A maize phytochrome-interacting factors protein ZmPIF1 enhances drought tolerance by inducing stomatal closure and improves grain yield in Oryza sativa

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Summary
Phytochrome-interacting factors (PIFs) play major roles in regulating plant growth and development, but their roles in drought stress remain elusive. Here, we cloned and characterized a maize (Zea mays) PIF transcription factor, ZmPIF1. The expression level of ZmPIF1 was significantly induced by independent drought and abscisic acid (ABA) treatments. The ZmPIF1 transgenic rice and Arabidopsis displayed water saving and drought resistance, which were associated with reduced a stomatal aperture and transpiration rate. Moreover, the ZmPIF1 transgenic rice were hypersensitive to exogenous ABA, while the endogenous ABA level was not significantly changed, suggesting that ZmPIF1 was a positive regulator of the ABA signalling pathway. Digital gene expression (DGE) results further indicated that ZmPIF1 participated in ABA signalling pathway and regulated the stomatal aperture in rice. In addition, grain yield and agronomic traits analysis over 4 years showed that ZmPIF1 was able to increase the grain yield through an increase in tiller and panicle numbers in transgenic rice. Overall, ZmPIF1 plays an important role in the ABA-mediated regulation of stomatal closure to control water loss. ZmPIF1 can enhance water saving and drought resistance and improve the crop yield in rice, illustrating the capacity of ZmPIF1 for crop improvement.

Keywords: drought tolerance, water saving, morphological character, physiological trait, transcription factor, stomata.

Introduction
Global warming is expected to increase the frequency and intensity of droughts worldwide. Additionally, water scarcity is a major agricultural problem, restricting crop expansion and reducing crop yield. Of the total human water consumption, 70% is agricultural water. Thus, adaptation and mitigation strategies should focus on researching the mechanism of crop water saving and drought resistance to reduce current and future drought risks. In fact, even a small improvement in crop water saving will result in large reductions in crop loss (Claeys and Inze, 2013).

Stress avoidance and stress tolerance are two different mechanisms for dealing with low water availability, and both responses are related to abscisic acid (ABA)-dependent and ABA-independent mechanisms (Claeys and Inze, 2013; Lawlor, 2013). In plants, ABA is a main regulator of the transpiration rate and other mechanisms of water saving and drought resistance (Jones, 2015). It has been extensively shown that plants accumulate more ABA, which controls stomatal closure under drought conditions, thereby saving water (Murata et al., 2015).

Phytochrome-interacting factors (PIFs) are a subset of basic helix-loop-helix (bHLH) transcription factors. The first PIF gene cloned from bHLH transcription factors was PIF3 (Ni et al., 1998). Subsequently, PIF1, PIF4, PIF5, PIF6, PIF7 and PIF8 were identified (Leivar and Monte, 2014; Leivar and Quail, 2011). All of these PIFs contain a conserved bHLH domain that is responsible for DNA binding. These PIFs also contain an active phytochrome B (APB) motif, which is conserved in the N-terminal sequence and necessary for phytochrome B (phyB)-specific binding. Moreover, PIF1 and PIF3 also contain the active phytochrome A-binding (APA) region, which is necessary for phytochrome A (phyA) binding (Leivar and Monte, 2014; Leivar and Quail, 2011; Shen et al., 2008). Until now, there has been little information on PIFs in crop. Six phytochrome-interacting factor-like (PIL) homologs (OsPIL11-OsPIL16) have been found in rice by evaluating the rice databases (Nakamura et al., 2007). In addition, two other PIF proteins have been identified and characterized, including LjPIF4 in Lotus japonicas (Ono et al., 2010) and ZmPIF3 in maize (Gao et al., 2015; Kumar et al., 2016).

Activated phyB interacts with PIF1 and induces PIF1 phosphorylation and degradation under light irradiation (Krzymuski et al., 2014). It has been reported that phyB is able to induce tolerance to stresses by enhancing ABA sensitivity (Gonzalez et al., 2012; Stanlozi et al., 2008). The phyB mutants were able to reduce water loss and improve drought tolerance by regulating stomatal opening and density (Boccaandroni et al., 2009; Liu et al., 2012). In addition, PIF1 can indirectly inhibit the gibberellic acid (GA) pathway and plays an important role in regulating the expression of ABA biosynthesis-related genes and promoting ABA biosynthesis (Kim et al., 2008; Oh et al., 2009). PIF1 also interacts with the ABA-positive signalling component gene ABI5, which inhibits seed germination via ABA signalling (Kim et al., 2016). Thus, PIF1 can regulate endogenous ABA and ABA signalling, and ABA can regulate plant adaptation to drought stress (Osakabe et al.,...
2014). However, the role of PIF1 in drought stress remains poorly understood.

Although the regulatory functions of the PIFs have been widely explored in light responses, seed germination and hormone regulation, the roles of PIFs in the drought stress response remain elusive (Leivar and Quail, 2011; Oh et al., 2009). The rice PIF-like protein OsPIL1 is down-regulated under drought stress conditions (Todaka et al., 2012), and overexpression of both DREB1A and OsPIL1 enhances drought tolerance in Arabidopsis (Kudo et al., 2016), while ZmPIP2 regulates plant responses to drought stresses (Gao et al., 2015). Thus, PIFs likely play complicated roles under drought stress. In this study, we focused on the role of PIFs in water saving, drought resistance and grain yield and suggest that maize ZmPIF1 is a promising candidate gene for transgenic breeding of water saving and drought-resistant plants for crop improvement.

