Genome-wide identification of XTH genes in *Liriodendron chinense* and functional characterization of *LcXTH21*

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*Liriodendron chinense* is a relic tree species of the family Magnoliaceae with multiple uses in timber production, landscape decoration, and afforestation. *L. chinense* often experiences drought stress in arid areas. However, the molecular basis underlying the drought response of *L. chinense* remains unclear. Many studies have reported that the xyloglucan endotransglucosylase/hydrolase (XTH) family plays an important role in drought stress resistance. Hereby, to explore the drought resistance mechanism of *L. chinense*, we identify XTH genes on a genome-wide scale in *L. chinense*. A total of 27 XTH genes were identified in *L. chinense*, and these genes were classified into three subfamilies. Drought treatment and RT-qPCR analysis revealed that six *LcXTH* genes significantly responded to drought stress, especially *LcXTH21*. Hence, we cloned the *LcXTH21* gene and overexpressed it in tobacco via gene transfer to analyze its function. The roots of transgenic plants were more developed than those of wild-type plants under different polyethylene glycol (PEG) concentration, and further RT-qPCR analysis showed that *LcXTH21* highly expressed in root compared to aboveground organs, indicating that *LcXTH21* may play a role in drought resistance through promoting root development. The results of this study provide new insights into the roles of *LcXTH* genes in the drought stress response. Our findings will also aid future studies of the molecular mechanisms by which *LcXTH* genes contribute to the drought response.

**KEYWORDS**

*Liriodendron chinense*, XTH family, drought stress response, genome identification, root development
Introduction

Plants are continuously exposed to various types of abiotic stress because they are sessile, and drought stress has a negative effect on the growth, yield, and cultivation of plants. Given that water scarcity reduces plant performance, improving the drought resistance of plants is a major goal of breeding efforts (Boyer, 1982; Bray et al., 2000; Cushman and Bohnert, 2000; Mittler, 2006). Plant improvement of drought resistance via molecular breeding approaches is a clear trend that further promotes the development of modern agriculture. With the various genome resources available, mining and utilizing genes that provide high resistance to drought stress will promote the development of plant molecular breeding (Parmar et al., 2017; Wai et al., 2020).

In recent years, identification and functional analysis of various abiotic stress-responsive genes and transcription factors and their applications in breeding stress-tolerant plants were favored (Roy et al., 2011; Sharma et al., 2017). For example, overexpression of HhGRAS14 in Arabidopsis thaliana significantly improved the drought tolerance of transgenic plants (Ni et al., 2022). And orphan gene PpARDT was found to be involved in drought tolerance potentially by enhancing ABA response in Physcomitrella patens (Dong et al., 2022). MdFLP enhanced drought tolerance by regulating the expression of MdNAC019 in self-rooted apple stocks (Wang et al., 2022).

The bZIP transcription factor ABP9 in maize is involved in the regulation of drought resistance, and overexpression of OsNAC10 in rice increased grain yield under drought stress (Jeong et al., 2010; Wang et al., 2017). The overexpression of a rice OsSaltT in tobacco showed increased root growth and resulted in improved drought tolerance (Kaur et al., 2022). In Manihot esculenta, MeRSZ21b was found to be involved in drought tolerance, and plants overexpressed MeRSZ21b gene had longer roots than WT (Chen et al., 2022).

Generally, there are gene families related to abiotic stress in plant genomes. The xyloglucan endotransglucosylase/hydrolase (XTH) family is a typical example (Eklöf and Brumer, 2010). XTH family belongs to the glycoside hydrolase 16 family (GH16), and XTH genes can be divided into four groups: I/II III-A, III-B, and the early diverging group (Campbell and Braam, 1999b). Increasing evidence has revealed that genes in the XTH family play an important role in drought stress. For example, plants overexpressing GmXTH23 had stronger drought tolerance and greater root lengths than wild-type (WT) plants (Long, 2020). Following overexpression of the hot pepper gene CaXTH3 in tomato, half of the stomata of transgenic plants were open under drought stress, and most of the stomata of WT plants were closed, a condition that was suggestive of transgenic plants having a higher drought tolerance than WT plants (Choi et al., 2011). XTH genes have also been shown to be involved in plant growth. For example, GUS staining of A. thaliana has shown that AtXTH genes might be expressed throughout all growth stages (Becnél et al., 2006). The expression levels of DcXTH2 and DcXTH3 increased dramatically during flowering, confirming that XTH genes play a role in petal growth (Harada et al., 2011). In addition, some plants use xyloglucan as a storage polysaccharide for embryonic development (Reid, 1985; Buckeridge et al., 2000). Due to their important roles in drought stress and growth, XTH family members have been identified in various plants, including Arabidopsis (33 genes), Oryza sativa (29 genes), Hordeum vulgare (24 genes), Populus spp. (41 genes), Ananas comosus (24 genes), and Schima superba (34 genes) (Yokoyama and Nishitani, 2001; Yokoyama et al., 2004; Geisler-Lee et al., 2006; Fu et al., 2019; Li et al., 2019; Yang et al., 2022).

