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ORIGINAL PAPER

The molecular basis of the interaction between Brassinosteroid induced and phosphorous deficiency induced leaf inclination in rice

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Abstract

The phosphorous deficiency in arable land limits crop production globally. Plants developed a set of coordinated biochemical and developmental responses to cope with Pi deficiency during evolution. One of typical developmental responses to Pi deficiency is the induction of leaf erectness, which reduced the light capture ability and inhibited photosynthesis to conserve Pi in rice. It has been revealed that Pi deficiency induced leaf inclination by regulating the expression of BR pathway genes. However, how canonic BR signaling coordinates Pi deficiency responses in rice lamina joint development was not clear. Understanding mechanism underlying Pi-deficiency-induced leaf inclination enable us to breed new rice cultivars with increased Pi efficiency. Here we reported the molecular mechanism underlying the interaction of phosphorous deficiency-induced and BR-induced leaf inclination. We showed that BR deficiency can attenuate the leaf inclination by compromising Pi deficiency-induced *BU1* expression and that constitutively activated or repressed BR signaling resulted in the insensitivity of Pi deficiency-induced leaf inclination. Furthermore, we compared expression profile of WT and BR signaling constitutively activated or repressed transgenic plants under normal and deficient phosphorous conditions by RNA-seq analysis. Our work revealed the complexity of Pi deficiency stress-induced and BR induced leaf inclinations in rice.

Keywords Plant architecture · Leaf inclination · Lamina joint · Phosphate starvation response · BR signaling

Introduction

Plant architecture, including plant height, tiller number, tiller angle and leaf angle, is an important agronomic trait, which determines grain yield in crops (Wang et al. 2018). The plant architecture is regulated by internal developmental programs and external environmental factors (Wang et al. 2018). Phosphorus is one of the essential macronutrients in plant growth and development. Although the total abundance of phosphorus in soil is not deficient, the phosphorus that can be absorbed and utilized by plants is usually low, (Vance 2010). On the one hand, the phosphorus deficiency has become one of the important factors limiting crop yields and require the input of phosphorus fertilizer to sustain the crop yields. On the other hand, the overload phosphorus fertilizer has caused environmental concerns due to the low solubility and high chemical reactivity of phosphorus in soil. To improve the phosphate (Pi) using efficiency of crops is becoming more and more important for the sustainable agriculture development (Lopez-Arredondo et al. 2014).

To adapt to the Pi limited environment, plants have developed various mechanisms to increase effective absorption of phosphorus by roots from soil and to improve the using efficiency of phosphorus in plant during evolution (Lopez-Arredondo et al. 2014). The adaptation to the phosphorus deficiency mainly depends on the phosphorus starvation response (PSR) (Heuer et al. 2017), which can integrate the phosphorus homeostasis regulated by phosphorus level information of different parts of the plant and minimize the stress from the deficient phosphorus conditions (Bustos et al. 2010). MYB transcription factor family genes *OsPHR1*, *OsPHR2* and *OsPHR3* are core regulatory components of PSR regulation system (Guo et al. 2015). PHRs can bind to

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the P1BS (PHR1 binding site, GNATATNC) *cis*-elements of PSR genes to activate or repress their expression and regulate Pi homeostasis in plant (Wu and Wang 2008; Guo et al. 2015). The loss function of PHRs repressed the Pi starvation response and disrupted Pi homeostasis in rice (Wu and Wang 2008; Guo et al. 2015). On the contrary, when these transcription factors overexpressed in rice, for example *OsPHR2,* it would lead to Pi over-accumulation in the shoots under Pi-sufficient conditions (Wu and Wang 2008; Bustos et al. 2010). Recently, the SPX domain (SYG1/Pho81/XPR1) proteins were identified as negative regulators for PHRs and play critical roles in maintaining Pi homeostasis of plant (Lv et al. 2014; Shi et al. 2014; Wang et al. 2014). It has been found that the PSR is regulated by the delicate system of SPXs-PHRs (Wild et al. 2016). The SPXs interact with PHRs under Pi sufficient conditions, which prevents the nuclear localization of PHRs and their binding activity to P1BS element of the promoters of PSR genes under Pi deficiency conditions (Lv et al. 2014; Shi et al. 2014; Wang et al. 2014). When Pi is deficient, the SPXs cannot maintain their interaction with PHRs, which in turn leads to the nucleus enrichment of PHRs and the binding of PHRs to the P1BS element of downstream PSR genes (Lv et al. 2014; Shi et al. 2014; Wang et al. 2014). The expression of *OsSPXs* except *OsSPX4* is in response to the change of Pi conditions to maintain Pi homeostasis (Lv et al. 2014; Shi et al. 2014; Wang et al. 2014). However, the stability of SPX4 is dependent on the Pi availability. Furthermore, promoters of most SPXs genes contain P1BS element. The expression levels of these genes are unregulated in Pi deficiency conditions, which in turn inhibits the function of PHRs. Recently, the SPX protein has been indicated to be a receptor of the endogenous Pi signaling molecule InsP8 (Wild et al. 2016; Dong et al. 2019). The endogenous level of InsPs responses to the change of Pi availability in environment and modulates the interaction between SPXs and PHRs (Wild et al. 2016), which mediates the association and disassociation between SPXs and PHRs.

Phytohormones were shown to be associated with the absorption and use of phosphorus (Lin et al. 2014). Phytohormones determine the use or utilization of phosphorus in plants by regulating the development of plant roots, modulating the symbiotic relationship between plants and microbiome in soil, and coordinating the above-ground part to under-ground parts of plant (Lopez-Arredondo et al. 2014). The developmental and biochemical mechanisms mediated by phytohormones action developed during evolution are able either to inhibit the unnecessary utilization and remobilize internal phosphorus, or promote absorption of external phosphorus, which enables plants to adapt to phosphorus limited environment (Chiou and Lin 2011). The changes in phosphorus availability can alter the synthesis, perception and transport of plant hormones while the phosphorus effectiveness can directly affect the metabolism and signal conduction of plant hormones in plants (Chiou and Lin 2011) Brassinosteroid (BR) plays an important role in plant development. The working model of BR biosynthesis and signaling has been well established in the past two decades (Nolan et al. 2019). BES1/BZR1 are key transcription factors in the BR signaling pathway in plants. When BR binds to its receptor BRI1, the glycogen synthesis kinase BIN2 would be dephosphorylated and inactivated, thereby inhibiting the phosphorylation of BZR1/2. The dephosphorylated BZR1/2 would accumulate in nucleus and regulate the downstream genes transcription (Nolan et al. 2019). The plant mainly improves the absorption and utilization of available phosphorus in the surface soil by inhibiting the growth of the main roots and promoting the formation of lateral roots and root hairs. The observations of phosphorus limited induced root system defects were attenuated in *bes1/bzr2-D* or *bzr1-D* indicating that BR is involved in the regulation of the plant adaptation to the phosphorus-limited environment (Singh et al. 2014).

