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Molecular evolution and functional characterization of chitinase gene family in *Populus trichocarpa*

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ABSTRACT

Chitinases, the chitin-degrading enzymes, have been shown to play important role in defense against the chitin-containing fungal pathogens. In this study, we identified 48 chitinase-coding genes from the woody model plant *Populus trichocarpa*. Based on phylogenetic analysis, the *Populus* chitinases were classified into seven groups. Different gene structures and protein domain architectures were found among the seven *Populus* chitinase groups. Selection pressure analysis indicated that all the seven groups are under purifying selection. Phylogenetic analysis combined with chromosome location analysis showed that *Populus* chitinase gene family mainly expanded through tandem duplication. The *Populus* chitinase gene family underwent marked expression divergence and is inducibly expressed in response to treatments, such as chitosan, chitin, salicylic acid and methyl jasmonate. Protein enzymatic activity analysis showed that *Populus* chitinases had activity towards both chitin and chitosan. By integrating sequence characteristic, phylogenetic, selection pressure, gene expression and protein activity analysis, this study shed light on the evolution and function of chitinase family in poplar.

1. Introduction

Chitin, a linear homopolymer of β-1,4- linked *N*-acetyl glucosamine residues, is a major component of fungal cell walls and arthropod exoskeletons (Austin et al., 1981; Collinge et al., 1993; Passarinho and de Vries, 2002). Chitinases (EC 3.2.1.14) catalyze the hydrolytic cleavage of the β-1,4-glycoside bond in chitin (Kasprzewska, 2003). In fungi and arthropods, chitinases play important roles during their growth and development. For example, in fungi, chitinases participate in cell wall structure remodeling and daughter cell separation (Cohen-Kupiec and Chet, 1998; Patil et al., 2000; Shimono et al., 2002). In insects, these enzymes take part in the shedding of old cuticle and turnover of peritrophic matrix (Merzendorfer and Zimoch, 2003). Although plants lack chitin, chitinases are found to be widely distributed in plant kingdom, such as the bryophyte *Physcomitrella patens*, lycophyte *Pteris ryukyuensis*, gymnosperm *Pinus strobus*, and angiosperm *Arabidopsis thaliana* (Wan et al., 2008; Kobaru et al., 2016; Onaga and Taira, 2008; Wu et al., 1997; Verburg and Huynh, 1991).

In plants, chitinase genes usually form multigene families. For example, 67, 42 and 49 chitinase genes were found in the woody plants *Eucalyptus grandis, Vitis vinifera* and *Camellia sinensis*, respectively (Tobias et al., 2017; Zheng et al., 2020; Bordoloi et al., 2021). The nonwoody plants *A. thaliana*; *Oryza sativa*, and *Gossypium raimondii* has 24, 37 and 47 chitinase genes, respectively (Passarinho and de Vries, 2002; Xu et al., 2007; Xu et al., 2016). Based on the characteristic catalytic domain, chitinases are grouped into glycoside hydrolase family 18 (GH18) and glycoside hydrolase family 19 (GH19) (Henrissat, 1991). GH18 chitinases are widely distributed in bacteria, fungi, yeast, viruses, plants and animals, whereas GH19 members are mainly found in plants (Passarinho and de Vries, 2002). Until now, all plants are reported to have both GH18 and GH19 chitinases (Passarinho and de Vries, 2002; Kobaru et al., 2016; Xu et al., 2007; Xu et al., 2016).

Based on the ability of degrading chitin, plant chitinases have been considered as a defense mechanism against fungal pathogen. In vitro,

Abbreviations: GH18, glycoside hydrolase family 18; GH19, glycoside hydrolase family 19; LRT, likelihood ratio test; SA, salicylic acid; MeJA, methyl jasmonate; ML, maximum-likelihood; CHI, chitinase; MRCA, most recent common ancestor.

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purified plant chitinases inhibited fungi spore germination and hypha growth. For example, chitinases from thornapple, tobacco, and wheat can inhibit spore germination and hyphal growth of *Trichoderma hamatum* and *Phycomyces blakesleeanus* (Broekaert et al., 1988); combinations of purified pea chitinase and β-1,3-glucanase strongly inhibited the growth of eight tested fungi (Mauch et al., 1988); a purified *A. thaliana* chitinase protein is an effective inhibitor of the growth of *Trichoderma reesei* (Verburg and Huynh, 1991). In vivo experiments showed overexpression of plant chitinase enhanced the transgenic plant resistance to fungal pathogen. Transgenic rice expressing a bitter melon class I chitinase gene conferred enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani* (Li et al., 2009); transgenic wheat expressing a rice chitinase gene exhibited enhanced resistance to stripe rust disease (Huang et al., 2013); overexpression of a chitinase gene from *Leymus chinensis* improves resistance to both pathogen and saline-alkali stress in transgenic tobacco and maize (Liu et al., 2020). Besides the antifungal function, plant chitinases have also been shown to be involved in many other aspects, such as anti-herbivore defense, abiotic stress tolerance, symbiotic associations, plant growth and development (Zhong et al., 2021; Takenaka et al., 2009; Wu et al., 2009; Salzer et al., 2000; Calabrese et al., 2017; Sánchez-Rodríguez et al., 2012; Grover, 2012).

Populus trichocarpa is the most important woody model plant with genomics currently available (Tuskan et al., 2006). Compared to annual herbaceous plants, the perennial woody plants have much longer generation time. The constantly changing abiotic and biotic stresses during their long-life cycle confront woody plants with more complex challenges. Woody plants might have evolved unique traits to cope with these stresses. Some of these traits might be related to the functional evolution of the resistance-related gene families. In a previous study, 37 chitinase genes were identified from *P. trichocarpa* by keyword search (Jiang et al., 2013). In this study, re-identification by TBLASTN and manual re-annotation were performed to confirm the chitinase genes in *P. trichocarpa*, and identified 48 *Populus* chitinase genes. By integrating gene sequence, gene structure, protein domain, molecular evolution, gene expression pattern and protein activity analyses, we provide detailed characterization of chitinase gene family in *P. trichocarpa*.

2. Materials and methods

2.1. Identification of chitinase genes from the Populus genome

To identify the *Populus* chitinase gene family, TBLASTN was conducted to search against the *Populus* genome version 3.0 (https://phytozome.jgi.doe.gov/pz/portal.html) using *Arabidopsis* chitinase protein sequences as templates (Passarinho and de Vries, 2002). The identified *Populus* chitinase candidates were then verified using the HMMER web server (https://www.ebi.ac.uk/Tools/hmmer/) (Potter et al., 2018) to confirm the presence of chitinase protein domain in their protein structures.

2.2. Phylogenetic analysis of chitinase gene family

The full-length GH18 and GH19 chitinase protein sequences were aligned by MUSCLE program in MEGA 6.0 software with default parameters, respectively (Tamura et al., 2013). The alignments were then trimmed manually. The best models were identified using the Models module in MEGA 6.0. The maximum-likelihood trees were then constructed in MEGA 6.0 using WAG + G (the GH18 chitinase trees) or WAG + G + I (the GH19 chitinase trees) model with pairwise deletion of gaps, and were tested with 1,000 bootstrap replicates.

2.3. Molecular evolution analysis

The chitinase coding sequences were translated into protein sequences, aligned using the MUSCLE program in MEGA 6.0 and reverse translated into the corresponding nucleotide alignment. To evaluate variation in selective pressures among different *Populus* chitinase groups, the branch models of CODEML in PAML package were used to estimate ω value [ratio of nonsynonymous substitutions (d_N) and synonymous substitutions (d_S)] under two assumptions: a one-ratio model that assumes the same ω ratio for all branches; and a three-ratio model (GH18 chitinases) or four-ratio model (GH19 chitinases) that allows different ω ratio for the groups (Yang, 2007). To verify which of the models best fits the data, likelihood ratio tests (LRTs) were performed by comparing twice the difference in log-likelihood values between pairs of the models using a χ^2 distribution.

