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Understanding the salt overly sensitive pathway in *Prunus*: Identification and characterization of *NHX*, *CIPK*, and *CBL* genes

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Abstract

Salinity is a major abiotic stress factor that can significantly impact crop growth, and productivity. In response to salt stress, the plant Salt Overly Sensitive (SOS) signaling pathway regulates the homeostasis of intracellular sodium ion concentration. The *SOS1*, *SOS2*, and *SOS3* genes play critical roles in the SOS pathway, which belongs to the members of Na^+/H^+ exchanger (*NHX*), CBL-interacting protein kinase (*CIPK*), and calcineurin B-like (*CBL*) gene families, respectively. In this study, we performed genome-wide identifications and phylogenetic analyses of *NHX*, *CIPK*, and *CBL* genes in six Rosaceae species: *Prunus persica*, *Prunus dulcis*, *Prunus mume*, *Prunus armeniaca*, *Pyrus ussuriensis* × *Pyrus communis*, and *Rosa chinensis*. *NHX*, *CIPK*, and *CBL* genes of *Arabidopsis thaliana* were used as controls for phylogenetic analyses. Our analysis revealed the lineage-specific and adaptive evolutions of Rosaceae genes. Our observations indicated the existence of two primary classes of CIPK genes: those that are intron-rich and those that are intron-less. Intron-rich CIPKs in Rosaceae and Arabidopsis can be traced back to algae CIPKs and CIPKs found in early plants, suggesting that intron-less CIPKs evolved from their intron-rich counterparts. This study identified one gene for each member of the SOS signaling pathway in *P. persica*: *PpSOS1*, *PpSOS2*, and *PpSOS3*. Gene expression analyses indicated that all three genes of *P. persica* were expressed in roots and leaves. Yeast two-hybrid-based protein–protein interaction analyses revealed a direct interaction between PpSOS3 and PpSOS2; and between PpSOS2 and PpSOS1C-terminus region. Our findings indicate that the SOS signaling pathway is highly conserved in *P. persica*.

Abbreviations: BLASTP, Protein Basic Local Alignment Serach Tool; CBL, calcineurin B-like; CIPK, CBL-interacting protein kinase; EC, electrical conductivity; NHX, Na^+/H^+ exchanger; QDO, quadruple dropout; SOS, Salt Overly Sensitive.

1 | INTRODUCTION

Salinity is a major abiotic stress and a severe threat to food security in different parts of the world, mainly in arid and semiarid regions. The presence of high concentrations of NaCl in the soils leads to a reduction of water potential and reduced availability of water, which, in turn, leads to osmotic stress in plants (Acosta-Motos et al., 2017). During salinity stress, higher accumulations of some ions like Na^+ and Cl^- cause toxicity in plants by negatively impacting cellular homeostasis. Ions like Na^+ and Cl^- from soil can pass into plants via peripheral root cells (Isayenkov & Maathuis, 2019). An increase in ion concentration in plant cells leads to osmotic stress, ionic stress, and reactive oxygen species production. It is known that an increased level of Na^+ inhibits photosynthesis, hampers the activity of various enzymes, damages cellular membrane structures, and impairs metabolic networks leading to inhibition of plant growth, development, and productivity (Acharya et al., 2021; Tester & Davenport, 2003). It is well known that salinity tolerance ability varies among plant genotypes (Sandhu et al., 2017, 2020) and that some plants, such as spinach, have the ability to maintain nitrogen (N) and potassium (K) homeostasis despite of a high tissue accumulation of chloride (Cl) and sodium (Na), even under K deficiency (Ferreira et al., 2020; Uçgun et al., 2020). Salt-tolerant genotypes are better equipped with different types of molecular components that sense salt, transduce salt-induced signals, and activate transporters that help to maintain ion homeostasis (Acharya, Sandhu, Dueñas et al., 2022; Kaundal et al., 2021).

To mitigate the effects of salinity stress, plants have evolved various mechanisms that regulate intracellular Na levels, such as actively exporting Na^+ from the inside of cells to the exterior and compartmentalizing sodium within the cell. These two processes require the transport of Na^+ across membranes and are catalyzed by ion transporters, including Na^+/H^+ antiporters (sodium–proton exchangers: NHX proteins) (Assaha et al., 2017; Munns & Tester, 2008; Zhu, 2003). The NHX proteins are primarily localized in vacuoles, endosomes, and plasma membranes. Arabidopsis encodes eight NHX proteins (NHX1–NHX8). AtNHX1, AtNHX2, AtNHX3, and AtNHX4 are localized in vacuoles (Vac class); AtNHX5 and AtNHX6 are localized in endosomes (Endo class); and AtNHX7 (SOS1) and AtNHX8 are localized in plasma membranes (PM class) (Almeida et al., 2017). In addition to ion homeostasis, the *NHX* genes are also involved in pH homeostasis and membrane vesicular trafficking (Almeida et al., 2017).

Calcium signals are sensed and transduced by various Ca^{2+} sensors, including calcineurin B-like proteins (CBLs) (Kudla et al., 2010). Arabidopsis possesses 10 *CBL* genes (Kolukisaoglu et al., 2004). CBL-interacting protein kinases (CIPKs) are known to interact with CBLs to transduce Ca^{2+} signals in response to various environmental stresses or other stimuli. In Arabidopsis, 26 *CIPK* genes have been identified (Kolukisaoglu et al., 2004). CIPK proteins are known to play signaling roles for the transport of Na^+, K^+, and NO_3^-. Previous phylogenetic analyses revealed that most CIPKs are primarily divided into two groups: intron-less and intron-rich clades (Ye et al., 2013). CBLs and CIPKs of green algae, early diverging land plants, and advanced land plants have been identified and characterized (Kleist et al., 2014; Weinl & Kudla, 2009). The first example of a CBL-CIPK signaling was discovered in the Salt Overly Sensitive (SOS) pathway of Arabidopsis, which plays a crucial role in the plant's ability to tolerate salinity stress (Zhu, 2003). The SOS signaling pathway in *Arabidopsis thaliana* consists of three core signaling components: SOS3 (also known as CBL4), SOS2 (also known as CIPK24), and SOS1 (also known as NHX7) (Zhu, 2003).

In response to the perception of salinity stress, intracellular Ca^{2+} level increases that is mediated by an extracellular salt sensor, mono-cation induced $[Ca^{2+}]_I$ increases 1 (MOCA1) (Jiang et al., 2019). Increase of intracellular Ca^{2+} in response to salinity stress activates SOS signaling pathway. In brief, SOS3 (CBL4) binds to Ca^{2+} and activates SOS2 (CIPK24) protein kinase upon interaction. Subsequently, SOS2 activates SOS1 (NHX7) by phosphorylation that, in turn, extrudes Na^+ from inside of the cell (Quintero et al., 2011).

Rosaceae is an important family that has ~100 genera and 3000 species (Hummer & Janick, 2009; Soundararajan et al., 2019). The genus *Prunus* comprises more than 400 species, which include economically important crops and ornamental plants like almond (*Prunus dulcis*), peach (*Prunus persica*), plum (e.g., *Prunus mume*), and apricot (*Prunus armeniaca*) (Potter et al., 2007; Shi et al., 2013; Shulaev et al., 2008; Soundararajan et al., 2019). Multiple reports indicate that Rosaceae species show varied tolerance in response to salinity (Acharya, Sandhu, Dueñas et al., 2022; El-Motaium et al., 1994; Sandhu et al., 2020; Shao et al., 2020; Tian et al., 2018;

Core Ideas

- The study performed phylogenetic analyses of *NHX*, *CIPK*, and *CBL* genes in six Rosaceae species.
- Analysis showed an orthologous relationship between the Rosaceae and Arabidopsis genes.
- Candidate *PpSOS1*, *PpSOS2*, and *PpSOS3* genes were identified in *P. persica*.
- Protein–protein interaction revealed a direct interaction between PpSOS3 and PpSOS2; and between PpSOS2 and PpSOS1.
- The SOS signaling pathway is highly conserved in *P. persica*.

Toro et al., 2021; Zrig et al., 2011). Despite the economic importance of the Rosaceae family, there has been a lack of detailed study on the identification, phylogenetic analysis, and characterization of genes associated with the SOS signaling pathway (NHXs, CIPKs, and CBLs) within this plant family.

For this study, six species of the Rosaceae were chosen for evolutionary analyses of NHX, CIPK, and CBL family members in comparison to *Arabidopsis thaliana*: *Prunus persica*, *Prunus dulcis*, *Prunus mume*, *Prunus armeniaca*, *Pyrus ussuriensis* × *Pyrus communis*, and *Rosa chinensis*. Expression status of members of the SOS family genes, *PpSOS1*, *PpSOS2*, and *PpSOS3* of *Prunus persica* were examined under control and in response to salinity stress. Additionally, the SOS pathway genes of *Prunus* were cloned, and the interaction status between PpSOS3 and PpSOS2; and PpSOS2 and PpSOS1 were examined using the yeast two-hybrid assay.

2 | MATERIALS AND METHODS

2.1 | Genome databases and plant species

Genome-wide protein sequences of *Prunus persica* (v2.1) were downloaded from Phytozome (v13). Sequences of other five Rosaceae species were downloaded from NCBI, including *Prunus mume* (v1.0, accession #PRJNA246160), *Prunus armeniaca* (accession # PRJEB37669), *Rosa chinensis* (v1.0, accession #PRJNA438537), *Pyrus ussuriensis* × *Pyrus communis* (assembly ASM893209v1.0, accession #PRJNA494996), and *Prunus dulcis* (v2.0, accession #PRJNA631757).

2.2 | Prediction of *NHX*, *CIPK*, and *CBL* genes

A previously established protocol was employed to predict *NHX* genes from different species of the Rosaceae species (Wu et al., 2019). All eight *A. thaliana* NHX proteins, AtNHX1 to AtNHX8 (Table S1), were obtained from the *A. thaliana* genome database (TAIR10) at Phytozome (Goodstein et al., 2012) and used as queries to search in the target protein databases using the Protein Basic Local Alignment Search Tool (BLASTP). NHX proteins that showed *E*-values < $1e^{-10}$ were predicted as NHXs (Wu et al., 2019) (Table S1).

