Overexpression of OsTF1L, a rice HD-Zip transcription factor, promotes lignin biosynthesis and stomatal closure that improves drought tolerance

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Summary
Drought stress seriously impacts on plant development and productivity. Improvement of drought tolerance without yield penalty is a great challenge in crop biotechnology. Here, we report that the rice (Oryza sativa) homeodomain-leucine zipper transcription factor gene, OsTF1L (Oryza sativa transcription factor 1-like), is a key regulator of drought tolerance mechanisms. Overexpression of the OsTF1L in rice significantly increased drought tolerance at the vegetative stages of growth and promoted both effective photosynthesis and a reduction in the water loss rate under drought conditions. Importantly, the OsTF1L overexpressing plants showed a higher drought tolerance at the reproductive stage of growth with a higher grain yield than nontransgenic controls under field-drought conditions. Genomewide analysis of OsTF1L overexpression plants revealed up-regulation of drought-inducible, stomatal movement and lignin biosynthetic genes. Overexpression of OsTF1L promoted accumulation of lignin in shoots, whereas the RNAi lines showed opposite patterns of lignin accumulation. OsTF1L is mainly expressed in outer cell layers including the epidermis, and the vasculature of the shoots, which coincides with areas of lignification. In addition, OsTF1L overexpression enhances stomatal closure under drought conditions resulted in drought tolerance. More importantly, OsTF1L directly bound to the promoters of lignin biosynthesis and drought-related genes involving powx/PRX38, Nodulin protein, DHHC4, CASPL5B1 and AAA-type ATPase. Collectively, our results provide a new insight into the role of OsTF1L in enhancing drought tolerance through lignin biosynthesis and stomatal closure in rice.

Introduction
Drought is an abiotic stress that induces a range of molecular, biochemical and physiological responses in plants, which in turn can result in a reduction in crop productivity (Pandey and Shukla, 2015). Consequently, there is considerable interest in developing drought-tolerant crops that do not exhibit a yield penalty under normal growth conditions. Many studies have identified transcription factors (TFs) to be involved in the drought tolerance mechanisms. Modifying their activity may provide a means to increase the capacity of a plant to avoid drought stress. For example, a number of TFs from rice (Oryza sativa) have been shown to enhance drought tolerance using an overexpression strategy (Hu et al., 2006; Jeong et al., 2010, 2013; Lee et al., 2015, 2016, 2017; Oh et al., 2009; Park et al., 2015; Redillas et al., 2012).

All eukaryotic organisms have homeodomain (HD) type TFs that are characterized by 60 conserved amino acids, and plant-specific homeodomain-leucine zipper (HD-Zip) TFs contain both a homeodomain and a leucine zipper domain (Ariel et al., 2007). These TFs play important roles in plant growth and development, as well as in stress responses. Among them, HD-Zip class I TFs were well studied in stress tolerance mechanisms, such that overexpression of HaHB4, HaHB13, AtHB13 and Zmhdz10 in Arabidopsis enhances tolerance to drought and high salinity (Cabello and Chan, 2012; Cabello et al., 2007; Dezar et al., 2005; Manavella et al., 2006; Zhao et al., 2014). Furthermore, a member of HD-Zip class IV, Arabidopsis EDT1/HDG11 was identified as a key player to improve drought tolerance. Overexpression of AtEDT1/HDG11 enhances tolerance to drought stress in rice, Arabidopsis, tobacco, cotton, pepper and Chinese kale via reduction in stomatal density, acceleration of stomatal closure and improvement of root system (Yu et al., 2008, 2013, 2016; Zhu et al., 2015; Zhu et al., 2016). However, the underlying molecular mechanisms of their drought tolerance are not well understood.

The rice HD-Zip class IV consists of 11 members, which are named as Rice outermost cell-specific (Roc) (Javelle et al., 2011) and O. sativa transcription factor 1 (OsTF1) (Yang et al., 2002). Ito et al. (2002, 2003) demonstrated the epidemis–predominant expression of five Roc genes, Roc1, 2, 3, 4 and 5, suggesting a potential role in epidermal differentiation. OsTF1, another member of the HD-Zip class IV, exhibited a similar expression pattern during early embryogenesis (Yang et al., 2002). Of the 11 class IV genes, only Roc 4 and 5 have been functionally characterized so far to be associated with flowering time (Wei et al., 2016), and with leaf rolling (Zou et al., 2011), respectively. Thus, it remains
elusive to understand roles of the HD-Zip class IV in development and abiotic stress responses of rice.

Many kinds of literature suggest that abiotic stress tolerance is connected to plant lignification. In A. thaliana, overexpression of OsSOD from Potentilla atrosanguinea and/or RarPXX from Rheum austral (Gill et al., 2010; Shafi et al., 2014). In maize (Zea mays), drought-tolerant ibred lines with high expression levels of lignin biosynthetic genes such as cinnamyl alcohol dehydrogenase (CAD), cytochrome protein 96A8 (COMT) or S-adenosyl-L-methionine synthase (SAMS) (Hu et al., 2009) show highly lignified shoots, suggesting a correlation between drought tolerance and shoot lignification. Overexpression of the IbLEA14 gene increases the lignin content in transgenic sweet potato (Ipomoea batatas) calli and enhances tolerance to drought and salt stress due to an up-regulation of lignin biosynthetic genes (Park et al., 2011). In wild watermelon (Citrusllus lanatus), which is notably drought-tolerant, increase in expression levels of lignin biosynthetic genes has been observed, which may represent a survival strategy during progressive drought stress (Yoshimura et al., 2008). Embolism resistance is an indicator of plant response to drought and adaptive strategies under drought conditions (Lens et al., 2013). Greater lignin content in the xylem improves resistance to drought-induced embolism (Peireira et al., 2018), while mutants with reduced lignin content showed increased embolism vulnerability (Awad et al., 2012; Coleman et al., 2008; Voeller et al., 2011). Thus, it appears that high levels of lignification in plants can lead to increased drought tolerance, although the associated regulatory networks have not been well elucidated.

