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# Overexpression of the phosphatidylinositol synthase gene (*ZmPIS*) conferring drought stress tolerance by altering membrane lipid composition and increasing ABA synthesis in maize

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## ABSTRACT

**Phosphatidylinositol (PtdIns) synthase is a key enzyme in the phospholipid pathway and catalyses the formation of PtdIns. PtdIns is not only a structural component of cell membranes, but also the precursor of the phospholipid signal molecules that regulate plant response to environment stresses. Here, we obtained transgenic maize constitutively overexpressing or underexpressing *PIS* from maize (*ZmPIS*) under the control of a maize ubiquitin promoter. Transgenic plants were confirmed by PCR, Southern blotting analysis and real-time RT-PCR assay. The electrospray ionization tandem mass spectrometry (ESI-MS/MS)-based lipid profiling analysis showed that, under drought stress conditions, the overexpression of *ZmPIS* in maize resulted in significantly elevated levels of most phospholipids and galactolipids in leaves compared with those in wild type (WT). At the same time, the expression of some genes involved in the phospholipid metabolism pathway and the abscisic acid (ABA) biosynthesis pathway including *ZmPLC*, *ZmPLD*, *ZmDGK1*, *ZmDGK3*, *ZmPIP5K9*, *ZmABA1*, *ZmNCED*, *ZmAAO1*, *ZmAAO2* and *ZmSCA1* was markedly up-regulated in the overexpression lines after drought stress. Consistent with these results, the drought stress tolerance of the *ZmPIS* sense transgenic plants was enhanced significantly at the pre-flowering stages compared with WT maize plants. These results imply that *ZmPIS* regulates the plant response to drought stress through altering membrane lipid composition and increasing ABA synthesis in maize.**

*Key-words*: PtdIns synthase; transgene.

*Abbreviations*: CDP-DG, CDP-diacylglycerol; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGK, diacylglycerol kinase; MGDG, monogalactosyldiacylglycerol; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; PI4K, phosphoinositide 4-kinase; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PIS, phosphatidylinositol synthase; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdIns (4,5)$P_2$, phosphatidylinositol 4, 5-bisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; WT, wild type.

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## INTRODUCTION

Drought is one of the major environmental stresses that adversely affect plant growth and crop yield (Boyer 1982). Because of the accelerated increases in world population and a global water source shortage, it is necessary to breed drought-tolerant crops and improve crop yield by the development of biotechnological methods. Maize, one of the most important food and feeding crops, is susceptible to drought during its growth and development, especially at the flowering stage (Shaw 1977). Therefore, it is of strategic significance to improve the drought resistance of maize.

In response to drought stress, some genes are up-regulated, which can alleviate the detrimental effect of stress, lead to the adjustment of the cellular milieu and thus contribute to plant tolerance (Mahajan & Tuteja 2005). To resist and adapt to stress, drought-tolerant plants often display specific traits such as improved water use efficiency and/or osmotic adjustment (Moore *et al.* 2008; Bargmann *et al.* 2009; Yang *et al.* 2010). Moreover, drought tolerance of plants depends on complex signalling networks (Mahajan & Tuteja 2005). In previous studies, many signal transduction pathways that are activated in response to osmotic stress have been reported (Xiong, Schumaker & Zhu 2002). Of these pathways, the lipid signalling events, especially phospholipid signalling ones, arouse our attention because of the lipid molecules' dual function of a structural role and a signal-transducing property.

Drought stress has a striking effect on the metabolism of membrane lipids, which is generally observed as changes in the membrane lipid composition (Repellin *et al.* 1997; Gigon *et al.* 2004; Torres-Franklin *et al.* 2007). The membrane lipids in plants include a wide range of molecules, such as sphingolipids, galactolipids and phosphoglycerolipids (Testerink & Munnik 2011). Besides being involved in the reorganization of cellular components, membrane lipids have a role in cell signalling and allowing the plant to cope with stress conditions (Welti *et al.* 2002; Wang 2004). Drought-induced modifications in the plant membrane lipid composition correlated well with

the intensity of drought stress and the sensitivity or tolerance of plants to drought stress. Drought-tolerant plants display several characteristics that indicate their capacities to adapt to water stress shown by unchanged or even increased levels of unsaturated polar lipids implying higher membrane fluidity and physiological efficiency (de Monteiro Paula *et al*. 1990; Repellin *et al*. 1997; Torres-Franklin *et al*. 2007; Guerfel *et al*. 2008). Drought-tolerant plants also display an increase in the digalactosyldiacylglycerol:monogalactosyldiacylglycerol (DGDG:MGDG) ratio of leaf lipids. In numerous plant species, drought has been reported to stimulate DGDG biosynthesis and induced accumulation of DGDG in extrachloroplastic membranes that contribute to drought tolerance in plants (de Monteiro Paula *et al*. 1990; Gigon *et al*. 2004; Larsson, Nystrom & Liljenberg 2006; Torres-Franklin *et al*. 2007).

Phospholipids, one of the main structural components of membranes, can also act as signalling precursors or signalling molecules to regulate plant growth, development and response to environmental stimuli (Laxalt & Munnik 2002; Xue, Chen & Mei 2009). As the precursor of inositol-containing phospholipids in plant cells, phosphatidylinositol (PtdIns) accounts for 15–20% of the total phospholipids in the plasma membrane and outer mitochondrial membrane. It can be converted into various lipids by alternative pathways. The inositol head of PtdIns can be successively phosphorylated at the 3, 4 or 5 positions by a series of distinct kinases to generate a variety of polyphosphoinositides, such as PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(4, 5)$P_2$, PtdIns(3, 5)$P_2$ and PtdIns(3, 4)$P_2$, many of which have emerged as second messengers known to regulate important physiological processes (König *et al*. 2008; Darwish *et al*. 2009; Heilmann 2009; Munnik & Testerink 2009). For example, PtdIns(4, 5)$P_2$ can not only be hydrolysed by phosphoinositide-phospholipase C (PI-PLC) to generate the second messengers diacylglycerol (DAG) and $InsP_3$, but it is also emerging as a signalling molecule in response to various stresses (Delage *et al*. 2012a). In plants, $InsP_3$ can be converted to inositol hexakisphosphate ($InsP_6$), which seems to stimulate the release of $Ca^{2+}$ from an intracellular store (Lemtiri-Chlieh *et al*. 2003). DAG is also rapidly converted to phosphatidic acid (PtdOH) by phosphorylation via the diacylglycerol kinase (DGK). PtdOH is an important intermediate involved in the biosynthesis of phospholipids and galactolipids at the endoplasmic reticulum (ER) and the plastid (Athenstaedt & Daum 1999; Arisz 2010). It is an essential signal in the plant response to abiotic or biotic stress (Meijer *et al*. 2001; Munnik 2001; Wang 2005; Wang *et al*. 2006; Testerink & Munnik 2011). Alternatively, PtdIns might also be converted into a number of other products by various lipid-modifying enzymes, such as PLA1, PLA2, PLC and inositol-phosphoceramide synthase (Beisson *et al*. 2003). These phospholipases, various lipid kinases and/or phosphatases control the spatial and temporal production of lipid mediators involved in plant responses to specific biotic and/or abiotic stresses. Recently, several articles have examined the function and regulatory role of phospholipid molecules and related enzymes in plant growth and development, stress response and hormonal signalling (Xue *et al*. 2009). For example, the plant PLC and phospholipase D (PLD) have been linked to salt stress, water deficiency and abscisic acid (ABA) signalling (Hirayama *et al*. 1995, 1997; Frank *et al*. 2000; Munnik *et al*. 2000; Katagiri, Takahashi & Shinozaki 2001; Sang *et al*. 2001; Zonia & Munnik 2004; Zhai *et al*. 2005; Mane *et al*. 2007; Hong *et al*. 2008; Sui *et al*. 2008; Tasma *et al*. 2008; Wang *et al*. 2008; Bargmann *et al*. 2009). Consistent with this, many of lipid kinases including PI3K, PI4K and PIP5K have been found to regulate various types of physiological process (Mikami *et al*. 1998; Lin *et al*. 2004; Lee *et al*. 2007; Leshem, Seri & Levine 2007; Lou, Gou & Xue 2007; Ischebeck, Stenzel & Heilmann 2008; Kusano *et al*. 2008; Mikami, Saavedra & Sommarin 2010; Mei *et al*. 2012; Delage *et al*. 2012b). Similarly, several isomers of inositol polyphosphate 5-phosphatases (5PTases) have also been shown to be involved in water stress signalling and ABA signalling in plants (Lin *et al*. 2005; Gunesekera *et al*. 2007; Ananieva *et al*. 2008; Perera *et al*. 2008).

In eukaryotic cells, the PtdIns synthesis is catalysed by PtdIns synthase (PIS, EC 2.7.8.11) using CDP-diacylglycerol (CDP-DG) and myo-inositol as substrates at the first step of the PI signalling pathway. The genes encoding PISs have been characterized in *Saccharomyces cerevisiae* (Nikawa, Kodaki & Yamashita 1987), rat (Tanaka *et al*. 1996), man (Lykidis *et al*. 1997) and plant (Collin *et al*. 1999; Xue *et al*. 2000), respectively. The PISs from plants have been reported to be involved in drought stress response (Das *et al*. 2005; Löfke *et al*. 2008; Sui *et al*. 2008; Zhai *et al*. 2012). Our previous work has showed that overexpression of *ZmPIS* in tobacco plants altered the membrane lipids composition and improved drought stress tolerance (Zhai *et al*. 2012).

In order to deeply elucidate the function of PIS in plant drought stress tolerance and the underlying molecular mechanism, in this study, the *ZmPIS* gene coding a PIS from maize under the control of a maize ubiquitin promoter was introduced into the elite maize (*Zea mays* L.) inbred line DH4866 by an *Agrobacterium*-mediated method. Based on the electrospray ionization tandem mass spectrometry (ESI-MS/MS)-based lipid profiling assay and the liquid chromatography-based ABA content determination, it was found that the overexpression of *ZmPIS* resulted in elevated levels of phospholipids, galactolipids and ABA in *ZmPIS* sense transgenic maize leaves under drought stress conditions compared with these levels in controls. Using real-time RT-PCR, elevated transcriptional levels of several genes related to the PI pathway and other downstream responsive genes involved in multiple drought stress pathways were detected, which might explain the regulatory mechanism of *ZmPIS* gene during drought stress. Moreover, the *ZmPIS* sense plants displayed enhanced osmotic adjustment ability, reduced cellular membrane damage, a higher photosynthesis rate, and a higher grain yield under drought stress at the pre-flowering stages. These results revealed in part the molecular mechanism by which *ZmPIS* regulates the plant response to drought stress, suggesting the potential use of *ZmPIS* in breeding drought tolerance crops.

## MATERIALS AND METHODS

### Plant materials

The plant material used in this study was the maize (*Z. mays L.*) elite inbred line DH4866. Seeds of DH4866 were surface

sterilized with 70% (v/v) ethanol for 7 min and 0.1% (w/v) mercuric chloride for 8–10 min. After the seeds were rinsed five times with sterile water, the seeds were geminated on moistened filter paper in glass vessels at 28 °C in the darkness for 2–3 d. After the emergence of plumule and radicle, the etiolated seedlings were transferred to solidified modified MS medium (Li *et al*. 1990) and cultured under the same conditions. When the plumule of the seedling was 3.0–4.0 cm in length, the coleoptiles were excised, and the central portions of exposed meristems were vertically cut with a sterile scalpel. The injured shoot tips (exposed apical meristems) were used as the target tissue for *Agrobacterium* infection.

