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Assessment of Drought Tolerance Based Impacts with Over-expression of *ZmLTP3* in Maize (*Zea mays* L.)

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Numerous studies showed that lipid transfer proteins (LTPs) play important roles in flower, development, cuticular wax deposition and pathogen responses; however, their roles in abiotic stresses are relatively less reported. This study characterized the function of a maize *LTP* gene (*ZmLTP3*) during drought stress. *ZmLTP3* gene was transferred into maize inbred line Jing2416; subsequently the glyphosate and drought tolerance of the over-expression (OE) lines were analyzed. Analysis showed that OE lines could significantly enhance drought tolerance. Transgenic maize lines OE6, OE7 and OE8 showed lower cell membrane damage, higher chlorophyll contents, higher protective enzymes activities, better growth and development under drought condition. The results strongly indicated that overexpression of *ZmLTP3* could increase drought tolerances in maize.

Keywords: maize, lipid transfer protein, *ZmLTP3*, abiotic stress tolerance

Introduction

Lipid transfer proteins are a kind of basic proteins with small molecular mass (about 6 to 10 kD). They are named by their abilities to bind and transfer various lipids (Kader 1996). Each LTP has 8 conserved Cys residues (8 CM), these 8 Cys residues form 4 disulfide bonds, which endows LTPs with the characters of improved tolerance to abiotic stresses including high temperature and denaturation (Edstam et al. 2014). The LTP molecules contain 4 or 5 α-helices. The α-helices further form a tunnel-like hydrophobic cavity, which various lipid molecules can bind to (Kader 1997; Edstam et al. 2014).

Based on molecular mass, the LTPs was classified into 2 groups, the first group, LTP1, has higher molecular masses (about 10 KD) and more amino acids residues (90 ~ 95) (Edstam et al. 2011). The second group, LTP2, is smaller than LTP1, with about 70 residues and about 7 kD molecular masses (Castro et al. 2003). These two group members share about 30% sequence identity and similar folding properties (Trevor and Jocelyn 2008). Additionally, at the amino terminal both families contain a signal peptide (Suelves and Puigdomenech 1997; Garcia-Garrido et al. 1998). Recently, a modified classification

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system was presented based on Cys residues spacing, the conserved intron position and the post-translational glycosylphosphatidylinositol (GPI)-anchor addition. In this classification system, the LTPs are classified into 10 groups (Edstam et al. 2011).

As plant LTPs have the ability to transfer lipids, they are initially thought to be involved in the synthesis of bio-membrane system (Kader et al. 1984). Later, the findings of the signal peptide and the extracellular position indicate further details of their functions. Now numerous functions have been suggested for LTPs in plant physiology, such as cutin biosynthesis and transport, β-oxidation, pollen development, cell wall elongation, plant signaling and stresses (Sterk et al. 1991; Cameron et al. 2006; Patkar and Chattoo 2006; Kirubakaran et al. 2008; Trevor et al. 2008; Debono et al. 2009; Lee et al. 2009; Fan et al. 2013; Huang et al. 2013; Edstam and Jocelyn 2014). However, their roles in abiotic stresses is relatively seldom reported. This study investigated the biological role of *ZmLTP3*, which was reported to be induced by various abiotic stresses (Sun et al. 2014). Over-expression of *ZmLTP3* constitutively enhanced the drought tolerance in transgenic maize seedlings.

Materials and methods

Plasmid construction and Plant transformation

The full length *ZmLTP3* cDNA was obtained via PCR from T vector and cloned into our previous modified plasmid pGreen0229 (Li et al. 2014) at MluI-NotI sites (Fig. 1).

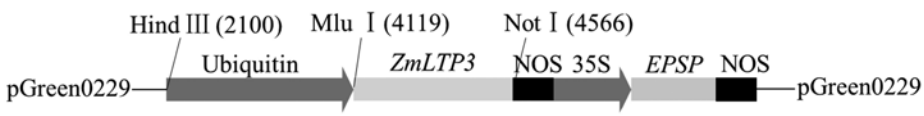

Figure 1. Construction of the *ZmLTP3* expression vector

Maize inbred line Jing2416 was used for transformation in this work. The procedures for the transformation method were conducted as described by Zou et al. (2014).