L. chinense is a relict species in the Magnoliaceae family that has been widely used in timber production, landscape decoration, and afforestation. Drought stress has become a major barrier restricting the cultivation of L. chinense (He and Hao, 1999). As mentioned above, XTH family has been widely reported to be involved in drought response. However, the precise role and molecular mechanisms of the XTH family under drought stress remains unclear in L. chinense. Hereby, in order to have a better insight into the roles of XTH genes in L. chinense, we identified LcXTH genes on a genome-wide scale, uncovered LcXTH genes in relation to drought resistance and characterized their function. Overall, our study provides new insights into the possible roles of XTH genes in L. chinense and will aid future studies of drought resistance mechanisms in woody plants.

Materials and methods

Plant materials and growth conditions

L. chinense material used in this study was obtained from Xiashu Forest Station at Nanjing Forestry University, Jurong, Jiangsu, China. In April 2021, leaves, roots and leaf buds were collected from a 30-year-old L. chinense tree originating form Songyang, Zhejiang Province, and immediately frozen in liquid nitrogen, and then stored at −80℃ until further use. L. chinense seeds were soaked in water for 2 days, transplanted to soil, and cultivated in 40 cm × 30 cm × 4 cm trays for 2 months with a 16-h/8-h light/dark photoperiod. All L. chinense seedlings were watered twice a week. To investigate the response of L. chinense to drought stress, seedlings were watered with 10% PEG (100 g/L) solution. Samples of L. chinense were taken at 0, 3, 6, 12, 24, and 48 h and immediately frozen in liquid nitrogen for RT-qPCR analysis. All seedlings were grown under the same conditions, with the exception of plants in the drought stress treatment.

Tobacco (Nicotiana benthamiana) was used for transgenic assays. Before sowing, seeds were sterilized with 75% (v/v) alcohol for 30 s, NaClO (v/v) for 15 min, and double-distilled
water four times. Seeds were then sown on 1/2 MS medium (Murashige and Skoog, 2006) and vernalized at 4°C in the dark for 2 days. These seeds were placed in an incubator (SANYO, Japan) under a photoperiod 16-h/8-h light/dark photoperiod at 23°C for 10 days; they were then transplanted into MS medium and cultivated for 20 days. WT tobacco was used as a control, and all seedlings were grown in the same environment. The medium of the 30-day-old transgenic and WT tobacco seedlings was removed, and seedlings were planted in trays. Transgenic and WT tobacco plants were treated with 10% PEG (100 g/L) solution for 5 days, and root length was measured.

Identification and characterization of XTH family members in L. chinense

The two XTH domains (PF00722 and PF06955) were used as queries to search the L. chinense genome with HMMER (v. 3.0). The default settings and cutoff values were set to 0.001 (Potter et al., 2018; El-Gebali et al., 2019). Potential sequences were filtered using the Conserved Domain Search Service website (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) (Marchler-Bauer and Bryant, 2004), and the candidate genes were identified using the SMART database (https://smart.embl.de/) (Letunic et al., 2021). Redundant genes were removed manually. The molecular weight (MW), isoelectric point (pI), and protein length were analyzed using the ExPASy website (https://web.expasy.org/protparam/) (Wilkins et al., 1999). Single peptides and the subcellular localization of LcXTH genes were predicted by SignalP (https://dtu.biolib.com/) for secondary structure prediction.

