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RESEARCH PAPER

Lipid transfer protein 3 as a target of MYB96 mediates freezing and drought stress in *Arabidopsis*

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Abstract

Several lipid-transfer proteins were reported to modulate the plant response to biotic stress; however, whether lipid-transfer proteins are also involved in abiotic stress remains unknown. This study characterized the function of a lipid-transfer protein, LTP3, during freezing and drought stress. *LTP3* was expressed ubiquitously and the LTP3 protein was localized to the cytoplasm. A biochemical study showed that LTP3 was able to bind to lipids. Overexpression of *LTP3* resulted in constitutively enhanced freezing tolerance without affecting the expression of *CBFs* and their target *COR* genes. Further analyses showed that *LTP3* was positively regulated by MYB96 via the direct binding to the *LTP3* promoter; consistently, transgenic plants overexpressing *MYB96* exhibited enhanced freezing tolerance. This study also found that the loss-of-function mutant *ltp3* was sensitive to drought stress, whereas overexpressing plants were drought tolerant, phenotypes reminiscent of *myb96* mutant plants and *MYB96*-overexpressing plants. Taken together, these results demonstrate that *LTP3* acts as a target of MYB96 to be involved in plant tolerance to freezing and drought stress.

Key words: *Arabidopsis*, drought stress, freezing tolerance, gene regulation, lipid-transfer protein 3, *MYB96*.

Introduction

Low temperature is the environmental factor that limits plant growth, development, and distribution. Most temperate plants have evolved mechanisms to adapt to the seasonal variations in temperature through cold acclimation in which a prior exposure to low non-freezing temperatures increases their ability to survive under subsequent freezing temperatures. Cold acclimation involves transcriptional regulation that induces cold-regulated (COR) genes, some of which encode proteins and enzymes that mediate lipid and sugar metabolism to protect plant cells from freezing injury. In *Arabidopsis*, most *COR* genes are directly regulated by the CBF/DREB (C-repeat-binding factor/dehydration-response element-binding protein) family of transcription factors (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006). Overexpression of *CBF1*-*CBF3* in *Arabidopsis* upregulates *COR* gene expression and concomitantly enhances freezing tolerance (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2004), and the constitutive expression of *COR* genes also enhances freezing tolerance (Artus *et al.*, 1996; Steponkus *et al.*, 1998). *CBF* expression is transcriptionally regulated by the transcription factors ICE1 (inducer of *CBF* expression 1), CAMTA3 (calmodulin-binding transcription activator 3), and MYB15 (Chinnusamy *et al.*, 2003; Agarwal *et al.*, 2006; Doherty *et al.*, 2009). ICE1 is negatively regulated by an E3 ubiquitin ligase, HOS1 (high expression of osmotically responsive genes 1) and positively regulated by a SUMO E3 ligase, SIZ1 (SAP and Miz), at the post-translational level (Dong *et al.*, 2006a; Miura *et al.*,, 2007). In addition, several other components have been identified that modulate freezing tolerance without affecting *CBF* expression (Xin and Browse, 1998; Gong *et al.*, 2005; Dong *et al.*, 2006b; Lee *et al.*, 2006; Knight *et al.*, 2008; Zhu *et al.*, 2008).

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Plant non-specific lipid-transfer proteins (LTPs) are small, basic, secreted proteins (Kader, 1996). The conserved features of plant LTPs include four disulphide bonds of eight cysteine residues and two pentapeptide consensus motifs (Maldonado *et al.*, 2002). These proteins are able to transfer several different phospholipids and can bind fatty acids. There are approximately 100 LTPs or putative LTPs in *Arabidopsis*, and four major groups are classified based on phylogenetic analysis (Chae *et al.*, 2009). Among them, group I includes 13 SCA (stigma/style Cys-rich adhesin)-like LTPs which are related to conventional LTPs. Several group I LTPs have been characterized. The overexpression of barley *LTP4* in tobacco (*Nicotiana tabacum*) enhances the plant's resistance to a bacterial pathogen (Molina and Garcia-Olmedo, 1997), and tobacco LTP2 has been shown to mediate cell-wall loosening *in vitro* (Nieuwland *et al.*, 2005). *Arabidopsis* LTP5 is implicated in pollen tube tip growth and fertilization (Chae *et al.*, 2009). Groups II and III are mostly as LTP-like proteins. They include DIR1 and AZI1 which function in plant defence responses (Jung *et al.*, 2009; Maldonado *et al.*, 2002). LTPG in group III is required for the export of lipids to the plant surface (Debono *et al.*, 2009). However, whether LTPs function in cold stress in plants remains unclear.

LTP3 from group I is induced by abiotic stress (Seo *et al.*, 2011). This study investigated the biological function of *LTP3* by altering its expression. The overexpression of *LTP3* was constitutively enhanced the freezing tolerance of *Arabidopsis*. Further investigation indicated that the expression of *LTP3* is positively regulated by MYB96, and *in vitro* and *in vivo* assays demonstrated that MYB96 could directly bind to the promoter of *LTP3*. Consistently, *MYB96*-overexpressing transgenic plants exhibited enhanced freezing tolerance that was similar to that of *LTP3*-overexpressing plants. In addition, similar to *myb96*, *ltp3* was more sensitive to drought stress than wild-type plants. These results demonstrate that *LTP3* is positively regulated by MYB96 and plays important roles in the response of *Arabidopsis* plants to abiotic stress.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 plants were used in this study. The plants were grown at 20 ± 1 °C under a 16/8 h light/dark cycle at 100 μmol m^{-2} s^{-1}, with 50–70% relative humidity in soil or on MS medium (Sigma) containing 2% sucrose and 0.8% agar. The *ltp3-1* (SALK_095248) and *myb96-2* (SAIL_815_G17) mutants were obtained from The *Arabidopsis* Information Resource (TAIR, http://arabidopsis.org).

For the drought-tolerance assay, water was withheld from plants that had grown in soil under normal conditions in a growth room for 2 weeks, after which, the plants were watered and the number of surviving plants was counted after 1 week. For water loss of detached leaves, leaves were detached from 3-week-old plants, placed on a Petri dish for 1, 2, 3, 4, 5, and 6 h, and weighed (Hua *et al.*, 2012). Three independent experiments were performed, with 30 plants for each line in each experiment.

Plants were treated with abscisic acid (ABA) by soaking 2-week-old seedlings in an aqueous 10 μM solution for 0, 1, 3, 6, and 12 h. Similarly, in treatment with methyl viologen (MV), an oxidative stress inducer (Dodge, 1971), plants were soaked in an aqueous 10 μM solution of MV for 0, 3, 6, and 12 h. There were three replications, each replicate contained 50 seedlings, and the study was repeated three times. For all studies involving RNA extraction, seedlings were then rinsed in sterile distilled water, frozen in liquid nitrogen, and stored at –80 °C.

Plasmid construction and plant transformation

MYB96 genomic DNA was amplified using primers MYB96-1 and MYB96-2 and cloned into pBI121 to generate *pSuper::MYB96*. *MYB96* cDNA was amplified using primers MYB96-3 and MYB96-2 and cloned into the binary vector pER8 (Zuo *et al.*, 2000) to generate *pER8::MYB96*. *MYB96* cDNA was amplified using primers MYB96-3 and MYB96-2 and cloned into pQE-80L (Qiagen) to generate MYB96-His-expressing vector. To construct *pSuper::MYB96-Myc*, *MYB96* cDNA was amplified using the primers MYB96-3 and MYB96-4 and subsequently cloned into the pSuper1300 vector (Yang *et al.*, 2010) containing a 6 × Myc tag.

