ABSTRACT

*Lilium Pumilum* with wide distribution is highly tolerant to salinity. The blue copper protein *LpCPC* (*Lilium pumilum Cucumber Peeling Cupredoxin*) gene was cloned from *Lilium pumilum*, which has the conserved regions of type I copper protein. Moreover, *LpCPC* has the closest relation to *CPC* from *Actinidia chinensis* using DNAMAN software and MEGA7 software. qRT-PCR indicated that *LpCPC* expression was higher in root and bulb of *Lilium pumilum*, and the expression of the *LpCPC* gene increased and reached the highest level at 12 h in bulbs under 20 mM NaHCO₃. The transgenic yeast was more tolerant compared with the control under NaHCO₃ stress. Compared with the wild type, overexpressing plants indicated a relatively lower degree of wilting. In addition, the chlorophyll content, soluble phenol content, and lignin content of overexpressing lines were higher than that of wild-type, whereas the relative conductivity of overexpressing plants was significantly lower than that of wild-type plants. Expression of essential genes including *NHX1* and *SOS1* in salt stress response pathways are steadily higher in overexpression tobacco than that in wild-types. Transgenic lines had much higher levels of *CCR1* and *CAD*, which are involved in lignin production, compared with wild-type lines. The yeast two-hybrid technique was applied to screen probable interacting proteins interacting with *LpCPC*. Eight proteins interacted with *LpCPC* were screened, and five of which were demonstrated to be associated with plant salinity resistance. Overall, the role of gene *LpCPC* is mediating molecule responses in increasing saline-alkali stress resistance, indicating that it is an essential gene to enhance salt tolerance in *Lilium pumilum*.

Introduction

The soil in the Northeast of China is heavily salinized. Lilies have high ornamental, medicinal, and edible value, but most species of lilies are extremely sensitive to saline stress. The *Lilium pumilum* is an excellent plant material because of its high salt tolerance and suitability for alkaline soils. We analyzed and compared the differential transcriptome of *Lilium pumilum* without treatment and under 20 mM NaHCO₃ treatment. The results indicated that the expression of the CPC gene was significantly increased under 20 mM NaHCO₃ treatment. CPC belongs to the blue copper protein. Copper ions are the important components of many biological enzymes in the metabolic processes of living organisms, which play an indispensable role in their lives. For example, it is related to many metabolic effects including plant photosynthesis. Copper-binding proteins control the uptake, transport, chelation, and efflux of copper ions, which indirectly or directly regulate the metabolic activities of organisms. It can be divided into three categories according to the valence state of copper ions: type I copper proteins, type II copper proteins, and type III copper proteins. As one of the members of the type I copper protein family, the blue copper protein was named because it has a dark blue absorption peak of Cu ion at 600 nm. Blue copper protein was found for the first time in *Thiobacillus ferrooxidans*. It contains small redox-active proteins with similar structural folds and contain a single copper ion, whose primary role is to transport electrons in bacteria and plants. According to the different residues of copper ion ligands, spectral properties, and structural differences in the structural domains of precursors and mature proteins, the blue copper protein family can be divided into two groups, Plastocyanin (Pc) and Phytocyanin (Ph). Plastocyanin is an electron transfer protein in photosynthesis, which is mainly responsible for the electron transfer from cytochrome f to photosystem I, thus converting light energy into chemical energy. In oxygenated organisms, Ph is the largest subfamily of blue-copper proteins that act as carriers of electron transfer between cytochromes and photosystems I, which can be classified into Stellacyanin (Sc), Plantacyanin (Pa), and Uclacyanin (Ua) according to their differences in copper-binding sites. Phycocyanin is mainly involved in response to light, oxidative stress, and aluminum toxicity, which plays an essential role in secondary cell formation in the biosynthesis of lignin. CPC plays an essential role in plant photosynthesis as the mobile electron carrier between the Photosystem I reaction center complex and the cytochrome b 6/f complex. In plants, CPC improves oxidative stress response through increasing peroxidase and catalase enzyme activities.
Oxidative stress-induced the overexpression of blue copper-binding protein AtBCB to confers oxidative aluminum resistance in Arabidopsis thaliana.\textsuperscript{17} The expression levels of several SC genes were significantly increased when induced by salt stress in the Capsicum annuum.\textsuperscript{18} PtSC18 could dramatically enhance the drought tolerance of transgenic plants by reducing water loss, regulating the H₂O₂ content, peroxidase, and enzymatic activities of catalase.\textsuperscript{19} BCP diminished aluminum stress through the formation of lignin or the effect of miR408.\textsuperscript{18,19} The tolerance of salt stress was enhanced through higher antioxidant enzyme activities and BCP transcription levels (Table 1).\textsuperscript{16,20,21}

NHXI (Vaccuolar Na⁺/H⁺ antiporter 1, Accession number: JX987081.1) and SOS1 (Plasma membrane Na⁺/H⁺ antiporter 1, Accession number: JK739016.1) are two key genes responding to salt tolerance. NHXI decreases the accumulation of Na⁺ ions in the cytosol by pumping Na⁺ in the vacuole. SOS1 is responsible for efflux Na⁺ from the cytosol to apoplasts. NHXI and SOS1 might be used as biomarkers to further investigate the interaction between genes and stress tolerance.\textsuperscript{22–24}