Transpirational water loss through stomata is a key determinant of drought tolerance (Matsuda et al., 2016; Xiong et al., 2002). Regulation of stomatal movement in this regard has been an active research area for drought tolerance. For example, overexpression of OsASR5, ONAC022, AtMYB61, BpiMYB46 and PtoMYB170 significantly enhanced drought tolerance in plants by regulation of stomatal closure under drought stress conditions (Guo et al., 2017; Hong et al., 2016; Li et al., 2017; Romano et al., 2012; Xu et al., 2017). Interestingly, overexpression of AtMYB61, BpiMYB46 and PtoMYB170 also increased lignin biosynthesis.

In this study, we generated overexpression and knockdown transgenic lines of OsTF1L, a rice homeodomain-leucine zipper transcription factor gene, and found that the overexpression lines enhanced drought tolerance and higher grain yield under both normal and drought conditions, whereas the knockdown lines remain susceptible to drought stress. OsTF1L overexpression elevates expression levels of lignin biosynthetic genes in shoots with a concomitant increase in the accumulation of lignin and enhances stomatal closure under drought stress conditions. This OsTF1L-mediated drought tolerance mechanism increases our knowledge about interrelations among lignification, stomatal closure and drought tolerance in rice.

**Results**

**Molecular characterization of OsTF1L**

Rice HD-Zip class IV has 11 members, which consists of the conserved domains such as HD, Zip, steriodogenic acute regulatory protein-related lipid transfer (START) and START-associated domains (SAD) (Figures 1a and S1). Spatial-temporal expression of OsTF1L (Oryza sativa transcription factor 1-like), which belongs to the OsTF1 subclade in a phylogenetic tree (Figure 1a), was determined by qRT-PCR and in situ hybridization. Based on qRT-PCR analyses, OsTF1L is expressed in all organs at various developmental stages (Figure 1b,c) and in situ hybridization analysis revealed that OsTF1L is expressed in the vasculature and outer cell layers, including epidermis (Figure 1d,e). To examine subcellular localization of OsTF1L, OsTF1L coding sequence was fused to the GFP (green fluorescent protein) reporter gene (Figure S2) under the control of the GOS2 (rice euakaryotic translation initiation factor 1-like gene) promoter and transformed into rice protoplasts (Figure 1f). Green fluorescent protein fluorescence was observed in the nucleus of protoplasts, as evidenced by co-localization of the GFP signal with DAPI (4',6-diamidino-2-phenylindole) nuclear DNA stain.

**OsTF1L modulates drought-inducible, stomatal movement and lignin biosynthetic genes**

To uncover functional roles of the OsTF1L, we generated overexpression and RNAi transgenic rice plants. Overexpression (OsTF1LOX) and RNAi knockdown (OsTF1LRNAi) plants were generated using the PGD1 (phosphogluconate dehydrogenase gene) and GOS2 promoter, respectively, which are constitutively active throughout the whole plant body (Figure S2). Thirty independent lines for each type were initially generated, and lines with somaclonal variations were eliminated by successive field selection through T3 generation. We then selected three independent, homozygous lines for each of OsTF1LOX (#17, 23 and 24) and OsTF1LRNAi (#2, 12 and 13) for further analysis. The expression levels of OsTF1L were higher in the OsTF1LOX lines by 3.2- to fourfold and lower in the OsTF1LRNAi lines by 10- to 15-fold, as compared to nontransgenic (NT) lines (Figure 2d). To identify OsTF1L target genes, we performed an RNA-seq analysis with shoots from OsTF1LOX (#23) and NT control plants grown under normal conditions. A total of 1743 genes were expressed at higher levels (>2-fold) in the OsTF1LOX shoots as compared to the NT controls, and they were categorized using GO analysis as follows: metabolic process (25%), response to stimuli (7%), catalytic activity (23%), and ion binding (15%; Figure S3a). We found that 59 drought-inducible genes were up-regulated in the OsTF1LOX shoots by filtering with a public database involving drought response genes (Chung et al., 2016; Table S2). These include PECTIN METHYLESTERASE INHIBITOR PROTEIN (PMI), LATE EMBRYOGENESIS ABUNDANT PROTEIN (LEA14), HEAT SHOCK PROTEIN (HSP70 and 90), Na+/H+ ANTPORTER (NHX4), APELATA 2ethylene response factor (ERF52 and 101) and CYTOCHROME P450 (CYP450). In addition, 25 genes related to stomatal movement were up-regulated in the OsTF1LOX shoots. These include ATP-BINDING CASSETTE SUBFAMILY G PROTEIN (ABC5G), PECTIN METHYLESTERASE (PM2, 6), Ca2+ P-TYPE ATPASE (ACA1), VACUOLAR CATIONPROTON EXCHANGER (CA X2), GLUTAMATE RECEPTOR LIKE (GLR1.1) and NAC DOMAIN-CONTAINING PROTEIN (NAC022) (Hong et al., 2016; Matsuda et al., 2016; Pandey et al., 2007; Table S3). We also found that 29 genes involved in lignin biosynthesis are up-regulated in the OsTF1LOX shoots. These include pEROXIDASE (poxN/PRX38, PRX22 and PRX2), CINNAMYL ALCOHOL DEHYDROGENASE (CAD6 and 7), CAFFEIC ACID O-METHYLTRANSFERASE (COMT5) and CINNAMATE-4-HYDROXYLASE (C4H) (Barros et al., 2015; Hirano et al., 2012; Marjamaa et al., 2009; Wang
et al., 2013; Xu et al., 2009; Table S4). Their increased and decreased levels of expression in the OsTF1L\textsuperscript{OX} and the OsTF1L\textsuperscript{RNAi} shoots, respectively, were validated by qRT-PCR (Figure 2a–c). From this information, we concluded that OsTF1L modulates expression of drought-inducible, stomatal movement and lignin biosynthetic genes.

