Expression analysis of PIN family genes in Chinese hickory reveals their potential roles during grafting and salt stress

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Grafting is an effective way to improve Chinese hickory while salt stress has caused great damage to the Chinese hickory industry. Grafting and salt stress have been regarded as the main abiotic stress types for Chinese hickory. However, how Chinese hickory responds to grafting and salt stress is less studied. Auxin has been proved to play an essential role in the stress response through its re-distribution regulation mediated by polar auxin transporters, including PIN-formed (PIN) proteins. In this study, the PIN gene family in Chinese hickory (CcPINs) was identified and structurally characterized for the first time. The expression profiles of the genes in response to grafting and salt stress were determined. A total of 11 CcPINs with the open reading frames (ORFs) of 1,026–1,983 bp were identified. Transient transformation in tobacco leaves demonstrated that CcPIN1a, CcPIN3, and CcPIN4 were localized in the plasma membrane. There were varying phylogenetic relationships between CcPINs and homologous genes in different species, but the closest relationships were with those in Carya illinoiensis and Juglans regia. Conserved N- and C-terminal transmembrane regions as well as sites controlling the functions of CcPINs were detected in CcPINs. Five types of cis-acting elements, including hormone- and stress-responsive elements, were detected on the promoters of CcPINs. CcPINs exhibited different expression profiles in different tissues, indicating their varied roles during growth and development. The 11 CcPINs responded differently to grafting and salt stress treatment. CcPIN1a might be involved in the regulation of the grafting process, while CcPIN1a and CcPIN8a were related to the regulation of salt stress in Chinese hickory. Our results will lay the foundation for understanding the potential regulatory functions of CcPIN genes during grafting and under salt stress treatment in Chinese hickory.

KEYWORDS
Carya cathayensis, PIN, transport, auxin, salt, grafting
Introduction

Auxin is the first discovered phytohormone (Went, 1935) and plays an important role during different growth stages of plants (Yu et al., 2022), including gametogenesis, seed germination, root elongation, vascular patterning, and blossoming (Zhao, 2010). In addition, an important function of auxin is its regulation of the responses of plant species to different biotic and abiotic stresses (Kapazoglou et al., 2020; Dastborhan et al., 2021; Mearaji et al., 2021; Zhang et al., 2022). The functioning of auxin is closely related to its homeostatic regulation, which involves biosynthesis, transport, and degradation (Zhang and Peer, 2017). Following biosynthesis, transport is the key step for auxin functioning (Gomes and Scortecci, 2021) because the site of action is usually different from that of synthesis.

Plants have evolved two methods for transporting auxin: the long-distance network (transporting auxin through phloem) and the cell-to-cell transport network (also named polar auxin transport) (Muday and DeLong, 2001; Robert and Friml, 2009). In plants, polar auxin transport is mainly mediated by AUXIN/LIKE AUXIN proteins (AUX/LAX, responsible for auxin influx), PIN-formed proteins (PIN, responsible for auxin efflux), PIN-LIKES (PILS, responsible for auxin transport between the cytosol and endoplasmic reticulum), and ATP-binding cassette subfamily B proteins (ABC, responsible for auxin influx/efflux) (Swarup and Peret, 2012; Mohanta et al., 2018; Sauer and Kleine-Vehn, 2019). PIN proteins play major roles in auxin efflux because their polar localizations are always consistent with the directionality of auxin (Zhou and Luo, 2018).

PIN is a gene family composed of multiple genes with similar or different structures and functions. AtPIN1 was the first discovered PIN protein in Arabidopsis. The mutant of PIN1, pin1, has no leaves or flowers in cauline inflorescence (Kuhlemeier and Reinhardt, 2001) and resembles pins, which is the reason why PIN1 and the subsequent proteins in this family were named PIN-formed proteins (PINs). PIN family proteins contain conserved N- and C-terminal transmembrane domains and intracellular central hydrophilic loops. There are two types of PIN proteins categorized according to the differences in structures of the central hydrophilic loop: canonical PIN proteins with a long central hydrophilic loop and non-canonical PIN proteins with a short hydrophilic loop. In Arabidopsis, canonical PIN proteins (AtPIN1, 2, 3, 4, and 7) are localized to the plasma membrane (PM) and mediate auxin efflux to the extracellular space (Bennett et al., 2014). Non-canonical PINs (AtPIN5 and 8) are localized to the endoplasmic reticulum (ER) and mediate intracellular auxin transport between the cytoplasm and ER (Mravec et al., 2009; Sauer and Kleine-Vehn, 2019). AtPIN6 possesses a long hydrophilic loop and is located on the PM and ER, and thus is classified as a non-canonical PIN (Bennett et al., 2014).