2.4. Expression of Populus chitinase genes under different treatments

To investigate the expression patterns of the *Populus* chitinase genes under normal and treatment conditions, seedlings of *Populus* were cultivated in potting soil at 25 °C under 14 h light/10 h dark conditions in a growth chamber for two months before treatment. The chitosan (acetylation $\leq$ 10%), chitin, salicylic acid (SA), and methyl jasmonate (MeJA) were all dissolved in water. The seedlings were then sprayed with 150 μg/mL chitosan, 150 μg/mL chitin, 5 mM SA, and 0.1 mM MeJA, respectively. After four hours of chitosan, chitin or MeJA treatment, or 24 h of SA treatment, the roots, stems and leaves of the *Populus* seedlings were collected. Total RNAs were extracted from these samples using an RNAprep Pure Plant Kit (Polysaccharides&Polyphenolics-rich) (TIANGEN, Beijing, China). The extracted RNAs were then reverse-transcribed into cDNA using an RNA PCR Kit (AMV) version 3.0 (TaKaRa, Dalian, China). Based on the *Populus* chitinase gene sequences, specific PCR primers were designed (Table S1). PCR reaction was performed in a volume of 20 μL containing 1.0 μL first-strand cDNA, 2.0 μL TaKaRa 10 × PCR buffer, 0.1 μL TaKaRa Ex Taq (5 U/μL), 0.8 μL dNTP (2.5 mM each), 0.4 pmol primer, and 15.7 μL H_2O. PCR conditions consisted of an initial denaturation of 3 min at 94 °C, followed by cycles of 30 s at 94 °C, 40 s at 58 °C and 30 s at 72 °C with a final extension of 3 min at 72 °C. In all PCR analyses, the Actin gene was used as an internal control. The PCR products were each analyzed using a 1% agarose gel and validated by DNA sequencing. Three biological replicates were used in all gene expression analyses.

2.5. Populus chitinase protein expression and purification

To investigate the enzymatic activities of the chitinase proteins, the nucleotide sequences corresponding to the proteins without their signal peptide sequences were subcloned into pET-32a (Novagen, Madison, WI, USA) vector to express 6 × His-tagged fusion proteins using specific primers containing restriction sites (Table S1). The recombinant plasmids were then transformed into *Escherichia coli* Origami B (DE3) competent cells. Overnight cultures of the bacteria harboring the recombinant plasmids were diluted 1:100, and grown until the optical density (A_{600}) reached 0.5. Synthesis of the recombinant chitinase proteins was induced by isopropyl-beta-d-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After incubation at 37 °C or 20 °C overnight, the bacteria were harvested by centrifugation at 8,000 × *g* for 3 min at 4 °C, resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.4), and disrupted by cold sonication. The soluble fraction was separated from the insoluble fraction by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatant which containing soluble recombinant protein was then mixed with Ni Sepharose High Performance affinity media (Amersham Pharmacia Biotech, Piscataway, NJ, USA) that had been preequilibrated with binding buffer for 40 min at room temperature. After the unbound contaminants were removed by washing with binding buffer, the bound proteins were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4).

2.6. Enzymatic activity characterization

The chitinase activity was determined by a modification of a previous method with colloidal chitin and chitosan (acetylation ≤ 10%) as the substrates (Imoto and Yagishita, 1971). The reaction mixture contained 550 μL 0.3% colloidal chitin or chitosan, 70 μL 300 mM sodium acetate buffer, pH 5.0, and 50 μL eluate. After incubation at 37 °C for 10 min, the reaction mixture was put on ice immediately to terminate the reaction. A 900 μL of color reagent solution (0.5 g/L potassium fericyanide dissolved in 0.5 M sodium carbonate) was then added to the reaction, and boiled for 15 min. After cooling and centrifugation at 12,000 × *g* for 2 min, the absorbance of the supernatant at A_{420} was measured. One unit of chitinase activity was defined as the amount of enzyme which produces 1 μmol of reducing sugar per min. Protein concentrations were determined by measuring absorbance at 280 nm.

3. Results

3.1. Sequence characteristics of Populus chitinase genes

Forty-eight full-length genes encoding putative chitinase proteins were identified from *P. trichocarpa* genome by TBLASTN search (Table S2). Two sequences (*PtCHI11_ps* and *PtCHI27_ps*) of them were considered to be putative pseudogenes based on the presence of frame shifts disrupting the coding regions, resulting in truncated proteins. After revising the frame shifts by deleting two or four nucleotides, these two full-length sequences were included in the phylogenetic and gene expression analyses. Twenty-seven and 21 proteins of the candidates were confirmed to contain glycoside hydrolase family 18 (GH18) or GH19 chitinase domain by HMMER web server (https://www.ebi.ac.uk/Tools/hmmer/) (Potter et al., 2018). Seven (PtCHI-like18, 20, 29, 36, 37, 38 and 39) of the *Populus* chitinases lacked the essential catalytic residues (Fig. S1), therefore they are considered as chitinase-like (CHI-like) proteins. In addition to the full-length chitinase genes, 15 partial chitinase fragments were identified in the *Populus* genome (Table S3). The length of these chitinase fragments ranged from 39 to 253 amino acids. These fragments contained partial GH18 domain, GH19 domain, or had the highest sequence similarity to chitinase according to BLASTP search in NCBI. In this study, these chitinase fragments were also considered to be pseudogenes.

Using the maximum-likelihood (ML) method, we constructed the phylogenetic relationships among the GH18 and GH19 chitinase genes, respectively (Fig. 1). On the ML trees, the GH18 and GH19 chitinase genes were grouped into three (groups I, II and III) and four (groups IV, V, VI and VII) groups, respectively. *Populus* groups I, II, III, IV, V, VI and VII chitinase genes contained 12, 5, 10, 10, 7, 2 and 2 members, respectively. Pairwise comparison within GH18 and GH19 families showed that they had pairwise protein sequence identity ranging 4.3% – 99.3% and 20.5% – 95.2%, respectively (Fig. S2).

Gene structure analysis showed that the seven chitinase groups have different exon–intron pattern (Fig. 2). For GH18 chitinase genes, except *PtCHI35* with two introns, all the other group I and II chitinase genes have no intron. However, highly variable gene structures were observed in *Populus* group III chitinase genes. Among the 10 group III chitinase genes, six have one intron, one has five introns, and the other four have six introns. For GH19 chitinase genes, two or three introns were observed in group IV; group V and VI chitinases only have one intron; the two group VII chitinases have two introns.

3.2. Domain architecture of Populus chitinases

Analysis of domain architecture showed that all the groups I and II chitinases only contain GH18 domain (Fig. 2C). Compared to groups I and II chitinases, highly variable domain architectures were observed in group III chitinases. Among the 10 group III chitinases, four (PtCHI8, 9, 40 and 41) only have GH18 domain. PtCHI-like36, 37, 38 and 39 contain both GH18 and protein kinase domain. In addition to GH18 domain, PtCHI10 and PtCHI11_ps proteins also contain partial LPXTG-anchored fibronectin-binding protein domain and adventurous gliding motility protein domain, respectively. Besides the GH19 domain, all the groups IV and V chitinases also possess carbohydrate-binding module family 18 (CBM18) domain. The groups VI and VII chitinases only have GH19