### *Agrobacterium* strain and plasmid

The *Agrobacterium tumefaciens* strain used in this experiment was LBA4404, which carries a mini-Ti plasmid pCAMBIA1300-pUbi::*ZmPIS* (+)/(–)-p35S::*als* that was constructed by our lab. The T-DNA region of this plasmid contained the sense or antisense *ZmPIS* gene (1131 bp from *Z. mays L.*, Accession No. AY370763) driven by the maize ubiquitin promoter and the mutational chlorsulfuron-resistance acetolactate synthase gene (*als*) from *Arabidopsis* driven by a CaMV35S promoter (Fig. 1a). The construction of this plasmid was carried out according to standard protocols described by Sambrook, Fritsch & Maniatis (2000). The recombined plasmid was introduced into the *A. tumefaciens* strain LBA4404 by the freeze-thaw method.

### Maize transformation and regeneration

Transgenic maize plants were generated by *Agrobacterium*-mediated transformation of injured maize shoot tips according to the methods described by Li *et al*. (2008a). At three-leaf stage, the transformation plantlets were screened with the herbicide Lvhuanglong® (included 15% chlorsulfuron, produced by Shenyang Pesticide Company, Shenyang, China) at a 25 mg $L^{-1}$ concentration. The resistant plantlets (T0) were transplanted into the field and self-pollinated to produce the next generation (T1). Many independent transformants were obtained. Seeds harvested from the T1 generations were sown and self-pollinated for the T2 generations. The transgenic-positive plants at every generation from T0 to T3 generations were obtained by herbicide screening and PCR assays.

### PCR analysis and Southern blotting of transgenic plants

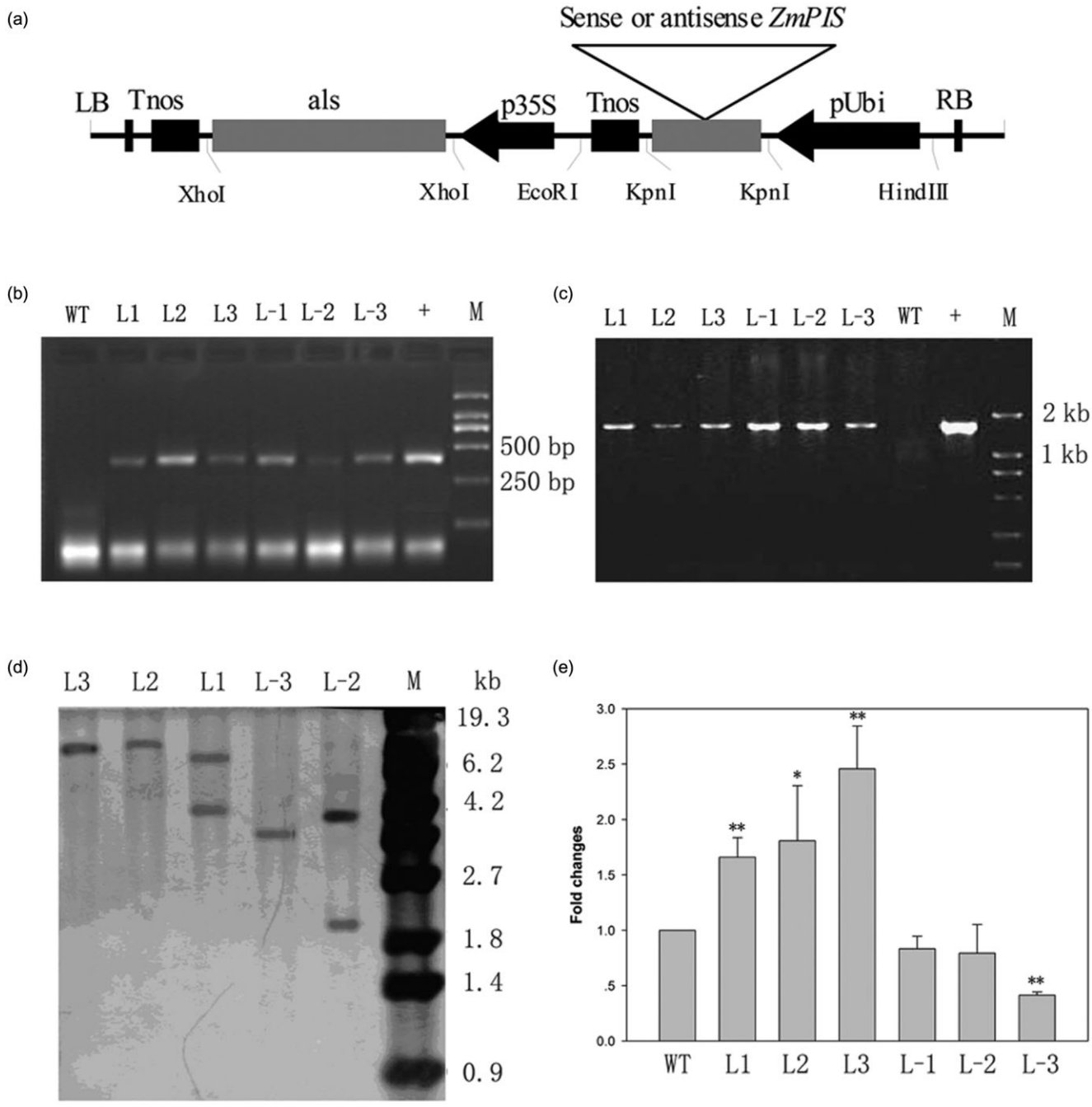
Genomic DNA was extracted from maize leaves by the cetyltrimethylammonium bromide (CTAB) method. To confirm the presence of the sense or antisense *PIS* gene in transgenic plants, two pairs of specific primer sequences were designed. The primers for the *ZmPIS* gene were the following: 5′ CTCACCAATTTAAGGGCAGAAC 3′ and 5′ TACATTTGAAACCAGTGGCTCG 3′. PCR assays were also performed to detect the presence of the *als* gene using primers 5′ GAGGACACGCTGAAATCACC 3′ and 5′ GCATCA GGGTTAGCAACAG 3′. The PCR annealing temperatures were 56 °C for the approximately 400 bp *PIS* gene fragments and the approximately 1700 bp *als* gene fragments. The genomic DNA of PCR-positive maize plants was digested with *EcoR*I, and then Southern blotting was performed using a digoxigenin (DIG)-labelled *PIS* specific probe as described in the DIG System Manual (Roche, Inc., Basel, Switzerland).

### RNA isolation and real-time RT-PCR

Total RNA was extracted from 0.1 g of leaves from transgenic or wild-type (WT) lines using the Trizol reagent (Sangon, Shanghai, China), and then was treated with RNase-free DNase to remove any genomic DNA contamination. cDNA synthesis was performed with the RT reagent kit (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol. For real-time RT-PCR, the gene-specific primer sequences of *ZmPIS* were used according to Sui *et al*. (2008). Real-time RT-PCR was performed on a ChromoTM 4 (MJ Research, Waltham, MA, USA) with the SYBR® RT-PCR Kit (Takara, Dalian, China) following a method described by Sui *et al*. (2008) The *ubiquitin* gene (GenBank Accession No. U29159) was used as an internal control, and the primers are shown in Supporting Information Table S1. The amplification conditions were the following: 2 min at 95 °C; 40 cycles of 20 s at 95 °C; 20 s at 60 °C; and 25 s at 72 °C. The relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Each experiment was repeated three times.

### Seedling growth and treatments

Seeds from the homogeneous transgene T3 generation and WT were sown into flower pots (diameter: 14.1 cm; height: 10.4 cm) fitted with homogenous soil under normal conditions (16 h light/32 °C, 8 h dark/25 °C) at 5 plants per plot. The pots were watered every day with sufficient quantities of water. At the three-leaf stage, healthy and uniform plants were selected for drought stress treatment. The solute potential ($\Psi s$) of leaves was measured as described by Gaxiola *et al*. (2001). Leaves from plants grown under three different hydration levels were harvested for analysis: before stress ($\Psi s$ in WT = –0.57 ± 0.04 MPa, $\Psi s$ in sense transgenic plants = –0.58 ± 0.01 MPa, $\Psi s$ in antisense transgenic plants = –0.58 ± 0.02 MPa); after stress ($\Psi s$ in WT = –0.68 ± 0.01 MPa, $\Psi s$ in sense = –0.71 ± 0.01 MPa, $\Psi s$ in antisense = –0.68 ± 0.00 MPa), which was attained 4 d after water stress; and rehydrated ($\Psi s$ in WT = –0.60 ± 0.01 MPa, $\Psi s$ in sense = –0.66 ± 0.02 MPa, $\Psi s$ in antisense = –0.59 ± 0.02 MPa), which was attained 1 d after rewatering. In each pot, one of the five plants was used to measure the solute potential, one plant was immediately frozen in liquid nitrogen for RNA extraction, and the third plant was used for lipid extraction. In all cases, the samples consisted of the third fully expanded leaf from the top of the seedlings. Each sample had three biological replicates for real-time RT-PCR and five biological replicates for ESI-MS/MS analysis of lipid molecular species.




## ESI-MS/MS analysis of lipid molecular species

Total lipids were extracted following a previously described procedure (Welti *et al.* 2002), and the lipid samples were analysed in the Kansas Lipidomics Research Center Analytical Laboratory by ESI-MS/MS.

## The relative expression assay of genes involved in the phospholipid signal pathway and ABA synthesis pathway

To analyse the effect of *ZmPIS* overexpression on the phospholipid signal and ABA synthesis, the relative expression assays of several genes encoding phospholipases, lipid kinases and phosphatases, and several ABA biosynthetic genes were performed. The GenBank accession number and the specific primers of these genes that were used for real-time RT-PCR are shown in Supporting Information Table S1. The total RNA extraction and cDNA synthesis, real-time RT-PCR, and the amplification conditions used the same method as previously described.

## ABA content determination

Seedlings at three-leaf stage from T3 transgenic and WT plants were subjected to drought stress as described above.

**Figure 1.** Schematic structure of the T-DNA region of plasmid pCAMBIA1300-pUbi::*ZmPIS* (+)/(–)-Tnos-p35S::*als* and molecular characterization of the expression of the *ZmPIS* transgene in transgenic maize. (a) The T-DNA region of the vector has a phosphatidylinositol synthase gene (*ZmPIS*, sense or antisense) driven by a maize-constitutive ubiquitin promoter (pUbi), and a mutational chlorsulfuron-resistance acetolactate synthase gene (*als*) from *Arabidopsis*, which is driven by the promoter CaMV35S (p35S). LB and RB mean the left and right T-DNA border sequences, respectively. (b) Polymerase chain reaction (PCR) analysis of T3 transgenic maize plants for the *ZmPIS* gene: M, DNA marker DL2000; WT, non-transformed control; +, the PCR result of plasmid pCAMBIA1300-pUbi::*ZmPIS* (+)/(–)-Tnos-p35S::*als*; L1-L3, PCR-positive *ZmPIS* sense plants; L-1–L-3, PCR-positive *ZmPIS* antisense plants. (c) PCR analysis of T3 transgenic maize plants for the *als* gene: M, DNA marker DL2000; L1–L3, PCR-positive *ZmPIS* sense plants; L-1–L-3, PCR-positive *ZmPIS* antisense plants; WT, untransformed control; +, the PCR result of plasmid pCAMNIA1300-pUbi::*ZmPIS* (+)/(–)-Tnos-p35S::*als*. (d) Southern blot analysis indicating the presence of the transgene *ZmPIS* in the genome of transgenic maize. In all cases, genomic DNA (20 $\mu$g) was digested with *Eco*RI and separated on a 0.8% agarose gel by electrophoresis. After blotting, the membrane was hybridized with a digoxigenin (DIG)-labelled specific probe as described in the DIG System Manual. M, λDNA/EcoT14I molecular weight marker; L1–L3, Southern blot of DNA from independent sense maize lines; L-2, L-3, Southern blot of DNA from independent antisense maize lines. (e) Analysis by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of the expression of *ZmPIS* in maize transgenic lines. Specific PCR products of approximately 400 bp were detected in the six transgenic lines. A 200 bp ubiquitin fragment was amplified by RT-PCR as an internal control. WT, wild-type plant; L1–L3, *ZmPIS* sense transgenic plants; L-1–L-3, *ZmPIS* antisense transgenic plants. The asterisks indicate a significant difference between the transgenic maize and the WT at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test.

