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Cloning and Expression Analysis of *PeDIR19* Gene in Poplar

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Abstract Dirigent (*DIR*) gene plays an important role in the formation of lignin and lignans by controlling the stereoselective coupling of pineperin alcohol monomers in plants during the polymerization of lignin and lignans. In this study, we take the 'Nanlin 895' (*Populus deltoides* × *P. euramericana* cv 'Nanlin 895') as the material, and clone the *PeDIR19* gene. *PeDIR19*, the whole length of 588 bp, has a length of 555 bp in ORF region and has only a length of 33 bp intron in the middle. PeDIR19 protein codes 185 amino acids and is a hydrophobic protein. It has four conservative motifs and has a highly conservative structural functional domain between 50 bp ~ 142 bp. *PeDIR19* gene was found to have the highest homology with *Populus trichocarpa PtDIR19* (*Potri.001G009100*), with only four bases differences. By phylogenetic analysis, *PeDIR19* gene belongs to *DIR*-B/D subfamily. Through tissue specific expression analysis of 'Nanlin 895' with different growth stages, it was found that *PeDIR19* gene expression level was the highest in the root during the tissue culture seedlings and in the stem during the pot seedlings. *PeDIR19* gene had a strong response after 1 d under the induction of Yangpan Erbaojun (*Marssonina brunnea*). Under the induction of salicylic acid, methyl jasmonate and abscisic acid stress, *PeDIR19* gene can strongly respond to the stress of methyl jasmonate and abscisic acid and weakly respond to the salicylic acid stress. This study provides a solid foundation for further study on the function identification of *DIR* gene in the biosynthetic pathway of plant resistance.

Keywords *PeDIR19*; Poplar; Expression analysis; Disease resistance

Poplar (*Populus*) is a perennial deciduous tree with a wide range of uses. It can be used not only as wood, but also as an industrial material. However, woody plants have a long growth cycle and are prone to stress such as pests and diseases (Jiang, 2016). As an important component of cell wall, lignin plays an important role in plant growth, tissue and organ development, lodge resistance and various biological and abiotic stress responses, providing a physical barrier for healthy plant growth (Liu et al., 2018).

DIR protein plays an important role in the synthesis of lignin monomer. In vitro, during the formation of In vitro, during the formation of terpineol from coniferyl alcohol, laccase, peroxidase and oxidase cannot be stereoselectively coupled through their own control, and can be randomly coupled to produce raceme-coupled mixtures of (+/-)8,8, (+/-)8,5 and (+/-)8-O-4. However, in plants, the coupling of bimolecular phenoxy groups has a clear control mode, resulting in optically active dimers (Davin et al., 1997). This indicates that the formation of lignin and lignans has selective specificity, and some substance mediates the reaction of bifolecular phenoxy in plants, so that they can be coupled in a correct way. Until 1997, Davin et al. (1997) isolated FiDIR protein in the study of lignin monomer coupling in Lianqiao. The biochemical experiments showed that FiDIR protein can trap (+) pinecroidel bimolecular phenoxy radical during the formation of (+) terpineol from (+) coniferyl alcohol, and guide the stereoselective coupling and specifically form (+) coniferyl alcohol. Subsequently, DIR gene has been reported in a variety of monocotyledon and dicotyledon plants, such as Ninanjie (*Arabidopsis thaliana*) (Gasper et al., 2016), rice (*Oryza sativa*) (Liao et al., 2017), wheat (*Triticum aestivum*) (Subramanyam et al., 2013), soybean (*Glycine max*) (Li et al., 2017), common flax (*Linum usitatissimum*) (Behr et al., 2018), etc.. It was found that this gene plays an important role in the formation of lignin and lignans. The increase of lignin can enhance the mechanical strength and hardness of plants and better resist the adverse external environment, while lignans can increase the resistance of plants to exogenous pathogens and enhance the disease resistance of plants.

Pickel et al. (2012) predicted the structure of *Arabidopsis thaliana* monomer AtDIR6 protein, which resembles epoxide ene cyclase and is an eight-strand reverse parallel β barrel with a central hydrophobic cavity that can be used for substrate binding. Gasper et al. (2016) obtained PsDRR206 protein from soybean that could form (+) -terpineol, and the crystal structure showed that PsDRR206 protein was a tightly packed trimer. The structure of β1 -- β2 ring at the active site of soybean PsDRR206 protein is significantly different from that of *Arabidopsis thaliana* AtDIR6 protein, but both contain an Ω ring containing a cluster of highly conserved amino acid residues. Multiple residues were mutated in AtDIR6 protein, and it was found that Asp137, Arg144 and Asp49 residues in AtDIR6 protein were essential for the formation of (+) and (-) terpineol. The enantioselective function of DIR proteins is the result of precise mapping of substrates (Halls et al., 2004). The AtDIR10/ESB1 protein with the DIR domain is essential for the formation of the correct Kjeldahl bands in the roots of Ninanjie. CASPs can accurately locate the formation of Casps by forming protein scaffolds in the plasma membrane. In the absence of AtDIR10/ESB1, disordered and defective Kelson bands can form (Hosmani et al., 2013). (+) -gossypol plays an important role in the antiviral of cotton (*Gossypium spp*). Studies have shown that the *GhDIR4* gene in cotton has a certain selective effect on the coupling of galactosyl, and the content of (+) -gossyol produced in the presence of this gene is greater than 80% (Effenberger et al., 2015). Soybean *Pdh1* gene plays an important role in soybean fruit cracking and is highly expressed in the pod wall. *Pdh1* gene can dry the pod wall and further promote fruit cracking, indicating that *Pdh1* gene is related to the synthesis of soybean pod cell wall (Funatsuki et al., 2014). Through microscopic observation, lignin content determination, proteomics and other methods, researchers found that *DIR* gene plays an important role in lignin formation during the hypocotyl development of Dama (*Cannabis sativa*) (Behr et al., 2018).

