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CLONING AND EXPRESSION PROFILE OF *PDPAPDOL3* GENE IN SHANXIN POPLAR (*POPULUS DAVIDANA* × *P. ALBA* VAR. *PYRAMIDLIS*) IN RESPONSE TO STRESS

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Abstract

In order to identify and verify crucial genes that regulate the stress response and resistance of poplar, RNA-seq data of poplar (*Populus davidiana* × *P. alba* var. *Pyramidlis*, cv 'Shanxin') leaves induced by *Trichoderma asperellum* and/or *Alternaria alternata* were screened. As a result, a key responding gene was identified, cloned and named as *PdPapDOL3*. In silico analyses showed that the coded protein *PdPapDOL3* was a non-transmembrane hydrophilic protein of the DOG1 transcription factor family. The tissue-specific expression profile of *PdPapDOL3* in poplar seedlings was investigated through real-time quantitative polymerase chain reaction (RT-qPCR). The results showed that *PdPapDOL3* was constititutively expressed in different compartments of poplar with the root demonstrating the highest expression. The differential regulation of *PdPapDOL3* expression induced by salt, alkali, polyethylene glycol (PEG), five soil-borne plant fungal pathogens or phytohormones for 48 hours was separately investigated. The results showed that the alkali induction caused the most evident change in *PdPapDOL3* expression. *Fusarium oxysporum*, *Cytospora chrysosperma* or *Alternaria alternata* induction significantly up-regulated *PdPapDOL3* expression in young tissues. *F. oxysporum* induction significantly up-regulated *PdPapDOL3* expression in mature leaf. *C. chrysosperma* and *A. alternata* significantly induced *PdPapDOL3* expression in the root. *PdPapDOL3* expression was induced by salicylic acid (SA) in all the investigated compartments of poplar. While induced by jasmonic acid (JA) or abscisic acid (ABA), *PdPapDOL3* expression was up-regulated in young tissues but down-regulated in the root. In this study, we report the tissue-specific expression patterns and the differential regulation profiles of the *PdPapDOL3* gene in poplar under various stress conditions. Our results lay a foundation for further elucidating the function of *PdPapDOL3* and provide insights into breeding novel stress-resistant poplar cultivars through modifying *PdPapDOL3* expression.

Key words: *PdPapDOL3*, Abiotic stress, biotic stress, Hormone induction, RT-qPCR.

Introduction

The *DOG1* (*DELAY OF GERMINATION1*) gene commonly exists in dicotyledons and monocotyledons, and is usually expressed in developing or mature seeds. There are five genes in the DOG1 family in *Arabidopsis* (DOG1, DOGL1-4) (Huo *et al*., 2016). The identification of *DOG1* for the first time was carried out by QTL analyses in a highly dormant *Arabidopsis* Cvi population (Alonso-Blanco *et al*., 2003). *DOG1* can result in a variety of transcripts and proteins through variable splicing at the 3 'and 5' ends. Through different unusual splicing, it can generate 5 different variants of transcripts, also termed as genetic subtype, namely DOG1-α, DOG1-β, DOG1-γ, DOG1-δ and DOG1-ε, which code three different subtypes of protein, namely DOG1-α, DOG1-β and DOG1-δ (Nakabayashi *et al*., 2015). A single DOG1 protein subtype is functional but not stable. Therefore, other protein subtypes are needed to maintain the stability of the protein. The self-connection of DOG1 proteins can significantly enhance its function (Bentsink *et al*., 2006; Nakabayashi *et al*., 2012; 2015).

Initially, it was suggested that *DOG1* might be a quantitative trait locus of the abscisic acid (ABA) biosynthesis pathway, participating in the ABA signaling pathway (Chen *et al*., 2021). However, in recent years, many studies have shown that *DOG1* is specifically related to seed dormancy and dormancy level regulation. It has been proposed that *DOG1* is a timer for seed dormancy release (Nakabayashi *et al*., 2012). *DOGl* not only participates in the induction of secondary dormancy, but also regulates the mechanical penetration of the endosperm cap, participating in the regulation of seed maturation and flowering. Moreover, it is related to gibberellin (GA), ABA and ABA-mediated sugar signaling pathway in terms of signal pathway regulation (Graeber *et al*., 2010; Footitt *et al*., 2011). In addition, TFIIS (a transcription elongation factor), CBFs (repeat binding factor), LDL1 and LDL2 (LYSINESPECIFIC DEMETHYLASE LIKE 1 and 2, which code *Arabidopsis* histone demethylases), and histone modifications all affect *DOG1* gene expression (Kendall *et al*., 2011; Nakamura *et al*., 2011; Mortensen *et al*., 2014; Vaistij *et al*., 2018). At present, *DOG1* has been cloned and studied in many plants, and has become a research hotspot in the field of seed dormancy regulation (Cyrek *et al*., 2016; Chen *et al*., 2021). However, there are few reports on the function of *DOG1* in the resistance of plants to stress.

'Shanxin' poplar (*Populus davidiana* × *P. alba* var. *pyramidalis*) is an excellent fast-growing hybrid poplar cultivar for afforestation and urban greening in the arid and cold areas of north China. *P. davidiana* was its female parent and *P. alba* var. *pyramidalis* was its male parent. Shanxin poplar has the excellent characteristics of fast-growing, strong adaptability and stress resistance, and has high economic and ornamental values (Zhang *et al*., 2018; Yao *et al*., 2018; Zhai *et al*., 2019). So far, bioinformatics analysess, the tissue -specific expression

profile, hormone-induced expression profile, and the response to biotic and abiotic stress of Shanxin poplar *DOG1* have not been reported. Previously, we constructed a set of transcriptomic data of Shanxin poplar inoculated with *Trichoderma asperellum* and/or *Alternaria alternata*. We screened the data for specifically induced genes under the inductions and identified *Potri.007G098800* (v3.1) as a key regulatory gene in response to the inductions. It was annotated as a camp-response element binding protein-related gene in the *P. trichocarpa* genome (v3.1) in Phytozome 13 (https://phytozome-next.jgi.doe.gov/). Its coded protein Potri.007G098800 had a complete DOG1 superfamily functional domain at 59th-123rd amino acids (175-369 bp of *Potri.007G098800*). BLASTx alignment of *Potri.007G098800* was performed and Xp_034907328.1 (PROTEIN DOG1-LIKE 3 of *Populus alba*) had the highest consistency. Hence, *Potri. 007G098800* was named *PdPapDOL3*. The constitutive expression pattern of *PdPapDOL3* was investigated by real-time quantitative polymerase chain reactions (RT-qPCR). The differential regulation of *PdPapDOL3* expression in the shoot tip, mature leaves and root under abiotic and biotic stresses and hormone induction for 48 hours (h) was further studied with untreated Shanxin poplars as control. The results of this study provided a basis for further revealing the function of *PdPapDOL3* in the tolerance to stress and response to hormone induction, and obtaining new poplar cultivars with improved resistance by genetic engineering.