The functions of PIN proteins have been well studied in Arabidopsis. AtPIN1 regulates the shoot apical meristem, root elongation, and the development of xylem (Bililou et al., 2005; Alabdallah et al., 2017). AtPIN2 influences root gravitropism and responds to salt stress (Wang et al., 2019; Gibson et al., 2020). AtPIN3 is mainly expressed in root and vascular tissues. It regulates auxin distribution in roots, participates in lateral root growth, and responds to light and gravity (Bililou et al., 2005; Zhai et al., 2021; Li et al., 2022). Similar to AtPIN3, AtPIN4 is related to light and gravity responses. In addition, AtPIN4 participates in auxin flow to the quiescent center (Bureau et al., 2010). AtPIN5 mediates not only the transport of auxin from the cytoplasm to the ER, but also regulates auxin homeostasis and metabolism (Mravec et al., 2009). AtPIN6 regulates lateral root formation (Sauer and Kleine-Vehn, 2019). The functions of AtPIN7 are similar to those of AtPIN3 and AtPIN4, and it participates in the gravimetric response of roots (Lewis et al., 2011). The function of AtPIN8 is the opposite of that of AtPIN5, transporting auxin from the ER to the cytoplasm (Ding et al., 2012). In addition, there is functional redundancy for some PIN proteins (Bililou et al., 2005; Wang et al., 2021). For example, the mutant genes pin1 and pin2 exhibit functional redundancy regarding the regulation of root meristem and root length (Vieten et al., 2005).

In addition to developmental functions, PIN family genes also participate in the regulation of abiotic stress (Yu et al., 2017). For example, in pepper (Capsicum annuum) and Sorghum bicolor, the expression profiles of PIN genes were changed under high salt treatment (Shen et al., 2010; Zhang et al., 2018). The process of grafting creates wounding stress, which also influences the expression of PIN genes (Melnyk, 2017; Sharma and Zheng, 2019). The overexpression of AtPINs-GUS in Arabidopsis shows that AtPINs are expressed in the grafted union, indicating their involvement in grafting regulation (Wang et al., 2014). Transcriptome sequencing of Arabidopsis grafting also supports this conclusion (Melnyk et al., 2018).

Chinese hickory (Carya cathayensis Sarg.), belonging to the Juglandaceae family, is one of the most important trees economically in the Zhejiang and Anhui Provinces of China. Nuts from Chinese hickory trees are rich in nutritional compounds, including polyunsaturated fatty acids, phenolics, and flavonoids (Huang et al., 2022). However, a long juvenile phase and narrow distribution have restricted the development of the Chinese hickory industry. Grafting is an effective way to solve these problems. In addition, soil salinization has limited the development of the Chinese hickory industry.

To date, members of the PIN gene family have not been identified, and their potential roles during grafting and salt stress treatment in Chinese hickory are still unknown. In this study, PIN family genes in Chinese hickory were cloned and structurally analyzed, and their expression profiles in response to grafting and salt stress treatment were determined. Our
results will lay the foundation for revealing the molecular mechanism of PIN family genes in regulating grafting and salt stress in Chinese hickory.

Materials and methods

Identification of PIN family genes in Chinese hickory

Candidate gene sequences of PIN in Chinese hickory (CcPINs) were identified from the published genome (Huang et al., 2019). The process used to identify genes was the same as that used in a previous study (Yang et al., 2021). Hidden Markov model (HMM) profiles of the PIN domain (PF03547) were used for analysis. TBtools (Toolbox for Biologists, v1.098745) was used for the preliminary screening of PIN genes (Chen et al., 2020). The identified CcPINs were named according to their phylogenetic relationships with the homologs in Arabidopsis (Supplementary Figure 1).

Structural analysis of CcPINs

In addition to predicting the functions of CcPINs in Chinese hickory, the PIN proteins in Chinese hickory and 14 other species were chosen to undergo phylogenetic relationship analysis. The PIN proteins of 11 species were obtained from previous studies. PINs of the other three species (including Juglans regia, Caryya illinoienses, and Nymphaea tetragona) were analyzed based on the genome in Phytozome v13. The identified CcPINs were named according to their phylogenetic relationships with the homologs in Arabidopsis (Supplementary Table 1).

