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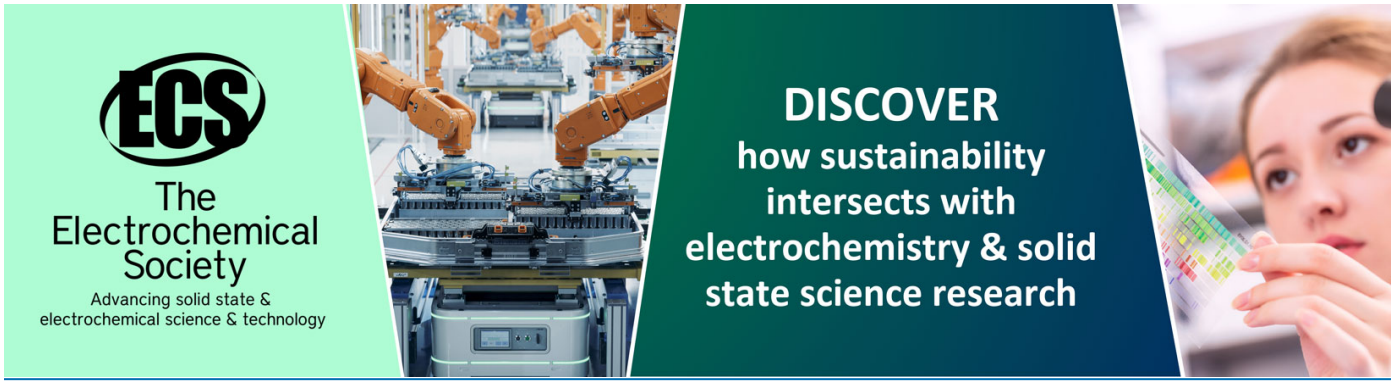

Cloning and bioinformatics analysis of CcPILS gene of Hickory (Carya cathayensis)

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Abstract. PILS is a key auxin efflux carrier protein in the auxin signal transduction. A CcPILS gene related to hickory (Carya carthayensis) grafting process was obtained by RACE techniques. The full length of CcPILS gene was1541bp contained a 1263bp length open reading flame (ORF). The CcPILS encoded 294 amino acids with molecular weight of 46 kDa, PI 5.38 and localized at endoplasmic reticulum membrane. The gene contained a central hydrophilic loop separating two hydrophobic domains of about five transmembrane regions each. The gene of CcPILS belonged to Clade III sub-family of PILS and its sequence had high homology with Arabidopsis. Real Time RT-PCR analysis showed that the gene expressions were weakly induced in bud, inflorescence, fruit, leaf and stem, while strongly in root. The expression levels were strongly induced and reached a peak at the third day of grafting in scion and rootstock of hickory, which were 1.45 and 3.45 times higher, respectively, compared to that of control. The results indicated that CcPILS may be involved in regulating the expression of genes related to auxin signal transduction during hickory graft process.

1. Introduction

Auxin plays an important role in plant growth and development [1-3]. Polarity transport is an important feature of auxin, and is commonly regulated by the synergy of auxin input carrier AUX/LAX protein family and the output carrier PIN-FORMED (PIN) protein family [4]. Barbez et al. (2004) found that another family of seven-member was also related to intracellular transport of auxin. Although the sequence similarity to PIN is very low (10-18%), it is still named as PILS (PIN-LIKES, PILS) because its topology is similar to that of PIN [5]. PILS can be divided into Clade I, Clade II and Clade III subfamilies. The PILS5 and PILS7 genes included in Clade III subfamily, play an important role in the process of vascular plant evolution [6].

Hickory (Carya cathayensis Sarg.) is an important woody oil plant and economic forest species in Zhejiang Province [7-10]. There are many reports on growth and development of hickory [11-13]. However, the grafting technique and quality improving of hickory is difficult. To understand the physiological and biochemical mechanisms of hickory graft, a series of studies have been carried out by Zheng et al. [15-17]. According to our transcriptomic data [17-18], we found that the expression of PILS gene was markedly induced in the graft process of hickory. In this study, the full-length cDNA of PILS gene was cloned according to the conservative fragment obtained from the transcriptomic data by RACE technique. The functional structure and regulation pattern of CcPILS was analyzed. The

expression levels of CcPILS were evaluated in the scion and rootstock during the graft process of hickory.