Material and Methods

Plant material and growth conditions: Four-week-old vegetatively propagated Shanxin poplar plantlets grown on woody plant medium (WPM) + NAA (0.1 mg/L) + 6-BA (0.5 mg/L), were cut and cultured in glass vessels containing 25 ml of liquid WPM + 0.1 mg/L IBA, cultured for two weeks for rooting. The six-week-old plantlets were then treated with or without various stress conditions and collected as samples. All poplar plants were placed in a growth chamber at 26°C with a 16/8h light/dark cycle during both the growth and the treatments.

Pathogen strains, preparation of fungal inoculum, and inoculation of plants: The strains of *Fusarium oxysporum* (Fo), *Sclerotinia sclerotiorum* (Ss), *Alternaria alternate* (Aa), *Rhizoctonia solani* (Rs) and *Cytospora chrysosperma* (Cc) were preserved in our laboratory. Each fungal strain was cultured on potato dextrose agar (PDA) medium in an incubator at 26°C in dark to obtain sufficient mycelia or spores. Collection of each fungal inoculum was performed under sterile conditions. Conidia of Aa, Ss, Fo and Cc were collected as inoculum by gently rinsing with liquid WPM and was adjusted to 1×10^5 cfu/mL conidia WPM suspension using a hemacytometer under a microscope. For Rs, total mycelia on 1×4 cm^2 square-shaped PDA culture was used as the inoculum.

Poplar plant treatments: All the treatments were carried out under sterile conditions with the Shanxin poplar plant cultured in a glass vessel and its root immersed in 25 ml of liquid WPM and incubated for 48 hours (Fig. 1). The untreated control plants were cultured in liquid WPM. Salt treatment was performed with WPM with 200 mM NaCl. Desiccation (drought) treatment was performed with WPM containing 30% (m/v) PEG6000. Liquid WPM adjusted to pH 10 by adding Na_2CO_3 was used for alkali treatment. Hormone inducing was separately performed using WPM containing 100 µM ABA, JA or SA. The ABA, JA and SA were all of HPLC-grade and purchased from Sigma-Aldrich. For each of *A. alternata*, *S. sclerotiorum*, *F. oxysporum* or *C. chrysosperma* inducing, 1×10^5 cfu/mL conidia WPM suspension was used to culture the poplar plant. For *R. solani* inducing, poplar plant was co-cultured in WPM containing the collected mycelia.

Collecting of plant samples: The shoot tip, the young stem (3^{rd}-5^{th} internodes), the young leaves between the 3^{rd}-5^{th} internode, the developing stem (7^{th}-9^{th} internodes), the mature leaves between the 7^{th}-9^{th} internodes, the lignified stem (11^{th}-13^{th} internodes), the relatively old leaves between the 11th-13th internodes of the stem and the root of untreated wild-type Shanxin poplar plants were harvested as samples (Fig. 2).

After treatment, the shoot tip, the mature leaves between the 7^{th}-9^{th} internodes of the stem and the root of the Shanxin plants undergone each treatment were separately collected. For each sample, the material from ten plants were pooled as one biological replicate.

Screening and cloning of *PdPapDOL3*: In a previous study, the transcriptome data of Shanxin poplar plants with *T. asperellum* (T), *A. alternata* (A), or dual inoculation of both T and A (TA) by co-culturing for 48 hours was constructed using Illumina technology (Biomarker Technologies Co, LTD. Beijing). Differentially expressed genes (DEGs) were identified according to Gang *et al*., (Gang *et al*., 2019), and DEGs that responded to the fungal inductions were selected as genes of interest. Among these DEGs was *Potri. 007G098800.1*, which showed significant regulation. The coding sequence of *Potri.007G098800.1* was obtained from Phytozome 13. A pair of primers for cloning the CDS of *Potri.007G098800.1* were designed using Primer Premier (Ver. 6.0) (Table 1). The total RNA of Shanxin poplar was extracted from leaves via the cetyltrimethylammonium bromide (CTAB) method as described by Gambino *et al*., 2008. Then, the first-strand cDNA was synthesized from 2 µg of total RNA using the PrimeScript RT reagent Kit (TaKaRa) following the manufacturer's instructions; the resulted cDNA was diluted by 10 fold with sterile water, then used as the template for the subsequent PCR assays. The amplification of *PdPapDOL3* sequence was performed using PrimeSTAR® Max (TaKaRa, Dalian). The PCR cycles were set as below: pre-denaturation at 98°C for 3 min, then 98°C 10 s, 60°C 8 s, 72°C 5 s for 35 cycles, and 2 min at 72°C in the end. Sequencing of the PCR product was performed to confirm the *PdPapDOL3* sequence. Sequencing was performed by Tsingke (Beijing).