Lignin deposition on plant cell walls is considered to be one of the mechanisms contributing to the development of large erect plants adapting to terrestrial habitats. In the process of lignification, as lignin penetrates, the cell wall stiffness is increased, with the mechanical support of the cell being improved, leading to that the compressive strength is enhanced, facilitating the formation of mechanical tissue. It figures prominently in plant support and drainage.\textsuperscript{25} Salt stress has an impact on the biosynthesis of secondary cell walls in plant cells.\textsuperscript{26} It is obvious that regulation of lignification by the accumulation of lignin in the cell wall has the possibility of raising the salinity tolerance of plants.\textsuperscript{27,28} In summary, lignification occurs as a common response to various environmental stresses and mechanical damage in many plant species, that are associated with plant resistance to stresses.\textsuperscript{29}

In this study, the LpCPC gene was selected and amplified from the Lilium pumilum bulb. The LpCPC gene belongs to the Phytocyanin’s subfamily of blue copper proteins, that are involved in plant photosynthesis, oxidative stress, and aluminum toxicity response, promoting plant lignin synthesis. The gene was characterized by its expression in different organs of the Lilium pumilum, the phenotypic analysis of the transgenic LpCPC yeast and overexpressing Nicotiana benthamiana under NaHCO₃ stress, and the determination of a series of physiological indicators, which provide a preliminary analysis of the gene function and a foundation for researches related to plant stress resistance gene.

### Materials and methods

**Cloning and bioinformatics analysis of LpCPC**

Lilium pumilum was grown in a controlled growth chamber at 25 ± 2°C with a 16 h light/8 h dark cycle. Screening of the LpCPC from the Lilium pumilum transcriptome and design the primer sequences using SnapGene software (Table 2). Total RNA from Lilium pumilum bulb was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized by reverse transcribing 500 ng of total RNA with Prime-Script Reverse Transcriptase (Tokara, Tokyo, Japan). The PCR amplification reaction using primers LpCPC F and LpCPC R was carried out with cDNA as a template. The PCR product was ligated into the pMD18-T vector (Takara, Tokyo, Japan), and then sequenced.

### Characterization of LpCPC expression in different organs using real-time quantitative PCR (RT-qPCR)

RNA was extracted from the roots, bulbs, leaves, flowers, and seeds of Lilium pumilum, then cDNA was obtained by reverse transcription. The qPCR primers (LpCPC qPCR-F and LpCPC qPCR- R) were designed based on the sequence of the LpCPC gene (Table 2). The primers of LpActin F and LpActin R was used as a control (Table 2). A 20 μl reaction system was made with UltraSYBR Mixture reagents, and qRT-PCR was performed according to the standard reaction condition designed procedure: pre-denaturation at 94°C for 10 min, denaturation at 94°C for 30s, annealing at 55–60°C for 30s, extension at 72°C for 1 min; 40 cycles in total. All tests were repeated in triplicate.

---

**Table 1. Functions of blue copper-binding protein.**

| Protein Name | Functions | Reference |
|--------------|-----------|-----------|
| AtBCB        | Enhanced constitutive lignin production; lipid peroxidation caused by aluminum stress was diminished because of the formation of lignin inclusion bodies \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \) as well as drought stresses; downregulated in fibers by \( \text{Al}^{3+} \) treatment | 18 |
| GhBCPs       | Up-regulated in fibers by high-salinity including \( \text{Cu}^{2+} \) and \( \text{Zn}^{2+} \) | 20 |
| PtSIC8       | Strongly responded to drought stress by improving water retention and reducing oxidative damage | 16 |
| OsSUL8       | Increase the number of panicle branches and the number of grains, resulting in better grain productivity | 19 |
| BcBCP1       | Increase osmotic stress resistance via increasing photosynthetic rates, antioxidant enzyme activities, and cytosol ascorbate peroxidase transcription | 21 |