DIR gene can also respond to insect and pathogen stress. In weevils induced or mechanically damaged spruce (*Picea asperata Mast*) bark, the DIR gene is expressed rapidly and strongly (up to 500 times) under weevils induction (Steven et al., 2006). Phytophthora infestans can cause root rot and seriously affect the normal growth of soybean. In highly resistant soybean inoculated with phytophthora infestans, the expression of soybean *GmDIR22* gene was up-regulated. GmDIR22 protein can effectively conjugate (+) coniferyl alcohol to (+) -terpineol after purification in vitro. Compared with the wild type, the total accumulation of lignans in soybean overexpressing *GmDIR22* gene was significantly increased, and the in vivo inoculation experiment showed that the resistance to soybean phytophthora was significantly enhanced (Li et al., 2017). Subramanyam et al. (2013) studied wheat *HfrDrd* gene and found that wheat *HfrDrd* gene was specifically induced by Hessian fly and highly expressed. Within two hours after the hessian fly eggs hatch, *HfrDrd* gene expression increased sharply. This suggests that the HfrDrd gene plays a role in early defense against Hessian fly larvae. In addition to that, Fusarium soybean (Seneviratne et al., 2015), Taixian (*Physcomitrella patens*) Maopan Baozijun (Reboledo et al., 2015), verticillium wilt of cotton (Guo et al., 2014), grapevine (*Vitis vinifera*) powdery mildew (Borges et al., 2013), Mianhua Dalilunzhijun (*Verticillium dahliae*) (Shi et al., 2012) and other pathogens can induce the high expression of *DIR* gene in corresponding plants.

At present, there are few studies on *DIR* gene and its disease resistance in poplar. In this study, a *PeDIR19* gene, which is highly homologous to *PtDIR19* of Maoguoyang (*Populus trichocarpa* Torr. & Gray), was cloned from 'Nanlin 895' poplar. In this study, sequence analysis and functional prediction of PeDIR19 gene were performed using bioinformatics technology, and tissue specificity analysis was performed for poplar with different growth cycles. At the same time, we also analyzed the expression changes of *Marssonina brunnea* and various hormones such as salicylic acid (SA), methyl jasmonate (MeJA) abscisic acid (ABA) under stress induction, providing reference and basis for further analysis of the biological function of poplar *PeDIR19* gene.

1 Results and Analysis

1.1 Full length of *PeDIR19* gene and cloning of CDS

Based on the sequence of Maoguoyang *PtDIR19* (Potri.001G009100) published in Phytozome, the full length and CDS region of PeDIR19 gene were obtained from 'Nanlin 895' poplar, with the lengths of 588 bp (Figure 1B) and 555 bp (Figure 1B), respectively. This gene has no 5 'and 3' terminal UTR and contains only a 33 bp intron at 395

bp encoding 185 amino acids (Figure 2). By Blast sequence alignment, the similarity of the CDS gene sequences of PeDIR19 and PtDIR19 of Maoguoyang was about 98%, with only 4 bases difference. The amino acid sequence similarity is about 99%, only 1 amino acid difference. The identification of the PeDIR19 protein domain through the online website SMART showed that the PeDIR19 protein had a conserved DIR domain, with a highly conserved structural function domain between the 50th and 142nd positions, which was consistent with the characteristics of the DIR gene family (Figure 3).

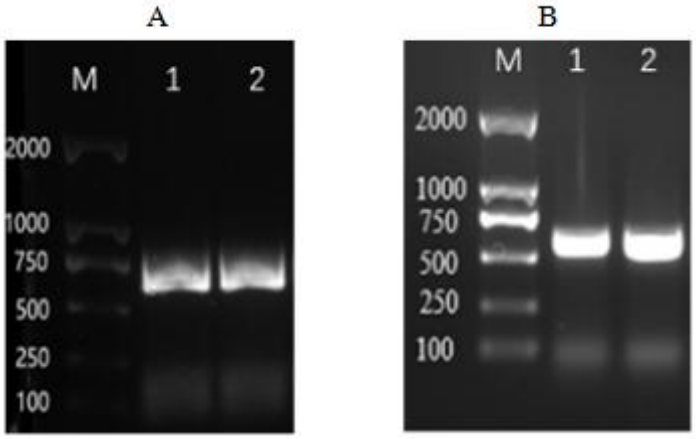

Figure 1 Cloning of *PeDIR19* gene

Note: A: *PeDIR19* gene full-length sequence amplification band; B: *PeDIR19* gene ORF amplification band; M: DL2000 bp DNA Marker