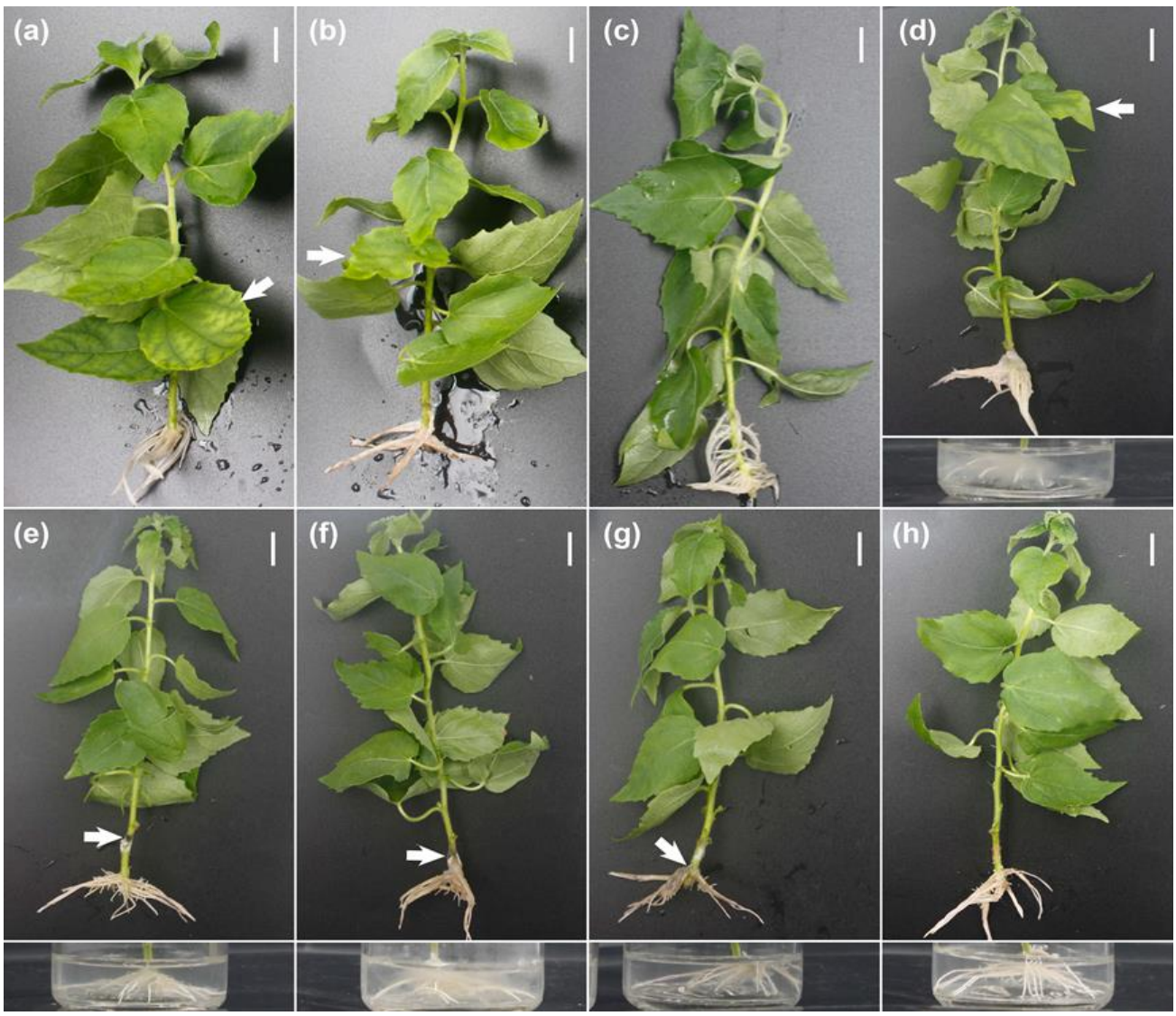

Fig. 1. Poplar plants treated with stress or pathogen fungi.
Six-week-old tissue-cultured 'Shanxin yang' poplar plants treated with abiotic stress or fungal infection for 48 hours. (a) Salt-treated plant. (b) Alkali-treated plant. (c) PEG-treated plant. Plant infected by (d) *F. oxysporum*. (e) *S. sclerotiorum*. (f) *A. alternata*. (g) *C. chrysosperma*. (h) *R. solani*. White arrows indicate yellowing of leaves in (a), (b), (d) and necrosis in (e), (f), (g). Bar = 1 cm.

Fig. 2. Sampled compartments for expression analyses of *PdPapDOL3* gene.
The different organs or tissues taken as samples from six-week-old tissue-cultured *P. davidiana* × *P. alba* var. *pyramidalis* are shown. The leaf samples (L1, L2, L3) corresponding to each stem sample is marked with white triangle. All indicated samples were collected from untreated plants. ST, L (L2) and R were collected from treated plants. Bar = 1 cm.

Sequence alignment and phylogenetic analyses: The open reading frames (ORFs) of *PdPapDOL3* was identified using the ORF finder tools of NCBI (http://www.ncbi.nlm.nih.gov/ gorf/gorf.html). Calculation of the molecular weight (MW) and isoelectronic point (pI) of the coded protein of PdPapDOL3 was performed using the ExPASy - ProtParam tool (http://web.expasy.org/protparam/). The conserved domain of PdPapDOL3 was predicted employing the NCBI CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/ wrpsb.cgi). Subcellular localization prediction of PdPapDOL3 was completed by Psort II (https://www. genscript.com/psort.html). Transmembrane region prediction for PdPapDOL3 was completed by TMHMM-2.0 (https:// services.healthtech.dtu.dk/ service.php?TMHMM-2.0). Hydrophobicity of PdPapDOL3 protein sequence was carried out by ProtScale (https://web.expasy.org/protscale/). The secondary structure of PdPapDOL3 was predicted online by SOPMA (https://npsa-

prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page= npsa_ sopma. html). Homologous protein sequences of PdPapDOL3 protein were obtained using NCBI BLASTx program (https://blast.ncbi. nlm.nih.gov/) and their motifs of 3 structural domains were found using MEME Suite 5.4.1 (https://meme-suite.org/ meme/). All homologous protein sequences of PdPapDOL3 were subjected to phylogenetic analysis by constructing a phylogenetic tree (Bootstrap=1000) by the neighbor-joining (NJ) method in Mega6.0 software.

Differential expression analysis of *PdPapDOL3*: The RT-qPCR reactions were performed using TransStart Top Green qPCR SuperMix (TransGen) following the manufacturer's instructions, with a LightCycler 96 (Roche). The genes *ACT7* (*PdPapACT7*), *EF1-α2* (*PdPapEF1-α2*) and *Tub-α* (*PdPapTUB-α*) of the 'Shanxin yang' poplar were used as reference genes to normalize the expression levels of *PdPapDOL3* gene. All primers are listed in Table 1. The specificity of each primer pair had been examined and confirmed through electrophoresis. The RT-qPCR cycles were set as: 95°C for 10 min for preincubation, followed by 45 cycles of 95°C for 5 s, 59°C for 15 s, 72°C for 10 s and 95°C for 10 s, 65°C for 60 s and 97°C for 1 s for melting. The RT-qPCR assays were performed with three biological replicates, each with three technical replicates.

Statistical analyses: The relative expression and the plant samples undergone treatments of *PdPapDOL3* gene compared with the mean expression of the three internal reference genes were calculated by $2^{-\Delta Ct}$ according to Riedel *et al*., (2014). Data analyses and graph visualization were performed using Microsoft® Excel 2019. Data were expressed as means (±SD). Two-way ANOVA followed by Fisher's exact test was performed to evaluate statistical significance ($p<0.05$).