Expression pattern analysis and GO annotation

The expression levels of LcXTH genes were evaluated using fragments per kilobase of transcript per million mapped reads (FPKM) values based on transcriptome data (https://www.ncbi.nlm.nih.gov/, PRJNA559687) from different tissues of L. chinense, and heat maps were constructed using TBtools (Chen et al., 2020). Gene Ontology (GO) analysis was performed using the clusterProfiler 4.0 (Wu et al., 2021).

Analysis of motifs and gene structure

The online software MEME (https://meme-suite.org/meme/doc/meme.html) was used to analyze the conserved motifs of XTH proteins with a maximum of 10 motifs (Bailey et al., 2015). The Gene Structure Display Server (http://gsds.gao-lab.org/) was used to analyze gene structure (Hu et al., 2014). The promoter sequence (2000 bp upstream of the start codon) of each LcXTH gene was extracted and then analyzed using PlantCARE online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/); the results were visualized using TBtools. The protein sequences were submitted to ESPript Web server (http://escript.ibcp.fr/ESPript/ESPript/) for secondary structure prediction.

Extraction of RNA and RT-qPCR analysis

A SteadyPure Plant RNA Extraction Kit (AG21019, Accurate Biotechnology, Hunan, Co., Ltd.) was used for RNA extraction following the user manual. The quality of RNA was assessed using a NanoDrop 2000 spectrophotometer. A260/A280 values ranged from 1.8 to 2.0, and values of A260/A230 ranged from 1.9 to 2.1; a total of 500 ng of RNA was used to synthesize complementary DNA (cDNA). RT-qPCR was used to analyze the expression profiles of LcXTH genes, and Actin97 was used as the reference housekeeping gene (Tu et al., 2019). The thermal cycling conditions for RT-qPCR were based on instructions provided in the SYBR Green Premix Pro Taq HS qPCR Kit (AG11701, Accurate Biotechnology, Hunan, Co., Ltd.). We used the 2−ΔΔCT method to calculate relative levels of expression (Livak and Schmittgen, 2001). Three biological replicates and technical replicates were conducted to ensure the accuracy of the results. And primers used in this study are listed in Table S1.
Full-length cDNA cloning of LcXTH21 and plant transformation

The coding sequence of LcXTH21 was obtained from the genome of L. chinense, and primers were designed using Oligo software (v. 7). The full-length cDNA of LcXTH21 was amplified, and an 876-bp open reading frame sequence was obtained (File S1). The cDNA of LcXTH21 was then cloned into the modified pBI-121 vector, which was digested with XbaI and BamHI QuickCut enzymes (Takara Biomedical Technology, Dalian, China). The transgene construct was introduced into Agrobacterium tumefaciens strain GV105, which was then transformed into tobacco using a leaf-disc infection method. We cut off the edge of wild-type tobacco leaves that had been cultured for about 30 days, put them in solid MS medium at 25°C for 2 days in the dark. Then we activated the transformed A. tumefaciens with liquid MS medium for 30 min to prepare an infection solution, then soaking, the leaves were cultivated continuously in the dark at 25°C for two days, then placed them at 25°C for 16 h in the light and 8 h in the dark. When callus grown on the edge of the leaves, we isolated callus and cultured on MS medium containing kanamycin. After 20 days of culture, PCR was used to determine whether the plants were positive, and positive plants were cultured until they reached maturity. The details can be seen in Figure S1.

Results

Identification and characteristics of LcXTH genes

HMM searches were used to identify XTH genes. We originally obtained 29 putative XTH genes. These 29 candidate genes were then submitted to the SMART database, and incomplete sequences were removed manually. Finally, 27 XTH genes were obtained, which were named LcXTH1–LcXTH27.

The characteristics and subcellular localization of LcXTH proteins were also predicted. The length of LcXTH proteins ranged from 243 to 337 amino acids, LcXTH26 and LcXTH27 were the largest proteins with 337 amino acids, and LcXTH17 was the smallest (243 aa). The theoretical pl values for LcXTH proteins ranged from 4.85 to 9.65, and the MW of these proteins ranged from 27.61 to 38.47 kDa. All LcXTH members were predicted to be localized to the cytoplasm. Details are provided in Table S2.