Transformants screening

After transformation, T_1 seeds were harvested and used for characterization of transformants in greenhouse. 200 mg/L glyphosate were sprayed to the three-leaf stage seedlings. Two weeks later, most seedlings died, only few seedlings survived which were possible transformants.

The glyphosate resistant seedlings were characterized by PCR. Genomic DNA was isolated from glyphosate resistant and inbred line Jing2416 (wild type, WT) seedlings. The forward primer (FP) and reverse primer (RP) sequences designed corresponding to partial *ZmLTP3* and NOS sequence were as follows:

FP, 5'- TGTGCAGAACCCATCTCTTATC -3',
RP, 5'- CGACAGCGAGAATCGGATATT -3'.

QualiPlate™ Kit for LibertyLink® PAT/EPSP was used to detect EPSP protein in leaf tissues of PCR positive seedlings according to the procedure of kit manual.

Drought tolerance assays

Maize growth and drought treatment were conducted as described by Zou et al. (2014) with minor modification. Maize seedlings including OE and WT with uniform size were selected at 3-leaf stage, and imposed to drought stress for 2 weeks. After 1 week, the photographs were taken. According to the previous characterizations, transgenic lines 6, 7 and 8 (OE6, OE7 and OE8) and WT were chosen for the onward morphological and physiological measurements. The morphological parameters of seedlings were determined at 14 days after drought treatment. The physiological parameters of seedlings were determined at 0, 7 and 14 days after stress, respectively. The above morphological and physiological parameters of OE and WT plants under normal condition as controls were also determined at the same time. Measurements of malonaldehyde (MDA) and relative electric conductivity ratio were performed according to the description of Zou et al. (2014). Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities and chlorophyll contents were determined as described by Kumar et al. (2008). The experiments were repeated for 3 times. Student's *t*-test and Tukey's multiple range tests (for one-way ANOVA) in SPSS version 22 were used for the data statistical analyses.

Results

Herbicide selection of transformants

Glyphosate was used to select the possible transgenic maize seedlings. After two weeks of glyphosate application, most maize seedlings exhibited phytotoxicity symptoms and finally died. Only few seedlings grew healthier without phytotoxicity symptoms, strongly indicating herbicide resistance (Fig. 2). These seedlings were likely positive transgenic lines and thus were used for the next characterizations.

Molecular characterization of transgenic maize

After selection based on herbicide resistance, 19 individual entries were obtained from T_1 seeds. PCR analysis of the 19 lines was performed for further confirmation. PCR result suggested that most herbicide resistant plants possessed the *ZmLTP3* gene (Fig. 3). The homozygous transgenic lines were obtained from the PCR positive plants for the following immunoassay. Immunoassay showed that all the homozygous plants exhibited a positive EPSP-specific reaction band (Fig. 4). This strongly confirmed the expression of the foreign genes in transgenic lines.

Figure 2. Selection of herbicide resistance in transgenic maize

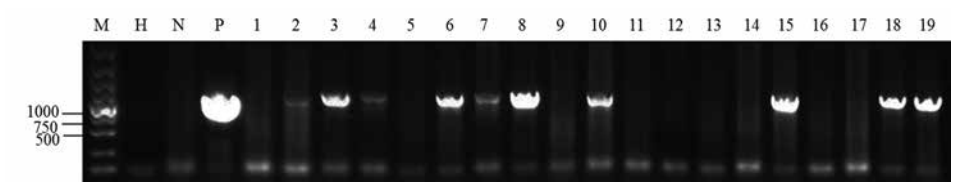

Figure 3. PCR analysis of the transgenic maize lines. M: Marker; H: dd H_2O; N: Non-transgenic maize; P: Plasmid control; 1–19: Herbicide resistance lines

Morphological assay of transgenic maize

Drought tolerance comparison was conducted between inbred line Jing2416 and three homozygous transformants (OE6, OE7 and OE8). The examination results showed that after drought treatment, WT and transgenic plants showed obvious phenotypic differences. Severe growth inhibition and leaf wilting were observed in the WT plants, whereas, all the transgenic lines grew better with less growth inhibition and leaf wilting, indicating their more drought tolerance than WT plants (Fig. 5).