Results and Analysis

Characterization of *PdPapDOL3*: The cDNA of *PdPapDOL3* was amplified by PCR, and an 840-bp sequence was obtained (Fig. 3A). The predicted coded protein of PdPapDOL3 contains 279 amino acid residues, and has a complete, conserved DOG1 (pfam14144, the only member of the Superfamily CL16653) domain located between the 175th to 369th amino acids of PdPapDOL3 (E-value = 5.21e-18) ((Fig. 3B). The molecular formula of PdPapDOL3 protein is $C_{1408}H_{2213}N_{403}O_{434}S_7$, the theoretical molecular weight is 31954.92 D, the theoretical isoelectric point is 5.92 and no transmembrane structure was identified. The instability index is 55.49, and the average hydrophilicity is -0.561. In sum, the PdPapDOL3 protein is predicted to be an unstable non - transmembrane hydrophilic acidic protein.

Prediction of the subcellular localization showed that the possibility of distribution in nucleus, cytoplasm, mitochondria, golgi body and peroxisome accounted separately for 47.8%, 30.4%, 13.0%, 4.3% and 4.3%. Therefore, PdPapDOL3 might play a role as a transcription factor in the nucleus.

The prediction of the secondary structure of PdPapDOL3 protein showed that the protein was mainly composed of irregular coil and *α*-helix motifs, of which irregular coil accounted for 21.86%, *α*-helix accounted for 71.68%, *β* -rotation accounted for 2.51% and extended chain accounted for 3.94%.

Table 1. PCR primer sequence.

Gene	Primer	GC%	Tm/°C	Product size/bp
DOL3	F---ATGTCCAAGCTGGCAAGTAG	52.0	67.8	840
	R---TCAGTGGTGGACGCGGCG	72.2	70.5	
	qF---AGCCTCTTATCAACCGTGTTC	47.6	58.3	184
	qR---CCTGACTTTGAGTAGAGCAAGTG	47.8	58.3	
ACT7	F---TCACTCATTGGAATGGAAGC	45.0	58.6	173
	R---GGAGCAAGAGCTGTGATCTC	55.0	57.7	
EF1-a2	F---GGAAGTGCAGGCTGAGTTG	57.9	59.6	176
	R---CACTAAGAAAGAGTATCTGGCCC	52.2	58.5	
Tub-a	F---TCAGCCACCTACTGTAGTACCTG	47.8	58.5	173
	R---CTTCCATGCCTTCACCAAC	52.6	59.1	

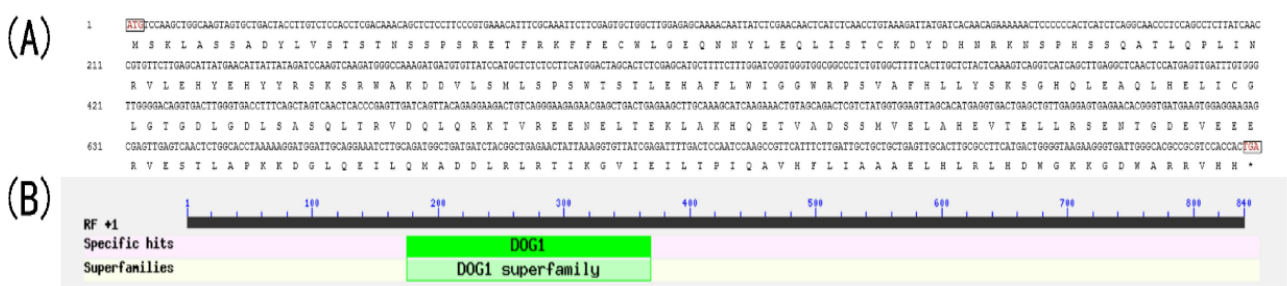

Fig. 3. Sequences and the predicted conserved domain of PdPapDOL3
Notes: (A) Nucleotide and amino acid sequences; (B) predicted conserved domain

