GsLTP, a Non-Specific Lipid Transfer Protein from *Glycine soja*, Enhances Drought and Salt Stress Tolerance in Transgenic Tobacco

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Abstract

Background: Lipid transfer proteins (LTPs) mainly exist in plant cell walls where they make an important impact in the transport of diverse lipophilic compounds. They have an important effect on the stress tolerance of plants by mediating plant responses to environmental stimuli and cell signal transduction pathways. Although the functions of several LTPs in different plant species have been identified, little is known about the biochemical and enzymatic activities of LTP family members in soybeans.

Results: Herein, *GsLTP* was identified from *Glycine soja* using soybean gene microarray expression and screened in a salt stress expressed sequence tag (EST) library. The 369 bp open reading frame (ORF) encodes a protein of 122 amino acids. The cDNA sequence shares similarity with *LTP* genes in other plants such as soybean (93%) and *Vigna* (72%). The function of the gene was characterized in transgenic tobacco. Various physiological characteristics under different environmental stresses were investigated. We discovered that overexpression of *GsLTP* enhanced tolerance to drought and salt stresses.

Conclusions: Our results indicate that *GsLTP* plays an important role in multiple abiotic signalling pathways, and provide a theoretical basis and practical gene resource for crop breeding.

Background

Abiotic stresses, including low temperature, drought and salinity, can significantly reduce crop yield and quality [1]. Therefore, improving resistance to such adverse environments is extremely important for agriculture. New genes associated with stress resistance have been identified, and transgenic plants carrying these genes exhibit greater tolerance to drought stress [2], salt stress [3], cold stress [4], heavy metals [5], paraquat and wounding stress [1].

Non-specific lipid transfer proteins (nsLTPs) are widely present in the plant kingdom and are involved in the binding and transport of various lipids. All known plant nsLTP precursors include an N-terminal signal peptide, indicating that nsLTPs are secreted [6]. Mature nsLTPs are small proteins characterized by an eight-cysteine motif (8CM) with the consensus sequence C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C [7,8]. The eight cysteines are part of the four disulfide bonds that stabilize the three-dimensional structure of the hydrophobic cavity. This allows different lipids and hydrophobic compounds to bind [8-10]. Based on sequence similarity, ten types of nsLTPs are divided from *Arabidopsis*, rice, *Solanaceae* and other plants [11].

Lipids play an important role in stress tolerance by mediating plant responses to environmental stimuli and cell signal transduction pathways, resulting in changes in energy storage and maintaining basic cellular functions. In these processes, lipid transformation proteins (LTPs) act as lipid transporters. Non-specific lipid transfer proteins (nsLTPs) can bind or transfer various hydrophobic molecules *in vitro*, such as fatty acids, fatty acyl-coenzyme A, phospholipids, glycolipids and cutin monomers [6]. *GsLTP* belongs to the nsLTP family members which play important roles in numerous physiological processes, such as cutin and wax biosynthesis, abiotic and biotic stresses, seed development and germination, sexual

reproduction, cell wall growth, nodulation, calmodulin (CaM) binding and responses to plant allergens [9,12,13].

Functions of LTPs under multiple abiotic stresses have been studied in various plants including sugarcane [14], *Triticum turgidum* [15], *Solarium tuberosum* [16] and *Setaria italica* [17]. NsLTPs from sugarcane respond to abiotic stresses and the signalling molecules salicylic acid and methyl jasmonate [14]. The wheat protein *TdLTP4* promotes tolerance to abiotic and biotic stresses in *Arabidopsis thaliana* [15]. Transgenic potato lines over-expressing *StnsLTP1* display enhanced cell membrane integrity under stress [16]. *SiLTP* enhances salt and drought stress tolerance in foxtail millet and may be partially upregulated by *SiARDP* [17]. Overexpression of a pepper nsLTP gene (*CALTP1*) in transgenic *Arabidopsis* increased the resistance of *Arabidopsis* against infection by *Pseudomonas syringae* pv. tomato and *Botrytis cinerea*. The tolerance of *Arabidopsis* to NaCl and drought stress at all vegetative growth stages was improved [18].