Leaf inclination is an important agronomic trait in rice, providing the foundation of shoot architecture and affecting grain yields in the field. BR positively regulates rice leaf inclination (Sun et al. 2015). Both the BR biosynthesis mutant *d2* and the signaling mutant *d61* showed erect leaves (Yamamuro et al. 2000; Hong et al. 2003). Other phytohormones (such as ABA) can affect leaf inclination by modulating the BR biosynthesis or signaling in rice (Li et al. 2019). BR regulates leaf erectness by affecting the abaxial sclerenchyma cell number of rice lamina joints. The *CYC U4;1* encoding U-type cyclin abundantly expresses in lamina joints. The BR regulated expression of *CYC U4;1* has been proposed to be essential step in regulating lamina joints development through controlling the abaxial sclerenchyma cell proliferation in rice (Sun et al. 2015). BR can regulate the transcriptional expression of *CYC U4;1* through BES1 to control the leaf erectness (Sun et al. 2015). Downstream of BR signaling, several transcription factors other than BZR1 has been found to be involved in the regulation cell division and elongation of lamina joint and affect the leaf inclination in rice. The HLH transcription factors BRASSINISTEROID UPREGULATED1 (BU1) (Tanaka et al. 2009), BU1-LIKE1 (BUL1) (Jang and Li 2017), and BUL1 COMPLEX1 (BC1) are able to enhance leaf inclination mainly by promoting cell elongation of lamina joint (Tanaka et al. 2009; Zhang et al. 2009a, b; Jang and Li 2017). Overexpressing *INCREASED LAMININAR INCLINATION1* (*ILI1*) and *ILI1 BINDING HLH1* (*IBH1*) in rice leads to leaf inclination (Zhang et al. 2009b). IBH1 can interact with BC1 and BUL1 and repress their function in regulating cell elongation (Jang and Li 2017). These regulatory networks of the transcription factors modulate the leaf inclination in rice. Under low-phosphorus conditions, the shoots of plants also reduce leaf size,

tiller number and leaf inclination to reduce the amount of phosphorus requirement. Recently, it has been reported that SPX1, a central regulatory element in the phosphorus signaling system, is involved in the regulation of leaf inclination in low-phosphorus stress. Under low-phosphorus conditions, SPX1 can interact with transcription factor RLI1 (Regulator of Leaf Inclination 1), thus repressing the regulation of RLI1 on the transcriptional expression of BU1 and BC1 in lamina joint cells (Ruan et al. 2018). Increasing expressions of *BU1* and *BC1* promote cell elongation of lamina joint and increase the leaf angle in rice. RLI1 positively regulates leaf angle by binding to the promoters of *BU1* and *BC1*, and activating their expressions. Pi deficiency can induce *SPX1/2* expression resulting in the elevation of SPX1/2 proteins, which can interact with RLI1 and inhibit the expressions of the downstream *BU1* and *BC1* genes. The reduction of downstream genes, such as *BU1* and *BC1* can lead to the restriction of cell elongation of lamina joint and induced leaf inclination in rice (Ruan et al. 2018).

It has been showed that the cellular basis of leaf inclination induced by BR deficiency is different from the cellular basis induced by Pi-deficiency stress. Pi-deficiency stress can inhibit both abaxial and adaxial cell divisions (Ruan et al. 2018). BR deficiency can induce abaxial cell division. Furthermore, leaf erectness induced by Pi-deficiency was mainly caused by the inhibition of lamina joint cell elongation in which leads to restricted lamina joint size. Meanwhile, exogenous BR treatments can induce leaf inclination in the plants grown under Pi-deficient condition. Therefore, the similarity and diversity of the cellular basis for leaf inclination regulated by Pi-deficiency stress and BR deficiency indicated the regulation complexity of leaf erectness in rice (Ruan et al. 2018). Here, we investigated the possible molecular basis for the difference of Pi deficiency stress-induced and BR induced leaf inclinations in rice. Better understanding of the molecular mechanism can help us develop low Pi tolerance rice varieties.

Results

BR deficiency attenuate the leaf inclination by comprimising Pi deficiency-induced *BU1* expression

Given the role of BR in the regulation of leaf inclination in rice, we tested whether Pi-deficiency can repress leaf inclination of the loss-of-function mutant *d2* and *d61* (Supplementary Fig. 1A). The overall plant morphology did not obviously change between two tested conditions (Fig. 1a). But plants grow in Pi-defecient condition showed significantly increase of leaf inclination (Fig. 1a). The PSR genes *IPS1* and *PT6* were selected as marker genes to verify the effectiveness of treatment (Secco et al. 2013). It showed that the expression levels of *IPS1* and *PT6* under Pi-deficient condition were about 1000 times higher than those under Pi-sufficient condition in wild type (WT) (Fig. 1b, c), which is consistent with previous work and verified the effectiveness of treatment conditions. The leaf angles of *d2* and *d61* were less to that of the wild type plants under Pi-sufficient condition (Fig. 1d, e). Under Pi-deficient condition, the leaf angles of *d2* and *d61* were still less than that of the wild type, similar to those under Pi-sufficient condition (Fig. 1e). However, the leaf inclination in *d2* and *d61* induced by Pi-deficiency was less than those in WT (Fig. 1e), which suggested that the disruption of BR biosynthesis or signaling can attenuate the Pi-deficiency induced leaf inclination. The Pi-deficiency induced expressions of *IPS1* and *PT6* were somewhat compromised in *d2* but not in *d61* (Fig. 1b, c). The expression of *OsDWARF4* was negatively regulated by BR pathway (Nakamura et al. 2009), while the expression of *OsBZR1* seems not subject to the negative feed-back regulation of BR pathway (Bai et al. 2007). Consistently, the disruption of BR biosynthesis or BR signaling resulted in the upregulation of *OsDWARF4* expression (Fig. 1f). The Pi-deficiency stress led to upregulation of *OsDWARF4* and *OsBZR1* in WT (Fig. 1f, g). On the contrary, the Pi-deficiency stress induced expressions of *OsDWARF4* were compromised in *d2* and *d61* (Fig. 1f). These results indicate that the Pi-deficiency stress induced the expressions of genes *OsDWARF4* which are regulated by BR require the intact BR biosynthesis or signaling. The Pi-deficiency stress induced expressions of *OsBZR1* in WT whereas Pi-deficiency stress repressed expressions of *OsBZR1* in *d2* and *d61* (Fig. 1g). Pi deficiency induced leaf inclination through repression of *RLI1* expression and in turn induced *BU1* expression (Ruan et al. 2018). The Pi deficiency repressed *RLI1* in *d2* and *d61,* which is similar to those in WT (Fig. 1h). However, the Pi deficiency induced *BU1* expression in WT but did not induce *BU1* expression in *d2* and *d61* (Fig. 1i). These results indicated that the less leaf inclination induced by Pi-deficiency in *d2* and *d61* may be partially through the disruption of the Pi deficiency-induced *BU1* expression.

Constitutively activated or repressed BR signaling resulted in the insensitivity of Pi induced leaf inclination

It has been showed that leaf inclination of rice grown under Pi-defecient condition still can be responsible to the exogenous BR application (Ruan et al. 2018). However, it is not sure whether leaf inclination in the plants with constitutively activated or repressed BR signaling be able to response to Pi-deficiency. The BR signaling specific activation of *CYC U4* in lamina joint is essential for BR induced leaf erectness in rice (Sun et al. 2015). We tested whether Pi-deficiency