**Table 2. Primers used in this study.**

| Primer Name   | Primer Sequence |
|---------------|-----------------|
| LpCPC F       | GAGTTACAACCTCACGATGGAGGCTC |
| LpCPC R       | TGACCAGTTCGTTCCACATC |
| LpCPC qPCR-F  | ACTCCAAACAGGAGTTGGTGC   |
| LpCPC qPCR-R  | AAAGCTCATCCCTGAGGCC   |
| LpActin F (Internal Reference) | GCATCACACCTTCTACAGC |
| LpActin R (Internal Reference) | GAAGACGATACACCTTCATAGA |
| LpCPC-EcoR1 -F | GAATCTGAGGAGGCTTACTCTC |
| LpCPC-Xhol -R | CTCGAGGTAAATGTTGAGTTGGATGAGTGGCAACAAAGAACAGG |
| NbSOS1 R      | AATCCTGAGGGACGCCG |
| NbActin-R     | TGGGTCTGAGGGCTGATGTTG |
| NbActin-F     | TCACTTGCCCATCAAGGATCTAT |
| CAD qPCR-F    | GGATGCTGCAACTCCTCCTTC |
| CAD qPCR-R    | TGGGTTGGCCATCAGTGATAA |
| CCR1 qPCR-R   | GGTGGCCGAGACATCCCCAA |
| NbhNHXI qPCR-R | TCACGAGGATTTGTCGTCAGCA |
| NbhNHXI qPCR-F | AGGACCGCCACCTGGATTTG |
| NbhSOS1 qPCR-R | ACACCTGAGGTCTTCTACATA |
| NbhSOS1 qPCR-F | CAAGCTAACCTTCTACATCA |
| LpCPC-Sal1-R  | TGGGCGAGGCCTCCACATC |
| LpCPC-Sal1-F  | GTCGACTGAATGGTGAAGTATGGGATGGAC |
Analysis of LpCPC expression under abiotic stress using RT-qPCR

*Lilium pumilum* seedlings were grown on MS medium containing 20 mM NaHCO₃ for 6h, 12 h, 24 h, 36 h, 48 h to apply salt stress. There were 18 seedlings divided into 6 groups with 3 repetitions of each group. RNA was extracted from the *Lilium pumilum*’s bulbs after stress treatment, then reverse transcribed to cDNA and quantified according to the qPCR reaction.

Analysis of LpCPC yeast expression vectors

The LpCPC-T plasmid was amplified using the primers containing restriction sites (LpCPC-EcoRI -F and LpCPC-XhoI -R) (Table 2) and the PCR product was subsequently ligated into the pMD18-T vector, before the pMD18-T and pYES2 plasmid were both digested with EcoRI and XhoI. Next, ligated them resulting in pYES2-LpCPC. Competent yeast strain INVSC1 was used to transform pYES2 or pYES2-LpCPC plasmids using the Gietz standard transformation methods.

After induction of protein expression in transgenic yeast cells, the obtained broth (OD₆₀₀ = 0.6) was subsequently diluted 10⁻¹, 10⁻², 10⁻³, 10⁻⁴. 3 μL spots were taken in SC-U + 2% glucose solid medium containing 0mM, 15 mM, 30 mM NaHCO₃, then the yeast was incubated for 3 days at 30°C for yeast stress treatment analysis.

Genetic transformation, identification, and quantitative analysis of Nicotiana benthamiana transgenic lines

PCR was carried out to amplify LpCPC-T plasmid with the primers of LpCPC F and LpCPC R before the products were ligated into the pCXSN plasmid, then PCR was applied again to identify whether the gene was positive or negative with primers of LpCPC F and NOS R (Table 2). The pCXSN-LpCPC plasmid was transformed into Agrobacterium tumefaciens *EH105* by electroporation transformation.

*Nicotiana benthamiana* leaves were placed in MS+0.5 mg/LBA+0.1 mg/LNAA medium for pre-culture 48 h. The leaves were infected by Agrobacterium tumefaciens *EH105* with pCXSN-LpCPC, before co-cultured in the dark for 48 h, and then transferred to the screening medium (MS+0.5 mg/LBA+0.1 mg/LNAA+50 mg/L Hygromycin) for further incubation. Germinated leaves were transferred to 1/2 MS medium to generate root. DNA of selected *Nicotiana benthamiana* leaves in favorable growth conditions were extracted and then identified by PCR using the LpCPC F and NOS R primers. qPCR was performed to detect the expression of the LpCPC gene in transgenic plants. Primers of NbActin-F and NbActin-R were used as control.

Analysis of salt resistance in transgenic Nicotiana benthamiana

Wild-type and transgenic plants with the same growth condition were watered with 40 mL 500 mM NaHCO₃ solution per day lasting 5 days to observe the phenotype. Chlorophyll was measured on *Nicotiana benthamiana* using a SPAD chlorophyll meter (SPAD-502Plus, Japan), repeating three times. The standard immersion method was used to measure the relative conductivity of *Nicotiana benthamiana* leaves. Stem segments and roots were taken for analysis of total soluble phenol and lignin content, as detailed in Ana.

The relative levels of the transcript were accumulated for the *NHX1* and *SOS1* genes by qPCR using the following primers: NbNHX1 qPCR-F, NbNHX1 qPCR-R, NbSOS1 qPCR-F, NbNHX1 qPCR-R, NbSOS1 qPCR-R (Table 2). The primers of *LpActin F* and *LpActin R* were used as a control. The expression of *CCR1* (Cinnamoyl-CoA Reductase 1, LOC107804645) and *CAD* (Cinnamyl Alcohol Dehydrogenase, EC 1.1.1.195) involving in lignin biosynthesis were detected by qPCR. The gene-specific primers of qRT-PCR analysis were listed in Table 2. The expression of genes was normalized by *NbActin* with the primers of *NbActin-F* and *NbActin-R*.

