected no differential expression between *Rht-B1b*-OE lines and TNLs. By contrast, another three homologs of *TB1* on homoeologous group 5 chromosomes, were upregulated by *Rht-B1b* overexpression, suggesting that these three TB1-like genes could be involved in regulation of tillering (Fig. 5A; Table S3). *D14* in rice inhibits tillering and participates in the conversion of strigalactone to the bioactive form (Arite et al., 2009), but the wheat homologs of *D14* were upregulated by *Rht-B1b* overexpression (Fig. 5A; Table S3). The mutant *d2*, which has a mutation in a BR biosynthetic enzyme gene encoding cytochrome P450, has been shown to reduce tiller angle in rice (Dong et al., 2016). A homolog of *D2* was upregulated by *Rht-B1b* overexpression (Fig. 5A; Table S3). *Rht-B1b* overexpression upregulated some genes encoding GA 2-oxidases (*GA2ox*), GA 13-oxidases (*GA13ox*) and GA receptor *GID1*, showing that it could trigger self-regulation of GA metabolism and signal transduction in tillers (Fig. 5A; Table S3). We also detected other hormone-related DEGs, such as the genes encoding IAA15, IAA18 (Jain et al., 2006) and the ethylene-responsive transcription factor AIL5 (Nole-Wilson et al., 2005) (Fig. 5A; Table S3). Based on the putative key DEGs related to tillering, we present a diagram to illustrate the regulatory network of *Rht-B1b* responsible for modulation of tiller number and angle (Fig. 5B).

Spike development

Transcriptome assays to uncover the molecular mechanism of DELLA in regulating spike development, especially floret fertility, were conducted using young spikes at the booting stage (Feekes 10.0, when spikes can be seen in the swollen section of the leaf sheath below flag leaves). A total of 229/150 DEGs were upregulated or downregulated in *Rht-B1b*-OE lines compared with TNLs (Table S4). Notably, a few DEGs were homologs of genes involved in floral development, such as *CBP1*, *HEC1*, *MSP1* and *TB1*. *CBP1* is a regulator of transcription initiation in central cell-mediated pollen tube guidance in *Arabidopsis* (Li et al., 2015). *HEC1* is required for female reproductive tract development and fertility (Gremski et al., 2007). *MSP1* is required to restrict the number of cells entering into male and female sporogenesis and initiates anther wall formation in rice (Nonomura et al., 2003). *TB1* is involved in apical dominance and the formation of female inflorescences (Dixon et al., 2018; Studer et al., 2017). Some homologs of *CBP1*, *HEC1*, *MSP1* and *TB1* were upregulated by *Rht-B1b* overexpression (Fig. 6A, Table S4). A *d10* mutant has been

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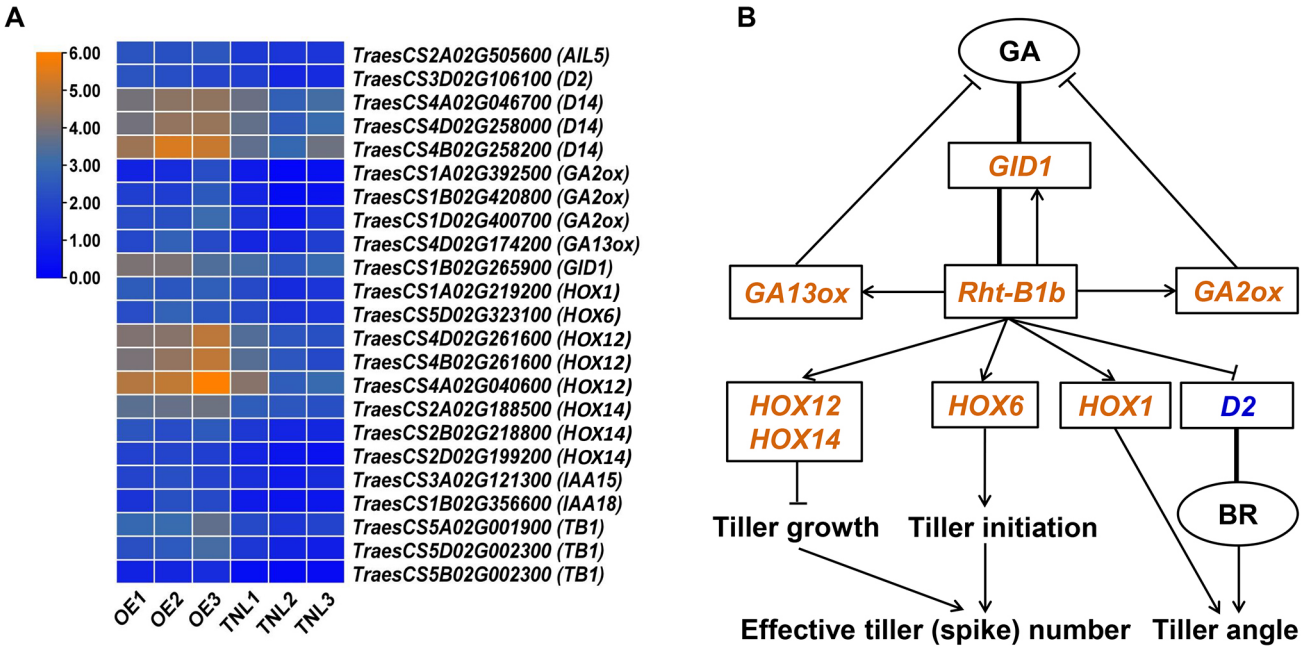