Plant nsLTPs are involved in a variety of physiological functions, such as sebum transport, cutin synthesis, cell wall extension, pollen development, pollen tube growth and guidance, stigma and pollen adhesion, plant signalling, biological stresses, abiotic stresses and seed maturation [15,19,20]. Evidence suggests that nsLTPs are expressed in a wide range of plant cells. They are involved in various physiological and biochemical processes, including cuticle synthesis and embryo development, adaption to a variety of stress environments, and resistance to microorganisms. However, the exact biological functions of these proteins have not been elucidated. At low temperatures and under drought and NaCl treatment, high expression of nsLTPs has been detected in plant stems of tomato [21], barley [22] and sunflower [23]. These results indicate that nsLTPs help plants adapt to adverse environmental conditions.

Glycine soja (*Glycine soja* Sieb. et Zucc), grown widely in Jilin Province in Northeast China, has a high tolerance to environmental challenges [24]. It is a model species for molecular mechanisms of low temperature and salt stresses. The ancestor of cultivated soybean is a highly adaptable plant species that grows well in desert conditions [8,25,26]. It is also an excellent genetic germplasm for mining abiotic resistance genes for agricultural crop breeding [27]. In one of our earlier studies in which *Glycine soja* was grown under 150mM NaCl and salinity responsive ESTs were identified. The expression of *GsLTP* was found to be induced to high salinity condition. In this study, we investigated the functions of *GsLTP* by analyzing the physiological responses of transgenic tobacco plants which were subjected to low temperature (4°C), salinity (300 mM NaCl) or drought (without water for 2, 4, 6, 8, 10, 12 or 14 days) treatment. Our results showed that *GsLTP* enhanced the salt tolerance and drought resistance of plants by regulating the synthesis of protective compounds. Thus, this study enhanced our understanding of the functions of *LTP* genes and the mechanisms by which plants tolerate multiple stresses. Moreover, it provides a theoretical basis and practical genetic resources for crop breeding.

Results

The identification of full-length *GsLTP*

The full-length sequence information of *GsLTP* was derived from extending the 254 bp partial EST to 689 bp by Phrap program. The extended *GsLTP* gene shares high similarity with genes in the *LTP* family, indicating its association with fatty acid transport in soybean.

Characterisation of *GsLTP* gene and protein sequences

After sequencing, multiple sequence alignment was performed with known *LTP* sequences. This comparison revealed high homology and the expected splice sites without nucleotide mutations. BLASTx analysis revealed that *GsLTP* is 369 bp in length, and encodes a predicted polypeptide of 122 amino acids. The molecular weight of the protein is ~13 kDa and the predicted isoelectric point (pI) is 8.74. Amino acid sequence analysis with the NCBI BLASTp program revealed similarity with *LTP* proteins in a various plants, further indicating membership of the *LTP* gene family. Therefore, the gene was named *GsLTP* and submitted to GenBank (accession number FJ825765) after confirming its sequence.

Identification of transgenic tobacco lines

To identify transgenic tobacco lines, total DNA was isolated and the inserted fragment was verified by PCR (Figure 1). The pBI121-*GsLTP* construct was successfully transferred into tobacco line #1, #3, #5, #6, #8, #10, #11, #12, #14, #15, #16, #17, #18, #19, #20, #21, #23, #24, #25, #26, #27 and #28. Total DNA was isolated from positive seedlings and digested with *Bam*HI for Southern blotting (Figure 2) and RT-PCR (Figure 3). Southern blot hybridization results showed that three transgenic tobacco lines yielded positive electrophoretic bands (lanes 4, 5 and 7), indicating that the *GsLTP* gene was successfully transferred into the plant genome. Conversely, tobacco lines without a hybridization signal indicated no gene integration. The signal in lanes 4 and 7 implied single-copy insertion into the genome. However, lane 5 has two hybridization bands, indicating the insertion of two gene copies. As expected, the positive control displayed one band, validating the appropriateness of the experimental procedure (Fig. 2). RT-PCR was also used to investigate gene transcription in plants. The successfully amplified fragments in lanes 3, 6, 8, 10, 12, 14 and 16 confirmed these independent lines harbor the target gene (Fig. 3). After reliable identification, two transgenic lines (#85 and #96) were chosen for phenotype experiments analysis, and transgenic line #96 were chosen for the chlorophyll, MDA and proline content of plants as well as conductivity of the leaves analysis.