Results

Isolation and characterization of ZmPIF1 encoding a phytochrome-interacting factor in maize

The clone of the target gene was 1704 bp in length, and a database search showed that the sequence was similar to those of other PIFs. Outside of the bHLH domain, the ZmPIF1 amino acid sequence also contained two characteristic domains, APB motif and APA motif (Figure S1). The Y2H study results indicated that the APB domain of ZmPIF1 interacts with maize phytochrome B1 (ZmPhyB1) and the APA domain of ZmPIF1 interacts with maize phytochrome A1 (ZmPhyA1) in yeast (Figure 1a). A phylogenetic analysis revealed that ZmPIF1 was closely related to ZmPIF3 in maize, PIF1 and PIF3 in Arabidopsis, and OsPIL15 and OsPIL16 in rice (Figure S2).

The full-length ZmPIF1 was introduced into the p2GW7 vector, and a ZmPIF1-green fluorescent protein (GFP) fusion protein was constructed to investigate the subcellular localization of ZmPIF1 (Gao et al., 2015). Transient expression assays suggested that the ZmPIF1-GFP fusion protein was localized in the nucleus (Figure 1b), which was corroborated by bimolecular fluorescence complementation (BiFC) tests. The results suggest that ZmPIF1 can interact with itself via BiFC and may form homodimers (Figure S3). Thus, ZmPIF1 is located in the nucleus. Different tissue expression patterns of ZmPIF1 were examined. As shown in Figure 1, the expression of ZmPIF1 was higher in pistils and leaves, while lower expression levels were observed in seeds, stamens and roots (Figure 1c). The expression level of ZmPIF1 under polyethylene glycol (PEG), salt, ABA and low-temperature treatments was examined and was significantly regulated by PEG, salt and ABA, while its expression did not change under cold temperature conditions (Figure 1d–g). Thus, ZmPIF1 may play important roles in drought, salt and ABA responses.

Expression of ZmPIF1 enhances tolerance to drought stress in rice

The expression patterns suggest that ZmPIF1 plays an important role in drought stress. To investigate whether the overexpression of ZmPIF1 can improve drought resistance in rice, the full-length of ZmPIF1 was transformed into ‘Wuyunjing’ rice, and eleven transgenic lines were screened by qRT-PCR. The qRT-PCR results indicated that the expression level of ZmPIF1 in transgenic plants could be detected, but not in the wild-type (WT) or the vector control (VC) (Figure S4a). The ZmPIF1 overexpression (OE) transgenic lines OE-1, OE-3 and OE-7 were chosen for all assays in this study, and there were no obvious differences in plant morphology between the transgenic lines and the two controls under normal conditions (Figure S4b–d).

To investigate the drought tolerance of ZmPIF1, two controls and ZmPIF1 transgenic rice were exposed to water deficit using a 20% PEG solution treatment. Under normal conditions, there were no differences in plant morphology between the two controls and ZmPIF1 transgenic rice (Figure 2a). After 4 days of PEG treatment, the majority of the two control plants began to wither, while a few of the leaves in the transgenic rice were rolled and wilted (Figure 2a). After recovery in a normal hydroponic solution, some of the transgenic rice survived and recovered, while most of the leaves of the two control plants were still rolled and wilted (Figure 2a). Ten days after recovery, the survival rates of the two controls and the transgenic rice were determined. The survival rate of the transgenic lines, at ~60%, was higher than those of the two control plants, which had almost completely died (Figure 2b).

Four physiological parameters, relative water content (RWC), chlorophyll content, chlorophyll fluorescence and cell membrane stability (CMS), were assayed (Figure 2c–f). In this study, no differences were observed in these four physiological parameters between the two controls and the transgenic lines under nonstressed conditions. Under PEG treatment, the physiological parameters of the two controls were significantly lower than those of the ZmPIF1 transgenic rice (Figure 2c–f). These results revealed that ZmPIF1 improves water retention and photosynthetic and cell membrane stability capabilities compared with the controls under PEG treatment in rice.

To further verify the drought tolerance of ZmPIF1, two controls and ZmPIF1 transgenic rice were exposed in soil to a water deficit. Before the drought treatment, the growth of two controls and transgenic rice was similar under normal conditions (Figure 2g, under normal conditions). After 7 days without watering, the leaves of the two control plants were rolled and wilted, while only a few leaves of the ZmPIF1 transgenic rice had begun to roll and wilt (Figure 2g, drought for 7 days). After 1 day of recovery, the two control plants still had drought phenotypes. Moreover, more than half of the transgenic rice were green and healthy (Figure 2g, 1 day after rewatering). After 10 days of recovery in water, the majority of the two control lines did not recover and only approximately 5% survived, while more than 90% of the transgenic plants survived (Figure 2h). Taken together, these results reveal that ZmPIF1 can increase drought tolerance in rice.

Expression of ZmPIF1 prevents water loss and participates positively in stomatal closure

Interestingly, when ZmPIF1 transgenic rice and WT rice were grown in different individual pots, the drought-resistant phenotype of the ZmPIF1 transgenic rice was more apparent compared with the WT phenotype. However, when the ZmPIF1 transgenic and WT rice were planted in the same pot, the drought-resistant phenotypes of the ZmPIF1 transgenic and WT rice were not obviously different (data not shown). We hypothesize that ZmPIF1 transgenic rice differs in terms of transpiration and may exhibit improved drought tolerance through water saving. To further investigate the involvement of ZmPIF1 in modulating water saving, a water loss assay was tested. Thirty-five well-grown ZmPIF1 transgenic rice and control seedlings, respectively, were transplanted into clear pots of the same size containing the same volume of hydroponic culture solution. After 3 days under normal conditions, the water levels of the ZmPIF1 transgenic lines were
obviously higher relative to those of the two controls (Figure 3a). These results demonstrate that ZmPIF1 can significantly improve water retention in rice seedlings.