LTP3 cDNA was amplified using the primers LTP3-1 and LTP3-2 and cloned into pSuper1300 to generate *pSuper::LTP3*. *LTP3* cDNA was amplified using the primers LTP3-1 and LTP3-8 and cloned into pGEX-4T-1 (Chen *et al.*, 2009) to generate the construct *LTP3-GST*. *LTP3* cDNA was amplified using primers LTP3-1 and LTP3-4 and fused in-frame with GFP in pSuper1300::GFP (Shi *et al.*, 2012) to generate *pSuper::LTP3-GFP*. For the molecular complementation assay, a 2.0-kb genomic fragment comprising the *LTP3* promoter and the coding region was cloned into pCAMBIA1300 (Cambia) to generate the *pLTP3::*LTP3 construct. All of the primers used are listed in Supplementary Table S1 (available at *JXB* online).

Agrobacterium tumefaciens GV3101 was transformed with the different constructs and used to transform wild-type, *myb96-2*, and *ltp3-1* plants via the floral dip method (Clough and Bent, 1998).

GUS activity

A 1.7-kb genomic fragment upstream of the *LTP3* ATG start codon was amplified by PCR using the LTP3-p1 and LTP3-p2 primers. The putative MYB96 binding sites were named X, Y, and Z, so the XYZ, YZ, and Z binding site regions were amplified using the primers LTP3-P11 and LTP3-P2, LTP3-P13 and LTP3-P2, and LTP3-P15 and LTP3-P2, respectively. These fragments were cloned into the plasmid pPZPGUS2 (Diener *et al.*, 2000) to generate different *pLTP3::GUS* constructs.

For transient GUS assay, *pLTP3::GUS* vectors and *pSuper::MYB96* along with *35S::LUC* were coinfiltrated into *Nicotiana benthamiana* leaves. The cotransformation of *pZPGUS2* and *pSuper::MYB96* along with *35S::LUC* was used as the control. The GUS activity was assayed using methylumbelliferyl glucoronide (Sigma Aldrich), and the LUC activity was used as an internal control. The GUS/LUC ratio was used to determine the promoter activity (Shi *et al.*, 2012).

Freezing tolerance, ion leakage, and proline content assays

The freezing-tolerance assay was performed as described (Shi *et al.*, 2012). The electrolyte leakage was measured as previously described (Lee *et al.*, 2002). The proline content was measured as described (Bates *et al.*, 1972).

Lipid-binding assay

Fluorescent binding assays were performed as described (Buhot *et al.*, 2004). The purified recombinant LTP3-GST protein was mixed with various concentrations of 2-*p*-toluidinonaphthalene-6-sulphonate (TNS) at 25 °C. This mixture was excited at 320 nm and the emission at 437 nm was recorded using a spectrofluorometer (Hitachi F-7000). Purified GST was used as a control. Kd value was calculated using Sigma Plot software.

RNA extraction and real-time PCR

Total RNA was extracted from 14-day-old seedlings grown on MS plates using TRIzol (Invitrogen), followed by treatment with DNase I (Takara). The treated RNA samples were subjected to first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega). Real-time PCR was performed using SYBR Green PCR Master Mix (Takara), SYBR Premix Ex Taq (Takara), and an Applied Biosystems 7500 real-time PCR system, as previously described (Huang *et al.,* 2010).

Recombinant protein purification and electrophoretic mobility shift assay

The MYB96-His vector was expressed in *Escherichia coli* BL21 (DE3). The recombinant protein was purified using Ni-agarose affinity. An electrophoretic mobility shift assay (EMSA) was performed as described (Shi *et al.,* 2012). The XYZ, XY regions, and XY mutant region of the LTP3 promoter were amplified using primers LTP3-P3 and LTP3-P4, LTP3-P3 and LTP3-P8, and LTP3-P5 and LTP3-P6, respectively. The biotin-labelled DNA fragments listed in Supplementary Table S1 were synthesized and used as probes, and the biotin-unlabelled DNA fragments of the same sequences were used as the competitors in this assay.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed as previously described (Gendrel *et al.,* 2005) using MYB96-MYC transgenic plants. Two-week-old MYB96-MYC seedlings were fixed in 1% formaldehyde for 15 min and neutralized with 0.125 M glycine for an additional 5 min under vacuum. After being washed twice with cold sterilized water, the tissues were homogenized, and the chromatin was isolated and sonicated. The MYB96 protein was immunoprecipitated using an anti-Myc antibody (Sigma Aldrich). The enrichment of the DNA fragments was determined using quantitative real-time PCR (qRT-PCR) with the primers listed in Supplementary Table S1. The experiments were repeated three times with similar results.

Confocal laser microscopy

Protoplasts were prepared from leaves of 4-week-old plant under a 12/12 h lightdark cycle as described (Yoo *et al.,* 2007). The fluorescence of GFP in the transformed *Arabidopsis* protoplasts was visualized using a confocal laser-scanning microscope (LSM510, Carl Zeiss) at 2–4 days after infiltration.

Results

Expression pattern of LTP3 and localization of LTP3

LTP3 is induced by abiotic stress (Seo *et al.,* 2011), implying this gene might be involved in the plant responses to abiotic stress. To study the biological function of *LTP3*, this study first examined the tissue-specific *LTP3* expression in *Arabidopsis.* Total RNA was extracted from various tissues, including the roots, stems, leaves, flowers, and siliques, and the transcript levels were analysed by qRT-PCR. *LTP3* was strongly expressed in the cauline leaves, flowers, and siliques (Fig. 1A), a result that was verified by the GUS staining of the transgenic plants that expressed *GUS* driven by the *LTP3* promoter. The GUS activity was high in the leaves, flowers and siliques but was low in the roots, stems, and seeds (Fig. 1B).

Next, the expression patterns of *LTP3* were analysed by qRT-PCR under stress conditions. *LTP3* was found to be slightly upregulated by cold stress after 3–6 h treatment but was reduced thereafter (Fig. 1C). The transcript level of *LTP3* was dramatically increased by dehydration and ABA treatment (Fig. 1D and E).