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  1 ATGGCTAGATCAGTTCCCATCCTTGCTTCCAAATTCATCACTCTC  316 AGGGCGCAAGGGTTTTATGCACAAGCGTCGCAACAAGATATTGGG
    M  A  R  S  V  P  I  L  A  S  K  F  I  T  L      R  A  Q  G  F  Y  A  Q  A  S  Q  Q  D  I  G

 46 TTACTCCTCTCTTCCTTTGCCACAATCTTGGTCACTGGAGACCAG  361 TTATTGATGGCCATGAACTTTGCTTTTATTGAAGGTAAGTATAAT
    L  L  L  S  S  F  A  T  I  L  V  T  G  D  Q      L  L  M  A  M  N  F  A  F  I  E  G  K  Y  N

 91 GATCATGAATTTGTGAGAAGTTTGGACAGGAAGCTACTAGGCCTC  406 GGTAGCACTATTACTGTTCTAGGGAAGAACGCAGTGTTCTCGACG
    D  H  E  F  V  R  S  L  D  R  K  L  L  G  L      G  S  T  I  T  V  L  G  K  N  A  V  F  S  T

136 AAGAAAGAAAAGCTAAGCCACTTCAAGTTATATTGGCATGACATC  451 GTGAGAGAGATGCCGGTGATCGGAGGAAGCGGACTTTTCCGGTTT
    K  K  E  K  L  S  H  F  K  L  Y  W  H  D  I      V  R  E  M  P  V  I  G  G  S  G  L  F  R  F

181 CTTACTGGCCAGAACCCCAGCGCCGTCCAAGTTGTGCCACCGCCA  496 GCTAGAGGTTATGTTCAGGCGAGAACTCACAAGCTCGACATGGCC
    L  T  G  Q  N  P  S  A  V  Q  V  V  P  P  P      A  R  G  Y  V  Q  A  R  T  H  K  L  D  M  A

226 TCGAACACATCAAGAACAGCTTTTGGGTTAGTGAGAATGATCGAT  541 ACAGGAGACGCTACAGTTGAGTATAATGTCTACGTTTTTCATTAT
    S  N  T  S  R  T  A  F  G  L  V  R  M  I  D      T  G  D  A  T  V  E  Y  N  V  Y  V  F  H  Y

271 AACCCATTAACCTTAGGGCCTGAAATGAGCTCAAAGTTGGTAGGA  586 TGA
    N  P  L  T  L  G  P  E  M  S  S  K  L  V  G      *
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Figure 2 The sequence information of *PeDIR19* gene

Note: Black box: The intron of *PeDIR19* gene

Figure 3 The DIR domain structure of PeDIR19

1.2 Bioinformatics analysis of PeDIR19 protein

By predicting the sequence information of PeDIR19 protein on the online website ProtParam, the molecular formula of PeDIR19 protein was $C_{926}H_{1456}N_{248}O_{258}S_7$. The molecular weight of the protein was 20.4 kD, the theoretical isoelectric point (PI) was 9.56, and the instability index was 40.10, suggesting that it was an unstable protein. The average hydrophilic coefficient (GRAVY) is 0.068. PeDIR19 is predicted to be hydrophobic. Among

the amino acids of PeDIR19 protein, Leucine (Leu) accounted for 11.4%, followed by Ala (alanine) and Valine (Val), accounting for 8.2% each. As predicted by SignalP, amino acid sites 1-28 of PeDIR19 protein were signal peptides (Figure 4A). NetNGlyc predicted that there were two glycosylation sites in the 65th and 77th amino acid of PeDIR19 protein. As predicted by Cell-PLOc 2.0, PeDIR19 protein was localized to the cell membrane and chloroplast. The secondary structure of PeDIR19 protein predicted by PRABI-GERLAND showed 38% random curling, 35.87% extension, 21.2% α-helix, and 4.89% β-turning (Figure 4B). SWISS-MODEL was used to predict the tertiary structure of PeDIR19 protein (Figure 4C), which showed that the protein consisted of eight antiparallel chains forming a β bucket with a hydrophobic cavity in the middle, which could be used for substrate binding. It also further indicated that PeDIR19 was a hydrophobic protein.