The presence of the protein kinase domain (IPR000719) and the NAF domain (IPR004041) are the typical characteristics of CIPK proteins (Ye et al., 2013). For the prediction of CIPK proteins, the genome-wide protein sequences were searched for the presence of the NAF motif (PF03822) using HMMSCAN (Finn et al., 2011). Then, proteins containing the NAF domain were retrieved and used for INTERPROSCAN to examine the presence of the protein kinase domain (Finn et al., 2016). Only proteins with both protein kinase- and NAF domains were considered CIPK proteins (Table S2).

In order to identify CBL proteins containing four EF-hand motifs, we used a method previously described with slight modification (Mohanta et al., 2015). First, sequences of 10 CBL proteins of *A. thaliana* were used as a query, and Protein Basic Local Alignment Serach Tool (BLASTP) (E-value < $1e^{-5}$) was employed to search the plant protein databases (Mo et al., 2018). Then, the ScanProsite search contained within the INTERPROSCAN package (v74.0) was used to examine the presence of three canonical EF-hand motifs (PS50222), EF2, EF3, and EF4, within the identified proteins. Subsequently, identified sequences were retrieved to search (BLASTP) in the *Arabidopsis thaliana* database, and only top hits were considered as CBL proteins (Mo et al., 2018) (Table S3). Consequently, we performed multiple sequence alignments using protein sequence of CBLs along with 10 CBLs of Arabidopsis (as control) to verify the presence of all four EF-hand motifs: one unusual EF-hand motif and three canonical EF-hand motifs.

The predicted NHX, CIPK, and CBL proteins that were longer than 100 amino acids were used for the respective phylogenetic analyses. It should be noted that when multiple isoforms were identified for a single gene locus, the longest isoform was selected as the representative for the specific gene for the phylogenetic analysis. Additionally, for the phylogenetic analysis of CIPKs, protein sequences of *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Klebsormidium nitens*, *Physcomitrium patens*, and *Selaginella moellendorffii* were included.

2.3 | Phylogenetic analysis

The MAFFT (v7.427) was used for protein sequence alignment (v7.427) (Katoh & Standley, 2013), and TRIMAL (v1.4.1) was used to trim the regions that were not well aligned based on a gap threshold of 0.25 (Capella-Gutierrez et al., 2009). PROTTEST (v3.4.2) was used to determine the best-fit models of protein evolution, which were JTT+G for CIPK and CBL proteins; and WAG+G+F for NHX proteins according to Bayesian information standards (Darriba et al., 2011). To perform phylogenetic analyses, two runs with four chains per run in MRBAYES (v3.2.6) were executed till the standard deviation of split frequencies between runs dropped below 0.05. The first 25% of generations were discarded, and the remaining generations were used to build a 50% majority-rule consensus tree.

2.4 | Plant material and salt treatment

Peach variety "Nemaguard" was acquired from Burchell nursery and grown in 6-L pots containing a mixture of sand

and sandy loam soil in a 1:1 ratio. Each pot contained only one plant. For gene expression analyses, three plants were used per replication. Riverside city water supplemented with essential nutrients was used for watering (electrical conductivity = 1.36 dS m^{-1}) (Table S4). Nutrient-enriched irrigation water with added Na^+ and Cl^- was used for the saline treatment (electrical conductivity = 3.0 dS m^{-1}) (Table S4).

One-year-old plants were used for gene expression analyses. For the tissue-specific gene expression study and to study gene expression in response to salinity, three plants were used per replication, and three biological replicates were used for each study. Root and leaf samples were harvested 48 h after salt treatment. Samples from three plants were pooled and snap-frozen using liquid nitrogen and stored in a −80°C freezer until RNA extraction.

2.5 | RNA extraction and gene expression analyses

Total RNA was isolated from leaf and root tissues using TRIzol reagent (Invitrogen). To eliminate DNA contamination, RNA samples were treated with DNase I (Thermo Scientific). Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) assay was performed using iTaq Universal SYBR Green one-step-kit in a BioRad CFXS system machine (Bio-Rad Laboratories). qRT-PCR was performed in a total volume of 10 µL which included 100 ng of total RNA, 0.125 µL iScript Reverse transcriptase, 5 µL of 2x one-step SYBR Green reaction mix, and 0.75 µM of forward and reverse primers (Table S5). The qRT-PCR used was: 50°C for 10 min (cDNA synthesis), 95°C (initial denaturation); subsequently, 40 cycles of 95°C for 10 s (denaturation), 57°C for 30 s (annealing) and 68°C for 30 s (extension). *PpUbiquitin10* and *PpTEF2* genes were used as reference genes for normalization (Tong et al., 2009). To calculate the relative expression, the cycle threshold value of each gene to reference genes was used.

2.6 | AtSOS1 and PpSOS1 sequence alignment

First, AtSOS1 and PpSOS1 protein sequences were converted into FASTA format. Then multiple sequence alignments were performed using T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular) to obtain the result in fasta-aln format (Di Tommaso et al., 2011). Subsequently, the Boxshade was used to generate the final figure (http://www.ch.embnet.org/software/BOX_form.html).

2.7 | Gene cloning

The *Prupe.1G339200.1* was predicted as the *PpSOS1* gene of *P. persica* according to our phylogenetic and sequence analyses (Figure 1). Similarly, *Prupe.7G244500.1* (Figure 2) and *Prupe.2G310300.1* (Figure 3) were predicted as the candidate for *PpSOS2* and *PpSOS3* genes, respectively. The primers were designed based on predicted gene models (Phytozome portal v12.1) for cloning (Table S5).

The full-length CDS of *PpSOS1*, *PpSOS1* cDNA c-terminus (2977-3507 that corresponds from 993 to 1168 of the PpSOS1 protein), the full-length CDS of *PpSOS2*, and the full-length CDS of *PpSOS3* were cloned from leaf or root cDNA of *P. persica* in pCR8/GW/TOPO vector (ThermoFisher Scientific) according to manufacturer's instruction.

2.8 | Yeast two-hybrid assay (Y2H assay)

PpSOS2 was cloned in *Eco*RI/ *Pst*I restriction sites of the pGBKT7 vector, whereas *PpSOS3* or *PpSOS1Cter* were cloned in the *Eco*RI/ *BamH*I of the pGADT7-AD vector. Specific pairs of constructs (e.g., pGBKT7-*PpSOS2* + pGADT7-*PpSOS3*) were co-transformed into the *Saccharomyces cerevisiae* Y2HGold strain according to the manufacturer's protocol (Clontech Laboratories). Co-transformed yeasts were selected on SC-Trp-Leu plates, the double dropout medium. Subsequently, to check the interaction status of two pairs of proteins (PpSOS2 + PpSOS3; or PpSOS2 + PpSOS1 Cter), selected co-transformed yeasts were plated on SD-Leu, -Trp, -His, -Ade, medium, the quadruple dropout (QDO) medium, supplemented with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and Aureobasidin A (AbA) (QDO/X/A). To check the autoactivation status, each bait and prey construct was co-transformed with the corresponding empty vector used for the Y2H assay. Interaction between T-antigen and P53 proteins was used as a positive control, whereas T-antigen and Lam protein pair was used as a negative control for the Y2H assay. Colonies that showed blue color were considered as positive interaction (Zhao et al., 2021).

2.9 | Subcellular localization prediction

The Plant-mPLoc server (Chou & Shen, 2010) was used to investigate the subcellular localization of proteins.