Fig. 5. Differentially expressed genes between *Rht-B1b* overexpressing lines (OE1-3) and their transgenic null lines (TNL1-3) in tiller buds internodes based on transcriptome analysis. (A) Putative key downstream genes of *Rht-B1b*. The names of homologous genes are shown in brackets. These genes are differentially expressed between *Rht-B1b*-OE lines and TNLs according to transcriptome analysis and their homologs may be involved in modulating tiller initiation or growth. (B) A model of the mechanism underpinning *Rht-B1b* modulation of tillering. Orange and blue letters show genes upregulated and downregulated by *Rht-B1b*, respectively. BR, brassinolide; GA, gibberellin. Arrows show promotion of gene expression; lines with blunt ends show repression of gene expression; bold lines represent direct binding. Latin prefixes for species in gene names are not shown.

shown to exhibit enhanced branches and apical dominance in rice (Arite et al., 2007) and one of its homologs was downregulated by *Rht-B1b* overexpression (Fig. 6A). Rice *HOX12* negatively regulates panicle exsertion (Gao et al., 2016). A homolog of *HOX12* upregulated in *Rht-B1b*-OE lines likely represses spike elongation. GA signaling plays an important role in spikelet fertility in rice (Kwon and Paek, 2016). *Rht-B1b* overexpression led to upregulation of two GA 2-oxidase genes, suggesting that it could drive a feedback inhibition for GA homeostasis in young spikes (Fig. 6A; Table S4). In addition to GA-related DEGs, several DEGs encoded homologs of proteins involved in other hormone pathways, including ZmAO-2, ARF13, IAA18, SAUR32, SAUR36, SAUR72, ETR4, ACC oxidase 1, OsEIN2, ERF013, OsCKX3, OsABA8ox3 and AtAIB (Fig. 6A; Table S4). We propose a molecular regulatory model underlying *Rht-B1b* function in modulating spike growth and development (Fig. 6B).

