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

Isolation 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 software. 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 further functional analysis.

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

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 ($p = 0.2248$, a = 0.05). However, significant differences in chlorophyll content were observed under drought ($p = 0.0077$, a = 0.05) and salinity ($p = 0.0101$, a = 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 ($p<0.0001$, a = 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.

There was a significant difference between transgenic and control plants in terms of Pro content ($p<0.0001$, a = 0.05) under low temperature and salinity treatments, but the difference was less significant ($p = 0.0413$, a = 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	Treat	0.2248	<0.0001	0.2317	<0.0001
(4°C)	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 widely distributed in plants, animals and microorganisms. They are an important class of active proteins in plants, accounting for 4% of soluble protein in cells. *In vitro* studies showed that LTPs bind reversibly to and transport a variety of hydrophobic molecules such as fatty acids, phospholipids, glycolipids, acetyl coenzyme A, steroids, aromatic derivatives and cutin monomer lipid complexes [6]. LTP genes have been isolated from a various plants, including six in pepper and 15 in Arabidopsis [28]. In addition, recent rice genome sequence analysis identified 53 nsLTP genes in rice [29]. For example, *OsLTPL36* may be involved in lipid absorption and/or transport during seed development in rice [30]. These nsLTPs may be related to mobile signalling factors in systemic acquired resistance processes [31]. The functions of these nsLTPs are affected by low temperature, drought and salinity[32-34], and abscisic acid (ABA) and other abiotic factors can increase expression. These findings suggest that rice *LTP* gene expression and abiotic stress are closely correlated. However, the exact mechanism of *GsLTP* in stress responses remains unclear. Some studies indicate that LTPs and some known plant defense response elicitors share similar structural and functional characteristics, including receptor control of plant defense response mechanisms and disease resistance mechanisms[33]. According to previous studies, LTPs may be related to plant resistance. Our results showed that *GsLTP* enhanced the salt tolerance and drought resistance of transgenic tobacco.

In the present study, we obtained a 369 full-length sequence of the *GsLTP* gene that encodes a predicted polypeptide of 122 amino acids. Many plants contain multiple LTP gene family members. BLASTp analysis revealed a similarity between *GsLTP* and other LTPs, although with some differences between species.

Transgenic tobacco plants harboring the Gs*LTP* gene displayed significantly higher resistance to low temperature, drought and salt stresses. Overexpression of the LTP gene in tobacco may activate LTP synthesis and the expression of downstream genes. In transgenic plants, *LTP* gene overexpression can also improve salt tolerance, drought resistance, osmotic stresses, and ABA sensitivity. These results indicate that LTPs play important roles in osmotic stress signal transduction and responses, ABA signalling[34] and ethylene signalling[35]. Our studies on *GsLTP* expand our knowledge of plant responses to stresses. Plant resistance is a complex process mediated by the interaction of multiple genes[36], and this is the case for tolerance to drought, salinity and low temperature[24,37]. Even so, transferring a single gene into a plant can significantly enhance resistance in some cases[38]. It is generally believed that complex genetic transformation can greatly improve resilience in transgenic plants. In future studies, we will apply a multi-gene transformation to achieve even stronger stress resistance and agronomic traits.

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 M_0 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 M_1 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 300mMNaCl, 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 software. 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.

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 (Mg^{2+}-free), 3 μl of 25 mM Mg^{2+}, 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)TAAGTCTTTGTTCATTTGAGTAG -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 (pAL4404).

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. Approximate 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 μmol m^{-2} 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 T_3 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 [39], malondialdehyde (MDA) content (TBA assay) [40,41], proline (Pro) content (ninhydrin assay) [42], leaf membrane permeability (relative conductivity assay)[43] 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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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