Overexpression of *GsLTP* enhances drought and salt stress tolerance in transgenic tobacco but not improves low temperature resistance

Chlorophyll content, MDA and conductivity are three critical indexes of cell damage induced by the stresses. The results in Tables 1 and 2 reveal no significant difference between transgenic plants and non-transgenic plants regarding chlorophyll content under low temperature treatment ($p = 0.2248$, $\alpha = 0.05$). However, significant differences in chlorophyll content were observed under drought ($p = 0.0077$, $\alpha = 0.05$) and salinity ($p = 0.0101$, $\alpha = 0.05$) treatments. The chlorophyll content of transgenic seedlings was also significantly higher than that of control seedlings under drought treatment. We, therefore, concluded that transgenic seedlings displayed higher tolerance to drought conditions than control

seedlings. In addition, the MDA content of transgenic seedlings in each treatment was significantly higher than that of control seedlings ($p < 0.0001$, $\alpha = 0.05$). This suggests that transgenic seedlings were more resistant to osmotic stress than control seedlings. Under all treatments, there was no significant difference in the conductivity between transgenic and control seedlings. Thus, transgenic seedlings did not demonstrate higher tolerance to osmotic stress than control seedlings under any of the treatments.

Proline (Pro) is one of the common canonical amino acids which are critical to plants stress tolerance. There was a significant difference between transgenic and control plants in terms of Pro content ($p < 0.0001$, $\alpha = 0.05$) under low temperature and salinity treatments, but the difference was less significant ($p = 0.0413$, $\alpha = 0.05$) under drought treatment. Because the Pro content of transgenic seedlings was much higher than that of control seedlings, transgenic seedlings exhibited better resistance to osmotic stress.

Tobacco seedlings harboring the *GsLTP* gene displayed greater resistance than control seedlings based on membrane permeability and enzymatic activity. Based on chlorophyll content, the *GsLTP* gene significantly enhanced tolerance to drought but did not enhance tolerance to low temperature stress or salinity stress. Based on the MDA and Pro content, the *LTP* gene significantly enhanced resistance to low temperature, drought and salinity. The accumulation of these osmotic protective substances in transgenic seedlings in response to stress is an important biochemical indicator for measuring tolerance to low temperature, drought and salinity. Our results clearly showed that resistance of transgenic tobacco seedlings to drought and salinity was improved (Fig. 4). Compared with control seedlings, all physiological parameters tested were enhanced to various degrees in transgenic tobacco seedlings. Thus, *GsLTP* improved the tolerance of tobacco plants to drought and salinity stresses.

Table 1. Variance analysis of chlorophyll content, MDA content, Conductance and Pro content under different treatment.

Pr>F	Source	Chlorophyll content	MDA content	Conductance	Pro content
Low temperature (4°C)	Treat	0.2248	<0.0001	0.2317	<0.0001
	Data	<0.0001	<0.0001	0.0303	<0.0001
	Repeat	0.4631	0.9256	0.6446	0.8066
Drought treatment	Treat	0.0077	<0.0001	0.7990	0.0413
	Data	0.0001	0.0005	0.0076	<0.0001
	Repeat	0.5453	0.6738	0.8940	0.3755
Salt treatment	Treat	0.0101	<0.0001	0.0795	<0.0001
	Data	<0.0001	<0.0001	0.0041	<0.0001
	Repeat	0.3382	0.8806	0.7632	0.8378

Table 2. T-tests (LSD) for chlorophy content, MDA content, Conductance and Pro content in different treatment.

T-tests (LSD)	Treat	Low temperature (4°C)	Drought treatment	Salt treatment
Chlorophyll content	control	0.71847a	0.70110b	0.65486a
	LTP	0.73573a	0.89190a	0.94614a
MDA content	control	0.0129333a	0.0110952a	0.0123810a
	LTP	0.0078667b	0.0080000b	0.0071905b
Conductance	control	81.727a	73.631a	80.064a
	LTP	86.061a	76.024a	86.879a
Pro content	control	0.045333b	0.036000b	0.049238b
	LTP	0.077000a	0.052667a	0.082952a