The ABA content determination in leaves from plants grown under three different hydration levels was performed following a previously described method (Haver, Schuch & Lovatt 2003). Leaves frozen in liquid nitrogen freeze dried. Dried leaves were ground in 9 mL of extraction solvent 80% (v/v) methanol including 40 mg $L^{-1}$ butylated hydroxytoluene (BHT) and extracted at 4 °C overnight. Samples were centrifuged and the supernatant was recovered, the pellet was then resuspended in a further 2 mL of extraction solvent and centrifuged and the supernatants were combined. The extraction solvent was evaporated and the residue was resuspended in 8 mL of ammonium acetate (0.1 mol $L^{-1}$, pH 9.0). The supernatants were applied to a polyvinylpolypyrrolidone (PVPP) column below which was attached a [2-(diethylamino) ethyl-Sephadex__] (DEAE) column. The acidic hormones were eluted from the DEAE column with 25 mL of 1.5 mol $L^{-1}$ acetic acid and collected in a second Sep-Pak C18 cartridge (Waters Corp., Milford, MA, USA). Sep-Paks were washed with 5 mL distilled water and the hormones were eluted from the cartridges with 5 mL (acidic hormones) of 50% (v/v) methanol. The eluted solution was freeze dried and resuspended with 1 mL 50% (v/v) methanol. The extracted solution was separated on Waters (TM) Symmetry $RP_{18}$ (4.6 mm × 150 mm, 5 $\mu$m; Waters Corp.) at 35 °C and were analysed by liquid chromatography with Waters 2695 separation system. The mobile phase was methanol: 0.6% (v/v) acetic acid (40:60) at a flow rate of 0.5 mL $min^{-1}$.

## Drought stress treatment of pre-flowering maize plants

Three independent sense transgenic lines (L1, L2 and L3) and three independent antisense lines (L-1, L-2 and L-3) were selected for drought tolerance analysis. The drought stress experiments were carried out under natural conditions during the maize growing season in the campus-experiment field of Shandong University in Jinan, China. A transparent, plastic shed was used to protect plants from rain. Seeds of the T3 transgenic and non-transgenic lines were sown into flower pots (diameter: 35 cm; height: 25 cm) fitted with homogenous soil in May with 1 plant sown per plot; the plants were watered with sufficient quantities of water every day. At the 10-leaf stage, healthy and uniform plants were selected and divided into two groups. One group was served as non-stress control and the normal watering was maintained. Another group was subjected to drought stress treatment for 15 d by controlling the watering to maintain the soil water content at approximately 15%, and then this group was exposed to rehydration treatment (i.e. the plants were irrigated sufficiently). Physiological parameters including the photosynthetic parameters, photosystem II (PSII) activity, chlorophyll content, malondialdehyde (MDA) concentration, ion leakage, leaf relative water content (RWC), solute potential, and soluble sugar and proline contents were measured on 0, 1, 5, 10 and 15 d during the period of drought stress treatment. The samples tested were fully expanded leaves of similar sizes, ages and positions on the plants. Six plants were assayed for each line.

## Measurement of RWC and solute potential

The leaf discs from transgenic and WT plants were excised, and their fresh weights (FWs) were immediately recorded. The discs were then soaked in deionized water at 4 °C overnight and weighed (rehydrated weight, RW). Finally, their dry weights (DWs) were obtained after drying the leaf discs in an oven at 80 °C overnight. The RWC was calculated as $RWC\ (\%) = (FW - DW)/(RW - DW) \times 100\%$.

The solute potential ($\Psi s$) was measured using the protocol described by Gaxiola *et al*. (2001). The solute potential of maize leaf discs from transgenic and WT plants was measured with a cryoscopic osmometer (Model 210; Fiske, Norwood, MA, USA) and calculated with the formula: $\Psi s = -\text{moles of solute } (RK)$, where $R = 0.008314$ and $K = 298$ °C.

## Quantification of total soluble sugar and proline

The total soluble sugars were extracted from 100 mg of maize leaves by boiling water for 30 min twice and determined by anthrone reagent using glucose as the standard (Yemm & Willis 1954). To detect the amount of proline, 100 mg of maize

leaves were used according to the method described by Bates, Waldren & Teare (1973).

### Measurement of photosynthesis and chlorophyll fluorescence

In the drought stress experiment, net photosynthesis rate, transpiration rate and stomatal conductance were measured using the third fully expanded leaf from the top of the plant and a portable infrared gas analyser-based photosynthesis system (LI-6400; Li-Cor Inc., Lincoln, NE, USA). The photosynthetic photon flux density was set at 800 $\mu$mol $m^{-2}$ $s^{-1}$ by an internal 6400-02BLED source. All of the measurements were carried out at 0900–1100 h. During the measurements, the air relative humidity was approximately 50%, and the ambient $CO_2$ concentration was approximately 350 $\mu$mol $CO_2$ $mol^{-1}$.

After adaptation to the dark for 30 min, the chlorophyll activity in the third leaf from the top of the plant was measured as $F_v/F_m$ by using a pulse-modulation chlorophyll fluorometer (Mini-Pam; Walz, Effeltrich, Germany) at room temperature (Schreiber 1986).

### Determination of chlorophyll content

Approximately 0.1 g of leaves were excised from maize plants to measure chlorophyll content by the method described by Arnon (1949).

### Measurement of the MDA content and the cellular membrane damage

Maize leaf discs (200 mg) were used to measure the MDA content according to Quan *et al*. (2004). The leakage of electrolytes from leaf cells was measured with a conductivity meter by the method described by Shou *et al*. (2004) and implied leaf cell membrane damage of leaves.

### Agronomic traits of maize plants

A field experiment was separately carried out in 2008 and 2009 in the Experimental Field of Lvjia in Jinan, China (117°29′E, 36°54′N) under a removable rain-off shelter. The maize test plots were arranged according to a report described by Li *et al*. (2008a). Plants at the 10-leaf stage were subjected to drought stress for 6 weeks. The RWC of soil was controlled to 50–55% at a depth of 40 cm during stress. After exposure to drought stress conditions for 6 weeks, the plants were watered well, with the RWC of soil controlled to 80–88% at a depth of 40 cm during stress. The plant height, leaf numbers, and anthesis-silking interval (ASI) of 10 maize plants from each of the transgenic and WT lines were determined at the pollination stage. After harvesting, the seeds were dried naturally to a constant weight, and the number and weight of seeds per ear from each line were recorded.

In addition, to better investigate the drought tolerance and yield of transgenic *ZmPIS* maize, a parallel field experiment was performed in the Anningqu Experimental Field of Xinjiang Academy of Agriculture in the Xinjiang Uygur Autonomous Region (87°68′E, 43°77′N) in China under natural conditions in 2008 and 2009. Xinjiang is a typical arid and semi-arid agricultural area in China, and the amount of precipitation is less than 200 mm during the growth period of crops. Snowmelt is the main water source for land irrigation by farmers. Therefore, the drought stress experiment of transgenic *ZmPIS* maize under natural conditions was arranged in Xinjiang. The experiment fields were divided into two zones: one zone was for drought stress treatment and another zone was for growth of the control under sufficient water conditions. Each zone was arranged in a random complete block design with three replications. The moisture segregations with a width of 5 m were designed around each zone. The seeds from each homozygous line were sown in four rows with a 0.6 m distance between the rows and a 0.3 m spacing between plants. The length of each row was 5 m. Plants at the 10-leaf stage in the drought stress zone were exposed to drought stress without irrigation. Plants in the irrigation control zone were watered normally every 7–10 d. After harvesting, the grain weight per plant and the grain weight per ear were measured.

### Statistical analysis

All statistical analyses were conducted using Sigmaplot 10.0 software (Systat Software Inc., Chicago, IL, USA). All the physiological parameters had three replicates. All data were presented as the mean ± standard deviation (SD). Comparisons between the transgenic and WT plants were performed using the Student's *t*-test to determine statistical significance.

## RESULTS

### Molecular characterization of the transgenic maize plants

The transgenic maize plants from T0 to T3 generations were confirmed by PCR assay using the *ZmPIS* gene-specific primers (Fig. 1b,c). The PCR-positive plants of the T3 generation were further confirmed by Southern blot analysis. As shown in Fig. 1d, the endogenous *ZmPIS* gene fragments and the specific hybridization bands were detected. These data indicate that the sense or antisense *ZmPIS* transgenes were stably integrated into the genomes of the respective transgenic plants.

To estimate the relative expression levels of *ZmPIS* gene in the transgenic and WT lines, real-time RT-PCR analysis was performed. Obvious differences in the expression levels of *ZmPIS* gene were found between the different lines under normal conditions (Fig. 1e). The transcript levels of *ZmPIS* gene increased up to 1.70–2.46-fold in sense transgenic plants compared with those in WT while the transcript levels of *ZmPIS* gene in antisense plants were only 0.41–0.83-fold of WT. The significant differences in expression level of *ZmPIS* were observed between transgenic lines and WT, except for L-1 and L-2.

