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Overexpression of *LpCPC* from *Lilium pumilum* confers saline-alkali stress ($NaHCO_3$) resistance

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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 $NaHCO_3$. The transgenic yeast was more tolerant compared with the control under $NaHCO_3$ 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, indicatingthat it is an essential gene to enhance salt tolerance in *Lilium pumilum*.

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Lilium pumilum; blue copper protein; saline stress; yeast two-hybrid

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.[1,2] We analyzed and compared the differential transcriptome of *Lilium pumilum* without treatment and under 20 mM $NaHCO_3$ treatment. The results indicated that the expression of the *CPC* gene was significantly increased under 20 mM $NaHCO_3$ 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.[3] 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.[4] 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.[5,6] Blue copper protein was found for the first time in *Thiobacillus ferrooxidans*.[7] 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.[8]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).[9,10] 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.[11] 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.[12,13] 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.[14]

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.[15] In plants, CPC improves oxidative stress response through increasing

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peroxidase and catalase enzyme activities.[16] Oxidative stress-induced the overexpression of blue copper-binding protein AtBCB to confers oxidative aluminum resistance in *Arabidopsis thaliana*.[17] The expression levels of several SC genes were significantly increased when induced by salt stress in the *Capsicum annuum*.[18] *PtSC18* could dramatically enhance the drought tolerance of transgenic plants by reducing water loss, regulating the H_2O_2 content, peroxidase, and enzymatic activities of catalase.[16] *BCP* diminished aluminum stress through the formation of lignin or the effect of miR408.[18,19] The tolerance of salt stress was enhanced through higher antioxidant enzyme activities and *BCP* transcription levels (Table 1).[16,20,21]

NHX1 (Vacuolar 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. *NHX1* 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. *NHX1* and *SOS1* might be used as biomarkers to further investigate the interaction between genes and stress tolerance.[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.[25] Salt stress has an impact on the biosynthesis of secondary cell walls in plant cells.[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.[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.[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 toxin 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 $NaHCO_3$ 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.

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	18
GhBCPs	Up-regulated in fibers by high-salinity including Cu^{2+} and Zn^{2+} as well as drought stresses; downregulated in fibers by Al^{3+} treatment	20
PtSC18	Strongly responded to drought stress by improving water retention and reducing oxidative damage	16
OsUCL8	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 were used in this study.

Primer Name	Primer Sequence
LpCPC F	GAGTTACAACTTCAGATGGAGTCC
LpCPC R	CTCAAATCCACTCTAGACCAACAA
LpCPC qPCR-F	ACTCCAACACGAGGTTGGTC
LpCPC qPCR-R	AAAGCTCATCCCCTGAAGGC
LpActin F (Internal Reference)	GCATCACACCTTCTACAACG
LpActin R (Internal Reference)	GAAGAGCATAACCCTCATAGA
LpCPC-EcoRI -F	GAATTCATGGAGTCCATAACTCT
LpCPC-XhoI -R	CTCGAGTTAATGGTGATGGTGATGATGGACCAACAAAGAAGC
NOS R	AATCATCGCAAGACCGGC
NbActin-F	TGGTCGTACCACCGGTATTGTGTT
NbActin-R	TCACTTGCCCATCAGGAAGCTCAT
CAD qPCR-F	GGCATGTCCAACTACCCTCT
CAD qPCR-R	TGGGTTTGCCATCAGTGTAGA
CCR1 qPCR-F	GGTGCCGAGACATACCCAAA
CCR1 qPCR-R	TCAACGGATTGTCGTCAGCA
NbNHX1 qPCR-F	AGGCACGCTTTTGCAACTTT
NbNHX1 qPCR-R	ACACTGCGCCTCTCATCAAA
NbSOS1 qPCR-F	ACAGCTAGTTTTTCTCATCAA
NbSOS1 qPCR-R	TGACCAGTTCGTTCCACATC
LpCPC-Sal1-R	GTCGACTTAATGGTGATGGTGATGATGGAC

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 (Takara, 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 primes of *LpActin F* and *LpActin R* was used as a control (Table 2). [30] 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.