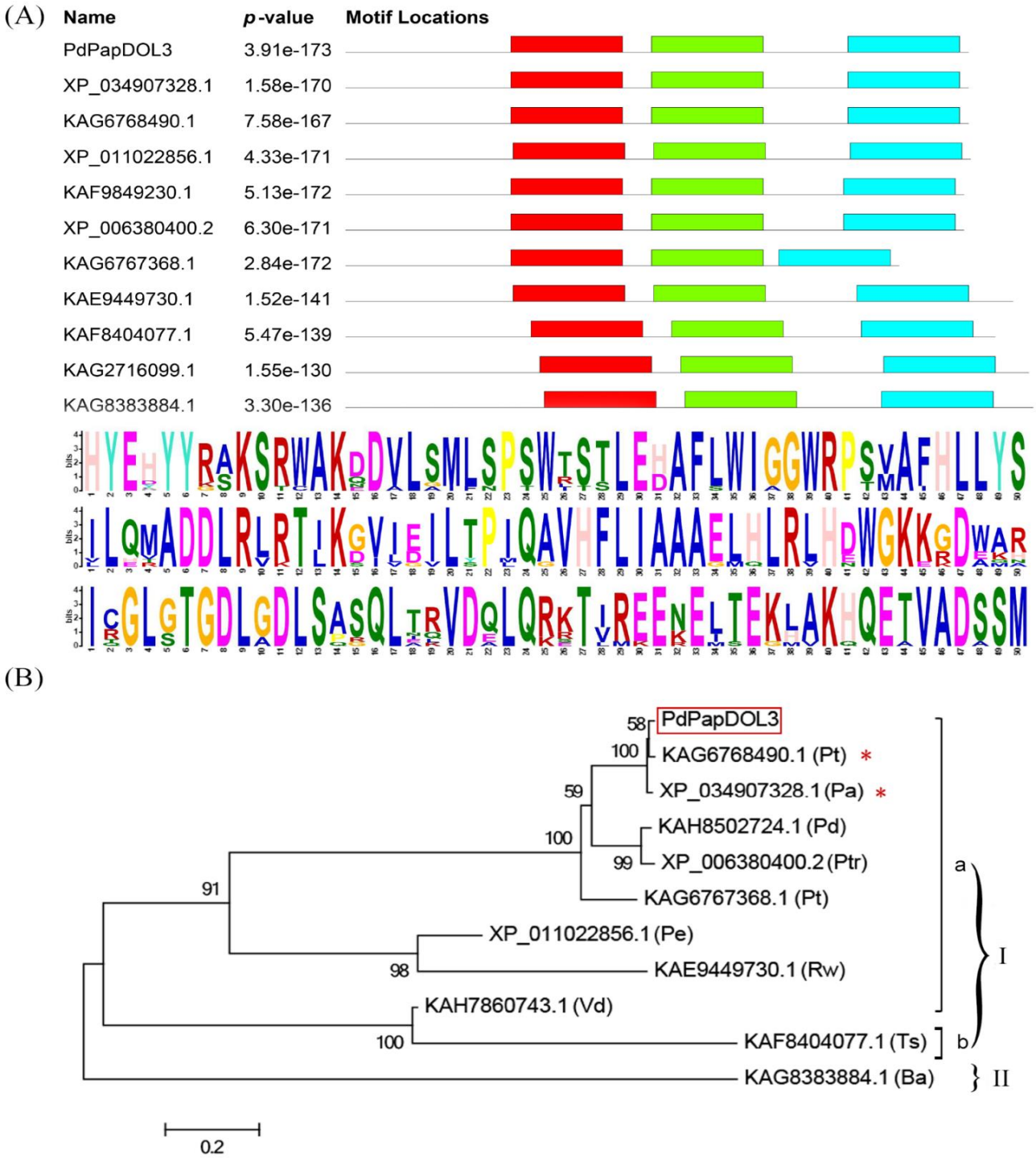

Fig. 4. Multiple sequence alignment, conserved motifs of PdPapDOL3, and phylogenetic analysis
Notes: (A) Sequence alignment; (B) phylogenetic tree. The red frame marked the PdPapDOL3 protein.
*Proteins phylogenetically close to PdPapDOL3.

Sequence alignments and phylogenetic analysis: The sequences of ten DOG1 domain-containing homologous proteins of PdPapDOL3 (from the species of 5 generas including *Populus*, *Rhododendro*, *Vaccinium*, *Buddleja* and *Tetracentron*) with the highest identity (Total Score > 352; Query Cover > 96%; E-value > 1e-118; Ident > 61.36%) were obtained by searching BLASTx in the Genbank database. All the obtained sequences were examined using the Simple Modular Architecture Research Tool (SMART; http://smart.embl.de) and PFAM (http://pfam.xfam.org), confirming the presence of characteristic DOG1 domains. Alignment of PdPapDOL3 and the 10 DOG1-containing sequences indicated that all the 11 sequences had three highly conserved motifs located the middle and C-terminal (Fig. 4A).

The ten selected DOG1 domain-containing homologous proteins were classified into two groups in the phylogenetic tree (Fig. 4B). Group II only contained KAG8383884.1 (*B. alternifolia*, Ba). Group I consisted of two subgroups, a and b. Subgroup b contained

KAH7860743.1 (*V. darrowii*, Vd) and KAF8404077.1 (*T. sinense*, Ts). However, *PdPapDOL3*, KAG6768490.1 (*P. tomentosa*, Pt), XP_034907328.1 (*P. alba*, Pa), KAH8502724.1 (*P. deltoids*, Pd), XP_006380400.2 (*P. trichocarpa*, Ptr), KAG6767368.1 (Pt), XP_011022856.1 (*P. euphratica*, Pe) and KAE9449730.1 (*R. williamsianum*, Rw) were classified as Subgroup a. KAG6768490.1 (Pt) and XP_034907328.1 (Pa) were phylogenetically closest to *PdPapDOL3*.

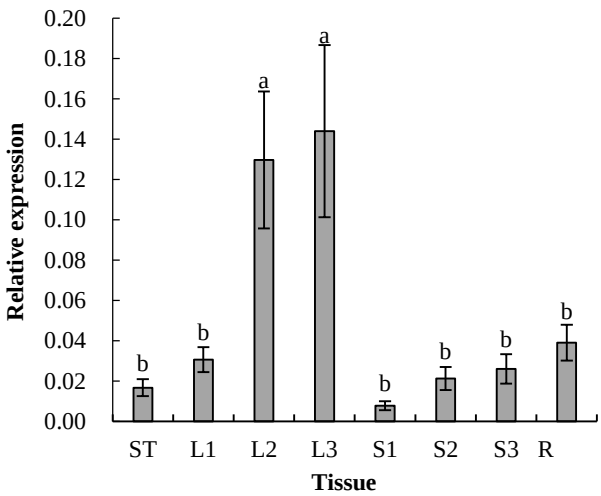

Fig. 5. Tissue-specific expression of *PdPapDOL3*
Notes: The x-axis represents different compartments of the poplar plant. ST, shoot tip. L1, young leaves. L2, mature leaves, L3, relatively old leaves. S1, young stem. S2, developing stem. S3, lignified stem (see methods). The y-axis shows the relative expression of *PdPapDOL3* normalized by the average expression of three internal reference genes, *PdPapACT7*, *PdPapEF1-α2* and *PdPapTUB-α*. Data are expressed as mean ± SD. Different lower-case letters represented the significance of difference (p<0.05) among different samples.

Tissue-specific expression of *PdPapDOL3*: The results of RT-qPCR revealed evident specificity of *PdPapDOL3* expression in different compartments of the poplar plant, as shown in Figure 5. *PdPapDOL3* showed characteristic high expression in the young leaf (L1), and up-regulated expression L2 (mature leaves) and L3 (relatively old leaves). The relative expression of *PdPapDOL3* in L2 and L3 were 4.23 and 4.70 times of L1. Similar up-regulated expression pattern of *PdPapDOL3* was also evident along the developmental gradient of the stem. The relative expression of *PdPapDOL3* in S3 and S2 were 3.37 and 2.75 times higher than S1 (p<0.05). Taken together, our results reflected constitutively high and characteristic expression patterns of *PdPapDOL3*. In particular, *PdPapDOL3* is preferentially expressed in older tissues.

Regulation of *PdPapDOL3* expression under stress

Tissue-specific regulation of *PdPapDOL3* expression under abiotic stress: To thoroughly investigate the expression of *PdPapDOL3* in response to abiotic stress, in this study we determined the regulation of *PdPapDOL3* expression in the shoot tip (ST), mature leaves (L, which are the same as L2 in untreated plants) and root (R) under salt (NaCl), alkali (Na_2CO_3) and desiccation (PEG) stress (Fig. 6). After 48 hours of treatment, the expression of *PdPapDOL3* in ST was significantly up-regulated only under salt stress (p<0.05, 9.42 times of the control). *PdPapDOL3* expression in leaves was significantly up-regulated after the salt and alkali stresses (p<0.05). The expression of *PdPapDOL3* in root was significantly up-regulated under salt or alkali stress (p<0.05), but reduced (although not statistically significantly) to 0.71 times of the control under desiccation stress (Fig. 6).