Grain size and weight

Considering that grain filling started at 7 days after flowering (DAF) and peaked at 20 DAF, transcriptome analysis of developing grains at these stages were performed to dissect the molecular mechanism underlying the *Rht-B1b*-driven reduction in grain size and weight (Li et al., 2019; Liu et al., 2016). Totals of 1446/2047 and 965/657 DEGs were upregulated or downregulated by *Rht-B1b* overexpression in grains at 7 and 20 DAF, respectively (Tables S5 and S6). Remarkably, homologs of a few genes related to grain size and/or weight, such as *BG1*, *OsSPL13*/*GLW7*, *ABI5*, *GW6*, *OsDWARF* and *OsDWF4* were differentially expressed between *Rht-B1b*-OE lines and TNLs (Fig. 7A; Tables S5 and S6). Among them, *BG1*, *OsDWARF*, *OsSPL13* and *OsDWF4* function as promoting factors of grain size in rice and some of their wheat homologs were downregulated by *Rht-B1b* overexpression in grains at 7 DAF (Fig. 7A; Table S5) (Li et al., 2018b; Liu et al., 2015; Mori et al., 2002; Si et al., 2016). *GW6* functions as a positive regulator of grain size and weight in rice, and its homologs were repressed by *Rht-B1b* overexpression in grains at both 7 and 20 DAF (Shi et al., 2020). ABI5 has been reported to act as a negative effector of seed size in *Arabidopsis* and its homologs were upregulated by *Rht-B1b* overexpression in grains at both 7 and 20 DAF (Fig. 7A; Tables S5 and S6) (Cheng et al., 2014). DELLA, encoded by *Rht-B1b*, is well known as a repressor of the GA pathway. As expected, we detected numerous DEGs involving the GA response (Fig. 7A; Tables S5 and S6). A few homologs of the genes related to GA metabolism, such as *GA 20-oxidase* (*GA20ox*) and *GA 3-oxidase* (*GA3ox*) were differentially expressed between *Rht-B1b*-OE lines and TNLs (Fig. 7A; Tables S5 and S6) (Luo et al., 2006). DEGs related to ABA and ETH metabolism were also identified (Fig. 7A; Tables S5 and S6).

As shown in the microscopic investigation, *Rht-B1b* overexpression led to reduced grain size by decreasing cell number in the grain pericarp. Maize *CKX2* negatively regulates cell division or number by catalyzing oxidative degradation of cytokinins (Bilyeu et al., 2001) and one of its homologs was upregulated by *Rht-B1b* overexpression in grains at 7 DAF (Fig. 7A; Table S5). By contrast, *IPT* genes promote the biosynthesis of *trans*-zeatin, a member of the cytokinin family (Miyawaki et al., 2006), and one plant-specific *IPT5* gene was downregulated in grains at 7 DAF (Fig. 7A; Table S5). Some DEGs encoded cyclin-dependent kinases (CDK: CDKB1; 1 and CDKB2; 2) and Cyclin (CycB1; 1, CycB1; 5 and CycH1; 1) that participate cell proliferation (Fig. 7A; Tables S5 and S6) (Fabian et al., 2000). Many DEGs related to carbohydrate metabolism were also identified in developing grains (Fig. 7A; Tables S5 and S6). IDD14a represses starch synthesis through activation of *QQS*, a negative regulator of starch accumulation in *Arabidopsis* (Seo et al., 2011), but its homolog was downregulated by *Rht-B1b*

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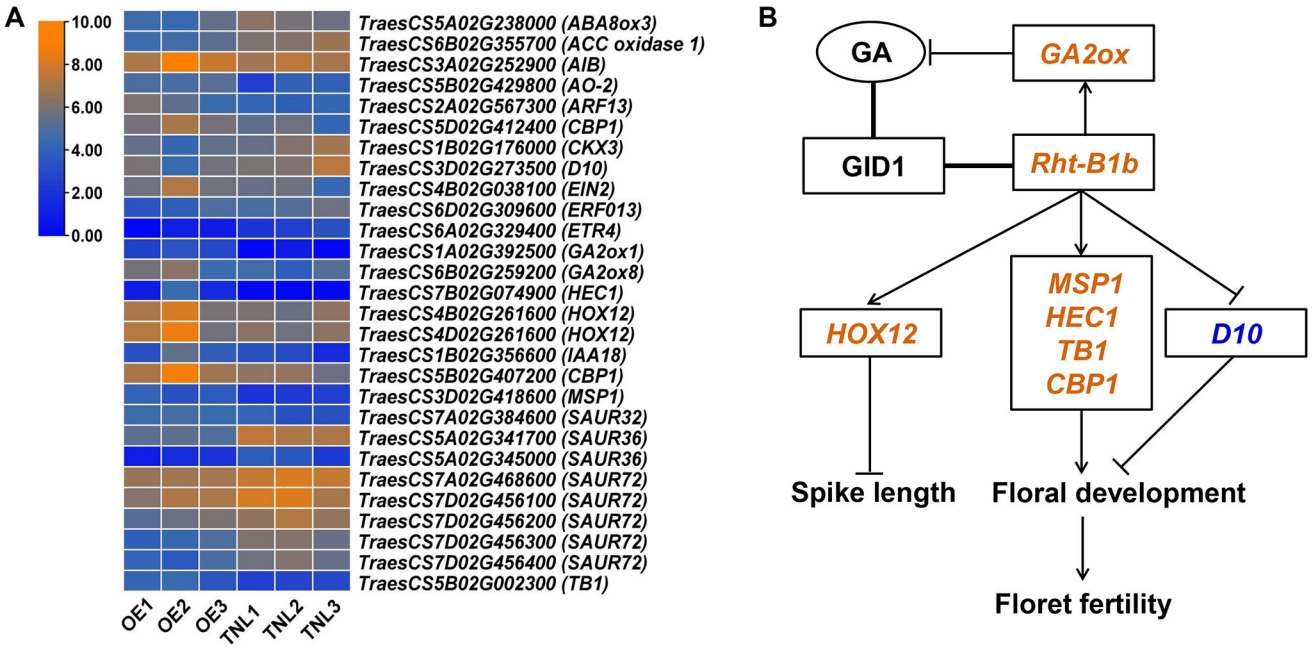