Note—control—wild type plant; LTP: Seedlings transformed with *LTP* gene

Discussion

LTPs are an important class of proteins in plants [6]. In the past decade, some progresses have been made in analyzing the expression profiles of LTPs in response to plant stresses [18,28,29,30]. It has been discovered that the expression levels of LTPs were induced to low temperature, drought and salinity [31-33]. Although these prior studies indicated that LTPs may participate in the physiological regulation of plant stress response, very limited studies have demonstrated the protection role of this LTPs in plants under stress growth conditions. In the present study, a 369 full-length sequence of the *GsLTP* gene that encodes a predicted polypeptide of 122 amino acids was obtained. Multiple tobacco plants harboring the *GsLTP* gene have been produced. The physiological analysis on these transgenic plants clearly showed that overexpression of *GsLTP* can exert a protection function to tobacco plants under drought and salinity growth conditions.

Under abiotic stress, e.g., drought and salinity, growth conditions, cell membrane stabilities may be decreased and permeability is increased. These alterations may lead to the release of cytoplasmic components to cell wall and degradation of chlorophyll. For example, the exosmosis of inorganic ions and soluble sugars in the cell leads to the increase of relative conductivity [34]. The higher concentration

of chlorophyll and low conductivity in the transgenic plants indicated that overexpression of *GsLTP* in tobacco protected the cell membrane and prevent chlorophyll degradation. Under abiotic stress condition, the destruction of the cell membrane is also related to the enhancement of the peroxidation of the cell membrane [34]. The lower accumulation of MDA, a main product of membrane lipid peroxidation, further indicated the less deleterious modification on the cell membrane in the transgenic plants. Pro is normally accumulated under abiotic stress and plays a positive role in maintaining water balance and cell membrane integrity in plants [35]. After the application of stress, the more significant accumulation of Pro in the transgenic tobacco indicated that the overexpression of *GsLTP* promote the biosynthesis of Pro. This might be another factor that contributed to the improved resistance to drought and salinity in the transgenic plants. Future more in-depth transcriptomic analysis, e.g., RNA Seq, on these transgenic plants will elucidate the molecular mechanisms imposing the resistance to drought and salinity.

The growth and yield of soybean are negatively affected by adverse growing environment such as drought and salinity. The combination of transgenic technology and traditional breeding is the most effective way to rapidly improve the resilience of soybean. In this study, only physiological indexes of transgenic tobacco were measured. In future studies, the *LTP* gene can be transferred into soybean and the related physiological parameters will be measured. Other components discovered from a more detailed transcriptomic analysis can also be used to improve abiotic stress resistance in soybean. Transformation strategies harboring *LTP* and other genes with known abiotic stress resistance ability may lead to more significant improvement in soybean.

Conclusion

We isolated the *GsLTP* from *G. Soja* and transformed it into tobacco. Transgenic plants were assessed for stress resistance by analyzing physiological parameters and biochemical indicators following low temperature, drought and salinity stress treatments. *GsLTP* improved tolerance to drought and salinity to different degrees. However, resistance to low temperature was not enhanced. In summary, the genetic transformation of *GsLTP* into plants can improve resistance to environmental stresses.

Methods

Plant materials and growing conditions

The seeds of *G.soja* G50109 were obtained from the Jilin Academy of Agricultural Sciences (Changchun, China). To remove waxiness and promote germination, treat seeds with sulfuric acid (98%) for 10 minutes, wash them in sterile water 5 times, and store them in darkness for at least 2 days. After that, wild soybean seedlings were transferred to soil and incubated for 19 days at 24-26°C under a 16 hours light / 8 hours dark cycle condition. Leaves and roots were collected and stored at -80°C after being snap-frozen in liquid nitrogen.

Wild-type (WT) *Nicotiana tabacum* Longjiang 911, from Mudanjiang Normal University, China, was used for transformation. Tobacco seeds were soaked for 3-5 h in water, sterilized with 70% ethanol for 30 s, treated with 0.1% mercuric chloride for 3 min, and rinsed with sterile water five times. Finally, all seeds were placed on M₀ medium (1/8 MS + 0.8% agar, pH 5.8). When young leaves reached 2-3 cm, edges were removed, leaves were cut into discs (7 mm diameter) and placed with adaxial sides facing downwards on M₁ medium (MS + 1.0 mg L6-BA + 0.1 mg LNAA + 0.75% agar, pH 5.8) for pre-culturing for 2 to 3 days. Leaf disc incisions began to swell and callus tissue started growing. WT and transgenic lines were treated with 0 mM or 300 mM NaCl, or subjected to drought stress, to analyze gene function.

