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BIOINFORMATICS AND EXPRESSION ANALYSIS OF PAL GENE FAMILY OF POPLAR PLANT UNDER DIFFERENT NITROGEN TREATMENTS

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ABSTRACT

Phenylalanine ammonia lyase is the crucial enzyme and rate-limiting enzyme in the biosynthesis pathway of phenylpropane. Lignin, phytoalexin and many kinds of active substances produced in this pathway, such as flavonoids and phenols, play a key role in lignification, stress resistance, insect resistance and disease resistance of plant cells. In this study, five *PAL* gene sequences of *Populus simonii* × *p.nigra* were studied, and the basic characteristics of their genes and proteins were analyzed. The tissue expression pattern of *PAL* gene of *Populus simonii* × *p.nigra* were tested by real-time quantitative. PCR under different nitrogen forms and nitrogen concentrations. The results showed that the physicochemical property of five proteins were similar. All the proteins except PtrPAL4 and PtrPAL5 were stained on chromosome 10, PtrPAL1, PtrPAL2 and PtrPAL3 were on chromosome 6, 8 and 16, respectively. The five proteins were mainly distributed in chloroplasts, and the three-level structures of the five proteins were consistent, and they were all homologous tetramers. The evolutionary relationship results show that the *PAL* gene of poplar is highly homologous to rice and Arabidopsis. When poplar was treated with 0.1 mmol · L^{-1}, 10 mmol · L^{-1} and 2 mmol · L^{-1}, the relative expression of *PAL* genes in stem was the highest under low nitrogen level, and was most obviously induced by ammonium nitrogen.

Key words: Poplar, *PAL* gene family, bioinformatics, Stem, Gene expression

INTRODUCTION

Phenylalanine. ammonia-lyase (PAL) is the crucial enzyme and rate-limiting enzyme for the biosynthesis of phenylpropanoid metabolic pathways (Wong *et al.* 2011; Chang *et al.* 2009). It connects primary metabolism and phenylpropanoid metabolism and catalyzes phenylpropane Enzymes that are the first step in metabolism are also the most studied enzymes in the phenylpropane metabolism pathway (Jiang and Yu 2001). PAL is widely present in various plants (LIU *et al.* 2007), and was first found in green plant barley (Koukol and Conn, 1961). With the rapid development of research, PAL is not only found in green plants, but also found in fungi, bacteria and algae (Ouyang and Xue 1988). Currently, PAL has been isolated and purified from various plants such as wheat and rice (Ouyang *et al.* 1985), and cDNA clones and sequence analysis of *PAL* genes from plants such as Arabidopsis and rice (Minami *et al.* 1989; Mauch-Mani and Slusarenko 1996). Immunocytochemical studies have shown that PAL is synthesized in fence cells and sponge cells, mainly in the cytoplasm and chloroplasts (Santiago 2000). Tissue blotting showed that *PAL* mRNA is often found in tissue cells near the epidermis and microtubules (Nakazawa *et al.* 2001). Nakushima found in the results of the subcellular localization of PAL that PAL activity was highest in the interstitial part of the cell. The results of electron microscopy showed that PAL was localized in the cell matrix, the secondary wall thickening Medium and the Golgi vesicles (Nakashima 1997).

The general feature of plant *PAL* gene structure is that it consists of a small multi-gene family. Generally, it contains 2-6 PAL genes in a group of chromosomes, and can be divided into 2-3 different groups or subgroups. For example, kidney bean has three genes (Cramer *et al.* 1989) , tomato has five genes (Fukasawa-Akada et al. 1996) , and pine has a PAL gene(Whetten and Sederoff 1992) . The PAL enzyme protein is an oligomer containing 4 identical subunits, each of which has a point of action (Camm and Towers 1973; Ouyang and Xue 1988). The amino acid composition varies with different sources. For example, the amino acid composition of rice PAL is lower than that of wheat, corn, and potato, while the neutral and alkaline components are higher than the latter three (Ouyang *et al.* 1985). PAL enzymes are acidic proteins with molecular weights between 220 and 230 kDa (Camm and Towers 1973; Given *et al.* 1988), but there are differences between species. For example, wheat is 280 kDa, rice is 230 kDa (Camm and Towers 1973), and strawberry is 266 kDa (Given *et al.* 1988) The optimum pH of PAL is between 8.0 and 9.5, and there are also differences between different species. For example, the optimum pH of PAL for sweet potatoes is 8.5 to 9.5, 9.2 for rice, and 8.8 for wheat (Cheng *et al.* 2003).

PAL, as the first key enzyme and rate-limiting enzyme in plant phenylpropanoid secondary metabolism process, has function of directing metabolic flow towards the phenylpropanoid secondary metabolism process (Zhang *et al.* 2008). Because phenylpropane metabolism can produce lignin, phytoalexins and many kinds of active substances like flavonoids, phenols, etc., PAL plays an crucial action in plant cell lignification, resistance to diseases, insects and stress (Geng *et al.* 2018; Chen *et al.* 2021). The principle of action is mainly because the formation of lignin can increase the thickness of cell walls, increase the degree of tissue lignification, and form a mechanical barrier for pathogenic bacteria invasion (Legrand *et al.* 1976). When subjected to cold stress, the PAL activity of citrus will increase, the extent of which depends on the degree of cold damage to the peel and the surrounding ethylene concentration (Lafuente *et al.* 2004). Studies on tobacco scab and TMV infection of tobacco have shown that changes in plant resistance and phenylalanine ammonia lyase activity are closely related (Chen *et al.* 1998; Legrand et al. 1976). PAL activity can also be used as a physiological index to measure the resistance of plants to stress. *PAL* gene can also be induced by external environment and being factors. When low temperature, plant damaged, light, toxin treatment, insect feeding, mineral nutrition; pathogen infection and exogenous plant hormones can all induce the expression of *PAL* gene at the transcription level (Leyva 1995; Liu and Cheng 2003; Qin *et al.* 2022). In the future, this study provides a reference for understanding the basic characteristics of *PALgenes* and verifying its function under nitrogen induction in poplar.