Plant material, treatment, and sampling

One-year-old Chinese hickory seedlings were used for tissue-specific expression analysis, while 2-year-old Chinese hickory seedlings were used as rootstocks for grafting and materials for salt stress treatment. Seedlings were planted in seedling pots with a diameter of 17 cm and height of 22 cm and cultivated in the greenhouse of Zhejiang A&F University. One seedling was planted in each pot with 5 L of soil. The soil formula included 50% peat, 13% pastoral soil, 15% organic fertilizer, 5% exocarp, 10% agricultural bran, 5% perlite, and 2% release fertilizer. The seedlings were grown under the following conditions: temperature of 25 ± 3°C, humidity of 60–70%, and photoperiod of 12-h light/12-h dark. The seedlings were watered every 5 days. Tobacco (Nicotiana benthamiana) was cultivated in the culture room at the temperature of 22°C, humidity of 60–70%, and photoperiod of 16-h light/8-h dark. For subcellular localization analysis, 45-day-old tobacco leaves were used.

For the grafting experiment, 2-year-old seedlings with similar phenotypes were used for rootstocks, and the grafting operation was conducted on April 20th, 2018. One-year-old branches (7–8 cm in length) with a new bud collection on the fruit-bearing trees were chosen as scions. For salt stress treatment, the 2-year-old seedlings were treated with 150 mm NaCl (Shanghai Hushi Laboratorial Equipment Co., Ltd., China) solution three times every 3 days, following the same method as a previous study (Chen et al., 2015). The equivalent water treatment was used for the control check (CK).

Graft unions were collected at 0, 3, 7, and 14 days after grafting, representing the initiation stage, isolation layer formation stage, callus formation and isolating layer disappearance stage, and vascular bridge formation and linkage stage during Chinese hickory grafting, respectively, as detected by cytological observation (Liu et al., 2009). In each treatment, three graft unions were mixed together and regarded as a single sample. For salt stress treatment, samples from different tissues, including roots, stems, and leaves, were collected after 0, 1, 3, and 10 days of treatment, representing the initial-, short-, medium- and long-term treatment stages as reported in similar studies of other woody plants (Chen et al., 2015; Zhao et al., 2015; Hang et al., 2020; Huang et al., 2020). The day on which the seedlings were treated with NaCl solution for the first time was regarded as day 0. For each tissue, collections from five different seedlings were mixed together and regarded as a single sample. For tissue-specific expression analysis, different samples, including roots, stems, leaves, and shoots, of 1-year-old Chinese hickory seedlings were collected in early spring (on April 16, 2022), representing the fast-growing stage of the seedlings. For each tissue, collections from five different seedlings were mixed together and regarded as a single sample. All the samples were wrapped in aluminum foil, immediately immersed in liquid nitrogen, and stored in an ultra-low temperature refrigerator (−80°C) for future use.

1 https://phytozome-next.jgi.doe.gov/
2 https://meme-suite.org/tools/meme
3 https://services.healthtech.dtu.dk/service.php?TMHMM-2.0
4 http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
RNA extraction, cDNA synthesis, and real-time quantitative PCR analysis

The total RNAs of Chinese hickory were extracted using a MiniBEST Universal RNA Extraction Kit [Code No. 9767, Takara Biomedical Technology (Beijing) Co., Ltd., China]. For the grafting experiment, the rootstocks and scions in a single sample were separated from each other, and the cells at the conjunction surfaces of the rootstocks and scions were scraped using blades, respectively. Total RNAs of the rootstocks and scions were extracted separately. For the salt stress experiment, the total RNAs of the roots, stems, and leaves collected at different time points were extracted separately. For the tissue-specific experiment, the total RNAs of the roots, stems, and leaves of 1-year-old seedlings were extracted separately. The cDNA used for cloning was synthesized using PrimeScriptTM 1st strand cDNA Synthesis Mix [Code No. 6215A, Takara Biomedical Technology (Beijing) Co., Ltd., China]. PrimeSTAR Max DNA Polymerase [Code No. R045Q, Takara Biomedical Technology (Beijing) Co., Ltd., China] was used for PIN gene cloning. The cDNA used for Real-Time Quantitative PCR analysis (qRT-PCR) was obtained using the PrimeScriptTM RT Master Mix [Code No. RR036A, Takara Biomedical Technology (Beijing) Co., Ltd., China].

Real-Time Quantitative PCR analysis primers were designed on the National Center for Biotechnology Information (NCBI) primer prediction website.5 The CcActin gene was used as the internal standard for normalization. The primers for qRT-PCR are listed in Supplementary Table 2. The methods used for qRT-PCR were followed according to a previously published study (Yuan et al., 2018). The qRT-PCR analysis was replicated at least three times.