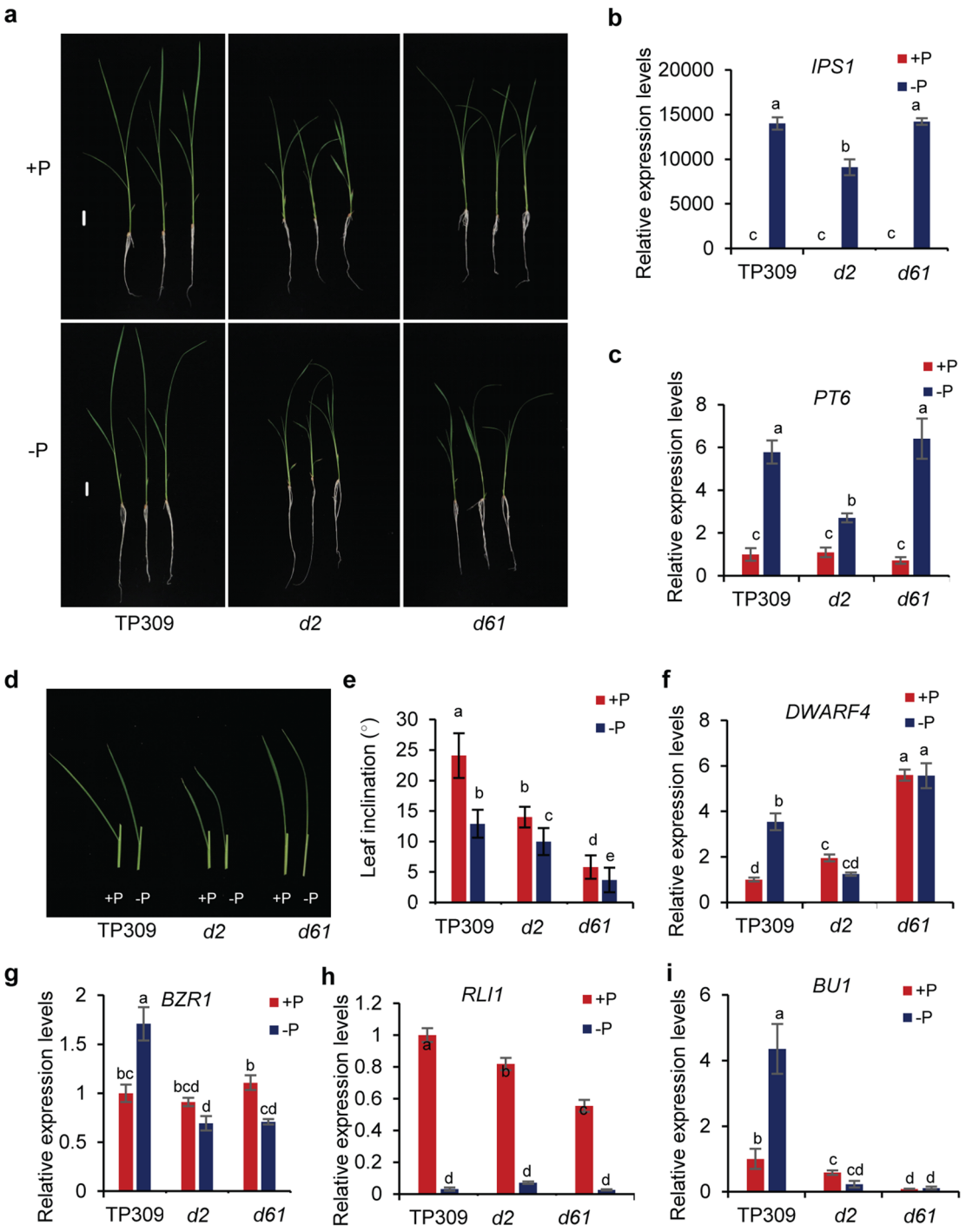


Fig. 1 Pi-deficient induced leaf erectness was compromised in BR deficiency mutants. **a** The phenotype of WT, *d2* and *d61* plants after 7 days treatment in deficient (0 μM) or sufficient (300 μM) Pi hydroponic culture solutions, respectively. Bar = 2 cm. **b**, **c** The expression of PSR marker genes in *d2* and *d61* to verify the effectiveness of Pi deficiency treatment. **d** The leaf angles of mutants after 7 days Pi-deficiency stress treatment. **e** The statistical analysis of leaf inclination for mutants after 7 days Pi-deficiency stress treatment, n = 10. **f–i** The expression of genes related to leaf inclination which were regulated by BR or Pi deficiency. Error bars are standard error. Different letters indicate significant differences, statistic analysis by one-way analysis of variance (ANOVA) using Duncan's multiple range test ($P < 0.05$)

can repress leaf inclination of the transgenic plants of gain-of-function *pDWF4::bes1-D* and *pDWF4::bin2-1*, which showed widder and narrower leaf angel than that in WT plants repectively (Fig. 2a). The Pi-defeciency can significantly induce leaf inclinationin in WT but neither in *pDWF4::bes1-D* nor in *pDWF4::bin2-1* plants (Fig. 2b, c and Supplementary Fig. 1B). The Pi deficiency-induced expressions of *IPS1* and *PT6* in WT confirmed the effectiveness of low phosphorus treatment (Fig. 2d, e). The expression of *CYC U4* in WT under Pi-suffecienct condition confirmed that the BR signaling is constitutively activated in *pDWF4::bes1-D* and constitutively repressed in *pDWF4::bin2-1* respectively (Fig. 2f). Taken together, these results suggested that either constitutive activation or repression of BR signaling can disrupt the Pi deficiency-induced leaf inclination. The expression of *CYC U4* in WT and in *pDWF4::bin2-1* is not response to Pi-deficiency stress, however, Pi-deficiency stress enhanced the expression of *CYC U4* in *pDWF4::bes1-D,* which is higher than that in WT under Pi-sufficient conditions (Fig. 2f). Moreover, the Pi-deficiency stress induced expression of *OsDWARF4* was compromised in *pDWF4::bin2-1* but not in *pDWF4::bes1-D* (Fig. 2g). Furthermore, we found that the Pi deficiency-induced upregualtion of *IPS1* was hyposensitive in *pDWF4::bes1-D* and hypersensitive in *pDWF4::bin2-1* compared to those in WT. However, the Pi deficiency-induced expression of *PT6* showed little different responsiveness in *pDWF4::bes1-D* or in *pDWF4::bin2-1* compared to those in WT (Fig. 2d, e). These results indicate that either constitutive activation or repression of BR signaling could affect part of the Pi deficiency-induced downstream genes.

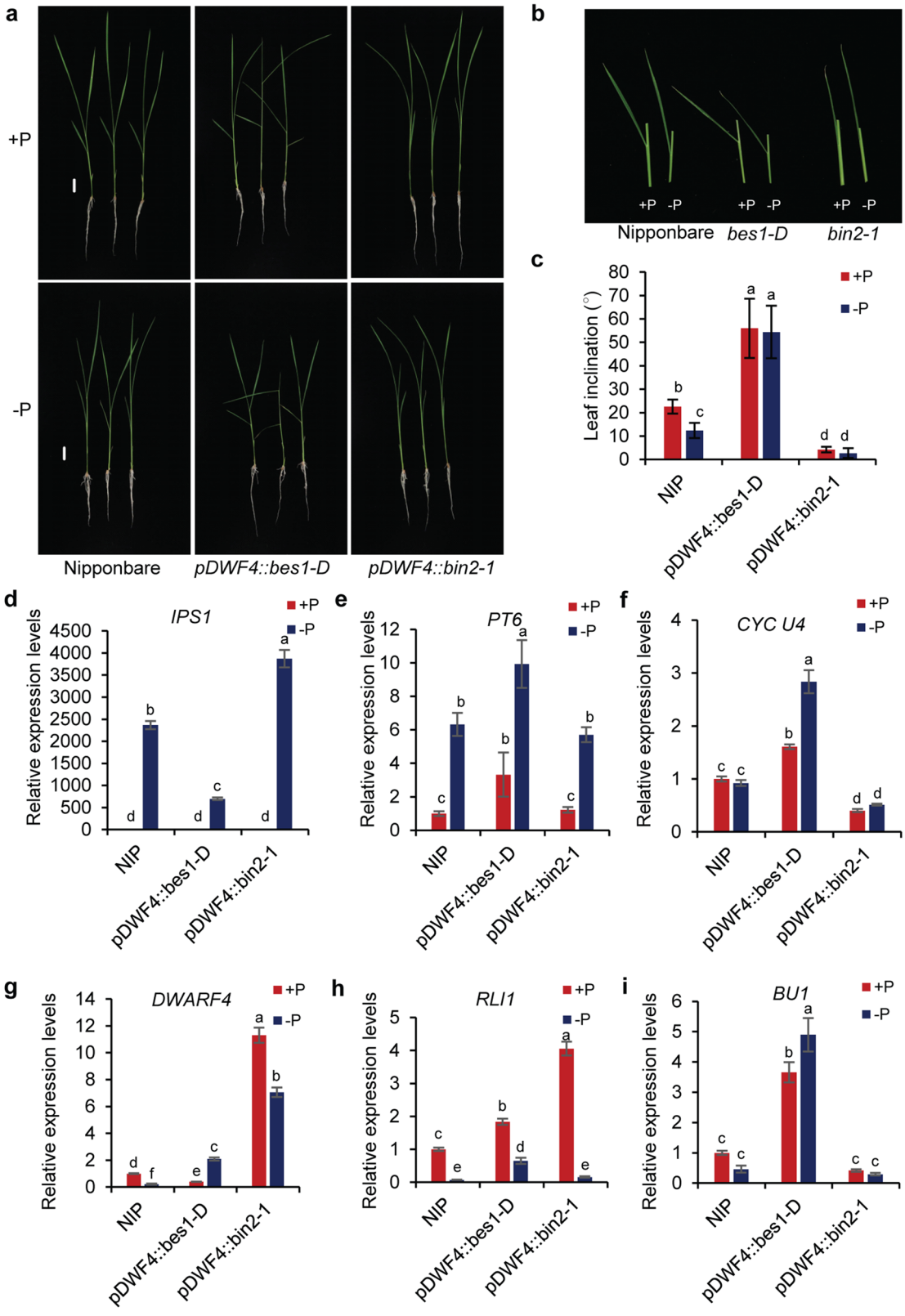
Under Pi-sufficient conditions, the expression of *RLI1* significantly increased in *pDWF4::bes1-D* and dramatically increased in, *pDWF4::bin2-1*(Fig. 2h). The Pi deficiency was still able to repress *RLI1* in *pDWF4::bes1-D* and in *pDWF4::bin2-1*, similar to those in WT (Fig. 2h), however, the fold change of Pi-deficiency induced down regulation of *RLI1* expression was less in *pDWF4::bes1-D* than that in WT whereas more in *pDWF4::bin2-1* than that in WT(Fig. 2h). In WT, the *BU1* expression was down-regulated in response to Pi-deficiency stress, which is consistent with the expression of RLI. The expression of *BU1* was upregulated in *pDWF4::bes1-D* and down regulated in *pDWF4::bin2-1* under Pi sufficient condition (Fig. 2i). When BR signaling is constitutive activated or repressed, the expression of *BU1* is not response to Pi-deficiency stress, which may explain the insensitivity of leaf inclination in response to the Pi-deficiency stress. Although the cellular basis of Pi deficiency-induced leaf inclination is different from BR deficiency-induced leaf inclination, it seems that leaf inclination induced either by Pi-deficiency or by BR deficiency is dependent on their regulatory role on the expression of *BU1*.