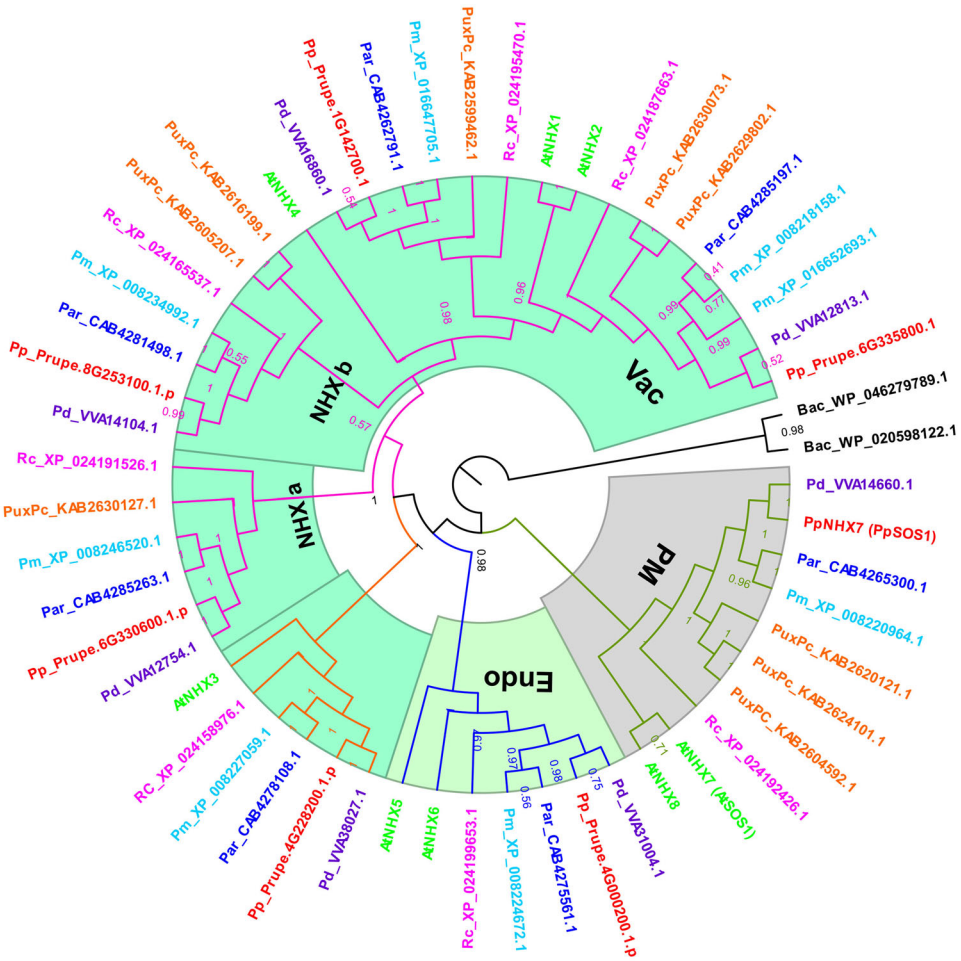

FIGURE 1 Phylogeny of the Na^+/H^+ exchanger (NHX) proteins identified in Rosaceae species and *Arabidopsis thaliana*. Clade posterior probabilities are indicated at nodes. Taxa are color coded for each species. Branches of clades of different groups are color coded. Different groups are named PM (plasma membrane), Endo (endosomal), and Vac (vacuolar). The bacterial proteins (outgroup) are in black. The taxa for plant species are indicated as: At, *Arabidopsis thaliana*; Bac, *bacteria*; Par, *Prunus armeniaca*; Pd, *Prunus dulcis*; Pm, *Prunus mume*; Pp, *Prunus persica*; PuxPc, *Pyrus ussuriensis* × *Pyrus communis*; Rc, *Rosa chinensis*. Rosaceae-specific NHX clades in the Vac category are indicated as "NHX a" and "NHX b."

3 | RESULTS

3.1 | Prediction of *NHX*, *CIPK*, and *CBL* genes of Rosaceae species

In silico approaches were employed to identify the *NHX*, *CIPK*, and *CBL* genes from the genomes of the six Rosaceae species: *Prunus persica* (Pp), *Prunus dulcis* (Pd), *Prunus armeniaca* (Par), *Prunus mume* (Pm), *Pyrus ussuriensis* × *Pyrus communis* (PuxPc), and *Rosa chinensis* (Rc) (see materials and methods). In this study, eight *NHX* genes, 26 *CIPK* genes, and 10 *CBL* genes of *A. thaliana* were used as queries for the prediction of *NHX*, *CIPK*, and *CBL* genes of the six above-described Rosaceae species (Tables S1–S3). Our analysis identified 7–9 members of the *NHX* gene family, 16–27 members of the *CIPK* gene family, and 8–10 members of the *CBL* gene family among six Rosaceae species (Table 1, Tables S1–S3).

3.2 | Evolution of *NHX* genes of Rosaceae species

NHX proteins of Rosaceae were grouped into three classes, the plasma membrane (PM) NHX, the vacuolar (Vac) NHX, and the endosomal (Endo) NHX as in Arabidopsis, and phylogenetic analysis showed that the predicted NHX proteins of Rosaceae species clustered together with AtNHXs with high posterior probabilities (Figure 1).

Our data indicate that in the Vac category, *P. persica*, *P. dulcis*, *P. armeniaca*, and *R. chinensis* have five NHXs each, *Pyrus ussuriensis* × *Pyrus communis* and *P. mume* have six NHX members each, and Arabidopsis has four NHX

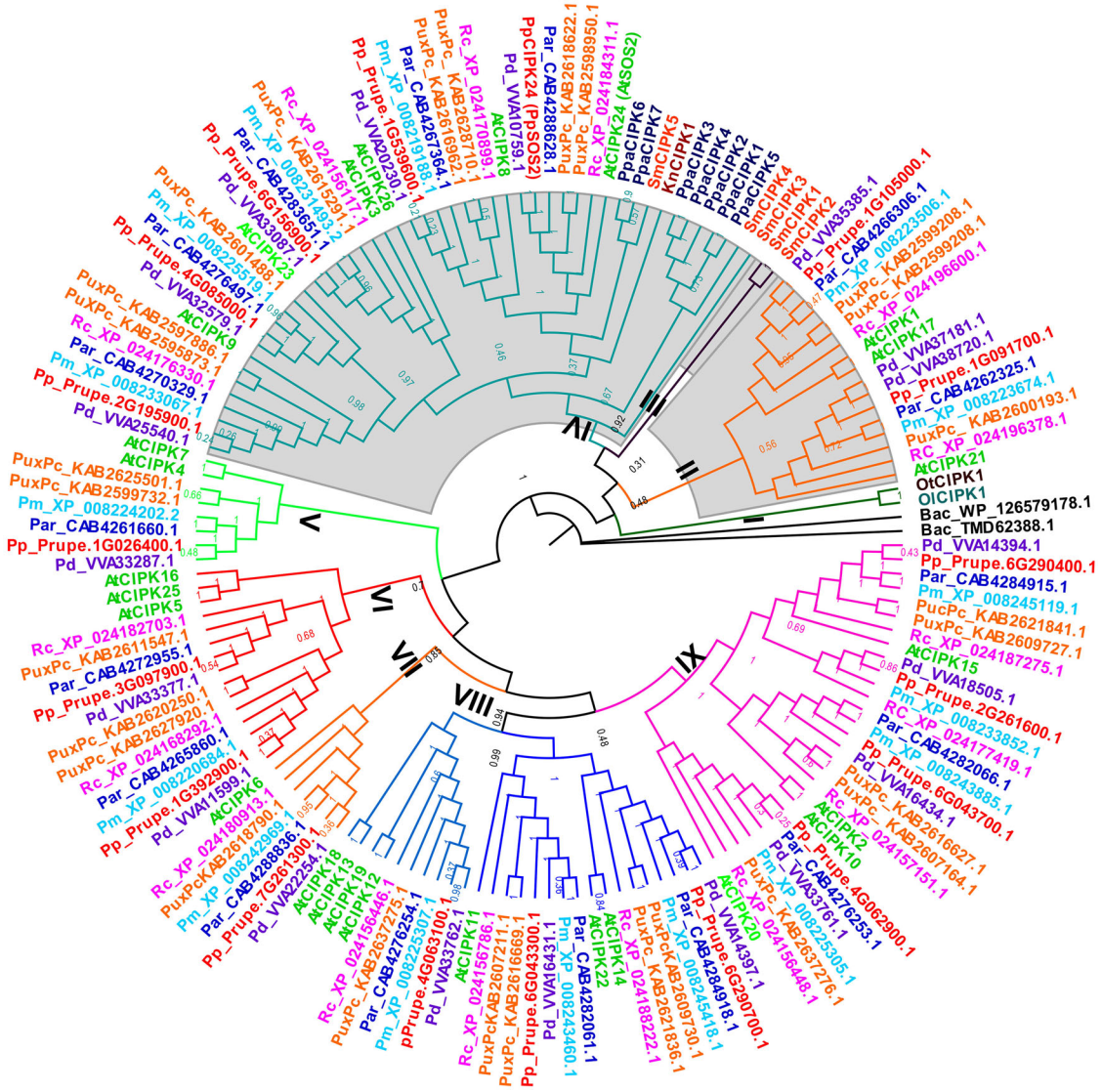

FIGURE 2 Phylogeny of the CBL-interacting protein kinase (CIPK) proteins identified in Rosaceae species and *Arabidopsis thaliana*. Clade posterior probabilities are indicated at nodes. Taxa are color coded for each species. Branches of clades of different groups are color coded. Different groups are numbered from I to IX. The bacterial proteins (outgroup) are in black. The taxa for plant species are indicated as: At, *Arabidopsis thaliana*; Bac, *bacteria*; Kn, *Klebsormidium nitens*; Ol, *Ostreococcus lucimarinus*; Ot, *Ostreococcus tauri*; Par, *Prunus armeniaca*; Pd, *Prunus dulcis*; Pm, *Prunus mume*; Pp, *Prunus persica*; Ppa, *Physcomitrium patens*; PuxPc, *Pyrus ussuriensis* × *Pyrus communis*; Rc, *Rosa chinensis*; Sm, *Selaginella moellendorffii*.

TABLE 1 The number of predicted Na^+/H^+ exchanger (*NHX*), CBL-interacting protein kinase (*CIPK*), and calcineurin B-like (*CBL*) genes of the six indicated genomes.

Species	*NHX* genes	*CIPK* genes	*CBL* genes
Prunus persica	7	18	8
Prunus dulcis	7	19	8
Prunus armeniaca	7	17	8
Prunus mume	8	16	10
Pyrus ussuriensis × *Pyrus communis*	9	27	9
Rosa chinensis	7	17	9