MATERIALS AND METHODS

Materials: The plant material hybrid *Populus simonii* × *p.nigra* selected in this experiment was cultivated at State Key Laboratory of Genetics and Breeding (Northeast Forestry University) in April 2019. The temperature in the seedling room was 25 ° C, the light intensity was 120 μmol photons m-2s-1, the light was 16h / 8h dark. The genome, amino acid sequences and CDs of five *PAL* gene family members of poplar (*Populus trichocarpa*) were obtained by the phytozome database. (https://phytozome.jgi.doe.gov/pz/portal.html.).

Analysis of Physicochemical Properties and Subcellular Localization of Poplar PAL Protein: We used the online software ProtParam tool (http://web.expasy.org/prot param.) to analyze theoretical isoelectric point, amino acid number, molecular weight, aliphatic amino acid index and hydrophobicity of PAL protein. The online software The subcellular localization was tested by the WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html.).

Tertiary structure prediction of poplar PAL protein: The software SWISS-MODEL (https://swissmodel.expasy.org/interactiv) was applied to upload the obtained amino acid sequence to the website to predict tertiary structure of the PAL protein.

Prediction of Poplar *PAL* Gene Gene Structure and Conserved Motif Identification: The PAL genomic and CDS sequence from *Populus trichocarpa* obtained on Phytozome were analyzed by the commonly used bioinformatics analysis software GSDS2.0(http://gsds.cbi.pku.edu.cn.). Upload protein sequences of 5 PAL family members of *Populus trichocarpa* to the conserved motif analysis software MEME (http://meme-suite.org/tools/meme.) to analyze conserved motifs. The conserved motif value is set to 10, and the parameters can be set to default.

Poplar PAL protein multiple alignment: The bioinformatics analysis software BioEdit was used for PAL sequences multiple alignment in Populus trichocarpa, and the parameters were set as default parameters.

Phylogenetic Analysis of Poplar PAL Protein: To build a phylogenetic tree using MEGA5 software, we selected a NJ (Neighbor-Joining) type phylogenetic tree with a bootstrap value set to 1000 repeats, and finally modified it using ITOL (https://itol.embl.de/) software.

Expression analysis of *PAL* gene family in poplar stems under different nitrogen treatments: The nutrient solution (LA + 1mM NH_4NO_3) was cultured in vermiculite to 25 cm in the vermiculite. After three days of nitrogen interruption, the NH_4NO_3 component was removed from the nutrient solution during nitrogen interruption, and other nutrients were normal. The black poplars were was simultaneously and separately treated with different nitrogen (NH_4CL, $NaNO_3$, NH_4NO_3), and each nitrogen concentration was set to 0.1mM, 2mM, 10mM, and the nutrient solution was poured every three days, each time being 200mL, and the treatment was three weeks harvest material. The stem RNA was extracted, reverse transcription was performed, and then the *PAL* gene relative expression in the stem was determined by fluorescent quantitative PCR experiments. The fluorescence quantitative kit was purchased from Kangwei Century Biological Co., Ltd. The reaction system of real-time PCR is water 6.4 µL, SYBR Mixture 10 µL, template 2 µL, upstream primer 0.8 µL, and downstream primer 0.8 µL; reaction program is 95 ° C 10 .min ; 95 ° C 15 s, 60 ° C 1. min, 40 cycles; 95 ° C 15 s; 60 ° C 1 min; 95 ° C .15 s; 60°C15s. Internal reference gene is *UBQ7* gene. Informations on gene quantitative primer is shown in Table 1.

Table 1 Informations on gene quantitative primer

Gene name	Upstream primer（5′-3′）	Downstream primer（5′-3′）
PtrPAL1	CTTGGAAGCAATTACCAAGCTACTT	ACTTCTCCGTTGGGACCAGTG
PtrPAL2	ACGCCCTGATGAATGGTGAG	CCTGCATTCCGTGATCCTGT
PtrPAL3	CTTGGAAGCTATTACCAGGCTCCTC	ACTTCTCCGGTGGGACCAGTG
PtrPAL4	TGTCATGGTGGAGTTGTCAG	AGTAGTAACACCATAACTGTCTGTG
PtrPAL5	GAGATGCTGGAAGCTATCACCAAGC	GGCTCTCCATTGGGTCCAACT
UBQ7	GGAACGGGTTGAGGAGAAAGAAG	GCAAGAACAAGATGAAGCACAGAGA