**OsTF1L directly regulates five target genes**

To examine the interaction between OsTF1L and promoters of the target genes, we performed chromatin immunoprecipitation (ChIP)-seq analysis on the OsTF1L\textsuperscript{OX} (myc-tagged OsTF1L overexpressor) and NT shoots. Two different antibodies, anti-myc and anti-RNA Pol II (Figure S3b), were used to probe interacting loci. Genomewide ChIP-seq analysis led us to identify 345 putative OsTF1L binding sites in the promoter regions (defined here as within a 3-kb region upstream from the transcription start site) of 514 genes. Of these, 336 genes were enriched by more than twofold compared to the NT controls in the RNA Pol II ChIP-seq analysis. Thus, by filtering with RNA Pol II ChIP results, we could select interacting loci that are transcriptionally active. Finally, by cross-referencing of the selected 336 genes with the RNA-seq results (>2-fold; Figure S3c), we identified five putative direct targets of OsTF1L: poxNPRX2B, Nodulin protein, AAA-type ATPase, DDHC4 and CASPL5B1 (Table 1). The genes were down- or up-regulated in OsTF1L\textsuperscript{RNAi} and OsTF1L\textsuperscript{OX} shoots, respectively (Figure S3d). The AAA-type ATPase gene was previously reported to be involved in drought tolerance, and other four genes play roles in lignification (Ito et al., 2000; Qi et al., 2013; Ranocha et al., 2010; Roppolo et al., 2011, 2014; Xia et al., 2013; Zhou et al., 2013). These results suggest that OsTF1L binds directly to promoters of the target genes and regulates their expression to enhance drought tolerance and lignin biosynthesis in rice.

The target genes were found to have potential HD-binding cis-elements AAATTAAA, AAATTAGT, TAAATGTA, CAATGATTG and TGCATTTA (Xu et al., 2014) in their promoters. The interactions between OsTF1L and promoter regions of the five target genes were confirmed by ChIP-qPCR analysis (Figure 3a–j). We further validated the binding activity of OsTF1L to the five target genes by a transactivation assay. Promoters of the five genes were fused to a firefly luciferase (fLUC) reporter and co-transfected into rice protoplasts with either the 35S::OsTF1L construct or an empty vector (Figure 3k). As a result, expression of the five reporter constructs was significantly elevated in the presence of the 35S::OsTF1L construct, but not in the presence of the empty vector (Figure 3l). These results suggest that the OsTF1L binds and activates five direct target genes involved in drought tolerance and lignification.

**OsTF1L overexpression in rice plants confers drought tolerance at the vegetative stage**

As a number of drought-inducible, stomatal movement and lignin biosynthetic genes were differentially expressed by OsTF1L overexpression, we first tested drought tolerance of the OsTF1L\textsuperscript{OX} and OsTF1L\textsuperscript{RNAi} lines after exposed to drought stress. Five-week-old plants were exposed to drought conditions for 3 days by withholding irrigation, where soil moisture content was uniformly decreased over time and reached approximately 10% of soil moisture content at 3 days after the onset of the drought treatments (Figure S4). The OsTF1L\textsuperscript{OX} plants showed delayed visual symptoms of drought-induced damage, such as leaf rolling.
Figure 2  qRT-PCR analysis showing the transcript levels of drought-inducible genes (a), stomatal movement related genes (b) and lignin biosynthetic genes (c) in 1-month-old OsTF1LOX and OsTF1RNAi shoots. Ubi1 was used as the reference gene. Data are shown as the mean ± SD of three biological and two technical replicates. (d) qRT-PCR analysis of OsTF1L expression in 5-week-old OsTF1LOX (#17, 23, 24), OsTF1RNAi (#2, 12, 13) and NT plants. Ubi1 was used as an internal control. Data bars represent the mean ± SD of two biological replicates, each of which had three technical replicates. Asterisks indicate significant differences compared with NT (\( P < 0.05 \), one-way ANOVA).

Table 1  List of putative target genes directly regulated by OsTF1L based on the chromatin immunoprecipitation (ChIP)-seq analysis using RNA Pol II ChIP-seq and the RNA-seq data

| Binding locus         | Gene ID   | Description | RPM (Normalization) | Log2 ratio | RNA-seq |
|-----------------------|-----------|-------------|---------------------|------------|---------|
|                       |           |             | NT-total     | OX-myc     | OX-Pol II | PKM (Normalization) |
| chr03-7132344-7133928 | Os03g0235000 | popN/PNX38  | 7.58        | 29.64      | 17.20    | 1.84     | 1.08     | 0.09 | 0.35     | 3.78 | 1.92   |
| chr01-4057257-40574276 | Os01g0923000 | DHHC4       | 11.57       | 47.32      | 28.60    | 1.94     | 1.24     | 0.14 | 0.38     | 2.64 | 1.40   |
| chr01-3640328-36404182 | Os01g0847300 | CASPL51     | 1.20        | 6.59       | 5.91     | 1.79     | 1.65     | 0.84 | 2.14     | 2.55 | 1.35   |
| chr03-3347905-33480620 | Os03g0802500 | AAA-type ATPase | 21.72   | 65.00      | 47.07    | 1.54     | 1.08     | 10.46 | 38.22   | 3.65 | 1.87   |
| chr12-2733005-2733766  | Os12g0637800 | Nodulin protein | 0.06  | 5.55       | 3.27     | 2.63     | 2.01     | 0.07 | 0.19     | 2.60 | 1.38   |

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and wilting, as compared with the NT and the OsTF1LRNAi plants. After rehydration, the OsTF1LOX plants rapidly recovered, whereas the NT and OsTF1LRNAi plants did not (Figure 4a). The OsTF1LRNAi plants remained as sensitive as the NT plants to the drought stress. Collectively, these results suggested that OsTF1L overexpression confers drought tolerance during vegetative growth.