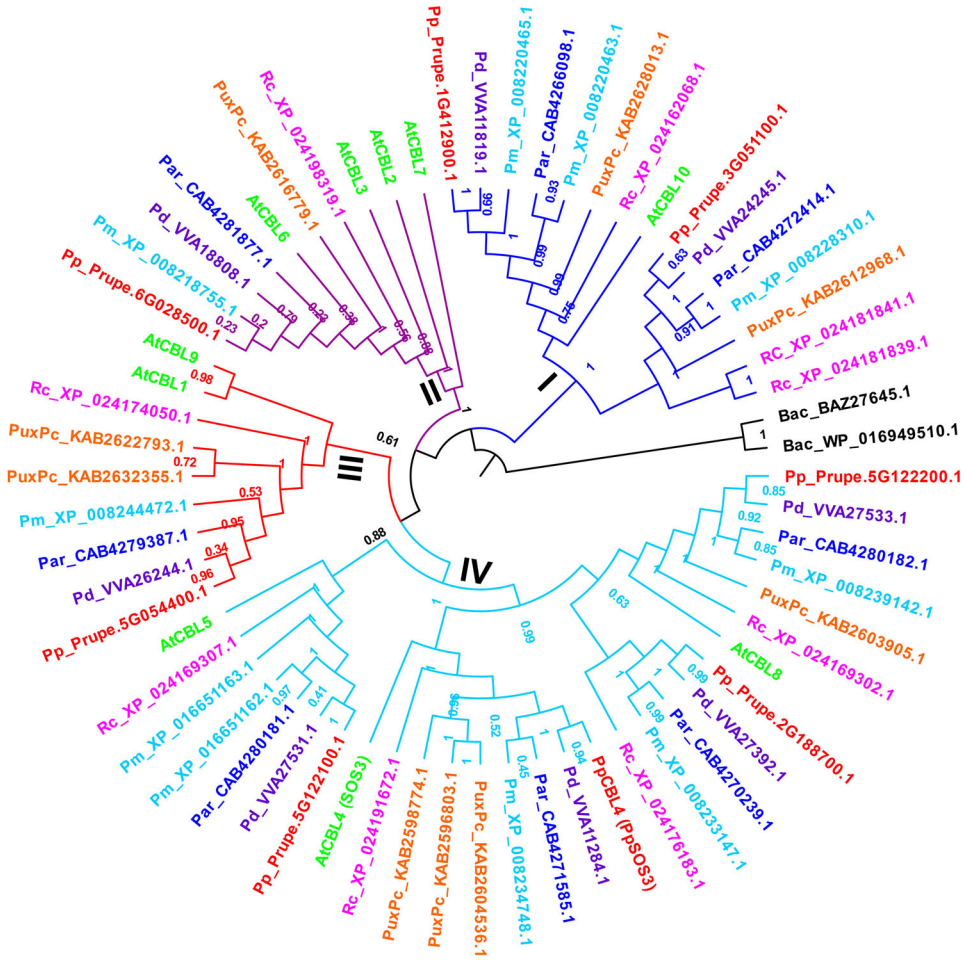

FIGURE 3 Phylogeny of the calcineurin B-like (CBL) proteins identified in Rosaceae species and *Arabidopsis thaliana*. Clade posterior probabilities are indicated at nodes. Taxa are color coded for each species. Branches of clades of different groups of CBLs are color coded. Different groups are numbered from I to IV. The bacterial proteins (outgroup) are in black. The taxa for plant species are indicated as: At, *Arabidopsis thaliana*; Bac, *bacteria*; Par, *Prunus armeniaca*; Pd, *Prunus dulcis*; Pm, *Prunus mume*; Pp, *Prunus persica*; PuxPc, *Pyrus ussuriensis* × *Pyrus communis*; Rc, *Rosa chinensis*.

members (Figure 1). In addition, Rosaceae species formed two exceptional clades within the Vac category, "Rosaceae NHX a" and "Rosaceae NHX b". In the clade "Rosaceae NHX a", six NHXs, one specific NHX from each corresponding Rosaceae species, were found to be clustered with a high posterior probability of 1. In the "Rosaceae NHX b" clade, one NHX from all examined Rosaceae species except for *PuxPc* with two NHXs (Figure 1). Rosaceae NHX proteins, which were grouped into two unique clades in the Vac category, were found to be specifically localized in the vacuole, as confirmed by the subcellular localization prediction results. It should be noted that these Rosaceae NHXs were found to be separated from Arabidopsis NHXs (Figure 1). Additionally, the Rosaceae NHX clades showed the closest phylogenetic connection with other Vac-type NHXs of Rosaceae and Arabidopsis NHXs.

Our phylogenetic analysis also indicated probable gene duplications/deletions in Rosaceae species in comparison to Arabidopsis. For example, in the PM category, *Pyrus ussuriensis* × *Pyrus communis* had three NHXs, PuxPc_KAB2620121.1, PuxPc_KAB2624101.1, and PuxPc_KAB2604592.1, whereas other Rosaceae species had only one NHX (e.g., Par_CAB4265300.1) (Figure 1). Among these three, PuxPc_KAB2624101.1_and PuxPc_KAB2604592.1 are paralogs and probably evolved due to gene duplication. *A. thaliana*, however, had two members, AtNHX7 and AtNHX8. Our analysis also revealed that *P. persica* had only one copy of NHX in the PM category, PpNHX7, which is the *SOS1* candidate of *P. persica* (*PpSOS1*) (Figure 1). PpSOS1 (PpNHX7) was evolutionarily closer to Pd_VVA14660.1 of *P. dulcis* whereas Par_CAB4265300.1 of *P. armeniaca* was closer to Pm_XP_008220964.1of *P. mume* (Figure 1) but distantly related to Rc_XP_024192426.1of *R. chinensis*.

In the Endo category, no NHX members were observed for *Pyrus ussuriensis* × *Pyrus communis* but the other

five species had one representative each, including *P. persica* (Pp_Prupe.4G000200.1) and *Prunus dulcis* (Pd_VVA31004.1). Arabidopsis, on the other hand, had two NHXs in Endo category: AtNHX5 and AtNHX6 (Figure 1). Additionally, Pp_Prupe.4G000200.1 of *P. persica* was closer to Pd_VVA31004.1of *P. dulcis* than Par_CAB4275561.1of of *P. armeniaca* or Pm_XP_008224672.1 of *Prunus mume*.

3.3 | Evolution of *CIPK* genes of Rosaceae species

To understand the evolutionary relationship of CIPKs of Arabidopsis and *Rosaceae* with CIPKs of algae and primitive plants, we included CIPK sequences of marine green algae, CIPK of *Ostreococcus lucimarinus* (OlCIPK1) and CIPK of *Ostreococcus tauri* (OtCIPK1); CIPK sequence of fresh water green algae, *Klebsormidium nitens* (KnCIPK1); CIPKs of ancient plants, *Physcomitrium patens* CIPKs (PpCIPK 1–PpCIPK7), and CIPKs of *Selaginella moellendorffii* (SmCIPK1–SmCIPK5) in our phylogenetic analysis.

Our analysis predicted 16–27 members of genes in the *CIPK* family among the six Rosaceae species, which was more than the number of genes identified for *NHX* or *CBL* family members (Table 1). A total of 18 and 19 *CIPK* genes were identified in *P. persica* and *P. dulcis*, respectively (Table 1, Table S2).

Our phylogenetic analysis showed two major classes of CIPKs, intron-rich CIPKs (9–14 introns) (shaded in gray) and intron-less CIPKs (0–1 intron) (Figure 2). Intron-rich CIPKs were divided into three groups, II–IV; and intron-less CIPKs were clustered into five groups, V–IX. Intron-rich CIPKs of Rosaceae grouped with intron-rich CIPKs of Arabidopsis, whereas all other Rosaceae CIPKs groups (V and IX) grouped with intron-less CIPKs of Arabidopsis. These findings indicated that the only CIPKs of Rosaceae of groups II–IV were intron-rich whereas CIPKs of other five groups were intron-less. To validate these findings, we analyzed the genomic sequence of the coding region for each CIPK in the Rosaceae family to determine the number of introns present. Sixty-four CIPKs of Rosaceae that were grouped with Arabidopsis intron-less CIPK clade, had no introns in the coding region, whereas the presence of one intron was observed in Rc_XP_024177419.1 and two introns in PuxPc_KAB2611547.1 (Table S6). Further, the number of introns ranged from 9 to 14 in Rosaceae *CIPKs* (Table S6) that were grouped with Arabidopsis intron-rich *CIPK* clade (Figure 2).

Ostreococcus tauri CIPK (OtCIPK1), *Ostreococcus lucimarinus* CIPK, OlCIPK1, all CIPKs of Physcomitrella and Selaginella clustered with intron-rich CIPKs of Arabidopsis and Rosaceae species (Figure 2). OtCIPK1 and OlCIPK1 clustered together as expected in group I. Selaginella CIPKs, SmCIPK1 and SmCIPK2, were closely related and clustered in group III, in comparison to SmCIPK3, SmCIPK4, and SmCIPK5 which were clustered in two sister clades of group IV. The CIPKs from PpaCIPK1 to PpaCIPK5 were clustered together with SmCIPK4 in one sister clade of group IV CIPKs, while PpaCIPK6 and PpaCIPK7 were clustered with SmCIPK5, as well as *Klebsormidium nitens* CIPK, KnCIPK1 in another sister clade of group IV. KnCIPK1 was found to be evolutionary closer to AtCIPK24 (AtSOS2), and AtCIPK8 (Figure 2). Additionally, all algal CIPKs, all CIPKs of Physcomitrella and Selaginella were evolutionarily linked with AtCIPK24 (AtSOS2) and AtCIPK8, indicating that intron-rich CIPKs of Arabidopsis and Rosaceae evolved from algal CIPKs and CIPKs of primitive plants. Additionally, group II CIPKs including AtCIPK1, AtCIPK17, and AtCIPK21 evolved from marine algae CIPKs, OtCIPK1, and OlCIPK1 (Figure 2). As anticipated, the evolutionary analysis revealed that AtCIPK24 and AtCIPK8 exhibit a closer evolutionary relationship to KnCIPK1, which aligns with expectations considering the close phylogenetic proximity between charophyte algae and land plants, their closest relatives.

AtCIPK24 (AtSOS2) was observed in a sister clade of group IV and, in that clade, a single specific CIPK was observed in each of the four Rosaceae species, Par_CAB4288628.1 (*P. armeniaca*), PpCIPK24 (*P. persica*), Pd_VVA10759.1 (*P. dulcis*), and Rc_XP_024184311.1 (*R. chinensis*); two CIPKs were observed for *Pyrus ussuriensis* × *Pyrus communis*, PuxPc_KAB2618622.1, and PuxPc_KAB2598950.1. In contrast, no CIPK was observed for *P. mume* in the clade where AtSOS2 belongs.

3.4 | Evolution of *CBL* genes of Rosaceae species

Our prediction analysis identified 8, 8, 8, 10, 9, and 9 *CBL* genes in *P. persica*, *P. dulcis, P. armeniaca*, *P. mume*, *Pyrus ussuriensis* × *Pyrus communis*, and *R. chinensis*, respectively (Table 1, Table S3). Phylogenetic analysis showed that Rosaceae CBLs formed clades with AtCBLs with strong posterior probability support (Figure 3). CBL proteins of the Rosaceae family were clustered into four groups, I, II, III, and IV (Figure 3). We also observed a strong clustering of all four groups of CBLs with a high posterior probability (Figure 3).