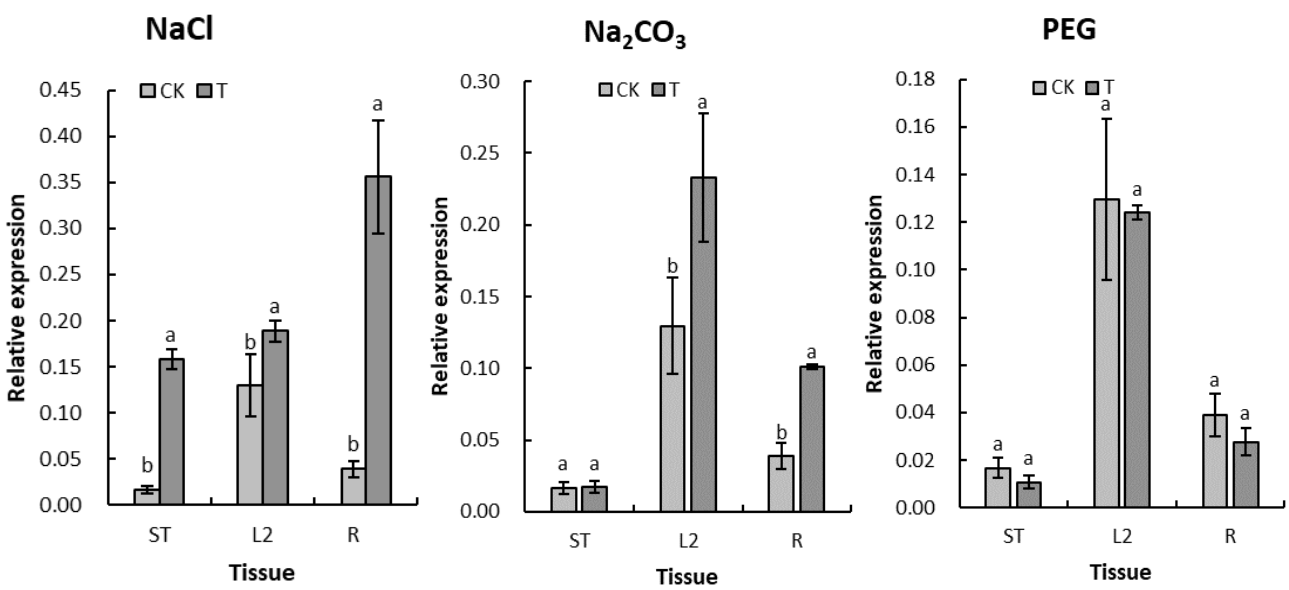

Fig. 6. Regulation of *PdPapDOL3* expression in the shoot tip, leaves and roots after abiotic stress induction
Notes: The x-axis represents different compartments of the poplar plant. The y-axis shows the relative expression of *PdPapDOL3* normalized by the average expression of three internal reference genes in ST, L (same as L2 in Fig. 5) and R after abiotic stress induction or not. Data are expressed as mean ± SD. Different lower-case letters represent significant difference (p<0.05) among different samples.

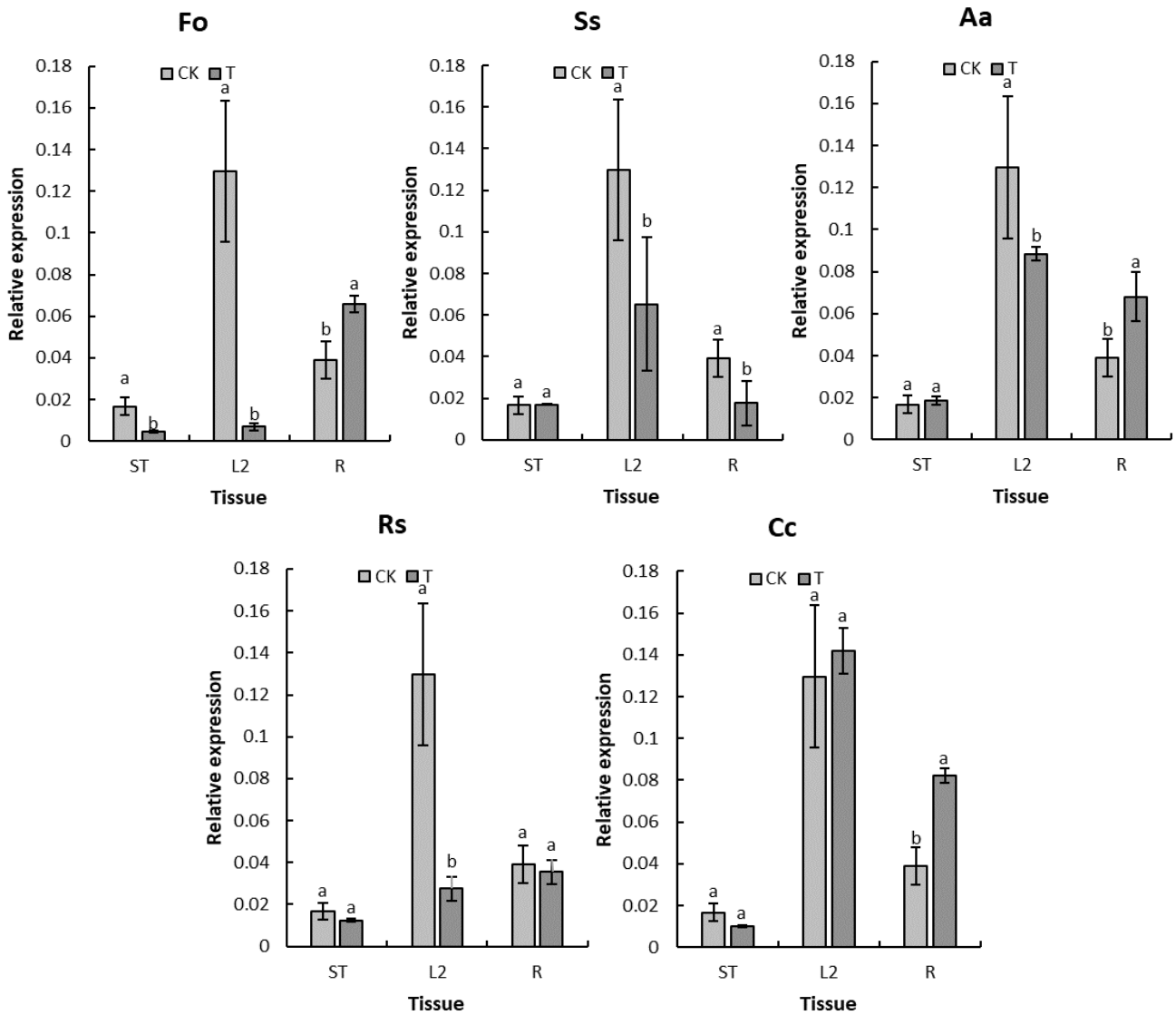

Fig. 7. Regulation of *PdPapDOL3* expression in the shoot tip, leaves and roots after fungal pathogen induction
Notes: The x-axis represents different compartments of the poplar plant. The y-axis shows the relative expression of *PdPapDOL3* normalized by the average expression of three internal reference genes in ST, L (same as L2 in Fig. 5) and R after biotic stress induction or not. Data are expressed as mean ± SD. Different lower-case letters represent significant difference (p<0.05) among different samples.