To validate the drought tolerance of the transgenic plants under delayed drought conditions, 6-week-old plants were transplanted to big pots (two plants per line) and exposed to drought stress by withholding irrigation for 12 days (Figure S5a). Soil moisture contents consistently decreased in the pots containing each of the different genotypes (Figure S5b).

Physiological changes were measured using the JIP test, which reflects the activity of the whole photosynthetic machinery affected by drought stress: $F_v/F_m$ (variable fluorescence and $F_m$: maximum fluorescence) value for photochemical efficiency of photosystem (PS) II, and the $P_{\text{total}}$ (performance index) value for photochemical efficiency of PS I and II (Redillas et al., 2011; Strasser et al., 2004). $F_v/F_m$ values of NT and OsTF1LRNAi plants were rapidly declined during 8 days after drought treatments, whereas those of OsTF1LOX plants remained unchanged (Figure 4b). In addition, the $P_{\text{total}}$ values of the OsTF1LOX plants were higher than those of the NT and OsTF1LRNAi plants during the drought treatments, indicating that the photochemical changes were more significant in OsTF1LOX plants.

**Figure 3** ChIP-qPCR and transient protoplast assays for OsTF1L interacting with promoters of the selected genes in ChIP-seq and RNA-seq analysis. (a–j) Two-week-old OsTF1LOX and NT shoots were used in the ChIP-qPCR experiments with an anti-myc antibody. (a–e) Promoter regions showing three PCR-amplified regions (P1–P3). (f–j) ChIP-qPCR data show each PCR amplification region of each gene. The relative enrichment was normalized with total input. Data are shown as the mean ± SD of three independent experiments. (k, l) Transient protoplast expression assay using a dual-luciferase reporter system. (k) Schematic diagram of the effector, internal control and five reporter constructs. (l) Relative fluc (fluc/luc) activity in rice protoplasts. Data are shown as the mean ± SD of three independent experiments. Asterisks indicate significant differences compared with NT ($P < 0.05$, Student’s $t$-test).
efficiencies of PS I and PS II were less damaged by drought in the OsTF1LOX lines than in the NT and OsTF1LRNAi plants. P(total) values of the OsTF1LRNAi plants decreased sharply up to 8 days after the beginning of the treatment as compared with the NT plants, indicating that the photochemical efficiency of PS I was more perturbed by drought in the OsTF1LRNAi plants than in the NT plants (Figure 4c). These opposite physiological phenotypes in the overexpression and RNAi lines are consistent with a role of OsTF1L in promoting drought tolerance.

OsTF1L overexpression significantly increases grain yield under field-drought conditions

We evaluated yield parameters of the OsTF1LOX lines and the OsTF1LRNAi plants grown in a paddy field under normal and drought stress conditions. The yield parameters were scored from 30 plants per transgenic line, with three replicates (Table 2 and Figure 4d). Under normal growth conditions, the yield parameters of the OsTF1LOX plants including the grain-filling rate and total grain weight were similar or slightly greater than the NT control plants, whereas those of the OsTF1LRNAi plants were lower. Interestingly, under drought stress conditions, the yield parameters of all the OsTF1LOX plants were significantly higher than those of the NT controls, while those of OsTF1LRNAi plants were lower. Thus, overexpression of OsTF1L enhanced drought tolerance and increased grain yield at the reproductive growth stage, in addition to increasing drought tolerance at the vegetative stage.

Overexpression of OsTF1L increases stomatal closure

As the RNA-seq data showed up-regulation of genes involved in stomatal movement, we further examined the stomatal status of OsTF1LOX and OsTF1LRNAi transgenic rice plants grown in field under both normal and drought-treated conditions. Leaf surfaces were observed using SEM to determine the numbers and size of stomata (Figure 5a). As shown in Figure 5b,c, there is no significant difference in the average number of stomata per square millimetre and length of stomatal cell on both the abaxial and adaxial surfaces of the OsTF1LOX, OsTF1LRNAi and NT plants. We next examined stomatal opening and closing in the OsTF1LOX, OsTF1LRNAi and NT leaves. As the stomata could be classified as the close and open (Figure 5d), the stomatal apertures were measured both before and after drought treatments (Figure 5e). Before drought

Figure 4 OsTF1L overexpression in rice confers drought tolerance. (a) Phenotypes of OsTF1LOX and OsTF1LRNAi transgenic rice plants under drought stress at the vegetative stage. Three independent homozygous OsTF1LOX and OsTF1LRNAi T3 lines and nontransgenic (NT) control plants were grown in soil for 5 weeks and exposed to drought for 3 days, followed by re-watering. (b) Chlorophyll fluorescence (Fv/Fm) of OsTF1LOX and OsTF1LRNAi transgenic rice plants and nontransgenic (NT) plants during a 12-day drought treatment. Fv/Fm values were measured in the dark to ensure sufficient dark adaptation. Data are shown as the mean ± SD (n = 30). (c) Performance index (PI(total)) of OsTF1LOX and OsTF1LRNAi transgenic plants and NT plants during drought conditions for 12 days. Data are shown as the mean ± SD (n = 30). (d) Agronomic traits of OsTF1LOX and OsTF1LRNAi transgenic rice plants grown in field under both normal and drought conditions. Spider plots of yield parameters with three independent homozygous T4 lines of OsTF1LOX and two independent homozygous T3 lines of OsTF1LRNAi under normal and drought conditions, respectively. Each data point shows a percentage of the means values (n = 30) listed in Table 2. Mean values from NT controls were set at 100% as a reference. CL, culm length; PL, panicle length; NP, number of panicles per hill; NTS, number of total spikelets; NSP, number of spikelets per panicle; NFG, number of filled grains; FR, filling rate; TGW, total grain weight; 1000 GW, 1000 grain weight.

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Each data value represents the mean (n = 30) for OsTF1LOX, OsTF1LRNAi and nontransgenic (NT) control rice plants (*P < 0.05 and **P < 0.01, one-way ANOVA).