Constitutively activated or repressed BR signaling altered expresion profile in lamina joint

To further analyze the effects of BR signaling on the expression profile of lamina joint, we used lamina joint from *pDWF4::bes1-D* and *pDWF4::bin2-1* for RNA-seq under Pi sufficient condition. By Illumina sequencing of mRNA libraries, we generated about 10G data for each samples FPKM (for fragment per kilobase of exon per million fragments mapped) from RNAseq data which were used to compare transcript abundance among different samples. We used fold change of >2 or <0.5 as cutoffs in all the three biological replicates. We identified 576 up-regulated and 167 down-regulated genes in *pDWF4::bes1-D* compared with those in WT (Supplementary Fig. 2A, B). We also identified 117 up-regulated and 63 down-regulated genes in *pDWF4::bin2-1* (Supplementary Fig. 2C-D). The Venn map showed that 3 identified up-regulated genes in *pDWF4::bes1-D* were down-regulated in *pDWF4::bin2-1* compared to those in WT (Fig. 3a) and that 3 identified down-regulated genes in *pDWF4::bes1-D* were up-regulated in *pDWF4::bin2-1* compared to those in WT (Fig. 3b). The 3 genes up-regulated in *pDWF4::bes1-D* but down-regulated in *pDWF4::bin2-1* compared with WT include *LOC_Os03g41480*, *LOC_Os07g01580* and *LOC_Os11g24374*. The 3 genes down-regulated in *pDWF4::bes1-D* but up-regulated in *pDWF4::bin2-1* compared with WT are *OsDWF4* (*LOC_Os03g12660*), *BRD1* (*LOC_Os03g40540*) and a gene encoding a glycine rich protein (*LOC_Os06g21250*). We also found 9 genes were up-regulated (Fig. 3c) and 40 genes were down-regulated both in *pDWF4::bes1-D* and in *pDWF4::bin2-1* (Fig. 3d). The Heatmap showed that most of these genes were insensitive to Pi-deficiency stress (Fig. 3e).

Pi deficiency-induced leaf inclination mainly dependent on the BR mediating regulation expression of downstream genes

To explore the BR signaling-dependent and BR signaling-independent mechanisms that regulate Pi deficiency-induced genes, we further compared the genes expression profile of Pi-surfficienct condition and Pi-deficient condition with three biological replicates. Using fold change of >2 or <0.5 as cutoff in all the three biological replicates, we identified 1721 Pi deficiency-induced differentially expressed genes (DEG, 689 up- and 1032 down-regulated) and 14,539 no differentially expressed genes (NDEG) in WT (Supplementary Fig. 3a), 924 Pi deficiency-induced DEG (458 up- and 466 down-regulated) and 16,317 NDEG in *pDWF4::bes1-D* (Supplementary Fig. 3B), and 1212 Pi deficiency-induced DEG (847 up- and 1365 down-regulated) and 13,560 NDEG in *pDWF4::bin2-1* respectively (Supplementary Fig. 3C). Among the up-regulated genes in response to Pi-deficiency


a
+P
-P
Nipponbare
pDWF4::bes1-D
pDWF4::bin2-1
b
+P -P +P -P +P -P
Nipponbare
bes1-D
bin2-1
c
Leaf inclination (°)
+P
-P
NIP
pDWF4::bes1-D
pDWF4::bin2-1
d
IPS1
Relative expression levels
e
PT6
f
CYC U4
g
DWARF4
h
RLI1
i
BU1

◂**Fig. 2** Constitutive activate or repress BR signaling suppress the Pi-deficiency induced leaf erectness. **a** The phenotype of WT (Nipponbare), *pDWF4::bes1-D* and *pDWF4::bin2-1* plants after 7 days treatment in deficient (0 μM)or sufficient (300 μM) Pi hydroponic culture solutions, respectively. Bar = 2 cm. **b** The leaf angles of mutants after 7 days Pi-deficiency stress treatment. **c** The statistical analysis of leaf inclination for mutants after 7 days Pi-deficiency stress treatment, n = 10. **d**, **e** The expression of PSR marker genes in *pDWF4::bes1-D* and *pDWF4::bin2-1* to verify the effectiveness of Pi deficiency treatment. **f**–**i** The expression of genes related to leaf inclination which were regulated by BR or Pi deficiency. Error bars are standard error. Different letters indicate significant differences, statistic analysis by one-way analysis of variance (ANOVA) using Duncan's multiple range test ($P < 0.05$)

stress, 40 and 1 genes were not in response to Pi-deficiency stress in *pDWF4::bes1-D* and in *pDWF4::bin2-1*, respectively (Fig. 4a). Among the down-regulated genes in response to Pi-deficiency stress, 46 and 7 genes were not response to Pi-deficiency stress in *pDWF4::bes1-D* and in *pDWF4::bin2-1* respectively (Fig. 4b). Heatmap showed that most of Pi-deficiency up-regulated genes showed insensitive to Pi-deficiency stress in *pDWF4::bes1-D* (Fig. 4c). It may be due to the less fold change of gene expression in response to Pi-deficiency stress in *pDWF4::bes1-D* comapred to those in WT. However, most of Pi-deficiency down-regulated genes showed insensitive to Pi-deficiency stress in *pDWF4::bes1-D* may be caused by the lower expression levels Pi-deficiency stress in Pi-sufficient conditions. Among the Pi-deficiency induced genes, we note that *Phosphate-Starvation Induced RING-Type E3 Ligase* (*OsPIE1*, *LOC_Os01g72480*) was not in response to Pi-deficiency stress in *pDWF4::bes1-D* (Fig. 4c). OsPIE1 has been reported to regulate Pi homeostasis at least partially by repressing OsSPX2 in rice (Yang et al. 2018). These results suggested that constitutively activated BR signaling inhibited the expression of Pi-deficiency stress induced genes.