Tissue-specific regulation of *PdPapDOL3* expression under biotic stress: After inoculated with each soil-borne plant pathogenic fungus for 48 h, the expression of *PdPapDOL3* in ST was significantly down-regulated after *F. oxysporum* inoculation (p<0.05, 0.28 times of the control). While *S. sclerotiorum* (Ss), *A. alternata* (Aa), *C. chrysosperma* (Cc) and *R. Solani* (Rs) did not induce significant change of *PdPapDOL3* expression (Fig. 7).

In L, the expression of *PdPapDOL3* was significantly down-regulated when inoculated with Fo, Ss, Aa or Rs (p<0.05, 0.05, 0.50, 0.68 and 0.21 times of the control, respectively). The expression of *PdPapDOL3* was upregulated only after Cc inoculation for 48 h (p>0.05), 1.09 times of that of the control group. (Fig. 7)

In R, the expression of *PdPapDOL3* was significantly up-regulated when the root was inoculated with Fo, Aa or Cc for 48 h (p<0.05, 1.68, 1.74 and 2.10 times of the control, respectively), but the changes caused by Rs was mild (Fig. 7).

Tissue-specific regulation of *PdPapDOL3* expression after SA, JA and ABA induction: We treated poplar plants with exogenous SA, JA or ABA and determined the regulation of *PdPapDOL3* expression in ST, L and R. After the 48-h induction separately with three plant hormones, the tissue-specific expression of *PdPapDOL3* in Shanxin poplar showed differential regulation (Fig. 8). *PdPapDOL3* expression in ST, L and R was significantly down-regulated by SA induction (p<0.05; 0.55, 0.21 and 0.19 times of control, respectively). After the 48-h JA induction, *PdPapDOL3* expression in ST, L and R was significantly down-regulated to 0.17, 0.25 and 0.21 times of control (p<0.05) (Fig. 8). After 48 hours of ABA induction, the expression of *PdPapDOL3* in ST, L and R were significantly regulated compared to control: *PdPapDOL3* expression in ST and L was significantly down-regulated, which was 0.43 and 0.24 times of that in the control (p<0.05), while the expression of *PdPapDOL3* gene in R was significantly up-regulated, which was 2.46 times that of control (p<0.05) (Fig. 8).

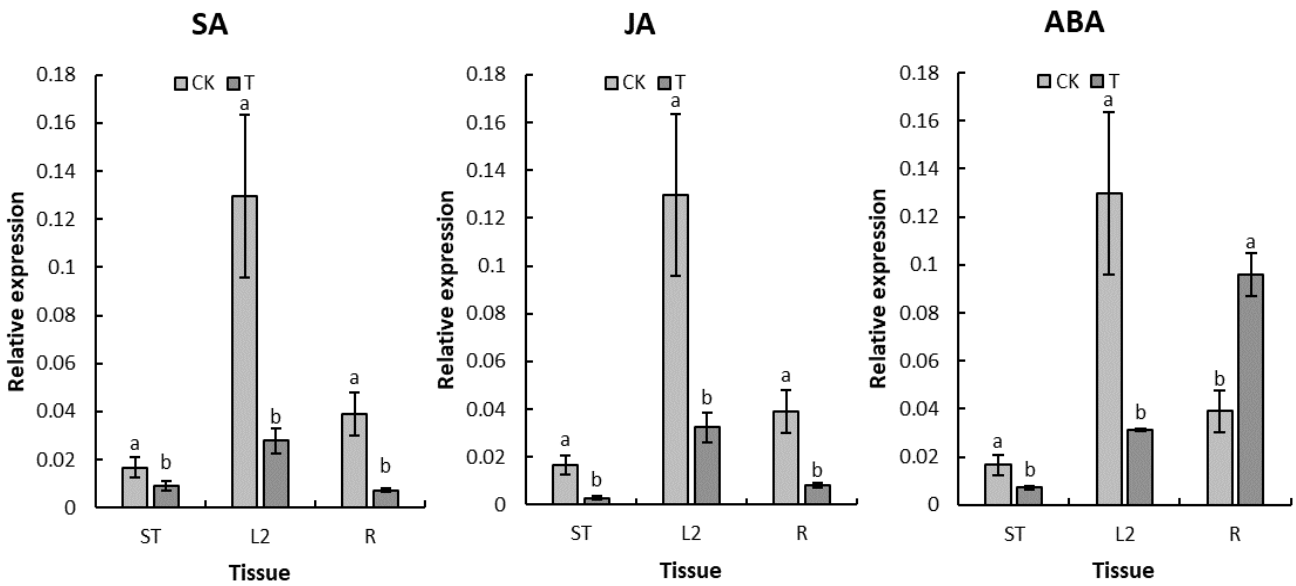

Fig. 8. Regulation of *PdPapDOL3* expression in the shoot tip, leaves and roots after hormone induction
Notes: The x-axis represents different compartments of the poplar plant. The y-axis shows the relative expression of *PdPapDOL3* normalized by the average expression of three internal reference genes in ST, L (same as L2 in Fig. 5) and R after hormone induction or not. Data are expressed as mean ± SD. Different lower-case letters represent significant difference (p<0.05) among different samples.

