Genome-Wide Identification of CBL-CIPK Gene Family in Honeysuckle (*Lonicera japonica* Thunb.) and Their Regulated Expression Under Salt Stress

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In plants, calcineurin B-like proteins (CBLs) are a unique group of Ca²⁺ sensors that decode Ca²⁺ signals by activating a family of plant-specific protein kinases known as CBL-interacting protein kinases (CIPKs). CBL-CIPK gene families and their interacting complexes are involved in regulating plant responses to various environmental stimuli. To gain insight into the functional divergence of CBL-CIPK genes in honeysuckle, a total of six LjCBL and 17 LjCIPK genes were identified. The phylogenetic analysis along with the gene structure analysis divided both CBL and CBL-interacting protein kinase genes into four subgroups and validated by the distribution of conserved protein motifs. The 3-D structure prediction of proteins shown that most LjCBLs shared the same Protein Data Bank hit 1uhnA and most LjCIPKs shared the 6c9Da. Analysis of cis-acting elements and gene ontology implied that both LjCBL and LjCIPK genes could be involved in hormone signal responsiveness and stress adaptation. Protein-protein interaction prediction suggested that LjCBL4 is hypothesized to interact with LjCIPK7/9/15/16 and SOS1/NHX1. Gene expression analysis in response to salinity stress revealed that LjCBL2/4, LjCIPK1/15/17 under all treatments gradually increased over time until peak expression at 72 h. These results demonstrated the conservation of salt overly sensitive pathway genes in honeysuckle and a model of Ca²⁺-LjCBL4/LjSOS3-LjCIPK16/LjSOS2 module-mediated salt stress signaling in honeysuckle is proposed. This study provides insight into the characteristics of the CBL-CIPK gene families involved in honeysuckle salt stress responses, which could serve as a foundation for gene transformation technology, to obtain highly salt-tolerant medicinal plants in the context of the global reduction of cultivated land.

**Keywords:** honeysuckle, calcineurin B-like protein, CBL-interacting protein kinase, salt stress, SOS pathway
INTRODUCTION

Calcium (Ca$^{2+}$), whose level fluctuates when cells undergo external changes, acts as a universal secondary messenger for numerous signals and confers specific cellular responses (Ma L. et al., 2019). Ca$^{2+}$ sensors or Ca$^{2+}$-binding proteins can sense changes in cytoplasmic Ca$^{2+}$ concentrations and transmit signals to regulate the function of the downstream proteins (such as transcriptional factors or membrane transporters) and elicit changes in cellular processes (such as gene expression or ionic fluxes) in response to environmental changes (Wang et al., 2020). In plants, there are three major classes of Ca$^{2+}$-binding proteins include calmodulin (CaM) and CaM-like proteins (CMLs), calcineurin B-like proteins (CBLs), and calcium-dependent protein kinases (CDPKs) (Tang et al., 2020).

CBL proteins contain four typical helix-loop-helix motifs (EF-hands) for calcium-binding. However, unlike CDPKs, CBL proteins lack kinase activity and function in calcium signal transduction by interacting specifically with a group of Ser/Thr protein kinases, namely CBL-interacting protein kinases (CIPKs)/SNF1-related kinase group 3 (SnRK3s) (Hrabak et al., 2003). After sensing the change of Ca$^{2+}$ level, CBLs physically interact with the NAF/FISL motifs at the C-terminal of CIPKs to activate CIPKs, and then, the activated CIPKs participate in calcium signaling by phosphorylating target proteins (Luan, 2009). Multiple experiments have proved the core role of the CBL-CIPK signal pathway in fine-tuning plants adaptive response to external environmental changes, including Arabidopsis, rice (Kolukisaoglu et al., 2004; Kanwar et al., 2014), maize (Chen et al., 2011), grapevine (Xi et al., 2017), turnip (Yin et al., 2017), eggplant (Li et al., 2016), and pineapple (Aslam et al., 2019). Among the 10 CBLs and 26 CIPKs in Arabidopsis, not only does each CBL interact with several CIPKs, but each CIPK interacts with one or more CBLs. Such interaction specificity and overlap between different members of CBL and CIPK family may confer both signaling specificity and functional synergism of CBL-CIPK complexes, forming a truly complex CBL-CIPK network when plants confronting a variety of external changes such as nutrient ions deprivation and abiotic stresses (Luan, 2009).