Some morphological parameters of the above plant materials were measured after 2 weeks' drought treatment. The results were shown in Table 1. After treatment the transgenic lines possessed increased plant height, main root length, base stem width, dry weight (DW) and fresh weight (FW), while the above parameters in WT plants were decreased. The three transgenic lines displayed no differences in the above parameters.

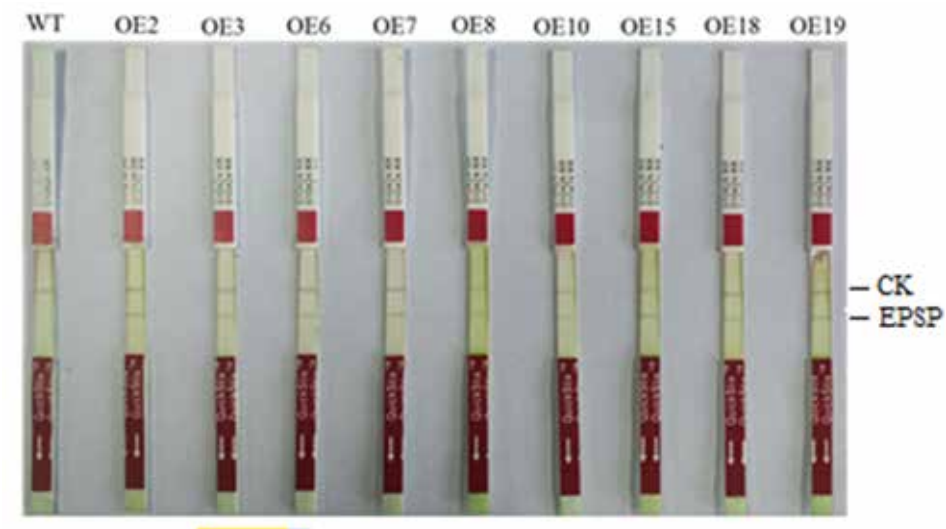

Figure 4. EPSP detection of the transgenic maize lines. WT: Wild type lines; OE2~OE19: Herbicide resistance lines