RESULTS

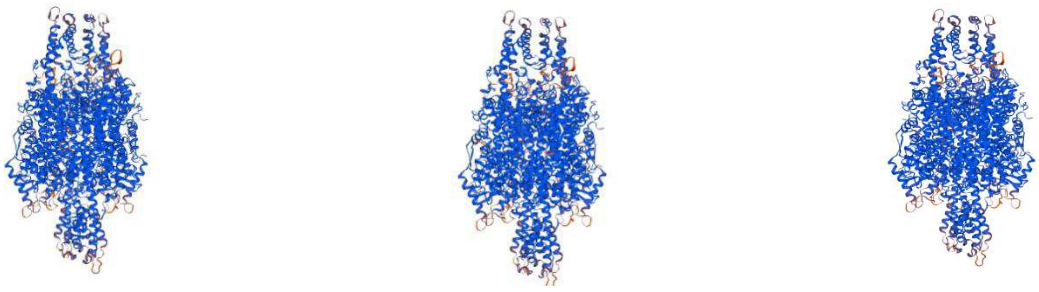
Physicochemical properties and subcellular. localization of PAL protein from *Populus Trichocarpa*: The physical and chemical properties, subcellular localization analysis results in *P. trichocarpa* PAL protein are shown in Table 2. It was found that the amino acid lengths of the five proteins of *P. trichocarpa* were not significantly different, and most of the lengths were between 707-715aa. Among them, PtrPAL3 has the largest number of amino acids, its molecular weight is the largest, which is 77875.7Da. PtrPAL5 with the smallest number of amino acids also has the smallest molecular weight, 77220.17 Da. The isoelectric points of the five proteins did not change much, ranging from 5.18-6.10. The highest isoelectric point was PtPAL1 and the lowest was PtrPAL4. The isoelectric point distribution of the five amino acid sequences were all in the acidic range. The aliphatic amino acid indexes of the proteins were all less than 100, ranging from 89.10-92.19, and the highest and lowest were PtrPAL2 and PtrPAL1. The average hydrophobicity (GRAVY) prediction results of the five P. euphratica PAL proteins were negative. All proteins except PtrPAL4 and PtrPAL5 are on chromosome 10, and PtrPAL1, PtrPAL2, and PtrPAL3 are on chromosomes 6, 8, and 16, respectively. We predicted the subcellular localization of the five proteins of *Populus trichocarpa* through online tools. The results show that the five proteins are mainly distributed in the chloroplast.

PAL Protein Tertiary Structure Prediction in *Populus trichocarpa*: In order to observe the structural characteristics of the five PAL proteins of Populus euphratica, we used the online tool SWISS-MODEL to predict the PAL protein tertiary structure. Figure 1 shows the tertiary structure of the five PAL proteins of *Populus euphratica*. The results show that the structural differences of the five proteins are not obvious, and are consistent with the tertiary structure of the PAL protein in parsley (Ritter and Schulz 2004) and in *Asarum* (LIN *et al.* 2017) PAL protein, which are homotetramers and all have " Hippocampus "shape.

Prediction of *PAL* gene gene structure and recognition of protein conserved motifs in *Populus trichocarpa*: The transcription and coding sequences of PAL family members were found in the Phytozome database, and the intron and exon structure of each member was analyzed by using the bioinformatics online software GSDS, as shown in Figure 2 and Table 3. The results show that all five members Introns, and the number of introns is one, and there are no significant differences in the similarity of the five gene structures. Conserved motifs of PAL family PAL family protein sequences were identified by using the MEME online tool, the maximum output set was 10 motif. As shown in Figure 2, the results show that the five family members have similar conserved domains.

Table 2 Physicochemical properties and subcellular localization of PAL protein from *Populus trichocarpa*

Gene name	Locus name Phytozome v3.0	Amino Acid no.	Molecular weight(Da)	Isoelectic points	Aliphatic index	GRAVY	Chromsome locaion	Cellular localization
PtPAL1	Potri.006G126800.1	714	77715.51	6.10	89.10	-0.202	Chr06:10249101..10252655	Chloroplast
PtPAL2	Potri.008G038200.1	711	77597.70	5.87	92.19	-0.168	Chr08:2123928..2128275	Chloroplast
PtPAL3	Potri.016G091100.1	715	77875.70	5.74	89.78	-0.180	Chr16:7482820..7486204	Chloroplast
PtPAL4	Potri.010G224100.1	711	77743.70	5.18	90.11	-0.209	Chr10:20815419..20819184	Chloroplast
PtPAL5	Potri.010G224200.1	707	77220.17	5.81	90.91	-0.192	Chr10:20822276..20825759	Chloroplast



Populus trichocarpa PAL1 protein *Populus trichocarpa* PAL2 protein *Populus trichocarpa* PAL3 protein