Soil salinization is one of the major environmental stress that reduces plant growth and productivity throughout the world, affecting an estimated 45 million hectares of irrigated land (Huang et al., 2019a). Under salt stress, extracellular high salt environment increased intracellular osmotic pressure and accumulated intracellular Na$^{+}$ to a toxic level (Cheeseman, 1988; Deinlein et al., 2014). Although osmotic pressure and Na$^{+}$ stress sensors in plants have not yet been identified, it seems that such stress signal transduction is closely related to Ca$^{2+}$ pathway (Cheeseman, 1988). The SOS (salt overly sensitive) pathway was originally discovered from the model plant Arabidopsis and was a well-studied Ca$^{2+}$-dependent CBL-CIPK module involved in salinity stress response regulation in plants (Olias et al., 2009). The SOS pathway, which involves two Ca$^{2+}$ sensor proteins, SOS3/AtCBL4 and SCaBP8/AtCBL10; the protein kinase SOS2/AtCIPK24; and the PM (plasma membrane)- Na$^{+}$/H$^{+}$ antiporter SOS1/AtNHX7 (Liu et al., 2020). The Ca$^{2+}$ signal induced by high-salt is decoded by SOS3 and SCaBP8. SOS3 recruit SOS2 to the PM and activate its kinase activity. SOS2/SOS3 complex then phosphorylates and activates downstream PM-binding AtNHX7/SOS1 to transport Na$^{+}$ out of the cell (Ji et al., 2013). Besides, AtCIPK24 can interact with AtCBL10, then phosphorylates and activates the vacuolar NHX, pumping excess Na$^{+}$ into the vacuole. The SOS pathway also exists in many other plants, such as rice, poplar (Tang et al., 2010), and spinach (Zhao et al., 2020). In rice, OsCIPK24/OsSOS2 interacts with OsCBL4/OsSOS3 regulating the activity of PM-located OsSOS1, thus participating in the regulation of plant salt tolerance (Pandey et al., 2015).

Lonicera japonica belongs to the honeysuckle family and is widely cultivated as an ornamental plant. Its dried flower buds have been prescribed in traditional Chinese medicine (TCM) to treat fever, influenza, sores and swelling for thousands of years (Pu et al., 2020). It is also a crucial antiviral drug used to treat the SARS coronavirus, influenza A viruses, the H1N1, H5N1, H7N9 flu virus, and Enterovirus 71 in recent years (Liu et al., 2005; Shang et al., 2011). It has further value as a component of cosmetic, healthy food, and beverages due to its unique aroma and pharmacological activity. Previously, Huang et al. found that the honeysuckle cultivar “Huajin 6” has high salt tolerance, but the physiological and molecular mechanisms are remained elusive until now (Huang et al., 2019b). Due to the great significance of CBL-CIPK networks in various physiological and stress resistance processes, it is of great significance to explore the salt tolerance mechanism of honeysuckle start from the analysis of CBL-CIPK gene.

In this study, we identified 6 CBL and 17 CIPK genes based on the genome sequences of honeysuckle. Various characteristics of these genes were analyzed, including physicochemical properties, phylogenetic relationship, conserved motifs, gene structures, cis-acting elements analysis, protein three-dimensional (3-D) structure prediction, and putative protein-protein interaction (PPI) prediction. Additionally, the gene expression patterns of CBL-CIPK genes were analyzed in different tissues and gradient salt stress. Our results could reveal the roles of CBL-CIPK genes in response to high-salt environments, and our study aimed to provide a valuable reference for further utilization of CBL-CIPK genes to develop salt-tolerant medicinal plants in the context of the global reduction of cultivated land.

MATERIALS AND METHODS

Identification of CBL-CIPK Genes in Honeysuckle

The published CBL and CIPK gene sequences of Arabidopsis and Oryza sativa were downloaded from Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org) and the rice genome database (http://rice.plantbiology.msu.edu/), respectively. The CBL and CIPK genes were further used as queries to search against Lonicera japonica genome databases to
identify CBL-CIPK genes from honeysuckle. Then, The EF-hand calcium-binding domain (PS50222) of CBL proteins was determined based on PROSITE (https://prosite.expasy.org/) and HMMER (https://www.ebi.ac.uk/Tools/hmmer/). Each CIPK protein was subjected to the PROSITE (https://prosite.expasy.org/), NCBI Conserved Domain database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterProscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) databases to confirm the presence of the kinase domain (PF00069) and the NAF domain (PF03822). Finally, Expert Protein Analysis System (ExPASy, http://web.expasy.org/compute_pi/) was used to predicate the isoelectric point (pI) and molecular weight (M.W) (Bjellqvist et al., 1993; Bassil et al., 2019). The palmitoylation sites and myristoylation sites of CBLs were determined with GPS-Lipid 1.0 (http://lipid.biocuckoo.org/index.php) (Ren et al., 2008; Xie et al., 2016).

Phylogenetic Analysis
The protein sequences of all the identified CBLs and CIPKs from *Lonicera japonica* (Lj), *Arabidopsis thaliana* (At), *Oryza sativa* (Os) were aligned using the Clustal-Omega (https://www.ebi.ac.uk/services). The whole phylogenetic trees were constructed by MEGA six using the Maximum likelihood (ML) method (Kumar et al., 2016). Pair distance among the CBL-CIPK genes was calculated by EMBOSS needle (https://www.ebi.ac.uk/Tools/psa/).