### Discussion

In this study, we found that overexpression of OsTF1L improves drought tolerance and grain yield of rice, providing a valuable candidate for crop biotechnology. The OsTF1LOX plants showed reduced and delayed drought-induced damage, compared with the control plants and recovered more rapidly after rehydration. They also showed decreased water loss and maintained normal photosynthesis efficiency under drought conditions. Although

| Normal condition | OsTF1LOX-17 | OsTF1LRNAi-12 | OsTF1LRNAi-13 | OsTF1LOX-23 | OsTF1LRNAi-2 | OsTF1LRNAi-17 | OsTF1LOX-24 | OsTF1LRNAi-13 | OsTF1LOX-23 | OsTF1LRNAi-2 |
|------------------|-------------|---------------|---------------|-------------|--------------|--------------|-------------|--------------|-------------|--------------|
| Culm length (cm) | 68.71       | 66.30**       | 63.14**       | 65.73       | 63.60**      | 56.44**       | 63.35       | 63.00       | 63.35       | 63.35       |
| Panicle length (cm) | 20.60 | 19.91         | 19.36         | 20.21       | 19.11        | 17.85         | 20.36       | 19.36       | 17.85       | 17.85       |
| No. of Panicles (hill) | 13.09 | 15.00**       | 12.95         | 13.33       | 13.37        | 11.53         | 12.97       | 12.95       | 11.53       | 11.53       |
| No. of total spikelets (hill) | 1484.30 | 1349.87** | 1430.75** | 1505.94 | 1302.56** | 759.94 | 875.80** | 1100.75** | 775.80** | 759.94 |
| No. of spikelets (panicle) | 114.21 | 91.04**       | 86.07**       | 114.94     | 91.04**      | 76.00         | 87.53       | 86.07**     | 76.00       | 76.00       |
| No. of filled grains | 1372.04 | 1124.63** | 929.70** | 1358.44 | 1124.63** | 613.33 | 661.69** | 929.70** | 613.33 | 613.33 |
| Filling rate (%) | 92.35 | 86.28**       | 84.02**       | 90.4*      | 16.62**      | 77.61         | 74.75       | 84.02**     | 77.61       | 77.61       |
| Total Grain weight (g) | 37.87 | 14.15**       | 28.11**       | 37.55      | 27.28**      | 14.29         | 16.62**     | 28.11**     | 14.29       | 14.29       |
| 1000 GW (g) | 27.69 | 23.76**       | 28.56**       | 28.82      | 28.82        | 24.38         | 28.82       | 28.56**     | 24.38       | 24.38       |

Overexpression of OsTF1L significantly increases lignin accumulation

Up-regulation of lignin biosynthetic genes by the OsTF1L overexpression led us to measure lignin levels in the OsTF1LOX, OsTF1LRNAi and NT plants (Figure 6a and Table 3). Lignin levels were higher by 1.8-fold in leaves and stems of the OsTF1LOX plants and lower in the corresponding tissues of OsTF1LRNAi plants than the NT control plants. In contrast, lignin levels in roots of the OsTF1LOX, OsTF1LRNAi and NT plants remained unchanged. Lignin-specific (Phloroglucinol-HCL) staining of those rice shoots confirmed the increased and decreased levels of lignin in the OsTF1LOX and OsTF1LRNAi plants (Figure 6b–g). In particular, the epidermis of the inflorescence stem, as well as the sclerenchyma tissues and vasculature of the leaf sheath of the OsTF1LOX plants, was highly lignified, whereas the corresponding tissues of the OsTF1LRNAi and NT plants remained less lignified. In situ hybridization data that OsTF1L is mainly expressed in the outer cell layers, including the epidermis, and the vasculature of the sheaths (Figure 1d) coincides with areas of increased lignification. Taken together, our results indicate that OsTF1L modulates lignification via up-regulation of lignin biosynthetic genes in rice, which contributes to the drought tolerance phenotype.
drought stress at the vegetative stage can affect rice growth and development, drought tolerance at the reproductive stages is more important for grain productivity (Ambavaram et al., 2014; Nuccio et al., 2015; Xiao et al., 2009; Yu et al., 2013). We observed that yield parameters of the OsTF1LOX plants were significantly higher under field-drought conditions, and saw no evidence of growth retardation or yield trade-off. In contrast, the OsTF1RNAi plants were drought-sensitive and had lower yield parameters than the NT control plants under both drought and normal field conditions.

Lignin is a biopolymer, composed of p-coumaryl, coniferyl and sinapyl alcohol monomers, which makes cell walls stiffer. This study identified that OsTF1L overexpression increased the degree of shoot lignification. Specifically, lignin levels in the OsTF1LOX plants were higher in the epidermis of the inflorescence stem, sclerenchyma tissues and vasculature of the leaf sheath, but were lower in the same tissues of the OsTF1RNAi than in the control plants. In situ hybridizations demonstrated that the OsTF1L is expressed in the outer cell layers including epidermis and vasculature of sheaths that coincides with areas of increased lignification in the OsTF1LOX plants. Despite the whole plant body overexpression of OsTF1L, enhanced lignification of the OsTF1LOX plants occurred only in the typically lignified tissues, rather than being ectopically expressed throughout the plant. These observations were supported by our RNA-seq and qRT-PCR analyses that lignin biosynthetic genes were up- and down-regulated in OsTF1LOX, OsTF1RNAi and NT plants under normal and drought stress conditions. Twenty stomata were measured for each plant. Ten plants were used for each line. Values are mean ± SD. Asterisks indicate significant differences compared with NT (P < 0.05, one-way ANOVA).