Analysis of LpCPC *expression under abiotic stress using RT-qPCR*

Lilium pumilum seedlings were grown on MS medium containing 20 mM $NaHCO_3$ 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-Eco*R I -F and *LpCPC-Xho* I -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 *Eco*R I and *Xho* I. 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.[31] After induction of protein expression in transgenic yeast cells, the obtained broth (OD_{600} = 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 $NaHCO_3$, 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 electroshock 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.[32]

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 $NaHCO_3$ 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.[33]

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-F, NbSOS1 qPCR-R* (Table 2). The primes 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 the 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-Eco*R1-*F* and *LpCPC-Sal*1-*R* (Table 2). The recombinant plasmids pGADT7-LpCPC were identified by double enzyme digestion (*Eco*R1and *Sal*1), then they were transformed into Y_2HGold 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.

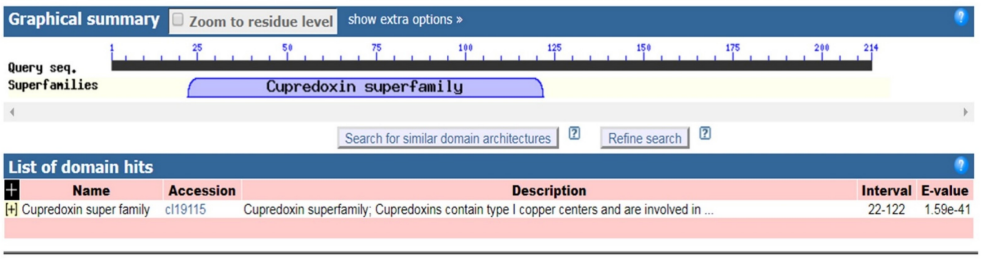

Figure 1. Analysis of the conserved domains of *LpCPC* genes.

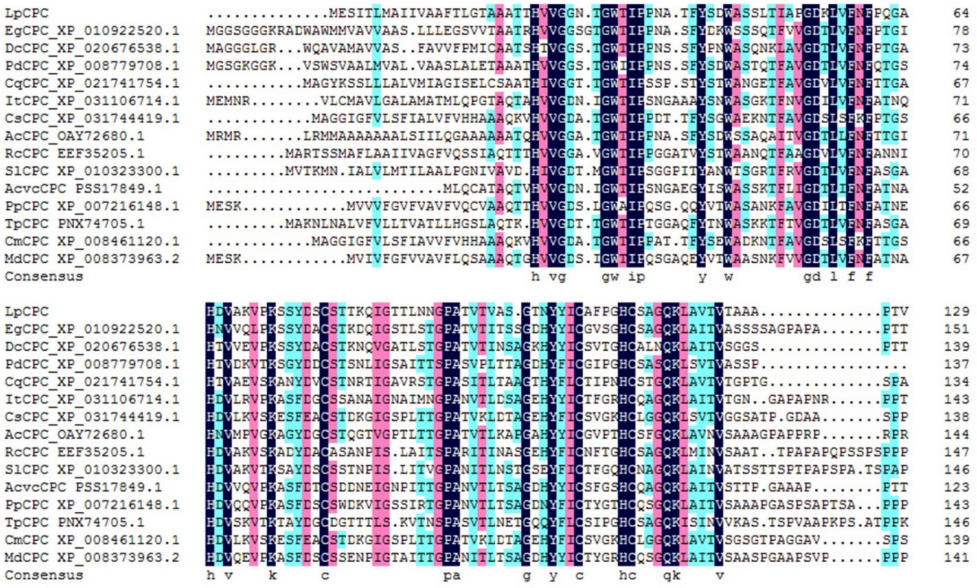

Figure 2. Protein alignment of the LpCPC deduced amino acid sequence with LpCPC proteins from other plant species. The amino acid sequence of this transcript was similar to that alignment of the CPC amino acid sequence from other species: Elaeis guineensis (African oil palm) XP_010922520.1; Dendrobium catenatum XP_020676538.1; Ananas comosus (pineapple) OAY72680.1; 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 pratensePNX74705.1).