The water loss rates of ZmPIF1 transgenic and WT rice were consistent with the water loss assay results. The leaves of ZmPIF1 transgenic rice lost less water than the WT control (Figure 3b). Subsequently, we measured the transpiration rates and stomatal conductance levels of rice leaves. The transpiration rate and stomatal conductance levels of transgenic rice were lower relative to the WT control (Figure 3c,d). We speculated that overexpression of ZmPIF1 in rice led to a lower rate of water loss.

In response to drought stress, stomatal movements are regulated to control water loss by transpiration (Murata et al., 2015). Thus, we measured the stomatal density, length and aperture in WT and ZmPIF1 transgenic rice. In ZmPIF1 transgenic rice, no significant alterations were observed in the density or length of stomata relative to WT rice (Figure 3i,m). More stomata were completely closed, and fewer stomata were completely open in the leaves of ZmPIF1 transgenic lines compared with WT rice (Figure 3i,j). The aforementioned results indicate that the reduction in ZmPIF1 stomatal aperture decreased the transpiration rate, which led to enhanced water saving and drought resistance in rice.

To verify the function of ZmPIF1-enhanced water saving and drought resistance in Arabidopsis, we constructed ZmPIF1 overexpression transgenic plants of in Arabidopsis. Six T3 transgenic lines were obtained, and two of them were used to verify the function of ZmPIF1 (Figure 4; Figure S5). A water loss assay was performed in Arabidopsis. Thirty well-grown ZmPIF1 overexpression Arabidopsis seedlings and WT seedlings were transferred into...
the same pot with 300 mL water. After 4 days, the water level was significantly higher in the pots containing the ZmPIF1 transgenic Arabidopsis compared with the WT plants (Figure 4a,b). Measurements of the water loss rate from the leaves showed that ZmPIF1 transgenic plants lost less water than WT plants (Figure 4c). Moreover, we measured the stomatal aperture, density and length of stomatal pores in the overexpression transgenic plants of ZmPIF1 (Figure 4d–g), and the results were consistent with those in rice. The density and length of stomata showed no significant alterations in ZmPIF1 transgenic Arabidopsis relative to in WT plants (Figure 4d–e). More stomata were completely closed and fewer stomata were completely open in the leaves of the ZmPIF1 transgenic lines compared with WT Arabidopsis (Figure 4f–g). These results also suggested that enhanced water saving and drought resistance in ZmPIF1 transgenic Arabidopsis were largely due to reducing stomatal opening.

Expression of ZmPIF1 increased ABA sensitivity in rice

Under drought stress, ABA promotes stomatal closure and decreases water loss (Desikan et al., 2004; Schroeder et al., 2001). Previous experiments have shown that ABA can induce the expression of ZmPIF1 (Figure 1g); therefore, we speculated that ZmPIF1 may be involved in the ABA pathway. In this study, we measured the transpiration rates and stomatal conductance levels of rice leaves treated with ABA. With ABA treatment, the transpiration rate and stomatal conductance levels of transgenic rice were significantly lower than that of the WT control (Figure 3f–h). We also compared the aperture of the stomata of WT and transgenic rice treated with ABA. The results showed that more stomata were completely closed and fewer stomata were completely open in the leaves of ZmPIF1 transgenic rice compared with WT (Figure 3k). These results indicated that the transgenic rice potentially showed a hypersensitivity to ABA.

To further verify whether the transgenic rice were sensitive to ABA, we measured the seed germination rates, root lengths and plant heights of WT and transgenic rice treated with ABA. In the absence of ABA, the seed germination rates of ZmPIF1 transgenic lines and WT were not significantly different (Figure 5a,b). With increasing ABA concentrations, the germination rates of WT seeds decreased from 99% (0 μM ABA) to 59% (5 μM ABA) and 37% (10 μM ABA) (Figure 5b). However, the seeds of transgenic rice showed a hypersensitivity to ABA. Almost 90% of ZmPIF1 transgenic rice seeds germinated with 0 μM ABA, but only 9% of

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Figure 3  ZmPIF1 enhanced stomatal closure and reduced transpiration in rice. (a) The phenotype of the ZmPIF1 transgenic rice showed decreased transpiration. Seedlings of wild-type, vector control and ZmPIF1 transgenic rice cultured after 3 days; water level of ZmPIF1 transgenic rice and control plants was marked with black lines. Bar = 10 cm. (b) Water loss assays for the leaves of the ZmPIF1 transgenic lines and wild type were performed within 6 h (n = 5). (c–h) The transpiration rate, stomatal conductance and photosynthetic rate of the ZmPIF1 transgenic lines and wild type: (c–e) no treatment and (f–h) 100 μM abscisic acid (ABA) treatment (n > 50). (i) Scanning electron microscopy images of three levels of stomatal opening. Bar = 20 μm. (j–k) The percentage of three levels of stomatal opening in ZmPIF1 transgenic lines and wild type: (j) no treatment and (k) 100 μM ABA treatments (n > 400). All tests were performed in 40-day-old well-watered plants in the glasshouse. Data represent the mean ± SE. **t-test, with P < 0.01; *t-test, with P < 0.05.
the ZmPIF1 transgenic rice germinated with 10 μM ABA (Figure S5a,b). Germination assays were also performed under mannitol and NaCl treatments. The same germination phenotypes of transgenic plants were shown in response to NaCl and mannitol (Figure S6). These results suggested that the transgenic rice seeds showed a hypersensitivity to ABA during the germination process.

For the root length and plant height measurements, the seedling growth rates of different genotypes were similar in the absence of ABA (Figure S5c,e). In response to different ABA concentrations (0, 5 and 10 μM), the root lengths and plant heights of the transgenic rice were more suppressed relative to those of WT (Figure S5c–f). These experiments demonstrated that root lengths and plant heights of transgenic rice showed a hypersensitivity to ABA during the postgermination process. These results indicated that ZmPIF1 is a positive regulator of ABA signalling.