To determine the subcellular localization of LTP3, The *pSuper::LTP3-GFP* construct was generated and

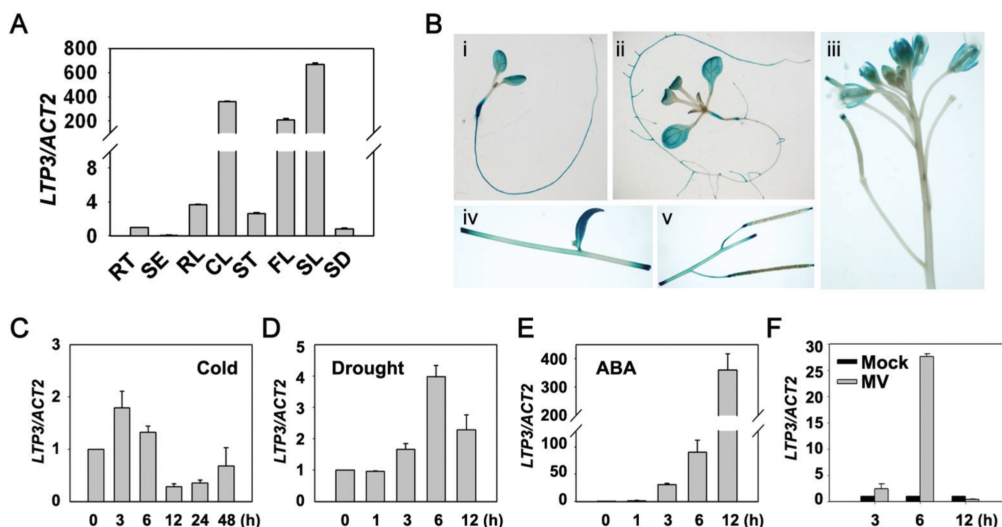

Fig. 1. Expression patterns of *LTP3*. (A) *LTP3* expression in various organs of *Arabidopsis* plants by qRT-PCR. Total RNA was isolated from roots (RT), four-leaf stage seedlings (SE), rosette leaves (RL), cauline leaves (CL), stems (ST), flowers (FL), mature siliques (SL), and seeds (SD). The data represent the means of three replicates ± SD. (B) *GUS* expression in seedlings at the two-leaf (i) and four-leaf (ii) stages, stem and cauline leaves (iii), inflorescence and flowers (iv), and mature siliques (v) of *LTP3::GUS* transgenic plants. (C–F) Expression of *LTP3* under cold (C), drought (D), ABA (E), and MV treatment (F) by qRT-PCR. Total RNA was extracted from plants treated with cold (4 °C), drought, abscisic acid (ABA, 10 μM), or methyl viologen (MV, 10 μM) for the indicated times. The transcription levels were determined by qRT-PCR. The data represent the means of three replicates ± SD.

transformed into *Arabidopsis* protoplasts. The GFP signal was examined using confocal microscopy. The green fluorescence of the protoplasts harbouring *pSuper::LTP3-GFP* was observed throughout the cytoplasm; the control protoplasts containing *pSuper::GFP* were localized ubiquitously (Fig. 2A).

LTP3 possesses lipid-binding activity

As LTP3 is a putative lipid-transfer protein, this study examined whether LTP3 exhibited lipid-binding activity using recombinant LTP3 and a fluorescent lipid substrate. The LTP3-GST protein was expressed in *E. coli*, purified using GST beads, and incubated with the fluorescent probe TNS, which emits an intense fluorescence when bound in a hydrophobic condition (Debono *et al.*, 2009). The fluorescence increased intensely with increasing concentrations of TNS, with a binding Kd of 9.8±4.7 μM (Fig. 2B). However, the fluorescence intensity did not increase with the TNS concentrations when a protein buffer or only GST was used. These results suggest that LTP3 protein has a lipid-binding activity *in vitro*.

Overexpression of LTP3 confers enhanced freezing tolerance

To dissect the biological function of *LTP3* further, a T-DNA insertion line of *LTP3* (SALK_095248), named *ltp3-1*, was obtained (Fig. 3A); qRT-PCR analysis showed that *ltp3-1* was a loss-of-function mutant (Fig. 3B). This study also generated transgenic plants that overexpressed *LTP3* driven by a Super promoter (*LTP3-OE*). A total of 20 independent transgenic plants were generated, and the T3 homozygous *LTP3-OE3* and *LTP3-OE4* plants were found to overexpress *LTP3* (Fig. 3B) and were chosen for further studies. Under normal growth conditions, the *ltp3-1* mutant plants and *LTP3-OE* plants were indistinguishable from the wild-type plants (Fig. 3C, Supplementary Fig. S1A). These results indicate that altered *LTP3* expression does not significantly affect plant growth and development under normal growth conditions.

To investigate the possible role of *LTP3* under stress conditions, this study first examined the freezing tolerance of the wild-type, *ltp3-1*, and *LTP3-OE* plants. Two-week-old

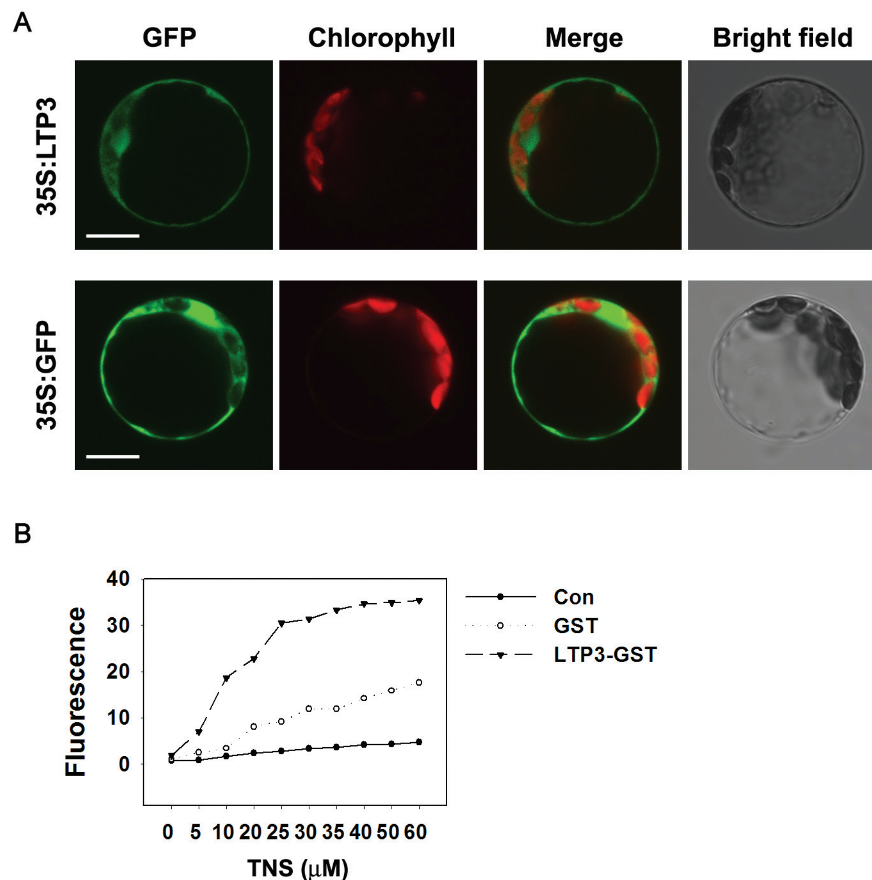

Fig. 2. Subcellular localization of LTP3 in *Arabidopsis* protoplasts and the lipid-binding activity of LTP3. (A) *pSuper::LTP3-GFP* and *pSuper::GFP* plasmids transformed into *Arabidopsis* protoplasts and signals detected using a confocal laser-scanning microscope. From left to right, green fluorescence signals, chlorophyll red autofluorescence, an overlay of the green and red signals, and bright-field images. Bar: 20 μm. (B) Purified recombinant LTP3-GST binds the TNS lipophilic probe. Recombinant LTP3-GST was expressed in *E. coli*, purified using GST beads, and incubated with increasing concentrations of TNS. Black triangles indicate recombinant LTP3 proteins (5 μM), white circles indicate GST only, and black circles indicate the protein buffer control.

