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Roles of a maize phytochrome-interacting factors protein ZmPIF3 in regulation of drought stress responses by controlling stomatal closure in transgenic rice without yield penalty

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Abstract

Key message **ZmPIF3 plays an important role in ABA-mediated regulation of stomatal closure in the control of water loss, and can improve both drought tolerance and did not affect the grain yield in the transgenic rice.**

Abstract Phytochrome-interacting factors (PIFs) are a subfamily of basic helix-loop-helix (bHLH) transcription factors and play important roles in regulating plant growth and development. In our previous study, overexpression of a maize PIFs family gene, ZmPIF3, improved drought tolerance in transgenic rice. In this study, measurement of water loss rate, transpiration rate, stomatal conductance, guard cell aperture, density and length of ZmPIF3 transgenic plants showed that ZmPIF3 can enhance water-saving and drought-resistance by decreasing stomatal aperture and reducing transpiration in both transgenic rice and transgenic *Arabidopsis*. Scrutiny of sensitivity to ABA showed that ZmPIF3 transgenic rice was hypersensitive to ABA, while the endogenous ABA level was not significantly changed. These results indicate that ZmPIF3 plays a major role in the ABA signaling pathway. In addition, DGE results further suggest that ZmPIF3 participates in the ABA signaling pathway and regulates stomatal aperture in rice. Comparison analysis of the phenotype, physiology, and transcriptome of ZmPIF3 transgenic rice compared to control plants further suggests that ZmPIF3 is a positive regulator of ABA signaling and enhances water-saving and drought-resistance traits by reducing stomatal openings to control water loss. Moreover, investigation of the agronomic traits of ZmPIF3 transgenic rice from four cultivating seasons showed that *ZmPIF3* expression increased the tiller and panicle number and did not affect the grain yield in the transgenic rice. These results demonstrate that ZmPIF3 is a promising candidate gene in the transgenic breeding of water-saving and drought-resistant rice plants and crop improvement.

Keywords Drought tolerance · Water-saving · Transcription factor · Stomata · *Oryza sativa* · Grain yield

Introduction

Water scarcity greatly affects plant growth and development and reduces crop yield. Under a water deficit, plants activate a diverse set of physiological, metabolic, and defense systems to survive and sustain growth, such as reducing water losses by adjusting stomatal movement (opening and closing) (Sirichandra et al. 2009). The majority of stomatal movement occurs through stomatal pores. Stomatal pores located in the epidermis of plant leaves control the uptake of CO_2 for photosynthesis and water loss during transpiration (Hetherington and Woodward 2003; Huang et al. 2009; Murata et al. 2015). Plant transpiration rates can be regulated by stomatal movement. Thus, stomatal pores are the primary defense mechanism to prevent water loss under drought stress and play a crucial role in drought tolerance.

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The plant stress hormone abscisic acid (ABA) can induce stomatal closure to maintain the water status in plant cells under water deficit conditions (Cutler et al. 2010; Murata et al. 2015). ABA is involved in the transcriptional and post-transcriptional regulation of some stress-responsive genes (Chinnusamy et al. 2004). Upon drought treatment, the ABA concentration quickly elevates to promote the binding of ABA to the receptors PYR/PYL/RCAR, which inhibits type 2C (PP2C) protein phosphatases and may result in release of SNF1-related type 2 protein kinases (SnRK2s) from the inhibition of PP2C protein (Ma et al. 2009; Yoshida et al. 2014). The activated SnRK2s can phosphorylate downstream ion channels and transcription factors that can bind the ABA response element and regulate the expression of ABA-responsive genes (Lee and Luan 2012; Boursiac et al. 2013). The regulation of several ion channels at the plasma membrane and tonoplast are related with ABA-induced stomatal closure. Therefore, ABA is a key mediator in drought stress response because it induces stomatal closing to minimize transpirational water loss (Cutler et al. 2010; Kim et al. 2010; Osakabe et al. 2014).

Phytochrome-interacting factors (PIFs) are a subset of basic helix-loop-helix (bHLH) transcription factors (Castillon et al. 2007; Leivar and Quail 2011). PIFs were first described as central players in transducing light signals perceived by the light sensing phytochrome (phy) photoreceptors (Castillon et al. 2007; Bae and Choi 2008; Leivar and Quail 2011; Leivar and Monte 2014). It has been reported that phytochrome B (phyB), the upstream regulator of PIFs, were able to reduce water loss and improve drought tolerance by regulating stomatal opening, density and stomatal length. Stomata of *phyB cop1* mutants opened less widely than those of *cop1* mutants, and stomata of the *pif3 pif4* mutants opened wider than those of the wild-type, indicating that COP1, together with PIFs, may act downstream of phyB in regulating stomatal opening (Wang et al. 2010). The rice deficient in phyB exhibited reduced stomatal density and stomatal length, which presumably results in a reduction of water loss and transpiration rate and thus enhanced drought tolerance (Liu et al. 2012). A further study found that phyB enhances drought tolerance by increasing stomatal sensitivity to ABA. PhyB mutants were less sensitive than the wild-type in diminishing stomatal conductance in response to exogenous ABA application (Staneloni et al. 2008; Boccalandro et al. 2009; Gonzalez et al. 2012).

Moreover, a recent study revealed that the PIFs-like protein OsPIL1/OsPIL13, acting as a key regulator of reduced internode elongation, was down-regulated under drought stress conditions in rice (Todaka et al. 2012). The rice PIF gene *OsPIF14* expression was shown to be modulated by drought (Cordeiro et al. 2016). It has also been reported that double overexpression of *OsPIL1* and *DREB1A* can improve drought tolerance in *Arabidopsis* (Kudo et al. 2016). In our previous studies, we cloned two maize PIF genes, ZmPIF1 and ZmPIF3, and found that these genes regulate plant response to drought (Gao et al. 2015, 2018). The solid evidence shows that *ZmPIF3* is involved in drought stress responses and has potential use for improving drought stress tolerance (Gao et al. 2015). Understanding the role of *ZmPIF3* in response to drought stress is a prerequisite for genetic improvement of drought stress tolerance. In this study, we further study the role of *ZmPIF3* in drought stress and suggest the maize ZmPIF3 protein can play an important role in an ABA-associated drought stress response by controlling stomatal closure.

Materials and methods

Plant materials and stress treatments

Rice (*Oryza sativa* L. Wuyunjing) for transgenic analysis was grown in a controlled environment chamber at 28/25 °C with a 16/8 h photoperiod and 70% relative humidity. Plasmid construction and rice transformation with *ZmPIF3* was conducted as stated in Gao et al. (2015). Three homozygous transgenic lines (OE3, OE5, OE11) with higher expression levels of the target gene were selected for phenotypic assays, and all seeds used for phenotypic assays were from the same harvest and stored under the same conditions. Wild-type *Arabidopsis thaliana* (Columbia 0 type) and homozygous transgenic *Arabidopsis* were placed in a climate chamber at 22 °C with 70% relative humidity and a 12/12 h photoperiod. All tests in this study were repeated a minimum of three times.