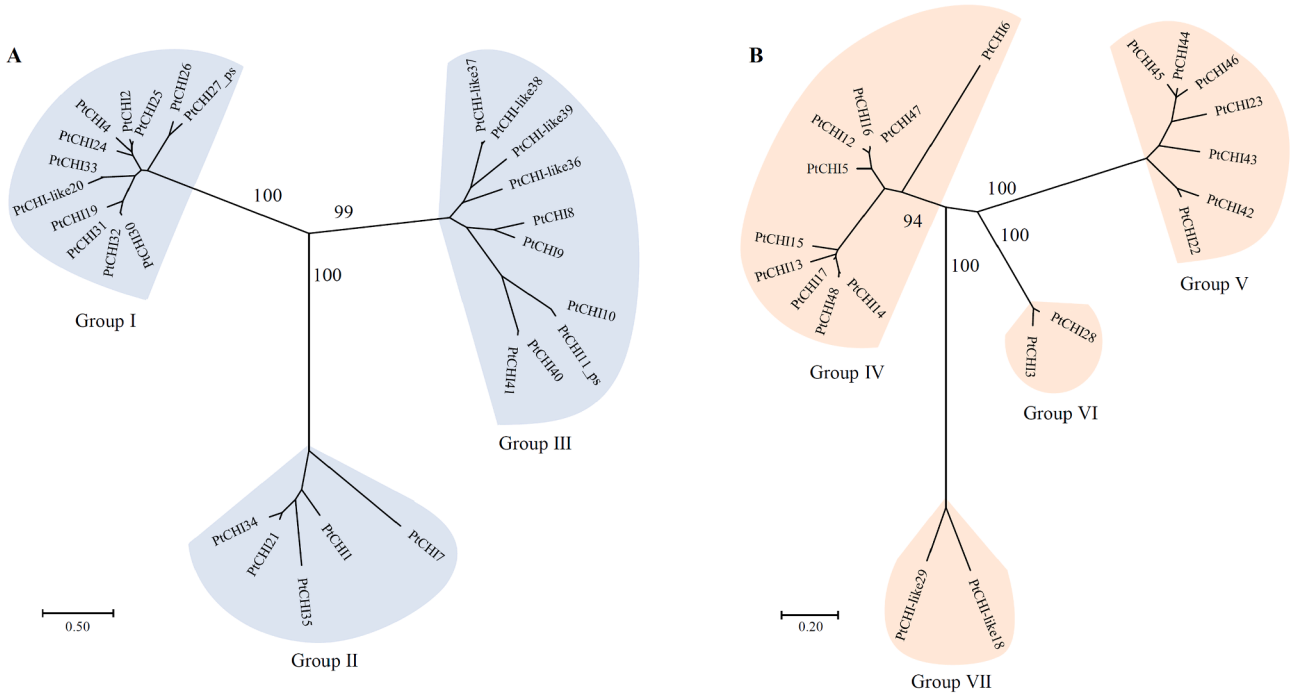

Fig. 1. Phylogenetic tree of the chitinases (CHIs) from *Populus trichocarpa* belonging to glycoside hydrolase family 18 (GH18, **A**) and glycoside hydrolase family 19 (GH19, **B**), respectively. The tree was constructed using the maximum-likelihood (ML) procedure. Numbers at the internal branches leading to the chitinase groups indicate the percentage bootstrap support from 1,000 replicates.

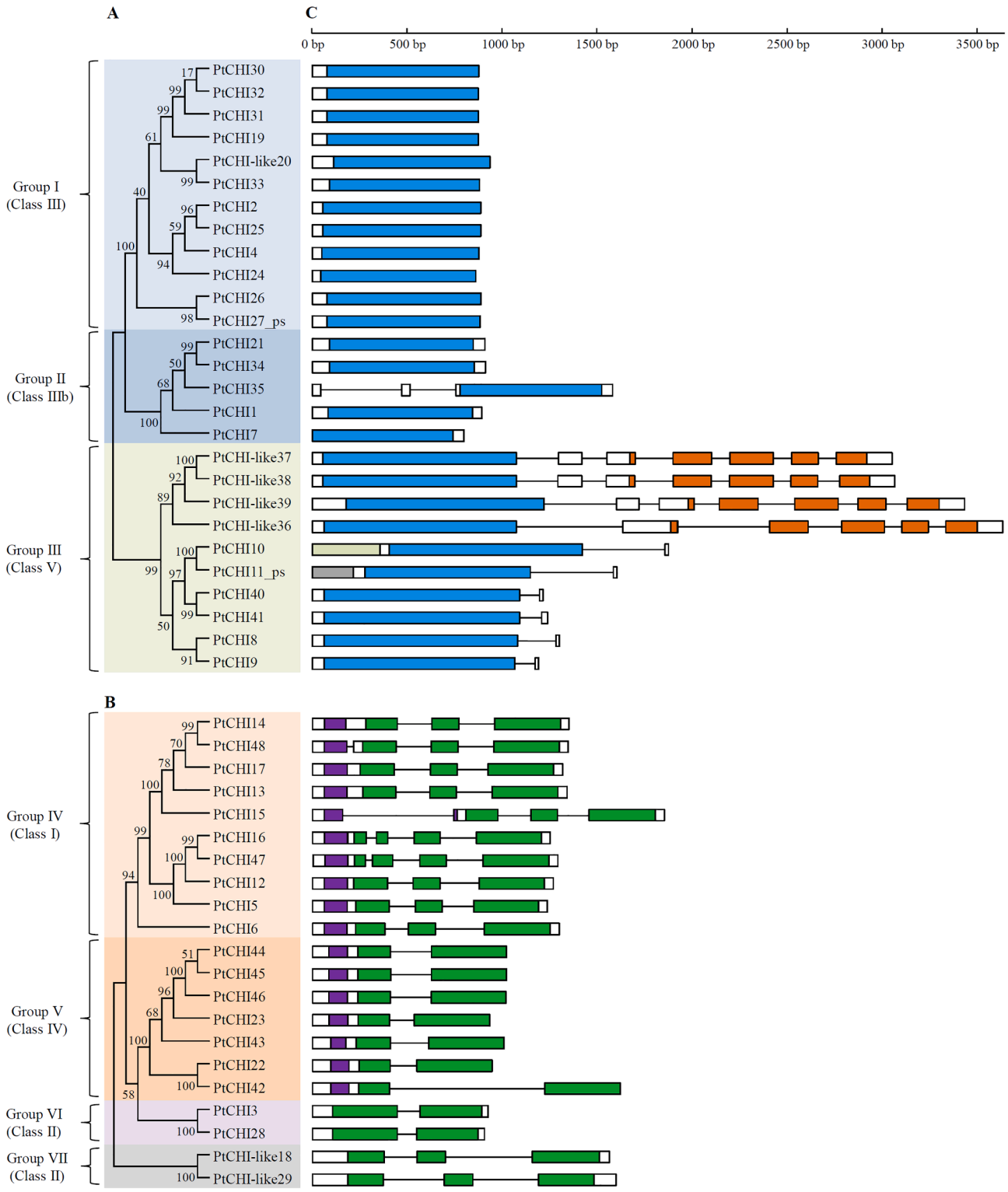

Fig. 2. Phylogenetic relationships of *Populus* GH18 (**A**) and GH19 (**B**) chitinases (CHIs), and gene structures and domain architectures (**C**). The numbers at the branches represent the support values calculated from 1,000 bootstrap replicates. Groups I, II, III, IV, V, VI and VII CHIs are shaded in light blue, blue, light olive, light orange, orange, light purple and gray, respectively. In (**C**), the GH18 domains are highlighted by blue boxes, GH19 domains are green boxes, protein kinase domains are orange boxes, LPXTG-anchored fibronectin-binding protein domain is light olive box, adventurous gliding motility protein domain is gray box, carbohydrate-binding module family 18 (CBM18) domains are purple boxes. Introns are shown as lines.

domain. Based on the classification of plant chitinases proposed by Taira (Taira, 2010), we classified groups I, II, III, IV and V as classes III, IIIb, V, I and IV, group VI and VII as class II, respectively.

3.3. *Duplication mechanism of Populus chitinase gene family*

We examined the location of the 48 chitinase genes on 19 *Populus* chromosomes. Forty-six chitinase genes were assigned to 13

chromosomes (Fig. 3), while the other two were on one as-yet-unattributed scaffold fragment (Table S2). The distribution of the chitinase genes on chromosomes was uneven: 33 chitinase genes were located to 6 chromosomes, 13 chitinase genes were located to 7 chromosomes, while the rest 6 chromosomes harbor no chitinase gene.

Three GH18 and two GH19 chitinase clusters with high density of chitinase genes (more than 4 genes) were found on the chromosomes. In addition, four GH18 and two GH19 chitinase pairs were arranged in tandem on the chromosomes (Fig. 3). In total, 22 GH18 and 15 GH19 chitinase genes were arranged in tandem repeats. This indicates that tandem duplication contributes significantly to the expansion of both *Populus* GH18 and GH19 chitinase gene families. Whole-genome analysis showed that the poplar genome underwent recent whole genome duplication about 60–65 million years ago (Tuskan et al., 2006). Paralogous segments created by this genome duplication event were identified in a previous genome annotation. Three GH18 duplicate pairs (*PtCHI2*/(*PtCHI24*, *25*, *26* and *27_ps*), (*PtCHI19* and *PtCHI-like20*)/(*PtCHI30*, *31*, *32* and *33*) and *PtCHI21*/(*PtCHI34* and *35*)), and two GH19 duplicate pairs (*PtCHI3/28* and (*PtCHI5* and *6*)/(*PtCHI12*, *13*, *14*, *15*, *16* and *17*)) were found to be each located in a pair of paralogous blocks on the chromosomes.

3.4. Selection pressure divergence of different Populus chitinase groups

The *Populus* GH18 and GH19 chitinase gene families were grouped into three and four groups, respectively. To determine whether there was difference in the selection pressure among different groups, we performed maximum-likelihood codon model analysis using PAML software for the GH18 and GH19 chitinases, respectively. Two assumptions were tested: a one-ratio model that assumes the same ω (d_N/d_S) ratio for different groups and a three- or four-ratio model in which the different groups of GH18 or GH19 chitinases were assigned to different ω ratios. The likelihood ratio test showed that the three- or four-ratio model rejected the null model (one-ratio model) (Table S4), indicating that different groups were under different selection pressure ($P < 0.0001$) (Fig. 4). For GH18 chitinases, the ω values for all the three groups were ≤ 0.1929, indicating all the three groups were under strong purifying selection pressure. Three of the four GH19 chitinase groups have ω values ≤ 0.1832, while group IV (ω value $= 0.4213$) showed more relaxed selection pressure compared to the other three groups.