## Drought stress-induced changes in the lipids of transgenic maize leaves

Our recent work showed that the overexpression of *ZmPIS* significantly increases the phospholipid and galactolipid levels in tobacco leaves under drought stress (Zhai *et al*. 2012). To determine whether the constitutive overexpression or suppression expression of *ZmPIS* results in altered levels and fatty acid composition of lipids in transgenic maize in response to stress, the total lipids were isolated from WT and transgenic maize under sufficient water supply, water deficit stress and rehydration conditions, respectively. The lipid profiling assay result is shown in Fig. 2 and Supporting Information Table S2. Under normal growth conditions, changes in the *ZmPIS* expression level did not result in variations in the level and fatty acid composition of most molecular species in the transgenic plants, except for the amount of PtdSer, which increased significantly in sense plants, and the level of PtdSer and PtdOH, which decreased significantly in antisense plants compared with WT plants. However, during drought stress, changes in the lipid species of transgenic plants differed from that occurred in the WT plants. In WT maize leaves, compared with the lipid levels in maize plants before stress, the amount of most molecular species including PtdEtn, PtdCho, PtdGro, PtdIns, PtdSer, DGDG and MGDG declined to different degrees, but the amount of PtdOH significantly increased (Fig. 2; Supporting Information Table S2). The levels of PtdEtn, PtdCho, PtdGro, PtdIns, PtdSer, DGDG and MGDG were decreased by 40, 17, 36, 40, 47, 6 and 18%, respectively. In antisense plants after stress treatment, the decrease in the amplitude of the previously described lipid molecules was similar to the amplitude found in the WT plants (Supporting Information Table S2). However, in *ZmPIS* sense transgenic plants, after exposure to the stress, the level of PtdGro, PtdSer and MGDG decreased less than those observed in the WT plant, whereas the levels of DGDG and PtdIns increased slightly compared with the levels detected in the sense plants before exposure to stress. The level of PtdOH increased by 90% from 0.38 to 0.72 nmol $mg^{-1}$ DW (Supporting Information Table S2) and was significantly higher than the level observed in the WT and antisense plants under stress. However, the amounts of PtdCho and PtdEtn were slightly lower than the amounts detected in the WT, which might contribute to PtdOH synthesis in the sense plants under stress conditions. PtdGro and PtdIns, the levels of which are higher in the sense plants under stress conditions, might be the other sources for PtdOH synthesis as well. These data suggested that the overproduction of PtdIns in *ZmPIS* overexpressors probably induced higher activities of phospholipases, such as PLC and PLD, compared with the activity found in the WT and antisense plants. Furthermore, in the *ZmPIS* overexpressors, many species that contain polyunsaturated acyl species, such as 34:4, 34:3 and 34:2 PtdGro, 34:3 and 34:2 PtdIns, 40:2 and 42:2 PtdSer, 34:3 and 34:2 PtdOH, 34:3 and 36:6 DGDG, and 36:6 MGDG, contributed the most to the higher level of unsaturated fatty acids during drought stress (Fig. 2). These data suggested that the overexpression of *ZmPIS* in maize plants under drought stress might lead to the increased synthesis of unsaturated phospholipid and galactolipid species, which are involved in the maintenance of membrane permeability and fluidity that might contribute to plant adaptation to drought stress.

Plant survival under water deficit conditions is most likely to depend not only on the protection of its essential cellular components but also on the ability to recover after drought relief (Torres-Franklin *et al*. 2007). Therefore, after rehydration, the extent to which the lipid molecular levels recovered from water stress in the WT and transgenic plants was analysed. In the *ZmPIS* overexpressors after rehydration, the levels of most lipid molecular species including PtdGro, PtdIns, PtdSer, DGDG and MGDG recovered to 79, 87, 56, 97 and 89%, respectively, of those before stress, which were higher than those in the WT plants (73, 66, 53, 90 and 77%, respectively, of the levels before stress). However, the degree to which the PtdEtn and PtdGro levels recovered was lower than the degree found in the WT, which might be related to the lower level of PtdOH in the sense plants because the high level of PtdOH after drought relief was unnecessary for plant survival (Supporting Information Table S2). These results suggested that overproduction of PtdIns in sense plants after rehydration led to the synthesis of unsaturated galactolipid and phospholipid species involved in the recovery of membrane compositions and contributed to an improved ability of plants to recover from drought relief.

## Drought stress-induced gene expression in *ZmPIS* transgenic maize

Changes in gene expression result in many biochemical and physiological changes, and the orchestration of these changes is necessary for the acquisition of stress tolerance (Reddy *et al*. 2011). To investigate whether the saturation changes of membrane lipids were correlated with changes in the expression levels of several genes encoding fatty acid desaturases in the *ZmPIS* overexpressors during drought stress, some genes were subjected to the expression profiling assay by real-time RT-PCR. The result showed that *ZmFAD7* and *ZmFAD8* genes that encode omega-3 fatty acid desaturase were downregulated in all maize plants under drought stress conditions and upon relief of drought (Fig. 3a,b). However, the two genes had relatively high transcript levels in the sense plants, indicating their positive effect in the synthesis of unsaturated fatty acids, which is consistent with previous report (Shimad *et al*. 2000; Wakita *et al*. 2001; Domínguez *et al*. 2010).

In addition, several key genes involved into phospholipid metabolism pathway including *ZmPIS*, *ZmPLC*, *ZmPLD*, *ZmDGK1*, *ZmDGK3* and *ZmPIP5K9* were found to be differentially expressed in transgenic plants. Figure 3d–i shows the expression levels of these genes in the leaves of maize plants under three different water supply conditions (before stress, after stress and rehydration). Before exposure to drought stress, the expression levels of these genes, except for *ZmDGK3*, were significantly higher in the sense plants than the WT plants, whereas in the antisense plants these gene expression levels were lower or equal to those of the WT plants. After drought stress, the expression levels of these

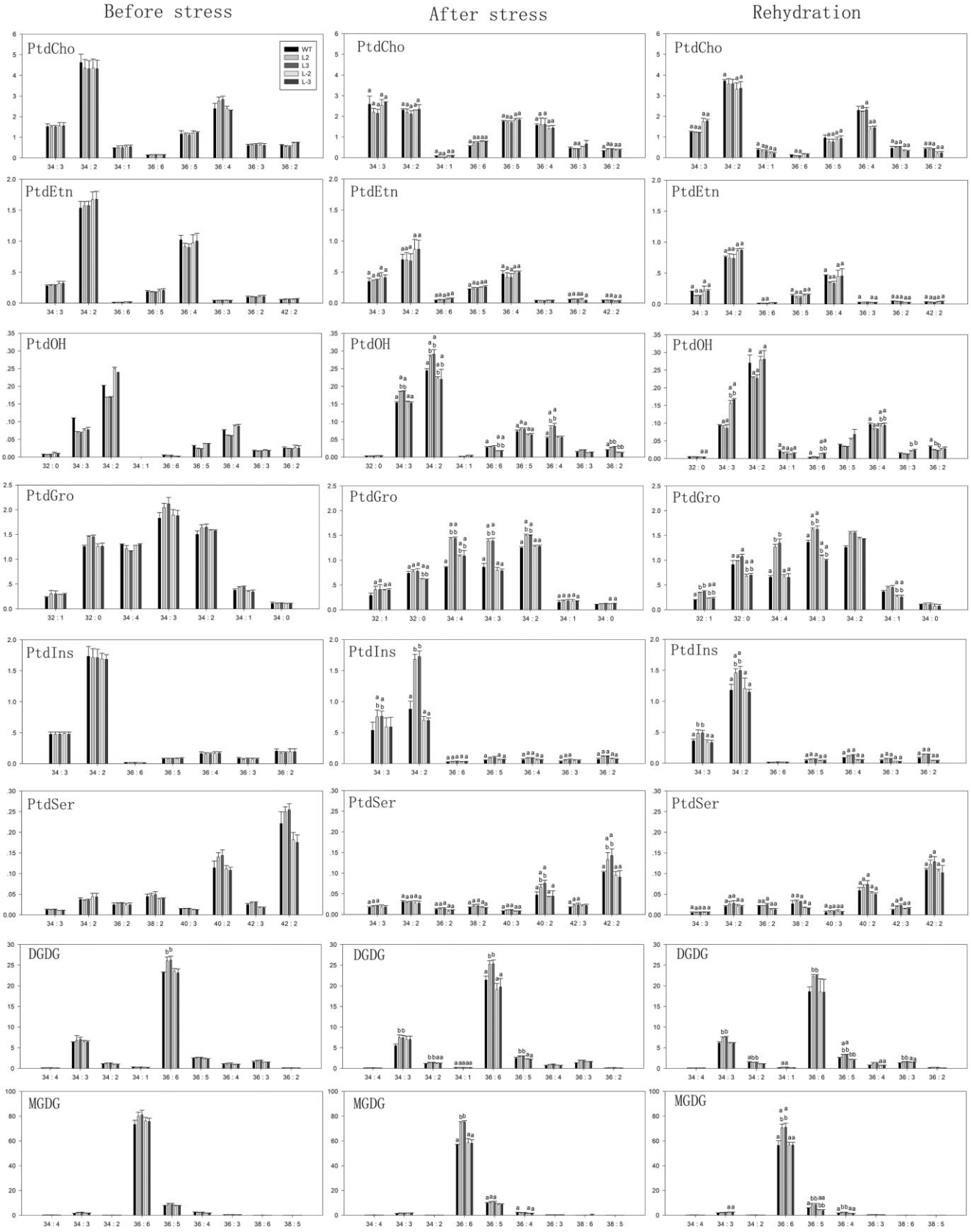

Before stress
After stress
Rehydration
PtdCho
PtdEtn
PtdOH
PtdGro
PtdIns
PtdSer
DGDG
MGDG
WT
L2
L3
L-2
L-3

**Figure 2.** Changes in the lipid molecular species during drought stress in maize plants. WT, wild-type maize; L2, L3, *ZmPIS* sense transgenic plants; L-2, L-3, *ZmPIS* antisense transgenic plants. The dry weight is the dry weight minus the lipids (i.e. dry weight after lipid extraction). Left panel, the lipid levels before stress; middle panel, the lipid levels after stress; right panel, the lipid levels after rehydration. Values are the means of five biological repeats ± SD. a, the value is different from that of the same genotype before stress; b, the value is different from that of WT plants under the same conditions ($P < 0.05$).

genes increased to higher levels in all lines compared with the levels before the stress treatment. However, the detected accumulation of these transcripts was significantly higher in sense plants than in the WT plants, especially for the *ZmPLD* gene, which increased to a level that was 18-fold greater than the level before stress treatment (Fig. 3f). When the plants were rehydrated, in all lines, the expression of most of these genes decreased and recovered to levels that were close to the levels before stress treatment, whereas the expression level of *ZmPIP5K9* did not change significantly after rehydration in sense plants. In contrast, the sense plants continued to display higher expression levels of these genes when compared with the control plants. Conversely, under drought stress and rehydration conditions, the antisense plants showed significantly or slightly lower expression levels of these genes than the levels found in the WT plants. These