Phenotype of transpiration assay

Forty-day-old wild-type, vector-only control and transgenic rice seedlings were planted in transparent pots filled with the same volume of hydroponic culture solution. There were 35 rice seedlings planted in each pot. Seedlings were cultured in a greenhouse without watering for 3 days. After 3 days, the water levels in the pots containing *ZmPIF3* transgenic and control rice were marked with black lines.

Thirty-day-old seedlings of WT (col) and T3 *ZmPIF3* transgenic *Arabidopsis* were transplanted into the same transparent pots (containing the same weight of soil), and 300 ml water was added after saturation of the soil with water. After 4 days, the water levels in the pots containing the *ZmPIF3* transgenic and WT *Arabidopsis* were marked with black lines. The remaining water in the WT and *ZmPIF3* transgenic *Arabidopsis* was measured after 4 days.

Measurements of water loss rate, transpiration rate, and stomatal conductance

Water-loss rates of detached leaves from *ZmPIF3* transgenic lines and wild-type plants were measured by monitoring the fresh weight loss at indicated time points. This assay was performed at room temperature (~ 23 °C) with 35% relative humidity. Water loss was calculated as the percentage of initial fresh weight at each time point. Transpiration rates and stomatal conductance were measured using a portable photosynthesis system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) in the morning (9–11 AM). Three measurements were made for each plant, and ten plants were used for both the wild-type and the transgenic plants. All parameters were measured in 40-day-old well-watered plants in a greenhouse.

Observations of leaf stomata by scanning electron microscopy (SEM)

Flag leaves of 40-day-old rice and leaves of 30-day-old *Arabidopsis* were detached from the plants of *ZmPIF3* transgenic lines and WT. The samples were immediately fixed with a 4% glutaraldehyde solution in 0.1 M phosphate-buffered saline (PBS; pH 6.8) to avoid any alterations. The stomatal pictures were obtained using an environmental scanning electron microscope (XL-30ESEM, Philips). Stomata were counted at random in eight visual sections on the abaxial epidermis, and final tallies were used to compute their densities. Length and apertures were measured randomly from a minimum of 140 stomata on the same specimens, using Image-Pro Plus6.0 software (Media Cybernetics, Singapore).

Germination assay and growth measurement

For testing the ABA sensitivity of transgenic plants at the germination stage, 35 surface-sterilized seeds were subsequently sown on sterile filter papers in square boxes supplemented with 0, 5, 10 μM ABA after 1 day of gemination. The germination rate of the treated seeds was calculated after 5 day. The filter papers were divided into four parts with black lines to sow the *ZmPIF3* transgenic lines and wild-type seeds, respectively.

To measure seedling plant height, 20 surface-sterilized seeds were transplanted into 96-well plates which were moved to the bottom of the plate after the shoot reached 2 cm in height. Seeds were grown in water containing 0, 5 μM ABA. After 7 days of growth, plant height was measured. To measure seedling root growth, 20 surface-sterilized seeds were vertically grown in water containing 0, 10 μM ABA. After 5 days of growth, root length was measured.

Quantification of the endogenous ABA content

Measurement of endogenous ABA levels of flag leaves was performed. 40-day-old seedlings of the *ZmPIF3* transgenic lines and the wild-type plants were used for ABA quantification. Briefly, rice leaves were ground in liquid nitrogen, homogenized in 1 ml distilled water, and then shaken at 4 °C overnight (~ 12 h). The homogenates were centrifuged at 12,000×*g* for 10 min at 4 °C, and the supernatant was used directly for ABA assays. ABA analysis was performed using the radioimmunoassay method (Zhang et al. 2015). The 450 μl reaction mixture contained 200 μl phosphate buffer (pH 6.0), 100 μl 1:5000 diluted antibody (Mac 252; Abcam, Cambridge, MA, USA) solution, 100 μl [^{3}H] ABA (~ 8000 c.p.m.; Sigma-Aldrich) solution, and 50 μl crude extract. The mixture was then incubated at 4 °C for 90 min, and the bound radio activity was measured in 50% saturated $(NH_4)_2SO_4$-precipitated pellets with a liquid scintillation counter (Beckman LS6500; Becton Dickinson, Franklin Lakes, NJ, USA).

DGE analysis

Flag leaves of 40-day-old *ZmPIF3* transgenic lines (OE3, OE5 and OE11) and wild-type seedlings (30 plants each) were harvested from a greenhouse, snap frozen immediately in nitrogen, and stored at − 80 °C until further processing. Three independent replicates were collected from each individual line. Total RNAs were extracted from the samples using RNAprep pure Plant Kit (Tiangen, China) according to the manufacturer's instructions. The process of digital gene expression (DGE) was conducted according to the standard protocol of the Beijing Genomics Institute (http://www.genomics.cn/index; Shenzhen, China).

Grain yield analysis

To evaluate yield components of transgenic plants under normal field conditions, four independent T3 (2014), T4 (2015), T5 (2016) and T6 (2017) homozygous lines of the *ZmPIF3* and wild-type plants were transplanted to a paddy field in Jiangsu Province, China. During the mature stage, yield parameters were scored for 3 (2014), 20 (2015), 15 (2016) and 40 (2017) plants per transgenic line and wild-type rice for statistical analysis. Tiller angle was measured between the main culm and the first side tiller. Tiller angles of the *ZmPIF3* and wild-type plants were calculated at 60 days and 120 days. The unit area yields of *ZmPIF3* transgenic and WT rice in the field were tested in 2017. The planting density was 30 plants/m^2. The unit area yields of ZmPIF3 transgenic and WT rice were calculated

for four replicates in different regions and > 100 plants per replicate.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA) and analyzed with a Student's *t* test. Significant differences are indicated by asterisks in the figures.

Results

ZmPIF3 reduces stomatal opening and decreases water loss in rice and *Arabidopsis*

To determine whether *ZmPIF3* was involved in regulating water loss, we performed a water loss assay with wild-type and transgenic plants. We hydroponically cultured 35 seedlings of *ZmPIF3* transgenic and wild-type rice transplanted into the same transparent pot filled with hydroponic culture solution. After 3 days, the water level of the *ZmPIF3* transgenic rice was significantly higher than the wild-type and vector control rice. Meanwhile, there were no significant differences in the most morphological traits of roots of the transgenic rice compared with the wild-type rice (Supplemental Fig. S1). These results demonstrate that *ZmPIF3* can significantly prevent water loss in rice seedlings (Fig. 1a). We also constructed transgenic *Arabidopsis* which overexpressed *ZmPIF3* (Supplemental Fig. S2). A water loss assay was also performed with the *Arabidopsis*. 30 seedlings of *Arabidopsis* overexpressing *ZmPIF3* and wild-type plants were transferred into the same pot respectively, and 300 ml of water was added. After 4 days, the water level of the *ZmPIF3* transgenic *Arabidopsis* was significantly higher than that of the wild-type plants (Fig. 2a, b). These results are consistent with those obtained in rice. Measurements of the water loss rate from the leaves showed that the leaves of the *ZmPIF3* transgenic plants lost less water than the leaves of wild-type plants in both rice and *Arabidopsis*. (Figs. 1b, 2c). We measured the transpiration rate and stomatal conductance of flag leaves in rice. The transgenic rice also exhibited a lower transpiration rate and stomatal conductance than the wild-type rice (Fig. 1c, d; Supplemental Figs. S3, S4). These results indicate that overexpression of *ZmPIF3* leads to a significant decrease in the rate of water loss in both rice and *Arabidopsis*.