In group I, Rosaceae CBLs were clustered into two clades (Figure 3). Although the first clade of group I did not contain any Arabidopsis CBL; while it contained one unique CBL for each corresponding Rosaceae species, with the exception of *R. chinensis*, which had two CBLs, Rc_XP_024181841.1 and Rc_XP_024181839.1 (Figure 3). The second clade of group I contained AtCBL10 and one specific CBL for corresponding Rosaceae species except for *P. mume* with two CBLs (Pm_XP_008220463.1and Pm_XP_008220465.1). In

the group II CBLs, all four different CBLs of Arabidopsis (AtCBL2, AtCBL3, AtCBL6, and AtCBL7) were present in the same clade, while only one specific CBL was observed for each corresponding Rosaceae species (e.g., Pp_XP_024181839.1) (Figure 3). In group III, Rosaceae CBLs were clustered together in a single clade, with each corresponding Rosaceae species having a unique CBL, except for *Pyrus ussuriensis* × *Pyrus communis* which had two CBLs (PuxPc_KAB2632355.1 and PuxPc_KAB2622793.1). This is similar to the situation in Arabidopsis where two CBLs (AtCBL1 and AtCBL9) were present in the same clade (Figure 3).

In group IV, Rosaceae CBLs were clustered in three clades (Figure 3). In this group, the clade containing AtSOS3 (AtCBL4), a key component of the SOS signaling pathway, was found to have one specific CBL protein for corresponding Rosaceae species except for *Pyrus ussuriensis* × *Pyrus communis* with 3 CBLs (PuxPc_KAB2598774.1, PuxPc_KAB2596803.1, and PuxPc_KAB2604536.1). The clade containing Arabidopsis CBL8 (AtCBL8) had two sister clades. The AtCBL8-containing sister clade had one specific CBLs from each Rosaceae species but the adjacent sister clade of AtCBL8-containing clade, wass specific for Rosacea specific CBLs (Figure 3). Furthermore, in the clade that included Arabidopsis CBL5 (AtCBL5), one specific CBL for each corresponding Rosaceae species was clustered, except for *P. mume* which had two CBLs, Pm_XP_016651162.1 and Pm_XP_016651163.1. However, no member was found for *Pyrus ussuriensis* × *Pyrus communis* in this clade (Figure 3).

3.5 | Identification of SOS genes in *P. persica*

The phylogenetic relationship of *P. persica* candidate gene(s) with specific Arabidopsis *SOS* gene was a key criterion to identify the *SOS* genes of *P. persica*. Based on our data, we identified *PpNHX7* (*Prupe.1G339200.1*), *PpCIPK24* (*Prupe.7G244500*), and *PpCBL4* (*Prupe.2G310300.1*) as the only *PpSOS1*, *PpSOS2*, and *PpSOS3* candidate, respectively, all with high clustering probability support value (Figures 1–3, Tables S1–S3).

3.6 | Gene expression analyses of *P. persica SOS* genes in response to salinity

To examine the expression status of *PpSOS1*, *PpSOS2* ,and *PpSOS3* genes, qRT-PCR was performed using RNA from leaves and roots of 1-year-old plants that were irrigated with control irrigation water (electrical conductivity [EC_{iw}] = 1.36 dS m^{-1}) or with saline (EC_{iw} = 3.0 dS m^{-1}) irrigation water (treatment) for 48 h (Table S4). Our qRT-PCR results indicated no significant difference in gene expressions of *PpSOS1*, *PpSOS2*, and *PpSOS3* in response to saline treatment in comparison to control in leaf or root (Figure 4). Our findings indicate that, in response to salt stress, SOS signaling pathway components, PpSOS1, PpSOS2, and PpSOS3, may be regulated by protein–protein interactions and/or protein phosphorylation.

3.7 | PpSOS3 interacts with PpSOS2

Our phylogenetic analysis identified PpCBL4 (Figure 3) and PpCIPK14 (Figure 2) as the only PpSOS3 and PpSOS2 candidates, respectively. We performed the yeast two-hybrid analysis to test protein–protein interaction between PpSOS2 and PpSOS3. Our yeast two-hybrid data showed that PpSOS3 interacts with PpSOS2 (Figure 5C).

3.8 | PpSOS2 interacts with PpSOS1

Our phylogenetic analysis identified one candidate each for *P. persica* SOS1 (PpNHX7) and SOS2 (PpCIPK24) (Figures 1 and 2; Tables S1 and S2). We hypothesized that if our identified candidates, PpSOS1 and PpSOS2, are true orthologs of AtSOS1 and AtSOS2, respectively, they are expected to interact. To test this hypothesis, before performing protein–protein interaction between PpSOS2 and PpSOS1 C-terminal fragment (PpSOS1 Cter), the PpSOS1 C-terminal fragment ($PpSOS1_{993–1168}$) was identified by sequence alignment that corresponds to $AtSOS1_{998–1146}$ (Figure S1). Subsequently, PpSOS1 Cter was cloned into yeast two-hybrid vector pGADT7-AD for the yeast two-hybrid assay. The yeast two-hybrid assay-based protein–protein interaction analysis showed that PpSOS2 interacts with C-terminal fragment of PpSOS1 (PpSOS1 Cter) (Figure 6C).

4 | DISCUSSION

In this study, we employed a bioinformatics approach to predict the *NHX*, *CIPK*, and *CBL* genes from six genomes of Rosaceae: *P. persica*, *P. dulcis*, *P. armeniaca*, *P. mume*, *Pyrus ussuriensis* × *Pyrus communis*, and *R. chinensis* (Figure 7). Our findings showed that the prediction approaches employed in this study are suitable for the identification and characterization of candidate genes from good-quality annotated genomes. Our phylogenetic analyses revealed Arabidopsis orthologs in Rosaceae species for the *NHX*, *CIPK*, and *CBL* gene families, allowing us to predict both lineage-specific and adaptive evolutions in a specific set of genes belonging to different families. Furthermore, we identified one respective gene for *AtSOS1*, *AtSOS2*, and *AtSOS3* in *P. persica*: *PpSOS1*,

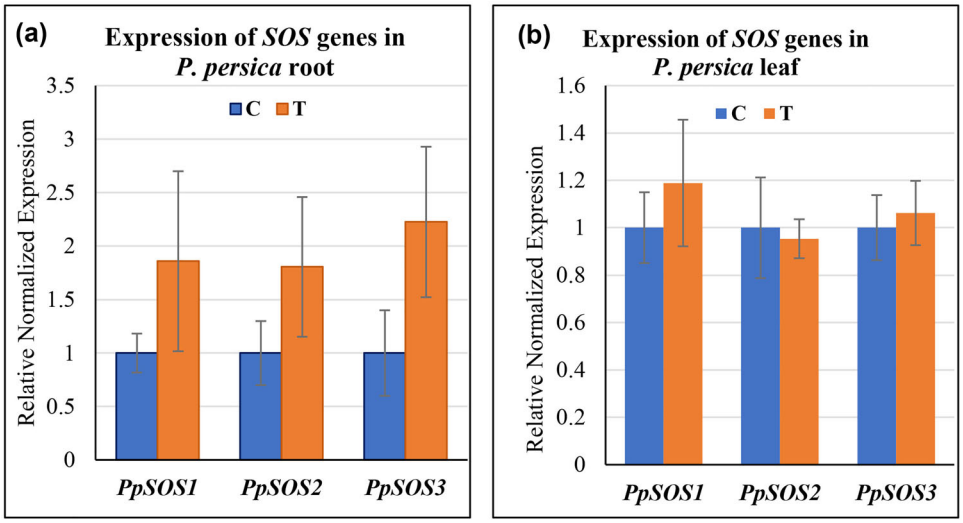

FIGURE 4 Expression analyses of the Salt Overly Sensitive (SOS) signaling genes in response to salinity by qRT-PCR. The *y*-axis shows relative normalized expression, and the *x*-axis indicates the SOS signaling genes, *PpSOS1*, *PpSOS2*, and *PpSOS3*. (Left panel) Expression of the *Prunus persica SOS* genes in roots in response to salinity stress. (Right panel) Expression of the *Prunus persica SOS* genes in leaves in response to salinity stress. C indicates control, and T indicates saline treatment. No significant differences were observed in gene expressions of *PpSOS1*, *PpSOS2*, and *PpSOS3* in response to saline treatment in comparison to control in leaf or root.