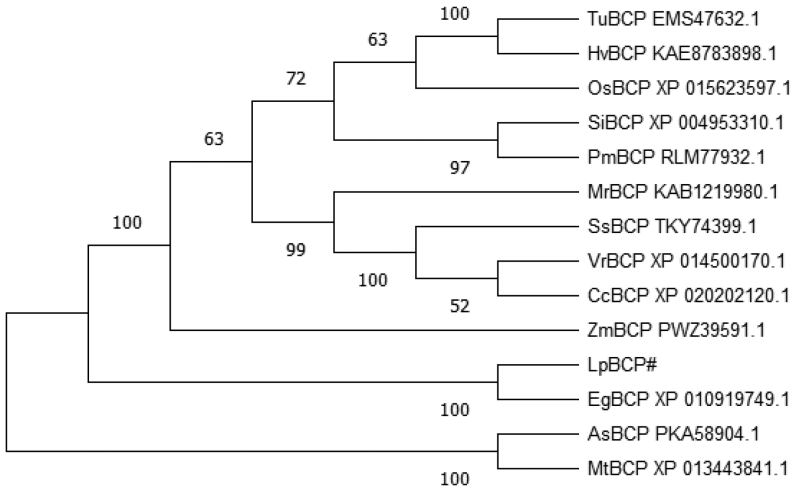

Figure 3. Phylogenetic tree of 14 selected plant CPC proteins. The MEGA3 program was used for the construction of phylogenetic trees. The bar represents 0.1 amino acid substitutions per site.

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* $NaHCO_3$ *stress*

To find out the expression of the *LpCPC* gene in *Lilium pumilum* under $NaHCO_3$ stress, qRT-PCR was performed on cDNA of the bulb after being treated by 20 mM $NaHCO_3$ for 6 h, 12 h, 24 h, 36 h, and 48 h. Under 20 mM $NaHCO_3$ treated, the expression of the *LpCPC* increased and reached the highest level at 12 h (Figure 5). The analysis revealed that $NaHCO_3$ stress promoted the expression of the *LpCPC*.

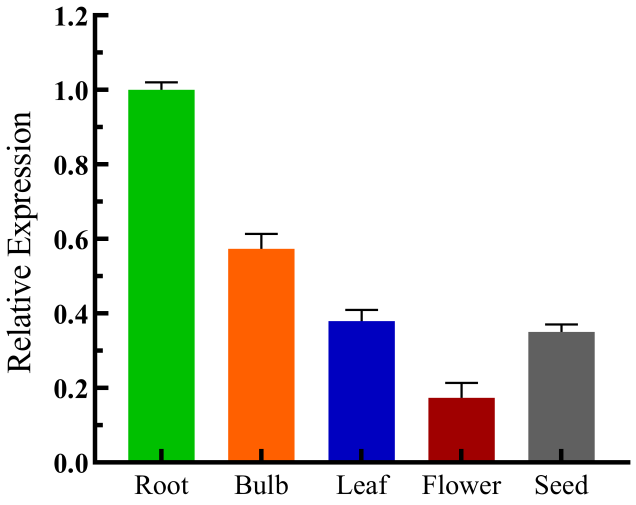

Figure 4. Real-time quantitative PCR analysis of the expression levels of *LpCPC* genes in different organs in *Lilium pumilum*. cDNA was obtained from the roots, stems, leaves, flowers, and seeds of *Lilium pumilum*, and the expression levels of *LpCPC* were detected by Real-time Quantitative PCR.

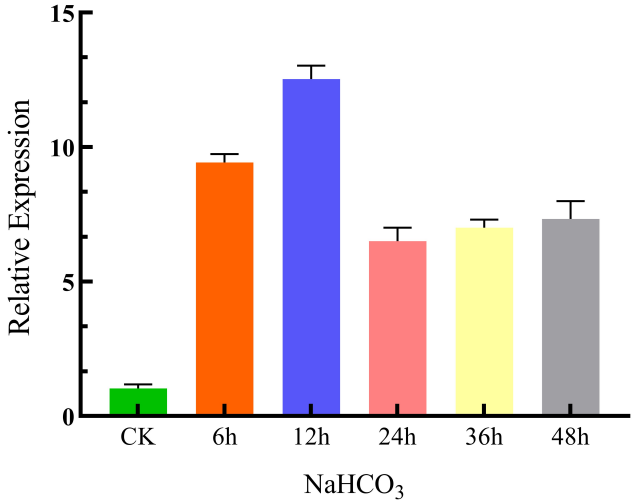

Figure 5. Real-time Quantitative PCR analysis of *LpCPC* expression in *L.Pumilum* under $NaHCO_3$ stress, qRT-PCR was performed on bulb cDNA from *L.Pumilum* with 20 mM $NaHCO_3$ treated for 6 h, 12 h, 24 h, 36 h, and 48 h. CK (No treatment) was used as a control.