Populus trichocarpa PAL4 protein *Populus trichocarpa* PAL5 protein

Figure 1. Tertiary structure of PAL protein structure

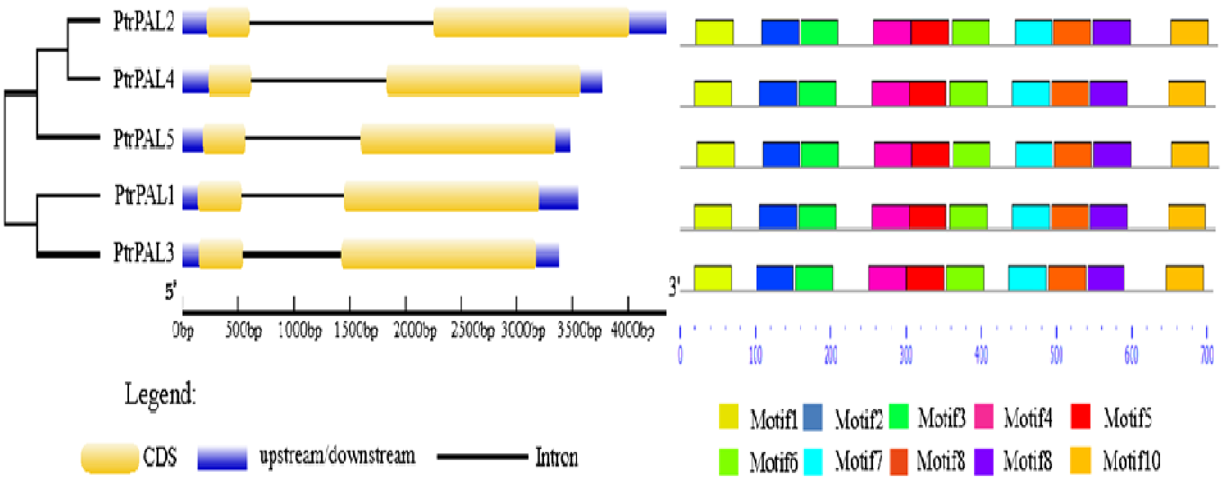


Figure 2. *PAL* gene structure and protein conserved motifs

Table 3. Conserved motifs

Motif1	🟨	DPLNWGMAAESLKGSHLDEVKRMIEEYRKPVVKLGGETLTIGQVTAIASR
Motif2	🟦	TTGFGATSHRRTKQGGELQKELIRFLNAGIFGNGTESSHTLPHSATRAAM
Motif3	🟩	RINTLLQGYSGIRFEILEAITKLLNHNITPCLPLRGTITASGDLVPLSYI
Motif4	🟪	NGTAVGSGLASMVLFETNVLAILSEVLSAIFAEVMQGKPEFTDHLTHKLK
Motif5	🟥	HHPGQIEAAAIMEHILDGSAYVKAAQKLHEIDPLQKPKQDRYALRTSPQW
Motif6	🟩	IEVIRTSTKMIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNTR
Motif7	🟦	RNPSLDYGFKGAEIAMASYCSELQFLANPVTNHVQSAEQHNQDVNSLGLI
Motif8	🟧	RKTAEAVDILKLMSTTFLVALCQAIDLRHIEENLKSTVKNTVSQVAKRVL
Motif9	🟪	GFNGELHPSRFCEKDLLKVVDREHVFSYIDDPCSATYPLMQKLRQVLVDH
Motif10	🟧	CRSYPLYKFVREELGTSLLTGEKVKSPGEEFDKVFTAICAGKJIDPMLEC

PAL protein Multiple sequence alignment in *Populus trichocarpa*: The protein sequences of PAL family members were collected. The online software Clustalx v1.83 was used for performing multiple sequence alignment analysis on amino acid sequence of PAL protein from *Populus euphratica*. As shown in Figure 3, the results show that the gene domains of the five PAL family members of Populus trichocarpa are highly conserved and contain the phenylalanine ammonialyase active center sequence GTITASGDLVPLSYVAG (200-216aa), which is consistent with (CHENG *et al.* 2003) [STG] [LIVM] [STG] [SAC] SG [DH] L. [PN] L [SA]. {2,3} [SAGVTL] phenylalanine ammonialyase tag sequence pattern.