Tobacco stem and root segments under the same growth conditions were stained by methods with phloroglucinol, then the results were observed using a light microscope. The details of staining methods are as follow. (1) Slicing the stems section with a blade, before sections with the same size were treated with a drop of concentrated hydrochloric acid, and then leave them for two minutes. This step is not required for root staining. (2) Stain of phloroglucinol was used to stain the sections of roots and stems for 3 minutes. (3) After the samples were successfully stained, the results were observed using a light microscope.

All data are presented as mean ± standard deviation (SD), n = 3. Significant differences were statistically analyzed by Tukey HSD test using GraphPad Prism 8 software (GraphPad, USA).

Screening of LpCPC interacting protein by yeast two-hybrid

LpCPC was cloned into the pGADT7 vector with the primers LpCPC-EcoRI-F and LpCPC-SalI-R (Table 2). The recombinant plasmids pGADT7-LpCPC were identified by double enzyme digestion (EcoR1 and SalI), then they were transformed into Y2HGold strain. The proteins interacting with pGADT7-LpCPC were screened from the *Lilium pumilum* Yeast cDNA library. Yeast DNA was extracted and sequenced to obtain the interaction protein sequences.

Results and analysis

Cloning and sequence analysis of LpCPC in *Lilium pumilum*

The LpCPC gene was successfully cloned from the leaf of *Lilium pumilum*. Small blue copper (type I) proteins are also called Cupredoxins. *LpCPC* has a highly conserved structural domain belonging to the Cupredoxin superfamily at amino acid position 22–122 (Figure 1), and further BLAST analysis indicated that LpCPC falls into the Stellacyanins.
Figure 1. Analysis of the conserved domains of LpCPC genes.

Figure 2. Protein alignment of the LpCPC deduced amino acid sequence with LpCPC proteins from other plant species. The amino acid sequence of this transcript was similar to that alignment of the CPC amino acid sequence from other species: Elaeis guineensis (African oil palm) XP_010922520.1; Dendrobium catenatum XP_020676538.1; Ananas comosus (pineapple) DQY72680.1; Phoenix dactylifera (date palm) XP_008779708.1; Chenopodium quinoa (quinoa) XP_021741754.1; Cucumis sativus (cucumber) XP_031744419.1; Cucumis melo (muskmelon) XP_008461120.1; Ricinus communis (castor bean) EEF35205.1; Ipomoea triloba (trilobed morning glory) XP_031106714.1; Prunus persica (peach) XP_007216148.1; Malus domestica (apple) XP_008373963.2; Actinidia chinensis var. Chinensis PSS17849.1; Solanum Lycopersicum (Lycopersicon esculentum) XP_010323300.1; Trifolium pratense PNX74705.1).

Figure 3. Phylogenetic tree of 14 selected plant CPC proteins. The MEGA3 program was used for the construction of phylogenetic trees. The bar represents 0.1 amino acid substitutions per site.
Homologous sequence alignment and evolutionary tree analysis of LpCPC

According to Blast results, the homologous sequence comparison of the data expounded that the black area in the figure is the highly conserved amino acid region, while the blue part is the similar amino acid sequence region (Figure 2). The gene was found to be homologous and have similar conserved regions to CPC in other plants, so the gene was named LpCPC. Based on a comparison of amino acid sequences and phylogenetic relationships, the results of the evolutionary tree analysis (Figure 3) showed that LpCPC is classified as a major evolutionary branch with Actinidia chinensis var. Chinensis, Solanum Lycopersicum and Trifolium pratense.

Analysis of LpCPC expression in different organs of Lilium pumilum

qRT-PCR was performed using cDNA from the roots, bulbs, leaves, flowers, and seeds of Lilium pumilum. The results revealed that the LpCPC was expressed at a higher level in the roots and bulbs, while the lowest level was in the flowers (Figure 4).

Analysis of LpCPC expression in Lilium pumilum under NaHCO₃ stress

To find out the expression of the LpCPC gene in Lilium pumilum under NaHCO₃ stress, qRT-PCR was performed on cDNA of the bulb after being treated by 20 mM NaHCO₃ for 6 h, 12 h, 24 h, 36 h, and 48 h. Under 20 mM NaHCO₃ treated, the expression of the LpCPC increased and reached the highest level at 12 h (Figure 5). The analysis revealed that NaHCO₃ stress promoted the expression of the LpCPC.

Identification of resistance in transgenic yeast under saline stress

The induced yeast solution was diluted ten times, one hundred times, one thousand times, ten thousand times, and one hundred thousand times in a turn. 3 µL of yeast solution was spotted in SC-U+2% glucose solid medium and SC-U+2% glucose solid medium containing 15 mM, 30 mM NaHCO₃ and incubated at 30°C for 3 days (Figure 6). The result indicated that there was no significant difference between transgenic and wild-type yeast in the growth under no saline stress. By contrast, under 15 mM, 30 mM NaHCO₃ stress conditions, the transgenic yeast grew better than the control yeast under 10⁻³ and 10⁻⁴ fold dilutions. When the concentration was increased to 30 mM, the control only had little growth, while the transgenic yeast could still produce colonies, demonstrating that the transgenic yeast was more tolerant under high NaHCO₃ stress compared with the control. So, the LpCPC gene involved in the NaHCO₃ stress reaction in transgenic yeast.