Stomatal movements are finely regulated to control water loss through transpiration in response to environmental changes (Murata et al. 2015). Therefore, we measured the stomatal aperture, density and length of stomatal pores in *ZmPIF3* transgenic rice and transgenic *Arabidopsis* (Figs. 1e–h, 2d–g). In wild-type rice, 9.5% of stomata were completely closed, whereas 33.1, 27.4 and 17.8% were completely closed in the OE-3, OE-5, and OE-11 *ZmPIF3* transgenic rice lines, respectively. In addition, 47.3% of stomata were completely open in the wild-type rice, compared with 9.4, 21.3, and 25.5% of open stomata in the respective *ZmPIF3* transgenic rice lines (Fig. 1f). We compared guard cell density and guard cell length in wild-type and transgenic rice. The *ZmPIF3* transgenic rice showed no significant difference in the density and length of stomatal pores relative to wild-type rice (Fig. 1g, h). In the transgenic *Arabidopsis*, the results were consistent with those in rice. The density and length of the stomata showed no significant alterations in *ZmPIF3* transgenic *Arabidopsis* relative to the WT plants (Fig. 2d, e). More stomata were completely closed and fewer stomata were completely open in the leaves of the *ZmPIF3* transgenic lines compared with the WT *Arabidopsis* (Fig. 2f, g). These results suggest that increased drought tolerance in *ZmPIF3* transgenic plants is largely due to reduced stomatal opening and decreased transpiration.

Overexpression of *ZmPIF3* increases ABA sensitivity in rice

ABA promotes stomatal closure to avoid water loss under drought stress (Evans et al. 2001; Desikan et al. 2004). Previous studies have shown that the expression of *ZmPIF3* was induced by ABA treatment (Gao et al. 2015); we speculated that *ZmPIF3* may be involved in ABA expression. In this study, wild-type and *ZmPIF3* transgenic rice seeds were germinated in square dishes containing a gradient of ABA concentrations (0, 5 and 10 μM). In the absence of ABA, seed germination of the different genotypes was similar (Fig. 3a, b). When ABA concentrations increased, germination rates of wild-type seeds concomitantly decreased, while the germination rates of the transgenic rice seeds showed a significant decrease (Fig. 3b). These results suggest that the germination process of the transgenic rice seeds is hypersensitive to ABA. To further clarify the effects of ABA, germination assays were performed under mannitol and NaCl treatments. When these seeds were transferred to 1/2 MS medium containing different concentrations of mannitol or NaCl, transgenic plants showed a lower seed germination percentage than that of wild-type plants (Supplemental Fig. S5). Therefore, these results suggest that *ZmPIF3* is positively involved in ABA-modulated germination processes.

We also investigated the ABA sensitivity of transgenic rice at the post germination stage. 2-day-old seedlings were transferred to medium containing different concentrations of ABA (0, 5 and 10 μM) and grown for another 7 and 5 days before plant height and root length, respectively, were assessed. In the absence of ABA, seedling growth of the different genotypes was similar (Fig. 3d, f). In the presence of different concentrations of ABA, root