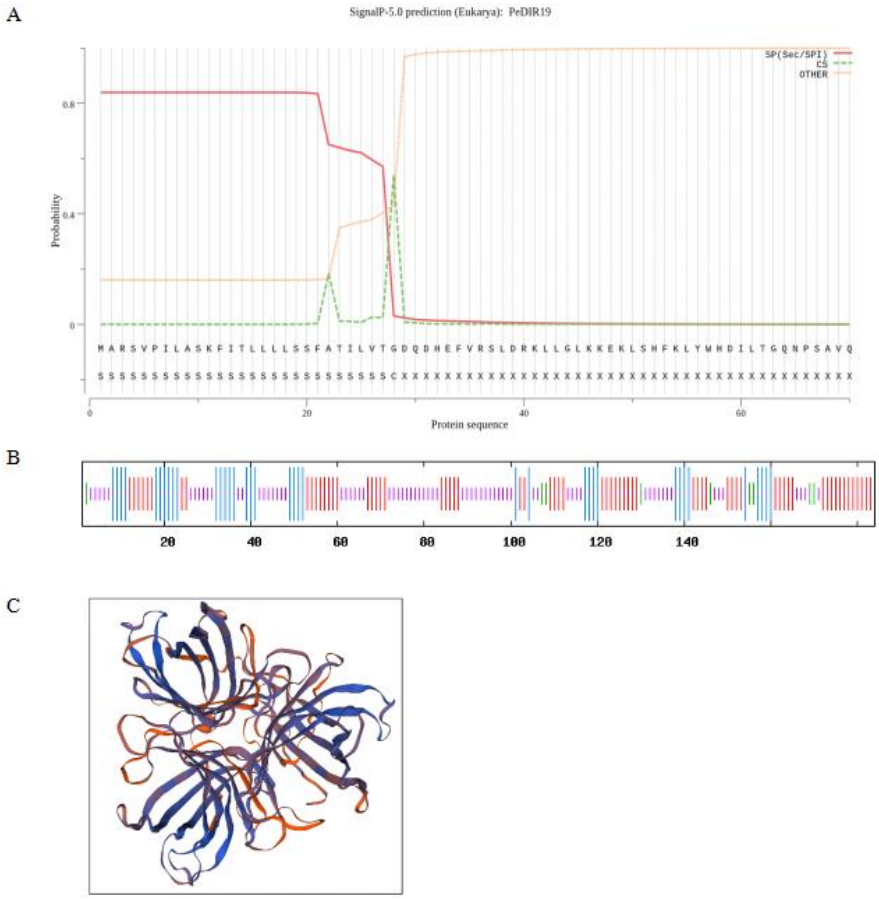

Figure 4 Analysis of physical and chemical properties and protein structure of PeDIR19
Note: A: The signal peptide prediction of PeDIR19 protein; B: The secondary structure prediction of PeDIR19 protein; C: The advanced structure prediction of PeDIR19 protein

1.3 Homology analysis of PeDIR19 protein

The PeDIR19 protein sequence was compared with NCBI blastp sequence. The results showed that the PeDIR19 protein of 'Nanlin 895' poplar had a high homology with that of black cottonwood (*Populus trichocarpa*) (Potri.001G009100.1), Xiaodianliu (*Salix brachista*) (KAB5572872.1), Mushu (*Manihot esculenta*) (XP_021606503.1), Xiangjiaoshu (*Hevea brasiliensis*) (XP_021639567.1) and walnut (*Juglans regia*) (XP_018834592.1). They are about 99%, 88%, 78% and 73% respectively. Multiple sequence alignment using ClustalX2.0 software showed that the PeDIR19 protein was highly conserved with DIR protein domains of other species, and there were 4 conserved motifs (Figure 5), indicating that all belonged to DIR family genes. In order to study the phylogenetic relationship between PeDIR19 protein and other species, MEGA was used to construct phylogenetic tree of PeDIR19 protein and some DIR protein sequences of *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Juglans regia*, *Salix brachista*, *Hevea brasiliensis* and *Manihot esculenta*. The results showed that the PeDIR19 gene belonged to the DIR-B/D family (Figure 6).