Identification of LpCPC overexpression in Nicotiana benthamiana

Overexpression Nicotiana benthamiana with LpCPC was quantified by qPCR. The result indicates that the expression of LpCPC in transgenic lines was higher than the wild-type lines (Figure 7), and transgenic lines #1, #2, and #3 were selected for the subsequent experiment.

Phenotypic analysis of tobacco plants under saline stress

Wild-type and LpCPC transgenic plants in the same growth condition were watered using 500 mM NaHCO₃ solution. The results demonstrated that with the accumulation of NaHCO₃, plants started to wilt. Compared with the wild type, transgenic plants indicated a relatively lower degree of...
wilting (Figure 8). According to the analysis of results, the transgenic plants had higher tolerance to salinity than wild-type plants.

**Analysis of the physiological indicators in Nicotiana benthamiana under NaHCO₃ stress**

Chloroplasts are destroyed under saline stress and chlorophyll concentration is immediately affected. Chlorophyll was measured on *Nicotiana benthamiana* leaves before and after NaHCO₃ treatment using a SPAD chlorophyll meter. The chlorophyll content of leaves was significantly reduced compared to that before salt stress treatment. Moreover, the chlorophyll content of transgenic plants was higher than that of wild-type (Figure 9). This revealed that chloroplasts of transgenic plants were less damaged under saline stress.

The relative conductivity of plants under stress was significantly higher compared to pre-treatment plants. However, the relative conductivity of transgenic plants was significantly lower than that of wild-type plants (Figure 10). This demonstrated that *Nicotiana benthamiana* plants transgenic the *LpCPC* gene were significantly less cell-damaging compared to wild-type *Nicotiana benthamiana* plants, which means that plants transgenic the *LpCPC* gene were more tolerant to salinity.

Measurement of total soluble phenol content in stems and roots of unstressed and post-stressed *Nicotiana benthamiana*. The content of soluble phenols increased with the accumulation of salinity stress. The total soluble phenol content of transgenic plants was higher compared with the wild type (Figure 11). It can be inferred that overexpression *Nicotiana benthamiana* has higher phenol oxidase and peroxidase activity, superior antioxidant capacity, and greater salinity tolerance.

Lignin content was measured in stems and roots of *Nicotiana benthamiana*. Lignin content increased with the accumulation of salinity stress. Moreover, transgenic plants had more lignin content compared to the wild type (Figure 12). It can be concluded that *Nicotiana benthamiana* overexpressing the *LpCPC* gene is more tolerant to salinity.

With the standard staining methods of phloroglucinol, analysis of stems and roots of NaHCO₃-treated *Nicotiana benthamiana* indicated that the xylem of plants overexpressing *LpCPC* gene was significantly larger than that of wild-type (Figure 13 and Figure 14).
Figure 9. Chlorophyll content change associated with stress response in wild type and transgenic lines. Plant in the pot was grown either with 500 mM NaHCO$_3$ or without, and samples were harvested 24 h later. Chlorophyll contents were detected in the leaves of wild-type and transgenic lines. Each data point is the average of five replicates, and error bars represent ± SE. Error bars indicate ±SD. *Significance at $P < .05$, **Significance $P < .01$, ***Significance $P < .001$.

Figure 10. Relative conductivity change associated with stress response in wild type and transgenic lines. Plant in the pot was grown either with 500 mM NaHCO$_3$ or without, and samples were harvested 24 h later. Relative conductivity was detected in the leaves of wild type and transgenic lines. ****Significance $P < .0001$.

Figure 11. Total soluble phenol content change associated with stress response in wild type and transgenic lines. Plant growth in the pot was grown either with 500 mM NaHCO$_3$ or without, and samples were harvested 24 h later. Total soluble phenol content was detected in the leaves of wild type and transgenic lines.
The expression levels of salt stress-related genes (NHX1 and SOS1) were determined under salt conditions. The results indicated that the expression of NHX1 and SOS1 were steadily increased in overexpression lines compared with that of WT (Figure 15).

Whether LpCPC transgenic lines could affect the potential lignin biosynthesis gene in tobacco or not. We found that the expression of CCR1 and CAD genes involved in lignin biosynthesis was altered in the transgenic lines (Figure 16). CCR1 and CAD, involved in lignin biosynthesis were significantly up-regulated in transgenic lines compared with wild-type lines.

**Screening of LpCPC interacting protein by yeast two-hybrid**

The positive protein was obtained from colonies grown on SD/-Trp-Leu-His-Ade+X-a-gal+AbA solid medium turned blue (Figure 17). Through BLAST these colonies in NCBI, eight proteins were successfully compared. Five of these have been demonstrated to be associated with plant salinity resistance (Table 3).