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 $NaHCO_3$, 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 $NaHCO_3$ 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 $NaHCO_3$ stress compared with the control. So, the *LpCPC* gene involved in the $NaHCO_3$ 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 $NaHCO_3$ solution. The results demonstrated that with the accumulation of $NaHCO_3$, plants started to wilt. Compared with the wild type, transgenic plants indicated a relatively lower degree of

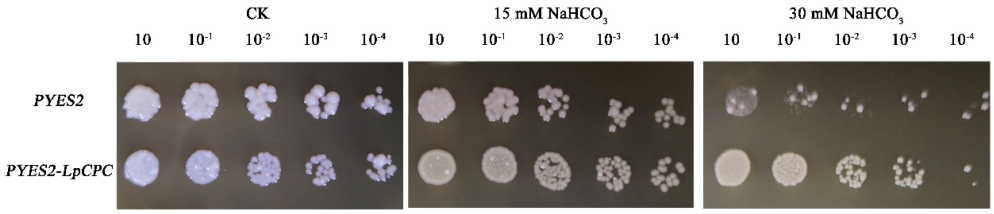

Figure 6. Resistance analysis of transgenic yeast under 0,15 and 30 mM $NaHCO_3$ stress growth of *LpCPC* transgenic yeast cells under $NaHCO_3$ stress. Ten-fold dilutions of yeast cells containing pYES2 (upper line) and pYES2-LpCPC vector (lower line) were spotted on solid YPG media supplemented with the indicated stresses and grew at 30°C for 3–7 d. No treatment is a control (CK).

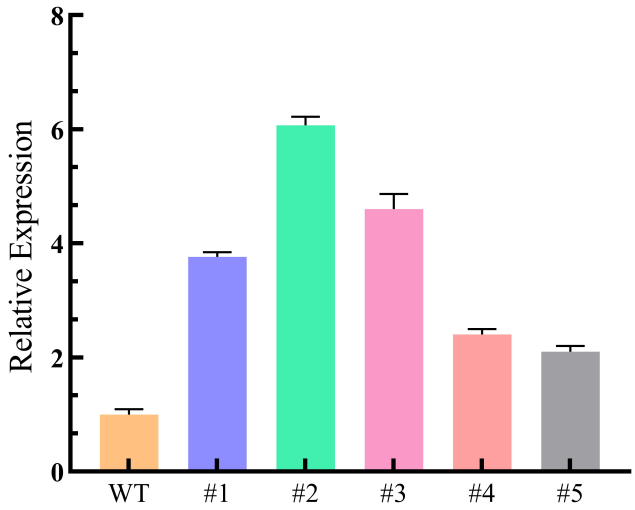

Figure 7. Identification of transgenic *Nicotiana benthamiana* lines by contrasting the expression of *LpCPC* in the wild-type and transgenic lines using RT-qPCR. WT: wild-type. #1–#5: *LpCPC* transgenic *Nicotiana benthamiana* lines.

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* $NaHCO_3$ *stress*

Chloroplasts are destroyed under saline stress and chlorophyll concentration is immediately affected. Chlorophyll was measured on *Nicotiana benthamiana* leaves before and after $NaHCO_3$ 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,[34] 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 $NaHCO_3$-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).

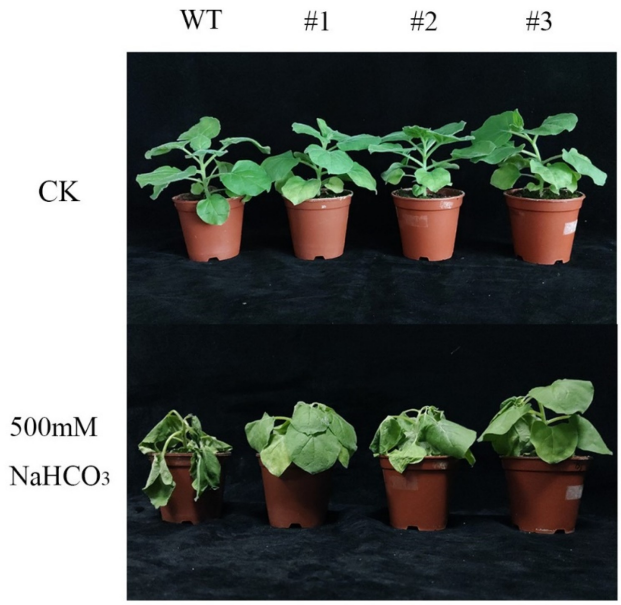

Figure 8. Plant growth between wild-type and *LpCPC* transgenic plants under 500 mM $NaHCO_3$ stresses. Plant growth in pot supplemented with 0 mM (CK), 500 mM $NaHCO_3$. WT: Wild-type. #1, #2, and #3: *LpCPC* transgenic lines.