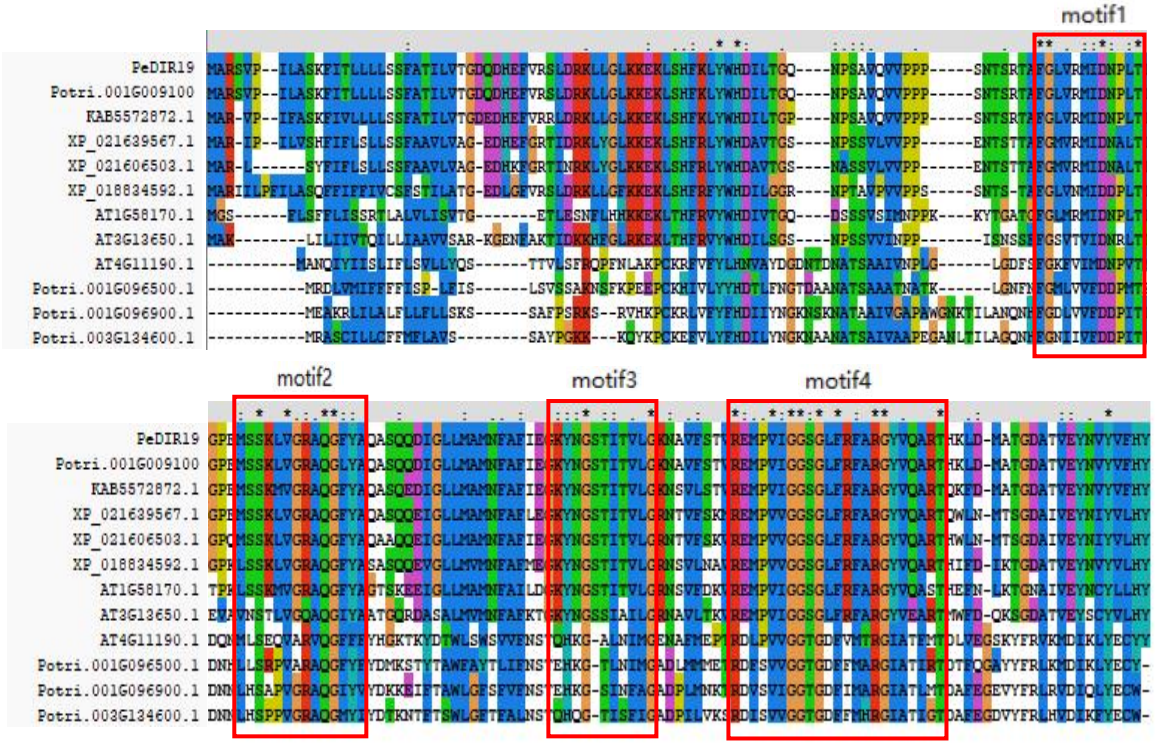

Figure 5 Multiple sequence alignment of PeDIR19 protein and other plants DIR protein sequences
Note: The red box represents the DIR protein conservative motif

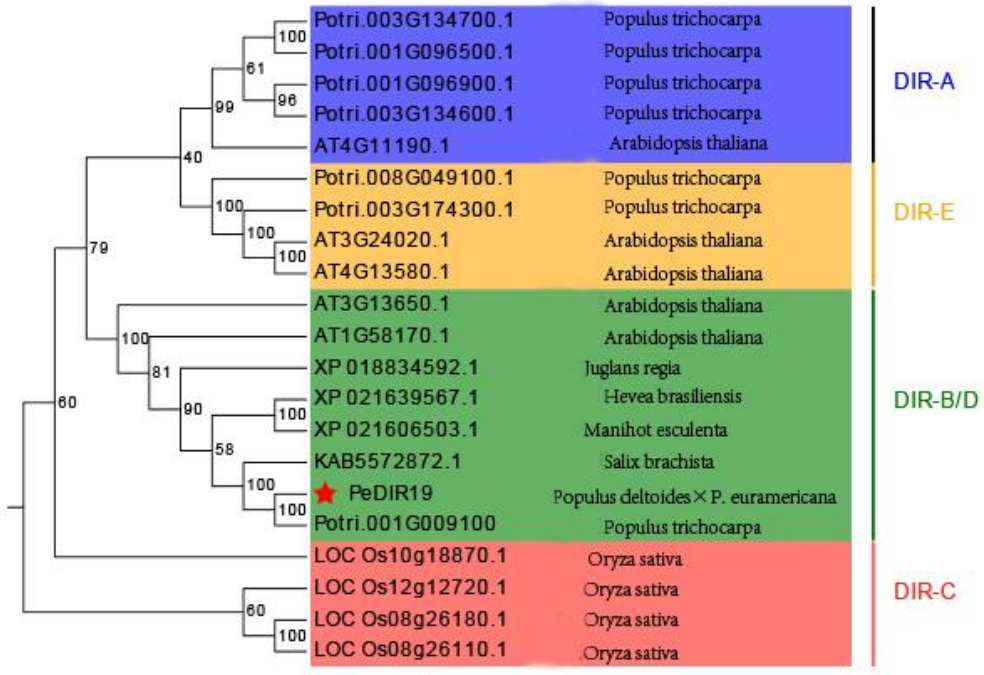

Figure 6 Phylogenetic tree analysis of PeDIR19 protein

1.4 Tissue-specific expression analysis of *PeDIR19* gene in different growth stages

Quantitative PCR analysis showed that PeDIR19 gene was expressed in all tissues of poplar. However, the relative expression levels of each tissue were different at different growth stages. In the tissue culture seedlings growing for one month, the expression level in root was the highest, followed by young and mature leaves, and the lowest in stem. However, in potted plants growing for three months, the expression level of PeDIR19 gene was the highest in roots, followed by in stems and the lowest in leaves (Figure 7).

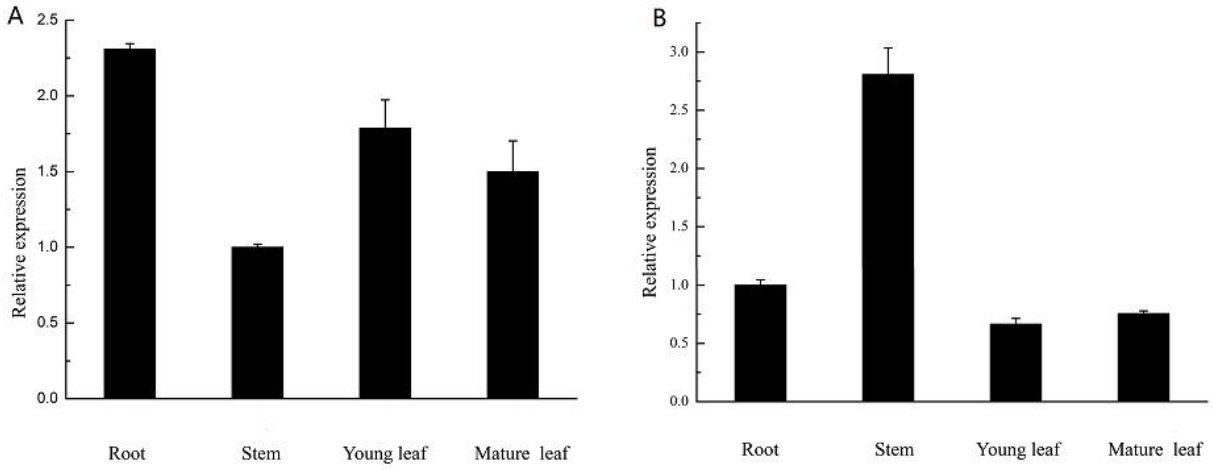

Figure 7 Tissue-specific expression analysis of *PeDIR19* gene in the stages of tissue culture seedlings and potted seedlings
Note: A: Expression differences of tissue culture seedings; B: Expression differences of potted seedings