**Discussion**

Biotic and abiotic stresses affect plants’ growth and development. Lilium pumilum is widely distributed and has strong resistance to disease, cold, and salinity. The expression of the LpCPC is significantly up-regulated in bulb transcriptome of Lilium Pumilum under 20 mM NaHCO₃. We would like to find what role does LpCPC plays in the salt tolerance of Lilium pumilum. The LpCPC belongs to the blue copper protein family, which is involved in the electron transfer in plant photosynthesis and oxidative stress response. Expression of the blue copper protein family can enhance plant tolerance to biotic and abiotic stresses. For example, the Arabidopsis thaliana’s blue-copper protein gene protects cell walls and cell membranes from aluminum toxicity through electron transfer reactions.¹⁴
In this study, the *LpCPC* gene was cloned from *Lilium pumilum*, which has the closest relation to *CPC* from *Actinidia chinensis*. The results of qRT-PCR revealed that the *LpCPC* gene was expressed at a higher level in the roots and bulbs, and it functions in the roots and bulbs, which are underground organs. Roots and bulbs are the first two organs to be exposed to salt stress, so the increased expression of *LpCPC* is conducive to plant resistance under salt stress. Under 20 mM NaHCO₃ treated, the expression of the *LpCPC* increased and reached the highest level at 12 h. It indicated that *LpCPC* may react immediately and perform its function when plants are exposed to salt stress.

The resistance experiment is carried out in the eukaryote (Yeast). Although some experimental operation is relatively simple, yeast containing the recombinant *LpCPC* gene grew better under saline stress than the control, suggesting that the *LpCPC* may be involved in the stress response in yeast, making the transgenic yeast more tolerant to salinity. The chlorophyll content of the transgenic plants after the stress treatment was higher than the wild type, indicating that the chloroplasts of the transgenic plants were significantly less damaged by the stress treatment compared to the wild type. The relative conductivity of the transgenic lines was lower than the wild-type, suggesting that the cell membrane disruption of the transgenic strain was lower than the wild-type. The expression of *NHX1* and *SOS1* (salt-related marker genes) in overexpression lines were higher than that in WT, indicating that overexpression lines improve the resistance of plants under salt stress.

---

**Figure 15.** qRT-PCR determination of representative gene expression in leaves. The relative expression level of *LpHNX1* and *LpSOS1* were detected in leaves of wildtype tobacco and transgenic tobacco seedlings exposed to 500 mM NaHCO₃ for 0 h, 6 h, 12 h, 24 h, 36 h, 48 h.

**Figure 16.** Expression of *CCR1* and *CAD*, potential lignin biosynthesis genes. The qPCR analysis the expression levels of *CCR1* and *CAD* genes related to lignin biosynthesis. Three biological replicates are used for each transgenic line and wild type.

**Figure 17.** Interactions between *LpCPC* and selected proteins. Yeast two-hybrid assays were performed by co-transforming strains that were spotted on SD-L-T/SD-L-T-H-A plates to examine potential direct interactions between the proteins. Yeast strains that contained "empty" BD or AD plasmids were used as negative controls. L: Leu, T: Trp, H: His, A: adenine, BD: pGADT7 vector, AD: pGADT7 vector, SD: synthetic dextrose.
Phenol is related to salt stress tolerance, and the highest phenol content under salinity stress was observed in the highest salt tolerance lines in cucumber.\textsuperscript{45} Plants exposed to stress are accompanied with an increase in lignin content.\textsuperscript{46} Multiple plants increase their tolerance to abiotic stresses through lignin accumulation.\textsuperscript{27–29,46} Silencing the \textit{AtBCB} gene leads to a reduction of lignin accumulation.\textsuperscript{47} Cinnamoyl-CoA reductase (CCR) and cinnamal alcohol dehydrogenase (CAD) related to lignin biosynthesis and its transcriptional regulation are uncovered based on Zhong (2015).\textsuperscript{48} \textit{LpCPC} may affect the content of the soluble phenol and lignin under stress, the content of the soluble phenol and the lignin was detected in the roots and stems of transgenic \textit{Nicotiana benthamiana} and wildtype. Overexpression of \textit{LpCPC} caused higher expression of both lignin biosynthesis genes. The conserved induction gene expression suggests that \textit{LpCPC} could retain functions in the regulation of lignin biosynthesis. Overexpression of \textit{LpCPC} in tobacco enhanced the lignification of the cell wall. These results showed that the transgenic plants had a higher tolerance than the wild type. These physiological indices showed that the \textit{LpCPC} transgenic lines were more tolerant than wild-type under NaHCO\textsubscript{3} treatment.