Fig. 6. Differentially expressed genes between *Rht-B1b* overexpression lines (OE1-3) and their transgenic null lines (TNL1-3) in young spikes based on transcriptome analysis. (A) Putative key downstream genes. The names of homologous genes are shown in brackets. These genes are differentially expressed between *Rht-B1b*-OE lines and TNLs according to transciriptome analysis and their homologs may be involved in modulating spike or floret development. (B) A simplified model of the mechanism underpinning *Rht-B1b* modulation of floret fertility and spike length. Black letters in the boxes show non-differentially expressed genes; orange and blue letters represent the genes upregulated and downregulated by *Rht-B1b*, respectively. GA, gibberellin. Arrows indicate promotion of gene expression; lines with blunt ends indicate repression of gene expression; bold lines represent direct binding. Latin prefixes of species in gene names are omitted.

overexpression (Fig. 7A; Table S6). Based on the above information, we present a molecular regulatory model underlying *Rht-B1b* modulation of grain size and weight (Fig. 7B).

Conjoint metabolome and transcriptome analysis of the effect of *Rht-B1b* on grain development

Grain metabolites were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) to decipher the underlying mechanism of *Rht-B1b* function in modulation of grain development. As in RNA-Seq assays, developing grains at 7 and 20 DAF were used for analysis. Only two metabolites, adenosine 5′-monophosphate (AMP) and guanosine-5′-monophosphate (GMP), had significantly different abundance between *Rht-B1b*-OE lines and TNLs in grains at 7 DAF, and were not involved in energy metabolism (Table S7). *Trans*-zeatin induces cell division and is formed from AMP and dimethylallyl pyrophosphate (Kakimoto, 2003). Some homologs of CKX1 and IPT5, key regulators of cytokinin metabolism, were detected in the transcriptome assays (Fig. 7A; Table S5). In the metabolome of grains at 20 DAF, 239/124 metabolites were upregulated or downregulated, respectively, in *Rht-B1b*-OE lines compared with TNLs (Table S8). *Rht-B1b*-OE lines had lower AMP abundance than did TNLs, showing that AMP was also a target metabolite of *Rht-B1b* in grains at 20 DAF (Table S8). Six DEGs involved in cytokinin metabolism or signal transduction were detected in the transcriptome assays (Fig. 7A; Table S6). GA homeostasis is tightly regulated by activation of GA 20-oxidases and GA 3-oxidases, and deactivation of GA 2-oxidases (Bao et al., 2020). GA7 is a bioactive GA isoform (Tudzynski et al., 2003; Tian et al., 2022) and its content was significantly increased in *Rht-B1b*-OE lines compared with the TNLs (Table S8), consistent with the *Rht-B1b*-upregulated *GA20-oxidase* and *GA3-oxidase* genes in grains according to RNA-Seq assays (Fig. 7A; Table S6). Starch is the major component contributing to wheat grain yield, accounting for approximately 70% of seed dry weight. ADP-glucose pyrophosphorylase (AGP) uses glucose 1-phosphate and ATP as substrates to produce ADP-glucose, which is the glucose donor for starch synthesis (Smidansky et al., 2002). *Rht-B1b*-OE lines had lower ADP-D-glucose (ADP-Glc) content than TNLs (Table S8). A few DEGs involving starch metabolism were also identified by transcriptome analyses (Fig. 7A; Table S6). Collectively, AMP, GA7 and ADP-Glc are likely key metabolites in regulating grain size and weight.