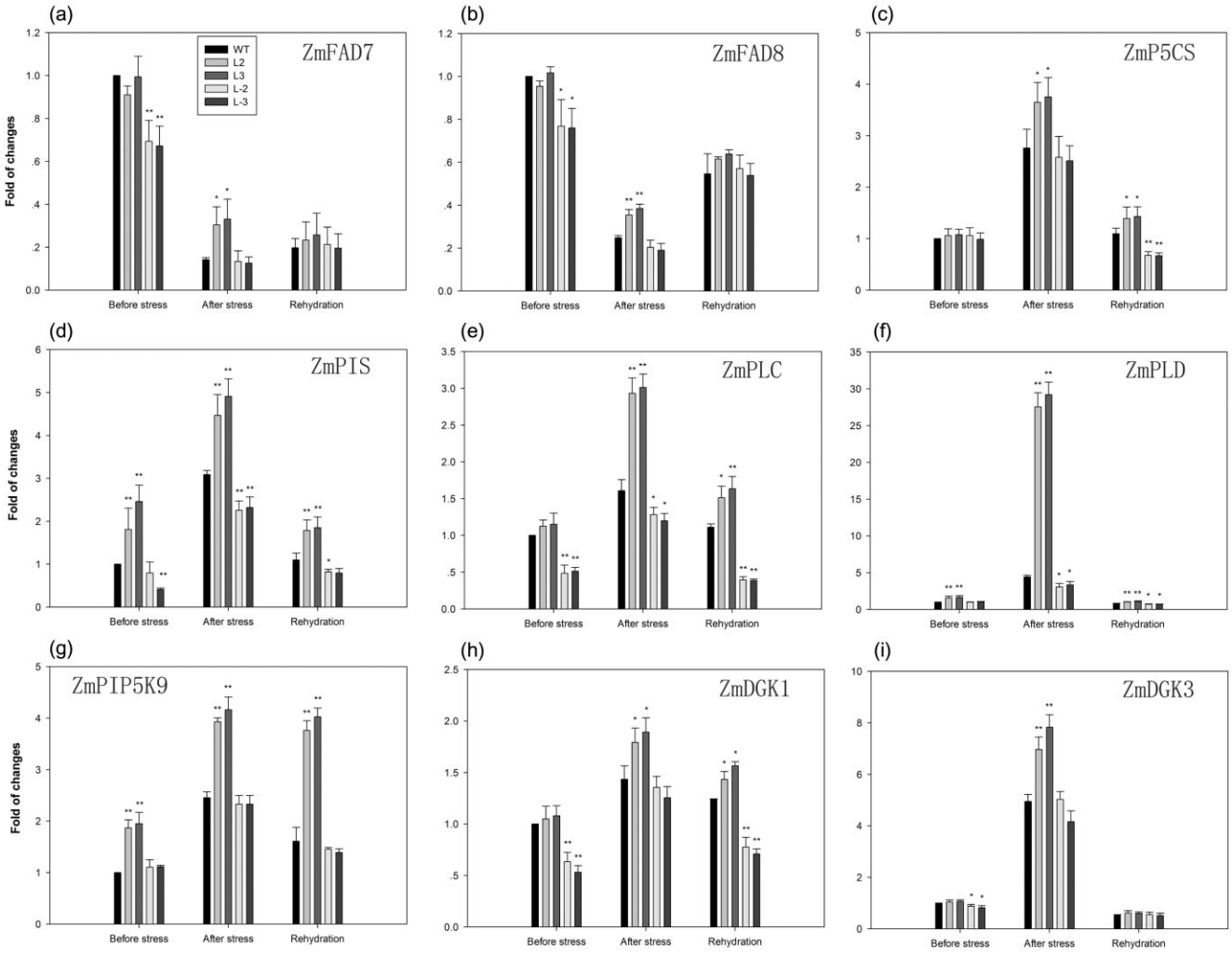



**Figure 3.** The expression pattern of genes involved in the phospholipid metabolism pathway and drought response. (a) The expression pattern of the *ZmFAD7* gene. (b) The expression pattern of the *ZmFAD8* gene. (c) The expression pattern of the *ZmP5CS* gene. (d) The expression pattern of the *ZmPIS* gene. (e) The expression pattern of the *ZmPLC* gene. (f) The expression pattern of the *ZmPLD* gene. (g) The expression pattern of the *ZmPIP5K9* gene. (h) The expression pattern of the *ZmDGK1* gene. (i) The expression pattern of the *ZmDGK3* gene. Total RNA was isolated from the leaves of maize plants under three different hydration levels (before stress, after stress and rehydration). WT, wild-type maize; L2, L3, *ZmPIS* sense transgenic plants; L-2, L-3, *ZmPIS* antisense transgenic plants. Values are the means ± standard deviation of three independent samples from each maize line. The asterisks indicate a significant difference between the transgenic maize and the WT at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test. The expression pattern of genes involved in the phospholipid metabolism pathway and drought response is shown with maize *ubiquitin* gene as an internal control.

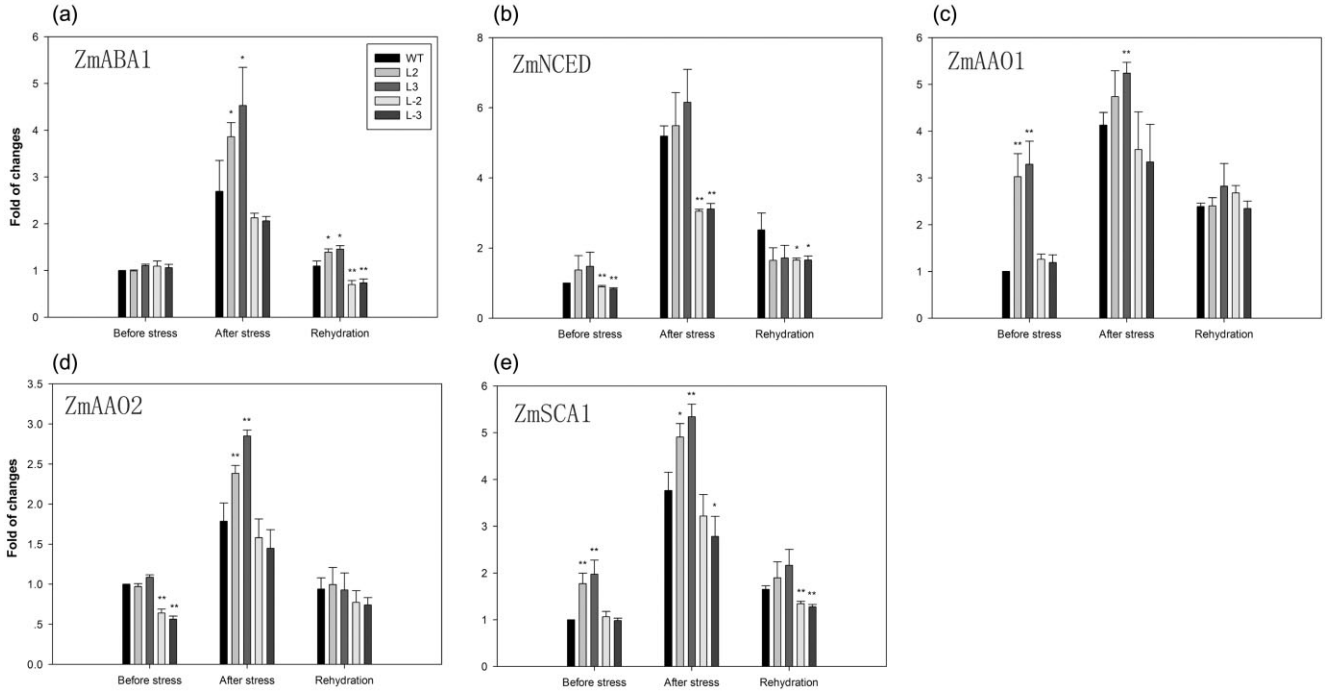



**Figure 4.** The expression pattern of genes involved in the abscisic acid (ABA) biosynthetic pathway. (a) The expression pattern of the *ZmABA1* gene. (b) The expression pattern of the *ZmNCED* gene. (c) The expression pattern of the *ZmAAO1* gene. (d) The expression pattern of the *ZmAAO2* gene. (e) The expression pattern of the *ZmSCA1* gene. Total RNA was isolated from the leaves of maize plants under three different hydration levels (before stress, after stress and rehydration). WT, wild-type maize; L2, L3, *ZmPIS* sense transgenic plants; L-2, L-3, *ZmPIS* antisense transgenic plants. Values are the means ± standard deviation of three independent samples from each maize line. The asterisks indicate a significant difference between the transgenic maize and the WT at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test. The expression pattern of genes involved in the ABA biosynthetic pathway is shown with maize *ubiquitin* as an internal control.

results implied that the overexpression of *ZmPIS* led to higher transcript levels of several genes coding phospholipases and phospholipid kinases, which might result in higher activity of these enzymes and the increased synthesis of phospholipids involved in drought stress response.

Because drought often triggers the production of the phytohormone ABA, we wanted to investigate whether the overexpression of *ZmPIS* affects the biosynthesis of ABA in maize under water deficit conditions. Therefore, the transcript levels of several ABA biosynthetic genes were examined, including *ZmABA1* (Zeathanxin epoxidase), *ZmMCSU* (molybdenum cofactor sulfurase), *ZmNCED* (9-cis-epoxycarotenoid dioxygenase), *ZmAAO1* (ABA-aldehyde oxidase), and *ZmAAO2*, *ZmSCA1* (short chain alcohol dehydrogenase1, ABA2). Drought stress induced the activation of these genes (Fig. 4). *ZmABA1*, *ZmNCED*, *ZmAAO1*, *ZmAAO2* and *ZmSCA1* were induced to higher levels in the sense plants than in the WT plants under water stress. Moreover, after rehydration, the higher expression levels *ZmABA1* and *ZmSCA1* were maintained compared with those in the WT (Fig. 4a,e). However, in the antisense plants, the drought-induced up-regulation of these ABA biosynthetic genes was less than that detected in the WT plants. The results indicated that drought stress might induce increased accumulation of ABA in the *ZmPIS* overexpressors, which might activate ABA-dependent stress-responsive genes and thus contribute to an enhanced adaptability of plants to environmental stimuli.

Drought-induced gene expression can be mediated either through an ABA-dependent or ABA-independent pathway (Yamaguchi-Shinozaki & Shinozaki 2005). The proline accumulation in plants can be regulated by both ABA-dependent and ABA-independent pathways (Mahajan & Tuteja 2005). In this study, the expression levels of the drought-responsive gene *ZmP5CS* (pyrroline 5-carboxylate synthase) in maize were analysed by real-time RT-PCR. As shown in Fig. 3c, under drought stress, the transcripts of this gene accumulated to higher levels in the sense plants than in the WT and antisense plants. The elevated transcript levels of *ZmP5CS* might produce increased accumulation of ZmP5CS protein in cells.

## The increased ABA content in sense transgenic plant leaves

To further investigate whether *ZmPIS* overexpression affects the ABA biosynthesis, the ABA content in plant leaves was determined. The result showed that when compared with WT, *ZmPIS* sense transgenic plants had significantly higher ABA content before and after stress and rehydration, whereas *ZmPIS* antisense transgenic plants had significantly lower ABA content (Fig. 5). This result was consistent with the expression pattern of ABA biosynthetic genes, suggesting that overexpression of *ZmPIS* indeed increases the ABA synthesis.

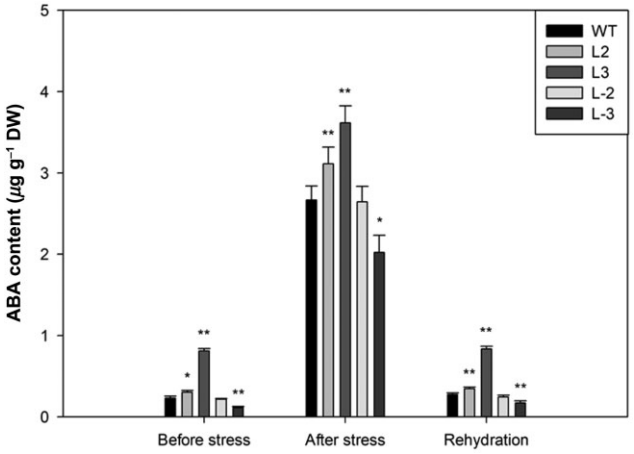



**Figure 5.** The content of abscisic acid (ABA) in maize leaves. WT, wild-type maize; L2, L3, *ZmPIS* sense transgenic plants; L-2, L-3, *ZmPIS* antisense transgenic plants. Values are the means ± standard deviation of three independent samples from each maize line. The asterisks indicate a significant difference between transgenic maize and the WT at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test.