Subcellular localization analysis

The coding sequence of CcPINs was cloned from the cDNA library of Chinese hickory and inserted into the modified pCAMBIA1300-GFP vector. Then, the obtained 35S:CcPINs-GFP was translated into Escherichia coli Trans1-T1 (TransGen Biotech Co., Ltd., China) and sequenced by Sangon Biotech (Shanghai) Co., Ltd., China. Plasmids from the identified strains were extracted and transformed into Agrobacterium strain GV3101 competent cells (AC1001, Shanghai Weidi Biotechnology Co., Ltd., China). Transient transformation of 45-day-old tobacco leaves was performed as previously described (Yamaji et al., 2009). The pm-rk (plasma membrane marker with red fluorescence, Nelson et al., 2007) was transiently co-transformed into tobacco leaves with CcPINs. After culturing for 48 h, the fluorescence signal in the transformed tobacco leaves was detected using a confocal microscope (LSM 880, Zeiss, Germany) with 488 and 594 nm argon lasers.

Statistical analysis and figure preparation

To calculate the relative expression levels of CcPIN genes, the 2−ΔΔCT method, introduced by Schmittgen and Livak (2008), was used. A one-way analysis of variance (ANOVA) and multiple comparisons [the least significant difference (LSD) method] were conducted using SPSS Statistics (version 17.0) to analyze the expression differences of CcPIN genes among different tissues and treatments.

The histogram and line figures were drawn using Microsoft Excel 2019. The phylogenetic tree was drawn using Mega X and embellished using Fig. Tree (v1.4.4). The figures illustrating motifs and gene structures were drawn using TBtools (Toolbox for Biologists, v1.098745). Finally, the figures were merged by Adobe Photoshop (version 2020).

Results

Identification of PIN family genes in Chinese hickory

Eleven CcPIN genes were identified, and the corresponding information is displayed in Table 1. The ORF length of the CcPINs ranged from 1,026 bp (CcPIN8a) to 1,983 bp (CcPIN3), encoding proteins with between 341 and 660 amino acids. The molecular weights of CcPINs ranged from 37,338.75 Da (CcPIN8a) to 72,046.05 Da (CcPIN3), and the predicted isoelectric points (pl) varied from 6.42 (CcPIN5) to 9.39 (CcPIN8a). The number of exons for CcPIN genes ranged from 5 to 7. To explore the potential locations where CcPINs perform their functions, several CcPINs were detected by subcellular localization. Transient transformation of CcPINs to tobacco leaves showed that CcPIN1a, CcPIN3, and CcPIN4 were expressed on the PM (Figure 1).

Phylogenetic relationships of PIN family proteins in Chinese hickory and other species

To predict the potential functions of PIN family genes in Chinese hickory, a phylogenetic tree was constructed to explore the phylogenetic relationships between the 11 PIN proteins in Chinese hickory and 121 PIN proteins from 14 other plant species. The 15 plant species used to construct
FIGURE 1. The subcellular localization of CcPIN1α, CcPIN3, and CcPIN4. The fluorescence images were captured in a dark field for green and red fluorescence, in a white field for the morphology of the cell, and in combination. GFP, green fluorescent protein fluorescence; RFP, red fluorescent protein fluorescence; Bright, bright field; Merged, GFP/RFP/bright field overlay. Bar = 20 µm.

The phylogenetic tree belonged to 5 types of charophytes, bryophytes, lycophytes, monilophytes, gymnosperms, and angiosperms according to the consensus phylogeny reported by Doyle (2018). Detailed information on the 132 PIN proteins is shown in Supplementary Table 1. A total of eight groups (I, II, III, IV, V, VI, VII, and VIII) were identified (Figure 2A). There was significant variation in the number of PIN proteins in the different groups. There was only one protein in Group I, three proteins in Group II, seven proteins in Group VI, and 11 proteins in Group VII. The number of proteins in Groups III, IV, and V was 22, 19, and 23, respectively, and the highest number of proteins was in Group VIII, with 46 (Figure 2B).

The number of PIN proteins varied in different species. There was only one PIN protein in Klebsormidium flaccidium and Picea abies, while the number of PIN proteins in the other 13 species changed from 4 to 15 (Figure 2B). In addition, there were more PINs (8–15) in angiosperm species than those in the other types (Figure 2B). Similar to C. illinoitensis and J. regia, the PINs in C. cathayensis were distributed in Groups III, IV, V, VII, and VIII, with the largest number of PINs in Group VIII. Most PINs in Oryza sativa, Zea mays, and S. bicolor were distributed in Groups III, IV, VI, VII, and VIII (Figures 2A,B).