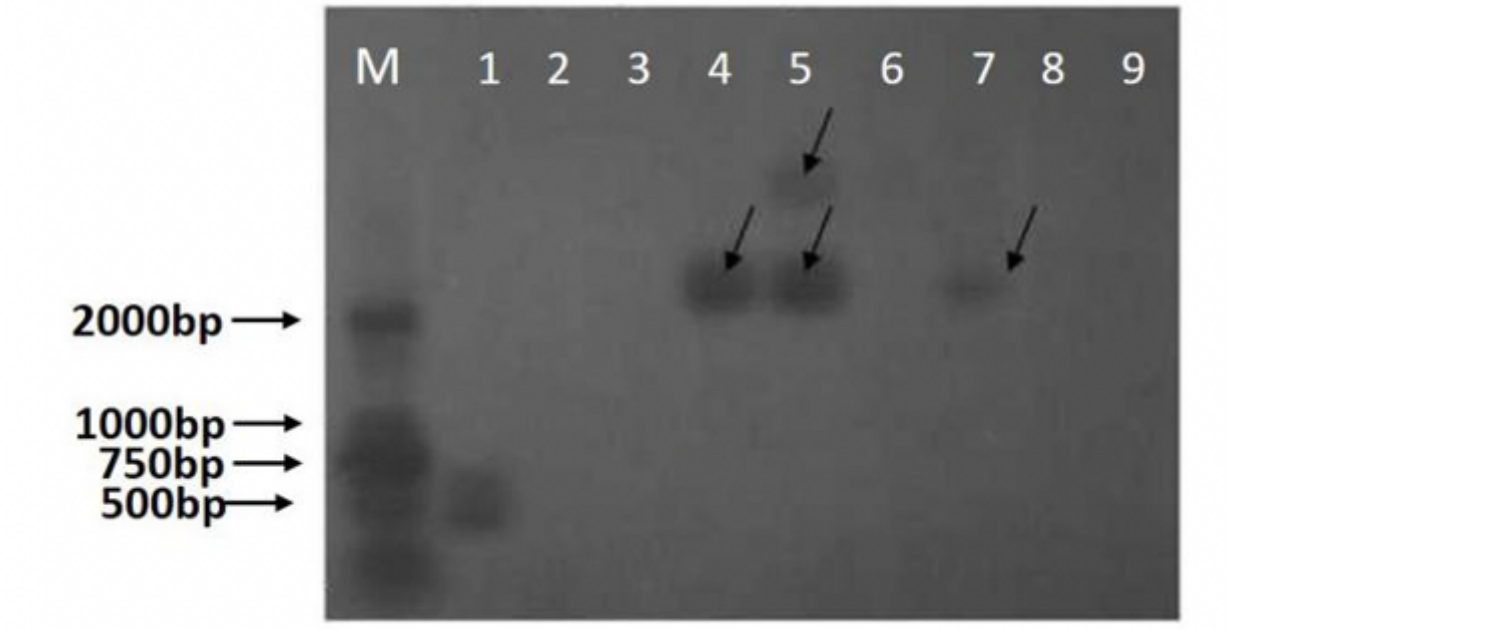

Figure 2

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

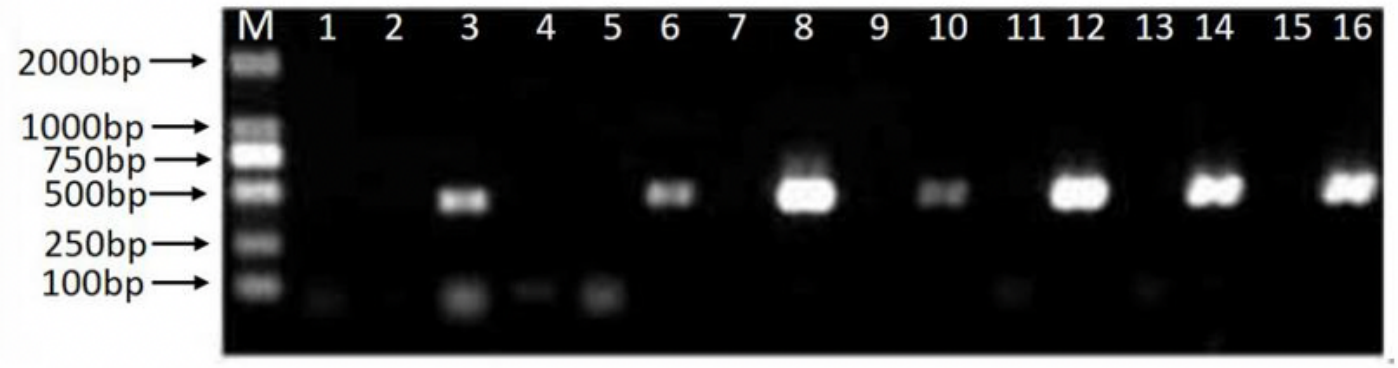

Figure 3

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