3.5. Gene expression pattern of Populus chitinase genes

The expression pattern of the *Populus* chitinase gene family in root, stem and leaf was investigated by reverse transcription polymerase chain reaction (RT-PCR) under normal growth condition and various treatments (chitosan, chitin, salicylic acid and methyl jasmonate) (Fig. 5, Fig. S3). Among the 48 *Populus* chitinase genes, 18 were constitutively expressed in all tissues under all growth conditions, five were not detected in any sample, and the rest 25 showed tissue- and/or

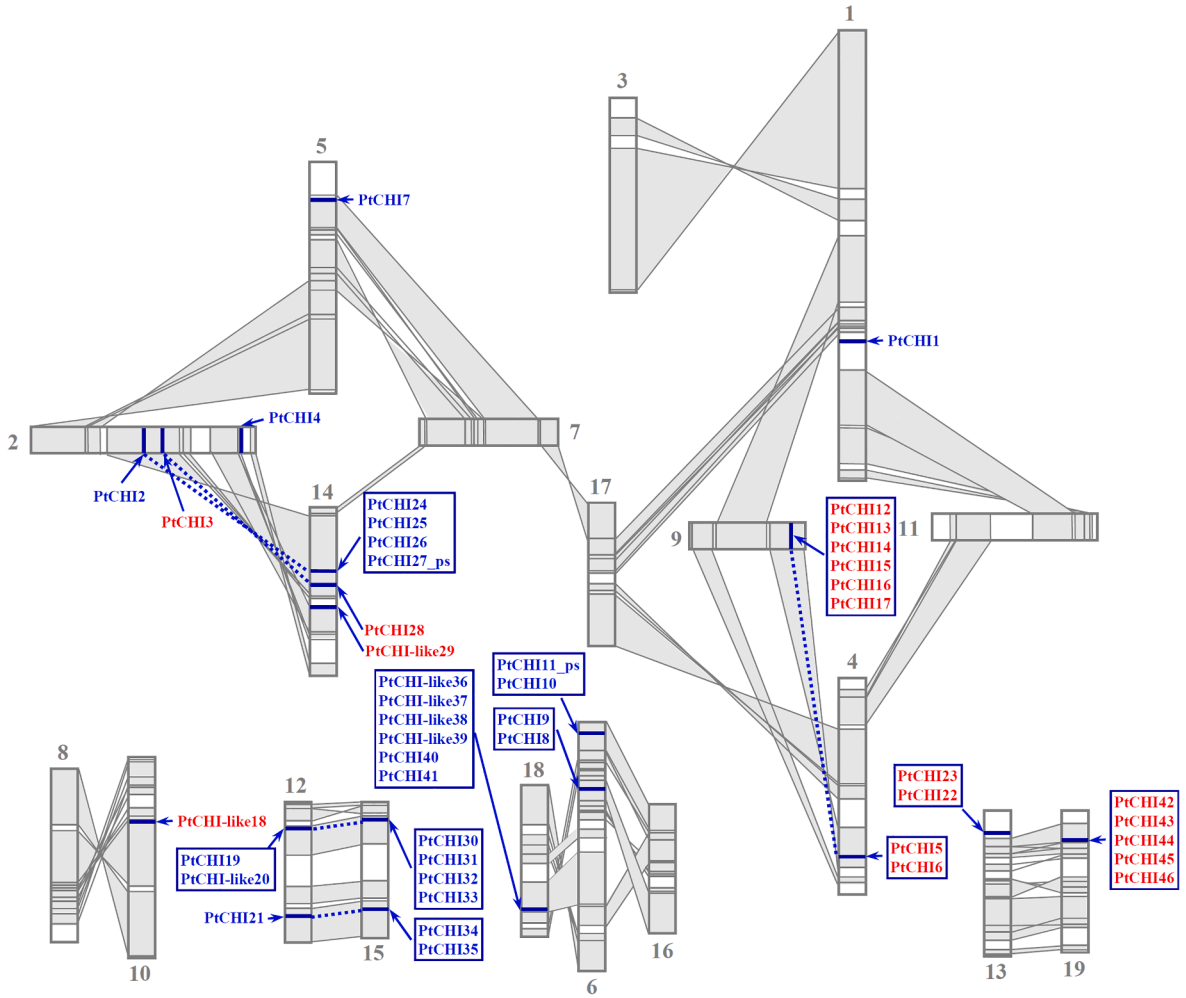

Fig. 3. Chromosomal localization of *Populus* chitinase (CHI) genes. Schematic view of chromosome-level reorganization by the most recent whole-genome duplication in *Populus* (adapted from Tuskan *et al.*, 2006). Genic synteny blocks are shaded gray and connected by lines. Paralogous CHI genes and clusters are indicated by dashed lines within the gray-shaded trapezoids. The chitinases belonging to GH18 and GH19 are represented by blue and red fonts, respectively.

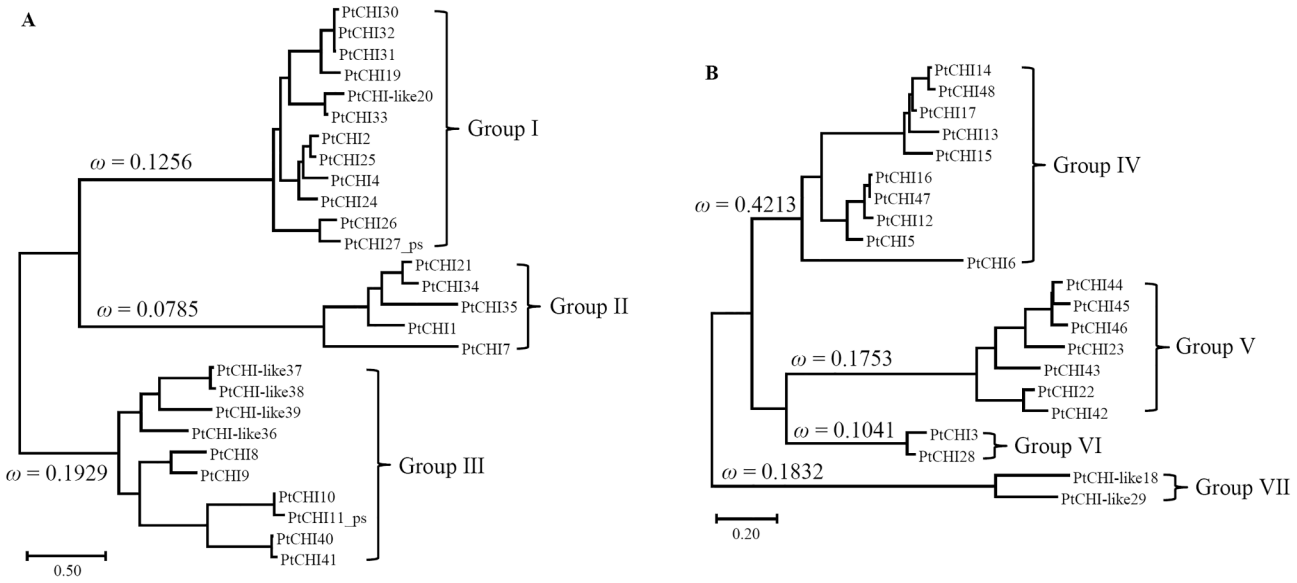

Fig. 4. Phylogenetic trees of GH18 (**A**) and GH19 (**B**) chitinases used for molecular evolution analyses. The trees were reconstructed using a maximum-likelihood (ML) procedure with 1,000 bootstrap replicates. Numbers at each branch in the phylogenetic trees are selection pressure values.