Discussion

Transcription factors are cis-acting elements that can identify the promoter region of target genes and play a crucial role in the biotic and abiotic stress response of plants (Baloglu *et al*., 2014). The basic leucine zipper (bZIP) family is one of the largest of transcription factor families, and is ubiquitous in eukaryotes. The bZIP gene family mainly plays a role in regulating plant growth, development and stress response (Ortiz-Espín *et al*., 2017). TGA transcription factors are the D subgroup of the bZIP (Basic Leucine Zipper, bZIP) family (Droge-Laser *et al*., 2018), which are widely expressed in different tissues of a variety of plants and can regulate stress tolerance or the growth and development of plants. Studies have shown that each of the CmTGA family proteins in melon (*Cucumis melo* L.) contains both the bZIP domain and the DOG1 domain, and the genes of this family are extensively involved in plant disease resistance (Tian, 2021). PdPapDOL3 has a complete conserved DOG1 domain. However, the tissue-specific expression pattern of *PdPapDOL3* and its regulation profiles while induced by various stresses and hormones have not been reported; therefore, we designed and performed this study. As a result, an 840 bp coding sequence of *PdPapDOL3* of Shanxin poplar was cloned; its encoded protein was an unstable non-transmembrane hydrophilic acid protein with a complete conserved DOG1 domain, and it is predicted to act as a transcription factor located in the nucleus.

Sequence alignment results showed that PdPapDOL3 and the ten DOG1 protein sequences of 5 plant genera including *Populus*, *Rhododendro*, *Vaccinium*, *Buddleja* and *Tetracentron* all had three highly conserved motifs. Phylogenetic analysis of homologous proteins showed that PdPapDOL3 was most closely related to KAG6768490.1 (*P. tomentosa*) and XP_034907328.1 (*P. alba*), suggesting that they might have similar functions. At present, there are relatively few studies on the function of DOG1 family genes in poplar. Hence, the results of our study on the regulation of *PdPapDOL3* expression will lay a foundation for exploring the function of the paralogs of *PdPapDOL3*.

Our study revealed that *PdPapDOL3* was constitutively expressed in young tissues, the leaves, the stem and the roots, with the leaves demonstrating the highest expression. With the aging of leaf from young to mature, *PdPapDOL3* expression increased gradually. *PdPapDOL3* expression in relatively old leaves was 40.41 times that in the shoot tip (p<0.05). In addition, *PdPapDOL3* expression increased with the development of stem and the increase of stem lignification. The pattern of *PdPapDOL3* expression in leaves and in the stem were the same.

During the growth and development of plants, they encounter various abiotic stresses such as drought, high salt, extreme temperature and nutrient deficiency. At this point, plants often respond to abiotic stress by regulating the expression of their own genes (Zou *et al*., 2012). In this study, it was revealed that the expression of *PdPapDOL3* changed under three abiotic stresses, namely salt, alkali and desiccation, among which salt had the greatest influence (Fig. 6). The expression of *PdPapDOL3* in the shoot tip, leaves and roots was 9.42, 1.45 and 9.11 times that of the control (p<0.05), respectively, after the salt treatment. These results suggested that it might be involved in the response to salt stress. However, under alkali stress, *PdPapDOL3* expression did not change significantly in the young, vigorously developmenting tissues, but only changed in the leaves and roots, which were 1.80 and 2.60 times of the control, respectively (p<0.05). Previous studies have shown that some bZIP proteins are involved in plants' response to environmental stress, including salt and drought stress (Tu *et al*., 2016). However, *PdPapDOL3* expression in the shoot tip, leaves and roots under the

desiccation (drought) stress did not consist with that of Tu (Tu *et al*., 2016), and slight down-regulation of *PdPapDOL3* expression was observed (p>0.05), which needed to be further investigated.

Hypersensitive response (HR) occurs when plants are infected by foreign organisms. Within a certain length of time post-infection, plants will develop resistance to the foreign organisms such as bacteria, fungi and filterable bacteria, so that plants can resist their infection (Glazebrook, 1999). *F. oxysporum* (Fo), *S. sclerotiorum* (Ss), *R. solani* (Rs), *A. alternata* (Aa) and *C. chrysosperma* (Cc) are five common soil-borne plant pathogens, which can cause varying degrees of root rot, stem rot, stem base rot, flower rot, sclerotium, brown spot disease, leaf blight and other disease symptoms under certain environmental conditions with a wide range of host. In this study, the tissue-specific expression of *PdPapDOL3* was altered by five soil-borne plant pathogenic fungi. Only the Aa inoculation up-regulated the expression of *PdPapDOL3* in the young tissues with vigorous development; the other four fungi down-regulated *PdPapDOL3* expression. Among them, Fo inoculation down-regulated *PdPapDOL3* expression the most, which was only 0.28 times that of the control (Fig 7). In the leaves, the expression of *PdPapDOL3* was significantly down-regulated when the root was inoculated with Ss, Fo, Aa and Rs (p<0.05); the expression of *PdPapDOL3* was the most down-regulated when inoculated with Fo, which was 0.05 times as much as that of the control. Only after Cc inoculation, *PdPapDOL3* expression was up-regulated (Fig 7). In the root tissue, *PdPapDOL3* expression decreased with Ss and Rs inoculation, while *PdPapDOL3* expression was upregulated by the Fo, Cc and Aa inoculations, and the upregulation of *PdPapDOL3* was the highest after the Cc inoculation, which was 2.10 times that of the control (Fig 7). These results suggested that *PdPapDOL3* has regulatory effects on the tissue infected by pathogenic fungi.

Plant hormones play an important role in coping with environmental stress. For example, SA and JA signals can participate in the regulation of plant growth, development and response to external stimuli by stimulating the establishment of plant defense mechanisms (Shine *et al*., 2019). ABA signal regulation is a complex biological process, which requires the participation of multiple signal factors, transcription factors and functional genes. Studies have shown that *AtbZIP1* can regulate the sensitivity of plants to ABA treatment and the expression of downstream ABA responsive genes by binding with ABRE elements, thus participating in the ABA signaling pathway of plants (Zimmermann *et al*., 2004). In this study, the expression of *PdPapDOL3* was significantly down-regulated by salicylic acid (SA) and jasmonic acid (JA). The expression of *PdPapDOL3* in the shoot tip and the leaf was significantly down-regulated by abscisic acid (ABA), while that in root was significantly up-regulated. These results indicate that *PdPapDOL3* may be involved in the signaling pathways of SA, JA and ABA, but whether it plays a positive or negative regulatory role remains to be further studied. The transcriptional of *PdPapDOL3* was increased by the pathogenic fungi Fo, Cc and Aa, which was consistent with the result of ABA induction. Whether the infection of these three fungi led to changes of ABA level in poplar roots and whether it was related to the ABA signaling pathway remains to be further researched.

This study will provide a basis for further revealing the function of *PdPapDOL3* and constructing poplar varieties with high resistance through molecular plant breeding.

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