Pi deficiency-induced expression PSR genes were attenulated by constitutively activated or repressed BR signaling

The observation that *OsPIE1* is not in response to Pi-deficiency stress in *pDWF4::bes1-D* promoted us to investigate that whether constitutively activated or repressed BR signaling alters the sensitivity of PSR genes. We further analyzed the genes in response to Pi-deficiency stress in *pDWF4::bes1-D* and in *pDWF4::bin2-1* as well as in WT. We identified 187 common up-regulated genes (Fig. 5a) and 230 common down-regulated genes in response to Pi-deficiency in WT, *pDWF4::bes1-D* and in *pDWF4::bin2-1* (Fig. 5b). Among the 187 genes, we find that the Pi-deficiency induced expression of several known PSR genes, such as *OsSPX2* (*LOC_Os02g10780*), were significant compromised in *pDWF4::bes1-D* (Fig. 5a). We further selected 76 conserved PSR genes (Supplemental Table 1) and 27 BR related genes (Supplemental Table 2) to compare their Pi-deficiency induced expression in WT, *pDWF4::bes1-D* and *pDWF4::bin2-1*. The Venn map showed that 26 conserved PSR genes and 1 BR related genes overlap with the common Pi-deficiency stress induced genes in WT, *pDWF4::bes1-D* and in *pDWF4::bin2-1* respectively (Fig. 5c). No Pi-deficiency stress down-regulated genes were found to overlap with the selected conserved PSR genes and BR related genes (Fig. 5d). However, the fold change of expression of these genes were significantly reduced in *pDWF4*::*bes1-D* but not in *pDWF4::bin2-1* compared with those in WT (Fig. 5e). These results suggested that constitutively activated BR signaling attenuated the sensitivity of PSR genes in response to Pi-deficiency stress in lamina joint.

The BR biosynthesis genes *D11* (*LOC_Os04g39430*), *BRD1* (*LOC_Os03g40540*) and *OsDWF4* (*LOC_Os03g12660*) can still response to Pi-deficiency stress in WT and *pDWF4::bin2-1* but not in *pDWF4::bes1-D* (Fig. 4c). We further noted that Pi-deficiency induced fold change of several BR related genes expression was altered when the BR signaling is constitutively activated or repressed. For example, the brassinolide (BL) unregulated gene *OsBLE3* was in response to Pi-deficiency stress in WT, *pDWF4::bes1-D* and *pDWF4::bin2-1*(Fig. 5a, b). It also showed that higher expression of *CYC U2;1* (*LOC_Os04g46660*) in *pDWF4::bin2-1* were downregulated by Pi-deficiency stress (Supplemental Dataset). These results suggested that disruption of BR signaling could modulate the sensitivity of BR related genes response to Pi-deficiency stress in lamina joint and indicated the possibility to attenuate the downside effect of Pi-deficiency stress on rice plant architecture by manipulating the BR regulated development of lamina joint.

Validation of the RNA-Seq by RT-PCR

To validate RNA-seq results, we selected 12 genes to perform RT-qPCR analysis (Fig. 6). It showed that Pi deficiency induced the expression of *OsGH3-2* and repressed the expression of *OsEXP1,* which is a member of α-Expansin (Lee and Kende 2002). The expression of *OsGH3-2* and *OsEXP1* in response to Pi-deficiency was more sensitive in *pDWF4::bin2-1* and less sensitive in *pDWF4::bes1-D*. *OsCesA4/BC7* (Zhang et al. 2009a) were unregulated in *pDWF4::bin2-1* under the normal condition compared with those in WT. The expression of *OsCesA4* in *pDWF4::bin2-1* under Pi-deficiency condition was significantly down-regulated compared with those under normal condition. A putative cellulose synthase-like family F gene, *CSLF8* (LOC_Os07g36630) also showed the similar expression pattern as *OsCesA4*. The expression of *OsCesA9/BC6* (Kotake et al. 2011) was unregulated in *pDWF4::bes1-D* and in

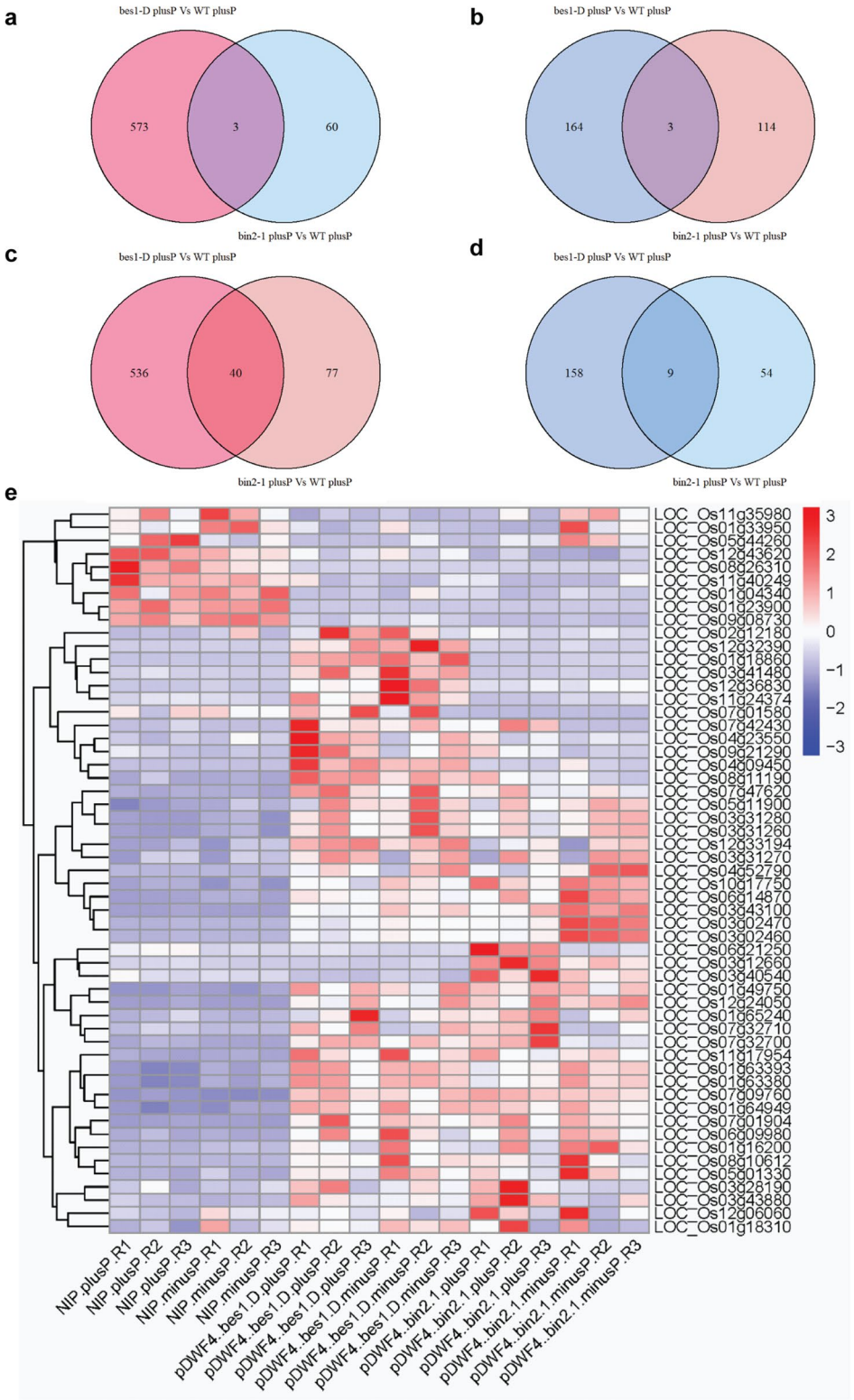


Fig. 3 BR signaling regulated genes is insensitive to the Pi-deficiency in lamina joint. **a** Up-regulated genes in *bes1-D* and Down-regulated genes in *bin2-1*. **b** Down-regulated genes in *bes1-D* and Up-regulated genes in *bin2-1*. **c** Up-regulated genes in *bes1-D* and in *bin2-1*. **d** Down-regulated genes in *bes1-D* and in *bin2-1*. **e** Heatmap of genes identified in (**a**–**d**)

pDWF4::bin2-1. The Pi-deficiency stress repressed expression of *OsCesA9* in *pDWF4::bin2-1*. These results indicated that modulation of cellulose synthesis are required for both Pi-deficiency stress induced and BR induced leaf inclination.