DISCUSSION

Dissection of phenotypic causes underpinning the increase in yield potential induced by GR genes

The introduction of *Rht-B1b* and *Rht-D1b* caused considerable increases in yield stability and potential and led to the wheat Green Revolution. However, the underlying mechanisms of GR genes in improving yield remained unknown. Here, we undertook a comprehensive investigation of the effects of *Rht-B1b* on agronomic traits and specified its function in modulating plant architecture and yield component traits. *Rht-B1b* significantly increases the number of effective tillers (fertile spikes) through improved tiller initiation. Several putative key genes for tillering capability function downstream of *Rht-B1b* (Fig. 5). We found that *Rht-B1b* overexpression increased GNS by enhancing floret fertility. A recent study showed that DELLA promotes floret fertility by interacting with OsMS188 in rice (Jin et al., 2022). We identified a group of DEGs involved in floret fertility in developing spikes between *Rht-B1b*-OE lines and TNLs by transcriptome assays. In terms of grain weight, *Rht-B1b* overexpression significantly decreased TGW, which was adverse to yield improvement. Thus, *Rht-B1b* enhances yield potential by increasing SN and GNS.

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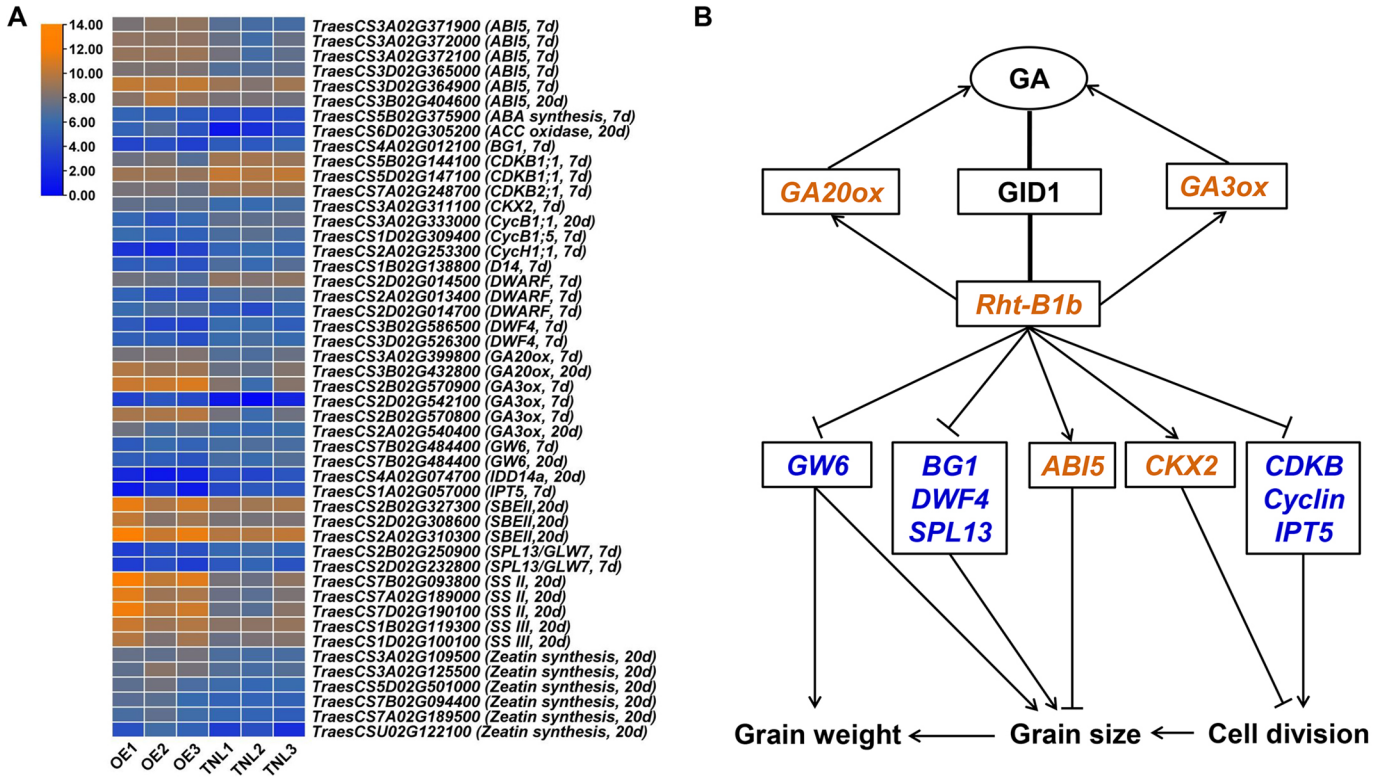