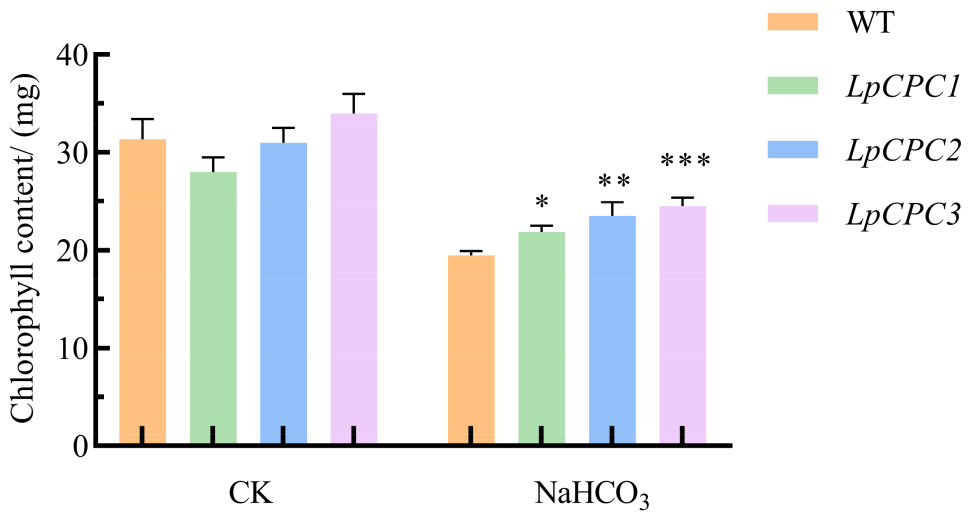

Figure 9. Chlorophyll content change associated with stress response in wild type and transgenic lines. Plant in the pot was grown either with 500 mM $NaHCO_3$ or without, and samples were harvested 24 h later. Chlorophyll contents were detected in the leaves of wild-type and transgenic lines. Each data 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.

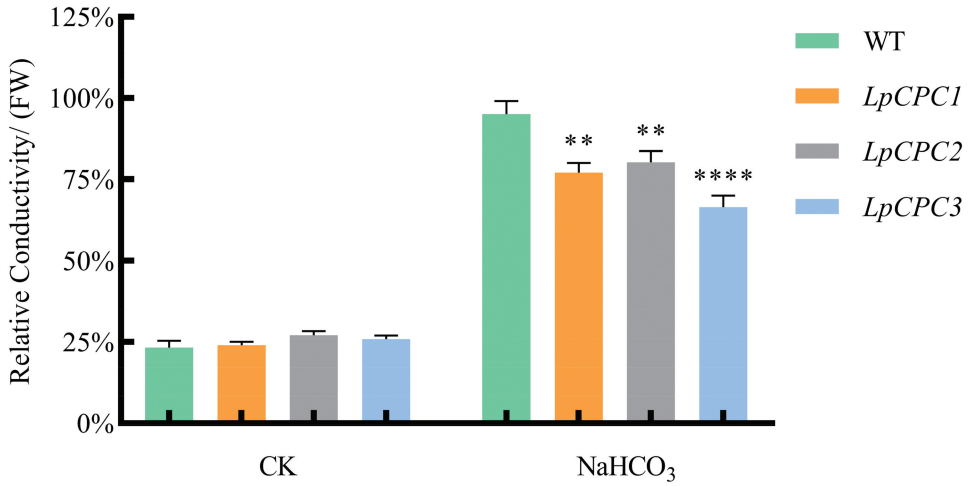

Figure 10. Relative conductivity change associated with stress response in wild type and transgenic lines. Plant in the pot was grown either with 500 mM $NaHCO_3$ or without, and samples were harvested 24 h later. Relative conductivity was detected in the leaves of wild type and transgenic lines. ****Significance P < .0001.