The expressions of *OsSPX1* and *OsSPX2* are essential to maintain Pi-homeostasis and regulate the Pi-deficiency-induced leaf inclination (Lv et al. 2014; Shi et al. 2014; Ruan et al. 2018).The expression of *OsSPX2*

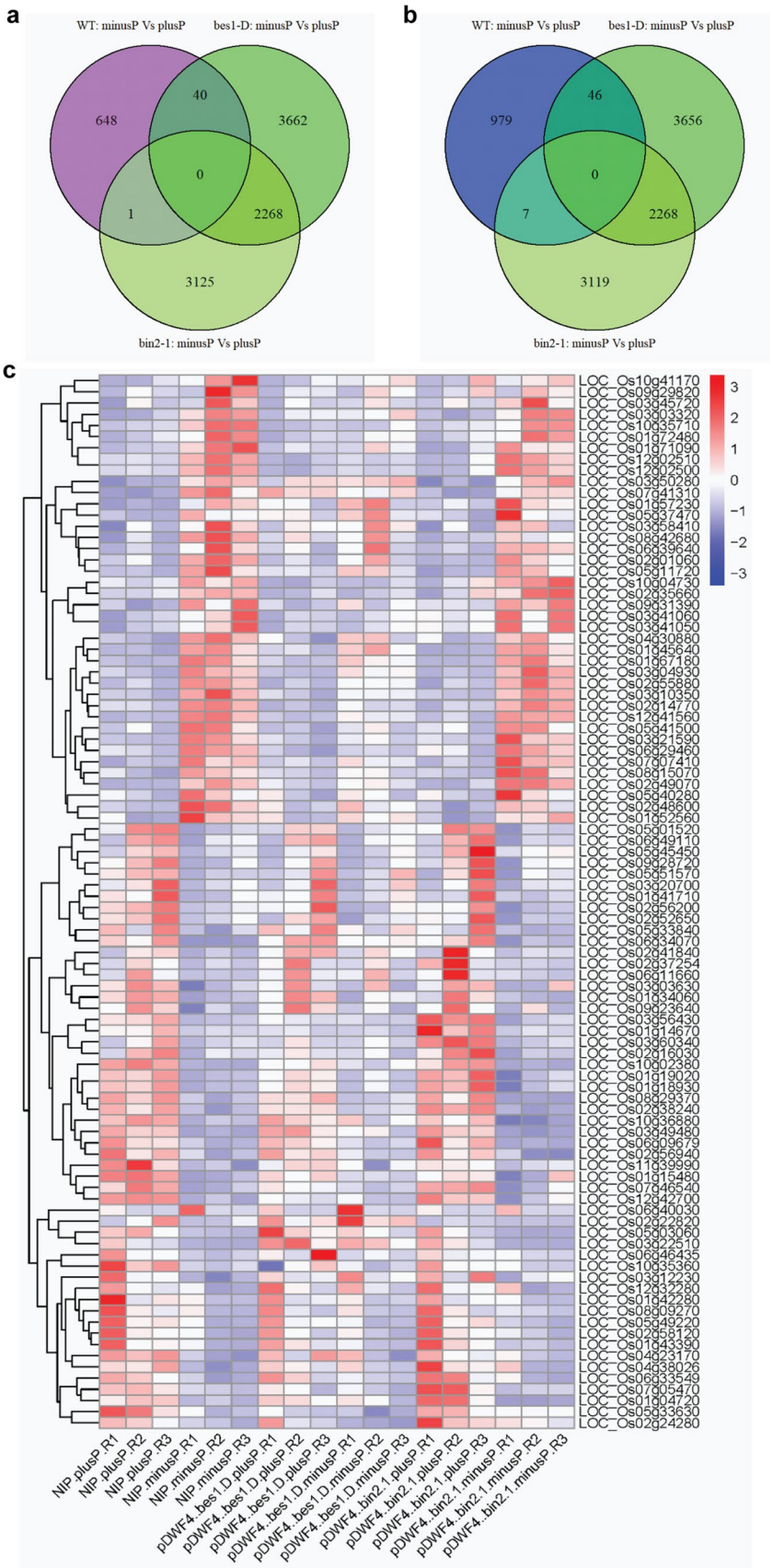


Fig. 4 Constitutive activate BR attenuated the Pi-deficiency induced gene expression in lamina joint. **a** Pi-deficiency induced up-regulated genes in WT but their expression are not response to Pi-deficiency in *bes1-D* and/or in *bin2-1*. **b** Pi-deficiency induced down-regulated genes in WT but their expression are not response to Pi-deficiency in *bes1-D* and/or in *bin2-1*. **c** Heatmap of genes identified in (**a**–**b**)