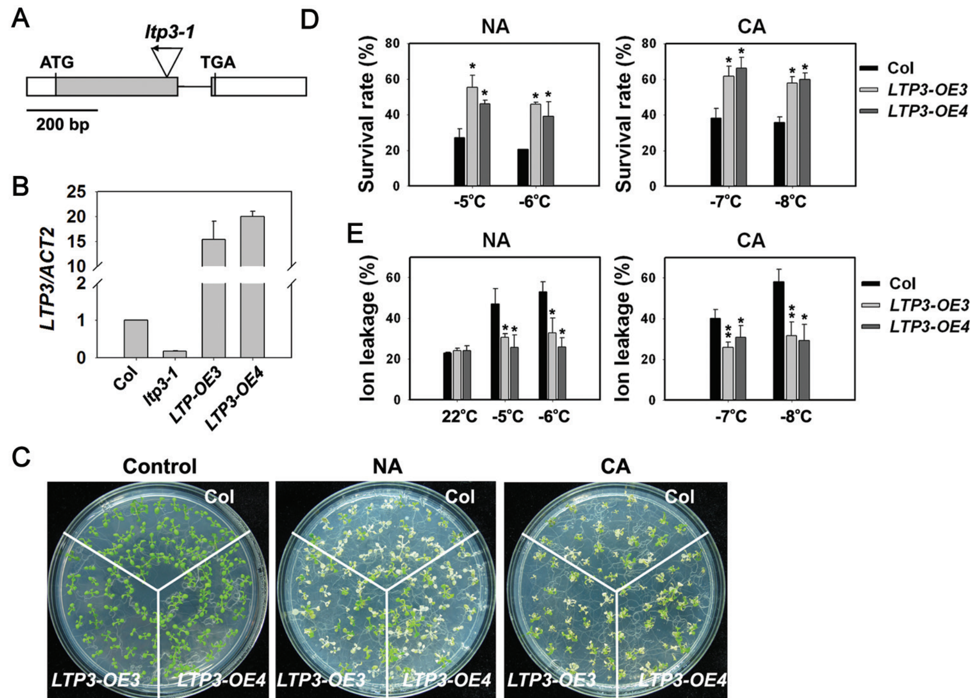

Fig. 3. Freezing-tolerance assay of *LTP3-OE* plants. (A) The genomic structure of *LTP3*. Exons, untranscribed regions, and intron, are indicated by grey boxes, white boxes, and lines, respectively. The T-DNA position in the *ltp3* mutant is shown. (B) *LTP3* expression in 2-week-old *ltp3-1* mutant and *LTP3*-overexpressing seedlings by qRT-PCR. The data represent the means of three replicates ± SD. (C) Freezing phenotypes of *LTP3-OE* transgenic plants. Two-week-old seedlings were treated at –5 °C for 1 h (non-acclimated, NA) or –7 °C for 1 h after 4 °C treatment for 4 d (acclimated, CA), and the pictures were taken after a 2-d recovery at 22 °C. (D) Survival rate of the seedlings in (C) after freezing. The surviving seedlings that could regrow were scored after a 2-d recovery. The data represent the mean values of three replicates ± SD; $^*P < 0.01$ (Student's t-test). At least three independent experiments were performed with similar results. (E) Ion leakage assay of the seedlings in (C) at the indicated freezing temperatures. The data are the mean values of three replicates ± SD; $^*P < 0.05$, $^{**}P < 0.01$ (Student's t-test).

LTP3-OE and wild-type seedlings grown on agar plates at 22 °C were subjected to a freezing treatment with or without cold acclimation, transferred to 4 °C for 12 h, and then returned to the normal growth conditions for 4 d and assayed. The survival rates of the non-acclimated *LTP3-OE* seedlings were much higher than the wild-type seedlings after treatment at –5 and –6 °C (Fig. 3C and D). Consistently, following a cold acclimation at 4 °C for 4 days, the *LTP3-OE* seedlings were more tolerant to freezing than wild type after treatments at –7 and –8 °C (Fig. 3C and D). However, the *ltp3-1* mutant resembled the wild-type plants with regard to freezing tolerance (Supplementary Fig. S1). Considering *LTP3* shares high sequence similarity with several other *LTP* genes (Supplementary Fig. S2A), including *LTP4*, *LTP8*, and *LTP12*, this study checked whether their expression is affected in *ltp3-1* mutant. Without cold treatment, expression of *LTP4* and *LTP12* was comparable to *LTP3* in the wild type and *ltp3-1* mutant. After cold treatment, the expression of *LTP4* and *LTP8* in *ltp3-1* was higher than that in the wild type, whereas *LTP12* expression in *ltp3-1* was lower than the wild type (Supplementary Fig. S2B). These results suggest that the normal response of *ltp3-1* mutant to freezing stress may due to the redundancy of *LTP* genes.

The electrolyte leakage as an indicator of membrane injury was measured in the non-acclimated and acclimated seedlings. The electrolyte leakage of the *LTP3-OE* seedlings was much lower than that of the wild-type seedlings in both non-acclimated and acclimated plants (Fig. 3E). These results indicate that the overexpression of *LTP3* results in a constitutive freezing tolerance of plants.

Expression of stress-responsive genes in LTP3-overexpressing plants

The CBF pathway is known to play an important role in cold stress signalling (Thomashow, 1999). This current study next determined whether the enhanced freezing tolerance of *LTP3-OE* was caused by altered expression of cold-responsive genes in the CBF pathway. qRT-PCR analysis showed that *CBF1*, *CBF2*, *CBF3*, and their target genes, such as *RD29A* and *COR47*, were induced by cold in both wild-type and *LTP3-OE* plants; however, no obvious differences in the expression levels were observed between the wild-type and *LTP3-OE* plants before and after cold treatment (Supplementary Fig. S3). Therefore, LTP3 appears to be involved in freezing stress independently of the *CBF* pathway.

Overexpression of LTP3 increases soluble sugar accumulation

Because soluble sugar and proline are important osmolytes that protect plants against cold stress (Nanjo *et al.*, 1999; Wanner and Junttila, 1999), the soluble sugar and proline contents were measured in wild-type and *LTP3-OE* plants. The *LTP3-OE* plants accumulated more soluble sugar than the wild-type plants at 22 °C (Supplementary Fig. S4A). Cold stress induced the accumulation of soluble sugar in the wild-type plants, and the increase in the soluble sugar content in the *LTP3-OE* plants was higher compared to the wild type after cold treatment (Supplementary Fig. S4A). In contrast, no notable differences in the proline levels were detected between the *LTP3-OE* and wild-type plants with or without cold treatment (Supplementary Fig. S4B).