Isolation and sequence analysis of *GsLTP*

Gene expression profiles were obtained with the soybean gene expression microarray. Salt stress expressed sequence tag (EST) library was used to determine gene expression during early osmotic stress. The *GsLTP* gene is highly similar to clone pTE-6424 in the NCBI database, as revealed by electronic extension using Phrap program (<http://www.phrap.org>). A BLAST analysis was carried out (<http://www.ncbi.nlm.nih.gov/blast/>) and the full-length sequence was obtained. The full sequence of the ORF was translated from the nucleic acid sequence by DNAMAN software (Version 4.0, Lynnon Biosoft, Quebec, Canada).

The full-length cDNA of *GsLTP* was obtained by homologous cloning. Total RNA from *G. soja* G50109 was extracted from soybean leaves using a GoldScript cDNA Synthesis Kit (Invitrogen) and converted into cDNA through reverse transcription. The full-length cDNA of *GsLTP* was amplified using gene-specific primers 5'-AGATGGCAGAGACATTCC-3' and 5'-TGCACCTTTCAATAC-3'. The reaction contained 2.5 µl of 10× PCR buffer (Mg²⁺-free), 3 µl of 25 mM Mg²⁺, 2 µl of 2.5 mM each dNTP, 1 µl of DNA template (DNA content <1 ng), and 0.2 µl of Taq polymerase (5 U/µl) or Pyrobest TM DNA polymerase. The PCR products were purified and cloned into the pGEM-T vector, and the Pyrobest TM DNA polymerase was used for PCR identification and DNA sequencing. Sequence alignment was performed using DNAMAN. Sequence similarity was verified by BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The vector was transformed to *Escherichia coli* DH5α and cultured on Luria Bertani (LB) solid medium containing ampicillin (100 mg/L).

Construction of the pBI121-*GsLTP* expression vector

The PCR-amplified *LTP* gene was cloned into the plant expression vector pBI121, and the insert was verified using forward (5'-TAGGATCC(*Bam*HI)TAAGTCTTTGTTCATTGAGTAG -3') and reverse (5'-TACGAGCTC(*Sac*I)ATATATAGAATTCTGCGACT-3') primers. PCR was performed as described above, and a 447 bp fragment was identified and purified as described above. Finally, the target plant expression vector harboring the *LTP* gene was transformed into *Agrobacterium tumefaciens* LBA4404 .

Tobacco transformation

The *A. tumefaciens* LBA4404 strain containing the pBI121-*GsLTP* vector was transformed into tobacco leaf disks following pre-incubation for 2-3 days. After co-culture for 3-4 days, leaf disks were degermed for 2-3 h with a liquid medium containing antibiotics that prohibit *Agrobacterium* growth. These leaf discs were dried on filter paper and then placed on shoot regeneration media for further selection. Five to six weeks later, small shoots of 3-4 cm in size were transferred to rooting medium. Approximately 14 days later, most seedlings developed a proliferate root system. These putative transgenic plantlets were then moved into a humid culture room without sealing for 1 week. Thereafter, seedlings were planted in flower pots (grass peat: sandy soil = 2:1) and grown in an artificial climatic cabinet (26°C, 126 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h day^{-1} illumination, 85-90% relative humidity).

Molecular biological identification

Genomic DNA was extracted from selected transgenic and wild type plants, and integration of the *GsLTP* gene was confirmed by PCR and Southern blotting using a probe consisting of a 375 bp fragment of the *GsLTP* gene generated from total DNA in plants digested with *Bam*HI. *GsLTP* mRNA transcription was determined by RT-PCR. The above experiments were carried out on the T₃ generation of all lines. A total of 180 seedlings were confirmed to be transgenic and used in further stress tolerance tests. The material did not been deposited in a publicly available herbarium.