**Conserved Motifs, Gene Structures, and Cis-Acting Elements Analysis**
Multiple Expectation Maximization for Motif Elicitation program (MEME version 5.0.5, http://meme-suite.org/tools/meme) was used to identify the conserved motifs of the CBL-CIPK proteins from honeysuckle (Bailey et al., 2009). The exon/intron organization for individual CBL-CIPK genes of honeysuckle were analyzed by the Gene Structure Display in GSDS, NCBI Conserved Domain database (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterProscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Hu et al., 2015). For flower stages including root, stem, mature leaf, young leaf, and flower were collected from 2-year-old honeysuckle grown at Shandong University of Traditional Chinese Medicine Medicinal Botanical Garden for quantitative real-time PCR (qRT-PCR). For salt stress, annual honeysuckle seedling plants were transplanted to plastic containers filled with quartz sand/vermiculite (1/3) in April 2021, one seedling per container. The seedlings were treated with the mixed solution containing NaCl (0, 100, 200, or 300 mM) and half-strength Hoagland’s nutrient solution. The seedlings were grown in climatic chambers (30°C day/25°C night, average RH of 65%, 300 μmol m−2 s−1 PAR, 14/10 h photoperiod). Samples for gene expression analysis were harvested at 0, 3, 6, 12, 24, 48 and 72 h after treatments and stored at −80°C until biochemical analysis. The roots were used for further study. Primers were designed using Primer Premier six and Oligo 7 with melting temperature of 58–62°C and production of 80–150 bp. The sequences of primers used in this study are listed in [Supplementary Table S1](#) (Huang et al., 2021).

**RESULTS**

**Identification of LjCBLs and LjCIPKs Genes**
A total of 6 CBL and 17 CIPK genes were finally obtained from the honeysuckle genome. All the LjCBLs contained the EF-hand while all the LjCIPKs possessed the conserved NAF/FISL motif at the C-terminal and the protein kinase domain at the N-terminal. These honeysuckle genes were named *LjCBL1-LjCBL6* and *LjCIPK1-LjCIPK17*, respectively, according to their chromosomal positions.

As shown in [Table 1](#), the deduced amino acid sequences of 6 LjCBL genes demonstrated great conservation in length, which ranged from 207 aa (*LjCBL5*) to 231 aa (*LjCBL2*), with an average length of 221 aa. The length of the LjCIPK genes was between 362 aa (*LjCIPK14*) and 519 aa (*LjCIPK3*), with an average length of 445 aa. The predicted M.W of the LjCBL proteins ranged from 23.82 to 26.62 kDa, and 41.06–58.35 kDa for CIPK proteins. The P1 of the LjCBL proteins ranged from 4.64 to 5.43, with an average P1 of 4.91, and 6.58 to 9.22 for CIPK proteins, with an average P1 of 8.45. Overall, the P1 of all LjCBL proteins was less than 7, while 88% of the LjCIPK proteins had a P1 of greater than 7. Therefore, the LjCBL proteins are rich in acidic amino acids and the LjCIPKs are rich in basic amino acids.

**Protein-Protein Interaction Network Analysis**
The PPI network of CBL-CIPK proteins was predicted using a model plant Arabidopsis on STRING protein interaction database (http://string-db.org) (Szklarczyk et al., 2019).

**Plant Material, Treatment, and qRT-PCR Analysis**
The honeysuckle cultivar ‘Huajin 6’ was used in this study. For tissue-specific expression analysis, five tissues (flowering stage) including root, stem, mature leaf, young leaf, and flower were collected from 2-year-old honeysuckle grown at Shandong University of Traditional Chinese Medicine Medicinal Botanical Garden for quantification real-time PCR (qRT-PCR). For salt stress, annual honeysuckle seedling plants were transplanted to plastic containers filled with quartz sand/vermiculite (1/3) in April 2021, one seedling per container. The seedlings were treated with the mixed solution containing NaCl (0, 100, 200, or 300 mM) and half-strength Hoagland’s nutrient solution. The seedlings were grown in climatic chambers (30°C day/25°C night, average RH of 65%, 300 μmol m−2 s−1 PAR, 14/10 h photoperiod). Samples for gene expression analysis were harvested at 0, 3, 6, 12, 24, 48 and 72 h after treatments and stored at −80°C until biochemical analysis. The roots were used for further study. Primers were designed using Primer Premier six and Oligo 7 with melting temperature of 58–62°C and production of 80–150 bp. The sequences of primers used in this study are listed in [Supplementary Table S1](#) (Huang et al., 2021).

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Phylogenetic Analysis

To determine evolutionary relationships and functional associations, multi-species phylogenetic trees using the identified CBL and CIPK full-length protein sequences from *Lonicera japonica* (Lj), *Arabidopsis thaliana* (At), *Oryza sativa* (Os) were constructed (Figure 1). Multiple protein sequence alignment showed that both CBLs and CIPKs were clustered into four subgroups. There are two LjCBL (LjCBL1/3) proteins in Group I, one LjCBL (LjCBL5) protein in group II, one LjCBL (LjCBL6) protein in group III, and two LjCBL (LjCBL2/4) proteins in group IV. Among CIPK subgroups, the Group I had the largest number of members, with eight LjCIPK (LjCIPK2/3/6/7/10/11/13/15) proteins. Group iii had the fewest members, with only one LjCIPK (LjCIPK17) protein. Group ii and Group iv contained three LjCIPK (LjCIPK14/16/18) proteins respectively.