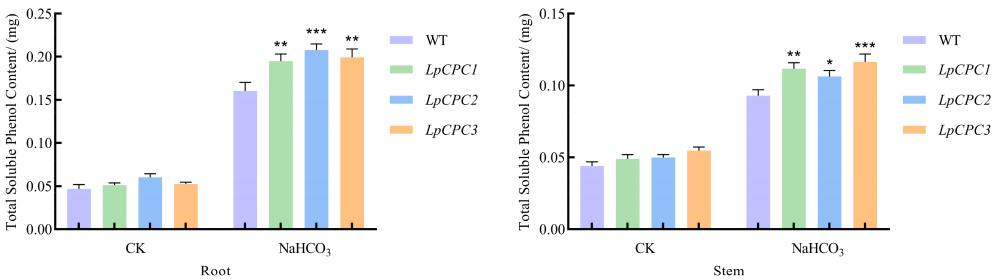

Figure 11. Total soluble phenol content change associated with stress response in wild type and transgenic lines. Plant growth in the pot was grown either with 500 mM $NaHCO_3$ or without, and samples were harvested 24 h later. Total soluble phenol content was detected in the leaves of wild type and transgenic lines.

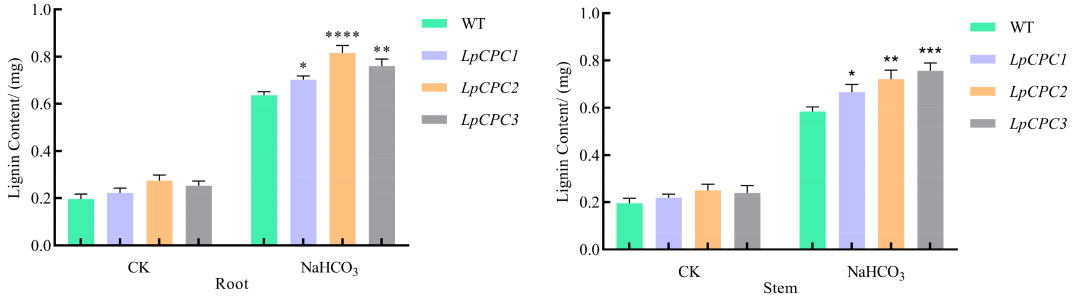

Figure 12. lignin content change associated with stress response in wild type and transgenic lines. Plant growth in the pot was grown either with 500 mM $NaHCO_3$ or without, and samples were harvested 24 h later.

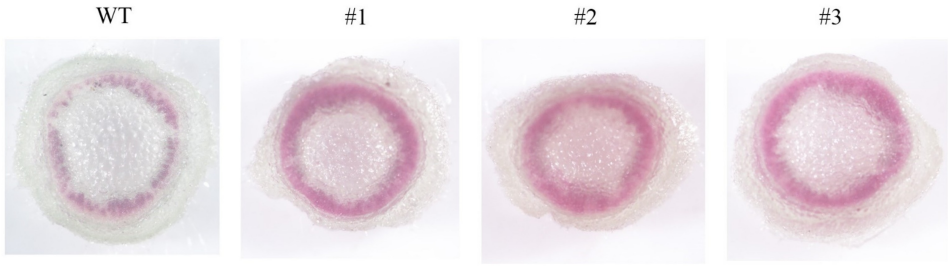

Figure 13. Lignin staining analysis of the cross-section of tobacco stem by phloroglucinol-HCl. WT: wild type; #1, #2, #3: staining of roots in *Nicotiana benthamiana* with over-expressing gene *LpCPC*.

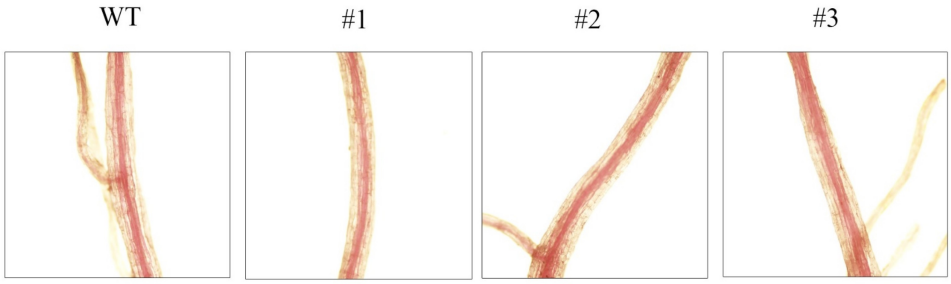

Figure 14. Lignin staining analysis of the cross-section of tobacco root by phloroglucinol-HCl. WT: wild type; #1, #2, #3: staining of roots in *Nicotiana benthamiana* with over-expressing gene *LpCPC*.