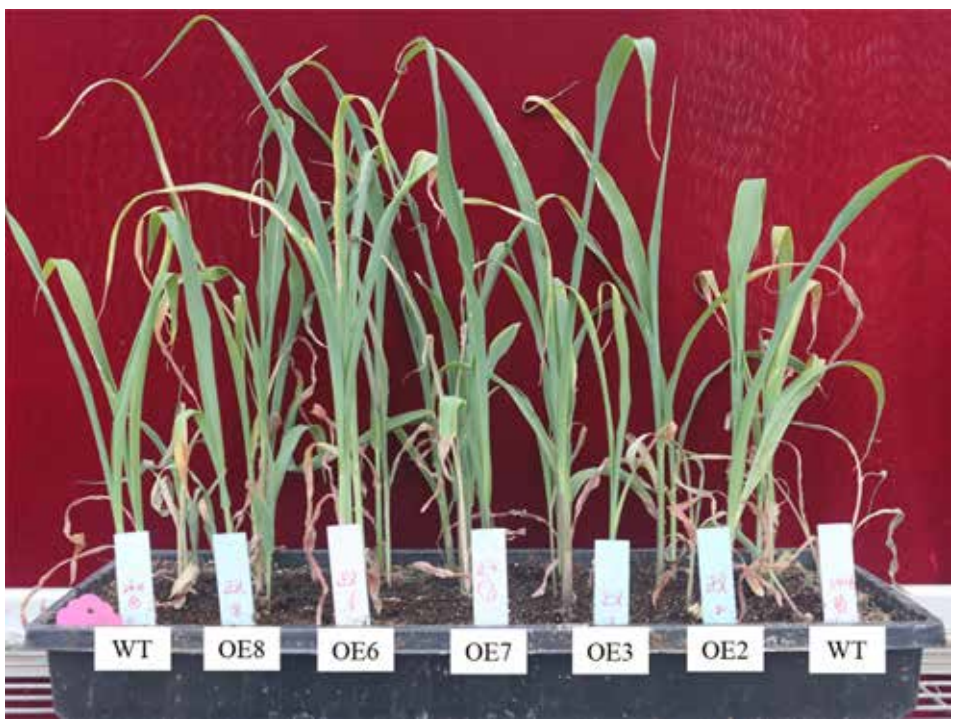

Figure 5. Phenotype of the WT and transgenic maize lines under drought stress. WT: Wild type lines; OE2, OE3, OE6, OE7, OE8: Transgenic lines

Table 1. Partial morphological analysis of transgenic and WT plants under drought stress

Lines	Plant height (cm)	Base stem width (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
WT	22.81±2.56 a	0.31±0.02 a	16.82±1.41 a	1.11±0.09 a	0.21±0.01 a
OE 6	32.57±2.14 b	0.47±0.03 b	25.81±2.16 b	2.42±0.21 b	0.27±0. 02 b
OE 7	33.69±2.61 b	0.43±0.03 b	27.57±1.97 b	3.02±0.12 b	0.27±0.02 b
OE 8	35.33±3.02 b	0.49±0.04 b	28.01±2.01 b	3.51±0.24 b	0.31±0.02 b

Data represent the means ±SE of three experimental replicates; values with different letters in the same row are significantly different ($P<0.05$).

Under normal condition, the above parameters of WT and transgenic showed no significant differences (data not shown). These results showed that *ZmLTP3*-overexpression lines had higher drought tolerance than the WT plants.

Chlorophyll contents assay in transgenic maize

Chlorophyll a and b are most important pigments for plant photosynthesis. Their contents determine the plant photosynthesis capacity. As shown in Table 2 and 3, no significant differences in chlorophyll contents were observed between the WT and transgenic plants under normal conditions before the start of the treatments (0 d). When treated with drought stress, chlorophyll a and b content decreased in all experiment plants, but the

Table 2. Chlorophyll a contents of transgenic and WT plants under drought stress

Lines	Chlorophyll a (mg/g FW)		
	0 d	7 d	14 d
WT	3.45±0.28 a	2.33±0.17 a	2.14±0.19 a
OE 6	3.39±0.17 a	3.21±0.12 b	2.98±0.21 b
OE 7	3.41±0.20 a	3.30±0.15 b	3.02±0.11 b
OE 8	3.43±0.21 a	3.32±0.21 b	3.11±0.26 b

Data represent the means ±SE of three experimental replicates; values with different letters in the same row are significantly different ($P<0.05$).

Table 3. Chlorophyll b contents of transgenic and WT plants under drought stress

Lines	Chlorophyll b (mg/g FW)		
	0 d	7 d	14 d
WT	1.28±0.11 a	1.02±0.08 a	0.98±0.07 a
OE 6	1.11±0.09 a	1.56±0.12 a	1.33±0.10 b
OE 7	1.29±0.11 a	1.66±0.17 b	1.35±0.11 b
OE 8	1.23±0.10 a	1.62±0.14 b	1.39±0.09 b

Data represent the means ±SE of three experimental replicates; values with different letters in the same row are significantly different ($P<0.05$).

transgenic plants could keep significantly higher chlorophyll a and b content compared to WT plants. There were no significant differences in chlorophyll contents among transgenic lines. Under normal conditions and at stages equivalent to those used in the treatments, the chlorophyll a and b contents in WT and transgenic showed no significant differences (data not shown). This showed that transgenic lines could keep higher chlorophyll contents under drought treatment, so as to maintain higher photosynthesis capacities.