In addition to lignin biosynthetic genes, many drought-inducible and stomatal movement genes were up-regulated in OsTF1LOX plants (Tables S2 and S3). The drought-inducible genes include PMEI, a gene for an enzyme that inhibits demethylsterification of pectin, which improves drought tolerance when overexpressed (An et al., 2008). LEA, HSP, NHX, CYP450 and AP2/ERF are among the up-regulated drought-inducible genes, which were reported to improve drought tolerance when overexpressed (Brini et al., 2007; Oh et al., 2009; Sato and Yokoya, 2008; Tamiru et al., 2015; Xu et al., 1996). Furthermore, up-regulation of stomatal movement genes contributes to OsTF1L-mediated drought tolerance.

Figure 5 Overexpression of OsTF1L Increasing Stomatal Closure. (a) Scanning electron microscopy images (600×) of the abaxial and adaxial leaf epidermis, where stomata are marked by black arrowheads. Scale bar represents 100 μm. (b) Average stomata numbers per square millimetre calculated from 10 plants for each line. Values are mean ± SD. (c) Comparison of the stomata length between NT and transgenic plants. Twenty stomata were measured for each plant. Ten plants were used for each line. Values are mean ± SD. (d) Scanning electron microscopy images (2000×) of two levels of stomata opening. Scale bar represents 10 μm. (e) The percentage of two levels of stomatal apertures in the leaves of OsTF1LOX, OsTF1RNAi and NT plants under normal and drought stress conditions. Twenty stomata were measured for each plant. Ten plants were used for each line. Values are mean ± SD. Asterisks indicate significant differences compared with NT (P < 0.05, one-way ANOVA). (f) Relative water content of detached leaves. For each replicate, fully expanded ten leaves of 3-month-old mature plants were used for each line. Data are shown as the mean ± SD of three independent lines and two experimental replicates. Asterisks indicate significant differences compared with NT (P < 0.05, one-way ANOVA).
drought tolerance. The reduced culm number 1 (rcn1) mutant encoding OsABCG5 shows rapid water loss more than the wild-type plants by deficiency of stomatal closure during drought stress (Matsuda et al., 2016). The rice NAC gene, ONAC022, was also identified as a positive role in drought tolerance through modulating a stomatal closure (Hong et al., 2016). The up-regulation of stomatal movement genes is consistent with our observation that OsTF1LOX plants showed high ratio of the reduced rates of water loss under drought conditions. Taken together, these data demonstrate that OsTF1LOX plants modulate multiple molecular mechanisms for drought tolerance including stomatal closure and lignin biosynthesis.

To further understand the OsTF1L-mediated drought tolerance mechanisms in rice, we identified five direct target genes of OsTF1L by the ChIP-seq and RNA-seq analysis: paxN/PRX38, DHHC4, Nodulin protein, CASPL5B1 and AAA-type ATPase. These genes were up-regulated during transient expression of OsTF1L in rice protoplasts and reported to have roles in lignin biosynthesis and drought tolerance. The paxN/PRX38 is responsible for the last step of lignin biosynthesis (Ito et al., 2000). The mutant in the AtPAT10 (Protein S-acyltransferases 10), a member of the DHHC family, was shown to result in reduction in lignified xylem (Qi et al., 2013; Zhou et al., 2013). The WAT1, an Arabidopsis homolog of our rice Nodulin gene, has been shown to reduce lignification in secondary cell wall (Ranocha et al., 2010). In addition, CASPARIAN STRIP MEMBRANE DOMAIN PROTEINS (CASPs) are known to mediate the deposition of Caspian strips in the endodermis by recruiting the lignin polymerization machinery (Roppolo et al., 2011, 2014). This raises a possibility that CASP5B1, a member of the CASP-like protein family, might have some roles in lignin biosynthesis. Additionally, overexpression of SKD1, a member of the AAA-type ATPase proteins, enhanced tolerance to drought and reduced accumulations of reactive oxygen species (ROS; Xia et al., 2013).

In conclusion, OsTF1L has a biological role for drought tolerance by modulating drought-inducible, stomatal movement and lignin biosynthetic genes. We found that overexpression of the OsTF1L gene increases drought tolerance capacity in rice and OsTF1L binds to promoters of five direct target genes, which are associated with lignin biosynthesis and drought tolerance. This study provides novel insights into the function of the HD-Zip class IV family in rice, further indicating its potential importance for crop biotechnology.

Experimental procedures

Plasmid construction and Agrobacterium-mediated rice transformation

For the overexpression of genes in rice, the full-length OsTF1L cDNA (AK100441; Os08g0292000) was amplified by RT-PCR according to the manufacturer’s instructions (Promega, Madison, WI). The coding sequence was inserted into the pE3c vector, which is flanked with a 6 x myc tag coding sequence (Dubin et al., 2008). Finally, the OsTF1L-6 x myc sequence from the

Table 3: Lignin content of leaf, stem and root cell walls of 1-month-old plants

| Sample (mg/g cell wall) | Leaf  | Stem  | Root  |
|-------------------------|-------|-------|-------|
| NT                      | 0.58 ± 0.00 | 0.47 ± 0.10 | 1.35 ± 0.07 |
| OsTF1LOX                | 1.04 ± 0.15 | 0.85 ± 0.10 | 1.37 ± 0.21 |
| OsTF1LRNAi              | 0.50 ± 0.19 | 0.37 ± 0.17 | 1.38 ± 0.04 |

Each data value represents the mean ± SD (n = 30) of 10 plants and three technical replicates.