Multiple sequence alignment, motif, and gene structure analysis of CcPINs

Multiple sequence alignment of CcPIN proteins is shown in Figure 3, which displays the high level of conservation in the N-terminal and C-terminal regions. There were short sequences in the middle region for CcPIN5, CcPIN8a, and CcPIN8b, with long sequences for the other CcPINs. In addition, “F165,” the site controlling the distribution of PIN proteins on PM, the phosphorylation site “TPRXS,” and the conserved “NPXXY” site were detected (Figure 3). Motif analysis of the eleven CcPIN proteins was performed using the MEME Suite, with the eight conserved motifs set. Motif 5 was not detected in short CcPINs (CcPIN5 and CcPIN8) (Figures 4A,B). The conserved element “NPXXX” was found in motif 5 (Figure 4D), and the phosphorylated site “TPRXS” of MPK 4/6 kinases...
FIGURE 2
Phylogenetic analysis of PIN proteins from 15 species. (A) The phylogenetic tree. (B) The number and distribution of PINs in different species. Kf, Klebsormidium flaccidum; Pp, Physcomitrella patens; Sm, Selaginella moellendorffii; Cf, Cystopteris fragilis; Pa, Picea abies; At, Arabidopsis thaliana; Pt, Populus trichocarpa; Ar, Amborella trichopoda; Jr, Juglans regia; Ci, Carya illinoinensis; Cc, Carya cathayensis; Nc, Nymphaea tetragona; Os, Oryza sativa; Zm, Zea mays; Sb, Sorghum bicolor.
TABLE 1 Information on CcPIN genes and properties of their deduced proteins in Chinese hickory.

| Gene name | Locus ID | Genomic location | Exon number | ORF (bp) | Length (aa) | pI | Mol wt (Da) | Ortholog with Arabidopsis |
|-----------|----------|------------------|-------------|----------|-------------|----|-------------|---------------------------|
| CcPIN1a   | CCA1233S0022 | scaffold50249:506539-510501 | 6 | 1,809 | 602 | 8.81 | 65,648.89 | AtPIN1 |
| CcPIN1b   | CCA0709S0034 | scaffold23721:408391-412462 | 6 | 1,779 | 592 | 8.95 | 63,882.92 | AtPIN1 |
| CcPIN2a   | CCA0888S0033 | scaffold31775:857604-860474 | 6 | 1,524 | 507 | 8.76 | 55,524.96 | AtPIN2 |
| CcPIN2b   | CCA0674S0073 | scaffold21864:981746-985490 | 6 | 1,926 | 641 | 9.22 | 69,513.49 | AtPIN2 |
| CcPIN3    | CCA1410S0065 | scaffold60275:585325-589367 | 6 | 1,983 | 660 | 8.45 | 72,066.84 | AtPIN3 |
| CcPIN4    | CCA0857S0074 | scaffold29914:809434-813085 | 6 | 1,932 | 643 | 9.16 | 70,068.83 | AtPIN4 |
| CcPIN5    | CCA0888S0013 | scaffold31775:259812-263129 | 5 | 1,086 | 361 | 9.19 | 59,537.80 | AtPIN5 |
| CcPIN6a   | CCA0944S0041 | scaffold35145:386749-392264 | 7 | 1,653 | 550 | 9.26 | 60,544.85 | AtPIN6 |
| CcPIN6b   | CCA1391S0033 | scaffold58974:378190-384204 | 7 | 1,629 | 542 | 9.26 | 59,537.80 | AtPIN6 |
| CcPIN8a   | CCA0779S0095 | scaffold26004:732921-735316 | 6 | 1,026 | 341 | 9.39 | 37,338.75 | AtPIN8 |
| CcPIN8b   | CCA1094S0128 | scaffold43199:1045218-1047610 | 6 | 1,104 | 367 | 9.39 | 40,253.74 | AtPIN8 |

pI denotes isoelectric point, and Mol wt denotes molecular weight.

was found in Motif 8 (Figure 4D). Transmembrane region prediction using TMHMM showed that there were N-terminal or C-terminal transmembrane regions for all CcPINs, with the total number ranging from 6 to 10 (Supplementary Figure 2). The coding sequences of CcPINs were compared with their corresponding DNA sequences on the genome. The numbers of exons in CcPINs ranged from five to eight (Figure 4C).