Protective enzymes activities assay in transgenic maize

Stresses trigger the production of active oxygen which can cause oxidative stress. Some antioxidants, such as SOD, POD and CAT can inhibit or delay the oxidation stress by scavenging free radicals. As shown in Table 4, 5 and 6, the activities of protective enzymes exhibited no obvious differences in both transgenic and WT plants without stress. After drought treatment, all the enzymes activities in all the examined plants increased. While the transgenic plants had significantly higher increase compared to WT plants. Under normal condition, the activities of the enzymes in WT and transgenic showed no significant differences (data not shown). This result indicated that transgenic plants could keep activities of antioxidant enzymes to higher levels under drought treatment thus reduce the oxidative damages in a certain degree.

Table 4. Superoxide dismutase activities in transgenic and WT plants under drought stress

Lines	Superoxide dismutase activities (U mg^{-1} protein)		
	0 d	7 d	14 d
WT	2.33±0.15 a	3.01±0.25 a	3.49±0.24 a
OE 6	2.43±0.18 a	3.65±0.29 b	4.61±0.36 b
OE 7	2.36±0.17 a	3.68±0.26 b	4.83±0.39 b
OE 8	2.41±0.17 a	3.78±0.31 b	4.93±0.47 b

Data represent the means ±SE of three experimental replicates; values with different letters in the same row are significantly different ($P<0.05$).

Table 5. Catalase activities in transgenic and WT plants under drought stress

Lines	Catalase activities (U mg^{-1} protein)		
	0 d	7 d	14 d
WT	84.26±7.56 a	227.44±19.25 a	312.75±22.47 A
OE 6	84.34±7.25 a	274.27±21.45 b	425.61±34.12 B
OE 7	85.98±6.25 a	291.42±20.21 b	453.25±39.36 B
OE 8	87.40±6.82 a	299.54±23.57 b	477.01±36.85 B

Data represent the means ±SE of three experimental replicates; different capital and small letters in the same row represent are significant differences at $P<0.01$ and $P<0.05$, respectively.

Table 6. Peroxidase activities in transgenic and WT plants under drought stress

Lines	Peroxidase activities (U mg^{-1} protein)		
	0 d	7 d	14 d
WT	8.12±0.52 a	11.26±1.05 a	13.32±0.98 a
OE 6	8.24±0.68 a	14.56±1.21 b	18.41±1.34 b
OE 7	8.03±0.57 a	15.72±1.37 b	19.37±1.49 b
OE 8	8.01±0.63 a	15.83±1.34 b	21.05±1.76 b

Data represent the means ±SE of three experimental replicates; values with different letters in the same row are significantly different ($P<0.05$).

Membrane stability assay in transgenic maize

MDA and ion leakage ratio are often used to reflect the degree of membrane injury under stresses in plants. In this study, these two important parameters were measured to judge the cell injury under drought. We found that the two parameters exhibited no statistically significant differences in all the examined plants under normal condition. Under stress condition, the both parameters increased in all the examined plants, but their accumulation levels in transgenic plants were significantly lower than that in WT plants (Table 7 and 8). Under normal condition, the two indexes in WT and transgenic showed no significant differences (data not shown). This result indicated that transgenic plants could keep higher cell membrane stability under drought treatment.

Table 7. MDA content in transgenic and WT plants under drought stress

Lines	MDA (nmol·g^{-1})		
	0 d	7 d	14 d
WT	3.10±0.21 a	8.16±0.68 a	17.08±1.42 A
OE 6	2.96±0.23 a	7.14±0.55 b	12.56±1.04 B
OE 7	3.02±0.23 a	6.48±0.46 b	11.80±0.98 B
OE 8	3.08±0.27 a	6.43±0.51 b	10.50±1.01 B

Data represent the means ±SE of three experimental replicates; different capital and small letters in the same row represent are significant differences at $P<0.01$ and $P<0.05$, respectively.