Figure 6: Accumulation of lignin in OsTF1LOX, OsTF1LRNAi transgenic rice and control plants. (a) Lignin contents in 2-month-old OsTF1LOX, OsTF1LRNAi and nontransgenic (NT) rice plants. Data bars represent the mean ± SD of three biological replicates (n = 3), each of which had two technical replicates. Asterisks indicate significant differences compared with NT (P < 0.05, one-way ANOVA). (b–g) Transverse hand sections of phloroglucinol-HCl stained 3-month-old mature shoots of NT (left), OsTF1LOX (middle) and OsTF1LRNAi (right) plants. (e–g) are enlarged views of the dotted line boxes in (b), (c) and (d), respectively. ls, leaf sheath; is, inflorescence stem; p, pith; va, vascular bundles; ep, epidermis; sc, sclerenchyma tissues.
pE3c-OsTF1L plasmid was subcloned into the p700 rice transformation vector (Lee et al., 2016) carrying the PGD1 promoter (Park et al., 2010) using the Gateway system (Invitrogen, Carlsbad, CA). This construct was named OsTF1L<sup>OX</sup> and was used for constitutive overexpression (Figure S2). For the suppression of OsTF1L expression in rice, 321 bp from the OsTF1L coding region (2103–2423 bp upstream of start codon ATG) was inserted into two sites of the p700-GOS2-RNAi vector (Lee et al., 2016), separated by a β-glucuronidase (GUS) reporter gene sequence (Figure S2). For transient expression of OsTF1L-GFP in rice protoplasts, the OsTF1L coding sequence without the stop codon was inserted into the p600 rice transformation vector (Lee et al., 2015) between the GOS2 promoter (de Pater et al., 1992) and the GFP coding sequence. The vectors were introduced into wild-type rice (<i>Oryza sativa</i> L. var. Japonica cv. Ilmil) using the <i>Agrobacterium tumefaciens</i> (strain LBA4404)-mediated co-cultivation (Jang et al., 1999). For each construct, thirty independent lines were initially generated. Transgene expression levels in T<sub>0</sub> transgenic lines were determined using the qRT-PCR and selected the best five overexpressing lines for OsTF1L<sup>OX</sup> or five down-regulating lines for OsTF1L<sup>RON</sup>. At T<sub>1</sub> generation, homozygous lines were selected through growth in phosphinothricin-containing MS media. Transgenic lines with somaclonal variations were eliminated by successive field selection through the T<sub>3</sub> generation. Finally, we obtained three independent homozygous lines for each of OsTF1L<sup>OX</sup> (#17, 23 and 24) and OsTF1L<sup>RON</sup> (#2, 12 and 13) and used for further analysis.

### qRT-PCR analysis

For gene expression analysis, cDNA was synthesized with oligo-dt primers using a first-strand cDNA synthesis kit (Fermentas, Burlington, ON). qRT-PCR was carried out using 2× qRT-PCR Pre-mix with 20 × EvaGreen<sup>TM</sup> (SolGent, Seoul, Korea). The amplification reactions were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 58°C for 40 s, 72°C for 20 s, in a 20 μL mix containing 1 μL of 20 × EvaGreen<sup>TM</sup>, 0.25 μM primers and 10 ng cDNA. qRT-PCR analysis was performed using a Stratagene Mx3000p instrument and Mx3000p software, v.2.02 (Stratagene, La Jolla, CA). The rice ubiquitin1 (AK121590) transcript was used as a normalization control, and three biological and two technical replicates were analysed for all quantitative experiments. To investigate the spatial and temporal expression patterns of OsTF1L, total RNA was extracted from shoots and roots of 10-, 15-, 30- and 60-day-old NT rice plants at the vegetative stages, and from leaves, roots, node, stems, sheathes and flowers at the reproductive stages. To measure OsTF1L expression levels in OsTF1L-overexpressing and knockdown plants, and to validate the RNA-seq data, total RNA samples were extracted from the shoots of 2-week-old transgenic and NT rice seedlings. All primer pairs are listed in Table S1.

### Measurement of chlorophyll fluorescence

To measure F<sub>Fm</sub> and P<sub>total</sub> values, chlorophyll fluorescence was measured using the Handy-PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments, King’s Lynn, Norfolk, UK) under dark conditions to ensure sufficient dark adaptation (at least 1 h; Redillas et al., 2011). OsTF1L<sup>OX</sup>, OsTF1L<sup>RON</sup> and NT plants were grown in a glasshouse at 28–30°C for 6 weeks. Ten leaves from each plant were analysed and values were calculated using Handy PEA software (version 1.31) and Bilozyer 4HP software (v4.0.30.03.02).

### Drought stress treatment at the vegetative stage

Transgenic and NT rice plants were germinated on Murashige-Skoog (MS) media in a growth chamber in the dark at 28°C for 3 days, followed by light conditions at 30°C for 2 days. Ninety seedlings of each transgenic line and NT control plants were transplanted to soil pots (4 × 4 × 6 cm; 3 plants per pot) and grown for 5 weeks in a glasshouse (16 h light/8 h dark) at 28–30°C. Drought stress was performed by withholding water from the pots for 3 days and irrigation was performed. Soil moisture was measured throughout the experiment to confirm similar water-deficit conditions using a soil moisture sensor (SM150, Delta-T Devices) for 3 days. Before drought treatment and after 2 and 3 days without watering, 10 pots per genotype were measured, respectively. For the IJP test (Redillas et al., 2011; Strasser et al., 2004), 6-week-old transgenic and NT plants were exposed to drought in large soil pots (6 kg of soil) for 12 days using a Handy-PEA fluorimeter.

### Agronomic trait analysis of rice plants grown in a paddy field

To evaluate the agronomic traits of the transgenic and nontransgenic (NT) rice plants, NT plants and three independent T<sub>3</sub> homozygous transgenic lines were planted in a paddy field at the Kyungpook National University, Gunwi (128:34E/36:15N), Korea. The experiment included three replicates where three different plots were planted in a randomized design. The yield components were of 30 plants per line from the three different plots for normal field conditions were then measured. To make the drought field conditions, plants were grown in a tank with a rain-off shelter to cover rice plants from rain. To evaluate the yield parameters of the transgenic plants under drought field conditions, drought stress was imposed both before and after the heading stages, by removing the water. When the NT plants showed visual symptoms of drought stress (e.g. leaf rolling), they were irrigated and the same drought treatment repeated. After two drought stress treatments, the irrigated plants were grown until harvesting while maintaining irrigation. The yield components of 30 plants per line from three different plots for the drought field conditions were measured.