The conservation of sequence among the CBL-CIPK genes was also confirmed by the identities and similarities of amino acid sequences (Supplementary Table S1 Sheet 3 and Sheet 4). The results showed that the identity of different LjCBLs ranged from 29.6 to 89.4% and the identity of different LjCIPKs ranged from 33.6 to 82.7%. The LjCBLs clustered into the same subgroup display higher identities of sequence in amino acid level (LjCBL1/LjCBL3 = 89.4% and LjCBL2/LjCBL4 = 82.2%), whilst the LjCBL genes in different subgroups exhibit lower identities. The sequences of LjCIPK10/LjCIPK15 and LjCIPK4/LjCIPK9 have higher identities (82.7 and 72.1%), which were the members of a close evolutionary relationship.

Chromosomal Location, Ka/Ks Ratio Calculation

As shown in Figure 2, six LjCBL genes were mapped onto five of total nine honeysuckle chromosomes while seventeen LjCIPK genes were mapped onto eight of total nine chromosomes, indicating a diverse distribution. LjCIPK1 and LjCIPK2 on chromosome 1 overlapped, but the two genes are located far away in the phylogenetic tree, indicating that their biological functions may be different. The same overlap was also observed on chromosome 1 (LjCIPK3 and LjCIPK4) and chromosome 2 (LjCIPK5 and LjCIPK6). In the present study, only one (LjCIPK10/LjCIPK15) duplicated gene pair was identified (Supplementary Table S1 Sheet 5). Ka/Ks ratio between LjCIPK10 and LjCIPK15 is 0.0561, implying that the genes...
had undergone strong purifying selection pressure, which reduces the rate of change in amino acid profile.

**Gene Structure and Conserved Motifs Analysis**

Members with close evolutionary relationships shown uniform or similar gene structure and motif composition (Figure 3B). The gene structure analysis along with the phylogeny results showed that the genes with a similar intron/exon pattern clustered near to each other in the same groups. LjCBL genes contained 8 exons and seven to nine introns. Group i/ii/iii of LjCIPK genes possessed one to three exons and one to two introns, whilst Group iv of LjCIPK genes had 13–14 exons and 12–13 introns. In general, the exon length, exons/intron number were moderately

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**FIGURE 1** | Phylogenetic tree of the CBL (A) proteins and CIPK (B) proteins from Lonicera japonica (Lj), Arabidopsis thaliana (At), Oryza sativa (Os). The different colored arcs indicate different subgroups. Proteins from honeysuckle, Arabidopsis, and rice are denoted by green triangles, blue circles, and purple squares, respectively. Details of CBL and CIPK protein sequence from three species are listed in Supplementary Table S1 Sheet 1.

**FIGURE 2** | Physical mapping of honeysuckle CBL-CIPK genes. The numbers of CBL-CIPK genes are indicated on the right of each chromosome. The scale on the left represents chromosome length.
conserved among the various subgroups, indicating similar biological function.

To further investigate the characteristic region of CBL-CIPK proteins, the motif distributions were investigated (Figure 3C). A total of six putative motifs were identified in LjCBL proteins, and 15 motifs were identified in LjCIPK proteins. In the LjCBL family, three motifs (motif 1, 3, and 5) existed in all the members, whilst motif 6 was only detected in Group I. Motif 2 existed in all subgroups except group III. In the LjCIPK family, seven motifs (motif 1, 2, 3, 4, 9, 10, and 13) existed in all the members, including the NAF motif (motif 10) and Protein kinase domain (motif 1, 2). Motif 11 was existed in both Group i and ii, while motif 15 was only detected in Group iv, suggesting that they might perform group-specific functions. The results indicate that members of the CBL-CIPK family belonging to the same subgroup have very similar motif types and numbers, but there are also differences in motif patterns among members of the same subgroup.

Cis-Acting Elements Analysis

To further investigate the potential regulatory mechanism of the CBL-CIPK genes in honeysuckle, the cis-acting regulatory elements of the 2000 bp upstream region from the translation initiation site of the CBL-CIPK genes were surveyed (Table 2). In the CBL-CIPK promoters, seven hormone-related (e.g., ABRE, TCA-element, CGTCA-motif), seven stress-related (e.g., LTR, ARE, WUN-motif, MBS), and twelve development-related (e.g., G-box, GT1-motif) elements were identified. There were half of the LjCBL genes that contained more than five types of hormone-related cis-acting elements, but only LjCIPK9 in the LjCIPK family contained so many types. Among stress-related cis-acting elements, ARE and STRE were found in most LjCBL and LjCIPK promoters. LjCBL4 contained nine stress-response elements, including four ARE, one WUN-motif, and four STRE. LjCIPK13 contained fourteen stress-response elements, including five STRE, four ARE, two WUN-motif, 2 W box, and one TC-rich repeats, which was the largest number of stress-response elements contained within one gene. LjCIPK6/8/16 contained ten stress-response elements respectively, which is slightly less than LjCIPK13. Among development-related cis-acting elements, the circadian, TCT-motif, and GCN4 motif were only predicted in CIPK genes, but not found in CBL genes. These results implied that both LjCBL and LjCIPK genes could be involved in hormone signal responsiveness and stress adaptation.