Protein–protein interaction network prediction of CcPINs

The PPI network was predicted between the homologs of CcPINs in Arabidopsis and other proteins in Arabidopsis by the String database, with the minimum required interaction score set to a high confidence score of 0.700, and the maximum number of interactors was limited to no more than 10. The PPI network consisted of 17 nodes and 39 edges (Figure 5A). Seven nodes were representative of PIN proteins. The corresponding information is shown in Supplementary Table 3. Different line colors represent protein-protein associations.

The nodes were annotated using Gene Ontology (GO) (Figure 5B and Supplementary Table 4). The results showed that most of the predicted proteins contributed to biological processes, such as “Cellular response to stimulus” (GO:0051716), “Auxin-activated signaling pathway” (GO:0009734), “Tissue development” (GO:0009888), and “Xylem and phloem pattern formation” (GO:0010051). The proteins also played a role in multiple cellular components including the “Plasma membrane” (GO:005886), “Auxin polar transport” (GO:0009926), and “Cytoplasm” (GO:0005737). In addition, the molecular function of the proteins included “Auxin transmembrane transporter activity” (GO:0080161), “Auxin efflux transmembrane transporter activity” (GO:0010329), “Auxin influx transmembrane transporter activity” (GO:0010328), and “Auxin binding” (GO:0010011).

Cis-acting regulatory elements affecting the promoters of CcPIN family genes

Regulatory elements that are cis-acting on the promoters of genes were the sites for expression regulation of genes. To explore how the expression of CcPINs was regulated by the different transcriptional factors, the section 2,000 bp upstream of the start codon (ATG) of CcPINs was used to query for cis-acting element detection using PlantCare. Five types of cis-acting elements, including a binding site element, hormone responsiveness, light responsiveness, plant development, and stress responsiveness, were detected, with numbers of 9, 71, 147, 28, and 40, respectively (Figure 6).
Within each type, specific cis-acting elements were detected. For example, five types of hormone-responsive elements, including gibberellin-responsive elements (GARE-motif, P-box, TATC-box, and TGA-element), abscisic acid-responsive elements (ABRE), MeJA-responsive elements (CGTCA-motif, TGACG-motif), and salicylic acid-responsive elements (TCA-element), were detected. The number of cis-acting elements for a certain CcPIN gene ranged from 15 (CcPIN8a) to 36 (CcPIN4), where light-responsive elements were the most abundant. In addition, five types of stress-responsive elements were detected on the promoters of CcPINs.

**Tissue-specific expression profiles of CcPINs in Chinese hickory**

To explore the relative expression of PIN genes in different tissues, tissue-specific expression profiles of CcPIN genes in roots, stems, leaves, and shoots of Chinese hickory were obtained by qRT-PCR. The results showed that CcPIN1a, CcPIN2a, CcPIN5, and CcPIN8a were highly expressed in stems, CcPIN4 and CcPIN6a showed the highest expression levels in shoots, and the highest expression of CcPIN3 was in leaves (Figure 7). The tissue-specific expression patterns of CcPIN
genes showed that they might play different roles in different tissues in Chinese hickory.

Expression profiles of CcPIN genes during grafting and salt stress in Chinese hickory

To investigate whether CcPINs regulated grafting in Chinese hickory, the expression levels of CcPINs were measured at different stages of grafting (Figure 8). In scions, compared with 0 days after grafting, the expression level of CcPIN1a was significantly upregulated over sevenfold and eightfold at 3 and 7 days after grafting, respectively. However, at 14 days after grafting, the expression was decreased but still significantly higher than at 0 days after grafting. The expression of CcPIN3 and CcPIN6a was downregulated in scions. The expression levels of CcPIN4 and CcPIN8a significantly changed in rootstocks after grafting. The highest expression for CcPIN4 was at 14 days after grafting in rootstocks, while CcPIN2a exhibited the highest expression at 3 days after grafting. CcPIN5 showed stable expression during Chinese hickory grafting.

To investigate how CcPINs respond to salt stress, the expression of CcPINs in the roots, stems, and leaves of Chinese hickory was determined at different time points in the salt stress treatment. CcPINs showed different expression profiles in different tissues (Figure 9). In roots, CcPIN1a was significantly upregulated after the salt stress treatment; CcPIN2a, CcPIN3, and CcPIN4 were downregulated at 3 days after the salt stress treatment; and CcPIN8a was downregulated at 1 day after the salt stress treatment. In stems, CcPIN1a, CcPIN3, and CcPIN4 were upregulated at 1 day after the salt stress treatment, while CcPIN3 and CcPIN4 were downregulated at 3 days after the salt stress treatment. The expression levels of CcPIN5, CcPIN6a, and CcPIN8a were similar to those after the CK treatment. In the leaves, most of the CcPIN genes were downregulated at 3 days after the salt stress treatment, while the expression of CcPIN8a was upregulated. The different expression profiles of CcPINs indicated that some of them might take part in the regulation of salt stress in Chinese hickory.