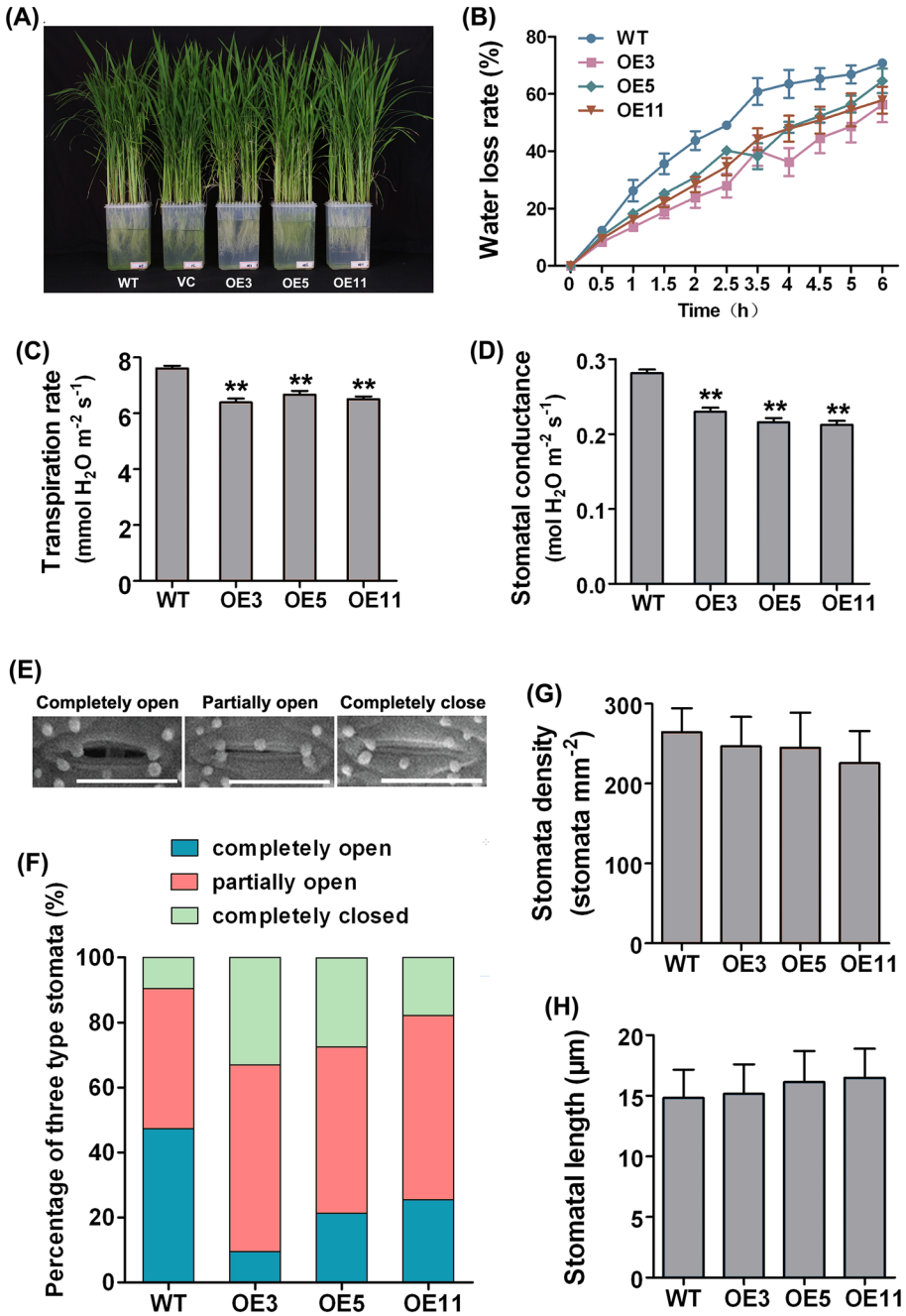

Fig. 1 *ZmPIF3* enhanced stomatal closure and reduced transpiration rates in rice. **a** The phenotypes of the *ZmPIF3* transgenic lines, wild-type and vector controls reduce transpiration. Seedlings of the wild-type, the vector-only control and *ZmPIF3* transgenic plants were transplanted into the same transparent pots filled with the same volume of hydroponic culture solution. After 3 days, the water levels of *ZmPIF3* transgenic plants and control plants were marked with black lines. **b** Water loss assays on the leaves of the *ZmPIF3* transgenic lines and wild-type plants were performed within 6 h (n = 5). **c** Transpiration rate (n = 10). **d** Stomatal conductance (n = 10). **e** The aperture of stomata observed with a scanning electron microscope. Bar = 20 μm. **f** The percentage of three levels of stomatal openings in *ZmPIF3* transgenic lines and wild-type plants (n > 140). **g** Stomatal density (n > 140). **h** Stomatal length (n > 140). WT, wild-type; VC, vector control; OE3, OE5 and OE11, three *ZmPIF3* transgenic lines. All parameters were measured in 40-day-old well-watered plants in a greenhouse. Data represent the mean ± SE. ***t* test, with $P < 0.01$; **t* test, with $P < 0.05$

and shoot growth of the transgenic rice was more severely inhibited than in the wild-type (Fig. 3c–f). These results suggest that overexpression of *ZmPIF3* can increase ABA sensitivity at post germination stages and indicate that *ZmPIF3* is a positive regulator of ABA signaling in rice.

We further investigated endogenous ABA levels in wild-type control and transgenic rice. Under normal conditions, the endogenous ABA level was not significantly different between the wild-type and transgenic rice (Fig. 3g). This result suggests that *ZmPIF3* is not involved in ABA synthesis but contributes significantly to the ABA signaling pathway.

ZmPIF3 affects differential gene expression in rice

To explore the molecular mechanisms of water-saving and drought tolerance underlying *ZmPIF3* transgenic rice, DGE profiling was performed to determine the differential gene expression profiles between wild-type and transgenic lines at the seedling stage (Supplemental Fig. S6). Under normal

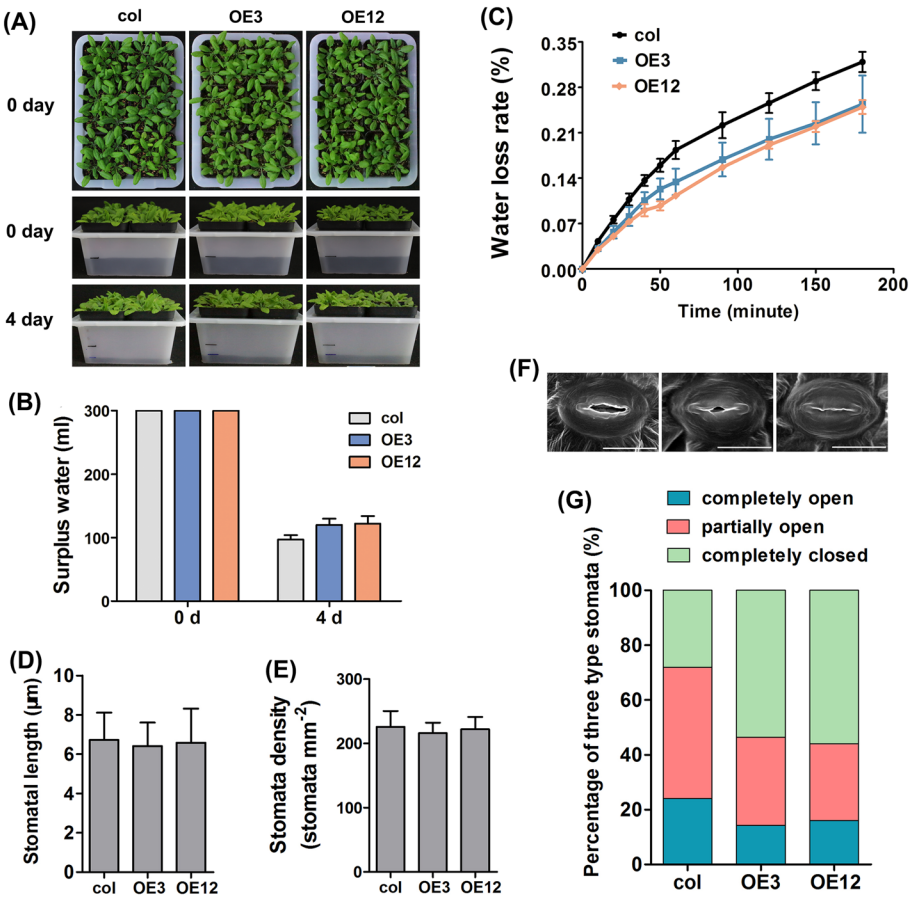

Fig. 2 *ZmPIF3* enhanced stomatal closure and reduced transpiration in *Arabidopsis*. **a** The phenotypes of *ZmPIF3* transgenic *Arabidopsis* and wild-type (col) plants with reduced transpiration (n = 30). Seedlings of wild-type (col) and *ZmPIF3* transgenic *Arabidopsis* were transplanted into the same transparent pots (containing the same weight of soil) and supplemented with 300 ml water after saturation of the soil with water. After 4 days, the water levels of *ZmPIF3* transgenic *Arabidopsis* and wild-type (col) plants were marked with black lines. **b** Surplus water of wild-type (col) and *ZmPIF1* transgenic *Arabidopsis* after 4 days. **c** Water loss assays on the leaves of *ZmPIF1* transgenic lines and wild-type (col) plants were performed within 200 min for *Arabidopsis* (n > 5). **d** Stomatal length (n > 200). **e** Stomatal density (n > 200). **f** The aperture of stomata observed with a scanning electron microscope in *Arabidopsis*. Bar = 20 μm. **g** The percentage of three levels of stomatal openings in *ZmPIF3* transgenic lines and wild-type (n > 200). col: *Arabidopsis thaliana* L. Heynh, Columbia; OE3, OE12, two *ZmPIF3* transgenic lines. Data represent the mean ± SE

growth conditions, a total of 35,073 genes were differentially expressed, with 4002 genes exhibiting a 1.5-fold up-regulation and 2988 genes exhibiting a 1.5-fold down-regulation in *ZmPIF3* transgenic rice compared with the wild-type. An important number of genes involved in plant hormone pathways and abiotic stress as well as those related to stomata were found to be differentially regulated in the leaves of *ZmPIF3*-transgenic rice compared with wild-type rice (Table 1; Supplemental Fig. S7).