Alteration in LTP3 expression affects plant responses to drought and oxidative stress

To investigate the role of *LTP3* in other abiotic stress, this study next examined the effect of *LTP3* on drought and oxidative stress. Two-week-old seedlings of *ltp3-1, LTP3-OE*, and wild type were exposed to dehydration by withholding water for 2 weeks. The plants were then watered and cultured continually for 1 week and the plant survival rates were measured. The *ltp3-1* mutant was more sensitive to drought than the wild type, whereas the *LTP3-OE* plants exhibited an enhanced tolerance to drought (Fig. 4A and B). Moreover, *ltp3-1* lost more water, whereas *LTP3-OE* lost less water than the wild type in the water loss assay of detached leaves (Fig. 4C). To determine whether the drought sensitivity of

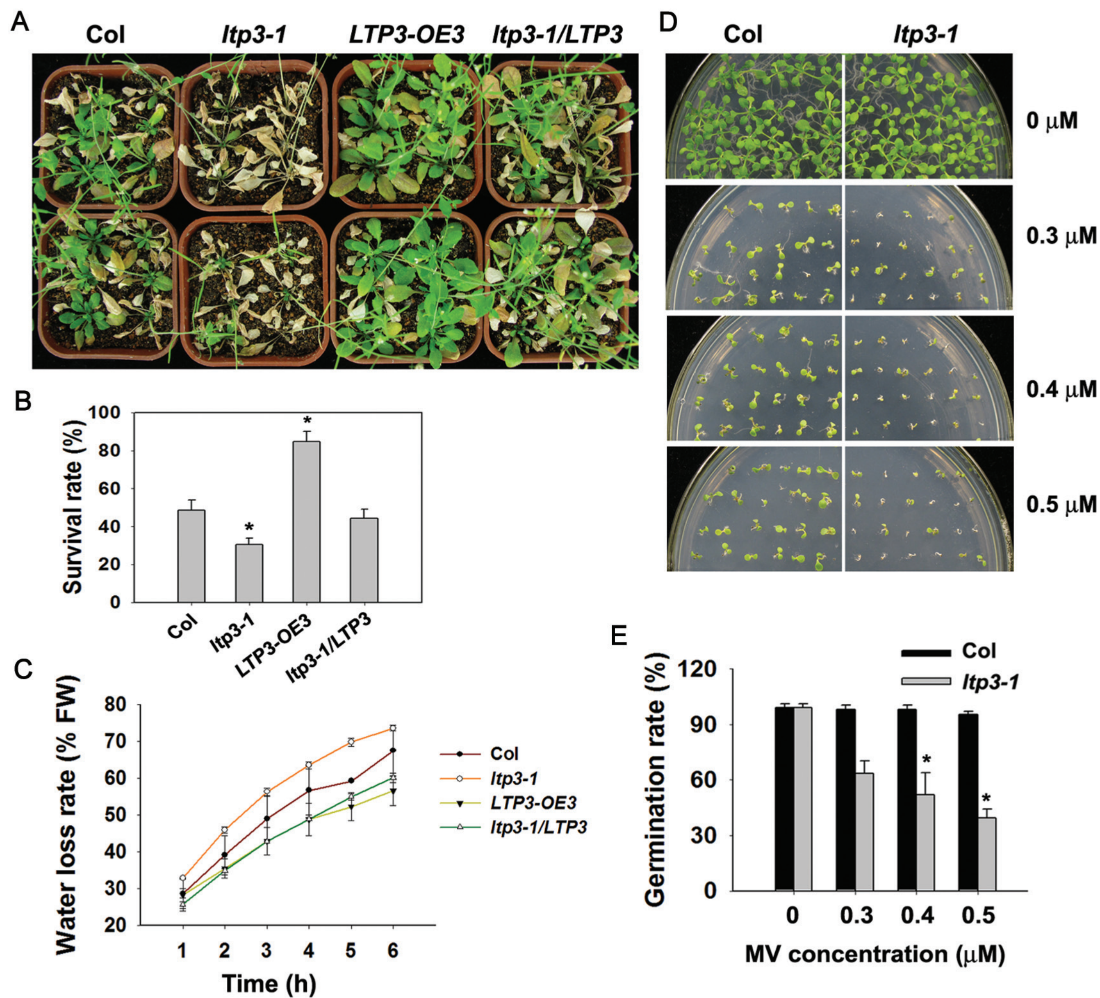

Fig. 4. Phenotypes of *ltp3-1* and *LTP3-OE* plants under drought and oxidative stress. (A) Drought-tolerance assay of the wild type, *ltp3-1, LTP3-OE*, and *ltp3-1/LTP3* complemented plants. The plants were grown in soil, deprived of water for 2 weeks, and then watered. Similar results were observed in three independent experiments. (B) Survival rate of the plants in (A) under drought stress. The data presented are the means ± SD (n = 30); $^*P$ < 0.05 (Student's t-test). Similar results were observed in three independent experiments. (C) Water loss of detached leaves of the wild type, *ltp3-1*, *LTP3-OE*, and *ltp3-1/LTP3* complemented plants. The data presented are the means ± SD of three replicates (n = 30 for each experiment); $^*P$ < 0.05 (Student's t-test). (D, E) Phenotype of *ltp3-1* plants under methyl viologen treatment. The wild-type Col and *ltp3-1* seeds were germinated at 22 °C on MS medium containing 0–0.5 μM methyl viologen. The photographs in D were taken on day 7 after stratification. The germination rates (E) are the means ± SD of three experiments (n = 30 for each experiment); $^*P$ < 0.05 (Student's t-test).

ltp3-1 was caused by the disruption of *LTP3*, a 2.0-kb wild-type genomic fragment containing *LTP3* under the control of its own promoter was transformed into the *ltp3-1* mutant. The transgene fully restored the drought-sensitive phenotype of the *ltp3-1* mutant (Fig. 4A and C), indicating that the *ltp3* mutation is responsible for the drought sensitivity. Taken together, these results demonstrate that *LTP3* positively regulates the plant response to drought stress.

The *ltp3-1* mutant exhibited a hypersensitive phenotype when germinated on MS media containing different concentrations of MV, an oxidative stress inducer (Fig. 4D), and the germination rates of *ltp3-1* were much lower than the wild type on MV-containing media (Fig. 4E). These observations, together with the strong induction of *LTP3* by MV (Fig. 1F), suggest that LTP3 plays a positive role in the plant responses to oxidative stress.

MYB96 directly regulates LTP3 expression

To identify direct upstream regulators of *LTP3*, this study searched the publicly available data and found that *LTP3* expression is decreased in a *myb96-1* mutant and increased in a *myb96-1D* mutant that overexpresses *MYB96* (Seo *et al.*, 2011). This study obtained a loss-of-function mutant allele, named *myb96-2*, from TAIR and generated *MYB96*-overexpressing (*MYB96-OE3* and *MYB96-OE4*) lines by transforming the *pSuper::MYB96* plasmid into the Col-0 background (Fig. 5A). The expression of *LTP3* was dramatically downregulated in the *myb96-2* mutant and upregulated in the *MYB96-OE* plants compared to the wild type (Fig. 5A). Further *MYB96*-overexpressing lines were generated, in which *MYB96* was driven by an inducible promoter (Zuo *et al.*, 2000). *MYB96* was dramatically upregulated after treatment with β-oestradiol, and *LTP3* was induced