## Sense transgenic plants are more tolerant to drought stress at the pre-flowering stage

Under normal growth conditions, no apparent phenotypic differences were observed between WT and sense transgenic maize plants (Fig. 6a), which suggested that the increases in the *ZmPIS* expression levels in transgenic maize did not have a negative effect on the growth and development of maize plants under normal conditions. However, the antisense plants appeared to be slightly shorter than the WT plants. When transgenic and WT maize plants at the pre-flowering stage were exposed to drought stress treatment, the plants wilted and showed different degrees of damage; the sense transgenic plants grew better and were less withered than the WT and antisense transgenic plants after stress treatment (Fig. 6b). At the pre-flowing stage, delayed plant growth was observed in all lines after 6 weeks of exposure to drought stress. However, the drought stress had less influence on the growth of *ZmPIS* sense transgenic plants with more green leaves than other plants (Table 1). These apparent phenotypic differences suggested that the sense transgenic plants had more tolerance to drought.

## Sense transgenic plants retain more solutes and water under drought stress conditions

To investigate the difference in drought tolerance between the WT and transgenic plants, the leaf solute potential ($\Psi$) of WT and transgenic lines under normal and drought stress conditions was detected during drought treatment. Under normal conditions, there were no obvious differences in the $\Psi$-values of WT and transgenic lines (Fig. 7a). However, under drought stress conditions, the $\Psi$-values of sense transgenic lines were significantly lower than those of WT and antisense lines. In particular, on the 10th day of drought treatment, $\Psi$ of the sense line L3 declined to –0.99 MPa, whereas the values of the WT and the antisense transgenic line L-3 were –0.87 and –0.77 MPa, respectively. After 15 d of exposure to stress, the values of $\Psi$ increased in all lines likely because of solute degradation in leaf cells by severe drought treatment. However, the differences in $\Psi$ between the WT and transgenic lines remained significant. The results

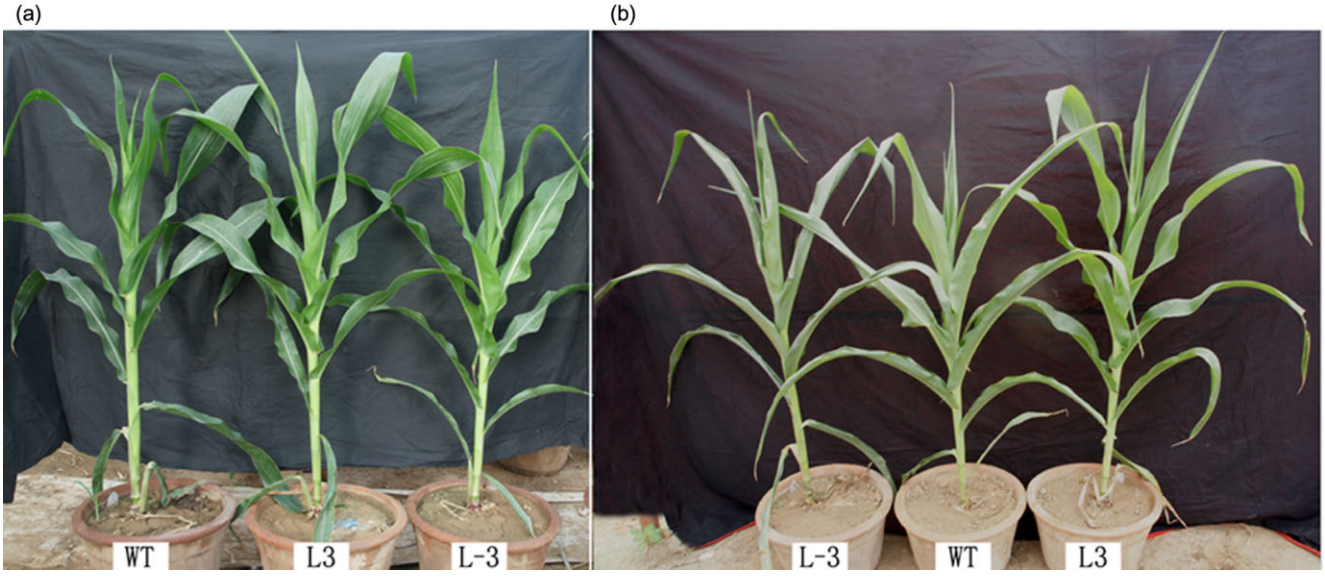



**Figure 6.** The growth status of WT and transgenic maize plants before (a) and after drought stress (b). Under normal growth conditions, no apparent phenotypic differences were observed between the WT and sense transgenic plants. The antisense plants appeared to be slightly shorter than the WT plants. After drought stress, the sense transgenic plants showed enhanced growth and were less withered than the WT and antisense transgenic plants. WT, wild-type maize; L3, *ZmPIS* sense transgenic maize; L-3, *ZmPIS* antisense transgenic maize.

**Table 1.** Agronomic traits of maize plants that suffered from drought stress in the field

| Locations | Traits | | WT | L1 | L2 | L3 | L-1 | L-2 | L-3 |
|---|---|---|---|---|---|---|---|---|---|
| Jinan | Plant height (cm) | Well watered | 178.4 ± 4.2 | 180.9 ± 5.0 | 183.6 ± 4.3 | 184.9 ± 5.3 | 179.4 ± 5.4 | 177.8 ± 4.4 | 177.4 ± 4.1 |
| | | Drought | 160.3 ± 6.3 | 163.8 ± 4.8 | 165.1 ± 4.3 | 167.7 ± 4.5 | 154.3 ± 3.5 | 153.1 ± 3.4 | 151.9 ± 3.1 |
| | Green leaf number/plant | Well watered | 16.7 ± 0.4 | 16.3 ± 0.6 | 17.0 ± 0.5 | 17.5 ± 0.6 | 16.4 ± 0.3 | 16.2 ± 0.3 | 16.0 ± 0.3 |
| | | Drought | 13.0 ± 0.6 | 14.0 ± 0.8* | 14.3 ± 0.5** | 14.9 ± 0.8** | 12.2 ± 0.2 | 12.0 ± 0.3 | 11.7 ± 0.1* |
| | ASI | Well watered | 2.1 ± 0.1 | 2.2 ± 0.3 | 2.1 ± 0.2 | 2.0 ± 0.2 | 2.1 ± 0.2 | 2.2 ± 0.3 | 2.3 ± 0.1 |
| | | Drought | 3.1 ± 0.5 | 2.6 ± 0.5 | 2.4 ± 0.3 | 2.2 ± 0.3* | 3.9 ± 0.4 | 4.1 ± 0.3* | 4.4 ± 0.5* |
| | Grain number/ear | Well watered | 294.8 ± 8.2 | 306.6 ± 7.6 | 309.2 ± 8.7 | 313.2 ± 8.3 | 295.3 ± 7.9 | 290.6 ± 7.4 | 285.5 ± 8.3 |
| | | Drought | 232.6 ± 10.4 | 260.8 ± 7.5* | 269.9 ± 7.0** | 278.1 ± 7.6** | 191.6 ± 7.8** | 186.7 ± 8.8** | 180.3 ± 8.3** |
| | Grain weight/ear (g) | Well watered | 80.3 ± 5.6 | 83.5 ± 6.1 | 85.8 ± 6.8 | 87.3 ± 6.3 | 76.4 ± 4.6 | 73.5 ± 4.3 | 70.5 ± 4.8 |
| | | Drought | 52.1 ± 3.2 | 61.6 ± 4.5* | 66.2 ± 5.1** | 71.2 ± 4.1** | 46.0 ± 3.5 | 42.3 ± 3.1* | 39.6 ± 3.2** |
| | 100 grain weight (g) | Well watered | 26.7 ± 3.6 | 27.1 ± 5.2 | 27.7 ± 4.7 | 28.4 ± 4.5 | 25.9 ± 3.9 | 25.3 ± 3.3 | 24.9 ± 3.4 |
| | | Drought | 24.3 ± 5.7 | 24.9 ± 3.6 | 25.8 ± 4.2 | 26.7 ± 4.6 | 23.7 ± 2.3 | 22.7 ± 2.3 | 22.2 ± 2.3 |
| Xinjiang | Grain weight/ear (g) | Well watered | 213.3 ± 17.9 | 206.9 ± 18.2 | 213.7 ± 18.6 | 216.5 ± 18.0 | 179.4 ± 5.4 | 177.8 ± 4.4 | 177.4 ± 4.1 |
| | | Drought | 109.2 ± 15.7 | 120.9 ± 16.3** | 125.8 ± 18.3** | 129.5 ± 17.5** | 97.3 ± 6.5 | 92.0 ± 8.4 | 89.9 ± 6.8 |

A field experiment was carried out in the Experimental Field of Lvjia in Jinan under a removable rain-off shelter. The maize test plots were arranged according to a report described by Li et al. (2008b). After exposure to drought stress conditions for 6 weeks, the plants were then well watered. Plant height, leaf numbers, and the ASI of 10 maize plants from each of the transgenic and WT lines were determined at pollination stage. After harvesting, the seeds were dried to constant weight naturally, and the number and weight of seeds per ear from each line were recorded, respectively. Another field experiment was performed in the Anningqu Experimental Field of Xinjiang Academy of Agriculture in Xinjiang Uygur Autonomous Region in China under natural conditions. The experiment fields were divided into two zones: one was for treatment of drought stress; another was the control under well-watered conditions. Each zone was arranged in a random complete block design with three replications. After harvesting, grain weight per plant and grain weight per ear were measured, respectively. The data were from the 2009 field experiment. Values of transgenic lines were significantly different from those of WT plants at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test.
ASI, anthesis-silking interval