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-α-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 $NaHCO_3$. 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.[44]

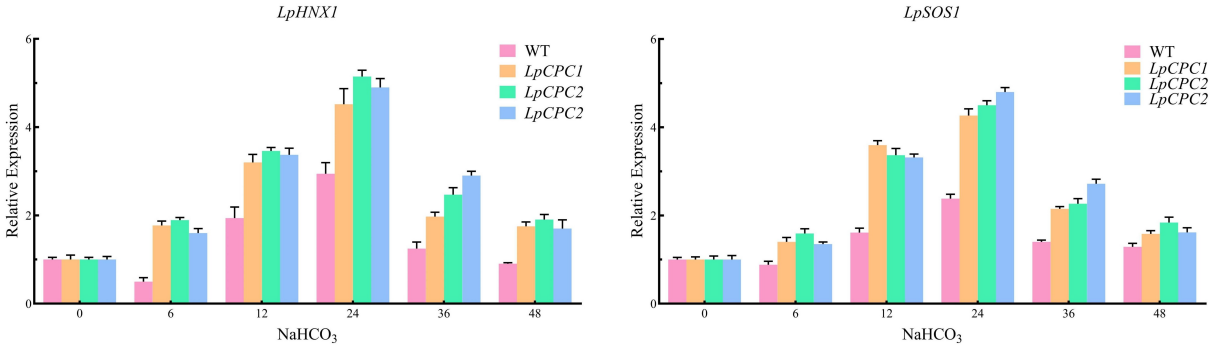

Figure 15. qRT-PCR determination of representative gene expression in leaves. The relative expression level of *LpHNX1* and *LpSOS1* were detected in leaves of wildtype tobacco and transgenic tobacco seedlings exposed to 500 mM $NaHCO_3$ for 0 h, 6 h, 12 h, 24 h, 36 h. 48 h.

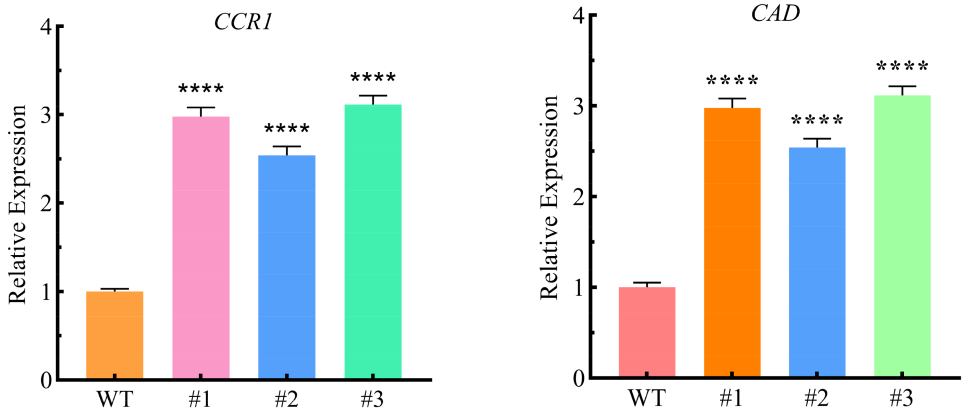

Figure 16. Expression of *CCR1*and *CAD*, potential lignin biosynthesis genes. The qPCR analysis the expression levels of *CCR1* and *CAD* genes related to lignin biosynthesis. Three biological replicates are used for each transgenic line and wild type.

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: pGBKT7 vector, AD: pGADT7 vector, SD: synthetic dextrose.

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 $NaHCO_3$ 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.

Table 3. Interacting proteins with 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

Phenol is related to salt stress tolerance, and the highest phenol content under salt stress was observed in the highest salt tolerance lines in cucumber.[45] Plants exposed to stress are accompanied with an increase in lignin content.[46] Multiple plants increase their tolerance to abiotic stresses through lignin accumulation.[27–29,46] Silencing the *AtBCB* gene leads to a reduction of lignin accumulation.[47] Cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) related to lignin biosynthesis and its transcriptional regulation are uncovered based on Zhong (2015).[48] *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 *Nicotiana benthamiana* and wildtype. Overexpression of *LpCPC* caused higher expression of both lignin biosynthesis genes. The conserved induction gene expression suggests that *LpCPC* could retain functions in the regulation of lignin biosynthesis. Overexpression of *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 *LpCPC* transgenic lines were more tolerant than wild-type under $NaHCO_3$ treatment.