OsbZIP88 (LOC_Os12g40920), a rice bZIP transcription factor, was predicted to be an ABA responsive element binding factor (Ji et al. 2009). The expression level of *OsbZIP88* was elevated to 3.3-fold in *ZmPIF3* transgenic rice. *OsRAB16C* (LOC Os11g26760) has been shown to be a downstream gene in the ABA signaling pathway (Chen et al. 2012). In this study, expression levels of *OsRAB16C* were increased to 5.5-fold in *ZmPIF3* transgenic rice (Table 1). Moreover, expression levels of *OsNCED1, OsNCED2, OsNCED3, OsNCED4, OsNCED5*, which are the key genes of ABA biosynthesis, were not found to be significantly different in *ZmPIF3* transgenic and wild-type tice (Saika et al. 2007; Zhu et al. 2009). Some ABA signaling genes, such as the PYR/PYL/RCAR, PP2Cs, and SnRK2s genes, were also unchanged in *ZmPIF3* transgenic rice under normal conditions (data not shown).

Transcript levels of many well-known drought resistance-related genes including *OsRAB16D* (LOC Os11g26780), *OsPR4b* (LOC_Os11g37960) and *OsPR4c* (LOC_Os11g37950) were also elevated to 4.4-, 2.6- and 2.3-fold, respectively, in *ZmPIF3* transgenic rice relative to wild-type rice (Chen et al. 2012; Wang et al. 2011). Further scrutiny of the differentially expressed genes identified a set of

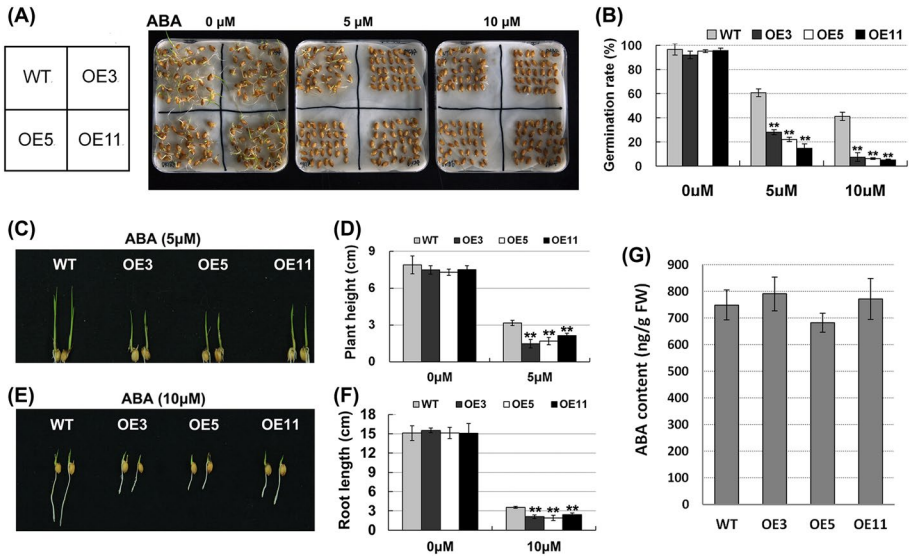

Fig. 3 Increased ABA sensitivity of *ZmPIF3* transgenic rice at the germination and seedling stages. **a** Germination phenotypes of *ZmPIF3* transgenic lines and wild-type seeds on wet filter paper containing 0, 5, or 10 μM ABA for 5 days (n = 35). **b** The germination rates of *ZmPIF3* transgenic lines and wild-type seeds subjected to ABA treatment (n = 35). **c** Phenotypes of plant height of *ZmPIF3* transgenic lines and wild-type plants transplanted into water containing 5 μM ABA for 7 days (n = 20). **d** The plant height of the lines grown in normal and 5 μM ABA-containing water for 7 days (n = 20). **e** Phenotypes of root length of *ZmPIF3* transgenic lines and wild-type plants transplanted into water containing 10 μM ABA for 5 days (n = 20). **f** The root length of the lines grown on normal and 10 μM ABA-containing water for 5 days (n = 20). **g** The ABA content of *ZmPIF3* transgenic lines and wild-type plants (n = 15). WT, wild-type; VC, vector control; OE3, OE5 and OE11, three *ZmPIF3* transgenic lines. **b, g** Data represent the mean ± SD. **d, f** Data represent the mean ± SE. ***t* test, with $P < 0.01$; **t* test, with $P < 0.05$. All tests were repeated a minimum of three times

EXPANSIN family genes associated with stomata aperture, including *OsEXPA2* (LOC_Os01g60770), *OsEXPA4* (LOC_Os05g39990), *OsEXPB2* (LOC_Os10g40710), *OsEXPB3* (LOC_Os10g40720), *OsEXPB4* (LOC_Os10g40730), *OsEXPB7* (LOC_Os03g01270), *OsEXPB11* (LOC_Os02g44108), that were up-regulated at least 1.5-fold (Liu et al. 2012). These results suggest that *ZmPIF3* elevated the transcript levels of many stress-resistance and stomata-related genes under normal conditions, thus improving the drought tolerance of the transgenic rice by reducing stomatal aperture.

In addition, other plant hormone pathways and abiotic stress genes also showed up-regulation in *ZmPIF3* transgenic rice relative to wild-type rice. For example, brassinosteroid insensitive 1 BRI1 (LOC_Os11g47240), which is involved in the brassinosteroid pathway, was up-regulated and had a higher level in *ZmPIF3* transgenic rice compared to wild-type rice. Meanwhile, the expression levels of the jacalin-like lectin domain containing protein SalT (LOC_Os01g24710) was up-regulated to 3.6-fold in the transgenic rice compared to wild-type.

The grain yield of the *ZmPIF3* transgenic rice

Because grain yield is the ultimate parameter for crops, we examined several agronomic traits of the *ZmPIF3* transgenic rice under normal conditions for four cultivating seasons (2014, 2015, 2016 and 2017) (Table 2; Fig. 4; Supplemental Table S1). Fewer plants were scored in 2014, while the 2015, 2016 and 2017 data exhibited greater statistical rigor. The *ZmPIF3* transgenic rice exhibited increased tiller and panicle number. Tillering in rice is one of the most important agronomic traits related to grain production (Li et al. 2003). At the mature stage, the average tiller and panicle number of the *ZmPIF3* transgenic rice plants grown in the field was significantly higher compared to the wild-type controls in 2014, 2015, 2016 and 2017 (Table 2; Supplemental Table S1). The filling rate of the *ZmPIF3* transgenic rice was decreased, but the reduction appeared to be balanced by the increase in the number of tillers and panicles (Table 2; Supplemental Table S1). Moreover, the unit area yield of the *ZmPIF3* transgenic rice was measured in 2017. The unit area yield of the *ZmPIF3* transgenic rice did not decrease compared to the wild-type rice (Fig. 5). An additional interesting phenotype of the *ZmPIF3* transgenic rice is their significantly wider tiller angle versus that of the wild-type rice, which was also confirmed in three independent lines of *ZmPIF3* transgenic rice (Fig. 6d–i). Morphological comparison of *ZmPIF3* transgenic rice and wild-type rice demonstrate a difference in tiller angle both in pots and paddy fields at 60 and 120 days (Fig. 6a–c). These results clearly indicate that *ZmPIF3* is important for grain yield production by increasing the number of panicles.