Table 8. Ion leakage ratio in transgenic and WT plants under drought stress

Lines	Ion leakage ratio (%)		
	0 d	7 d	14 d
WT	19.62±1.61 a	49.82±4.59 a	77.88±5.53 A
OE 6	19.10±1.51 a	38.18±2.93 b	60.04±4.29 B
OE 7	20.58±1.68 a	37.52±2.79 b	59.42±4.54 B
OE 8	19.72±1.33 a	37.18±2.93 b	57.14±4.09 B

Data represent the means ±SE of three experimental replicates; different capital and small letters in the same row represent are significant differences at $P<0.01$ and $P<0.05$, respectively.

Discussion

Growth inhibition is the common response to drought stress and also an important index of plant tolerance (Nonami and Boyer 1990). In this study, plant height, main root length, base stem width, FW and DW were compared between transgenic and non-transgenic plants. Results implied that the measured morphological parameters in transgenic lines were better than that in non-transgenic plants under drought condition, suggesting that transgenic maize could maintain better growth status under drought stress. Furthermore, the relative higher chlorophyll contents in transgenic lines may be one of the reasons of higher biomass and better growth status in transgenic lines.

LTPs mainly exist in the epidermal tissue in the shoots, so as to transfer lipids to the plant surface (Canevascini et al. 1996). Studies have shown that the structure and components of plant cell wall and membrane system change under stresses (Iraki et al. 1989). LTPs could repair the injured cell membrane and cell wall under stresses, thus maintain the stability of cell structure (Sterk et al. 1991). Abiotic stresses often lead to oxidative damage and thus result in MDA accumulation and ion leakage increase. MDA content, as well as relative electrical conductivity are usually considered as important parameters in regard to oxidative damage (Mittova et al. 2004). In this study, ion leakage ratio and MDA content were significantly lowered in transgenic plants under drought stress condition, suggesting that the higher stability of cell membrane in transgenic plants under drought stress.

Stress treatments often cause the excessive generation of antioxidants, such as SOD, POD and CAT which play vital roles in scavenging ROS (Noctor and Foyer 1998). In this study, activities of all the measured three antioxidants increased more in transgenic lines than that in the WT plants under drought condition. Meanwhile, MDA content and ion leakage ratio decreased more in transgenic lines than that in WT plants. This indicated that higher activities of SOD, POD and CAT could help plants to reduce oxidative damage resulted from stress and withstand stress.

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References

Cameron, K.D., Teece, M.A., Smart, L.B. 2006. Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. Plant Physiol. **140**:176–183.

Canevascini, S., Caderas, D., Mandel, T., Fleming, A., Dupuis, I., Kuhlemeier, C. 1996. Tissue-specific expression and promoter analysis of the tobacco *Ltp1* gene. Plant Physiol. **112**:513–524.

Castro, M.S., Gerhardt, I.R., Orru, S., Pucci, P., Bloch, C. 2003. Purification and characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds. J. Chromatogr. B. **794**:109–114.

Debono, A., Yeats, T.H., Rose, J.K., Bird, D., Jetter, R., Kunst, L., Samuels, L. 2009. Arabidopsis LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. Plant Cell. **21**:1230–1238.

Edstam, M.M., Viitanen, L., Salminen, T.A., Edqvist, J. 2011. Evolutionary history of the non-specific lipid transfer proteins. Mol. Plant. **4**:947–964.

Edstam, M.M., Laurila, M., Höglund, A., Raman, A., Dahlström, K.M., Salminen, T.A., Edqvist, J., Blomqvist, K. 2014. Characterization of the GPI-anchored lipid transfer proteins in the *moss Physcomitrella patens*. Plant Physiol. Biochem. **75**:55–69.

Fan, Y., Du, K., Gao, Y., Kong, Y., Chu, C., Sokolovc, V., Wang, Y. 2013. Transformation of *LTP* gene into *Brassica napus* to enhance its resistance to *Sclerotinia sclerotiorum*. Russ. J. Genet+. **49**:380–387.