Further studies are necessary to investigate the response mechanisms of the \textit{LpCPC} gene involving in plant salinity stress and the synergistic relationships between genes. Eight positive interacting proteins were obtained using yeast two-hybrid, and five of these have been proven to be associated with plant salinity resistance. \textit{Gh_D01G0234 (RPL14B)} knockdown significantly affected the cotton seedling’s performance under salt stress conditions as evidenced by a significant reduction in various morphological and physiological traits.\textsuperscript{49} The overexpression of \textit{RPL6} resulted in tolerance of moderate (150 mM) and high (200 mM) levels of salt (NaCl).\textsuperscript{50} \textit{Strictosidine synthases (Str)}, the key gene of terpenoid indole alkaloid biosynthetic pathway in response to salinity stress in Catharanthus roseus.\textsuperscript{51} High Mobility Group (HMG) proteins are relatively abundant small proteins related to chromatin. The overexpression of \textit{HMGB1} decreased the seed germination rate in the presence of NaCl stress. Expression of \textit{HMGB2} and \textit{HMGB3} was significantly down-regulated under salt stress.\textsuperscript{52} OsHMGB707 regulates rice tolerance by encouraging the expression of stress-related genes.\textsuperscript{53} SNF1 (SnRK2) are important components of the signaling pathways of salinity stress in plants. The \textit{AtSnRK2} family comprises kinase proteins activated by abscisic acid (ABA). SnRK2s participate in the modulation of root system architecture in response to salinity stress.\textsuperscript{54} Transcript factor R2R3-MYB participates in salt tolerance in Arabidopsis.\textsuperscript{39}

Overexpression lines of \textit{LpCPC} can increase lignin and phenol content. High content of lignin and phenol content can improve the lignification of the cell wall, which resulted in improved tolerance to salt stresses. Another mechanism of \textit{LpCPC} regulating plant salt tolerance was involved in the ABA

---

**Table 3. Interacting proteins with \textit{LpCPC} using yeast two-hybrid.**

| Protein name                        | Functions                                                                 | Reference |
|-------------------------------------|---------------------------------------------------------------------------|-----------|
| Ribosomal protein                   | Regulate protein synthesis; maintain ribosome’s function; involved in salt tolerance through signal transduction | 35        |
| SnRK2                              | Respond to drought, salt, and cold stress by phosphorylating targeted protein | 36        |
| HMG-box (high mobility group) DNA-binding family protein | Affect plant growth and stress tolerance including saline through modulation of transcription at target genes | 37, 38    |
| MYB protein                         | Enhances salt tolerance by elevating alternative respiration via transcriptionally upregulating | 39        |
| PQ-loop repeat family protein transmembrane family protein | Involved in physiological processes, including senescence, environmental adaptation, and host-pathogen interaction | 40        |
| Regulatory particle non-ATPase      | Against environmental stress through autophagic | 41        |
| Strictosidine synthase 3            | The key enzyme of monoterpenoid indole alkaloid biosynthesis; related to saline tolerance | 42        |
| N-terminal nucleophile aminohydrolases | Respond to metabolic salvage of purine compounds | 43        |
pathway, salt increased the expression of R2R3MYB, MYB may regulate the transcription of LpCPC, LPCPC may interact with SNRK2 to improve the salt tolerance of plants through the ABA pathway (Figure 18).

Conclusion
In this paper, the LpCPC was cloned based on the transcriptomic data of Lilium pumilum. Subsequently, bioinformatic analysis and analysis of gene expression patterns were performed. The LpCPC gene was transformed into yeast and Nicotiana benthamiana to obtain transgenic strains and plants. The phenotype of transgenic yeast and plants under salinity was analyzed. What's more, physiological index analyses including chlorophyll content, relative conductivity, and lignin content revealed that overexpression of the LpCPC gene could improve the salt resistance of transgenic plants. Overexpression tobacco has consistently higher expression of key genes such as NHX1 and SOS1 involving in salt stress response pathways. When compared with wild-type lines, transgenic lines displayed considerably greater amounts of CCR1 and CAD, which are implicated in lignin synthesis. Eight positive interacting proteins were derived from two yeast-hybrids, ribosomal protein, SNF1-related protein kinase, HMG-box (high mobility group) DNA-binding family protein, MYB protein 1, and Strigoidine synthase 3 were shown to be associated with resistance to plant salinity.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This work was supported by the Heilongjiang Province Nature Science Foundation (LH2019C01) and Fundamental Research Funds for the Central Universities (257202DY18)

ORCID
Yi Dong https://orcid.org/0000-0001-9898-9571
Shumei Jin https://orcid.org/0000-0002-5258-789X