Table 1 Expression of plant hormone signal transduction, stress and stomatal-related genes identified by DGE tag profiling

Gene ID	Description	Fold change
Plant hormone signal transduction		
LOC_Os12g40920	bZIP transcription factor domain containing protein, expressed (bZIP88)	3.293973
LOC_Os11g26760	Dehydrin, putative, expressed (OsRAB16C)	5.47032
LOC_Os02g43330	Homeobox associated leucine zipper, putative, expressed	2.091922
LOC_Os01g50910	Late embryogenesis abundant protein, group 3, putative, expressed (OsLEA14a)	2.654911
LOC_Os10g02880	*O*-Methyltransferase, putative, expressed	5.643856
LOC_Os01g06560	Transcription factor HBP-1b, putative, expressed	3.906891
LOC_Os08g31340	Heavy metal-associated domain containing protein, expressed	1.596367
LOC_Os03g18490	RPGR, putative, expressed	2
LOC_Os05g46480	Late embryogenesis abundant protein, group 3, putative, expressed (OsLEA19a/LEA3)	− 1.5381
LOC_Os11g47240	Leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed (BRI1)	2.463405
LOC_Os06g48200	Glycosyl hydrolases family 16, putative, expressed (BRII)	2.095672
LOC_Os09g26780	Zinc-finger protein, putative, expressed (JAZ)	2.303534
Stress-related genes		
LOC_Os11g26780	Dehydrin, putative, expressed (OsRab16D)	4.392317
LOC_Os01g24710	Jacalin-like lectin domain containing protein, expressed (SalT)	3.612443
LOC_Os04g14680	OsAPx3—peroxisomal ascorbate peroxidase encoding gene 5,8, expressed	2.649093
LOC_Os07g02440	Peroxidase precursor, putative, expressed (OsPOD1)	1.842979
LOC_Os08g43334	HSF-type DNA-binding domain containing protein, expressed (OsHsfB2b)	3.529698
LOC_Os11g37960	WIP4—wound-induced protein precursor, expressed (OsPR4b)	2.571935
LOC_Os11g37950	WIP3—wound-induced protein precursor, expressed (OsPR4c)	2.281286
LOC_Os01g51990	AN1-like zinc finger domain containing protein, expressed (OsSAP13)	− 4.52356
LOC_Os09g35030	Dehydration-responsive element-binding protein, putative, expressed (OsDREB1A)	− 2.47573
LOC_Os09g35010	Dehydration-responsive element-binding protein, putative, expressed (OsDREB1B)	− 2.81871
LOC_Os08g43334	HSF-type DNA-binding domain containing protein, expressed	3.529698
LOC_Os01g50910	Late embryogenesis abundant protein, group 3, putative, expressed	2.654911
LOC_Os07g34520	Isocitrate lyase, putative, expressed (ICL)	2.137504
LOC_Os01g58420	AP2 domain containing protein, expressed (OsAP37)	− 1.61333
Stomatal related gene		
LOC_Os01g60770	Expansin precursor, putative, expressed (OsEXPA2)	2.5025
LOC_Os05g39990	Expansin precursor, putative, expressed (OsEXPA4)	1.657112
LOC_Os10g40710	Expansin precursor, putative, expressed (OsEXPB2)	2.472068
LOC_Os10g40720	Expansin precursor, putative, expressed (OsEXPB3)	3.562242
LOC_Os10g40730	Expansin precursor, putative, expressed (OsEXPB4)	5.47032
LOC_Os03g01270	Expansin precursor, putative, expressed (OsEXPB7)	2.917538
LOC_Os02g44108	Expansin precursor, putative, expressed (OsEXPB11)	2.760812
LOC_Os02g40240	Receptor kinase, putative, expressed (LP2)	− 1.90689
LOC_Os11g32100	Inducer of CBF expression 1, putative, expressed (OsSCRM1)	1.514573
LOC_Os10g40090	Expansin precursor, putative, expressed (OsEXPB9)	− 1.73697

Selected up-regulated and down-regulated genes in *ZmPIF3* transgenic rice relative to wild-type plants. Genes with at least a 1.5-fold change in the *ZmPIF3* transgenic rice are shown

Table 2 Agronomic traits of *ZmPIF3* transgenic plants grown in the paddy field conditions in 2015, 2016 and 2017

Lines	No. of tillers per plant	Panicle number per plant	Panicle length (cm)	No. of grains per panicle	Filled grains per panicle	Seed-setting rate (%)	1000-grain weight (g)	Grain yield per plant (g)
2015								
WT	8.96 ± 1.97	8.92 ± 2.00	15.16 ± 1.93	152.20 ± 28.57	136.70 ± 25.12	90.32 ± 1.79	25.47 ± 0.23	20.70 ± 3.00
VC	10.04 ± 2.49	9.96 ± 2.56	15.58 ± 1.60	160.40 ± 15.74	134.40 ± 10.43	83.10 ± 4.34**	26.55 ± 0.25**	20.11 ± 2.97
ZmPIF3								
OE3	12.23 ± 3.53**	12.17 ± 3.06**	15.93 ± 1.58**	170.00 ± 16.17	140.00 ± 12.53	83.40 ± 1.49**	26.81 ± 0.10**	31.18 ± 5.63*
OE5	10.96 ± 2.27**	10.88 ± 2.27**	16.37 ± 1.52**	177.40 ± 34.43	124.10 ± 25.96	73.22 ± 3.27**	25.15 ± 0.10*	20.46 ± 4.11
OE11	11.42 ± 2.56**	10.96 ± 2.20**	16.34 ± 1.29**	183.33 ± 25.36*	133.10 ± 19.56	73.67 ± 6.73**	28.62 ± 0.05**	26.65 ± 3.90
2016								
WT	10.53 ± 1.38	10.53 ± 1.38	17.28 ± 0.25	141.43 ± 9.59	134.00 ± 9.86	95.09 ± 3.20	24.25 ± 0.18	29.07 ± 5.33
ZmPIF3								
OE3	13.37 ± 2.11**	13.3 ± 2.18**	16.59 ± 0.57**	167.27 ± 21.47**	155.40 ± 17.48**	92.95 ± 10.71	26.74 ± 0.09**	36.06 ± 9.99
OE5	12.63 ± 2.09**	12.6 ± 2.10**	16.29 ± 0.38**	152.83 ± 21.59	140.90 ± 23.07	92.43 ± 9.88*	26.22 ± 0.09**	34.59 ± 6.42
OE11	13.23 ± 2.11**	13.00 ± 2.15**	16.56 ± 0.20**	150.57 ± 19.74	140.27 ± 18.51	92.91 ± 5.18**	28.83 ± 0.16**	36.59 ± 5.61*
2017								
WT	12.34 ± 2.36	12.13 ± 2.45	15.13 ± 1.52	91.00 ± 10.08	80.44 ± 12.05	89.98 ± 4.58	27.33 ± 0.11	24.78 ± 1.99
ZmPIF3								
OE3	13.88 ± 3.31*	13.68 ± 3.07*	13.85 ± 1.38*	113.28 ± 15.64**	94.59 ± 15.67**	83.07 ± 6.87**	28.09 ± 0.02**	25.22 ± 1.44
OE5	14.51 ± 2.99**	14.30 ± 2.72**	13.86 ± 2.58*	122.50 ± 28.46**	88.59 ± 17.30*	80.49 ± 7.95**	27.18 ± 0.05	25.00 ± 1.91
OE11	14.07 ± 3.60**	13.59 ± 3.12*	13.77 ± 2.11*	104.27 ± 14.58*	89.63 ± 14.57*	85.86 ± 5.72*	30.53 ± 0.16**	24.69 ± 1.91