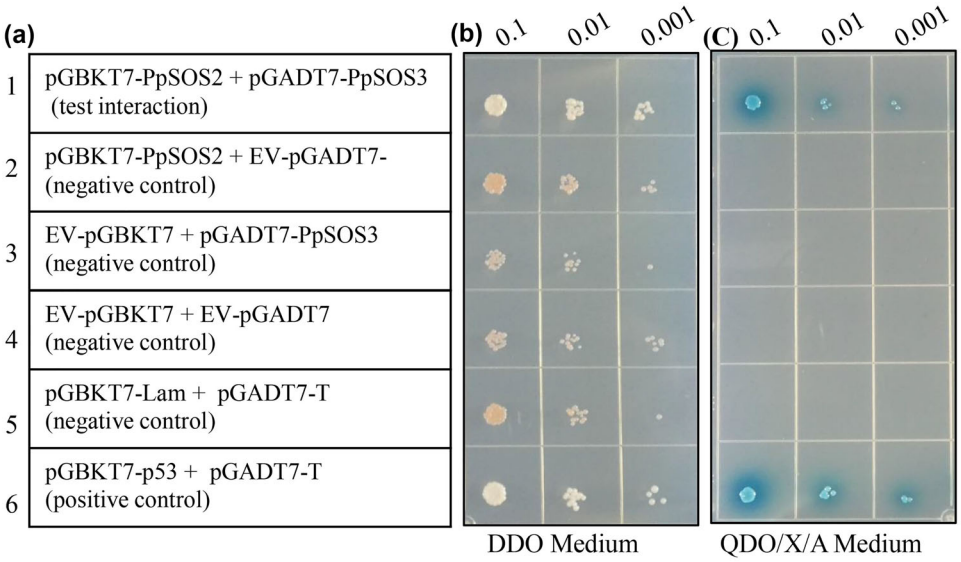

FIGURE 5 PpSOS3 interacts with PpSOS2 in yeast. Pairwise protein–protein interactions between the indicated proteins were performed using the yeast two-hybrid assay as shown in the panel (a). Transformed yeast clones containing both pGBKT7 and pGADT7 and constructs were tested in three 10-fold serial dilutions (OD 600 = 0.1, 0.01, and 0.001), as indicated in panel (a) and (c). Panel b shows co-transformed yeast growth in double dropout (DDO) media, SD/Leu/Trp. Panel c shows the interaction status of PpSOS3 with PpSOS2, which was determined by growth assay of yeast cells on interaction selective Quadruple Dropout media containing X-α-Gal and Aureobasidin, SD/-Leu/Trip/-His/X/A (QDO/X/A). PpSOS2 interacts with PpSOS3 (panel c1). Empty vectors (EV) were transformed to check the auto-activation status. The Lam and T-antigen protein pair was used as a negative control, whereas the P53 and T-antigen protein pair was used as a positive control.

PpSOS2, and *PpSOS3* (Figure 7). Our protein–protein interaction data revealed that *SOS* genes of *P. persica* are true orthologs of Arabidopsis genes.

Our computational analyses identified *NHX* genes in the range of 7–9, *CIPK* genes in the range of 17–27, and *CBL* in the range of 8–10 (Table 1). *CIPK* and *CBL* genes have been identified from algae to eudicots. For example, one *CIPK* and one *CBL* gene in Chlorophyta; seven *CIPKs* and five *CBLs* in Bryophyta; five *CIPKs* and four *CBLs* in Pteridophyta; 26 *CIPKS* and seven *CBLs* in gymnosperm; 34 CIPKs and 11

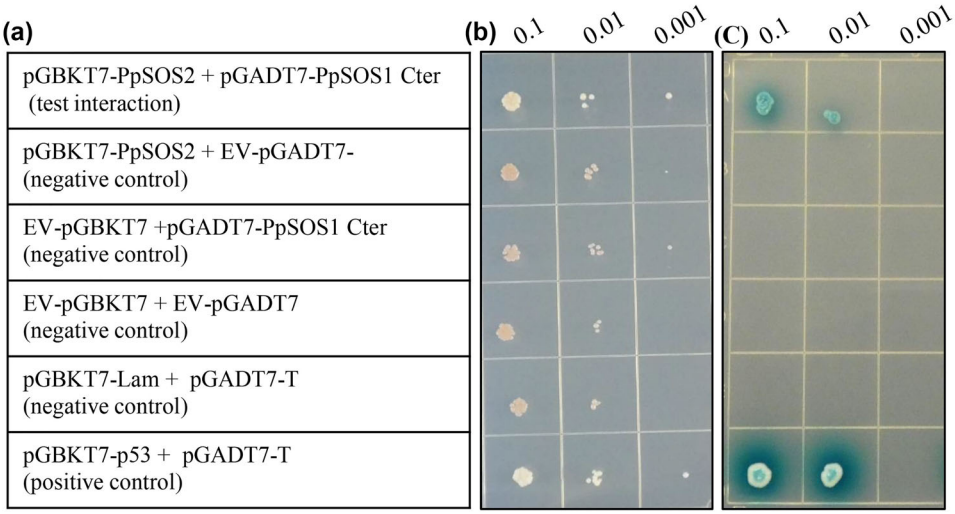

FIGURE 6 PpSOS2 interacts with PpSOS1 Cter in yeast. Pairwise protein–protein interactions between the indicated proteins were performed using the yeast two-hybrid assay as shown in the panel (a). Transformed yeast clones containing both pGBKT7 and pGADT7 and constructs were tested in three 10-fold serial dilutions (OD 600 = 0.1, 0.01, and 0.001), as indicated in panel (b) and (c). Panel b shows co-transformed yeast growth in double dropout (DDO) media, SD/Leu/Trp. Panel c shows the interaction of PpSOS2 with PpSOS1 Cter, which was determined by growth assay of yeast cells on interaction selective Quadruple Dropout media containing X-α-Gal and Aureobasidin, SD/-Leu/Trip/-His/X/A (QDO/X/A). PpSOS2 interacts with PpSOS1-Cter (panel c1). Empty vectors (EV) were transformed to check the auto-activation status. The Lam and T-antigen protein pair was used as a negative control, whereas the P53 and T-antigen protein pair was used as a positive control.

CBLs in monocotyledons; and 26 CIPKs and 10 CBLs in eudicotyledons (Edel et al., 2017). The above described facts indicate that as species evolved the ratio between *CIPK* and *CBL*::1:1 in Chlorophyta, 7:5 in Bryophyta, to 26:10 in eudicotyledons. It is generally believed that number of genes of a family may evolve via tandem duplications or segmental duplications and followed by losses that reshape the number of genes of a specific species (Cannon et al., 2004). In our study, among *Prunus* species, we observed eight *CBLs* (except for *P. mume* with 10 CBLs) and 16−19 *CIPKs* (Table 1). The *Vitis vinifera* genome, which did not undergo recent whole genome duplication, contains 20 *CIPK* genes and nine *CBL* genes similar to the number of CIPK and CBL genes we observed in *Prunus* (Jiao et al., 2012; Xi et al., 2017). The *P. persica* genome did not undergo recent whole genome duplication after the gamma whole genome triplication of the core eudicots as *Vitis vinifera,* which explains why different species of *Prunus* have similar number of *CIPKs* and *CBLs* as observed in *Vitis vinifera*. Similar numbers of *CIPKs* and *CBLs* observed in *Vitis* and *Prunus* genomes indicate that there was no significant impact of tandem and segmental duplications on the two respective gene families after separation of *Rosaceae* and *Vitaceae* (Qiu et al., 2022). Arabidopsis had similar number of *CBLs* as observed in *Prunus* or *Vitis vinifera*, but Arabidopsis had a much higher number of *CIPKs,* probably due to stronger evolutionary pressure on retention of the duplicate *CBL* genes compared to *CIPK* genes (Qiu et al., 2022). Both *Malus* and *Prunus* are part of *Fabidae* but evolutionarily separated. Apple (*Malus domestica*) has undergone recent whole genome duplication (Velasco et al., 2010). Apple has 34 *CIPK* genes and 11 *CBLs*. These facts suggest that, despite whole genome duplication, apple has only 11 *CBLs* probably due to gene-loss events (Qiu et al., 2022).

Phylogenetic analyses of NHX proteins revealed two unique clades (NHX a and NHX b) containing NHXs from all six Rosaceae species, but not from Arabidopsis, in the Vac category (Figure 1). The subcellular localization prediction (Chou & Shen, 2010) of these clustered proteins indicated their vacuolar localization, indicating the lineage-specific evolution of these Rosaceae-specific novel NHXs. Identification of functions of the NHX proteins of Rosaceae species observed in two unique clades of the Vac category would be interesting. Similarly, a unique NHX clade was observed in Amaranthaceae, *Gossypium hirsutum*, *Vitis vinifera*, *Populus trichocarpa*, which were separated from Arabidopsis NHXs (Wu et al., 2019). These findings imply that during evolution, specific *NHX* genes were lost in some lineages, like in Arabidopsis. Based on their predicted cellular localization and predicted function as sodium/proton antiporters, these unique NHX genes may be involved in ion homeostasis and/ or pH homeostasis in plants.

We observed three members of NHXs of *Pyrus ussuriensis* × *Pyrus communis* (PuxPc) in the PM (plasma membrane) category, which are orthologous to Arabidopsis SOS1, suggesting that PuxPc_KAB2598774.1, PuxPc_KAB2596803.1, and PuxPc_KAB2604536.1 may play important roles in response to salinity stress. Our findings predict that *Pyrus*