Fig. 7. Differentially expressed genes between *Rht-B1b* overexpressing lines (OE1-3) and their transgenic null lines (TNL1-3) in developing grains based on transcriptome analysis. (A) Putative key downstream genes of *Rht-B1b*. The names of homologous genes are shown in brackets. These genes are differentially expressed between *Rht-B1b*-OE lines and TNLs according to transciriptome analysis and their homologs may be involved in modulating grain development. (B) A model for *Rht-B1b* modulation of grain development. Black letters in the boxes show non-differentially expressed genes; orange and blue letters show the genes upregulated and downregulated by *Rht-B1b*, respectively. GA, gibberellin. Arrows show promotion of gene expression; lines with blunt ends show repression of gene expression; bold lines represent direct binding. Latin prefixes of species in gene names are not shown.

Although *Rht-B1b* promotes tiller bud initiation, it represses tiller outgrowth and consequently reduces ear-bearing tiller rate. Many studies have shown that nitrogen concentration has significant effects on tiller bud growth but does not influence tiller bud initiation (Huang et al., 2021; Lin et al., 2005). Appropriate increases in nitrogen fertilizer are necessary for the wheat varieties carrying GR genes to enhance earbearing tillers. We observed that *Rht-B1b* reduced grain size but had little effect on cell size in the pericarp, suggesting that *Rht-B1b* caused a decreased cell number and a concomitant reduction in grain size. By contrast, *Rht-B1b* decreased plant height by reducing cell size in stem internodes. Transcriptome analyses showed a large difference in the regulatory pathways underpinning the effect of *Rht-B1b* on plant height and grain size (Figs 4 and 7). Thus, *Rht-B1b* modulates plant height and grain size or weight through different pathways, providing useful cues to overcome the adverse effect of *Rht-B1b* on grain size and weight by modifying the downstream targets.

Integration of regulatory networks underlying the effect of GR genes in modulating different traits

We probed the molecular mechanisms underlying pleiotropic effects of *Rht-B1b* on agronomic traits. *Rht-B1b* regulated many homologs of key genes that have been functionally validated to control target traits (Figs 4–7). Numerous genes modulating different traits downstream of *Rht-B1b* were also identified. Most of the previously identified key genes have been not reported to work downstream of DELLA-encoding genes, so the resultant transcriptome information is also very helpful to broaden the regulatory networks underlying plant architecture and yield formation in other plants. Homologs of *OsHOX12* were regulated by *Rht-B1b* in tillers, stem internodes and young spikes, suggesting that they acted as integrators to orchestrate the growth and development of these organs (Figs 4 and 5) (Hu et al., 2020; Shao et al., 2018). Homologs of *OsD14*, a key regulator of plant architecture and yield (Arite et al., 2009; Zhou et al., 2013), were differentially expressed between *Rht-B1b*-OE lines and TNLs in tillers and developing grains (Figs 5 and 7). *TaD14-4D*, reported to be involved in strigolactone signaling, is associated with yield-contributing traits in wheat (Liu et al., 2021). *OsDWARF*, encoding a brassinosteroid biosynthetic enzyme, promotes elongation of leaves and stems in rice (Hong et al., 2002) and its homologs were differentially expressed between *Rht-B1b*-OE lines and TNLs in stem internodes and developing grains (Figs 4 and 7). GR genes encode truncated DELLA proteins, which still function in GA signal transduction but avoid GA-induced degradation because of an inability to interact with the GA receptor GID1 (Murase et al., 2008; Shimada et al., 2008). We observed several DEGs between *Rht-B1b*-OE lines and TNLs encoding GA-responsive proteins and GA metabolism enzymes in all investigated organs (stem internodes, tillers, young spikes and developing grains). These results suggest that *Rht-B1b*-modulated spatiotemporal GA homeostasis and signal transduction is a major cause of the resulting phenotypic variation. The above DEGs downstream of *Rht-B1b* were detected in multiple tissues, so they probably function in both plant morphogenesis and yield formation. As such, the DEGs homologous to *OsHOX12*, *OsD14* and *OsDWARF* as well as