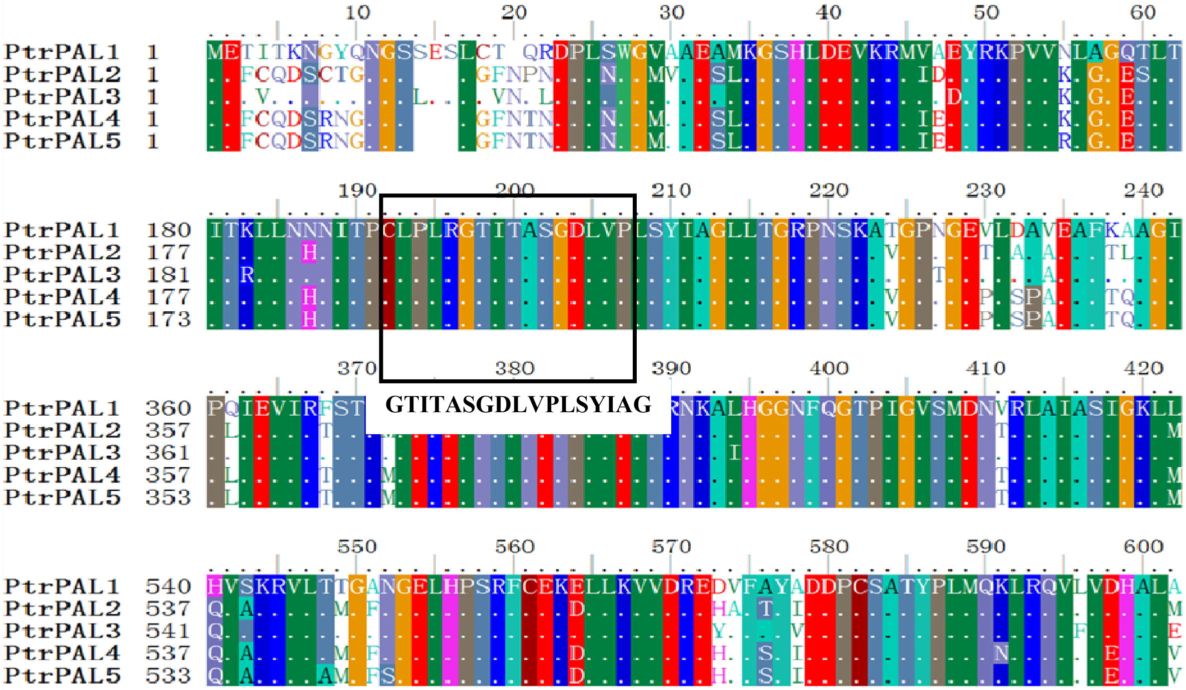


Figure 3. Multiple alignments of PAL amino acid sequence in *Populus trichocarpa*

Phylogenetic analysis of PAL protein in *Populus trichocarpa*: In order to further reveal the phylogenetic relationship of PAL, the protein sequences of 5 Papaver populus, 9 rice and 4 *Arabidopsis* PAL family members were collected, and phylogenetic tree of homologous sequences was constructed by using the commonly used bioinformatics analysis software MEGA5. As shown in Figure 5, the results show that the 18 PAL proteins are divided into three subfamilies. Subgroup I contains PTrPAL2, PTrPAL4, OsPAL2, OsPAL9, PTrPAL3, PTrPAL1, and OsPAL6. Among them, the relationship between PTrPAL2 and PTrPAL4 is closer, PTrPAL1 and OsPAL6. Kinship is closer. Subgroup II includes AtPAL4, PTrPAL5, OsPAL5, OsPAL8, and OsPAL1. Among them, AtPAL4 and PTrPAL5 are more closely related. Subgroup III includes AtPAL1, AtPAL3, AtPAL2, OsPAL7, OsPAL3, and OsPAL4. In summary, the evolutionary relationship results show that the PAL gene of poplar is highly homologous to rice and *Arabidopsis*.

Expression analysis of *PAL* gene family in poplar stems under different nitrogen treatments: The following figure shows the relative expression level of the *PAL* genes under the three forms of nitrogen treatment. Most of the results show that the relative expression levels under the ammonium nitrogen treatment are the highest, and the relative expression levels under the nitrate nitrogen treatment are the lowest. As you can see from Figure 6 and the relative expression of *PAL1* under ammonium nitrogen treatment is higher than that of normal nitrogen treatment, the difference is significant, about 1.2 times. Nitrogen and ammonium nitrate were both about 1 and 1.1 times; while the relative expression of *PAL1* gene in high nitrogen treatment was all reduced, and nitrate nitrogen was the lowest. As shown in Figure 7, compared with the normal 2 mmol • L^{-1} nitrogen concentration, the relative expression of *PAL2* gene was highest under low nitrogen conditions with 0.1 mmol • L^{-1} nitrogen concentration, and the difference was significant. Nitrogen was about 1.3 times, nitrate nitrogen and ammonium nitrate were both about 1.2 times, and the relative expression levels were reduced under high nitrogen conditions with a nitrogen concentration of 10 mmol • L^{-1}. The relative expression level of *PAL3* gene was highest under ammonium nitrogen treatment in Figure 8, and the expression level under low nitrogen treatment was about 1.3 times that under normal nitrogen treatment, while the relative expression amount under high nitrogen treatment decreased. As shown in Figures 9 and Figures 10, *PAL4* and *PAL5* relative expression levels were still highest under the treatment with ammonium nitrogen, and the relative expression levels under low nitrogen treatment were higher than those under normal nitrogen treatment, both were 1.3 times.