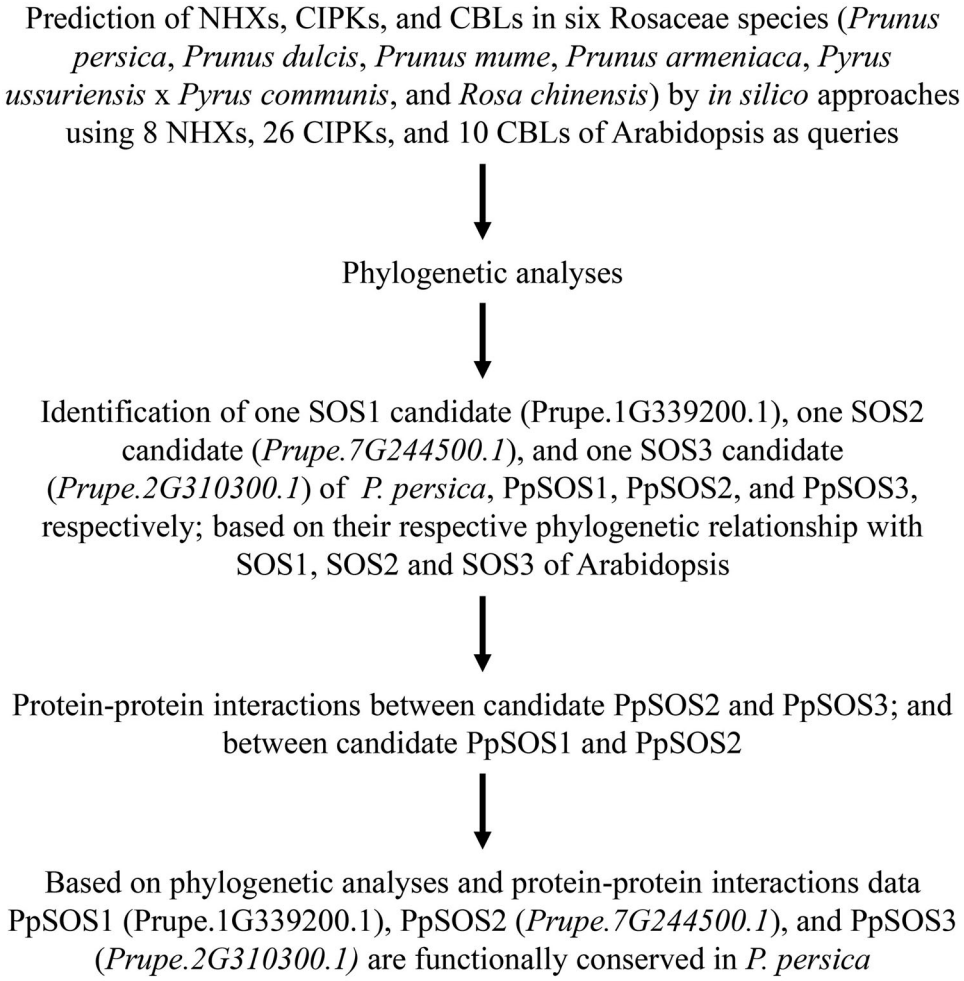

FIGURE 7 A schematic diagram shows the identification of NHXs, CIPKs, and CBLs of *Prunus persica* and validation of candidate Salt Overly Sensitive (SOS)-signaling candidate of *P. Persica* employing in silico approaches, phylogenetic analyses, and protein–protein interaction analyses. CBL, calcineurin B-like; CIPK, CBL-interacting protein kinase; NHX, Na^+/H^+ exchanger.

ussuriensis × *Pyrus communis* may be tolerant to salt stress. It should be also noted that hundreds of molecular components contribute to the salinity tolerance ability of a specific plant type. Future investigations may answer this data-driven hypothesis (Figure 1).

Our phylogenetic analysis failed to identify an AtSOS2 ortholog in *P. mume* (Figure 2). Similarly, a separate study that analyzed CIPKs in *P. mume*, Arabidopsis, and rice did not show any CIPKs of *P. mume* in the clade containing *AtSOS2* and *OsSOS2* (Li et al., 2019). These findings infer that *P. mume* may be a salt-sensitive species because of the lack of the *SOS2* gene, an important component of the SOS signaling pathway. It is worth mentioning that in Arabidopsis, in addition to SOS2 (AtCIPK24), CIPK8 (AtCIPK8) plays a positive regulatory role in providing salinity tolerance (Yin et al., 2020). Our phylogenetic analysis indicated that Pm_XP_008219188.1 is an ortholog of AtCIPK8 in *P. mume* (Figure 2). As SOS2 is absent, Pm_XP_008219188.1 may play an important role in providing some salinity tolerance in *P. mume*. It is possible that the absence of an identified AtSOS2 ortholog in *P. mume*, both in our study and in previous studies by other groups, may be due to incomplete sequence coverage of *P. mume*.

A rooted phylogenetic tree of Rosaceae CBLs was constructed, which exhibited two clades within the group I CBLs (Figure 3). Arabidopsis CBL10 was clustered in one clade with other Rosaceae species, most of which had one *AtCBL10* ortholog, except for *P. mume*, which had two, Pm_XP_008220463.1 and Pm_XP_008220465.1 (Figure 3). The second clade contained only Rosaceae species with no Arabidopsis homolog. Duplication of genes plays a role in adaptive evolution, and maintenance of a duplicated gene pair indicates the adaptive benefit of paralogous genes (Kondrashov, 2012). For example, *Eutrema salsugineum* (a halophyte), a salt-tolerant relative of Arabidopsis, has duplicated CBL10 compared to a single copy in Arabidopsis (Monihan et al., 2019). By employing molecular approaches including individual down-regulation

of a specific CBL10 copy (*EsCBL10a* or *EsCBL10b*) or down-regulation of both copies, the authors identified that each CBL copy plays a unique role and both are essential to provide higher salinity tolerance in response to salt stress in *E. salsugineum* (Monihan et al., 2019). These observations suggest that the presence of two *AtCBL10* orthologs, an ortholog of *AtCIPK8* and an ortholog of *AtSOS1*, may provide tolerance to salt in *P. mume* in the absence of *SOS2* and may warrant future investigations. Our phylogenetic analysis also identified a Rosaceae-specific clade in group I CBLs. Functional characterization of these genes would reveal their specific role(s). Another example of adaptive evolution we observed in SOS signaling components of *Pyrus ussuriensis* × *Pyrus communis* (PuxPc). Phylogenetic analysis of Rosaceae CBLs identified three copies of AtSOS3 orthologs in *Pyrus ussuriensis* × *Pyrus communis* in group IV CBLs: PuxPc_KAB2598774.1, PuxPc_KAB2596803.1, and PuxPc_KAB2604536.1 (Figure 3). In addition, for the same genome, we observed three orthologs of AtSOS1, PuxPc_KAB2620121.1, PuxPc_KAB2624101.1, and PuxPc_KAB2604592.1 (Figure 1), and two orthologs of AtSOS2, PuxPc_KAB2618622.1CIPK20 and PuxPc_KAB2598950.1 (Figure 2). As the *Pyrus ussuriensis* × *Pyrus communis* genome contains multiple copies of various SOS pathway genes, we expect it to be more salt-tolerant in comparison to the other four Rosaceae species. A future investigation would answer our phylogenetic analysis-based prediction.

Arabidopsis has four group II CBL members: AtCBL2, AtCBL3, AtCBL6, and AtCBL7 (Figure 3). These CBLs have been shown to be involved in a wide range of biological functions. For example, AtCBL2 plays a role in response to ABA and seed germination (Batistič et al., 2012). Arabidopsis CBL2 and/or CBL3 are known to play roles in magnesium homeostasis (Tang et al., 2015), seed size and embryonic development (Eckert et al., 2014), ABA-mediated stomatal movement (Song et al., 2018), and enabling the plant to adapt to a low-potassium environment (Tang et al., 2020). Arabidopsis CBL6 has been implicated in response to cold stress (Lee et al., 2005). In addition to many other functions, Arabidopsis CBL7 plays roles in hypocotyl elongation (Liu et al., 2022) and alkali tolerance (Yang et al., 2019). Like Arabidopsis, other plant species possess more than one group II CBLs. For example, two in tomato, five in soybean, and three in *Populus* (Xi et al., 2017; Zeng et al., 2017). In contrast, in group II, all six Rosaceae species are represented by only one specific CBL each (Figure 3). A previous study reported that group II CBLs of diploid Amaranthaceae species have one CBL member (Zhao et al., 2021). It appears that Rosaceae retained only one group II CBL gene, which could be a result of higher selective pressure on the retention of duplicated *CBL* genes. It is also possible that group II CBL of Rosaceae species may play multiple biological roles. Additionally, it cannot be ruled out that the *CBL* genes from other groups of Rosaceae may perform necessary biological roles for compensation.

Many plants, including Arabidopsis (Ji et al., 2013; Liu et al., 2000; Shi et al., 2000), rice (Martínez-Atienza et al., 2007), and wheat (Sathee et al., 2015), have been shown to induce *SOS* gene expression in response to exposure to salinity. In our study, we observed the expression of all three *SOS* genes of *Prunus persica*, *PpSOS1*, *PpSOS2*, and *PpSOS3*, both in roots and leaves (Figure 4). We did not observe significant differences in gene expressions of *PpSOS1*, *PpSOS2*, and *PpSOS3*, in response to salinity (3.0 dS m^{-1}) in leaves or roots (Figure 4). In response to salt stress, SOS signaling pathway components, PpSOS1, PpSOS2 and PpSOS3, may be regulated by protein–protein interactions and protein phosphorylation rather than salinity-induced transcriptional regulation. It cannot be ruled out that one or more factors might have impacted the expression of the *SOS* genes of *P. persica* in response to salinity, including components that regulate the SOS signaling network (Lang et al., 2017). For example, NaCl-induced secondary messengers like Ca^{2+}, H_2O, or NO have been shown to enhance the expression of *SOS1*, *SOS3*, and *CIPK* genes of *Glycyrrhiza uralensis* (Lang et al., 2017). The electrical conductivity of saline solution or level of salinity regulates expression of *SOS* genes. For instance, a moderately tolerant rice genotype showed upregulation of *SOS2* at higher level of salt concentration (200 mM NaCl) but not at the lower level (100 mM NaCl) (Sathee et al., 2015). Salt tolerant and sensitive plant genotypes show differential expression status of *SOS* genes in response to salt treatment. For example, *SOS3* has been shown to be induced in salt-tolerant rice genotype but not in salt-sensitive rice genotype (Sathee et al., 2015). A time kinetics of gene expression analyses of *SOS3* gene of *Tamarix hispida*, *ThSOS3*, indicated down regulation at 6 h, slight upregulation at 12 h, down regulation at 24 h, and upregulation of the gene at both 48 and 72 h which indicated duration of salinity treatment has impact *SOS* genes of some plant species (Liu et al., 2020). A previous study examined the expression status of *TaSOS1* in response to four salinity levels, from 0 to 72 h, in four genotypes of wheat, including two cultivated wheat cultivars Mahuti (tolerant) and Alamut (susceptible) (Ramezani et al., 2013). For the susceptible cultivar, the authors observed higher expression levels of *TaSOS1* in response to 200 mM NaCl in comparison to 100 and 50 mM, in contrast the tolerant genotype showed significantly lower expression of *TaSOS1* in response to 200 mM NaCl (Ramezani et al., 2013). These observations indicated that plant genotype acts as a determinant of *SOS* expression status irrespective of salinity tolerance level. These few above-described examples suggest that one or more factors might have contributed to significant induction of SOS gene expression in response to salinity. These factors include genotype, time point of tissue harvest, salinity level, accumulation status of secondary messengers, impact of