To more systematically evaluate the function of ZmPIF1, we analyzed the endogenous ABA levels in transgenic and WT rice, which were not significantly different under normal conditions (Figure S5g), suggesting that ZmPIF1 is not involved in ABA synthesis but contributes significantly to the ABA signalling pathway.

ZmPIF1 affects the expression of ABA-induced, stress-responsive and stomata-related genes

We performed a digital gene expression (DGE) analysis to determine the differential gene expression between WT and the transgenic rice at the seedling stage (Figure S7). Interestingly, the involvement of the hormone pathways, abiotic stress and stomatal-related genes were modulated in leaves of ZmPIF1-transgenic rice relative to WT (Table 1, Figure S8).

The expression levels of genes involved in the ABA signalling network were altered in ZmPIF1 transgenic rice compared with WT plants. The expression levels of OsABI5 and OsZIP88, which were predicted to be ABA-responsive binding factors, were elevated in ZmPIF1 transgenic rice (Table 1). The expression levels of OsRAB16A and OsRAB16C, which have been reported to be the downstream of ABA signalling (Hong et al., 2009; Wang et al., 2007), were increased in ZmPIF1 transgenic rice (Table 1). However, the expression levels of OsNCED1-5, which are the key genes in ABA biosynthesis (Zhu et al., 2009), did not significantly change in ZmPIF1 transgenic and WT rice (data not shown).

The transcript levels of many well-known drought resistance-related genes, including OsRAB16A, OsRAB16B, OsRAB16D, OsPR4b and OsPR4c, were up-regulated in ZmPIF1 transgenic rice compared with WT (Table 1) (Du et al., 2010; Wang et al., 2007, 2011). Overexpression of Leaf Panicle 2 (LP2), which is a leucine-rich repeat receptor-like kinase, leads to a decrease in stomatal closure (Wu et al., 2015). In the present study, the LP2 expression level was decreased in ZmPIF1 transgenic rice. To investigate the differentially expressed genes, some EXPANSIN family genes related to stomata aperture were identified. The expression levels of these genes, including OsEXP2A, OsEXP4A, OsEXP2B, OsEXP3B, OsEXP4B, OsEXP8B, OsEXP8B6 and OsEXP8B7, were up-regulated at least 1.5-fold (Table 1) (Liu et al., 2012).
**ZmPIF1 increases the grain yield in rice**

Grain yield is one of the most important agronomic traits of crops. Thus, we examined several agronomic traits of WT and ZmPIF1 transgenic rice over 4 years of cultivation (2014, 2015, 2016 and 2017). The 4-year data sets were consistent, but the number of scored plants was reduced in 2014, while the data for 2015, 2016 and 2017 showed greater statistical rigour (Table 2; Table S1). In 2015, 2016 and 2017, ZmPIF1 transgenic rice showed an increased grain yield relative to WT because of the increased numbers of tillers and panicles (Table 2; Table S1). Moreover, the unit area yield of ZmPIF1 transgenic lines was higher than in the WT control in 2017 (Figure 7). An additional interesting phenotype of ZmPIF1 transgenic plants was that their tiller angles were significantly wider than those of WT plants (Figure S9). It is speculated that ZmPIF1 is important for grain yield production by increasing the numbers of panicles.

**Discussion**

**ZmPIF1 plays a positive role in drought tolerance**

The overexpression of some stress-related genes can improve the environmental stress tolerance of plants (Tang et al., 2012). As previously discussed, the expression of ZmPIF1 was strongly up-regulated by PEG and salt stress (Figure 1d,e). Although PIFs have recently attracted much interest, their functions in abiotic stress remain largely unexplored. Some data have shown that PIF4 expression may be regulated by high temperature in Arabidopsis (Franklin et al., 2011; Koini et al., 2009; Stavang et al., 2009). Todaka et al. (2012) reported that drought down-regulates the PIF-like gene OsPIL1. In our previous study, the maize PIF gene ZmPIF3, a homolog of ZmPIF1, was found to function in the response to abiotic stress (Gao et al., 2015). Following exposure to drought stress, the survival rates of the ZmPIF1 transgenic rice were higher than in the two control plants grown in a hydroponic solution or soil (Figure 2). The RWC, chlorophyll content, chlorophyll fluorescence, and CMS of the ZmPIF1 transgenic rice were significantly increased under stress conditions. These results suggested that PIFs may be involved in regulating plant growth and development, as well as in responding to drought stress.

**ZmPIF1 positively regulates ABA-dependent drought tolerance by regulating the stomata aperture**

The drought responses of plants are complicated and modulated by multiple molecular pathways. Stomatal pores are involved in drought tolerance (Hetherington and Woodward, 2003; Schroeder et al., 2001). Stomatal responses can regulate water loss from plants to control the utilization of water (Murata et al., 2015). The ZmPIF1 transgenic rice and Arabidopsis all showed lower water loss rate than WT (Figure 3a,b; Figure 4a–c) and significantly promoted stomatal closure and a reduced stomatal...
Selected up-regulated and down-regulated genes in \textit{ZmPIF1} transgenic rice relative to wild-type plants. Genes with at least a 1.5-fold change in the \textit{ZmPIF1} transgenic rice are shown.