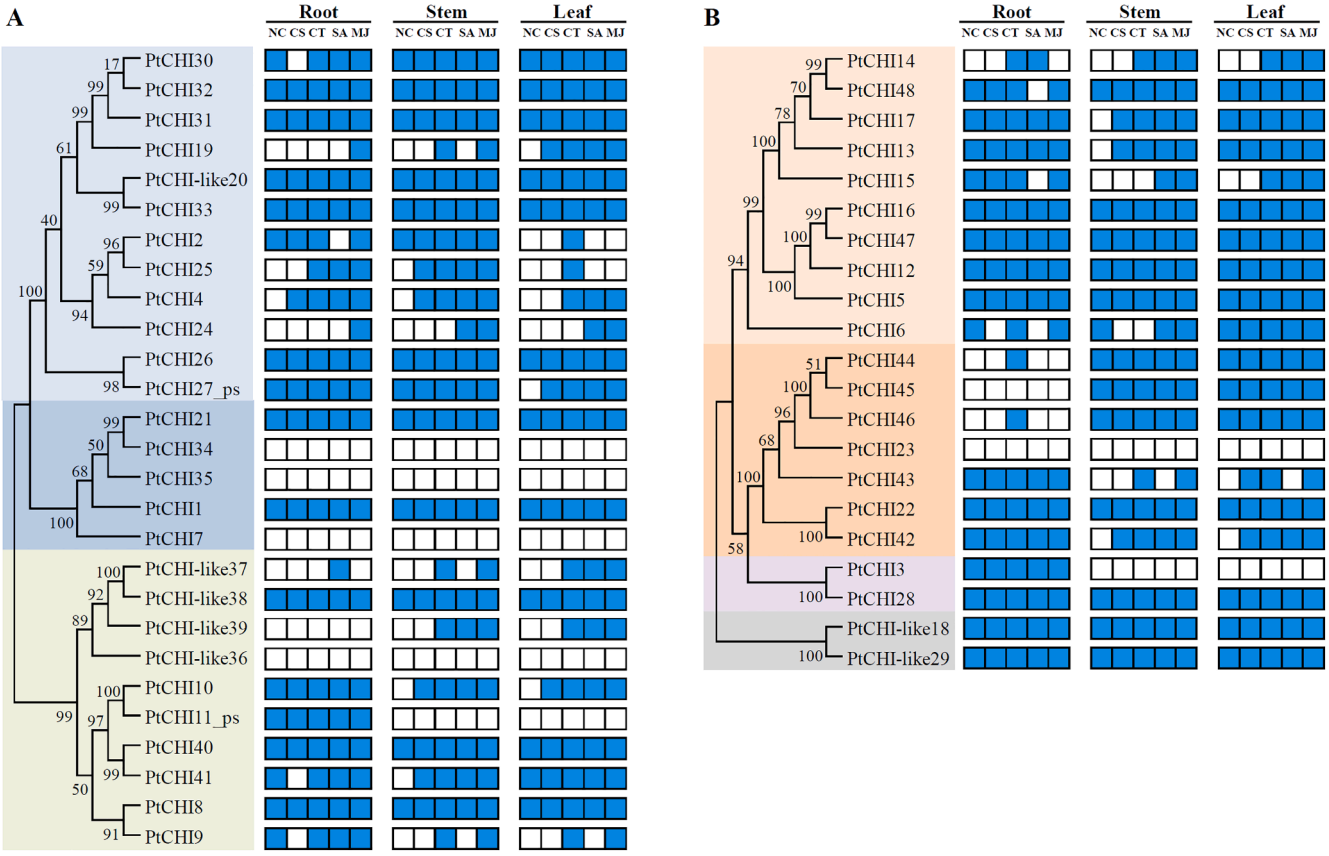

Fig. 5. Gene expression patterns of *Populus* chitinases belonging to GH18 (**A**) and GH19 (**B**). The phylogenetic relationships are the same as in Fig. 2. Numbers at branches indicate the support values calculated from 1,000 bootstrap replicates. Groups I, II, III, IV, V, VI and VII CHIs are shaded light blue, blue, light olive, light orange, orange, light purple and gray, respectively. The blue boxes indicate positive detection of gene expression in the corresponding tissue under normal growth condition (NC) and following chitosan (CS), chitin (CT), salicylic acid (SA) and methyl jasmonate (MJ) stress treatments.

treatment-specific expression pattern. The *Populus* chitinase gene family appears to be inducibly expressed in response to some treatments. Nineteen genes were not expressed in some tissues under normal growth condition, but expressed in response to treatments. Four *Populus* chitinase genes showed tissue-specific gene expression pattern. For example, *PtCHI-like39* and *PtCHI45* were not expressed in root, but expressed in stem and leaf; *PtCHI3* and *PtCHI11_ps* were expressed in root, but not in stem and leaf.

3.6. Enzymatic activity of the Populus chitinase proteins

Three chitinases (PtCHI30, PtCHI21 and PtCHI43) were over-expressed in *E. coli* and purified. PtCHI30, PtCHI21 and PtCHI43 is from group I, II and V, respectively. The specific activities of the three recombinant chitinases towards chitin and chitosan were assayed. All the three recombinant chitinases showed activities towards both chitin and chitosan. Of the three chitinases, PtCHI21 had the lowest activity towards chitin (4.96 ± 0.23 μmol /min per μmol) but the highest activity towards chitosan (422.19 ± 28.58 μmol /min per μmol), while PtCHI43 had the highest activity towards chitin (98.02 ± 11.38 μmol /min per μmol) but the lowest activity towards chitosan (13.88 ± 0.37 μmol /min per μmol). PtCHI30 showed median activities towards both chitin (24.18 ± 8.32 μmol /min per μmol) and chitosan (32.92 ± 2.66 μmol /min per μmol).

3.7. Expansion of chitinase gene family in Populus and Arabidopsis

The *Arabidopsis thaliana* genome contains 24 chitinase genes (Passarinho and de Vries, 2002). Of the 24 *Arabidopsis* chitinase genes, 10 have GH18 domain, and 14 have GH19 domain. Joint phylogenetic analysis of *Arabidopsis* and *Populus* chitinases found that *Arabidopsis* contains 1, 9, 1, 9, 2 and 2 groups I, III, IV, V, VI and VII chitinases, respectively (Fig. 6). However, we did not find any group II chitinase in *Arabidopsis*.

To compare the chitinase gene expansion in *Arabidopsis* and *Populus*, we identified the nodes that led to *Populus*- and *Arabidopsis*-specific clades, which represent the divergence points between these two species (circles in Fig. 6). These nodes also represent the most recent common ancestors (MRCA) of *Populus* and *Arabidopsis* chitinases. Gene losses could have occurred in either *Populus* or *Arabidopsis* after their split, and resulted in clades containing only *Arabidopsis* or *Populus* chitinases (arrows in Fig. 6). We found that six and four clades contained only *Populus*