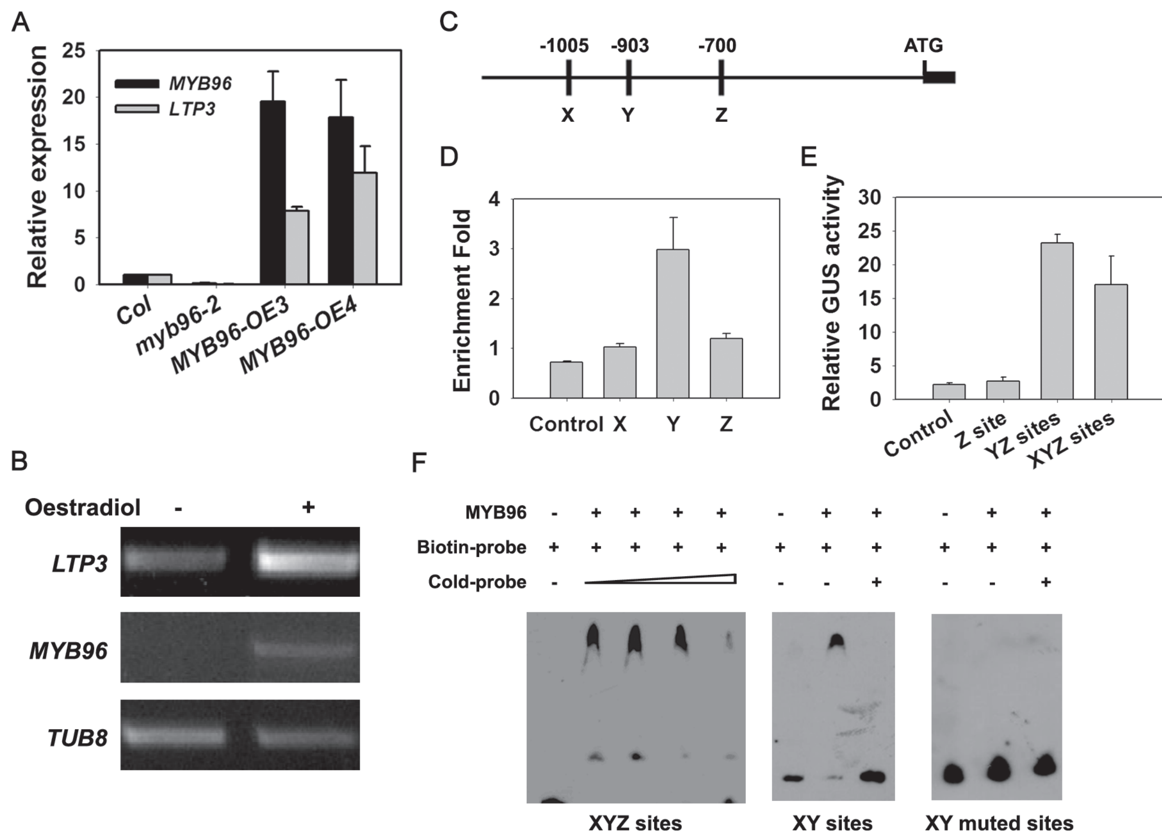

Fig. 5. *LTP3* is a direct target of MYB96. (A) Expression of *LTP3* and *MYB96* in 2-week-old wild-type Col, *myb96-2*, and *MYB96-OE* plants by qRT-PCR. The data are the means of three replicates ± SD. (B) Expression of *LTP3* and *MYB96* in the transgenic plants expressing *MYB96* under an oestradiol-inducible promoter with or without 5 μM β-oestradiol treatment for 6 h. (C) Schematic diagrams showing the promoter structure of *LTP3*. The 1.5-kb upstream sequence is shown. X, Y, and Z show the putative MYB96 binding sites, and the translation start site (ATG) is shown at position +1. (D) ChIP assay of MYB96-MYC binding to the *LTP3* promoter. The MYB96-binding sites are indicated in (C) and the primers used for the ChIP-qRT-PCR are listed in detail in Supplementary Table S1. A coding region of *LTP3* was amplified as a control. The data are the means of three replicates ± SD from one experiment. At least three independent experiments were performed with similar results. (E) Transient expression of different *pLTP3::GUS* vectors with *pSuper::MYB96* in *Nicotiana benthamiana* leaves. The cotransformation of *pZPGUS2* with the *pSuper::MYB96* vector was used as the control. The data are the means of three replicates ± SD. (F) EMSA assay for MYB96 binding to the promoters of *LTP3*. Each biotin-labelled DNA fragment was incubated with the MYB96-His protein. A competition assay for the labelled promoter sequences was performed by adding an excess of unlabelled wild-type or mutated probes.

accordingly (Fig. 5B). These results imply that *LTP3* might be a direct target of the MYB96 protein.

MYB96 can bind to the LTP3 promoter in vivo

MYB proteins can recognize the consensus CAGTTN motif in the promoters of their target genes (Urao *et al.*, 1993). A sequence analysis revealed that the promoter region of *LTP3* includes three CAGTTA elements, named the X, Y, and Z sites, respectively, within a 1.1-kb region upstream of the translation start site (Fig. 5C). ChIP was performed to determine whether MYB96 was able to bind directly to the *LTP3* promoter *in vivo*. This study generated transgenic plants that expressed the MYB96-Myc fusion protein under the Super promoter, and the protein-DNA complexes were purified using an anti-Myc antibody. qRT-PCR was then used to detect whether the MYB96 protein can bind to the *LTP3* promoter. As shown in Fig. 5D, the MYB96 protein strongly interacted with the fragment that contained the Y binding site but not the fragments that contained the X or Z binding sites or the exon region, implying that the Y binding site is important for the binding of MYB96 to the *LTP3* promoter.

Then the tobacco transient cotransformation system that expressed the *pSuper::MYB96* and *pLTP3::GUS* constructs was used to detect whether MYB96 can regulate *LTP3* expression. *pLTP3::GUS* constructs driven by a series of *LTP3* promoter fragments that contained different MYB96 binding sites were generated. In the presence of MYB96, the GUS activity increased significantly when the *LTP3* promoter included the YZ or XYZ binding sites. However, a low GUS activity was detected in the *LTP3* promoters that only contained the Z site or that did not contain an MYB binding site (Fig. 5E). Together, these results demonstrate that the MYB96 protein can specifically bind to the *LTP3* promoter *in vivo.*

MYB96 can bind the LTP3 promoter in vitro

An EMSA was performed to test whether the MYB96 protein binds directly to the *LTP3* promoter *in vitro*. Recombinant MYB96-His protein from *E. coli* was purified and incubated with biotin-labelled probes. A shifted DNA-binding band was detected with the addition of MYB96-His and labelled DNA probes containing XYZ or XY sites, but no band was detected in the absence of the MYB96-His protein. The DNA binding was abolished with increasing amounts of unlabelled probe (Fig. 5F). To determine the binding specificity, the mutated XY-containing probe in which the CAGTTA sequence was changed to ACGTAT was used; no DNA binding was detected after incubation with the MYB96-His protein (Fig. 5F). These results indicate that MYB96 specifically binds *in vitro* to the *LTP3* promoter containing the CAGTTA element.

MYB96-OE plants show LTP3-OE-like enhanced freezing tolerance

MYB96 is induced by different stress conditions, including ABA, drought, and salt stress (Seo *et al.*, 2009). Therefore, this study addressed whether *MYB96* was induced by cold stress. qRT-PCR analysis showed that *MYB96* could be slightly induced by cold conditions: the expression after 3-h cold treatment increased 2-fold over the expression at 0 h, and the expression decreased thereafter, returning to the basal level after 12 h under cold conditions (Fig. 6A).