Proteins Structures Analysis

To gain more insight into the putatively functional mechanism of CBL-CIPK proteins in honeysuckle, all the proteins were modeled by I-TASSER (Figure 4). The 3-D structures were construed based on the best structural templates and crystal structures from Protein Data Bank (PDB). The parameters of the best PDB structure (Table 3) illustrated that the models were constructed with high credibility since all of them had a C-score varied from −2.63 to 0.26. Most LjCBL proteins shared the same PDB hit 1uhnA (The crystal structure of the calcium-binding protein AtCBL2 from Arabidopsis thaliana) and LjCIPK proteins shared the 6e9Da (Crystal structure of...
KA1-autoinhibited MARK1 kinase). The same PDB hit indicating that their 3-D structures were similar.

**Protein-Protein Interaction Prediction**

To further explore the potential function of LjNHX members, the PPI network was constructed with STRING database, which was based on either known experimental or predicted interactions. Because the PPI network of *Lonicera japonica* is not available in the STRING database so far, we used the homolog gene between *Arabidopsis thaliana* and *Lonicera japonica* to search in the database. As shown in Figure 5, no immediately interacted relationship was predicted among LjCBLs or LjCIPKs. However, LjCBL and LjCIPK proteins have significantly more interactions. Individual LjCBL6 protein was hypothesized to interact with LjCIPK5/8/12/13/14/17 and CIPK1, and these proteins were clustered into red cluster in the PPI network. LjCBL4 protein was hypothesized to interact with LjCIPK7/9/15/16 and SOS1/NHX1, and these proteins were clustered to green cluster. However, individual

**Expression Patterns of LjCBLs and LjCIPKs**

To investigate the potential roles of LjCBLs and LjCIPKs in different tissues, their expression patterns across five tissues were described using three categories of GO classification: molecular function (MF), biological processes (BP) and cellular components (CC). The results of GO analysis showed that the proteins involved in the network were mainly localized to the cytoplasm, membrane, nucleus, and intracellular membrane-bounded organelle. Regarding MF, most of the proteins possessed protein serine/threonine kinase activity, catalytic activity, ion binding, and ion transmembrane transporter activity. Regarding BP, most of the proteins mainly participate in intracellular signal transduction, plant development process and respond to external stimuli.

**TABLE 2 |** Kinds and amounts of hormone-, stress-, and development-related cis-acting element in the promoters of CBL-CIPK genes of honeysuckle.

| Functional class | Elements | Function | LjCBLs | LjCIPKs |
|------------------|----------|----------|--------|---------|
| Hormone          | ABRE     | ABA-responsive element | 0 1 1 1 | 1 1 1 1 |
|                  | TCA-element | salicylic acid-responsive element | 3 4 5 6 | 5 6 7 8 |
|                  | P-box    | gibberellin-responsive element | 0 1 1 0 | 0 1 1 0 |
|                  | TATC-box | gibberellin-responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | CGTCA-motif | MeJA-responsive element | 2 2 2 2 | 2 2 2 2 |
|                  | TGAGG-motif | MeJA-responsive element | 2 2 2 2 | 2 2 2 2 |
|                  | TGA-element | auxin-responsive element | 1 1 1 1 | 1 1 1 1 |
| Stress           | ARE      | anaerobic induction | 0 2 0 4 | 0 2 0 4 |
|                  | LTR      | low-temperature responsiveness | 3 4 5 6 | 3 4 5 6 |
|                  | MBS      | MYB binding site involved in drought-inducibility | 0 0 0 0 | 0 0 0 0 |
|                  | TC-rich repeats | defense and stress responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | Box W     | WRKY Transcription factor binding site | 0 0 0 0 | 0 0 0 0 |
|                  | WUN-motif | wound-responsive element | 1 1 1 1 | 1 1 1 1 |
|                  | STRE     | Stress response element | 1 1 1 1 | 1 1 1 1 |
| Others           | MyB      | Transcription factor | 1 1 1 1 | 1 1 1 1 |
|                  | AE-box   | light-responsive element | 0 0 0 0 | 0 0 0 0 |
|                  | GATA-motif | light-responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | G-box    | light-responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | GT1-motif | light-responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | TCT-motif | light-responsive element | 1 1 1 0 | 1 1 1 0 |
|                  | Box 4    | light responseess | 1 1 1 0 | 1 1 1 0 |
|                  | MRE      | MYB binding site involved in light responseess | 0 0 0 0 | 0 0 0 0 |
|                  | circadian | circadian control | 0 0 0 0 | 0 0 0 0 |
|                  | CAT-box  | meristem expression | 0 0 0 0 | 0 0 0 0 |
|                  | GCN4_motif | endosperm expression | 0 0 0 0 | 0 0 0 0 |
|                  | O2-site  | zein metabolism regulation | 0 0 0 0 | 0 0 0 0 |