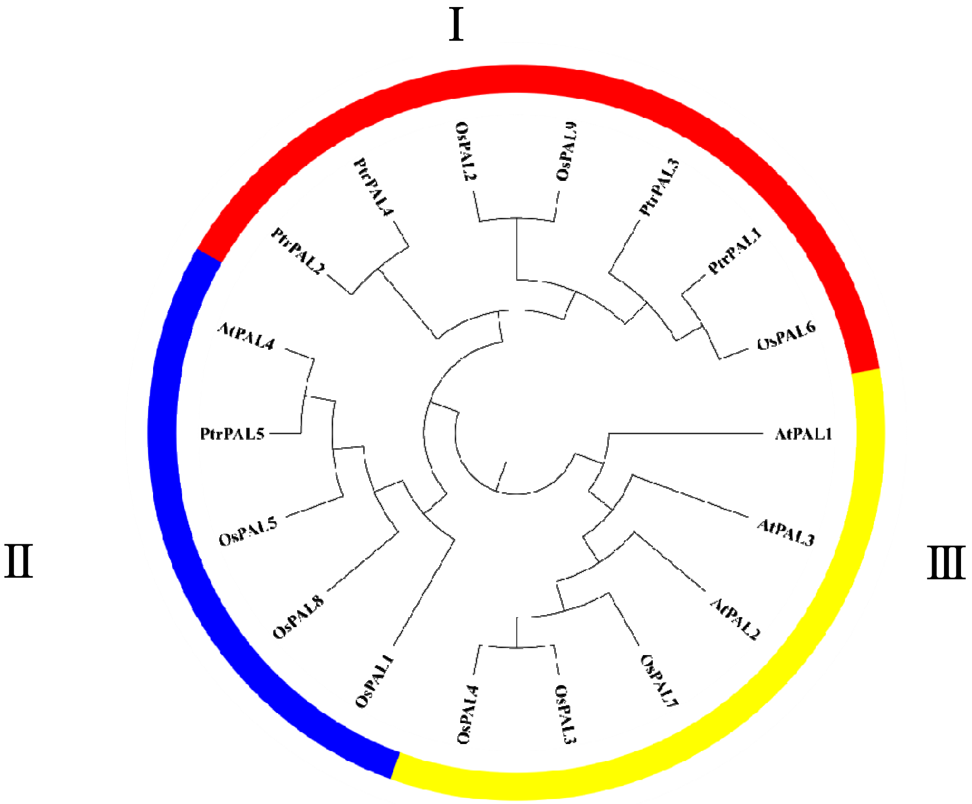


Figure. 4. Phylogenetic evolution of *Populus tomentosa* PAL

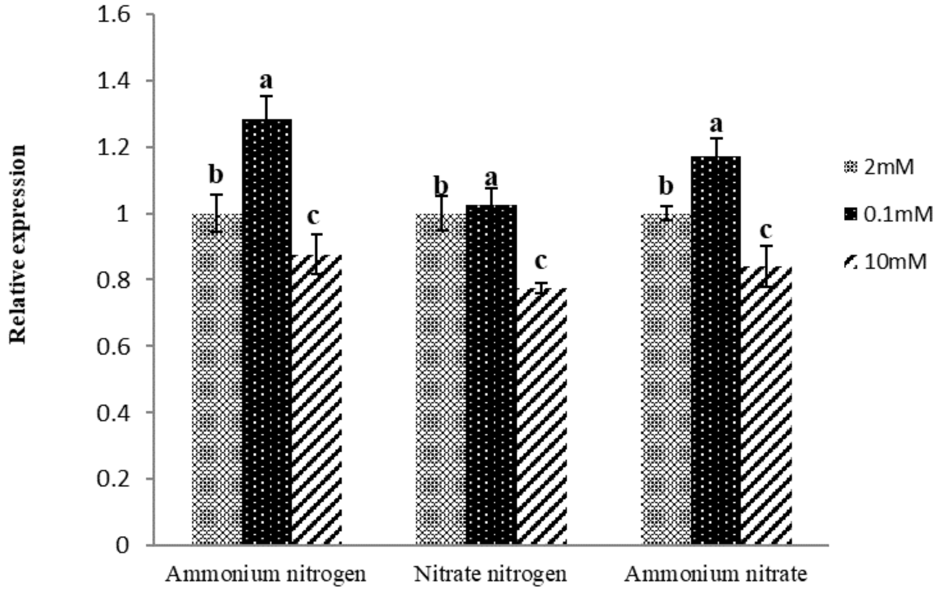


Figure 5. Effects of different nitrogen forms on poplar seedlings on stem *PAL1* gene expression

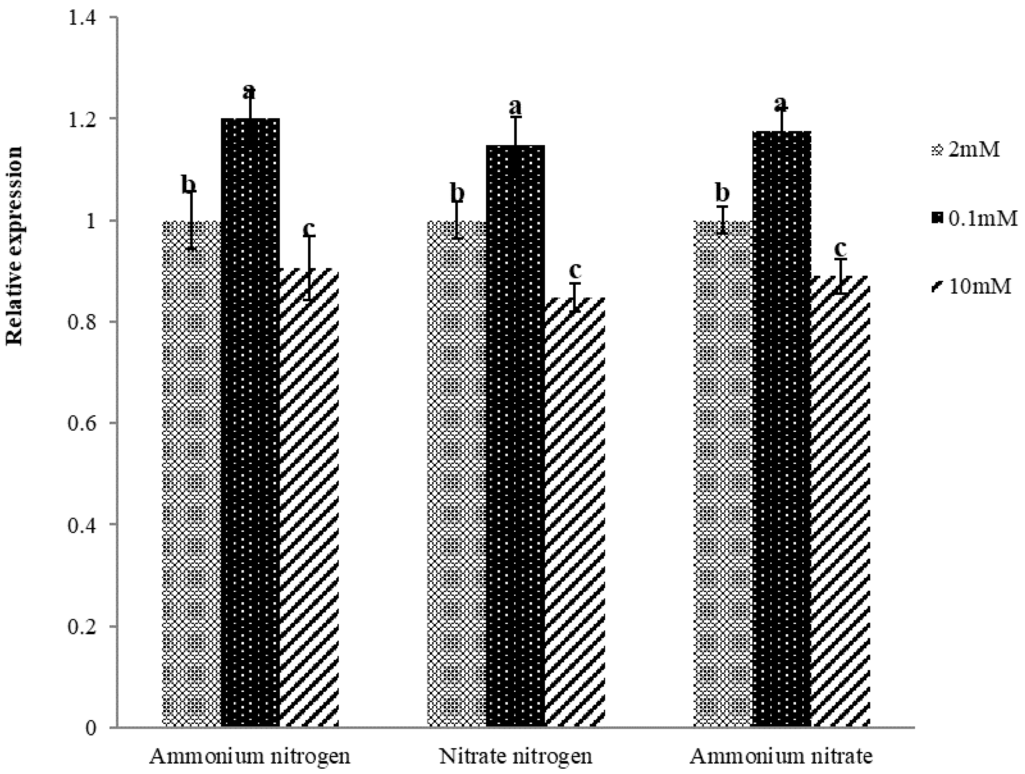


Figure 6. Effects of different nitrogen forms on poplar seedlings on stem *PAL2* gene expression