Chromosome location and homology analysis

The chromosomal location of genes is determined by prior evolutionary events. We thus investigated the chromosomal locations of LcXTH genes. LcXTH genes were randomly distributed on nine chromosomes (Figure S2). However, because the genome assembly was incomplete, the specific chromosomal locations could not be determined for two genes: LcXTH26 and LcXTH27. Most LcXTH genes were clustered on chromosomes 13, 14, and 17; chromosome 17 had 13 genes; and chromosomes 1, 3, 5, 9, and 16 had only one gene.

Syntenic within the LcXTH family was analyzed to clarify the evolutionary relationships among LcXTH genes, and three homologous pairs (LcXTH08-LcXTH12, LcXTH09-LcXTH14, and LcXTH02-LcXTH05) were identified (Figure S2). The identity of LcXTH08-LcXTH12, LcXTH09-LcXTH14, and LcXTH02-LcXTH05 was 81.30%, 67.25%, and 82.8%, respectively. The substitution rates (Ks/Ka) of these three gene pairs were calculated to assess whether LcXTH genes have been subjected to selection (Table S3). The substitution rates ranged from 0.099 to 0.114, which indicated that they have experienced purifying selection. These homologous pairs (LcXTH08-LcXTH12, LcXTH09-LcXTH14, and LcXTH02-LcXTH05) diverged approximately 66.42, 188.19, and 67.38 million years ago, respectively. We also evaluated the density of these genes across the entire genome. The density of these genes was high in regions with related homologous genes.

Tandem duplication is an important mechanism underlying the expansion of gene families, and tandemly duplicated genes often occur in clusters (Kozak et al., 2009). Hence, we calculated the protein similarity matrix to investigate the identity of LcXTH genes in three gene clusters (Figure S3 and Table S4). The identity of the LcXTH genes on chromosome 13 was 60.54%, and the identity of the three LcXTH genes on chromosome 14 ranged between 58.46% and 73.29%. Chromosome 17 contained 13 LcXTH genes, and the identity ranged from 47.16% to 96.59%. We speculate that the high identity of these closely arranged genes indicates that they are products of tandem duplication; generally, tandem duplication might be the major force driving the expansion of LcXTH genes.

Phylogenetic analysis of XTH proteins

To further investigate the evolutionary relationships among LcXTH family members, we constructed a phylogenetic tree using 113 XTH proteins. (Figure 1). Phylogenetic analysis revealed that all XTH proteins were classified into four groups (group I/II, group III-A, group III-B, and the early diverging group). In L. chinense, most LcXTH genes (24) were categorized into group I/II. Only one gene (LcXTH03) was classified in group III-A. The remaining genes (LcXTH26 and LcXTH27) were grouped into III-B. No genes were classified into the early diverging group in L. chinense, which might stem from the incomplete genome annotation or gene loss.
Structure and motif patterns of LcXTH genes

To investigate the structure of LcXTH proteins, we submitted the protein sequences to the SMART database to identify conserved domains. We analyzed the phylogenetic analysis of LcXTH proteins and two domains (Glyco_hydro_16 and XET_C) were identified in all LcXTH proteins (Figure 2A and Figure 2B). XET_C is a unique domain among GH16 family members, and the proteins in this family had a common structure known as the \( \beta \)-jellyroll fold (Atkinson et al., 2009; Eklöf and Brumer, 2010; Behar et al., 2018). We also performed conserved motif analysis on LcXTH proteins (Figure 2C) and found that the motif composition of LcXTH family members was similar. As shown in the schematic, the Glyco_hydro_16 domain included motifs 10, 6, 8, 3, 4, 2, and 1, and the XET_C domain included motifs 9, 5, and 7. Motifs 1, 3, 4 \((\text{ExDxE})\), and 5 were conserved in all LcXTH proteins.

Previous studies have shown that the exon distribution of AtXTH genes is conserved within each subfamily in \textit{A. thaliana} (Yokoyama and Nishitani, 2001; Yokoyama and Nishitani, 2008). We used the online tool GSDS 2.0 to analyze the exon–intron organization of the 27 LcXTH genes (Figure 2D). There were three or four exons in LcXTH genes, and the ExDxE domain was randomly distributed in these genes, with exception of the fourth exon. The signal peptides of LcXTH proteins were predicted. A total of 23 LcXTH proteins had signal peptides, and they were all located on the first exon. These short amino acid sequences might be responsible for transmembrane transport and have secretory functions.