**TABLE 2** Kinds and amounts of hormone-, stress-, and development-related cis-acting element in the promoters of CBL-CIPK genes of honeysuckle.
Figure 4 | Structural analysis of six LjCBL (A) and seventeen LjCIPK (B) modeled proteins. The α-helix, β-strand, and random coil are marked by red, yellow and blue, respectively. The parameters of the best PDB structure for LjCBLs and LjCIPKs are listed in Table 3. Details of secondary structure are shown in Supplementary Figures S3, S4.
honesuckle tissues (root, stem, mature leaf, young leaf, flower) were analyzed using qRT-PCR. As illustrated in Figure 6A, the examined genes were expressed in all selected tissue samples, although their expression levels differed among tissues. Hierarchical cluster analysis divides the tissue expression data of CBL into two categories. In the first group, LjCBL6, LjCIPK4/17 exhibited a high level of expression in mature leaf and root, while LjCBL1/2/4, LjCIPK1/7/11/13/16 exhibited low levels of expression in almost all tissues. In the second group, LjCBL3, LjCIPK3/10 had a high level of expression in almost all tissues. Notably, LjCBL3 and LjCIPK3/9/10/14/15 showed high specific expression in mature leaves. These results collectively illustrate that LjCBL and LjCIPK genes are important for honesuckle growth and development, and different genes may have functional variations.

To determine which LjCBL and LjCIPK genes contribute to salt stress tolerance, the time-course change in expression levels of genes was analyzed in the roots of the "Huajin 6" cultivar under different concentrations of NaCl by RT-qPCR. As shown in Figure 6B, the expression of LjCBL2/4, LjCIPK1 under all treatments gradually increased over time until peak expression at 72 h. When exposed to moderate (200 mM NaCl) and high salt stresses (300 mM NaCl), the expression of LjCBL4 was 7.54- and 16.6-fold higher at 72 h than under control conditions, respectively. Notably, LjCIPK11/13 rapidly increased the expression level at 3 h and maintained a lower level from 12 or 24 h, then significantly increased their expression level over a 48-h period.

**DISCUSSION**

Honesuckle plays an irreplaceable role in the development of TCM. The completion of the whole-genome sequencing of honesuckle (unpublished) makes it possible to analyze various gene families of honesuckle by bioinformatics. Here, a total of six LjCBL genes and 17 LjCIPK genes have been identified and characterized. There are 10 LjCBL genes and 26 LjCIPK genes in Arabidopsis (Arabidopsis thaliana), 10 OsCBL genes and 33 OsCIPK genes in rice (Oryza sativa) (Kolukisaoglu et al., 2004; Kanwar et al., 2014), seven VvCBL genes and 20 VvCIPK genes in Grapevine (Vitis vinifera) (Xi et al., 2017), 10 PtCBL genes and 27 PtCIPK genes in poplar (Populus trichocarpa) (Tang et al., 2010), five SmCBL genes and 15 SmCIPK genes in eggplant (Solanum melongena) (Li et al., 2016), eight ZmCBLs and 43 ZmCIPK in maize (Zea mays) (Chen et al., 2011), 19 BrCBL and 51 BrCIPK genes in Turnip (Brassica rapa) (Yin et al., 2017). It is well known that gene duplication and loss events during evolution lead to these differences in the number of CBL-CIPK genes among various species (Tian et al., 2017). The presence of multiple CBL-CIPK genes in the genome of the honesuckle indicates the functional diversity of the CBL-CIPK gene family.

CBL proteins share an overall structural homology consisting of four elongation factor EF-hand domains responsible for binding Ca\(^{2+}\). Differences in the EF-hand motifs may result in different ways of binding to Ca\(^{2+}\) (Raymond, 1998; Kim et al., 2000; Tang et al., 2020). As shown in Table 1, EF-hands of LjCBLs were highly conserved in honesuckle, indicating the functional conservation of binding Ca\(^{2+}\) ions. CIPK consists of a conserved
N terminal catalytic domain for kinase activity and a less conserved C terminal regulatory region containing the NAF motif/FISL motif for interaction with CBL as well as the protein phosphatase interaction motif for interaction with type-2C protein phosphatases (PP2C) and is separated by a junction domain (Albrecht et al., 2001; Liu et al., 2019). Once signal transduction begins, Ca^{2+}-bound CBL interacts with CIPK via the NAF domain and releases the inhibitory effect of the C terminus, leading to activation of the kinase (Guo et al., 2001; Tang et al., 2020). It has been shown that the putative NAF domains are highly conserved in the CIPK gene families, which is specific to mediate the interaction between CBL and CIPK.

**FIGURE 5** | Protein-protein interaction (PPI) prediction of LjCBLs and LjCIPKs (A) PPI network. Line thickness indicates the strength of data support. The network is clustered into 3 clusters, which are represented with red, green, and blue nodes, respectively (B) Gene Ontology (GO) analysis of the genes from the PPI network.
Motif analysis showed that the NAF domain was located in motif 10 of the LjCIPKs and it existed in all the detected CIPKs in honeysuckle. Similar results were found in pepper (Capsicum annuum) (Ma X. et al., 2019) and canola (Brassica napus) (Zhang et al., 2014). These results indicated the CIPK family genes in honeysuckle are relatively conserved during evolution.