| Gene ID | Description | Fold change |
|---------|-------------|-------------|
| LOC_Os01g64000 | bZIP transcription factor, putative, expressed (OsAB5/OsbZIP10) | 1.584962501 |
| LOC_Os12g40920 | bZIP transcription factor domain containing protein, expressed (bZIPB) | 3.663107306 |
| LOC_Os11g26760 | Dehydrin, putative, expressed (OsRAB16C) | 4.36923381 |
| LOC_Os11g26790 | Dehydrin, putative, expressed (OsRAB16A/OsRAB21) | 2.584962501 |
| LOC_Os11g47240 | Leucine-rich repeat receptor protein kinase EXS precursor, putative, expressed (BRI1) | 2.571528352 |
| LOC_Os06g48200 | Glycosyl hydrolases family 16, putative, expressed (BRIII) | 3.353943953 |
| LOC_Os02g43330 | Homeobox associated leucine zipper, putative, expressed | 1.972316441 |
| LOC_Os04g59900 | HLH transcription factor (OsSil1) | 3.415037 |
| LOC_Os11g39000 | Helix-loop-helix DNA-binding domain containing protein, expressed (OsSil2) | 4.196397 |
| LOC_Os10g20880 | O-methyltransferase, putative, expressed | 3.832890014 |
| LOC_Os01g24710 | Jacalin-like lectin domain containing protein, expressed (Salt) | 3.310785373 |
| LOC_Os01g06560 | Transcription factor HBP-1b, putative, expressed | 4.087462841 |
| LOC_Os08g31340 | Heavy metal-associated domain containing protein, expressed | 1.846087317 |
| LOC_Os03g18490 | RPRG, putative, expressed | 2.874469118 |
| LOC_Os07g03730 | SCP-like extracellular protein, expressed | -3.115538327 |
| LOC_Os10g36180 | Expressed protein (OsRD29) | 2.662950103 |
| LOC_Os11g26780 | Dehydrin, putative, expressed (OsRab16D) | 3.841032254 |
| LOC_Os11g26790 | Dehydrin, putative, expressed (OsRAB16A/OsRAB21) | 2.584962501 |
| LOC_Os11g26750 | Dehydrin, putative, expressed (OsRAB16B) | 2.983594355 |
| LOC_Os01g05910 | Late embryogenesis abundant protein, group 3, putative, expressed (OsLEA14a) | 1.767165832 |
| LOC_Os11g37960 | WIP4—Wound-induced protein precursor, expressed (OsPR4b) | 2.52118263 |
| LOC_Os11g37950 | WIP3—Wound-induced protein precursor, expressed (OsPR4c) | 4.377933505 |
| LOC_Os07g48100 | Peroxidase precursor, putative, expressed (POX1_1) | 1.674759171 |
| LOC_Os07g24400 | Peroxidase precursor, putative, expressed (OsPDI1) | 1.722466024 |
| LOC_Os01g39900 | AN1-like zinc finger domain containing protein, expressed (OsSAP13) | -4.6235916956 |
| LOC_Os09g35030 | Dehydration-responsive element-binding protein, putative, expressed (OsDREB1A) | -2.767148182 |
| LOC_Os09g35010 | Dehydration-responsive element-binding protein, putative, expressed (OsDREB1B) | -3.004175126 |
| LOC_Os08g43334 | HSF-type DNA-binding domain containing protein, expressed (OsHsfR2b) | 2.130629443 |
| LOC_Os04g14680 | OsAPX3—Peroxisomal Ascorbate Peroxidase encoding gene 5,8, expressed (ROS-related genes) | 1.628031123 |
| LOC_Os01g60770 | Expansin precursor, putative, expressed (OsEXPA2) | 2.444085868 |
| LOC_Os05g39990 | Expansin precursor, putative, expressed (OsEXPA4) | 1.900422334 |
| LOC_Os10g40710 | Expansin precursor, putative, expressed (OsEXPB2) | 1.865982652 |
| LOC_Os10g40720 | Expansin precursor, putative, expressed (OsEXPB3) | 3.759334072 |
| LOC_Os10g40730 | Expansin precursor, putative, expressed (OsEXPB4) | 4.544220516 |
| LOC_Os10g40700 | Expansin precursor, putative, expressed (OsEXPB6) | 2.911463325 |
| LOC_Os03g01270 | Expansin precursor, putative, expressed (OsEXPB7) | 1.959358016 |
| LOC_Os02g44108 | Expansin precursor, putative, expressed (OsEXPB11) | 2.637429921 |
| LOC_Os02g40240 | Receptor kinase, putative, expressed (LP2) | -2.321928095 |
| LOC_Os10g40909 | Expansin precursor, putative, expressed (OsEXPB9) | -1.736653594 |
| LOC_Os01g68598 | Expessed protein (EPFL9) | -1.823122238 |
| LOC_Os11g32100 | Inducer of CBF expression 1, putative, expressed (OsSAP13) | 2.280107891 |

Plants regulate gene expression levels and stomatal apertures in response to drought stress mainly by enhancing ABA biosynthesis and signalling (Cutler et al., 2010; Pizzio et al., 2013). As previously discussed, the \textit{ZmPIF1} expression level was rapidly induced by ABA (Figure 1g), and \textit{ZmPIF1} transgenic rice had higher survival rates after exposure to drought stress, which promoted stomatal closure (Figure 2; Figure 3c,e-j). Therefore, further exploration of the relationship between ABA and \textit{ZmPIF1} was essential. \textit{ZmPIF1} transgenic rice were tested for ABA responsiveness, and they exhibited a hypersensitive to ABA during germination and postgermination processes and in the seedling stage (Figure 3f–h, k; Figure 5). Moreover, the endogenous ABA levels in transgenic rice were also tested under normal conditions (Figure 5g). However, the endogenous ABA level of \textit{ZmPIF1}
transgenic rice was not significantly altered. Thus, ZmPIF1 was not involved in ABA biosynthesis. We hypothesized that ZmPIF1 participates in the ABA signalling pathway to reduce the stomatal aperture and inhibit the transpiration rate, thereby enhancing water saving and drought-tolerance in the transgenic rice.

Digital gene expression profiling can assist in screening for key genes and possibly mechanisms. Under normal conditions, the DGE profiles of the ZmPIF1 transgenic and WT control rice were compared to identify differentially expressed genes. The expression levels of the ABA biosynthesis-related genes OsNCED1-5 were not significantly changed in ZmPIF1 transgenic or WT rice (Zhu et al., 2009), consistent with the endogenous ABA content (Figure 5g), which further suggested that ZmPIF1 was not involved in the ABA biosynthesis pathway.