Analysis of stress tolerance and *GsLTP* function

Three groups of seedlings at a similar developmental stage (30 transgenic seedlings and 30 untransformed control seedlings per group) were subjected to low temperature (4°C), salinity (300 mM NaCl) and drought (without water for 2, 4, 6, 8, 10, 12 or 14 days). Chlorophyll content [36], malondialdehyde (MDA) content (TBA assay) [37,38], proline (Pro) content (ninhydrin assay) [39], leaf membrane permeability (relative conductivity assay) [40] and morphology were investigated for each treatment. Physiological indices and gene functions were statistically analyzed using SAS6.12.

Abbreviations

LTPs: Lipid transfer proteins

ORF: open reading frame

EST: expressed sequence tag

nsLTPs: Non-specific lipid transfer proteins

MDA: malondialdehyde

Pro: proline

ABA: abscisic acid

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: DC and JF conceived and designed research. JL and LC conducted experiments. JF contributed new reagents or analytical tools. JW and QM analyzed data. JF wrote the manuscript. All authors read and approved the manuscript.

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References

1. Wang W, Vinocur B, Altman A: **Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance.** *Planta* 2003, **218**(1):1-14.
2. Liu Y, Zhang L, Meng S, Liu Y, Zhao X, Pang C, Zhang H, Xu T, He Y, Qi M *et al*: **Expression of galactinol synthase from *Ammopiptanthus nanus* in tomato improves tolerance to cold stress.** *J Exp Bot* 2020, **71**(1):435-449.
3. Yang Z, Sheng J, Lv K, Ren L, Zhang D: **Y2SK2 and SK3 type dehydrins from *Agapanthus praecox* can improve plant stress tolerance and act as multifunctional protectants.** *Plant science : an international journal of experimental plant biology* 2019, **284**:143-160.
4. Liu S, Fukumoto T, Gena P, Feng P, Sun Q, Li Q, Matsumoto T, Kaneko T, Zhang H, Zhang Y *et al*: **Ectopic expression of a rice plasma membrane intrinsic protein (OsPIP1;3) promotes plant growth and water uptake.** *The Plant journal : for cell and molecular biology* 2019.
5. Gouiaa S, Khoudi H: **Expression of V-PPase proton pump, singly or in combination with a NHX1 transporter, in transgenic tobacco improves copper tolerance and accumulation.** *Environmental Science and Pollution Research* 2019, **26**(36):37037-37045.
6. Carvalho-Ade O, Gomes V: **Role of plant lipid transfer proteins in plant cell physiology-a concise review.** *Peptides* 2007, **28**(5):1144-1153.