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the genes involved in GA metabolism and GA signal transduction are likely more important regulators of plant architecture and yield component traits than the others. Overall, the findings provide informative gene resources for synergistic improvement of wheat yield component traits.

MATERIALS AND METHODS

Transgenic experiments

Fielder, a wheat cultivar with *Rht-B1b* (a dwarfing allele at the *RHT-B1*) and *Rht-D1a* (wild-type allele at *RHT-D1*), was used as a transgenic recipient. The complete coding sequence (CDS) of *Rht-B1b* (GenBank: MG681100.1) was cloned into the entry vector pDONR207 and then transferred into the destination vector pUbiGW by the Gateway cloning method (Xie et al., 2021). The resultant construct was transformed into immature embryos to generate overexpression lines of *Rht-B1b* by the *Agrobacterium tumefaciens* (strain EHA105)-mediated method (Ishida et al., 2015). The *Rht-B1b*-OE lines were identified by genomic PCR. The expression levels of target genes in *Rht-B1b*-OE lines were also validated by qPCR assays using the primers and the procedure reported by Xu et al. (2019). CRISPR/SpCas9 was used to create knockout lines of *Rht-B1b*. The single guide RNA (sgRNA) (PAM-guide sequence 5′-GGAGCCGTTCATGCTGCAG-3′) was designed to target conserved regions of *Rht-B1b* and cloned into the vector pWMBX110-SpCas9 (Liu et al., 2020). Genetic transformation was performed by infecting immature embryos of Fielder with *Agrobacterium tumefaciens* strain EHA105 carrying the destination constructs. The resultant mutant lines were identified by genomic and sequencing. Considering that *Rht-A1* and *Rht-D1* have highly similar sequences to *Rht-B1*, their corresponding regions with the sgRNA-targeting site of *Rht-B1* were also isolated and sequenced as above to determine whether there were off-target events (Fig. S1A). Primers for vector construction and transgenic plant identification are listed in Table S9.

Plant material and phenotype evaluation

Rht-B1b overexpression and knockout lines were grown in the field at Changping district in Beijing (40°2′N, 116°2′W) during the 2021 crop season and Jinan in Shandong province during the 2022 crop season. They were sown in 1.5-m rows with three replications in February and were harvested in June.

Plant height was measured from the ground to spike (awns excluded) at the grain fill stage. Tiller and spike number per plant, SN (spike number per m^2), spike length (awns excluded), floret and spikelet number per spike, and flag leaf size were also investigated. Tiller number per plant was counted at the heading stage. Tiller angle was defined following the method described by Zhao et al. (2020). GNS was deduced from measures on 30 spikes. TGW, grain length and grain width were scored with a Wanshen SC-G seed detector (Hangzhou Wanshen Detection Technology Co.). Analysis of variance (ANOVA) was performed using SAS 9.2 software.