To explore the role of *MYB96* in freezing tolerance, the survival rates of the wild-type and *MYB96-OE* seedlings after freezing treatment were determined. The non-acclimated *MYB96-OE* lines were more tolerant to freezing than the wild type after treatment at –5 °C (Fig. 6B). The survival rates for the *MYB96-OE3* and *MYB96-OE4* seedlings were 61 and 59%, respectively, whereas only 27% of the wild-type plants survived (Fig. 6C). A similar freezing-tolerant phenotype was observed in the acclimated *MYB96-OE* plants after being subjected to a freezing treatment (Fig. 6B and C). The electrolyte leakage was dramatically reduced in the acclimated and non-acclimated *MYB96-OE* plants versus the wild-type plants after the freezing treatment (Fig. 6D).

Furthermore, the expression patterns of *CBF1-CBF3* in the *MYB96-OE* plants were not substantially altered compared to the wild type (Fig. 6E). Thus, *MYB96* is involved in the plant response to freezing stress and is largely independent of the *CBF* genes.

Overexpression of LTP3 restores sensitivity to drought stress in the myb96 mutant

To investigate the genetic interaction of *LTP3* and *MYB96* further, this study generated *myb96-2 LTP3-OE* plants by transforming the *pSuper::LTP3* plasmid into *myb96-2* plants. The expression of *LTP3* in the *myb96-2 LTP3-OE* mutant was higher than that in the *myb96-2* plants, as determined by qRT-PCR (Fig. 7A). Notably, the constitutive expression of *LTP3* fully restored the drought-sensitive phenotype of *myb96-2* into the drought-tolerant phenotype of the wild-type plants (Fig. 7B and C). These results indicate that MYB96 acts upstream of *LTP3* to function in drought tolerance.

Discussion

This study found that the overexpression of *LTP3* could constitutively enhance the tolerance to freezing stress. Under cold treatment, there are numerous physiological and biochemical changes in plants, including the expression of *CBF* genes, *CBF* regulons, a transient increase in the ABA level, changes in the membrane lipid composition, the accumulation of compatible osmolytes such as soluble sugars and proline, and increased levels of antioxidants (Thomashow, 1999; Xin and Browse, 2000). The expression of *CBF1–CBF3* and their regulons was not notably affected in the *LTP3-OE* plants; however, the accumulation of soluble sugars in the *LTP3-OE* plants was dramatically increased compared to the wild-type plants. Therefore, the *LTP3*-mediated freezing tolerance appears to be at least partially attributed to the accumulation of soluble sugars but is uncoupled from the CBF pathway.

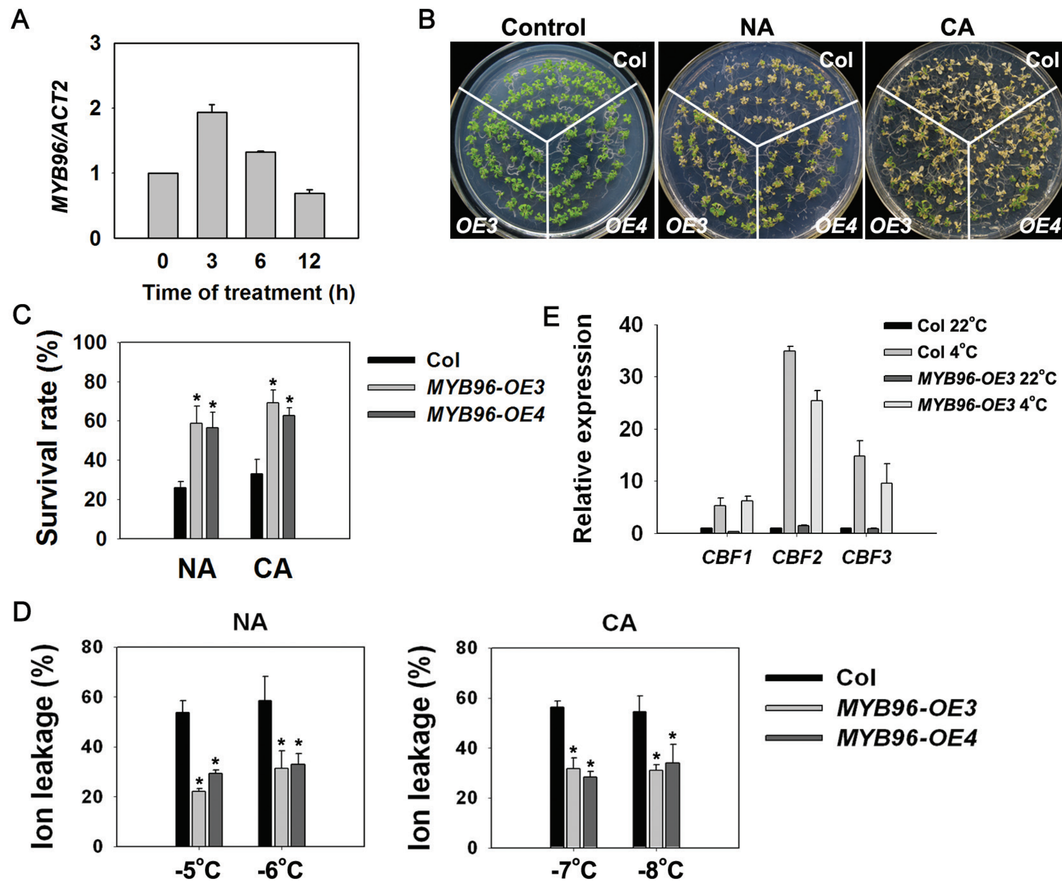

Fig. 6. Freezing tolerance is enhanced by the overexpression of *MYB96*. (A) Expression of *MYB96* under cold by qRT-PCR. Total RNA was extracted from plants treated at 4 °C for the indicated times. The data represent the means of three replicates ± SD. (B) Freezing phenotypes of *MYB96-OE* transgenic plants. Two-week-old light-grown seedlings were treated at –5 °C for 1 h (NA) or –7 °C for 1 h after 4 °C treatment for 4 d (CA), and the surviving seedlings were scored after a 2-d recovery. (C) Survival rate of the seedlings in (B) after freezing. The data represent the means of three replicates ± SD; *$P < 0.01$ (Student's t-test). At least three independent experiments were performed with similar results. (D) Ion leakage assay of the seedlings in (B) at the indicated freezing temperatures. The data are means of three replicates ± SD; *$P < 0.01$ (Student's t-test). (E) Expression of *CBF* genes in *MYB96-OE* plants by qRT-PCR. Two-week-old seedlings grown at 22 °C were treated at 4 °C for 3 h. The data represent the means of three replicates ± SD.