Protein myristoylation and palmitoylation are two critical modifications necessary for protein stability, aggregation, and trafficking (Kim et al., 2000; Hemsley and Grierson, 2008). Myristoylation is an irreversible protein modification in which myristate, a 14-carbon saturated fatty acid, is covalently attached through an amide bond to an N-terminal glycine residue in a co-translational process (Ishitani et al., 2000). In honeysuckle, LjCBL1/2/4/6 starts from a conserved N-myristoylation site, similar to the CBL protein structures from canola (Brassica napus) (Zhang et al., 2014) and rice (Oryza sativa) (Gu et al., 2008), which may play a role in the membrane targeting of the CBL-CIPK complex. Palmitoylation is the reversible addition of fatty acids to proteins, which increases their membrane affinity (Hemsley and Grierson, 2008). In Arabidopsis, the S-acylation-dependent (S-acylation, also known as palmitoylation) association of AtCBL2 with the vacuolar membrane is essential for ABA responses (Hrabak et al., 2003). Consistent with the results of Arabidopsis and canola (Brassica napus) (Zhang et al., 2014), all the six LjCBL proteins possess typical palmitoylation sites at the N-terminal.

The phylogenetic analysis serves as an excellent method to analyze evolutionary relationships among genes. Phylogenetic analysis of LjCBL and LjCIPK genes in honeysuckle, together with Arabidopsis and rice, classified both CBLs and CIPKs mainly into four different groups. Similar results have also been found in eggplant (Solanum melongena) (Li et al., 2016) and canola (Brassica napus) (Zhang et al., 2014). The results also demonstrated that the CBL-CIPK were unevenly distributed among subfamilies, and the CBL-CIPK gene family members in honeysuckle, rice, and Arabidopsis subfamilies. Consistent with the current information of plant evolution, the phylogenetic tree of LjCBLs and LjCIPKs were more closely related to Arabidopsis (dicot) compared to rice (monocot). However, several CBL-CIPK presented in Arabidopsis but was absent in honeysuckle, which mainly because of gene loss. The evolutionary characteristics of exon/intron structure provide strong evidence for phylogenetic grouping (Boudet, 2001). For LjCBLs, all of the CDS was discontinuous by the presence of introns. Whereas, CIPK genes were divided into an exon-poor clade (Group i/ii/iii) and an exon-rich clade (Group iv), similar to those in Arabidopsis (Mao et al., 2016), pepper (Ma X. et al., 2019), and canola.
2019), and cotton (Cui Y. et al., 2020). Combined with the evolutionary analysis of CIPK in plants, our results indicate that intron gain or loss events were the major driving factors for the gene structural evolution of the CIPK gene family before eudicot-monocot divergence (Zhu et al., 2016; Cui Y. et al., 2020). The structural diversification among the CBL-CIPK gene families may allow CBL-CIPK genes to function differently (Yin et al., 2017).

Gene duplication provides opportunities for the new gene production and its functional divergence in the process of gene family expansion and evolution. The paralogous genes were generated during the divergent evolution from a common ancestral gene through duplication events (segmental or tandem duplication) (Xi et al., 2017). It has been shown that the expansion of the CIPK gene family in Gossypium hirsutum and Gossypium barbadense mainly due to whole-genome duplication and segmental duplications (Cui Y. et al., 2020). In honeysuckle, only one CIPK paralogous pair (LjCIPK10/ LjCIPK15) was identified and were generated by segmental duplication events, as the genes present on different chromosomes. The Ka/Ks ratio is used to identify whether selective pressure existed on amino acid substitutions (Nekrutenko et al., 2002). Our results suggested that the function of the duplicated CIPK genes in honeysuckle did not diverge much during their evolution course, and purifying selection could mainly contribute to the maintenance of function in CIPK gene families. Noticeably, the duplicated genes of LjCIPKs were only detected in the intron-poor clade, a similar phenomenon has been observed in Grapevine (Vitis vinifera), indicating that the intron-poor clade of the LjCIPK gene family may play a more specific role to fulfill the specific characteristics of honeysuckle (Xi et al., 2017).

As a key molecular switch, cis-acting regulatory elements participate in the transcriptional regulation of gene activities that control various biological processes (Wu et al., 2019). Hormones, such as ABA, salicylic acid (SA), auxin (IAA), and gibberellin, play critical roles in several developmental stages and stress response (Gallego-Giraldo et al., 2008; Mishra et al., 2014; Li et al., 2019; Zhang and Li, 2019). Here, seven regulatory elements related to hormones were identified in the promoters of CBL-CIPK genes. ABRE (ABA-responsive element), which belongs to the so-called G-box family, contains an AGCT core, a sequence known to be recognized by plant bZIP proteins. ABRE was found in two-thirds of LjCBL and 13 out of 17 LjCIPK genes, respectively, which was also identified in both Arabidopsis and rice (Gomez-Porras et al., 2007), suggesting that the most CBL-CIPK genes might be involved in the ABA signal pathway, which mainly controls stomatal closure, seed and bud dormancy, and physiological responses to cold, drought, and salinity stress (Mishra et al., 2014). Furthermore, seven stress-responsive regulatory elements were identified namely ARE, W box, LTR, STRE, MBS, TC-rich repeats. Similar elements were found in CBL-CIPKs from Grapevine (Vitis vinifera) and BnaNHXs from oilseed rape (Brassica napus) (Cui J.-q. et al., 2020). Interestingly, W box (TTGACC) was identified in half of the CBL-CIPK genes from honeysuckle. W box is recognized by the family of WRKY transcription factors, which is involved in certain developmental processes and stress response, such as drought stress response in tomato (Ahammed et al., 2020), salt response in Arabidopsis (Xu et al., 2018) and populus (Jiang et al., 2020). In general, the identified cis-acting elements here help in understanding their roles in the developmental and various biotic stress-related mechanisms.