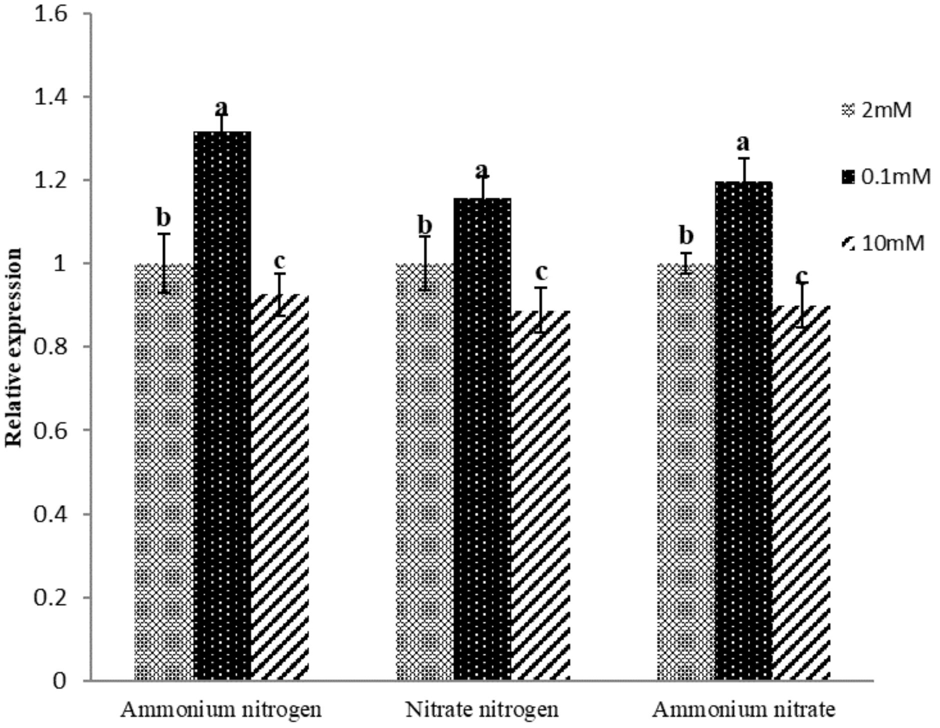


Figure 7. Effects of different nitrogen forms on poplar seedlings on stem *PAL4* gene expression

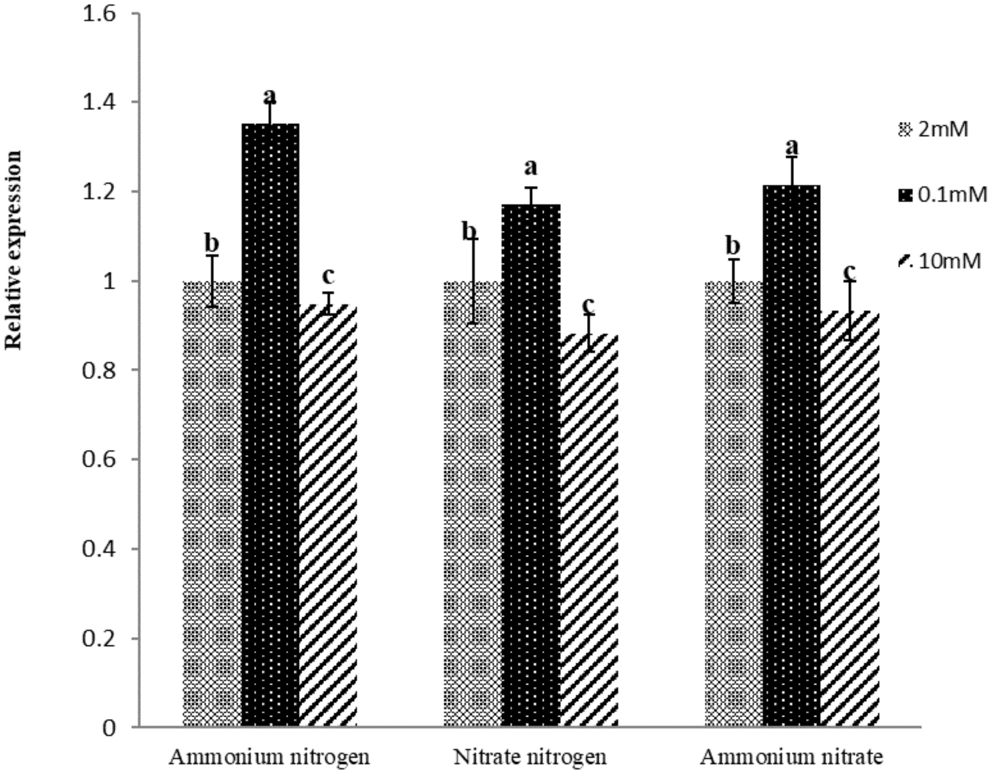


Figure 08. Effects of different nitrogen forms on poplar seedlings on stem *PAL5* gene expression