Little is known about how plants perceive changes in temperature or transfer a temperature signal. One hypothesis is that the fluidity of the cell membranes plays a key role in the perception of cold temperatures and the subsequent signal transduction (Murata and Los, 1997). The unsaturation of the lipids in thylakoid membranes has been shown to protect the photosynthetic machinery from photoinhibition at low temperatures (Nishida and Murata, 1996). The PLDα protein is the key enzyme involved in the biosynthesis of lipids. PLDα-deficient mutants, with lower levels of phosphatidylcholine and higher levels of phosphatidic acid than the wild type, display a destabilization of the membrane bilayer structure and improved tolerance to freezing stress (Welti *et al.*, 2002). Because cellular membranes are thought to be the primary sites of damage due to cold stress, changes in the membrane behaviour under cold conditions must be critical to the development of freezing tolerance. Several plant LTPs are known to interact with lipids and fatty acids *in vitro* without ligand specificity (Zachowski *et al.*, 1998; Guerbette *et al.*, 1999; Hamilton, 2004). They may function as lipid carriers between intracellular organelles (Kader *et al.*, 1984), and play a role in the delivery of wax components during the assembly of the cuticle (Sterk *et al.*, 1991; Yeats and Rose, 2008; Debono *et al.*, 2009). This study found that the overexpression of *LTP3* results in a constitutively enhanced tolerance to freezing stress. As LTP3 is able to bind to lipids in an *in vitro* assay and it is localized in cytosol, it is hypothesized that LTP3 might act as a co-signal of lipids and bind and transfer these lipids from cytosol to the cell membrane or cell wall to form cuticular wax, thus protecting the plants against adverse environmental conditions, including freezing and drought stress. It is also possible that certain changes in the content and species of lipids might occur when overexpressed *LTP3* gene in *Arabidopsis,* thereby affecting the responses of *Arabidopsis* plants to environmental stimuli. The type of lipids to which LTP3 binds and transfers remains to be investigated.

Several MYB proteins are reported to be involved in abiotic stress in *Arabidopsis*. For instance, MYB15 is reported to regulate *CBF* expression, thereby modulating freezing tolerance (Agarwal *et al.*, 2006). MYB44 plays a role in an

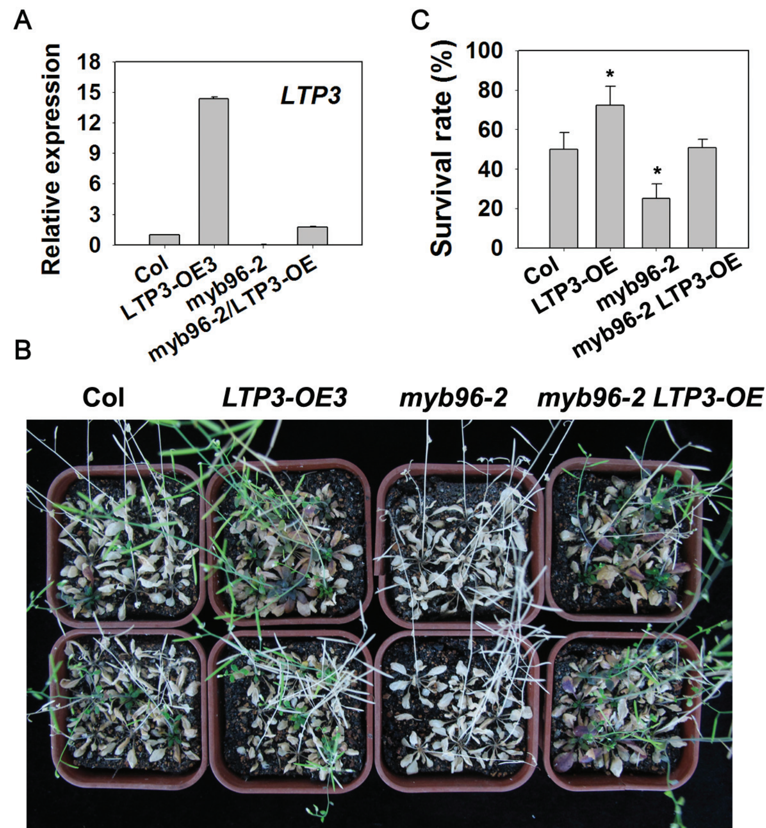

Fig. 7. Phenotypes of *myb96-2 LTP3-OE* plants under drought stress. (A) *LTP3* expression in 2-week-old wild-type Col, *LTP3-OE3, myb96*-2, and *myb96-2 LTP3-OE* plants, as determined by semi-quantitative RT-PCR. (B) Drought-tolerance assay of the wild-type Col, *LTP3-OE3, myb96-2*, and *myb96-2 LTP3-OE* plants. The plants were grown in soil, deprived of water for 2 weeks, and then watered. Similar results were observed in three independent experiments. (C) Survival rate of the plants in (B) under drought stress. The data presented are the means ± SD (n = 30); *P < 0.05 (Student's t-test). Similar results were observed in three independent experiments.

ABA-mediated signalling pathway that confers enhanced abiotic stress tolerance via the enhancement of stomatal closure (Jung *et al.*, 2008), and MYB60 acts to regulate stomatal movement (Cominelli *et al.*, 2005). MYB96 is involved in multiple physiological processes, including stomatal aperture, root development, pathogen infection, and cuticular wax accumulation. MYB96 is also suggested to act in the ABA-signalling pathway to regulate stomatal movement, thus regulating plant drought tolerance (Seo *et al.*, 2009). It has also been shown that MYB96 participates in disease resistance through the salicylic acid-signalling pathway (Seo and Park, 2010). A recent study showed that MYB96 regulates cuticular wax biosynthesis by directly binding to the promoters of genes that encode very-long-chain fatty acid-condensing enzymes (Seo *et al.*, 2011).

This study provides several lines of evidence showing that *LTP3* is the direct target of the MYB96 protein in the positive regulation of the plant responses to freezing and drought stress. *LTP3* expression was upregulated in the *MYB96-OE* plants and downregulated in the *myb96* mutant. Further study showed that MYB96 directly bound to the promoter of *LTP3 in vitro* and *in vivo*. Consistent with this, the overexpression of *LTP3* and *MYB96* displayed similar enhanced freezing tolerance and drought tolerance, whereas the *ltp3-1* mutant showed *myb96*-like drought-sensitive phenotypes. Furthermore, the overexpression of *LTP3* in the *myb96* mutant compromised the drought-sensitive phenotype of the *myb96* mutant. These results add more evidence to support the previous finding that MYB96 transcription activator confers drought tolerance by modulating cuticular wax biosynthesis (Seo *et al.*, 2011). It is noteworthy that overexpression of *MYB96* causes severe morphological defects such as dwarfed stature with altered leaf morphology, whereas overexpression of *LTP3* resembles the wild type in normal conditions. This difference might be because that *LTP3* is just one of MYB96 targets. The accumulation of cuticular wax in *MYB96*-overexpressing plants might also account for its enhanced freezing tolerance; however, this study did not observe obvious changes in accumulation of cuticular wax on the leaf surface of *LTP3*-overexpressing plants. Further investigation of molecular mechanism of *LTP3* will shed more light on its function in environmental stress responses.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Response of *ltp3-1* mutant to cold stress.

Supplementary Fig. S2. The effect of *LTP3* on expression of its homologues.

Supplementary Fig. S3. Expression of cold-responsive genes in *LTP3-OE* plants.

Supplementary Fig. S4. Soluble sugar content and proline contents in *LTP3-OE* plants.

Supplementary Table S1. Primer sequences used in this study.

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