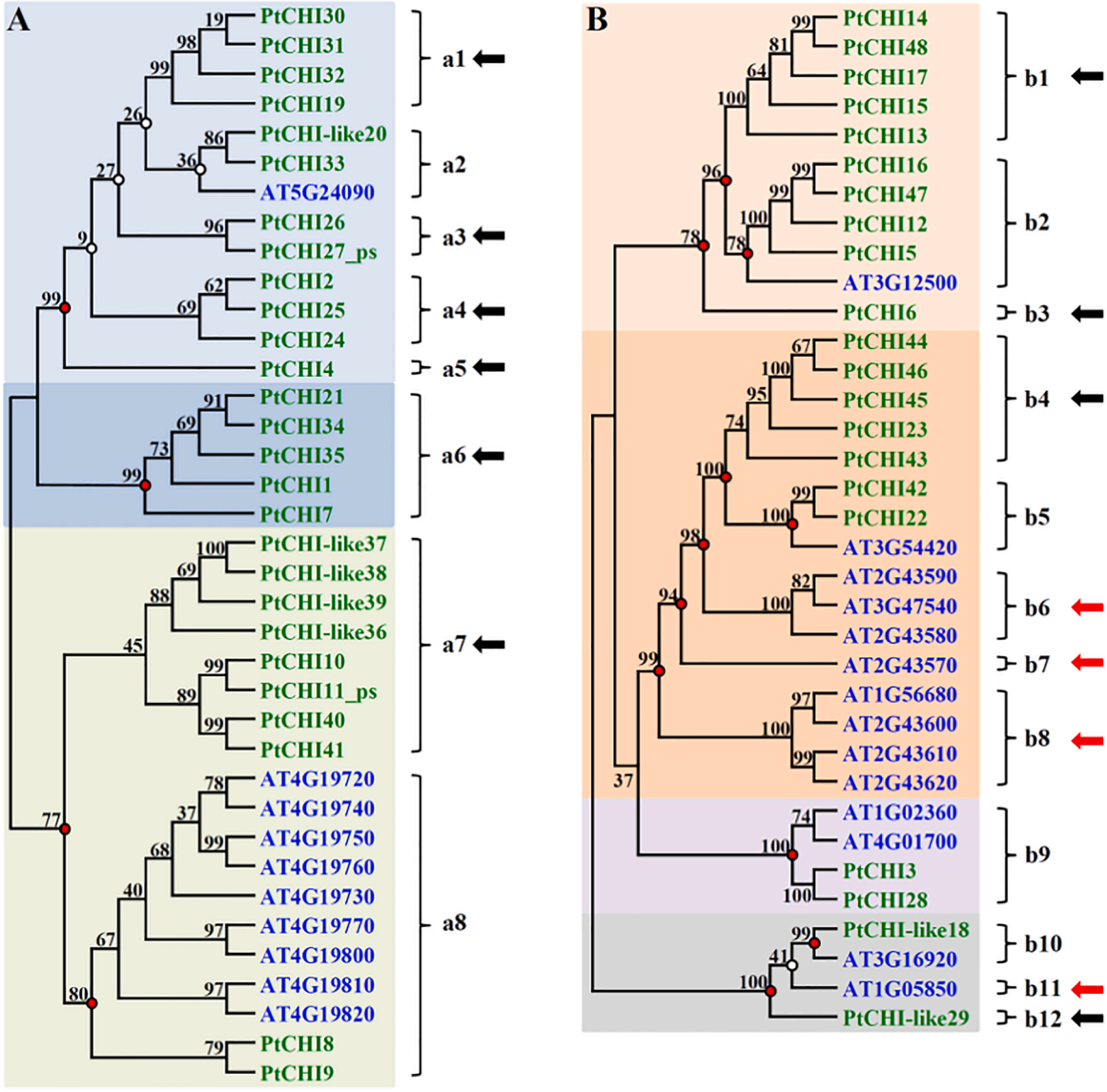

Fig. 6. Phylogenetic relationships of *Populus* and *Arabidopsis* chitinases (CHIs). The phylogenetic tree from GH18 (**A**) and GH19 (**B**) are constructed separately. Numbers on branches indicate the support values calculated from 1,000 bootstrap replicates. Groups I, II, III, IV, V, VI and VII CHIs are shaded light blue, blue, light olive, light orange, orange, light purple and gray, respectively. The nodes that represent the most recent common ancestors before the *Populus* and *Arabidopsis* split are indicated by red circles (bootstrap support > 50%) and white circles (bootstrap support < 50%). Clades that contain only *Populus* or *Arabidopsis* CHIs are indicated by black or red arrows, respectively.

chitinase genes in the GH18 and GH19 phylogenetic tree, respectively, indicating gene losses in *Arabidopsis* in these clades (Fig. 6, black arrows). However, while four clades containing only *Arabidopsis* chitinases were found in the GH19 phylogenetic tree (Fig. 6, red arrows), we did not find any clade containing only *Arabidopsis* chitinase in the GH18 phylogenetic tree. The number of clades indicated that there were at least four ancestral GH18 chitinase genes and eleven GH19 chitinase genes before the *Populus-Arabidopsis* split. In addition, four clades (Fig. 6A; clades a1, a2, a3 and a4) in the GH18 phylogenetic tree and one clade (Fig. 6B; clade b11) in the GH19 phylogenetic tree had low bootstrap support (< 50%). If we assume these less well-supported nodes are correct, there were eight GH18 and twelve GH19 ancestral chitinase genes.

Populus and *Arabidopsis* had at least 5, 1, 2, 3, 5, 1 and 3 ancestral group I, II, III, IV, V, VI and VII chitinase genes, respectively (Fig. 7). However, the chitinase genes exhibited group- and species-specific gene gain and gene loss pattern. For group I and II chitinases, while *Populus* gained seven and four genes, respectively, *Arabidopsis* lost four and one genes, respectively. This resulted in the large expansion of group I and II chitinase genes in *Populus*, and the retention of only one group I chitinase and loss of group II chitinase gene in *Arabidopsis*. For group III chitinases, both *Populus* and *Arabidopsis* gained eight genes after their split, and only *Arabidopsis* lost one gene. For group IV chitinases, while *Populus* gained seven genes, *Arabidopsis* lost two genes. The gene gain and loss numbers of groups V, VI and VII chitinases between *Populus* and *Arabidopsis* is similar.

4. Discussion

Chitinases form large gene families in plants, and have been shown to play multiple roles. In this study, we identified 48 full-length chitinase genes from *P. trichocarpa*. Of the 48 *Populus* chitinase genes, 37 (77%) were related to tandem duplication, indicating that tandem duplication is an important engine generating new gene copies in the *Populus* chitinase gene family. This gene duplication pattern is similar to other pathogen defense-related gene families, such as NBS-LRR, Thaumatin, Germin, PR1, and Major Latex Protein/PR10 families (Cannon et al., 2004). Of the 48 full-length *Populus* chitinase genes, two were found to be pseudogenes. In addition, 15 partial chitinase fragments were identified in the *Populus* genome. Thus, both gene gain and gene loss play important roles in shaping the *Populus* chitinase gene family structure.

Joint phylogenetic analysis showed that after *Populus* and *Arabidopsis* split, they have undergone different gene gain and gene loss events. In groups I and IV chitinases, *Arabidopsis* only retained one chitinase while *Populus* has 12 and 10 chitinases, respectively; in clade a7, *Populus* has eight chitinases while *Arabidopsis* has lost its corresponding ortholog; in clade a8, when *Populus* only has two chitinases, *Arabidopsis* expanded to nine chitinases; *Arabidopsis* has lost its group II chitinase ortholog in *Populus*; in group V, *Populus* and *Arabidopsis* underwent multiple independent gene loss events. The difference of gene gain and gene loss among different species can be driven by different environment selection pressure (Marri et al., 2006; Koskiniemi et al., 2012). In nature, *Populus* and *Arabidopsis* have to confront with different pathogens (Duplessis et al., 2009; Urban et al., 2002; Andargie and Li, 2016). The substantial gene gain and gene loss pattern difference between *Populus* and *Arabidopsis* might be the result of adaptation evolution in defense against different pathogens.

Group I and II chitinases only have chitinase domain, while divergent protein domain architecture was found in group III chitinases. PtCHI-like36, 37, 38 and 39 contain both GH18 chitinase domain and protein kinase domain (STKc). Kim *et al.* identified a chitinase-related receptor-like kinase (CHRK1) from tobacco (Kim et al., 2000). CHRK1 lacked the essential glutamic acid residue required for chitinase activity and did not show catalytic activity for chitin substrates. Upon infection by fungal pathogen or tobacco mosaic virus, the *CHRK1* expression was strongly induced. In addition, CHRK1 is involved in cell proliferation/differentiation and cytokinin homeostasis (Lee et al., 2003). The *Populus* CHRKs also lacked the essential glutamic acid active residue indicating that they do not have chitinase activity. *PtCHI-like36* expression was not detected in any sample we analyzed, indicating it might be a pseudogene or has function under specific condition. *PtCHI-like37* and *PtCHI-like39* were not expressed in all the three tissues under normal condition, but can be induced by treatments, such as chitin, salicylic acid and methyl jasmonate, indicating their roles upon stress conditions. *PtCHI-like38* was constitutively expressed in all the samples analyzed, thus might function in development regulation. BLASTP analysis only found GH18/STKc protein architecture from eudicot, indicating CHRK originated in eudicots. However, since we did not find proteins with GH18/STKc architecture in Brassicales plants, such as *A. thaliana*, *Brassica rapa* and *Boechera stricta*, the orthologous *CHRK1* gene might have been lost in the ancestor of Brassicaceae plants.