Microscopic imaging

Considering that the uppermost internodes is the most important determinant for the difference in plant height between among *Rht-B1b*-OE and *Rht-B1b*-OE lines and TNLs (Fig. 1D), we used the internodes to image their cells in order to uncover the micromorphological cause. The uppermost internodes of the *Rht-B1b*-OE and *Rht-B1b*-KO lines and TNLs at the heading stage were fixed in FAA solution (70% alcohol, 5% acetic acid and 0.02% formaldehyde), embedded in paraffin, longitudinally sectioned and stained with Saffron-solid Green dye (G1053, Servicebio). The stem internode cells were imaged with a digital Panoramic MIDI scanner (3DHISTECH), and cell length and width were scored using ImageJ (https://imagej.nih.gov/ij/index.html). The area of grain coats (harvested at 20 days after flowering) was scored with a Wanshen SC-G seed detector (Hangzhou Wanshen Detection Technology Co.). Developing florets were photographed using a stereo microscope M205C (Leica Microsystems). Pollen grains were stained using 1% iodine-potassium iodide (I2-KI) solution and imaged using an SMZ800N Nikon stereomicroscope. Fresh grains of the *Rht-B1b*-OE and *Rht-B1b*-KO lines and TNLs at 14 DAF were harvested and the grain pericarps were imaged with an S-3400 scanning electron microscope (Hitachi). Cell length was measured in a minimum of 100 cells per sample using ImageJ. Three biological replicates per sample were used for each experiment.

RNA-Seq assays

The *Rht-B1b*-OE lines and TNLs were used for RNA-Seq experiments. Total RNA was extracted from tiller buds at tillering, stem internodes at Feekes 7, uppermost internodes at Feekes 10.1, spikes at Feekes 10.0, and grains at 7 and 20 DAF. The mRNA was purified from total RNA using poly-T oligo-attached magnetic beads (S1419S, NEB). RNA extraction and library preparation were conducted by Novogene (http://www.novogene.com/).

Raw reads were first filtered through Trimmomatic 0.38 to get clean reads. Reference genome and gene model annotation files were downloaded from the EnsemblPlants database (https://plants.ensembl.org/Triticum_aestivum/Info/Index). Clean reads were aligned to the reference genome using HISAT2 (v2.0.5) and read counts were calculated using FeatureCounts v1.5.0. Differential expression analyses of contrasting lines were performed using the DESeq2 R package (1.16.1). Genes were assigned as DEGs with an adjusted *P*-value <0.05 and $|\log_2(\text{FoldChange})| \geq 1$. Heatmaps were produced to display expression patterns of DEGs using TBtools (Chen et al., 2020).

Grain metabolome analyses

Grains of the *Rht-B1b*-OE lines and TNLs were collected at 7 and 20 DAF for metabolome analyses. Three grains of each line were bulked for metabolite extraction. Three biological replicates per sample were prepared. The grains were frozen in liquid nitrogen and ground to fine powder. The homogenate was resuspended with prechilled 500 µl 80% methanol and 0.1% formic acid by vortexing. Samples were incubated on ice for 5 min and then centrifuged at 15,000 rpm (20,000 ***g***) for 10 min at 4°C. The supernatants were diluted to a final concentration containing 53% methanol using ultrapure water. The samples were subsequently transferred to fresh tubes and centrifuged at 15,000 ***g*** for 20 min at 4°C. Finally, filtrates were injected into the Q Exactive HF-X LC-MS/MS system (Thermo Fisher Scientific). Grains from 7 and 20 DAF plants were used for central carbon metabolism and non-targeted metabolomics assays, respectively (Dunn et al., 2011; Want et al., 2010). Data files generated by the LC-MS/MS system were processed using the SCIEX OS (https://sciex.com/) to integrate and correct the peaks. The KEGG database was used to determine the most important biochemical metabolic and signal transduction pathways (Kanehisa and Goto, 2000).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.C.; Methodology: D.X., S.C.; Validation: D.X., Y.B., X.L., C.J., Q.H., X.T., Q.C.; Formal analysis: D.X., S.C.; Investigation: D.X., Y.B., X.L., C.J., Q.H., X.T., Q.C.; Resources: S.C.; Data curation: D.X., Y.B., W.C.; Writing - original draft: S.C.; Writing - review & editing: W.C., W.M., Z.N., X.F., Z.H., X.X.; Visualization: D.X., S.C.; Supervision: S.C.; Project administration: S.C.; Funding acquisition: D.X., S.C.

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Data availability

RNA-Seq data have been deposited in the National Center for Biotechnology Information under BioProject number PRJNA936995.

Peer review history
The peer review history is available online at https://journals.biologists.com/dev/lookup/doi/10.1242/dev.201601.reviewer-comments.pdf

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