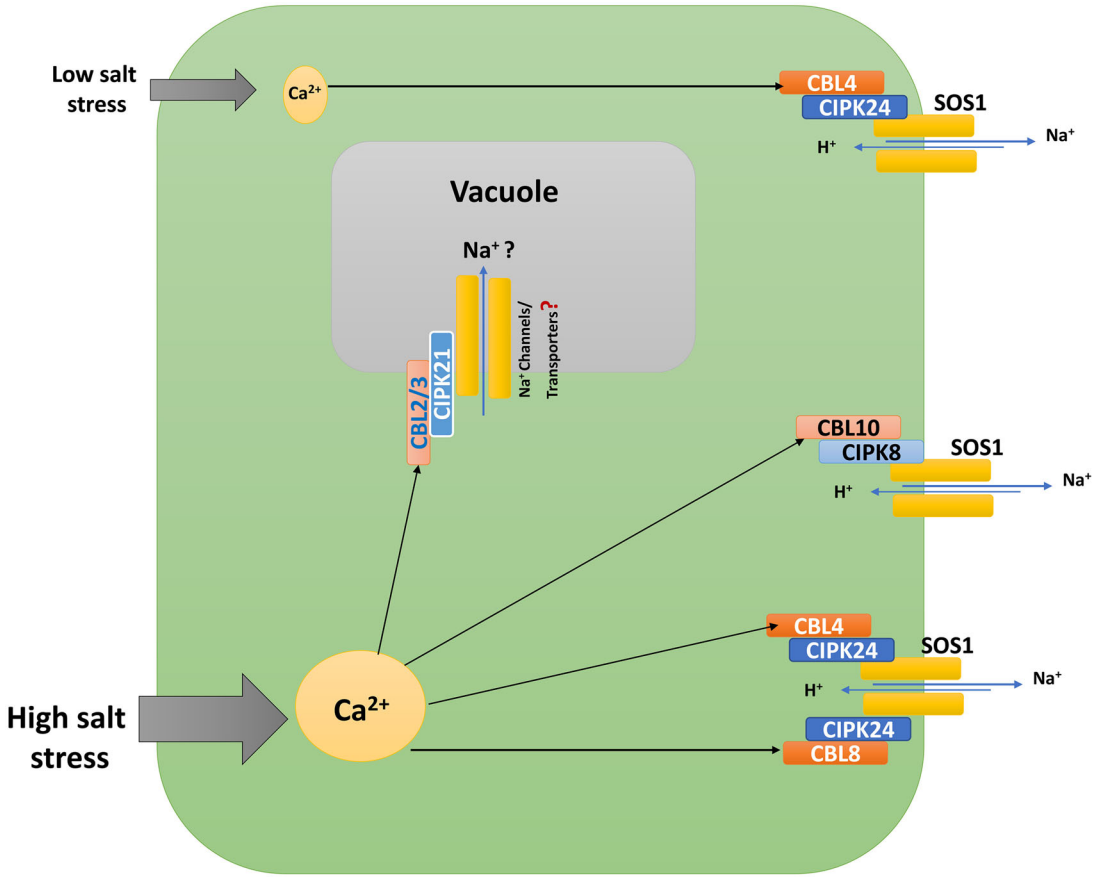

FIGURE 8 Model showing involvement of different CIPKs and CBLs in salinity response. The figure indicates roles of CIPKs, and CBLs in salinity tolerance based on published reports discussed in the discussion section. CBL, Calcineurin B-like; CIPK, CBL-interacting protein kinase; SOS, Salt Overly Sensitive.

abiotic factor(s), plant age, expression status of *SOS* gene regulators or the status of specific *cis*-elements in the promoters of respective *SOS* genes. A detailed analysis of *P. persica SOS* gene expression in response to salinity may reveal additional factors that play critical roles.

Under salinity stress, intracellular calcium levels (Ca^{2+}) increase and bind to the SOS3 protein, activating it (Jiang et al., 2019). Activated SOS3 then interacts with SOS2, a protein kinase that activates SOS2. Subsequently, SOS2 activates SOS1 by phosphorylation (Quintero et al., 2011), which helps extrude sodium from the inside of the cell in order to reduce the elevated level of sodium inside the cell. These facts indicate that SOS signaling proteins transduce salt stress signal through protein–protein interactions. Accordingly, one could perform protein–protein interactions between candidate- SOS3 and SOS2 proteins; and protein–protein interactions between candidate- SOS2 and SOS1 proteins; positive protein–protein interaction data may indicate the active status of specific pair of proteins. Our phylogenetic analyses of NHXs, CIPKs, and CBLs of *P. persica* identified one gene each for *SOS1*, *PpSOS1* (*PpNHX7*) (Table S1, Figure 1); one gene for *SOS2*, *PpSOS2* (*PpCIPK24*) (Table S2, Figure 2); and one gene for *SOS3*, *PpSOS3* (*PpCBL4*) (Table S3, Figure 3). Using the yeast two-hybrid protein-protein interaction assay method, we observed interaction between PpSOS3 and PpSOS2 proteins (Figure 5) and between PpSOS2 and PpSOS1 proteins (Figure 6). Our protein-protein interactions data indicated that PpSOS1, PpSOS2, and PpSOS3 are active SOS signaling proteins. In addition, our finding suggests that PpSOS2 may activate PpSOS1 by PpSOS2-mediated phosphorylation, as observed in Arabidopsis (Quintero et al., 2011).

It is well known that SOS signaling components, SOS3 (AtCBL4), SOS2 (AtCIPK24), and SOS1 (AtNHX7) play vital roles in salinity tolerance (Figure 8). AtCBL10 also contributes to salinity tolerance with SOS2 and SOS1. Apart from Arabidopsis' CIPK24 (SOS2), several other CIPKs have been discovered to play significant roles in salinity response. Similarly, beyond Arabidopsis' CBL4 (SOS3), additional CBL proteins have been identified that contribute to enhancing salinity tolerance (Figure 8). CIPK8 of Arabidopsis has been shown to function with CBL10 (but not with SOS3/CBL4) and SOS1 to provide salinity tolerance (Yin et al., 2020). Loss-of-function mutant of AtCIPK21 is hypersensitive to

high salt stress. CIPK21 has been shown to interact with CBL2 or CBL3 and preferably localizes to tonoplast in response to salt stress (Figure 8). It has been hypothesized that salt stress-mediated increase of calcium is sensed by CBL2/CBL3 that in turn activates CIPK21, which facilitates localization of CIPK21 to the tonoplast. In the vacuolar membrane, activated CIPK21 interacts and activates unidentified Na^+ channels/ transporter, which in turn promotes the sequestration of Na^+ from the cytosol to the vacuole during salt stress (Pandey et al., 2015). Under conditions of severe salt stress, the activation of CBL8 initiates a cascade of events. CBL8, once activated, in turn activates CIPK24 (SOS2). The subsequent activation of SOS2 enables it to interact with SOS1, and as a result, SOS1 transporter effectively detoxifies toxic levels of Na^+ within the cell (Steinhorst et al., 2022). Different signaling components, CBLs and CIPKs, contribute to salinity tolerance mediated by Na^+/H^+ antiporter (SOS1) at the plasma membrane or other Na^+ channel/ transporters at the vacuolar membrane (Figure 8).

5 | CONCLUSIONS

Bioinformatic and phylogenetic analyses of the NHX, CIPK, and CBL genes of six Rosaceae species indicated that CIPKs of algae and primitive plants are evolutionarily linked to AtCIPK24 (SOS2) and AtCIPK8 and corresponding Rosaceae CIPKs that belong to intron-rich CIPKs class. Additionally, we found that intron-poor CIPKs of Rosaceae and Arabidopsis evolved from intron-rich CIPKs. Our findings revealed both adaptive and lineage-specific evolution of these genes in the Rosaceae family. Based on adaptive evolution data, we predict that *Pyrus ussuriensis* × *Pyrus communis* hybrid plants are more salt tolerant than the other species evaluated in this study. However, further experimental verification is needed to test this hypothesis. Our phylogenetic analysis of SOS signaling components of *P. mume* did not identify any *PmSOS2* candidate, which suggested that *P. mume* may be a salt-sensitive species. Nevertheless, *P. mume* has an ortholog of *AtCIPK8*, PM_XP_008219188.1; two orthologs of *AtCBL10*, Pm_XP_008220463.1 and *PmCBl2* Pm_XP_008220465.1; and an ortholog of *AtSOS1*, Pm_XP_008220964.1, which might interact to provide salinity tolerance to *P. mume*. Experimental verification is needed to examine these two hypotheses regarding the salinity tolerance ability of *P. mume*. Incomplete sequence coverage of *P. mume* is a potential explanation for why our group, as well as others, were unable to identify an AtSOS2 ortholog in this species. We identified only one gene for *SOS1* (*PpSOS1*), one gene for *SOS2* (*PpSOS2*), and one gene for *SOS3* (*PpSOS3*) in *P. persica*. We observed positive protein–protein interactions between PpSOS1 Cter and PpSOS2; and PpSOS2 and PpSOS3 in yeast which indicated that *PpSOS1*, *PpSOS2*, and *PpSOS3* are true orthologs of Arabidopsis *SOS* genes. The identified *NHX*, *CIPK*, and *CBL* genes of Rosaceae species are a great resource for future research and may be useful for biotechnological uses.

AUTHOR CONTRIBUTIONS

Biswa R. Acharya: Conceptualization; formal analysis; methodology; writing—original draft; writing—review and editing. **Chaoyang Zhao**: formal analysis; methodology; writing—review and editing. **Lorenso Antonio Rodriguez Reyes**: writing—review and editing. **Jorge F.S. Ferreira**: funding acquisition; writing—review and editing. **Devinder Sandhu**: Conceptualization; formal analysis; funding acquisition; methodology; supervision; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data used in this study are included in the article and supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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