Populus chitinase genes showed highly divergent expression pattern,

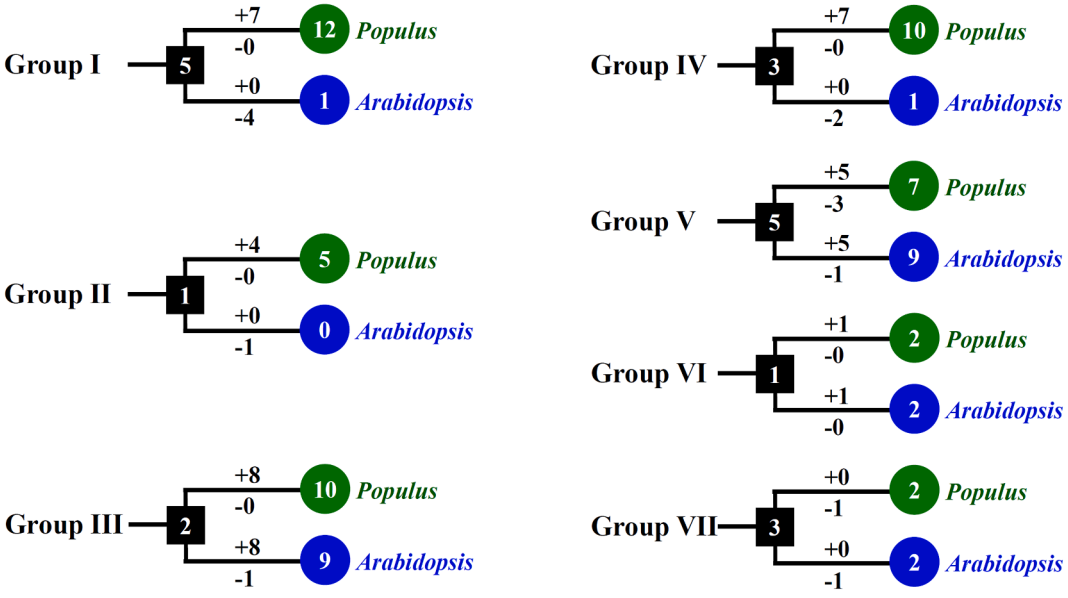

Fig. 7. The copy number changes of *Populus* and *Arabidopsis* chitinase genes. Numbers in circles and rectangles represent the numbers of chitinase genes in extant and ancestral species, respectively. Numbers on branches with plus and minus symbols represent the numbers of gene gains and losses, respectively.

both constitutive and inducible. The 18 constitutively expressed *Populus* chitinase genes might play roles in some basic physiological processes, or constant defense against fungal pathogens. Twenty *Populus* chitinase genes were only expressed upon treatment (chitin, chitosan, salicylic acid, or methyl jasmonate) in at least one of the three tissues (root, stem and leaf). Salicylic acid and methyl jasmonate are defense-related signaling molecules (Schenk et al., 2000). Chitin is the major component of fungal cell wall, and has been shown to induce defense-related cellular responses in many plants. For example, phytoalexin formation is induced by chitin in suspension-cultured rice cells (Yamada et al., 1993); chitin treatment elicits rice resistance against rice blast fungus (Tanabe et al., 2006); a plant immune receptor was activated by chitin in *Arabidopsis* (Liu et al., 2012). Chitosan is the deacetylated form of chitin and acts as a defense elicitor against fungal pathogens in plants. In pea, chitosan application enhanced the phytoalexin production and inhibited fungal growth (Hadwiger and Beckman, 1980); in parsley cell suspension cultures, chitosan elicited the deposition of callose on the cell wall (Conrath et al., 1989); chitosan treatment protected *Arabidopsis* from *Botrytis cinerea* and elicited expression of defense-related genes (Povero et al., 2011). In *Populus*, these induced chitinase genes might respond to fungal pathogen attack. While 18 *Populus* chitinase genes were induced by chitin, only nine were induced by chitosan, indicating *Populus* chitinase gene family is more likely a chitin-induced gene family rather than chitosan-induced.

Protein sequence analysis showed that most of the group I and II GH18 chitinases have conserved catalytic motif (DxDxE) (Umemoto et al., 2015), indicating they have the ability to hydrolyze chitin (Fig. S1A). This is consistent with our protein activity assay result that recombinant PtCHI30 (from group I) and PtCHI21 (from group II) have activity towards chitin and chitosan. For group III GH18 chitinases, except the chitinase-related receptor-like kinases and the pseudogene *PtCHI11_ps*-encoding protein, all the other chitinases have the conserved catalytic motif (DxDxE), indicating they might also have chitinase activity. For GH19 chitinases, most of them have both chitin-binding domain and chitinase domain. Except PtCHI6, all the double domain-containing group IV chitinases have the three catalytic sites (Fig. S1B) (Landim et al., 2017), and thus might have chitinase activity. This is supported by the degradation of chitin and chitosan by the recombinant PtCHI43 (from group V) protein. PtCHI3/28/-like18/-like29 only have chitinase domain. While PtCHI3 and PtCHI28 have the three catalytic sites, PtCHI-like18 and PtCHI-like29 have lost two of the three catalytic sites, indicating they cannot metabolize chitin. In the joint phylogenetic tree of *Populus* and *Arabidopsis*, PtCHI-like18 and PtCHI-like29 grouped together with AT3G16920 and AT1G05850 (Fig. 6). These two proteins from *Arabidopsis* have been shown to function in the cellulose assembly in *Arabidopsis*. Thus, PtCHI-like18 and PtCHI-like29 might be involved in the cellulose synthesis in *Populus*.

In conclusion, by exploring analyses of gene sequence, gene expression and protein activity, our study provides new insight into the evolution and function of chitinase gene family in a woody plant, and found that the *Populus* chitinase family has experienced comprehensive functional diversification. Integrated analysis of different plant groups, including algae, moss, fern, gymnosperm and angiosperm plants will help to understand the evolution of chitinase family in the whole plant kingdom.

5. Data availability

The data that support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Yuan-Jie Zhang: Investigation. **Lin-Ling Ren:** Investigation. **Xiao-Yang Lin:** Investigation. **Xue-Min Han:** Investigation. **Wei Wang:** Investigation. **Zhi-Ling Yang:** Conceptualization, Methodology, Formal analysis, Visualization, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

ZLY designed the study, conducted data analysis, and wrote the manuscript. YJZ, LLR, XYL, XMH and WW performed the experiments. All authors read and approved the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146329.

References

Andargie, M., Li, J., 2016. *Arabidopsis thaliana*: a model host plant to study plant–pathogen interaction using rice false smut isolates of *Ustilaginoidea virens*. Front. Plant Sci. 7, 192.
Austin, P., Brine, C., Castle, J., Zikakis, J., 1981. Chitin: new facets of research. Science 212, 749–753.
Bordoloi, K.S., Krishnatreya, D.B., Baruah, P.M., Borah, A.K., Mondal, T.K., Agarwala, N., 2021. Genome-wide identification and expression profiling of chitinase genes in tea (*Camellia sinensis* (L.) O. Kuntze) under biotic stress conditions. Physiol. Mol. Biol. Plants 27, 369–385.
Broekaert, W., Van Parijs, J., Allen, A., Peumans, W., 1988. Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. 33, 319–331.
Calabrese, S., Kohler, A., Niehl, A., Veneault-Fourrey, C., Boller, T., Courty, P.-E., 2017. Transcriptome analysis of the *Populus trichocarpa–Rhizophagus irregularis* mycorrhizal symbiosis: regulation of plant and fungal transportomes under nitrogen starvation. Plant Cell Physiol. 58, 1003–1017.
Cannon, S.B., Mitra, A., Baumgarten, A., Young, N.D., May, G., 2004. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol. 4, 1–21.
Cohen-Kupiec, R., Chet, I., 1998. The molecular biology of chitin digestion. Curr. Opin. Biotechnol. 9, 270–277.
Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinases. Plant J. 3, 31–40.
Conrath, U., Domard, A., Kauss, H., 1989. Chitosan-elicited synthesis of callose and of coumarin derivatives in parsley cell suspension cultures. Plant Cell Rep. 8, 152–155.
Duplessis, S., Major, I., Martin, F., Séguin, A., 2009. Poplar and pathogen interactions: insights from *Populus* genome-wide analyses of resistance and defense gene families and gene expression profiling. CRC Crit. Rev. Plant Sci. 28, 309–334.
Grover, A., 2012. Plant chitinases: genetic diversity and physiological roles. CRC Crit. Rev. Plant Sci. 31, 57–73.
Hadwiger, L.A., Beckman, J.M., 1980. Chitosan as a component of pea-*Fusarium solani* interactions. Plant Physiol. 66, 205–211.
Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. 280, 309–316.
Huang, X., Wang, J., Du, Z., Zhang, C., Li, L., Xu, Z., 2013. Enhanced resistance to stripe rust disease in transgenic wheat expressing the rice chitinase gene *RC24*. Transgenic Res. 22, 939–947.
Imoto, T., Yagishita, K., 1971. A simple activity measurement of lysozyme. Agr. Biol. Chem. 35, 1154–1156.
Jiang, C., Huang, R.F., Song, J.L., Huang, M.R., Xu, L.A., 2013. Genomewide analysis of the chitinase gene family in *Populus trichocarpa*. J. Genet. 92, 121–125.
Kasprzewska, A., 2003. Plant chitinases-regulation and function. Cell. Mol. Biol. Lett. 8, 809–824.
Kim, Y.S., Lee, J.H., Yoon, G.M., Cho, H.S., Park, S.-W., Suh, M.C., Choi, D., Ha, H.J., Liu, J.R., Pai, H.-S., 2000. CHRK1, a chitinase-related receptor-like kinase in tobacco. Plant Physiol. 123, 905–916.
Kobaru, S., Tanaka, R., Taira, T., Uchiumi, T., 2016. Functional analyses of chitinases in the moss *Physcomitrella patens*: chitin oligosaccharide-induced gene expression and enzymatic characterization. Biosci. Biotechnol. Biochem. 80, 2347–2356.
Koskiniemi, S., Sun, S., Berg, O.G., Andersson, D.I., 2012. Selection-driven gene loss in bacteria. PLoS Genet. 8.