All CBL-CIPK gene expression displayed tissue specificity, which indicated the distinct effect of CBL-CIPK genes. Interestingly, LjCBL3 and LjCIPK3/9/10/14/15 were highly expressed in mature leaves. Same result was found in cassava (Manihot esculenta), MeCBL1/9 and MeCIPK23 have higher transcriptional levels in the mature stage (Mo et al., 2018). Since CBL-CIPK is a modulator system for efficient nutrient acquisition, it is speculated that different tissues have different requirements for nutrient ions, resulting in tissue-specific expression of related genes (Dong et al., 2021; Verma et al., 2021). CBL and CIPK genes have been reported to enhance salinity tolerance in different species by establishing the homeostasis of macro-nutrients in the cytosol and subcellular compartments, such as in pigeon pea (Cajanus cajan) (Meng et al., 2021) and cotton (Gossypium hirsutum) (Sun et al., 2021).

Our study revealed that in honeysuckle, several CBL-CIPK genes express differentially at different time intervals under salinity stress. The SOS pathways, consisting of SOS3, SOS2, and SOS1, have been well defined as crucial pathways to control cellular ion homeostasis, by extruding Na⁺ to the extracellular space, thus conferring salt stress resistance in plants. As shown in Table 1, LjCBL4 exhibited orthologous relationships with AtSOS3 (At5g24270), while LjCIPK16 exhibited orthologous relationships with AtSOS2 (At5g35410). As shown in Figure 1, there is a close evolutionary relationship between LjCBL4 and SOS3, LjCIPK16 and SOS2. Interestingly, SOS1, a plasma membrane Na⁺/H⁺ antiporter, was predicted to interact with LjCIPK15/16 and LjCBL4 in the PPI network, while NHX1, the Na⁺/H⁺ antiporter located on tonoplast, was also predicted to interact with LjCIPK16 and LjCBL4, which indicated the key role of LjCIPK16 and LjCBL4 proteins in the salt tolerance of honeysuckle. Moreover, LjCBL4, LjCIPK16 and SOS1 were clustered into green cluster in the PPI network, indicating that they may act in the same pathway. As shown in Figure 6, expression profile analyses revealed that LjCBL4 and LjCIPK16 were upregulated under salt stress. The SOS pathway genes upregulated in response to salinity stress were confirmed in many plants, such as spinach (Spinacia oleracea) (Zhao et al., 2020) and poplar (Populus trichocarpa) (Tang et al., 2010). This result suggested that the Ca²⁺-SOS3-SOS2-cation/H⁺ antiporters (NHXs/AKTs) pathway might be not only a common but also an essential pathway regulating plant salt stress resistance (Zhao et al., 2020). This result also demonstrated the conservation of SOS pathway genes in honeysuckle and a model of Ca²⁺-LjCBL4/ LjSOS3-LjCIPK16/LjSOS3 module-mediated abiotic stress signaling in honeysuckle is proposed.

At the same time, the functions of LjCBL2/5, LjCIPK11/11/13/15/17 in the response of honeysuckle to salt stress are worth further exploring. Notably, LjCIPK1 and LjCIPK15 show greater enhanced expression under salt stress, even higher than LjCIPK16. LjCIPK15 exhibited orthologous relationships with AtCIPK6 (At4g30960). In Arabidopsis, overexpression of AtCIPK6 increased plant tolerance to salt stress (Chen et al.,
CONCLUSION

In the present study, a total of six LjCBL and 17 LjCIPK genes were identified. The phylogenetic analysis divided both CBL and CIPK genes into four subgroups and the same clade had similar motif compositions and gene structures. cis-Acting elements analysis implied that both LjCBLs and LjCIPKs are involved in stress adaptation. PPI network analysis results showed that LjCBL4 is hypothesized to interact with LjCIPK7/9/15/16 and SOS1/NHX1. The salt-induced expression patterns confirmed that the expression levels of LjCBL2/4, LjCIPK1/15/16/17 were affected by salinity. The theoretical foundation was established in the present study for the further functional characterization of the CBL-CIPK gene families in honeysuckle. However, extra works are required to decipher the interaction networks between LjCBLs and LjCIPKs, and the regulation mode of CBL-CIPK complex’s response to salt stress.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article and its Supplementary Materials, further inquiries can be directed to the corresponding author, Dr. Jia Li (ljyl7172@163.com).

AUTHOR CONTRIBUTIONS

Conceptualization and Analysis, LH; Software, ZL; Original Draft Preparation, CL and QF; Data Curation, GP and QL; Review & Editing, ZL; Funding Acquisition, JL; LH and ZL contributed equally and share first authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.751040/full#supplementary-material.

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