OE3, OE5 and OE11: three *ZmPIF3* transgenic plants. Values are mean ± SD (n > 15)

WT wild type, *VC* vector control

*, ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively

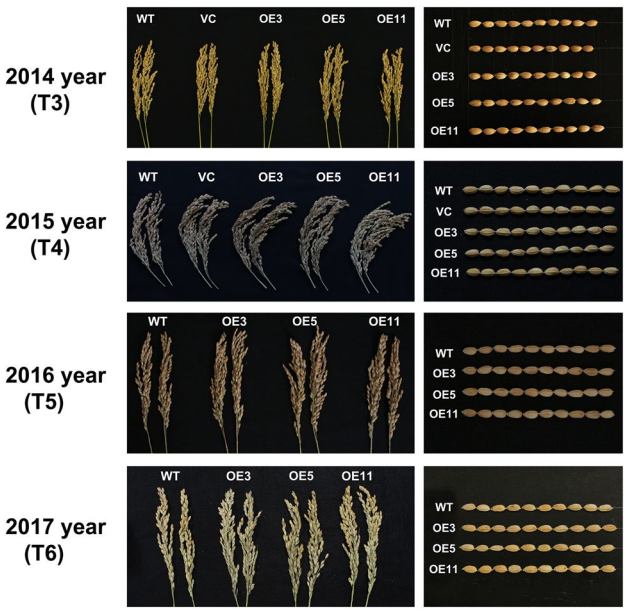

Fig. 4 Panicle phenotype and seed morphology of *ZmPIF3* transgenic lines and wild-type plants. Agronomic traits of the *ZmPIF3* transgenic plants under normal conditions for three cultivating seasons (2014, 2015, 2016 and 2017)

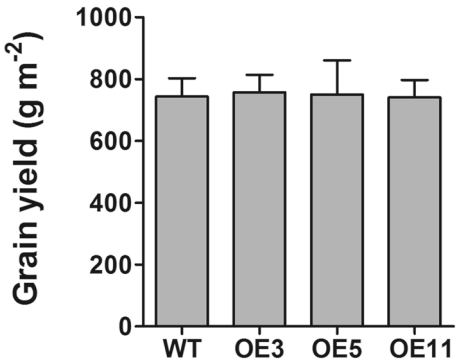

Fig. 5 The unit area yield of *ZmPIF3* transgenic and wild-type rice in 2017. The planting density was 30 plants/m^2. The unit area yields were calculated for four replicates in different regions and > 100 plants per replicate. Data represent the mean ± SE

Discussion

ZmPIF3 is a positive regulator of drought stress tolerance dependent on ABA signaling to regulate stomata aperture

The plant drought response is a complex process regulated by multiple molecular and cellular pathways. The plant stress hormone ABA is a key mediator in drought stress response because it induces stomatal closing to

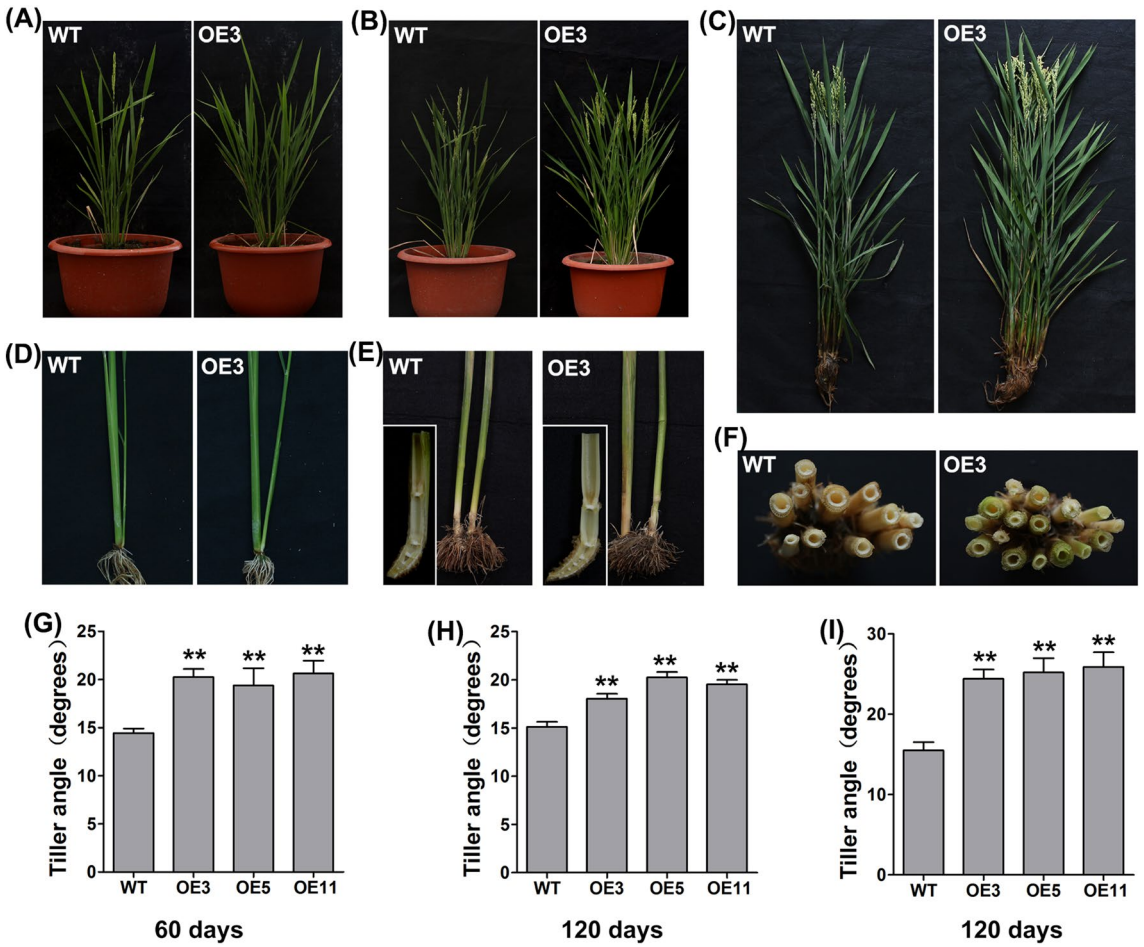

Fig. 6 *ZmPIF3* transgenic plants showed wider tiller angles and more panicles. **a** Phenotypes of *ZmPIF3* transgenic plants and wild-type plants at 90 days in pots. **b** Phenotypes of *ZmPIF3* transgenic plants and wild-type plants at 120 days in pots. **c** Phenotype of *ZmPIF3* transgenic plants and wild-type plants at 120 days in paddy fields. **d** Phenotypes of the angle between the main culm and the first side tiller of *ZmPIF3* transgenic plants and wild-type plants at 60 days. **e** Phenotypes of the tiller base (left panels) and the angle between the main culm and the first side tiller of *ZmPIF3* transgenic plants and wild-type plants at 120 days. **f** Phenotypes of the tiller base of *ZmPIF3* transgenic plants and wild-type plants at 120 days. **g** Tiller angle of *ZmPIF3* transgenic plants and wild-type plants at 60 days. **h** Tiller angle of *ZmPIF3* transgenic plants and wild-type plants at 120 days in pots. **i** Tiller angle of *ZmPIF3* transgenic plants and wild-type plants at 120 days in paddy fields. **g**–**i** Data represent the mean ± SE. ***t* test, with $P < 0.01$; **t* test, with $P < 0.05$