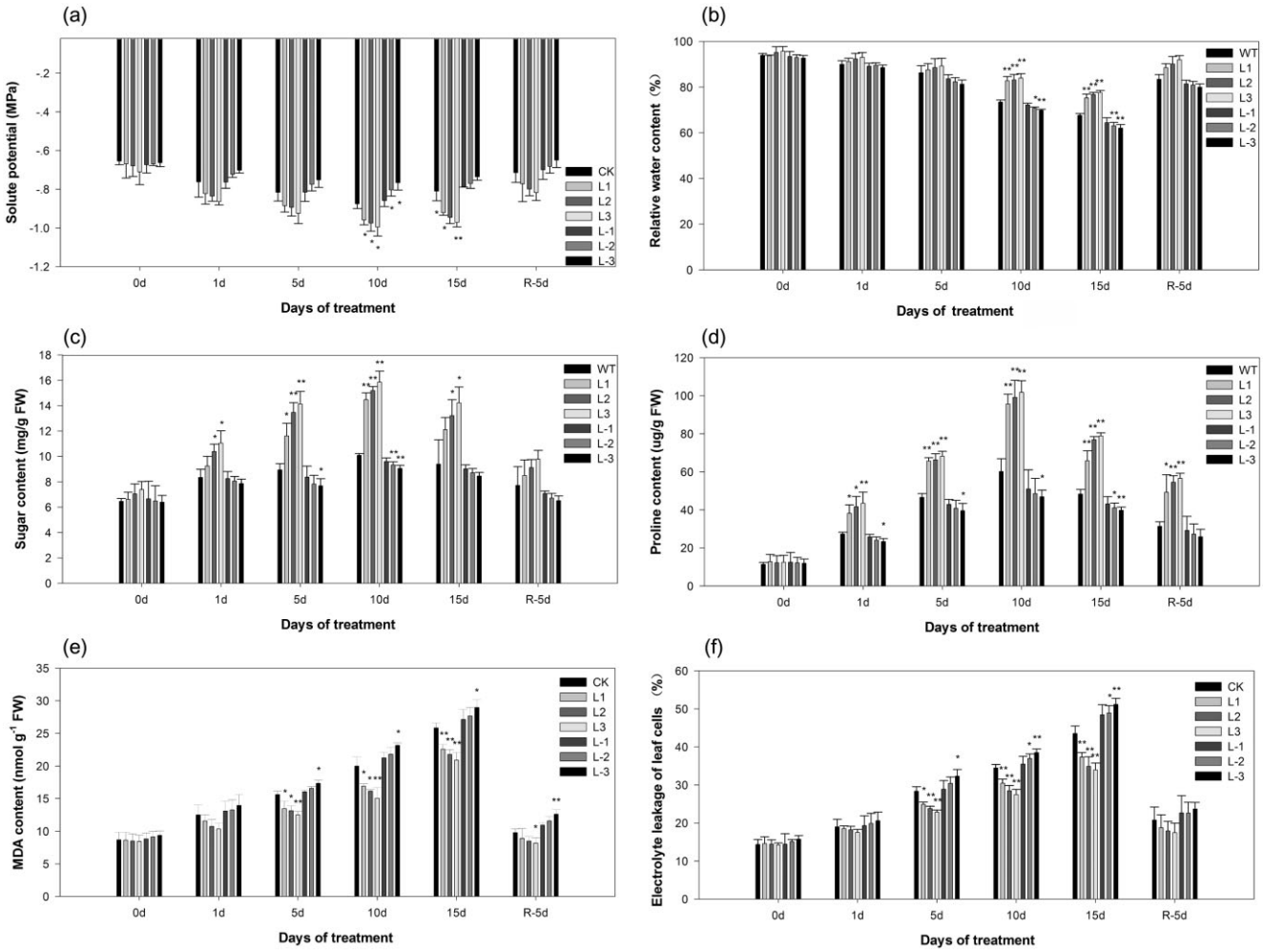



**Figure 7.** Effects of drought stress on the solution potential (a), relative water content (b), total soluble sugars (c), proline acids (d), malondialdehyde (MDA) levels (e), and membrane ion leakages (f) in maize leaves. Values are the means ± standard deviation of three samples. The asterisks indicate a significant difference from the WT under drought conditions at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test.

suggested that *ZmPIS* overexpression conferred a higher solute accumulation in the leaf cells of maize plants to cope with drought stress.

To detect the water loss of transgenic maize leaves, RWC of the leaves from sense, antisense transgenic and WT plants was measured under drought stress treatment. As shown in Fig. 7b, there were no significant differences between the transgenic and WT plants before stress treatment. After stress treatment, the RWC declined in the leaves of all lines. However, the WT and *ZmPIS* antisense transgenic plants showed more water loss than *ZmPIS* sense transgenic plants did under drought stress. Obvious differences were observed on the 10th and 15th days of exposure to stress. After 15 d of stress treatment, the leaf RWC of *ZmPIS* sense lines L1, L2 and L3 declined to 75.2, 76.7 and 77.6%, whereas the leaf RWC of the WT and *ZmPIS* antisense lines was 67.6%, and 62.1–64.4%, respectively.

After drought treatment, the $\Psi$ and RWC of all lines gradually recovered during the 5 d rewatering experiment, but the *ZmPIS* sense transgenic lines still had a lower $\Psi$ and a higher RWC than the WT and *ZmPIS* antisense transgenic lines (Fig. 7a,b).

## The *ZmPIS* sense transgenic line showed increased accumulations of total soluble sugar and proline

To further study the impact of other factors, such as the osmotic adjustment ability, in transgenic plants, the total soluble sugar and proline contents of leaves in the WT and transgenic plants were determined at different time points during stress treatment. Under normal conditions, no differences in the total soluble sugar and proline contents of WT, sense and antisense transgenic plant leaves were observed (Fig. 7c,d). However, the total soluble sugar and proline content of leaves of WT, sense and antisense transgenic lines was significantly different under drought stress. The total soluble sugar content of leaves was significantly higher in the three sense transgenic lines than in the WT and three antisense lines on the 5th, 10th and 15th days of drought stress,

and peaked (15.47, 15.19 and 15.86 mg $g^{-1}$ FW, respectively) on the 10th day of stress, whereas the WT line and antisense lines peaked at 10.08 and 9.05–9.61 mg $g^{-1}$ FW, respectively, at this time point. When all the lines were exposed to sufficient irrigation conditions for 5 d following drought treatment, the total soluble sugar content in the leaves of all lines gradually decreased, but the total soluble sugar contents remained higher in the L1, L2 and L3 than that in the WT and antisense lines. These results showed that the rate of sugar synthesis was higher in the sense lines than in the WT and antisense lines, suggesting the sense lines suffered from less damage and less reduction in photosynthesis under drought stress. The proline contents of leaves from the WT and transgenic plants followed a similar trend as described for the total sugar content. For example, the proline content in the sense transgenic line L3 peaked at 101.77 $\mu g$ $g^{-1}$ FW after 10 d of stress treatment, which was higher than the peak of the WT line (60.17 $\mu g$ $g^{-1}$ FW) and the antisense lines (46.88–51.05 $\mu g$ $g^{-1}$ FW). The result indicated that the induction of *ZmP5CS* during drought stress indeed led to an increased accumulation of proline. Moreover, the changes in proline content of different lines suggested the better drought tolerance of *ZmPIS* sense transgenic lines.

### Reduced cellular membrane damage in *ZmPIS* overexpressors

As the overexpression of *ZmPIS* led to the increased lipid levels of maize plants, we expect that drought stress induces less membrane injury in the *ZmPIS* overexpressors. As important markers for oxidative stress and the oxidation of membrane lipids, the MDA content and the electrolyte leakage (%) of leaf cell from maize plants were measured under drought and rehydration treatments. As shown in Fig. 7e,f, the MDA content and electrolyte leakage (%) of leaf cells increased gradually with increasing drought treatment in all lines. However, the damage to sense transgenic plants was much less than the damage to WT and antisense transgenic plants. For example, after 15 d of drought treatment, the MDA content and electrolyte leakage (%) in sense transgenic lines were 20.90–22.57 nmol $g^{-1}$ FW and 33.9–37.3%, respectively, less than those in the WT line (25.83 nmol $g^{-1}$ FW and 43.5%), while those in the antisense transgenic lines (27.15–28.96 mol $g^{-1}$ FW and 48.7–51.2%) were higher than those in the WT line (Fig. 7e,f). After 5 d of rehydration, the membrane ion leakage and MDA levels of leaves from sense transgenic plants were consistently closer to the initial levels observed under well-watered conditions. These results suggested that *ZmPIS* sense transgenic plants suffered from less cellular membrane damage during drought treatment because of enhanced lipid levels within membranes, which help to maintain the cellular membrane stability.

### *ZmPIS* sense transgenic plants displayed a higher rate of photosynthesis under drought stress

To evaluate the effect of drought stress on plant photosynthesis, the net photosynthesis rate (A), stomatal conductance (gs), and transpiration rate (Tr) of leaves from WT and transgenic plants were respectively measured during drought and rewatering treatment. The sense lines displayed a significantly higher A, gs and Tr than the WT and antisense lines did during drought treatment and an improved ability to recover after rewatering (Supporting Information Fig. S1a,b.c).

Under stress conditions, the chlorophyll content was also significantly higher in the sense plants than in the WT and antisense lines at the same time point (Supporting Information Fig. S1d). The $F_v/F_m$ represents the maximal efficiency of PSII photochemistry. After drought stress treatment, the $F_v/F_m$ declined faster in the WT and antisense lines than in the sense lines, which suggested that drought stress led to less damage to the photochemical activity of *ZmPIS* sense transgenic plants (Supporting Information Fig. S1e).

### Reproductive development and yields of maize plants after drought stress

Plants at the pre-flower stage were subjected to drought stress and then watered well. When the plants were at the pollination stage, the plant height, plant green leaf number, and ASI were recorded. As shown in Table 1, under drought stress, the differences in plant heights among different lines were not obvious, but sense transgenic lines had more green leaves than the WT and antisense lines did. Under drought stress, the development of male and female organs in maize plants was also seriously affected. The amount of pollen was decreased, and the ASI was prolonged. Nevertheless, the ASI was shorter in the sense transgenic maize than in the WT and antisense transgenic maize. From these results, it can be deduced that the reproductive development of sense transgenic lines was less inhibited by drought stress compared with the WT and antisense transgenic lines.

The yields of maize plants under drought stress in the Lvjia Experiment Field in 2009 are shown in Table 1. No difference in the 100 grain weights of WT and transgenic lines was observed. However, the grain number and grain weight for each ear were greater in the sense transgenic lines than those in the WT and antisense lines. The gain number for each ear of the sense lines was 12.1, 16.0 and 19.6% higher than the WT line, whereas the grain weight for each ear was 18.2, 27.1 and 36.7% higher in the sense lines than in the WT line. In contrast, the grain number and grain weight in each ear were significantly lower in the antisense transgenic lines than in the WT. The results suggested that the sense *ZmPIS* lines had higher yields than WT and antisense lines, which was further supported by the results from the Xinjiang field experiment (Table 1).

## DISCUSSION

### *ZmPIS* is involved in plant responses to drought stress in maize

Phospholipids have been revealed to have important roles in the adaptation of plants to stress (DeWald *et al*. 2001; König, Mosblech & Heilmann 2007; Löfke *et al*. 2008; Perera *et al*.

2008; Yang *et al*. 2008). PtdIns, one of the major phospholipids, has emerged as a signalling role and as precursors of several types of second messengers in eukaryotes. Realization of this important role for PtdIns has brought additional attention to the function of PIS, which catalyses PtdIns synthesis. Several reports have indicated the involvement of PIS during stress responses in plants (Lin *et al*. 2004; Das *et al*. 2005; Sui *et al*. 2008; Zhai *et al*. 2012). Here, transgenic maize plants overexpressing *ZmPIS* or underexpressing *ZmPIS* were obtained in order to unveil the role of *ZmPIS* gene in maize responses to drought stress. In our results, the transcript levels of the *ZmPIS* gene were altered to various levels in the transgenic lines (Fig. 1e). In the *ZmPIS* sense lines, the transcript levels of the *ZmPIS* gene were significantly enhanced compared with the WT, and the *ZmPIS* sense transgenic plants displayed improved drought tolerance compared with the WT and antisense transgenic plants. It could be concluded that *ZmPIS* is involved in plant responses to drought stress in maize.

## Overexpression of *ZmPIS* in maize alters the membrane lipid composition

Overexpression of *ZmPIS* resulted in elevated levels of most phospholipids and galactolipids in maize under drought stress (Fig. 2, Supporting Information Table S2). The PtdIns content was higher in the sense maize plants than in the WT by 1.18 nmol $mg^{-1}$ DW, which was significantly higher than the extent of PtdIns increases in the *ZmPIS*-overexpressing tobacco plants under drought stress treatment (0.56 nmol $mg^{-1}$ DW) (Zhai *et al*. 2012). The overproduction of PtdIns might lead to the increased expression of *ZmPLC*, *ZmDGK* and *ZmPLD* in sense transgenic plants (Fig. 3e,f), resulting in the hydrolysis of PtdCho and PtdEtn and the increased levels of PtdOH (Fig. 2, Supporting Information Table S2); because PtdOH can be synthesized from two pathways: the PLC/DGK pathway and the PLD pathway (Sui *et al*. 2008). Moreover, the increased amounts of PtdIns and *ZmPIP5K9* expression might supply sufficient substrates such as PtdIns(4,5)$P_2$ for phospholipases (Ischebeck *et al*. 2008; Stenzel *et al*. 2008) (Fig. 4g).