Structure-based sequence alignment

To further characterize LcXTH proteins, two fully resolved structures of PttXET16-34 (PDB ID: 1UN1) and TmNXG1 (PDB ID: 2UWA) were used to characterize the secondary structures of XTH proteins with ESPript software. The schematic of the secondary structures shows that all LcXTH proteins contained the conserved ExDxE domain (Figure 3). The first glutamic acid residue (E) acts as a catalyzed nucleophile, which typically initiates enzymatic reactions, and the second E residue acts as a base that activates the entering substrate (Fu et al., 2019). The N-glycosylation domain \((\text{NXT/S/Y})\) is thought to be critical for protein stability and is indicated in the figure. This N-glycosylation site was conserved in all group I/II proteins, but it was missing in nearly all group III-A XTH proteins (Eklöf and Brumer, 2010). However, the N-glycosylation site was observed in all LcXTH members, including LcXTH03 (a member of group III-A). The N-glycosylation sites of LcXTH proteins in group III were shifted by approximately 20 amino acids from the ExDxE domain to the C-terminus.
The architecture of proteins was conserved within specific groups; for example, other conserved domains adjacent to the ExDxE domain were identified in LcXTH proteins, which were referred to as loop 1, loop 2, and loop 3. Previous studies have demonstrated that the extension of loop 2 plays a key role in determining the activity of XTH proteins (Baumann et al., 2007). In this study, Loop 2 was significantly shorter in groups I/II and III-B than in group III-A, suggesting that the difference in the length of loop 2 among subfamilies of *L. chinense* might partly account for the differences in the classification of these proteins and their functions. The sequence DWATRGG of loop 3 was present in most group I/II proteins; however, this sequence was replaced by SWATEN in group III-A members.

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Tissue expression patterns and GO analysis of LcXTH genes

We analyzed the expression patterns of LcXTH genes across several tissues (including bracts, sepals, petals, stamens, pistils, leaves, and shoots) (Figure 4A). LcXTH genes showed tissue-specific expression patterns. LcXTH04, LcXTH12, LcXTH08, LcXTH25, and LcXTH26 were highly expressed in bracts, and the expression levels of these genes in other tissues were low. LcXTH07, LcXTH16, and LcXTH27 were significantly expressed in leaves. The expression levels of LcXTH03, LcXTH18, and LcXTH10 were high in pistils, suggesting that they are involved in pistil development. The expression levels of almost all LcXTH genes were low in sepals, petals, and stamens, suggesting that LcXTH genes were not expressed during flowering. The expression patterns of the three tandem arrays on chromosomes 13, 14, and 17 were not consistent. LcXTH13, LcXTH19, LcXTH20, LcXTH21, LcXTH22, and LcXTH24 were significantly expressed in shoots and had similar expression patterns, indicating that their functions might be redundant. However, the expression patterns of the other genes on chromosome 17 differed, suggesting that they might have acquired new functions. In addition, LcXTH01, LcXTH02, LcXTH11, and LcXTH17 were not expressed in any of the tissues examined in this study; this indicates that they are not expressed or have specific expression patterns that could not be detected in this study.

GO analysis was performed to clarify the functions of LcXTH genes. All LcXTH genes encoded proteins with xyloglucan xyloglucosyl transferase and hydrolase activity, and they were all localized to the cell wall and the apoplast, which was consistent with the subcellular localization prediction (Figure 4B). All LcXTH genes were predicted to be involved in cellular glucan metabolic process and carbohydrate metabolic process.

Cis-element prediction and RT-qPCR analysis

To determine the expression patterns and regulatory characteristics of LcXTH genes, the 2000-bp upstream sequence of the translation initiation site (ATG) was extracted, and cis-elements were predicted (Figure 5A). A large number of cis-elements were involved in plant growth and development, stress responses, and phytohormone responses (Figures 5B, C). In the first category, the main cis-elements were motif CAT-box (33.64%), motif CCAAT-box (27.1%), motif O2-site (23.36%), and GCN4-motif (11.21%). Cis-elements involved in stress responses mainly included the ARE motif (40.78%), MBS motif (30.1%), LTR motif (14.56%), TC-rich repeats (7.77%), and GC-motif (5.83%). In the third category, the main cis-elements were abscisic acid-responsive element (ABRE, 58.48%), methyl jasmonate-responsiveness element (CGTCA, 14.8%), and auxin-responsive element (TGA-element, 7.97%). The most abundant phytohormone response element is associated with abscisic acid (ABA)-responsiveness, indicating that LcXTH genes might be regulated by ABA.