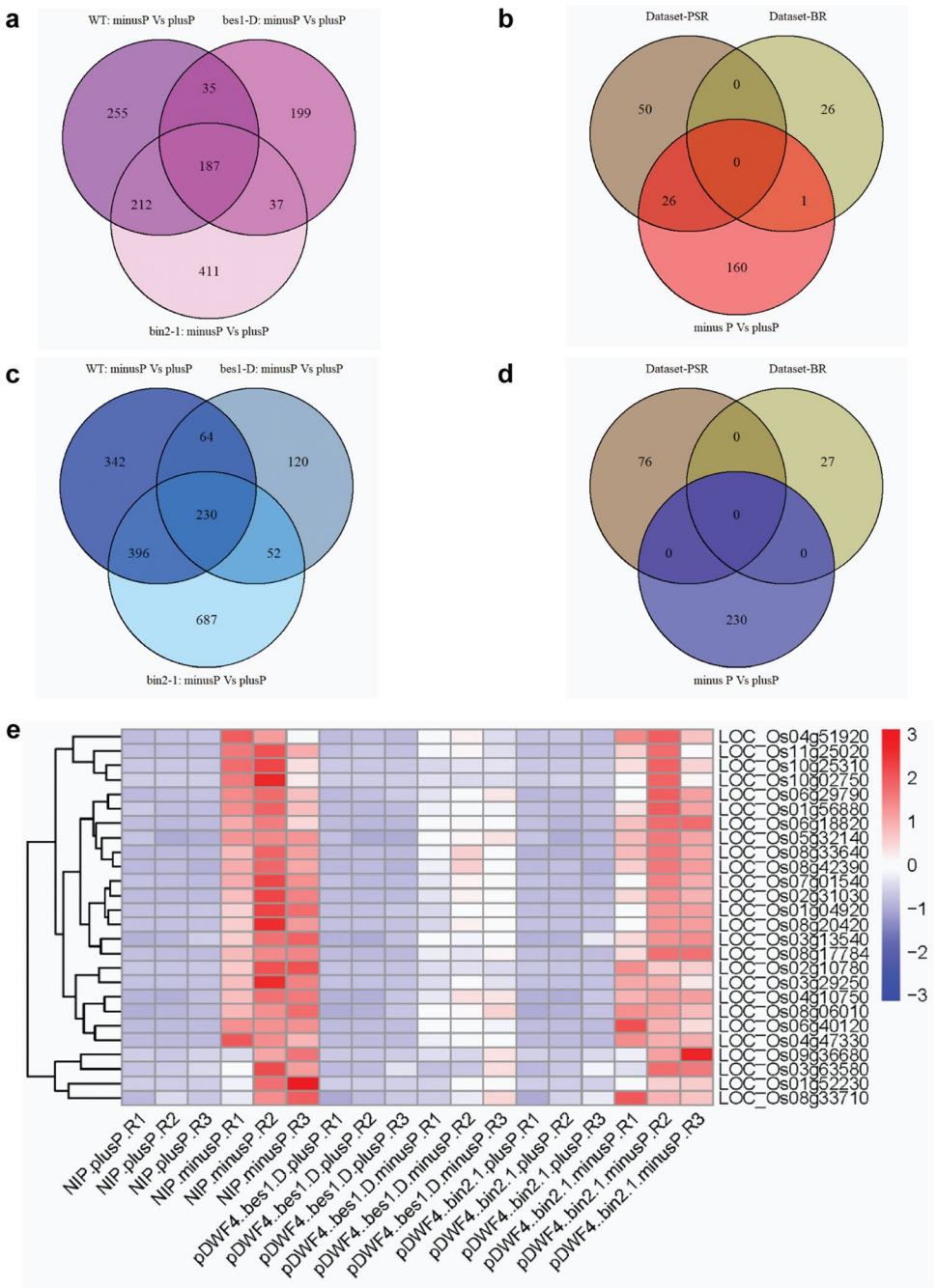


Fig. 5 The Pi-deficiency induced expression of PSR genes is compromised when constitutive activate BR signaling. **a** Up-regulated genes in WT, *bes1-D* and in *bin2-1*. **b** Overlapped up-regulated genes in A overlapped compare to PSR and/or BR related genes. **c** Down-regulated genes in WT, *bes1-D* and in *bin2-1*. **d** Overlapped down-regulated genes in B overlapped compare to PSR and/or BR related genes. **e** Heatmap of 26 PSR genes identified in (**b**)

was in response to Pi-deficiency in *pDWF4::bes1-D* and in *pDWF4::bin2-D* as well as in WT. However, the fold change of *OsSPX2* in *pDWF4::bes1-D* was much less than those in *pDWF4::bin2-1* and in WT. The GRF can bind to the intron of KNOX families of transcription factors and regulate plant architecture. This modulation was found in both monocot and dicot plants (Kuijt et al. 2014). Similar scenarios as *OsSPX2* have been observed for the expressions of *OsGRF8* and *OSH71*/*OsKn2*. On the contrary, the Pi-deficiency stress slightly induced expression of *OsBZR1* in *pDWF4::bes1-D* but not in *pDWF4::bin2-1* as well as in WT. However, the Pi-deficiency stress-induced expression of *BC1* was compromised in *pDWF4::bin2-1* but not in *pDWF4::bes1-D* compared to those in WT. It has been reported that *SLG* can modulate BR homeostasis in rice and that overexpression of *SLG* lead to the increase of leaf angle (Feng et al. 2016). We found that the Pi-deficiency induced down-regulation of *SLG* in *pDWF4::bes1-D* and in *pDWF4::bin2-1* as well in WT. Multiple abiotic stress

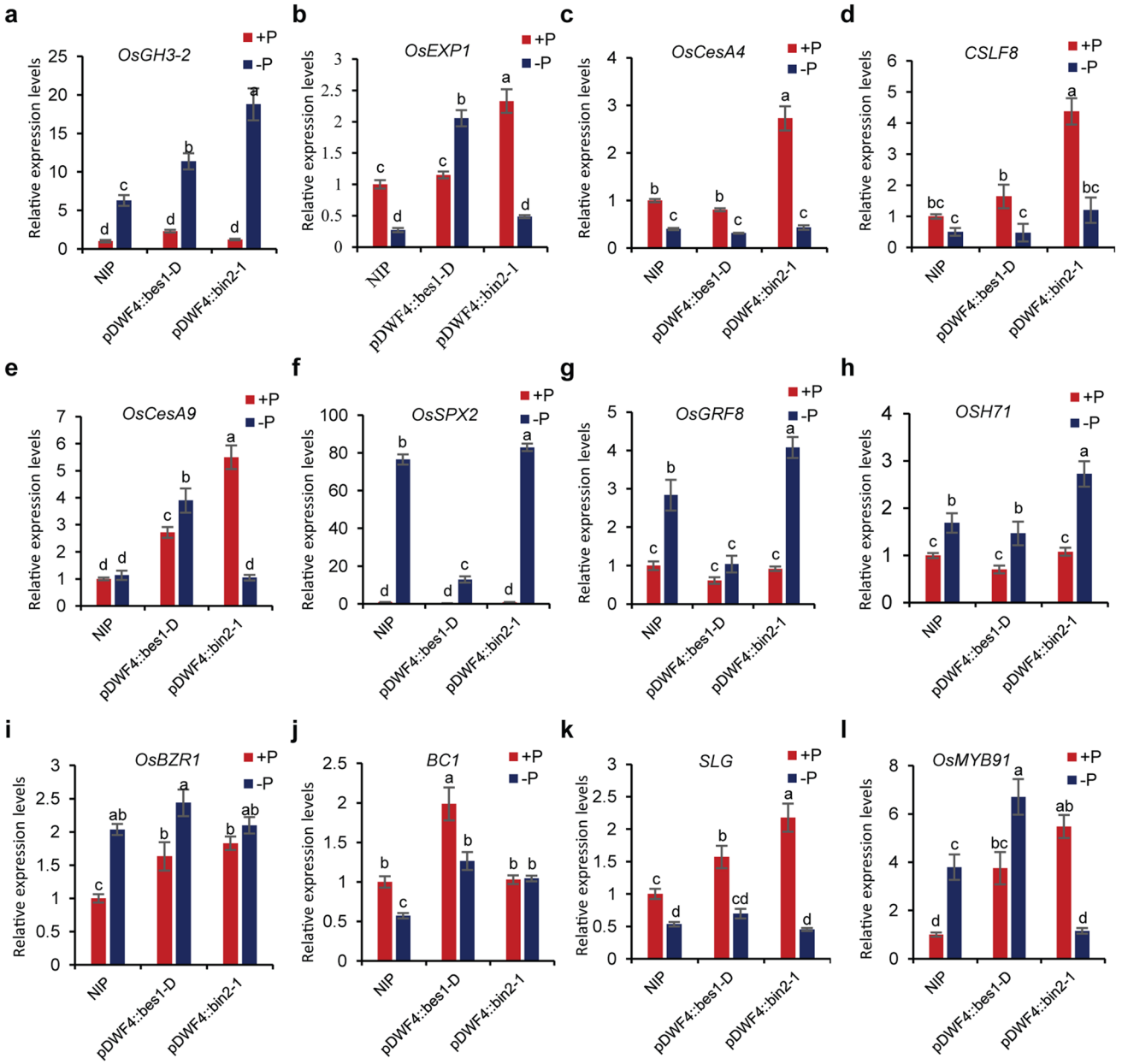


Fig. 6 Validation expression of genes by RT-PCR. Relatively expression levels of selected genes, which are normalized to the expression of *actin*. **a** *OsGH3-2* (*LOC_Os01g55940*), **b** *OsEXP1* (LOC_Os04g15840), **c** *OsCesA4* (LOC_Os01g54620), **d** *CSFL8* (LOC_Os07g36630), **e** *OsCesA9* (L OC_Os09g25490), **f** *OsSPX2* (LOC_Os02g10780), **g** *OsGRF8* (LOC_Os11g35030), **h** *OSH71*(LOC_Os05g03884), **i** *OsBZR1* (LOC_Os07g39220), **j** *BC1* (LOC_Os09g33580), **k** *SLG* (LOC_Os08g44840), L *OsMYB91* (LOC_Os12g38400). Error bars are standard error, n = 3. Different letters indicate significant differences, statistic analysis by one-way analysis of variance (ANOVA) using Duncan's multiple range test ($P < 0.05$)

stimuli and exogenous application of phytohormones can induce the expression of *OsMYB91*(Zhu et al. 2015). The Pi deficiency induced the expression of *OsMYB91* in WT and *pDWF4::bes1-D* but not in *pDWF4::bin2-1*.