Further studies are necessary to investigate the response mechanisms of the *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. *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.[49] The overexpression of *RPL6* resulted in tolerance of moderate (150 mM) and high (200 mM) levels of salt (NaCl).[50] *Strictosidine synthases (Str)*, the key gene of terpenoid indole alkaloid biosynthetic pathway in response to salinity stress in Catharanthus

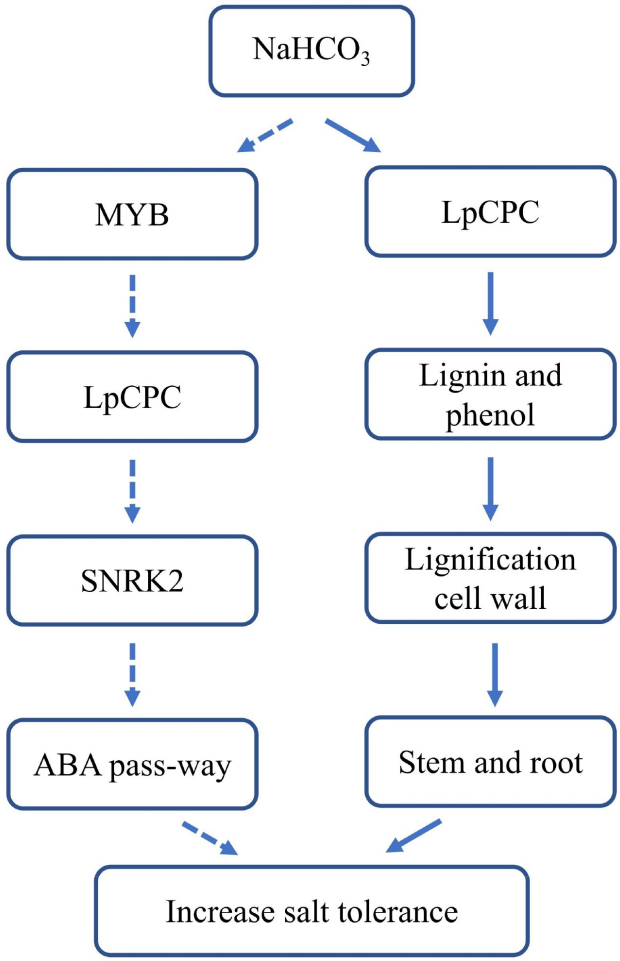

Figure 18. A mechanism model for the LpCPC regulating responded and tolerated to salt stress. A mechanism of LpCPC regulating plant salt tolerance was that LpCPC increased the lignification of the cell wall. The gene *LpCPC* would be directly activated after the treatment of $NaHCO_3$, then contents of lignin and phenol are increased in plants, finally increased the thickness of cell wall in the stem and root. As a result, the tolerance of saline was improved. Another mechanism was involved in the ABA pathway. After $NaHCO_3$ treatment, *MYB* expression was activated, then *MYB* expression increased the expression of gene *LpCPC*. Lp*CPC* interacting with *SNRK2*. As a result, saline tolerance was enhanced after the activation of gene *SNRK2* involving in the ABA pass-way. The solid line represented the experimental verification of the molecular pathway, and the dotted line represented the putative molecular pathway.

roseus.[51] High Mobility Group (HMG) proteins are relatively abundant small proteins related to chromatin. The overexpression of *HMGB1* decreased the seed germination rate in the presence of NaCl stress. Expression of *HMGB2* and HMGB3 was significantly down-regulated under salt stress.[52] OsHMGB707 regulates rice tolerance by encouraging the expression of stress-related genes.[53] SNF1 (SnRK2) are important components of the signaling pathways of salinity stress in plants. The *AtSnRK2* family comprises kinase proteins activated by abscisic acid (ABA). SnRK2s participate in the modulation of root system architecture in response to salinity stress.[54] Transcript factor R2R3-MYB participates in salt tolerance in Arabidopsis.[39]

Overexpression lines of *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 *LpCPC* regulating plant salt tolerance was involved in the ABA

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, bioinformatics 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 Strictosidine synthase 3 were shown to be associated with resistance to plant salinity.

Disclosure statement

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