To clarify the roles of LcXTH family members in drought resistance, we used BLASTp to identify stress-related LcXTH genes based on previous studies, and genes containing plant defense and stress elements (TC-rich motifs) were screened out.
A total of eight LcXTH genes (LcXTH07, 15, 18, 19, 20, 21, 25, and 27) were identified, and their expression patterns under drought stress were clarified. The RT-qPCR results revealed that there were significant differences in the expression of these genes at different times under drought stress (Figure 6). LcXTH07 expression was up-regulated at 0 and 6 h, down-regulated at 6 and 24 h, and highest at 48 h. Some genes exhibited similar expression patterns. For example, the expression patterns of LcXTH18, 19, 20, and 25 did not change significantly during 0 and 6 h, but significantly increased at 12 h. LcXTH21 exhibited the most rapid and strongest response to drought stress, and it was the most highly expressed gene. Some genes exhibited opposite expression patterns; the expression levels of LcXTH15 and LcXTH27 changed slightly, indicating that they might not play important roles in the response to drought stress.

Overexpression of LcXTH21 in tobacco promoted root development under drought stress

In light of the strong response of LcXTH21 under drought stress, we cloned LcXTH21 and overexpressed it in tobacco to analyze its function; the characteristics of transgenic and WT tobacco plants were noted. Then, we cultivated transgenic plants and WT on 1/2 MS medium containing different PEG concentration. With the increase in PEG concentration, the root length decreased to varying degrees. Ten-day-old transgenic plants on 1/2 MS medium had an average root length of 1.26 cm, which was 68% longer than that of WT plants (Figures 7A, B). Under 5% PEG, the average root length in transgenic plants decreased by 52%, however, the WT root length decreased by 79% (Figures 7C, D). When the PEG
concentration was increased to 10%, the average root length of transgenic tobacco and WT decreased by 95% and 154%, respectively (Figures 7E, F). Next, we treated thirty-day-old seedlings with 10% PEG for 5 days, and the root length were compared. The average root length was 2.11-fold that of WT plants (Figures 7G, H). To investigate the expression level between root and aboveground, we took samples of root and aboveground for RT-qPCR analysis. The results shown that the expression level of root was 42.06-fold than that of aboveground tissues/organs (Figure 7I). The plant hormone ABA plays a key role in regulating the resistance of plants to drought stress, whereas the NCED enzyme is a key rate-limiting ABA biosynthetic enzyme (Leung and Giraudat, 1998; Zhang et al., 2009; Zhu et al., 2017). Hence, to further study the relationship of the LcXTH21 genes and ABA signaling, we performed RT-qPCR analysis to detect the expression of NCED gene in thirty-day-old WT and transgenic tobacco (Figure 7J). The results showed that the relative expression level of NCED in transgenic plants was about 45-fold higher than that in WT controls, thereby suggesting that LcXTH21 might contribute to drought resistance by promoting ABA biosynthesis.

Discussion

The evolution and structure of LcXTH family members

The evolution of the XTH family has received wide research interest. XTH genes were first detected in Zygnematophyceae and non-charophycean taxa; bacterial licheninases have long been considered non-plant ancestors of the XTH family because of their sequence similarities (Barbeyron et al., 1998; Michel et al., 2001; Del Bem and Vincentz, 2010; Behar et al., 2018). The expansion of XTH genes has also received much research interest. The relaxation of substrate specificity, including the broader specificity of group I/II members, might have contributed to the expansion of group I/II members (Shinohara and Nishitani, 2021). Tandem duplication is one of the important mechanisms underlying the expansion of XTH family members, and tandem duplications comprise 4.56% of the genome of L. chinense (Chen et al., 2019). We detected a large number of tandemly duplicated LcXTH genes, accounting for 66.7% of all LcXTH genes, indicating that tandem duplication is the main mechanism underlying the expansion of LcXTH family genes. All the tandem repeats identified in this study were group I/II members, indicating that tandem duplication has been a particularly important mechanism underlying the expansion of group I/II members. Previous studies have shown that some gene family members have gained new functions or undergone defunctionalization (Lynch and Conery, 2000). In this study, tandemly duplicated genes on chromosome 17 were of particular interest. The expression patterns of LcXTH13, 19, 20, 21, 22, and 24 were similar, and their functions might have been retained after genome duplication. The expression patterns of LcXTH16, 18, and 25 were inconsistent. The differential expression of these genes indicates that they might have gained new functions. LcXTH01, 02, 11, and 17 were not expressed in any tissues in this study, suggesting that they might have undergone defunctionalization.