Discussions

BR deficiency induces abaxial cell division and inhibits adaxial cell elongation in rice. Pi-deficiency stress inhibits both abaxial and adaxial cell divisions (Sun et al. 2015). Introducing *pDWF4::bes1-D* can enlarge leaf angle through regulation of the transcriptional expression of *CYC U4;1* which thus controls the leaf erectness. BR positively

regulates rice leaf inclination (Sun et al. 2015). It had been showed that Auxin and ABA can affect leaf inclination by modulating the BR biosynthesis or signaling in rice (Li et al. 2019). We find that Pi-deficiency induced the expression of *OsGH3-2* and repressed the expression of *OsEXP1*. *OsGH3* can transform IAA to an IAA-amino acid conjugate. It has been found up-regulating *OsGH3-1/LC1* and *OsGH3.13/TLD1* transcription in the lamina joint leads to increasing of leaf angle. Enhanced expression of *OsGH3-2* leads to the suppressed expression of expansin genes (Zhao et al. 2013). The sensetivity of expression of *OsGH3-2* responsing to Pi-deficiency can be subject to the modulation of BR signaing. It has been revealed that auxin and BR play various similar roles in plant development by regualting a subset of common downstream genes. Pi-deficiency is able to mediate the regualtion of auxin biosynthesis and transport. How Pi-deficiency regualte leaf inclination by regualtion of auxin biosynthesis and transport in lamina joint needs to be further investigated.

Pi deficiency-induced leaf erectness was mainly caused by the inhibition of lamina joint cell elongation, which is restricted by cell wall. Cell wall thickening is a prominent feature during younger stages of lamina joint development, which declines over time. In response to Pi deficiency, cell wall-related genes were down-regulated which can lead to decrease of cell wall thickening, and thus decrease cell elongation. We find that expressions of *OsCesA4* and *CSLF8* in *pDWF4::bin2-1* under Pi-deficiency condition were significantly down-regulated compared with those under normal condition. It indicated that Pi deficiency not only inhibited lamina joint cell elongation but also induced cellular processes like cell wall lignification. Plants responding to developmental and environmental cues require to reprogram cell wall structure. The Pi-deficiency stress and BR deficiency signals might convergence on modulating the expression of cell wall related genes to control cell elongation in lamina joint. Therefore, the similarity and diversity of the cellular basis for the regulation of leaf inclination by Pi-deficiency stress and BR deficiency further emphasized the complexity of regulation of leaf erectness in rice. Local sensing of Pi deficiency can promote cytoplasmic accumulation of BES1 and BZR1, thus modulate the transcriptional of downstream genes and drive shallower RSA for forage of Pi from soil in *Arabidopsis* (Singh et al. 2014). When the Pi is adequate, BES1 and BZR1 restore nucleus localized and reprogram the transcription of downstream genes. This mechamism provides an efficient modularity of root development in repsonse to the change of avalibility of Pi. The Pi deficiency stress induced expression of Pi starvation-induced genes in *bzr1-D* is similar to those acting in wild-type in *Arabidopsis*. It showed PSR genes expressed higher sensitivity of responsiveness to Pi deficiency stress in *bzr1-D* than that in WT (Singh et al. 2014). Similar, local sensing of Pi in lamina joint might reprogram the transcriptional of downstream genes and optimize the leaf angle in rice. The deficiency of Pi promotes the leaf inclination and reduces photosynthesis, which decreases the demand of Pi supply. When sensing adequate Pi concentration, the leaf inclination can be restored and promoted. However, we observed that although Pi deficiency stress can still induce the expression of conserved PSR genes in *pDWF4::bes1-D* and *pDWF4::bin2-1*. The responsiveness to Pi deficiency stress becomes more insensitive in *pDWF4::bes1-D* plant compared to those in WT, while the responsiveness to Pi deficiency stress in *pDWF4::bin2-1* is similar as that in WT. It seems that BR signaling modulated PSR response in lamina joint of rice is different to those in root of *Arabidopsis*.

In order to improve grain yield by increasing the plant density of crop, it needs to optimise the coordination of photosynthesis and the absorbtion of nutrient from soil. To dissect the molecualr mechanism underlying the interaction of Pi-deficiency stress and BR deficiency-induced leaf inclination will provide an efficient modularization strategy to increase the plant density without yield penalty. It might due to BR regulated expression of *OsPIE1*, which acts partially through repression of *OsSPX2* and desensitizes Pi deficiency stress induced expression of PSR genes in lamina joint. Furthermore, leaf erectness induced by Pi-deficiency was mainly caused by the inhibition of lamina joint cell elongation resulting in restricted lamina joint size. Meanwhile, exogenous BR treatments can induce leaf inclination in the plants grown under Pi-deficient condition as well. Therefore, the similarity and diversity of the cellular basis for leaf inclination regulated by Pi-deficiency stress and BR deficiency indicated the regulation complexity of leaf erectness in rice.

Methods

Plant materials and growth conditions

The wild type rice cultivar used in this study is TP309 and Nipponbare (*Oryza sativa* Japonica). The BR biosynthesis mutant *d2* and the BR signaling mutant *d61* were in the TP309 background. The *d2* used in this study is *d2-1*, which contains a premature stop codon at residue 83 (Hong et al. 2003). The *d61* used in this study is *d61-1*, which contains change of threonine to isoleucine at residue 989 (Yamamuro et al. 2000). The *pDWF4::bin2-1* and *pDWF4::bes1-D* transgenic plants were in Nipponbare background. Surface-sterilized rice seeds were incubated in sterile water at 30 °C for 2 days in the dark. The germinated seeds were transferred to hydroponic culture medium in the 96-well plates and cultured at 30 °C with 16 h light/8 h dark photoperiod for 7 days. The seedlings were transferred to plastic container with 20 L hydroponic culture solution (300 μM Pi/0 μM

Pi) and grown at 30 °C for 7 days. Roots and lamina joints of the plant materials were collected respectively on the seventh day. The hydroponic solution was refreshed every 3 days. The hydroponic culture solution contained 1.25 mM NH_4NO_3, 0.35 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.39 μM $(NH_4)_6Mo_7O_{24}$, 9.0 uM $MnCl_2$ 20 μM H_3BO_3, 0.32 μM $CuSO_4$, 0.77 μM $ZnSO_4$, and 20 uM EDTA-Fe, 0.5 mM $NaSiO_3$, with or without 0.3 mM KH_2PO_4, resulting in the plus Pi (300 μM) and minus Pi (0 μM) conditions. The pH of the solution was adjusted to 5.5.

The measurement of leaf inclination

According to the described measurement methods in previous references (Sun et al. 2015), the angle between the second leaf lamina and sheath was measured with a protractor on the seventh day of low Pi treatment. The second leaf inclination was 180° minus the measured angle.

RNA extraction and sequencing

There are three biological replicates for every treatment condition. Total RNA was extracted from lamina joints using Trizol reagent (Invitrogen) according to the manufacture's protocol. The quality of the total RNA was detected using NanoDrop2000 and Agilent 2100 RNA 6000 Nano kit. The X-ten (Illumina) platform was used for RNA-Seq, and cDNA library construction and sequencing were done by Berry Genomics, Beijing, China.

RNAseq data analysis

The raw data size is at least 10 Gb for every sample, and the reads length is 150 bp. The raw RNAseq reads were processed by the fastQC using Trimmomatic tool to remove low quality reads and adapters. All analyzed reads were aligned to the rice reference genome and gene model annotation file (https://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/) using bowtie2 and tophat2 tools. And then the data was processed with cufflinks, cuffmerge and cuffdiff tools respectively, and based the cuffdiff results we collected genes whose $|\log2(\text{fold\_change})| \geq 1$ in different treatments and mutants as differential expression genes (DEGs).

qRT-PCR

cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). qRT-PCR was performed on a real-time PCR instrument (Bio-Rad CFX Connect Real-Time PCR Detection System) using ChamQ Universal SYBR qPCR Master Mix (Vazyme).

Statistical analysis

The data were analyzed using the online SAS software with one-way analysis of variance (ANOVA) (https://www.sas.com/en_us/software/university-edition/download-software.html#windows-setup). And the significant differences ($P < 0.05$) were observed using the Duncan's multiple range test.

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Author contributions LYZ and LJZ performed experiments, LYZ, MQ and GX analyzed data. LYZ, MQ and GX wrote the manuscript.

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