DISCUSSION

Nitrogen is a large nutrient necessary to plant growth and development (Jiao *et al.* 2018). Application of nitrogen fertilizer to increase the growth rate and growth of forest trees has been brought into wide public concern (Macdonald and Hubert 2002; Heilman and Norby 1998). The availability of nitrogen is the basis of plants because nitrogen affects most physiological processes in plant development (Stitt 1999). Nitrogen is the important environmental factor influencing the change of lignin content on plants (YU *et al.* 2003). Nitrogen fertilizer can change the content and composition of lignin (Pitre *et al.* 2007; Luo and Polle 2009). PAL is an enzyme that connects primary metabolism with phenylpropane metabolism, also catalyzes the first reaction in phenylpropane metabolism. It is the key enzyme and limiting velocity enzyme in phenylpropane metabolism pathway. It is also the most studied enzyme in plants. So it has an important role and function in the lignin synthesis pathway. Christina Fritz Used tobacco as experimental material and could induce some genes involved in phenylpropane metabolic pathways under nitrogen-deficient conditions, among them the *PAL* gene(Fritz *et al.* 2006).

There are five members in *PAL* gene family in *Populus trichocarpa*. In this study, the physicochemical properties of five proteins were analyzed. The five proteins contained similar numbers of amino acids, molecular weights, isoelectric points, and amino acid indexes, and all GRAVY values were similar. It is a negative number, only PtrPAL4 and PtrPAL5 are on the same chromosome, and all five proteins are distributed in the chloroplast (Table 1). The research of exon-intron gene structure will provide an key clue for studing gene evolution (Freeling 2009). Based on the structure analysis of the exon-intron tissue of the poplar *PAL* gene, the results show that each *PtrPAL* gene has an internal introns, and related studies have shown that the intron position of homologous *PAL* genes in *Arabidopsis* and poplars, the insertion of their symmetrical exons are very conserved, which indicating that all of *PAL* genes may have the same Ancestor (Yan *et al.* 2019), and the relationship between poplar and Arabidopsis protein is also closer. The tertiary structures of the five PAL proteins of the poplar are consistent, all of which are homotetramers. However, there is a significant difference in the length and sequence of all 50 PTRPAL PUTPAL, PtrPAL2, PtrPAL4, 50UTRs of PtrPAL5 longer, 215~227 nucleotides, while PtrPAL1 and PtrPAL3 have shorter 50UTRs, 123~121 nucleotides (Shi *et al.* 2010). Studies by Malys and McCarthy et al. Have shown that longer 50UTRs usually reduce Production of proteins (Malys and McCarthy 2011). However, PtrPAL2, PtrPAL4, and PtrPAL5 had higher protein-to-mRNA ratios, and PtrPAL1 and PtrPAL3 had lower protein-to-mRNA ratios in the stem xylem (Shi *et al.* 2013). This may be because the 50UTRs of PtrPAL2, PtrPAL4, and PtrPAL5 contain long sequences that can stimulates initiation of transcription or enabled the initiation process to bypass 50UTR. Therefore, if you want to regulate the activity of PAL, you can also regulate it at the protein level of plants (Araujo *et al.* 2012). The results of sequence analysis showed that the five *PAL* genes all contained a conserved active site Ala-Ser-Gly (A-S-G) (see Figure 3), indicating that the PAL protein in poplar has similar catalytic functions as the PAL proteins of other species.

The difference in expression of the PAL multigene family members is due to the difference in cis-elements contained in their promoters. It is precisely because of these cis-elements and corresponding transcription factors that different *PAL* genes can be expressed at different times and in different tissues (CHENG *et al.* 2003). Five *PAL* genes in poplar were divided into two groups through phylogenetic analysis and tissue specific expression level analysis (Tsai *et al.* 2006). Group A members (*PtrPAL2*, *PtrPAL4* and *PtrPAL5*) were expressed in the root tips and xylem, while group B Genes (*PtrPAL1* and *PtrPAL*3) are wider expressed (Tsai *et al.* 2006). In the stems of *Populus trichocarpa*, all *PtrPAL* genes have similar transcript abundances (Shi *et al.* 2013), which is consistent with the results of this study. There are no significant differences in the transcriptional abundances of the five genes in the stems of *Populus tomentosa* ((Figure 6-10). However, under the influence of nitrogen, the expression of five *PAL* genes was induced under low nitrogen in the stem, and the effect of ammonium nitrogen was most obvious. And in tobacco-based experiments, it was mentioned that under nitrogen-deficient conditions, some genes in the phenylpropane pathway are induced, including the *PAL* gene (Fritz *et al.* 2006). Studies have reported that group A members of the five *PtrPAL* are responsible for producing lignin (Shi *et al.* 2013). In the study by Li Mengchun (Li 2012) and others, it was mentioned that with the increase of nitrogen concentration, the lignin content of Populus popularis and 84K poplar (*Populus alba* × *P. glandulosa*) decreased. The authors measured the lignin content and showed that as the nitrogen concentration increased, the lignin content also showed a downward trend (data not published). In summary, there is a certain relationship between nitrogen and PAL and between nitrogen and lignin, and nitrogen can induce *PAL* gene expression in *Populus tomentosa*.

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Author contribution : C.J. Yang conceived and designed the experiments; Y.Q. Zhang and M. Jin performed the experiments and wrote the paper; Y.Q. Zhang analyzed the data; Y.B. Duan contributed financial support, reagents and materials.

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