Understanding the classification of subfamilies is important for studying the evolution of the XTH gene family. Previous studies of monocotyledonous rice and dicotyledonous A. thaliana have shown that group I and group II members are difficult to distinguish; they have thus been usually classified into one group (group I/II) (Yokoyama et al., 2004). However, both sequence analysis and catalytic measurements have confirmed the divergence of group III (Fanutti et al., 1993; Yokoyama et al., 2004). In this study, a total of 27 LcXTH genes were identified, and they were grouped into three subfamilies. None of these genes were classified in the early diverging group, which might
stem from an incomplete genome assembly or gene loss. Domain loop 2 in XTH family members plays an important role in subfamily classification, and previous studies have shown that the length of loop 2 contributes to the difference in the activity between XET and XEH members (Baumann et al., 2007). For example, the extension of loop 2 is thought to be the major structural change responsible for its endohydrolase activity in *Fragaria vesca* (Opazo et al., 2017). In this study, the extension of loop 2 was only observed in group III-A, and this might be an important factor contributing to the divergence of group III members. Glycosylation as one of key post-translational modifications can affect stability and the molecular weight of the target proteins (Ahmadizadeh et al., 2020; Heidari et al., 2020). In addition, proteins associated with the secretory pathway are firstly glycosylated in the endoplasmic reticulum. The above suggests that XET proteins may be involved in the secretory pathway. In this study, the N-glycosylation domain was observed in all LcXTH members, and previous research has demonstrated that the removal of this region significantly reduces the stability of some XET proteins (Campbell and Braam, 1999a; Kallas et al., 2005).

**The roles of LcXTH family members in abiotic stress and root development**

Stomatal closure is one of the first events that takes place in response to drought stress to prevent water evaporation,
and \(XTH\) genes play a crucial role in modifying the cell wall and altering its elongation under drought stress, which improves drought resistance (Kim et al., 2010). For example, overexpression of \(HvXTH1\) in barley resulted in the enlargement of the stomata of transgenic plants relative to those of WT plants under drought treatment, indicating that transgenic plants were more drought tolerant (Fu, 2019). Overexpression of \(XTH\) genes from rose (\(Rosa\) \(rugosa\)) increased the drought tolerance of transgenic China rose plants (Chen et al., 2016). In addition, the presence of xyloglucan in early land plants suggests that \(XTH\) gene family members have played a key role in the transition from wetter to drier habitats (Popper, 2008). In our research, the drought treatment and RT-qPCR analysis showed that six \(LcXTH\) genes significantly responded to drought stress. The expression patterns of \(LcXTH18\), 19, 20, and 25 were similar, suggesting that they exhibit similar responses to drought stress. ABA is an important signal in plants that mediates the response to drought stress. The most abundant phytohormone response element of \(LcXTH\) genes was associated with the ABA response (ABRE motif, 58.48%). Moreover, the augmented NCED expression levels detected in transgenic tobacco indicated that the increased drought stress resistance provided by the \(LcXTH21\) transgene probably involved the promotion of ABA biosynthesis.