1.5 Expression analysis of *PeDIR19* gene under pathogen stress and hormone treatment

Three-months-old 'Nanlin 895' poplar was treated by *Marssonina brunnea* SAMeJAABA, and the response of PeDIR19 gene was investigated by real-time fluorescence quantitative PCR. The results showed that the *PeDIR19* gene did not change much within 12 h after induction of *Marssonina brunnea*, but the expression level of *PeDIR19* gene was increased 3 times after induction 1 day, and the high expression level of *PeDIR19* gene remained until the 4th day. *PeDIR19* gene expression began to decrease on the 5th day (Figure 8A). Under ABA stress, the gene expression of *PeDIR19* was significantly increased, reaching the highest level at 9 h, which was 18 times of that before treatment, and then began to decline (Figure 8B). Under SA treatment, the response of *PeDIR19* gene was relatively weak. The expression level of *PEDIR19* gene decreased at 3 h of treatment, then increased slowly, and reached the highest expression level at 12 h, which was 1.6 times of that before treatment (Figure 8C). Under MeJA treatment, the response of *PeDIR19* gene was not obvious before 6 h of treatment, but the expression level of *PEDIR19* gene reached 3.5 times of that before 9 h of treatment (Figure 8D). The above results indicated that *PeDIR19* gene could be induced to express in response to Yangpan Erbaojun SAMeJAABA stress.

2 Discussion

Stereospecificity of synthetic reactions in organisms is considered to be a necessary prerequisite for the origin of life on Earth (Weber and Pizzarello, 2006). Temporal and spatial regulation of lignin biosynthesis is very important because lignification requires a large amount of carbon skeleton and polymerization energy, and is a process with high metabolic costs (Guo et al., 2020). *PeDIR19* belongs to the plant DIR family gene and is widely found in vascular plants. In the biosynthesis of lignin and lignans, DIR gene plays an important role in the stereoselective coupling of bilamolecular phenoxy radicals in plants, which can further promote the resistance of plants to various strains.

In this study, *PeDIR19* gene was cloned from poplar 'Nanlin 895' and was highly homologous to *PtDIR19* gene of Maoyangguo. At the same time, the structural functional domain analysis and Phylogenetic relatives relationship analysis of the gene showed that the PeDIR19 protein had a conserved DIR protein domain and was closely related to known DIR proteins in other species, indicating that the *PeDIR19* gene belonged to *DIR* gene family and *DIR* gene subfamily belonged to *DIR-B/D* gene subfamily. Bioinformatics analysis of PeDIR19 protein showed that PeDIR19 protein was hydrophobic and unstable with eight reverse parallel β chains. At the same time, tissue specific expression analysis showed that the relative expression level of *PeDIR19* gene was different in poplar tissues at different growth stages, suggesting that the functional expression of *PeDIR19* gene might be different in different growth cycles. At the stage of tissue culture seedling, the expression level in leaves was higher than that in stems. It was speculated that poplar was weak at seedling stage and leaves were more

susceptible to pathogen damage, so the expression level of *PeDIR19* gene in leaves was relatively high. This result was similar to that obtained by predecessors in Duruo (*Pollia japonica* Thunb.) (Wang, 2019), indicating the spatio-temporal diversity of *DIR* gene expression.