7. José-Estanyol M, Gomis-Rüth FX, Puigdomènech P: **The eight-cysteine motif, a versatile structure in plant proteins.** *Plant Physiol Biochem* 2004, **42**(5):355-365.
8. Liu F, Zhang X, Lu C, Zeng X, Li Y, Fu D, Wu G: **Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis.** *Journal of experimental botany* 2015, **66**(19):5663-5681.
9. Ji J, Lv H, Yang L, Fang Z, Zhuang M, Zhang Y, Liu Y, Li Z: **Genome-wide identification and characterization of non-specific lipid transfer proteins in cabbage.** *PeerJ* 2018, **6**:e5379.
10. Salminen TA, Blomqvist K, Edqvist J: **Lipid transfer proteins: classification, nomenclature, structure, and function.** *Planta* 2016, **244**(5):971-997.
11. Boutrot F, Chantret N, Gautier MF: **Genome-wide analysis of the rice and arabidopsis non-specific lipid transfer protein (nsLtp) gene families and identification of wheat nsLtp genes by EST data mining.** *Bmc Genomics* 2008, **9**(1):86.
12. Zhao J, Wang S, Qin J, Sun C, Liu F: **The lipid transfer protein OsLTPL159 is involved in cold tolerance at the early seedling stage in rice.** *Plant biotechnology journal* 2020, **18**(3):756-769.
13. Smart LB, Cameron KD, Bennett AB: **Isolation of genes predominantly expressed in guard cells and epidermal cells of *Nicotiana glauca*.** *Plant Molecular Biology* 2000, **42**(6):857-869.
14. Chen Y, Ma J, Zhang X, Yang Y, Zhou D, Yu Q, Que Y, Xu L, Guo J: **A novel non-specific lipid transfer protein gene from sugarcane (NsLTPs), obviously responded to abiotic stresses and signaling molecules of SA and MeJA.** *Sugar Tech* 2017, **19**(1):17-25.
15. Safi H, Saibi W, Alaoui MM, Hmyene A, Masmoudi K, Hanin M, Brini F: **A wheat lipid transfer protein (TdLTP4) promotes tolerance to abiotic and biotic stress in *Arabidopsis thaliana*.** *Plant Physiol Biochem* 2015, **89**:64-75.
16. Gangadhar BH, Sajeesh K, Venkatesh J, Baskar V, Abhinandan K, Yu JW, Prasad R, Mishra RK: **Enhanced Tolerance of Transgenic Potato Plants Over-Expressing Non-specific Lipid Transfer Protein-1 (StnsLTP1) against Multiple Abiotic Stresses.** *Frontiers in Plant Science* 2016, **7**(86).
17. Pan Y, Li J, Jiao L, Li C, Zhu D, Yu J: **A Non-specific *Setaria italica* Lipid Transfer Protein Gene Plays a Critical Role under Abiotic Stress.** *Frontiers in Plant Science* 2016, **7**(151).
18. Jung HW, Kim KD, Hwang BK: **Identification of pathogen-responsive regions in the promoter of a pepper lipid transfer protein gene (CALTPI) and the enhanced resistance of the CALTPI transgenic *Arabidopsis* against pathogen and environmental stresses.** *Planta* 2005, **221**(3):361-373.
19. Kader JC: **LIPID-TRANSFER PROTEINS IN PLANTS.** *Annu Rev Plant Physiol Plant Mol Biol* 1996, **47**(47):627-654.
20. Kie?Bowicz-Matuk AR, Pascal, Rorat T: **The organ-dependent abundance of a *Solanum* lipid transfer protein is up-regulated upon osmotic constraints and associated with cold acclimation ability.** *Journal of Experimental Botany* 2008, **59**(8):2191-2203.
21. Kahn TL, Fender SE, Bray EA, O'Connell MA: **Characterization of Expression of Drought- and Absciscic Acid-Regulated Tomato Genes in the Drought-Resistant Species *Lycopersicon pennellii*.** *Plant Physiology* 1993, **103**(2):597-605.

22. Molina A, Diaz I, Carbonero P, Garcíaolmedo F, Vasil IK: **Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens.** *Molecular & General Genetics Mgg* 1996, **252**(1-2):162-168.
23. Ouvrard O, Cellier F, Ferrare K, Tusch D, Lamaze T, Dupuis J-M, Casse-Delbart F: **Identification and expression of water stress-and abscisic acid-regulated genes in a drought-tolerant sunflower genotype.** *Plant molecular biology* 1996, **31**(4):819-829.
24. Ji W, Li Y, Li J, Dai CH, Wang X, Bai X, Cai H, Yang L, Zhu YM: **Generation and analysis of expressed sequence tags from NaCl-treated Glycine soja.** *BMC Plant Biology*,6,1(2006-02-22) 2006, **6**(1):4-4.
25. Cao L, Yu Y, Ding X, Zhu D, Yang F, Liu B, Sun X, Duan X, Yin K, Zhu Y: **The Glycine soja NAC transcription factor GsNAC019 mediates the regulation of plant alkaline tolerance and ABA sensitivity.** *Plant Molecular Biology* 2017, **95**(3):253-268.
26. Ge Y, Li Y, Zhu YM, Bai X, Lv DK, Guo D, Ji W, Cai H: **Global transcriptome profiling of wild soybean (Glycine soja) roots under NaHCO₃ treatment.** *Bmc Plant Biology* 2010, **10**(1):153.
27. Sun M, Sun X, Zhao Y, Zhao C, Duanmu H, Yu Y, Ji W, Zhu Y: **Ectopic Expression of GsPPCK3 and SCMRP in Medicago sativa Enhances Plant Alkaline Stress Tolerance and Methionine Content.** *Plos One* 2014, **9**(2):e89578.
28. Liu K, Jiang H, Moore SL, Watkins CB, Jahn MM: **Isolation and characterization of a lipid transfer protein expressed in ripening fruit of Capsicum chinense.** *Planta.* 2006, **223**(4):672.
29. Sarowar S, Kim YJ, Kim KD, Hwang BK, Ok SH, Shin JS: **Overexpression of lipid transfer protein (LTP) genes enhances resistance to plant pathogens and LTP functions in long-distance systemic signaling in tobacco.** *Plant Cell Reports* 2008, **28**(3):419-427.
30. Tapia G, Morales-Quintana L, Parra C, Berbel A, Alcorta M: **Study of nsLTPs in Lotus japonicus genome reveal a specific epidermal cell member (LjLTP10) regulated by drought stress in aerial organs with a putative role in cutin formation.** *Plant Molecular Biology* 2013, **82**(4-5):485-501.
31. Bogdanov IV, Shenkarev ZO, Finkina EI, Melnikova DN, Rumynskiy EI, Arseniev AS, Ovchinnikova TV: **A novel lipid transfer protein from the pea Pisum sativum : isolation, recombinant expression, solution structure, antifungal activity, lipid binding, and allergenic properties.** *Bmc Plant Biology* 2016, **16**(1):107.
32. Dani V, Simon WJ, Duranti M, Croy RR: **Changes in the tobacco leaf apoplast proteome in response to salt stress.** *Proteomics* 2005, **5**(3):737-745.
33. Gao C, Wang Y, Liu G, Yang C, Jing J, Li H: **Expression profiling of salinity-alkali stress responses by large-scale expressed sequence tag analysis in Tamarix hispid.** *In: Plant Mol Biol* 2008.
34. Luo P, Li Z, Chen W, Xing W, Yang J, Cui Y: **Overexpression of RmICE1, a bHLH transcription factor from Rosa multiflora, enhances cold tolerance via modulating ROS levels and activating the expression of stress-responsive genes.** *Environmental and Experimental Botany* 2020, **178**:104160.
35. Nounjan N, Nghia PT, Theerakulpisut P: **Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes.** *Journal of Plant Physiology* 2012, **169**(6):596-604.