The “balanced growth” hypothesis proposes that some plants can stimulate or maintain root growth while reducing shoot growth in response to drought stress (Bloom and Mooney, 1985). In \(Eucalyptus\) \(globulus\), a drought-tolerant clone was found to have higher root growth rate than a drought-sensitive one (Costa et al., 2004). Moreover, the development of the root system largely determines the performance of plants under drought conditions. Thus, increased root biomass is one of the primary mechanisms used by plants to avoid, or reduce, drought stress (Kashiwagi et al., 2005). For example, the overexpression of \(OsNAC5\) was found to enhance drought tolerance by increasing root diameter (Jeong et al., 2013). \(XTH\) gene family members have been widely studied considering the different roles they are known to play in root development. For example, seven \(XTH\) genes from rice were specifically expressed in the roots of seedlings (Yokoyama et al., 2004). \(AtXTH19\) and \(AtXTH23\) were involved in lateral root development via the \(BES1\)-dependent pathway, indicating that \(XTH\) genes play a role in root development (Xu et al., 2020). Later, the expression levels of \(AtXTH11\), \(AtXTH29\), and \(AtXTH33\) in the roots and aboveground organs were found to differ in \(A.\) \(thaliana\) plants subjected to high temperature and drought stress, thereby suggesting that these genes might mediate rapid responses to drought stress (De Caroli et al., 2021). In a study performed in grapevine, the transcription levels of \(VvXTH\) genes presented the largest changes in roots and leaves under drought and salt stress, indicating that \(VvXTH\) genes vigorously respond to abiotic stress in leaves and roots (Qiao et al., 2022).

Molecular breeding is a promising approach and great progress has been made in its use for improving the efficiency of plant breeding programs (Varshney et al., 2009). Combined with molecular marker-assisted selection, greater and faster genetic progress can be achieved. For example, in a study of \(Triticum\) \(aestivum\), Iquebal et al. (2019) found that the maximum number of drought responsive quantitative trait loci were detected at the seedling stage and further analyzed the regulatory networks of key candidate genes and their roles in responding to drought stress in order to identify putative markers for breeding applications. Besides, in a recent study, Chauhan et al. (2022) used morphological, biochemical, and molecular markers from \(Withania\) \(sомнiferа\) to assess the 25 accessions of Indian ginseng, and concluded that these markers could be used to select superior ginseng genotypes. In addition, \(XTH\) genes were also reported to be involved in other abiotic stress, such as salt, heat and cold stress (Han et al., 2017; Hidvégi et al., 2020; De Caroli et al., 2021). Hence, we suggest that \(LcXTH\) genes play roles in drought resistance and other abiotic stresses. In this study, we performed functional characterization of \(LcXTH21\), and transgenic tobacco showed higher drought resistance and more developed roots during seedling stage. Therefore, \(LcXTH\) genes could be potential functional markers when conducting marker-assisted-selection (MAS) for breeding varieties with high resistance to abiotic stress. As an example, we could select genotypes with high \(LcXTH21\) expression levels and these MAS genotypes could be expected to have higher resistance to drought stress at the adult stage. This procedure could represent an alternative way to breed \(L.\) \(chinense\) varieties with increased stress resistance associated with a highly developed root system in the coming decade.

**Conclusion**

In this study, 27 \(LcXTH\) genes were identified, and they were divided into three subfamilies. Tandem duplication was probably the major contributor to the expansion of the \(LcXTH\) family, and six \(LcXTH\) genes significantly responded to drought stress. Overexpression of \(LcXTH21\) in tobacco resulted in a more developed root system. In summary, these findings enhance our understanding of the \(LcXTH\) gene family and lay the foundation for further exploration on drought resistance mechanisms in \(L.\) \(chinense\).

**Data availability statement**

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA559687.
Author contributions

JW performed the experiments, analyzed the data and wrote the manuscript. YZ contributed to the data analysis and preparation of the manuscript. ZH took a role in experimental design and data analysis. ZH analyzed the data. LY, WL and ZC participated in plant sample collection and experimental assay. HL conceived the project, designed the experiments and revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.1014339/full#supplementary-material

Supplementary FIGURE 1

Different transgenic plant stages.

Supplementary FIGURE 2

Distribution and synteny of LcXTH genes in L. chinense chromosomes. Blue lines indicate synteny between LcXTH genes. Different colors indicate different chromosomes and syntenies. Gene densities are shown as red lines in the box. And the LcXTH genes were marked.

Supplementary FIGURE 3

The identity heat map of LcXTH genes.

Supplementary TABLE 1

The primer used in this study.

Supplementary TABLE 2

The characteristics and subcellular localization of LcXTH proteins.

Supplementary TABLE 3

The substitution rates (Ka/Ks) and diverging time of LcXTH gene pairs.

Supplementary TABLE 4

The protein similarity matrix of LcXTH genes.

Supplementary TABLE 5

The GO annotations of LcXTH genes.

FILE S1

The protein sequences used in phylogenetic tree constructing, and open reading frame sequence of LcXTH2L.

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