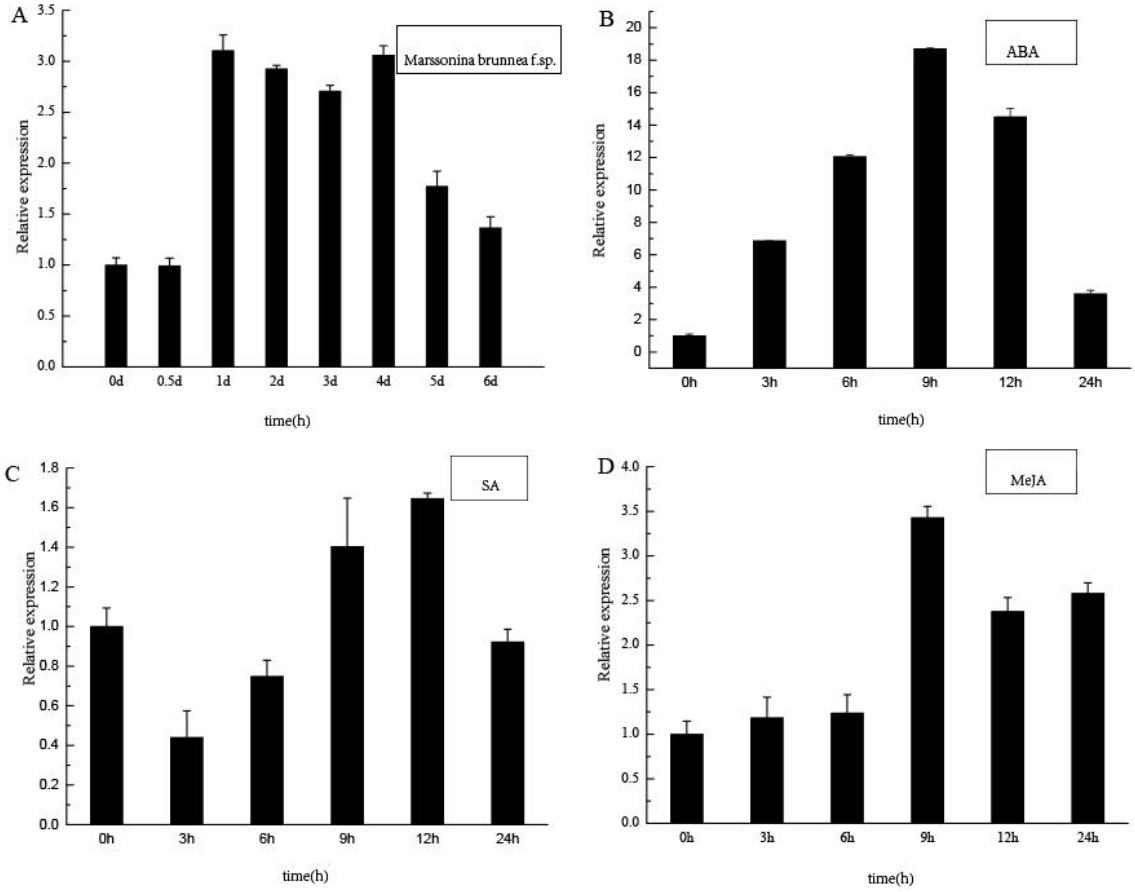

Figure 8 Expression changes under treatment with *Marssonina brunnea*, SA MeJA and ABA of *PeDIR19* gene

Note: A: Expression level of *PeDIR19* gene treated by *Marssonina brunnea*; B: Expression level of *PeDIR19* gene treated by ABA; C: Expression level of *PeDIR19* gene treated by SA; D: Expression level of *PeDIR19* gene treated by MeJA

Many studies have shown that plant *DIR* gene can further respond to various biological and abiotic stresses by regulating the biosynthesis of various hormones. Liu et al. (2018) treated cucumber with downocarb and found that *CsDIR16* gene of cucumber was related to residue of downocarb through high-throughput sequencing analysis. The expression of *CsDIR16* gene was also significantly up-regulated under MeJA and ABA stress induction (Liu et al., 2018). Liao et al. (2017) analyzed the expression of 49 *DIR* genes in rice through IAA and BAP induction, and found that 23 rice *OsDIRs* or *OSDIRs-like* genes responded. In this study, it was found that the *PeDIR19* gene could respond to the induction of *Marssonina brunnea* and the stress of SA, MeJA and ABA. However, the response degree to SA was relatively weak, and the response degree to ABA and MeJA was relatively strong. It was speculated that PeDIR19 gene may function mainly through MeJA and ABA signaling pathway.

In this study, we preliminarily studied the function of poplar *PeDIR19* gene, which provides a reference for further research on the function of poplar *DIR* gene. At the same time, poplar *DIR* gene family is large and the number of genes is large. The next research will use new molecular biology technologies, such as CRISPR-based multi-gene knockout technology, to conduct functional analysis of gene family members. In addition, the promoter of *DIR* gene and the corresponding upstream transcriptional regulatory factors were studied, and the changes of its downstream products were discussed. And the complete transcriptional regulatory network was systematically analyzed.

3 Materials and Methods

3.1 Materials of test

In this study, tissue culture seedlings and potted plants of poplar 'Nanlin 895' (*Populus deltoides* × *P. euramericana* cv 'Nanlin 895') were cultured in a greenhouse at 23℃ under 16 h light and 8 h darkness. Yangpan Erbaojun (*Marssonina brunnea*) was presented by Teacher Cheng Qiang from Nanjing Forestry University. The pEASY-T3 Cloning Vector was purchased from Trans, and the DH5α Escherichia coli demonstrator was purchased from Beijing Zoman Biotechnology Co., LTD. RNA and DNA extraction kit and plasmid lifting kit were purchased from TIANGEN Biotech (Beijing) Co., Ltd., reverse transcription kit was purchased from Takara Bio Companies, and real-time fluorescence quantitative kit was purchased from Nanjing Vazyme Biotech Co., Ltd.