36. LICHTENTHALER HK, Wellburn AR: **Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents.** In.: Portland Press Limited; 1983.

37. Dong W, Wang M, Xu F, Quan T, Peng K, Xiao L, Xia G: **Wheat oxophytodienoate reductase gene TaOPR1 confers salinity tolerance via enhancement of abscisic acid signaling and reactive oxygen species scavenging.** *Plant physiology* 2013, **161**(3):1217-1228.

38. Jia X, Xiaoguang D, Jun Y, Beeching JR, Peng Z: **Enhanced reactive oxygen species scavenging by overproduction of superoxide dismutase and catalase delays postharvest physiological deterioration of cassava storage roots.** *Plant Physiology* 2013, **161**(3):1517-1528.

39. Zhang T, Mo J, Zhou K, Chang Y, Liu Z: **Overexpression of Brassica campestris BclCE1 gene increases abiotic stress tolerance in tobacco.** *Plant Physiology and Biochemistry.*

40. Ben RW, Ben-Saad R, Meynard D, Verdeil JL, Azaza J, Zouari N, Fki L, Guiderdoni E, Al-Doss A, Hassairi A: **Ectopic Expression of Aeluropus littoralis Plasma Membrane Protein Gene AITMP1 Confers Abiotic Stress Tolerance in Transgenic Tobacco by Improving Water Status and Cation Homeostasis.** *International Journal of Molecular Sciences* 2017, **18**(4):692.

Figures

Figure 1

PCR identification of transgenic tobacco plants M: DL 2000 marker. +: positive control. -: negative control. 1-28: putative transgenic tobacco plants. 21: #85.26: #96

Figure 2

Southern blot analysis of transgenic tobacco plants M: DL 2000 marker. 1: positive control. 2: negative control (without hybridization signal). 3-9: putative transgenic plants. 4: #85.5: #96

Figure 3

RT-PCR identification of transgenic tobacco plants M: DL2000 marker. 1: control I: PCR product of wild-type tobacco RNA. 2: control II: PCR product of wild-type tobacco cDNA. 3: positive control: PCR product of pBI-GsLTP plasmid. 4: negative control: PCR product of water. 5, 7, 9, 11, 13, 15: PCR product results of all identified putative transgenic tobacco plants RNA. 6, 8, 10, 12, 14, 16: PCR product results of 6: #85.7, 8: #96

Figure 4

Phenotype experiments analysis of WT and plants under drought and 300mM NaCl treatment GsLTP#85 and GsLTP#96: transgenic tobacco plants

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