Garcia-Garrido, J.M., Menossi, M., Puigdimen, P., Martinez-Izquierdo, J.A., Delseny, M. 1998. Characterization of a gene encoding an abscissic acid inducible type 2 lipid transfer protein from rice. FEBS Lett. **428**: 193–199.

Huang, M.D., Chen, T.L., Huang, A.H. 2013. Abundant type III lipid transfer proteins in *Arabidopsis tapetum* are secreted to the locule and become a constituent of the pollen exine. Plant physiol. **163**:1218–1229.

Iraki, N.M., Singh, N.K., Bressan, R.A., Carpita, N.C. 1989. Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. Plant Physiol. **91**:48–53.

Kader, J.C. 1996. Lipid-transfer proteins in plants. Annu. Rev. Plant Phys. **47**:627–654.

Kader, J.C. 1997. Lipid transfer proteins: a puzzling family of plant proteins. Trends Plant Sci. **2**:66–70.

Kader, J.C., Julienne, M., Vergnolle, C. 1984. Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. Eur. J. Biochem. **139**:411–416.

Kirubakaran, S.I., Begum, S.M., Ulaganathan, K., Sakthivel, N. 2008. Characterization of a new antifungal lipid transfer protein from wheat. Plant Physiol. Bioch. **46**:918–927.

Kumar, P., Tewari, R.K., Sharma, P.N. 2008. Modulation of copper toxicity-induced oxidative damage by excess supply of iron in maize plants. Plant Cell Rep. **27**:399–409.

Lee, S.B., Go, Y.S., Bae, H.J., Park, J.H., Cho, S.H., Cho, H.J., Lee, D.S., Park, O.K., Hwang, I., Suh, M.C. 2009. Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein gene altered cuticular lipid composition, increased plastoglobules, and enhanced susceptibility to infection by the fungal pathogen *Alternaria brassicicola.* Plant Physiol. **150**:42–54.

Li, J., Guo, X.W., Zhang, Z.B, Sun, H.J., Zou, H.W., Luo, C., Huang, C.L., Yu, R., Wu, Z.Y. 2014. Studies on transferring *ATNCED3* into maize inbred line. Crops. **1**:58–62.

Mittova, V., Guy, M., Ta, M., Volokita, M. 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii.* J. Exp. Bot. **55**: 1105–1113.

Noctor, G., Foyer, C.H. 1998. Simultaneous measurement of foliar glutathione, gamma-glutamylcysteine, and amino acids by high performance liquid chromatography: comparison with two other assay methods for glutathione. Anal. Biochem. **264**:98–110.

Nonami, H., Boyer, J.S. 1990. Primary events regulating stem growth at low water potentials. Plant Physiol. **93**:1601–1609.

Patkar, R.N., Chattoo, B.B. 2006. Transgenic indica rice expressing nsLTP like protein shows enhanced resistance to both fungal and bacterial pathogens. Mol. Breeding. **17**:159–171.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., De Vries, S.C. 1991. Cell-specific expression of the carrot *EP2* lipid transfer protein gene. Plant Cell. **3**:907–921.

Suelves, M., Puigdomenech, P. 1997. Different lipid transfer protein mRNA accumulates in distinct parts of *Prunus amygdalus* flower. Plant Sci. **129**:49–56.

Sun, X.Y., Zhu, Y., Zhao, M.M., Li, Z.X., Zou, H.W. 2014. Cloning and characterization of a lipid transfer protein gene, *ZmLTP3*, from maize. J. Maize Sci. **22**:62–66.

Trevor, H.Y., Jocelyn, K.C. 2008. The biochemistry and biology of extracellular plant lipid transfer proteins (LTPs). Protein Sci. **17**:191–198.

Zou, H.W., Wang, X.H., Huang, C.L., Chen, J.S., Zhang, X.H., Luo, C., Yu, R., Wu, Z.Y. 2014. Stress-inducible expression of a gene encoding C-repeat binding factor 4 (CBF4) from Arabidopsis improved performance of transgenic maize under drought condition. Plant Omics J. **7**:94–101.