3.2 RNA extraction and target gene cloning

Total RNA was extracted from poplar leaves of 'Nanlin 895' by liquid nitrogen grinding, and the total RNA was extracted by Tianroot polysaccharide polyphenol plant total RNA extraction kit, and then reversed into cDNA using the reverse transcription kit of Takara Bio Companies. Total DNA was extracted with Tiangen DNA extraction kit. According to Maoguoyang *PtDIR19* (*Potri.001G009100*) sequence in Phytozome, specific primers for 5 'and 3' ends of *PeDIR19* in 'Nanlin 895' were designed (Table 1), and the CDS region and full-length fragment of *PeDIR19* gene were amplified. The PCR reaction procedure was: predenaturation at 95℃ for 5 min; Annealing at 95℃ for 30 s, annealing at 60℃ for 30 s, extension at 72℃ for 40 s, for 35 cycles; and the final extension is 10 min at 72℃. After amplification, agarose gel electrophoresis was performed, and the amplified target sequences were gelled and recovered. The cut and recovered fragment was ligated into the ASY-T3 Cloning Vector, transformed into DH5α Escherichia coli receptive state, and blue-white spot screening was performed. The positive monoclonal was selected for PCR detection of the bacterial solution, and the positive bacterial solution was sent to Tsingke Biotechnology Co., Ltd. for sequencing. After the sequencing results were returned, the sequencing results were compared with the sequences in the database, and the plasmid was extracted from the correct bacterial liquid by comparison. The plasmid was stored at -20℃ for subsequent use.

3.3 Analysis of physicochemical properties of PeDIR19 protein

The PeDIR19 protein domain and transmembrane structure were identified using the online website SMART (http://smart.embl-heidelberg.de/). The molecular weight, isoelectric point, instability index, hydrophilicity and other physical and chemical properties of PeDIR19 protein were predicted by ProtParam (https://web.expasy.org/protparam/). SignalP (www.cbs.dtu.dk/services/ SignalP/), NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and Cell PLoc2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) were used to predict protein signal peptides, glycosylation sites and subcellular localization, respectively. SOPMA (https://npsarabi.ibcp.fr/cgibin/npsautomat.pl?ageSAHLP/psahlp_equence.htmL) and SWISS MODEL (https://www.swiss model.expasy.org/) online website were used to predict the secondary and tertiary structure of PeDIR19 protein.

Table 1 Specific primers for *PeDIR19*

Primer names	Primer sequences (5'→3')
PeDIR19-F	ATGGCTAGATCAGTTCCCATCCTT
PeDIR19-R	TCAATAATGAAAAACGTAGACATTATAC
PeDIR19-qPCR-F	CATTAACCTTAGGGCCTGAAA
PeDIR19-qPCR -R	ATCTCTCTCACCGTCGAGAACA
actin-F	GACCTTCAATGTGCCTGCAA
actin-R	ACCATCACCAGAATCCAGCA

3.4 Sequence alignment and evolutionary tree analysis of PeDIR19 protein with other species

ClustalX2.0 was used to perform multiple sequence alignment of DIR related amino acid sequences of 'Nanlin 895' poplar, *Populus trichocarpa*, *Arabidopsis thaliana*, *Salix brachista*, *Manihot esculenta*, *Hevea brasiliensis* and *Manihot esculenta*. MEGA5 software and Neighbor-Joining statistical method were used to analyze the

phylogenetic analysis. Set the parameters to Bootstrap method and 1000 bootstrap to build the system evolution tree.

3.5 Tissue specific expression analysis of *PeDIR19* gene

Total RNA was extracted from the roots, stems, young leaves and mature leaves of tissue culture and potted poplar 'Nanlin 895'. 1 μg RNA was reverse-transcribed into cDNA and diluted 10 times. Using cDNA as template, tissue expression of *PeDIR19* gene was analyzed by fluorescent quantitative PCR, and actin gene was selected as an internal reference to obtain quantitative primers (Table 1). Fluorescence quantitative PCR reaction condition was 95 ℃ 30s; 95 ℃ 10 s, 60 ℃ 30 s, 40 cycles; 95 ℃ 15 s, 60 ℃ 60 s, 95 ℃ 15 s. All experiments were repeated three times.

3.6 Expression pattern analysis of *PeDIR19* gene induced by *Marssonina brunnea* and SA, JA, ABA stress

1 g SA was dissolved in anhydrous ethanol and diluted with water until the final concentration was 5 mmol/L. Weigh 0.22 g MeJA, dissolve it in anhydrous ethanol, and dilute it with water until the final concentration is 1 mmol/L. Weigh 0.052 g ABA, dissolve with sodium hydroxide, dilute with water to the final concentration of 200 μmol/L. Large tissue culture seedlings at 3 months of age were sprayed and sampled at 0 h, 3 h, 6 h, 9 h, 12 h and 24 h, respectively. After cultured in potato medium (PDA) for 10 days, all the tested *Marssonina brunnea* were added with sterile water, gently scraped spores with a coating rod, and filtered through cell sieve to make a spore suspension. The spore concentration was calculated according to the blood cell count plate and diluted into a spore suspension with a concentration of 1×10^6/ml. Tissue culture seedlings of 'Nanlin 895' poplar were sprayed at 3 months old, and humidity was maintained. Samples were taken at 0 d, 0.5 d, and 1 d to 6 d at 8 time points. Three biological replicates were performed on each sample.

Authors' contributions

LLL and WY were the executor of the experimental design and experimental research of this study. ZPJ, YXF and TWW completed the data analysis and wrote the first draft of the paper. SWB participated in the experimental design and analysis of the experimental results. ZGQ was the author and principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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