### Subcellular localization of OsTF1L

For transient expression experiments, plasmids containing the OsTF1L coding sequence fused to the GFP reporter coding sequence were transformed into isolated rice protoplasts using PEG-mediated transformation (Jung et al., 2015). For DAPI (Sigma, St. Louis, MO) staining, transformed rice protoplasts were incubated with 2 μg/mL DAPI for 2 min. GFP, chlorophyll and DAPI signals were detected using a Leica SP8 STED laser scanning confocal microscope (Leica, Solms, Germany).

### In situ hybridization

<i>In situ</i> hybridization experiments were performed as previously described (Lee et al., 2003), with minor modifications. Briefly, wild-type rice plants (<i>Oryza sativa</i> L. var. Japonica cv. Ilmil) were grown on MS media for 6 days, and shoots were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 20 min under vacuum at room temperature and for 12 h at 4°C without vacuum. Samples were dehydrated in a graded ethanol series, and finally embedded with paraplast (Sigma). The sections (8 μm) were made, and OsTF1L probes were prepared from the coding
region and 3' UTR (408 bp). Digoxigenin (DIG)-labelled OsTF1L antisense and sense probes were generated by in vitro transcription with DIG-labelled UTP (Roche, Mannheim, Germany) using the SP6 RNA polymerase and the T7 RNA polymerase, respectively (Roche).

**Phloroglucinol-HCl staining**

Hand-cut cross sections of 3-month-old OsTF1LOX, OsTF1LRNAi and NT stems were stained with phloroglucinol-HCl as previously described (Jensen, 1962).

**Lignin extraction and quantification**

Lignin of 1-month-old OsTF1LOX, OsTF1LRNAi and NT leaves, roots and stems were extracted and quantified as previously described (Moreira-Vilar et al., 2014). The lignin concentration was estimated using a standard curve generated with alkali lignin (Sigma-Aldrich, St. Louis, MO).

**RNA-seq**

Total RNA was extracted from shoots of 2-week-old transgenic and NT plants using the RNeasy plant mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instruction. RNA quality and purity were assessed with a Thermo Scientific Nanodrop 2000 and an Agilent Bioanalyzer 2100. RNA-seq libraries were prepared using the TruSeq RNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer’s instructions and sequenced in paired-end (NICEM, Seoul National University, Korea) using the Illumina HiSeq2000 (Illumina). Single-end sequences were generated and raw sequence reads were trimmed to remove adaptor sequences, and those with a quality lower than Q20 were removed using the clc quality trim software (CLCBIO). OsTF1L-bound peaks were identified using peak scoring algorithm MACS (http://liulab.dfci.harvard.edu/MACS). We defined target genes as those that contain ChIP-Seq peaks located within a 3-kb region upstream from the transcription start site. For ChIP-qPCR, the product of ChIP was analysed via quantitative PCR with a Mix3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA). The relative enrichment was normalized with total input. All primer sequences are listed in Table S1.

**Transactivation assay**

Full-length OsTF1L and 3-kb upstream sequences of target genes were amplified by PCR using high-fidelity DNA polymerase PrimeStar (TaKaRa, Tokyo, Japan). OsTF1L was cloned into a pBEST vector containing a 35S promoter by BamHI and PstI restriction enzyme using In-Fusion HD cloning system (TaKaRa). Target genes promoter region were cloned into linearized pGST6-LUC-NOS vector harbouring a GST6 enhancer/promoter-FIRELY LUCIFERASE by BamHI and NcoI restriction enzyme using In-Fusion HD cloning system (TaKaRa). 15 μL of vector solution including 3 μg of effector, 1 μg of reporter and 0.5 μg of internal control was transfected into isolated rice protoplasts using PEG (polyethylene glycol)-mediated transformation (Jung et al., 2015). The Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI) was used to measure the luciferase activity according to manufacturer’s manual, and the fluorescence values of luciferase were detected with an Infinite M200 System (Tecan, Seestrasse, Ménndorf, Switzerland). Three independent transfections for each sample were performed, and the value of firefly luciferase was normalized to that of renilla luciferase. The 35S::rLUC was used as an internal control.

**Scanning electron microscopy analysis**

Leaves of 1-month-old NT and transgenic plants were detached. Stomatal density and aperture were measured using scanning electron microscopy (SEM) (Hitachi TM3030Plus, Europark Fichtenhain, Krefeld, Germany). For stomatal density analysis, average stomata numbers per square millimetre were calculated from ten plants for each line. For comparison of the stomata length between NT and transgenic plants, twenty stomata were measured for each plant. Ten plants were used for each line. For stomatal aperture analysis, the two types (open and close) of stomata in the leaves of OsTF1LOX, OsTF1LRNAi and NT plants were counted under normal and drought stress (30 min) conditions. Twenty stomata were measured for each plant. Ten plants were used for each line.

**Accession numbers**

Genes from this article can be found in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: GSE93287 (RNA-seq), GSE93288 (Chip-seq), paxNP3 (Os03g0235000), PRX2 (Os01g0963000), PRX2 (Os03g0434800), CAD6 (Os04g0229100), CAD7 (Os04g0612700), COMTL5 (Os04g0175600), ABCG5 (Os03g0281900), CYP450 (Os10g0513900), ERF52 (Os05g0536250), ERF101 (Os04g0389000), HSP70 (Os03g0277300), LEA14 (Os01g0705200), NHX4 (Os06g0318500), PME1
(Os12g0283400), ABCG5 (Os03g0281900), PME2 (Os01g0311800), PME6 (Os01g0788400), ACA1 (Os03g0203700), CAX2 (Os02g0138900), GLR1.1 (Os09g0431100), ONAC022 (Os03g0133000), Nodulin protein (Os12g0637800), AAA-type ATPase (Os03g00802500), DHHC4 (Os01g0925300) and CASPL5B1 (Os01g0847300).

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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 section at the end of the article.