minimize transpirational water loss (Cutler et al. 2010; Kim et al. 2010; Osakabe et al. 2014). Stomatal responses can regulate water loss from plants to efficiently control the water status of plants and play a crucial role in drought stress tolerance (Murata et al. 2015; Schroeder et al. 2001; Hetherington and Woodward 2003; Huang et al. 2009). The *ZmPIF3* transgenic plants showed a lower water loss rate than the wild-type plants in both rice and *Arabidopsis* (Figs. 1a, b, 2a–c), and significantly promoted stomatal closure and reduced stomatal conductance and transpiration rate in both rice and *Arabidopsis* (Figs. 1c–h, 2). These adaptations help to better avoid dehydration when soil water becomes limiting. The results indicate that *ZmPIF3* plays an important role in the regulation of stomatal closure to control of water loss.

Plant response to drought is largely dependent on enhanced ABA biosynthesis and signaling to regulate both stomatal aperture and gene expression (Cutler et al. 2010; Pizzio et al. 2013; Seiler et al. 2014). In our previous study, we showed that expression of *ZmPIF3* is rapidly induced in response to ABA treatment, and the *ZmPIF3* transgenic rice exhibited a higher survival rate after exposure to drought stress (Gao et al. 2015). In this study, the *ZmPIF3* was significantly able to promote stomatal closure (Figs. 1f, 2g). Therefore, we attempted to investigate the functional relationship between ABA and *ZmPIF3*. *ZmPIF3* transgenic rice exhibited diminished

germination rates, plant height and root elongation than wild-type controls under ABA treatment (Fig. 3a–f). These results show that *ZmPIF3* transgenic rice exhibited hypersensitivity to ABA in both germination and seedling growth. Meanwhile, the endogenous ABA level in the *ZmPIF3* transgenic rice showed no significant change under normal conditions (Fig. 3g). These results show that *ZmPIF3* may be involved in the ABA signaling pathway but not involved in ABA biosynthesis. Transcriptomic comparisons could facilitate the identification of key genes and regulatory mechanisms. In this study, the expression level of ABA biosynthesis-related genes *OsNCED1, OsNCED2, OsNCED3, OsNCED4*, and *OsNCED5* (Saika et al. 2007; Zhu et al. 2009) were not found to be significantly changes in *ZmPIF3* transgenic and wild-type rice (data not shown). These results are consistent with the results for endogenous levels of ABA content (Fig. 3g), which further suggests that *ZmPIF3* is not involved in the ABA biosynthesis pathway. The ABA signaling pathway includes components such as cytosolic ABA receptors, PYL/RCARs, PP2Cs, SnRK2s, and basic leucine zipper (bZIP) transcription factors (Cutler et al. 2010; Umezawa et al. 2010; Kim et al. 2014). In our DGE results, expression of most PYL/RCARs, PP2Cs, and SnRK2s genes was not changed in *ZmPIF3* transgenic rice under normal conditions (data not shown). *OsbZIP88* encodes a rice bZIP transcription factor and can interact with *OsbZIP71* by forming heterodimers with *OsbZIP71*, while *OsbZIP71* may play an important role in ABA-mediated drought and salt tolerance in rice (Liu et al. 2014). The expression level of *OsbZIP88* was increased in *ZmPIF3* transgenic rice relative to wild-type rice. Moreover, the expression levels of *RAB16C*, a downstream gene in the ABA signaling pathway (Chen et al. 2012), were increased in *ZmPIF3* transgenic rice (Table 1). These results suggest that *ZmPIF3* participates as a positive regulator of the ABA signaling pathway.

EXPANSINS are cell wall proteins which mediate cell wall loosening and play an important role in regulation of stomatal aperture and stomatal density (Marowa et al. 2016; Zhang et al. 2011; Wei et al. 2011; Lü et al. 2013). In our DGE results, seven *EXPANSIN* genes were screened, and the expression of these genes was significantly up-regulated in *ZmPIF3*-transgenic rice relative to wild-type rice. These results are consistent with the lower transpiration phenotypes of *ZmPIF3*-transgenic lines (Fig. 1f), These results suggest that *ZmPIF3* may participate in regulating stomatal aperture. In addition, *ZmPIF3* transgenic rice also exhibited activated stress-responsive gene expression even in the absence of stress treatment, including genes such as *OsRAB16D, OsPR4b* and *OsPR4c* (Table 1) (Chen et al. 2012; Wang et al. 2011). The DGE analysis indicated that *ZmPIF3* promotes the closing of stomata and a decrease in the transpiration rate by participating in the ABA-dependent signaling pathway, which is responsible for enhanced drought tolerance in *ZmPIF3* transgenic plants.

ZmPIF3 transgenic rice improves the number of panicles without affecting the grain yield under normal conditions

The constitutive overexpression of stress-related genes often causes abnormal development and thus a loss in productivity (Dubouzet et al. 2003; Nakashima et al. 2007; Yu et al. 2013). Therefore, it is also important to develop transgenic plants that are tolerant to stresses but also maintain high yields under normal conditions. In rice, grain yield increases are caused by an increased number of tillers and panicles without a substantial change in the number of spikelets per panicle (Ekanayake et al. 1989; Ramegowda et al. 2014; Wei et al. 2014). Here, *ZmPIF3* did not affect rice grain yields under normal conditions and increased the number of panicles without a substantial change in the number of spikelets per panicle (Figs. 4, 5; Table 2; Supplemental Table S1). An additional phenotype of *ZmPIF3*-transgenic plants is their tiller angle is significantly wider than that of WT plants (Fig. 6). We speculated that the wider tiller angle of *ZmPIF3* transgenic plants was caused by more growth of the tiller compared with the wild-type plants. The yield results in multiple transgenic lines suggest the broader applicability of *ZmPIF3* in crop production.

In our previous study, we characterized a constitutively active form of the transcription factor *ZmPIF3* and showed a drought stress-resistant phenotype (Gao et al. 2015). In this study, comparison analysis of *ZmPIF3* transgenic rice and wild-type plants revealed that *ZmPIF3* is a positive regulator of ABA signaling and regulates drought stress resistance by reducing stomatal opening and transpiration rates. *ZmPIF3* not only improved drought tolerance, but also had no effect on the grain yield of rice, demonstrating that it is a promising candidate gene in transgenic breeding of drought resistance plants and crop improvement.

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Author contributions YG and JC conceived and designed the experiments; YG, MW, MZ, WJ, EL, NX and CZ performed the experiments; YG, DZ and JC analyzed the data and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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