Landim, P.G.C., Correia, T.O., Silva, F.D., Nepomuceno, D.R., Costa, H.P., Pereira, H.M., Lobo, M.D., Moreno, F.B., Brandão-Neto, J., Medeiros, S.C., 2017. Production in *Pichia pastoris*, antifungal activity and crystal structure of a class I chitinase from cowpea (*Vigna unguiculata*): Insights into sugar binding mode and hydrolytic action. Biochimie 135, 89–103.

Lee, J.H., Takei, K., Sakakibara, H., Cho, H.S., Kim, Y.S., Min, S.R., Kim, W.T., Sohn, D.Y., Lim, Y.P., Pai, H.-S., 2003. CHRK1, a chitinase-related receptor-like kinase, plays a role in plant development and cytokinin homeostasis in tobacco. Plant Mol. Biol. 53, 877–890.

Li, P., Pei, Y., Sang, X., Ling, Y., Yang, Z., He, G., 2009. Transgenic *indica* rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (*McCHIT1*) confers enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani*. Eur. J. Plant Pathol. 125, 533–543.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., 2012. Chitin-induced dimerization activates a plant immune receptor. Science 336, 1160–1164.

Liu, X., Yu, Y., Liu, Q., Deng, S., Jin, X., Yin, Y., Guo, J., Li, N., Liu, Y., Han, S., 2020. A Na_2CO_3-responsive chitinase gene from *Leymus chinensis* improve pathogen resistance and saline-alkali stress tolerance in transgenic tobacco and maize. Front. Plant Sci. 11, 504.

Marri, P.R., Hao, W., Golding, G.B., 2006. Gene gain and gene loss in *Streptococcus*: is it driven by habitat? Mol. Biol. Evol. 23, 2379–2391.

Mauch, F., Mauch-Mani, B., Boller, T., 1988. Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1, 3-glucanase. Plant Physiol. 88, 936–942.

Merzendorfer, H., Zimoch, L., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393–4412.

Onaga, S., Taira, T., 2008. A new type of plant chitinase containing LysM domains from a fern (*Pteris ryukyuensis*): roles of LysM domains in chitin binding and antifungal activity. Glycobiology 18, 414–423.

Passarinho, P.A., de Vries, S.C., 2002. Arabidopsis Chitinases: A Genomic Survey. The Arabidopsis book/American Society of Plant Biologists.

Patil, R.S., Ghormade, V., Deshpande, M.V., 2000. Chitinolytic enzymes: an exploration. Enzyme Microb. Technol. 26, 473–483.

Potter, S.C., Luciani, A., Eddy, S.R., Park, Y., Lopez, R., Finn, R.D., 2018. HMMER web server: 2018 update, 46 (2018) W200-W204.

Povero, G., Loreti, E., Pucciariello, C., Santaniello, A., Di Tommaso, D., Di Tommaso, G., Kapetis, D., Zolezzi, F., Piaggesi, A., Perata, P., 2011. Transcript profiling of chitosan-treated Arabidopsis seedlings. J. Plant Res. 124, 619–629.

Salzer, P., Bonanomi, A., Beyer, K., Vögeli-Lange, R., Aeschbacher, R.A., Lange, J., Wiemken, A., Kim, D., Cook, D.R., Boller, T., 2000. Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. Mol. Plant Microbe Interact. 13, 763–777.

Sánchez-Rodríguez, C., Bauer, S., Hématy, K., Saxe, F., Ibáñez, A.B., Vodermaier, V., Konlechner, C., Sampathkumar, A., Rüggeberg, M., Aichinger, E., 2012. Chitinase-like1/pom-pom1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in *Arabidopsis*. Plant Cell 24, 589–607.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., Manners, J.M., 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc. Natl. Acad. Sci. USA 97, 11655–11660.

Shimono, K., Matsuda, H., Kawamukai, M., 2002. Functional expression of chitinase and chitosanase, and their effects on morphologies in the yeast *Schizosaccharomyces pombe*. Biosci. Biotechnol. Biochem. 66, 1143–1147.

Taira, T., 2010. Structures and antifungal activity of plant chitinases. J. Appl. Glycosci. 57, 167–176.

Takenaka, Y., Nakano, S., Tamoi, M., Sakuda, S., Fukamizo, T., 2009. Chitinase gene expression in response to environmental stresses in *Arabidopsis thaliana*: chitinase inhibitor allosamidin enhances stress tolerance. Biosci. Biotechnol. Biochem. 73, 1066–1071.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tanabe, S., Okada, M., Jikumaru, Y., Yamane, H., Kaku, H., Shibuya, N., Minami, E., 2006. Induction of resistance against rice blast fungus in rice plants treated with a potent elicitor, *N*-acetylchitooligosaccharide. Biosci. Biotechnol. Biochem. 70, 1599–1605.

Tobias, P.A., Christie, N., Naidoo, S., Guest, D.I., Külheim, C., 2017. Identification of the *Eucalyptus grandis* chitinase gene family and expression characterization under different biotic stress challenges. Tree Physiol. 37, 565–582.

Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313, 1596–1604.

Umemoto, N., Kanda, Y., Ohnuma, T., Osawa, T., Numata, T., Sakuda, S., Taira, T., Fukamizo, T., 2015. Crystal structures and inhibitor binding properties of plant class V chitinases: the cycad enzyme exhibits unique structural and functional features. Plant J. 82, 54–66.

Urban, M., Daniels, S., Mott, E., Hammond-Kosack, K., 2002. *Arabidopsis* is susceptible to the cereal ear blight fungal pathogens *Fusarium graminearum* and *Fusarium culmorum*. Plant J. 32, 961–973.

Verburg, J.G., Huynh, Q.K., 1991. Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. Plant Physiol. 95, 450–455.

Wan, J., Zhang, X.-C., Stacey, G., 2008. Chitin signaling and plant disease resistance. Plant Signal. Behav. 3, 831–833.

Wu, H., Echt, C.S., Popp, M.P., Davis, J.M., 1997. Molecular cloning, structure and expression of an elicitor-inducible chitinase gene from pine trees. Plant Mol. Biol. 33, 979–987.

Wu, X.-F., Wang, C.-L., Xie, E.-B., Gao, Y., Fan, Y.-L., Liu, P.-Q., Zhao, K.-J., 2009. Molecular cloning and characterization of the promoter for the multiple stress-inducible gene *BjCHI1* from *Brassica juncea*. Planta 229, 1231–1242.

Xu, F., Fan, C., He, Y., 2007. Chitinases in *Oryza sativa* ssp. *japonica* and *Arabidopsis thaliana*. J. Genet Genomics 34, 138–150.

Xu, J., Xu, X., Tian, L., Wang, G., Zhang, X., Wang, X., Guo, W., 2016. Discovery and identification of candidate genes from the chitinase gene family for *Verticillium dahliae* resistance in cotton. Sci. Rep. 6, 1–12.

Yamada, A., Shibuya, N., Kodama, O., Akatsuka, T., 1993. Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetylchitooligosaccharides. Biosci. Biotechnol. Biochem. 57, 405–409.

Yang, Z., 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591.

Zheng, T., Zhang, K., Sadeghnezhad, E., Jiu, S., Zhu, X., Dong, T., Liu, Z., Guan, L., Jia, H., Fang, J., 2020. Chitinase family genes in grape differentially expressed in a manner specific to fruit species in response to *Botrytis cinerea*. Mol. Biol. Rep. 47, 7349–7363.

Zhong, X., Feng, P., Ma, Q., Zhang, Y., Yang, Y., Zhang, J., 2021. Cotton chitinase gene *GhChi6* improves the *Arabidopsis* defense response to aphid attack. Plant Mol. Biol. Rep. 39, 251–261.