OsABI5 is a rice bZIP transcription factor, and OsABI5-overexpression rice are hypersensitive to ABA. In Arabidopsis, in contrast, the ABA sensitivity of abi5-1 can be recovered by OsABI5 in a complementation test (Zou et al., 2008). OsbZIP88 is also a bZIP transcription factor that can interact with OsbZIP71 by forming heterodimers, while OsbZIP71 may be involved in salt and drought tolerance (Liu et al., 2014). Relative to WT plants, the expression levels of OsABI5 and OsbZIP88 increased in ZmPIF1 transgenic rice. Moreover, the expression levels of ABA signalling downstream genes RAB16A and RAB16C increased in ZmPIF1 transgenic rice (Table 1; Hong et al., 2009; Wang et al., 2007). These results suggested that ZmPIF1 participated downstream in the ABA signalling pathway through bZIP transcription factors to positively regulate the ABA signalling pathway.

EXPANSIN is a cell wall protein that is involved in regulating the stomatal aperture and stomatal density (Lü et al., 2013; Marowa et al., 2016; Wei et al., 2011; Zhang et al., 2011). Our DGE results revealed that eight EXPANSIN genes were up-regulated in ZmPIF1-transgenic rice (Table 1). The expression of LP2, a leucine-rich repeat receptor-like kinase, was decreased in ZmPIF1 transgenic rice. The expression of LP2 is down-regulated by drought, while transgenic plants overexpressing LP2 have increased stomatal apertures in leaves (Wu et al., 2015). The expression levels of stomatal-related genes also revealed that ZmPIF1 might participate in regulating the stomatal aperture. In addition, ZmPIF1 transgenic lines showed an induction of the expression of stress-related genes, such as OsRAB16A, OsRAB16B, OsRAB16D, OsPR4b and OsPR4c, even under normal condition (Table 1; Du et al., 2010; Wang et al., 2007). Taken together, the DGE analysis indicated that ZmPIF1 promoted closure of stomata and decreases in the transpiration rate by influencing the ABA-dependent signalling pathway, which enhances water saving and drought resistance in rice.

### ZmPIF1 transgenic rice have an increased grain yield

In crops, overexpression of stress tolerance genes may be lead to abnormal development and productivity loss (Dubouzet et al., 2003; Nakashima et al., 2007; Yu et al., 2013). Thus, it is also important to develop transgenic plants that can enhance stress tolerance and maintain grain yields. Under normal conditions, ZmPIF1 can enhance grain yields in transgenic rice. The increased grain yield was mainly attributable to the increase in the number of panicles, without a substantial change in the number of spikelets per panicle (Table 2; Figure 6; Figure 7; Table S1). An additional phenotype of ZmPIF1-transgenic rice was their tiller number, which was significantly higher than that of WT rice (Figure S9). Thus, the wider tiller angle of ZmPIF1 transgenic rice provided a larger growth space, promoting increased growth of the tillers relative to the WT rice. The grain yields were increased in ZmPIF1 transgenic lines, indicating a wider applicability of ZmPIF1 for crop improvement.

PIFs function as a signal hub in cells and participate in multiple signalling pathways. To date, many studies have shown that PIFs can affect brassinosteroid (BR; de Lucas and Prat, 2014; Oh et al.,

| 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 panicle (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             |
| ZmPIF1|                          |                          |                     |                          |                          |                       |                        |                          |
| OE1   | 13.87 ± 2.75**           | 13.83 ± 2.74**           | 15.63 ± 1.68*       | 145.60 ± 24.77           | 120.00 ± 26.70           | 81.61 ± 2.79**        | 27.54 ± 0.05**         | 27.08 ± 3.10*            |
| OE3   | 13.75 ± 2.98**           | 13.54 ± 3.15**           | 15.27 ± 2.27        | 166.60 ± 18.64           | 122.80 ± 14.10           | 75.94 ± 4.74**        | 25.99 ± 0.13**         | 25.83 ± 3.06             |
| OE7   | 13.17 ± 3.35**           | 12.17 ± 2.85**           | 15.87 ± 1.90**      | 167.00 ± 25.62           | 124.70 ± 19.28           | 73.62 ± 5.81**        | 25.17 ± 0.18**         | 25.59 ± 4.35             |
| 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             |
| ZmPIF1|                          |                          |                     |                          |                          |                       |                        |                          |
| OE1   | 12.60 ± 2.28**           | 12.53 ± 2.33**           | 16.44 ± 0.23**      | 138.27 ± 10.03           | 128.33 ± 9.90            | 93.06 ± 4.52**        | 28.66 ± 0.04**         | 35.82 ± 8.61             |
| OE3   | 13.80 ± 2.44**           | 13.73 ± 2.55**           | 16.49 ± 0.30**      | 148.07 ± 12.76           | 136.97 ± 12.87           | 92.35 ± 4.56**        | 27.41 ± 0.08**         | 38.44 ± 7.93             |
| OE7   | 13.60 ± 1.90**           | 12.63 ± 1.88**           | 16.71 ± 0.66**      | 145.97 ± 13.87           | 134.37 ± 17.05           | 92.00 ± 8.36**        | 25.57 ± 0.14**         | 30.73 ± 6.96             |
| 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             |
| ZmPIF1|                          |                          |                     |                          |                          |                       |                        |                          |
| OE1   | 14.01 ± 3.12*            | 13.62 ± 2.25*            | 14.83 ± 2.04        | 87.24 ± 12.63            | 71.12 ± 10.71*           | 85.23 ± 4.86*         | 30.39 ± 0.12**         | 25.63 ± 1.61*            |
| OE3   | 14.13 ± 3.13**           | 14.07 ± 2.22**           | 14.27 ± 2.27        | 106.34 ± 18.26**         | 80.59 ± 22.03            | 80.91 ± 7.13**        | 27.81 ± 0.06**         | 26.69 ± 1.56**           |
| OE7   | 13.35 ± 2.59*            | 13.04 ± 2.59*            | 14.51 ± 1.52        | 102.12 ± 16.82*          | 84.45 ± 14.11            | 83.38 ± 6.16**        | 27.43 ± 0.11           | 25.00 ± 2.28             |