References
1. Lei F, Zhang H, Long Y, Deng S, Zhang A. Characteristics and phylogenetic analysis of the complete chloroplast genome of Lilium concolor Salis. (Liliaceae) from Jilin, China. Mitochondrial DNA B Resour. 2022;7(1):30–31. doi:10.1080/23802359.2021.2006816.
2. Jin J, Liu H, Zhong C, Xie J, Qin Y, Liang X, Chen L, Cai P, Zhan S. Characterization of the complete chloroplast genome of an endangered ornamental and medicinal plant Lilium pumilum. Mitochondrial DNA B Resour. 2020;5(1):1111–1112. doi:10.1080/23802359.2020.1726228.
3. López-Millán AF, Duy D, Philippak P. Chloroplast iron transport proteins - function and impact on plant physiology. Front Plant Sci. 2016;7:178. doi:10.3389/fpls.2016.00178.
4. Komori H, Higuchi Y. Structure and molecular evolution of multieopper blue proteins. Biomol Concepts. 2010;1(1):31–40. doi:10.1515/bmc.2010.004.
5. Warren JJ, Lancaster KM, Richards JH, Gray HB. Inner- and outer-sphere metal coordination in blue copper proteins. J Inorg Bioch. 2012;115:119–126. doi:10.1016/j.jinorgbio.2012.05.002.
6. Rydén LG, Hunt LT. Evolution of protein complexity: the blue copper-containing oxidases and related proteins. J Mol Evol. 1993;36(1):41–66. doi:10.1007/BF02407305.
7. Cobley JG, Haddock BA. The respiratory chain of Thiobacillus ferrooxidans: the reduction of cytochromes by Fe(II) and the preliminary characterization of rusticyanin a novel "blue" copper protein. FEBS Lett. 1975:60(1):39–33. doi:10.1016/0014-5793(75)80411-x.
8. De Rienzo F, Gabdoulline RR, Menziani MC, Wade RC. Blue copper proteins: a comparative analysis of their molecular interaction properties. Protein Sci: A Publ Protein Soc. 2000;9(8):1439–1454. doi:10.1110/ps.9.8.1439.
9. Cao J, Li X, Lv Y, Ding L. Comparative analysis of the phyto-cyanin gene family in 10 plant species: a focus on Zea mays. Front Plant Sci. 2015;6:515. eCollection 2015. doi:10.3389/fpls.2015.00515.
10. Höhner R, Pribil M, Herbstová M, López LS, Kunz HH, Li M, Wood M, Svoboda V, Puthiyaveetil S, Leister D, et al. Phytocyanin is the long-range electron carrier between photosystem II and photosystem I in plants. Proc Natl Acad Sci U S A. 2020;117(26):15354–15362. doi:10.1073/pnas.2005832117.
11. Gough J, Chothia C. The linked conservation of structure and function in a family of high diversity: the monomeric cupredoxins. Structure (London, England: 1993). 2004;12(6):917–925. doi:10.1016/j.str.2004.03.029.
12. Nersissian AM, Shipp EL. Blue copper-binding domains. Adv Protein Chem. 2002;60:271–340. doi:10.1016/s0065-3233(02)60056-7.
13. Armstrong GD, Chapman SK, Sisley MJ, Sykes AG, Aitken A, Osheroff N, Margoliash E. Preferred sites on cytochrome c for electron transfer with two positively charged blue copper proteins, Anaebena variabilis phycocyanin and stellacyanin. Biochemistry. 1986;25(22):6947–6951. doi:10.1021/bi00370a031.
14. Van Gysel A, Van Montagu M, Inzé D. A negatively light-regulated gene from Arabidopsis thaliana encodes a protein showing high similarity to blue copper-binding proteins. Gene. 1993;136(1–2):79–85. doi:10.1016/0378-1119(93)90450-h.
15. Katoh S. Early research on the role of phycocyanin in photosynthesis. Photosynth. Res. 2003;76(1–3):255–261. doi:10.1023/A:1024924711453.
16. Lan Y, Zhang K, Wang Y, Wu J, Lin M, Yan H, Xiang Y. Comparative analysis of the stellacyanins (SCs) family and focus on drought resistance of PsSC18 in Populus trichocarpa. Gene. 2022;813:146106. doi:10.1016/j.gene.2021.146106.
17. Ezaki B, Katsuhara M, Kawamura M, Matsumoto H. Different mechanisms of four aluminum (Al)-resistant transgenes for Al toxicity in Arabidopsis. Plant Physiol. 2001;127(3):918–927. doi:10.1109/0425.10-1326-3.
18. Ezaki B, Sasaki K, Matsumoto H, Nakashima S. Functions of two genes in aluminum (Al) stress resistance: repression of oxidative damage by the AtBCB gene and promotion of efflux of Al ions by the NtGDI1 gene. J Exp Bot. 2005;56(420):2661–2671. doi:10.1093/jxb/er529.
19. Zhang JP, Yu Y, Feng YZ, Zhou YF, Zhang F, Yang YW, Lei MQ, Zhang YC, Chen YQ. MiR408 regulates grain yield and photosynthesis via a phycocyanin protein. Plant Physiol. 2017;175(3):1175–1185. doi:10.1104/pp.17.01169.
20. Zuan XM, Luo F, Li DD, Zhang J, Liu ZH, Xu WL, Huang GQ, Li XB. Cotton BCP genes encoding putative blue copper-binding proteins are functionally expressed in fiber development and involved in response to high-salinity and heavy metal stresses. Physiol Plant. 2011;141(1):71–83. doi:10.1111/j.1399-3054.2010.01420.x.
21. Wu H, Shen Y, Hu Y, Tan S, Lin Z. A phycocyanin-related early nodulin-like gene, BcBCP1, cloned from B. o. crassifolia enhances osmotic tolerance in transgenic tobacco. J Plant Physiol. 2011;168(9):935–943. doi:10.1016/j.jplph.2010.09.019.