Discussion

Chinese hickory is a native nut tree in China that is widely cultivated in Zhejiang and Anhui provinces. The nuts of Chinese hickory are not only rich in nutrition such as saturated and unsaturated fatty acids, proteins, essential amino acids for humans, and trace elements (Peng et al., 2013; Huang et al., 2022) but also have an important role in the medicinal field (Gao et al., 2020). High nutritional values have brought huge economic benefits for farmers. In 2021, the total output value of Chinese hickory in Lin’an distinct (Hangzhou, China) was 845 million RMB (∼123.3 million dollars) (Gu, 2022). However, the slow vegetative growth and narrow growing range have limited the development of Chinese hickory. Grafting is a technique that is commonly used in horticulture and has been applied to Chinese hickory to solve the difficulties in the Chinese hickory industry. In addition, it has been predicted that the salt in soil will be increased with increasing global temperatures...
Therefore, the study of grafting and salt stress in Chinese hickory is essential. PIN is an auxin efflux protein that participates in the regulation of plant grafting and salt stress (Wang et al., 2014; Chen et al., 2015). In the present study, we identified eleven PIN genes in Chinese hickory according to the published genome. The expression profiles in grafting and salt stress were determined to elucidate the function of CcPINs.

The number of identified CcPIN genes (11) was more than that in Arabidopsis (8). Phylogenetic analysis of 132 PIN proteins in 15 plant species was carried out to compare with CcPINs. On the phylogenetic tree, CcPINs were close to CilPINs in...
C. illinoinensis, which indicated that they might come from a common ancestor. To further study the similarity and diversity of CcPIN genes, an analysis of motif and intron/exon structure was executed. The exons of CcPINs were similar, and the various intron lengths were the main reason for different gene lengths, with similar results in other species (Wang et al., 2009; Hu et al., 2021).

PIN proteins play important roles in cellular auxin transport, and their function relies on corresponding sites in conserved regions (Zhou and Luo, 2018). In contrast, functional sites distributed in non-conserved regions might be unique in different species, and the phenomenon is related to sequence evolution (Zwiewka et al., 2019). In Arabidopsis, PIN genes consist of two conserved hydrophobic loops at the N- and C-terminus and a variable hydrophilic loop in the middle (Zhou and Luo, 2018). The analysis of multiple sequence alignments and motifs showed that there were similar motifs for CcPINs, and the motifs were distributed in a gene-conserved area. It was determined that F165 in AtPIN1 controls the polar distribution of the PIN protein (Sancho-Andrés et al., 2016). Functional F165 sites were also found in CcPIN proteins, indicating that they might perform the same function.

TPRXS, a phosphorylation site, appeared in motif 8 (Figure 3), and control of the polar distribution of PIN proteins was performed by phosphorylase kinase (Bassukas et al., 2022). The NPNXY site on the conserved domain is also related to...
PIN transport. Mutants of NPNXY in AtPIN1 contributed to the accumulation of the PIN1 protein on the endoplasmic reticulum (Mravec et al., 2009). The multiple sequence alignment results show that all the CcPINs contained NPXXY in the conserved region, which suggests that the NPXXY motif performed the functional equivalent in Chinese hickory.

The plant hormone auxin regulates various developmental processes through its asymmetrical distribution on the PM (Vanneste and Friml, 2009). The different auxin distribution is executed by PINs (Wisniewska et al., 2006), and PIN-mediated auxin polar transport is crucial for auxin homeostasis. The PPI network predicted the multiple protein interactions with CcPINs through their homologous AtPINs genes. GO annotation showed that the predicted proteins were involved in many processes, and they participated in the regulation of auxin homeostasis together.

PINs are auxin efflux proteins that play important roles in plant life (Yu et al., 2022). To further explore the function of CcPINs, the expression levels from different tissues, including roots, stems, leaves, and shoots in Chinese hickory, were analyzed by qRT-PCR. The results showed that there was a high tissue-specific expression in the leaves, stems, and shoots of Chinese hickory, and the results were in accordance with those from previous studies. As previously reported, D6 PROTEIN KINASE and PINOID (PID)/WAG kinases activated auxin efflux transport PINs and influenced stem
FIGURE 9
Relative RNA expression of CcPIN genes under CK (H2O) and salt stress treatments. 0, 1, 3, and 10 d represent 0, 1, 3, and 10 days after treatment, respectively. The sample from the CK group collected 0 days after treatment was regarded as the control sample. Different letters near the points indicate a significant difference in different treatments (P < 0.05).
generated in the developing xylem (Carraro et al., 2012). OsPIN5a and OSPIN5c in rice were highly expressed in leaves. PbPIN1-3, PbPIN2-2, 
PbPIN5-1, and PbPIN6 in pear trees were highly expressed in shoots (Qi et al., 2020). The tissue-specific transcriptional levels indicate that CcPINs participate in the regulation of hickory growth and development. Therefore, it is worthy of further research to clarify the detailed regulation process in Chinese hickory.