Values are the mean ± SD (n > 15). * and ** indicate significant differences at P < 0.05 and P < 0.01, respectively.
BR is associated with the plant panicle, and BRs are involved in panicle development in rice. Some BR signal transgenic plants appear to have more tillers, larger panicles, and more seeds per panicle than wild-type plants (Mori et al., 2002; Wu et al., 2008; Zhang et al., 2009). In the present study, the DGE results showed that BR signalling pathway genes, such as BRI1 (LOC_Os11g47240), BRII (LOC_Os06g48200), OsILI1 (LOC_Os04g54900) and OsILI2 (LOC_Os11g39000), were significantly induced in ZmPIF1 transgenic lines (Table 1). Therefore, we hypothesized that ZmPIF1 might affect number of panicles by influencing other signalling pathways such as BR.

In this study, ZmPIF1 was found to be a positive regulator of ABA signalling and to enhance water saving and drought resistance by reducing stomatal opening to control water loss. ZmPIF1 can enhance drought tolerance and improve the grain yield of rice, which indicates that ZmPIF1 plays important roles in drought tolerance and crop improvement. Further investigations are necessary to establish how ZmPIF1 responds to drought stress with ABA signalling-induced stomatal closure.

### Experimental procedures

#### Plant materials and stress treatments

Maize ‘Zhengdan 958’ (Zea mays, hybrid line) was germinated for 6 days. The seedlings were transferred to hydroponic growth conditions at 25 °C with a 16/8-h photoperiod. Four-leaf-stage maize seedlings were used for all the stress treatments. For the ABA treatment, twenty well-grown seedlings were treated for 0, 1, 3, 6, 12, 24 and 48 h with 100 μM ABA (n = 20). For high-salinity and PEG treatment, twenty well-grown seedlings were treated for 0, 1, 3, 6, 12, 24 and 48 h with 200 mM NaCl or 20% PEG6000 (n = 20). For the cold treatment, twenty well-grown seedlings were treated for 0, 1, 3, 6, 12, 24 and 48 h in a growth chamber at 4 °C for cold stress. (n = 20). All tests were repeated a minimum of three times.

Maize plants were grown in the field to obtain different tissues. At the jointing stage, total RNA from roots, stems and leaves was isolated separately. Stamens and pistils were sampled at heading stage.

The wild-type rice material ‘Wuyunjing’ (Oryza sativa L.) and transgenic rice were grown in a chamber at 28/25 °C with a 16/8-h light/dark cycle and 70% relative humidity. The wild-type Arabidopsis thaliana (Columbia 0 type) and transgenic Arabidopsis were placed in a climate chamber at 22 °C with 70% relative humidity and a 12-h light/12-h dark photoperiod. The growth and harvesting of transgenic lines were performed under the...
same conditions. All tests were repeated a minimum of three times.

Sequence homology and phylogenetic analyses
Based on the PIF3 and PIF1 gene sequences in Arabidopsis, a maize PIF gene, named ZmPIF1, was obtained from GenBank and MaizeGDB. The sequence of ZmPIF1 was analyzed at the websites (http://www.expasy.org/, http://www.plantgdb.org/, http://www.ncbi.nlm.nih.gov/ and http://www.maizegdb.org/). The phylogenetic analysis was generated with DNAMAN and MEGA version 6.

Yeast two-hybrid (Y2H) assays
A kit for two-hybrid analysis was obtained (Oebiotech, Shanghai, China). This kit contained all the tools essential for two-hybrid assay, including vectors: pGBK7T, providing the GAL4 DNA-binding domain, and pGADT7, providing the GAL4 activation domain. The following plasmids were constructed in this study: pGBK7T-ZmPhyA1, pGBK7T-ZmPhyA2, pGBK7T-ZmPhyB1, pGBK7T-ZmPhyB2 and pGADT7-ZmPIF1.

Subcellular localization
ZmPIF1 was fused upstream of the GFP gene in the p2GWF7 expression vector and transformed into living onion epidermal cells by biolistic bombardment with a GeneGu. The subcellular location of ZmPIF1 was measured as stated in Gao et al. (2015).

Gene expression quantified by qRT-PCR
Total RNAs were extracted using the manufacturer’s instructions of the TRizol reagent (Takara, China), and reverse transcription reactions were performed using manufacturer’s instructions of the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, IN, Germany). qRT-PCR was performed using a SYBR Green Master Mix kit (Roche, Mannheim, IN, Germany) and specific primers (Table S2) on an ABI 7300 system. Three separate biological replicates were carried out for the qRT-PCR experiments.

Rice and Arabidopsis transformation
To create overexpression constructs of ZmPIF1, a fragment of ZmPIF1 was cloned in maize. The ZmPIF1 fragment was then inserted into the binary plasmid p1011, which contains a ubiquitin promoter. This construct was introduced into Agrobacterium tumefaciens strain EHA105, and transgenic rice of ZmPIF1 were produced as described by Xu et al. (2005).

The ZmPIF1 fragment was subcloned into the vector LZ007, in which transgene expression is under control of the CaMV 35S promoter. Transformation of Arabidopsis was performed by the floral dip method (Clough and Bent, 1998) using Agrobacterium tumefaciens strain GV3101.