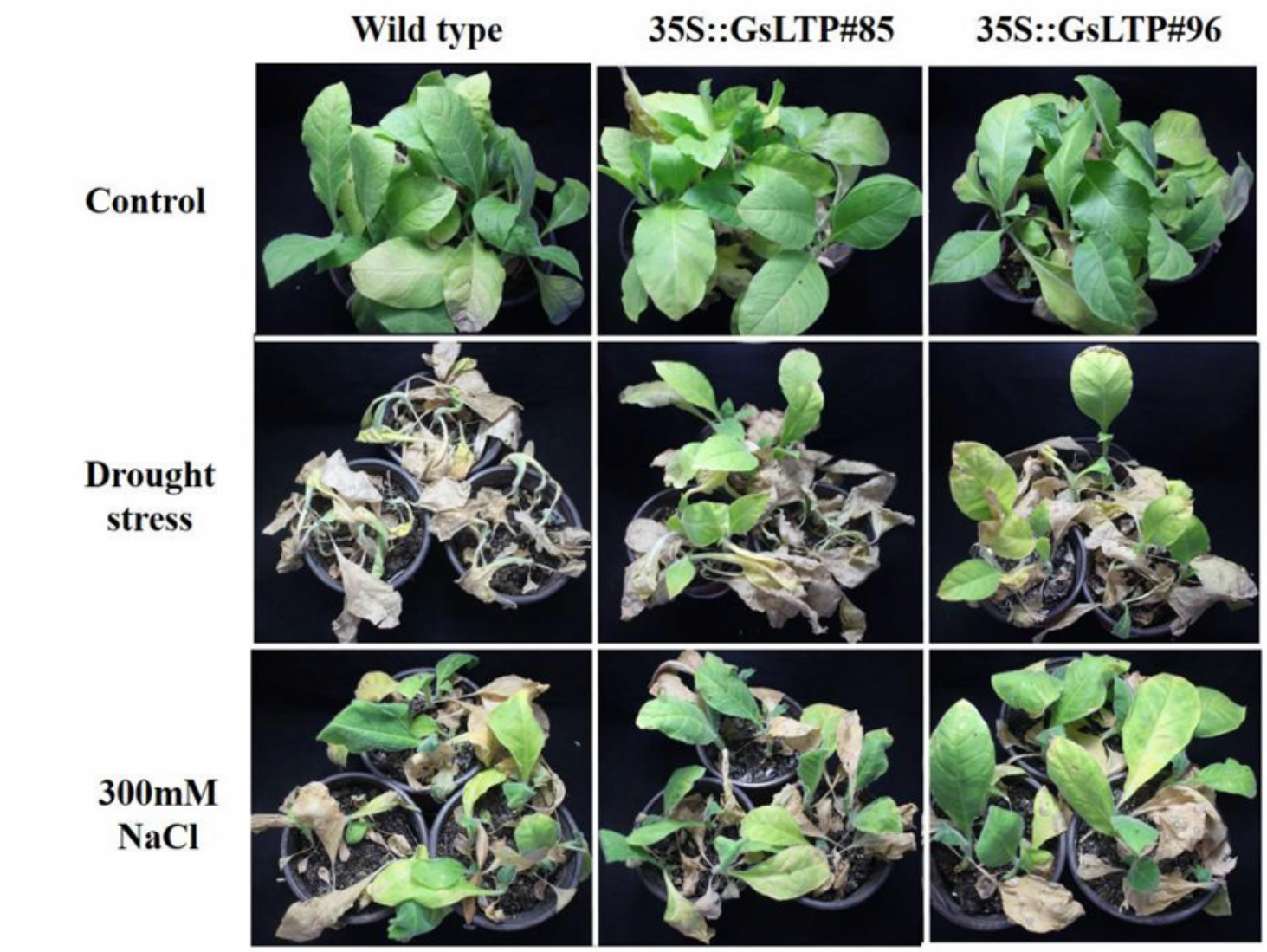

Figure 4

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

Supplementary Files

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