In the successful grafting of horticultural plants, the adhesion of different stems from rootstock and scion, namely the formation of the graft union, is necessary for grafting survival. During the process, callus formation and differentiation, and the reconnection of vascular tissues, are prominent (Melnyk et al., 2018; Sharma and Zheng, 2019). Auxin contributes to callus formation after a plant is wounded (Wulf et al., 2019). The imposed exogenous auxin also accelerates vascular reconnection (Wulf et al., 2019). In Chinese hickory grafting studies, morphological changes indicated that 3, 7, and 14 days after grafting were the crucial time points for isolation layer formation, callus formation, and vascular reconnection (Liu et al., 2009). Auxin-related genes, including CcIAAs and CcGH3, exhibited different expression levels at 0, 3, 7, and 14 days after grafting.

During Arabidopsis grafting, it has been proved that AtPIN1 participates in grafting with a high expression on the scion (Melnyk et al., 2018). To explore if auxin efflux proteins coding for CcPIN genes participated in the grafting process, the relative RNA expression of CcPINs was measured in rootstocks and scions at different time points by qRT-PCR. During the obtained results, the relative RNA expression level of CcPIN1a was upregulated 6–9-fold at the time points of 0, 3, 7, and 14 days after grafting. CcPIN1a is the ortholog of AtPIN1, indicating they might perform a similar function. Further study is necessary to elucidate the additional functions of CcPIN1a with respect to grafts. In addition to CcPIN1a, other PIN genes exhibited varied expression levels in grafting except for CcPIN5. The various expression profiles of CcPIN genes in different sections at the key stages of hickory grafting indicate their potential functions in grafting regulation.

When faced with salt stress, plants activate various mechanisms to resist the adverse environment. Auxin homeostasis and dynamic rearrangement are one of the regulatory pathways. A previous study showed that phospholipase D-derived phosphatidic acid combined with PID (PINOID/WAVY ROOT GROWTH) kinases enhanced the phosphorylation of PIN2, promoted auxin polar efflux ability, and contributed to the salt tolerance of Arabidopsis (Wang et al., 2019). PIN1 and PIN3 also participated in the process (Wang et al., 2019). During salt stress in watermelon, CcPINs performed functions of resisting salt with varying expressions (Yu et al., 2017). In the present study, samples of roots, stems, and leaves under salt stress were collected to explore the CcPINs’ expression profiles of anti-salt ability in Chinese hickory. As shown in Figure 9, the expression of CcPIN2a in roots and leaves was significantly downregulated, with variations similar to those in the previous report (Wang et al., 2019).

**Conclusion**

To investigate the potential roles of auxin efflux transporters during stress treatments, eleven CcPIN genes with an ORF of 1,026–1,983 bp were identified in Chinese hickory for the first time, and their structural characteristics and expression responses to grafting and salt stress were detected by qRT-PCR. CcPINs were localized on the PM and had the closest relationships with homologous genes in C. illinoinensis and J. regia. Different hormone- and stress-responsive elements were detected on the promoter of CcPINs, indicating their expression regulation by stress treatments. CcPINs performed different functions during grafting and salt treatment. CcPIN1a has the potential to respond to grafting, while CcPIN1a and CcPIN8a might be involved in salt-stress regulation. In addition, CcPINs displayed different expression levels in different tissues, suggesting their varying roles during growth and development. Further investigations might be conducted on the mechanism of CcPINs for grafting and salt stress regulation.

**Data availability statement**

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

**Author contributions**

HY, BZ, XW, and DY conceived and designed the concept of the manuscript. YY (1st author), JM, JC, YY (4th author), and KY performed the experiments. YG, XT, HL, and HY analyzed the data. YY (1st author), JM, YG, and JC performed the formal analysis. YY (1st author), JM, and HY drafted the manuscript. AS, XW, DY, RW, BZ, and HY revised and finalized the manuscript. All authors endorsed the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.999990/full#supplementary-material
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