The elevation in PtdOH levels might play a role in the activation of MGDG synthase, and result in increased accumulation of MGDG and DGDG (Dubots *et al*. 2010). Because the MGDG and the DGDG are the major components of the chloroplast envelope and thylakoid membrane, the decrease in their levels inevitably led to damage of the plastid membranes and the light-dependent reactions of photosynthesis. Therefore, the biosynthesis and levels of MGDG and DGDG in plant leaves could be important for plant's resistance to drought stress. In *ZmPIS* overexpressors, increased accumulation of MGDG and DGDG might help to maintain the membrane structure and reduce the damage to photosystems by drought stress. PtdGro is a major phospholipid in the chloroplast envelope and thylakoid membrane, and the elevation of its levels might also contribute to reduced damage to photosystems (Dubots *et al*. 2010). Consistent with this hypothesis, we detected a higher photosynthesis rate, increased chlorophyll content, and an enhanced photochemical activity of PSII in *ZmPIS* overexpressors. These results suggested that the overexpression of *ZmPIS* improved maize drought tolerance by maintaining higher levels of galactolipids.

Drought-tolerant plants were significantly enriched in unsaturated membrane lipids during osmotic stress (de Monteiro Paula *et al*. 1990; Repellin *et al*. 1997; Gigon *et al*. 2004; Larsson *et al*. 2006; Torres-Franklin *et al*. 2007). In *ZmPIS* overexpressors, a higher level of unsaturated fatty acids was detected during drought stress. We consistently observed higher expression levels of genes encoding fatty acid desaturases, reduced cellular membrane damage, higher total soluble sugar and proline contents, enhanced resistance to water loss and decreased solute potential. These results suggested that the *ZmPIS* overexpression plants increased the membrane stability by elevating the unsaturated fatty acid levels of membrane lipid composition, further contributing to the drought tolerance of plants.

## Overexpression of *ZmPIS* in maize altered the expression of genes involved in the PI signalling pathway

In eukaryotic cells, PtdIns synthesis is catalysed by PIS and initiates the PI signalling pathway. Thus, the overproduction of PtdIns likely affected the PI signalling system. Some key genes involved in PI signalling pathway, including *ZmPIPK9*, *ZmPLC*, *ZmDGK1*, *ZmDGK3* and *ZmPLD*, were induced to higher transcript levels in the *ZmPIS* overexpressors under drought stress (Fig. 3). Overproduction of PtdIns may induce higher activity of enzymes encoded by these genes. The subsequent formation of their products, for example, PtdIns (4, 5)$P_2$, DAG, $InsP_3$ and PtdOH, boosts the PI signalling cascades and activates a host of target enzymes connected to numerous cellular processes. Moreover, the formation of more $InsP_3$ and PtdOH may contribute to more substrates, for example, Ins and CDP-DG for PIS. Therefore, the regulatory loop of PI signalling pathway was increased by *ZmPIS* overexpression.

## Overexpression of *ZmPIS* increases the ABA synthesis in maize leaves

It is well known that ABA plays an important role in plant growth and development and is critical for controlling downstream stress responses (Tuteja 2007). ABA levels are induced strongly by drought stress and ABA signalling is closely linked to enzymes related to the phospholipid metabolism (Mikami *et al*. 1998; Munnik *et al*. 2000; Sang *et al*. 2001; Lin *et al*. 2004; Gunesekera *et al*. 2007; Lee *et al*. 2007; Hong *et al*. 2008; Perera *et al*. 2008; Sui *et al*. 2008; Tasma *et al*. 2008). Therefore, we speculated that overexpression of *ZmPIS* might affect the expression of ABA biosynthetic genes and the biosynthesis of ABA. We observed the induction of higher transcript levels of genes involved in ABA biosynthesis and the increased ABA content in leaves of *ZmPIS* overexpressor under drought stress, indicating that

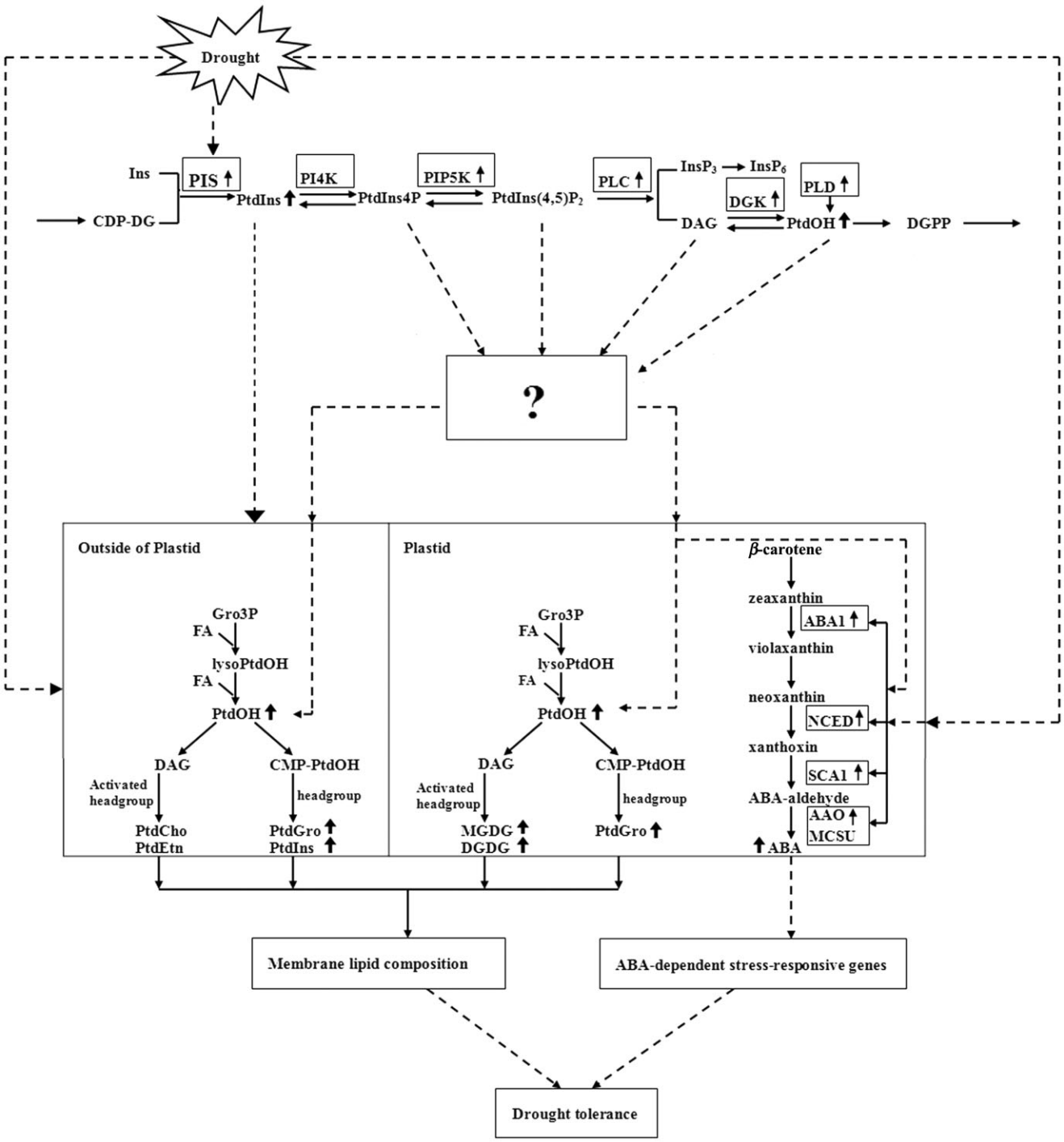



**Figure 8.** A speculative model of the increased activation of plant drought stress-responsive pathway by the overproduction of PtdIns. An increase in PtdIns could amplify the PLC/DGK signalling cascade by activating PIP5K, PLC and DGK. The increase in PtdOH could autoamplify PLD signalling. PtdIns can be signal molecular themselves or signal precursors to produce more signal molecules. Most of those not only can trigger a series of signal cascades and activation of stress-responsive genes, but also act as membrane lipid compositions that participate in membrane structure maintenance. The enhanced dual function of PtdIns and other phospholipid signal molecules resulted in physiological changes of *ZmPIS* overexpression plants adapting to stress, and finally contributing the increased drought tolerance. Ins, inositol; DGPP, diacylglycerol pyrophosphate; FA, fatty acid; Gro3P, glycerol 3-phosphate. Thin upward arrows stand for up-regulation of genes coding these enzymes; thick upward arrows stand for the increase of the composition content in leaves.

*ZmPIS* regulates maize response to drought stress by increasing ABA synthesis.

The transcript accumulation of *P5CS* was reported to be regulated in an ABA-dependent manner (Knight, Trewavas & Knight 1997; Silva-Ortega *et al.* 2008). Drought induces the expression of *P5CS*, which encodes a protein contributing dehydration tolerance. In a report by Takahashi *et al.* (2001), the hyperosmotic stress-responsive expression of some ABA-inducible genes including *AtP5CS1* was not influenced by the PI-PLC inhibitors, which showed these genes seem not to be regulated by the PI-PLC signalling pathway. Therefore, it is likely that there exists at least one PI-PLC-independent but ABA-dependent signalling transduction pathway, which can be induced by drought stress and result in the enhanced induction of *ZmP5CS* gene expression and the increased proline content. Moreover, the signalling pathway might also be related to the overproduction of PtdIns.

### *ZmPIS* overexpression in maize improves yields after drought stress treatment

The ultimate objective of crop transgenic technology is to obtain stress-tolerant crops with high yields. In this study, the yield of transgenic plants was also examined. We found that the ASI of sense transgenic maize lines was shorter than the ASI of WT plants after drought stress. Moreover, the grain yields were significantly higher in the sense transgenic lines than in the WT and antisense lines after 6 weeks of exposure to drought stress (Table 1). The Xinjiang field experiment also confirmed that the sense lines had higher yields than the WT lines under natural drought conditions. The increased yields were likely due to the improved photosynthetic performance observed in the sense transgenic plants than that in WT plants under drought conditions. This indicates that the *ZmPIS* gene could be used to improve the yields of a range of agriculturally important crops in arid and semi-arid regions or in areas where irrigation is difficult. This result provides a strategy for breeding new maize varieties with improved resistance to drought stress.

In summary, the overexpression of *ZmPIS* in maize plants effectively improves the drought tolerance of plants. In brief, overexpression of *ZmPIS* resulted in overproduction of PtdIns. PtdIns can be a signal molecular or a signal precursor to produce more signal molecules. Most of those as membrane lipids can participate in membrane structure maintenance and/or as signal molecules can trigger a series of signal cascades and activation of stress-responsive genes, further leading to the increased levels of unsaturated membrane lipids and the improved drought stress tolerance of plant (Fig. 8).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Effects of drought stress on net photosynthesis (a), stomatal conductance (b), transpiration rate (c), maximum efficiency of PSII photochemistry ($F_v/F_m$) (d), and chlorophyll content (e) of maize plants. The values are the means ± standard deviation of three samples. The asterisks indicate a significant difference from the wild type (WT) under drought conditions at $*0.01 < P < 0.05$ and $**P < 0.01$ using the *t*-test.
**Figure S2.** The growth status of WT and transgenic maize plants after drought stress in Xinjiang field experiment.
**Table S1.** Gene-specific primer pairs used in the RT-PCR experiments.
**Table S2.** Total amount of lipids in each head group class before and after drought stress, and rehydration in WT and transgenic maize plants.