Drought phenotype analysis
For PEG treatment at the seedling stage, 2-week-old T3 ZmPIF1 transgenic and two control rice were treated for 4 days with 20% PEG. Ten days after recovery, the survival rate of each line was measured. For drought treatment in soil, 40-day-old rice from each line were sown in individual pots. Water was withheld from the plants of each line for 7 days and then recovered. After 10 days of recovery in water, the survival rate of each line was determined.

Physiological measurements
The chlorophyll florescence, chlorophyll content, RWC and CMS values were measured as stated in Gao et al. (2015). All of the tests were measured under normal or stress conditions (20% PEG6000 for 2 days). These tests used 2-week-old rice of each line.

Phenotype of transpiration assay
Hydroponic cultured seedlings of T3 ZmPIF1 transgenic rice and two controls (40 days old) were transplanted into the same transparent pot filled with hydroponic culture solution. Thirty-five seedlings of each line were planted in each pot and grown under normal conditions. After 3 days, the water level of each line was marked with black lines.

Thirty-day-old seedlings of WT (col) and T3 ZmPIF1 transgenic Arabidopsis were transplanted into the same transparent pots (containing the same weight of soil) with addition of 300 mL water after saturation of the soil water. After 4 days, the water levels of ZmPIF1 transgenic Arabidopsis and WT were marked with black lines. The remaining water in the WT and ZmPIF1 transgenic Arabidopsis was measured after 4 days.

Measurements of water loss rate, transpiration rate and stomatal conductance
Leaves of 40-day-old rice of ZmPIF1 transgenic lines and WT were detached. Leaves of 30-day-old Arabidopsis of ZmPIF1 transgenic lines and WT were detached. The water loss rates of each line were calculated. A portable photosynthesis system (Li-Cor 6400; Li-Cor, Lincoln, NE) was used to test the stomatal conductance and transpiration rates in rice. Flag leaves of 40-day-old rice of each line were measured. For ABA treatment, thirty 40-day rice seedlings were treated for 3 h with 100 μM ABA (n = 40).

Scanning electron microscopy (SEM) images of stomata
Flag leaves of 40-day-old rice of ZmPIF1 transgenic lines and WT were detached. Leaves of 30-day-old Arabidopsis of ZmPIF1 transgenic lines and WT were detached and fixed in glutaraldehyde (2.5%), and images of the stomata were obtained by environmental scanning electron microscopy (XL-30ESEM, PHILIPS, Netherland). Stomatal densities, lengths and apertures were measured randomly using Image-Pro Plus6.0 software (Media Cybernetics, USA).

Germination assay and growth measurement
Thirty-five seeds were placed in square dishes supplemented with 0, 5 and 10 μM of ABA after gerninating for 1 day. After 5 days, the germination rate of each line was determined. Additionally, twenty seeds were transplanted into 96-well plates in which the bottoms were moved after the shoots reached 2 cm. Seeds were grown in water containing 0 and 5 μM ABA. After 7 days of growth, plant heights were measured. Concurrently, twenty seeds were grown in water containing 0, 10 μM ABA. After 5 days, root lengths were measured.

Quantification of the endogenous ABA contents
The ABA levels of ZmPIF1 transgenic lines and the WT were quantified as stated in Zhang et al. (2015). Flag leaves 40-day-old plants from each line were harvested to measure the endogenous ABA contents.
DGE analysis

Thirty well-grown WT and ZmPIF1 transgenic plant seedlings each were selected. The second fully expanded leaves of WT and ZmPIF1 transgenic seedlings were rapidly cut, frozen and stored. We established three replicates of the WT control and named them WT-1, WT-2 and WT-3. We also established OE1, OE3 and OE7 as three replicates of ZmPIF1 transgenic plants and designated OE1, OE3 and OE7 as F1-1, F1-2 and F1-3. Total RNAs were extracted in accordance with the instructions provided with the RNeasy Kit (Tiangen, China). DGE was performed in accordance with the standard protocol of Beijing Genomics Institute (http://www.genomics.cn/index; Shenzhen, China). 

Grain yield analysis

Thirty-day-old seedlings of ZmPIF1 and WT control were transplanted in a paddy field in Jiangsu Province, China. Agronomic traits were calculated for three replicates and 3 (2014), 20 (2015), and 2017. The planting density was 30/m2. The unit area yields of ZmPIF1 transgenic and WT rice were tested in 2017. The planting density was 30/m2. The unit area yields of ZmPIF1 transgenic and WT rice were calculated for 4 replicates in different regions and >100 plants per replicate.

Accession numbers

Sequence data for the genes described in this article can be found in the Maize Genome Initiative or GenBank/EMBL databases under following accession numbers: ZmPIF1 (GRMZM2G115960_T03), ZmPIF3 (GRMZM2G387528_T02), PIF1 (Q8GZM7.1) and PIF3 (Q80536.1).

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Sequence alignment of ZmPIF1 in other Zea mays and Arabidopsis PIFs family.

Figure S2 Phylogenetic tree analysis of ZmPIF1 from other PIFs family.

Figure S3 Confirmation of the subcellular localization of ZmPIF1 by BIFC in N. Benthamiana.

Figure S4 Molecular characterization and phenotypes of ZmPIF1 transgenic rice.

Figure S5 Expression patterns of ZmPIF3 in transgenic Arabidopsis.

Figure S6 The germination rates of ZmPIF1 transgenic rice and wild type seeds under NaCl and mannitol treatment.

Figure S7 Hierarchical clustering analysis of all DEGs.

Figure S8 Quantitative real-time PCR validation of the results of DGE tag profiling.

Figure S9 ZmPIF1 transgenic rice showed wider tiller angle and more number of panicle phenotype.

Table S1 Agronomic traits of ZmPIF1 transgenic plants grown in the paddy field conditions in 2014.

Table S2 Primer pairs used in quantitative real-time PCR.