2. Materials and methods

2.1 Materials

Hickory (Carya cathayensis Sarg.) trees were planted at the breeding base of Zhejiang A&F University, Lin'an, China. The leaves, buds, male inflorescences and fruits of 2-year-old hickory were collected for expression pattern analysis. The samples from rootstock and scion were collected at 0, 3, 7 and 14 days after grafting (DAG). After harvesting, the samples were freeze-clamped quickly at liquid N_2 temperature stored at -70°C for qRT-PCR analysis.

2.2 Methods

2.2.1 Rapid amplification of cDNA ends (RACE) of CcPILS. Total RNA was extracted by modified CTAB method. The first strand of cDNA was synthesized using M-MLV reverse transcriptase. The 5'RACE specific primers (5'-RACE) were designed according to the primers design principle of BDSMARTTM RACE cDNA Amplification Kit. According to conservative sequence of CcPILS，the primers were designed assymRev (5'TAGCTCCTCCCGAATCTGCTGAAG-3') and newpilsR (5'-TCCCGAATCTGCTGAAGAAATCCA-3'). The primers of synRev and newpilsR were used as GST and NGSP1, respectively, for Nested PCR. The 3'RACE specific primers were designed as newpilsF (5'-ATCTCCTCCTTATCATCGTCCCCG-3') and SymFor (5'-AGCACTTCAAGCA ACTGAG GAGGT-3'). The PCR amplification was performed according to the following conditions: 98°C 10s, 55°C10s, 72°C 1.5min, a total of 30 cycles. The PCR products of 5'RACE and 3'RACE were purified by 1.5% agarose gel electrophoresis. The product was ligated with pMD-18 vector and transformed into DH5α. The transformed DH5αwas plated on an LB plate and incubated overnight at 37°C. Monoclonal colonies were picked up for culturing large-scale and the positive clones were identified by PCR and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

2.2.2 Bioinformatics analysis. The amino acid sequences of *Arabidopsis thaliana* AtPILS1 (GI:1035997954), AtPILS2 (GI:75169666), AtPILS3 (GI:75169730), AtPILS4 (GI:75169729), AtPILS5 (GI:75205686), AtPILS6 (GI:75181312), AtPILS7 (GI:75171357) and *Daucuscarota* DcPILS3 (GI:1040866677), DcPILS6 (GI:1040913933), DcPILS7 (GI:1040905618) and *Malusdomestica* MdPILS3 (GI:1039893723), MdPILS5 (GI:658038089), MdPILS6 (GI:658061887)were obtained by BLAST searches in NCBI, aligned using ClustalX, and subjected to phylogenetic analysis using the Neighbor-Joining method with MEGA6 using 1,000 bootstraps. The physicochemical properties of the proteins were analyzed using the online tool ProtParam (http://web.expasy.org/protparam/). The hydrophobicity of the proteins was analyzed using the ProtScale program (http://web.expasy.org/protscale/). The amino acid transmembrane region prediction was performed using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). The subcellular location of the gene was predicted using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/). The protein secondary and tertiary structure predictions were performed using the Protein Expert System (http://www.expasy.org/).

2.2.3 Quantitative RT-PCR analysis. The total RNA was extracted and then the cDNA was synthesized. Quantitative RT-PCR analysis was carried out by TAKARASYBR Premix ExTaq^{TM} (perfect real time) kit. The RT-PCR primers were designed as symFor (5'-AGCACTTCAAGCAACTGAGGAGGT-3') and symRev (5'-TAGCTCCTCCCG AATCTGCTGAAG-3') according to the sequence of CcPILS. CcACTIN was used as an internal standard. The sequences of primer pairs were (5'-GTGAACGGGAAATTGTC-3') and actin Rev (5'-AGAGATGG CTGGAAGAGG-3'). PCR was performed as follows: 94 °C for 1min, 94°C5 s,

60°C 34 s for 35 cycles. The relative RNA levels for CcPILS were calculated from cycle threshold (C_T) values according to the $2^{-\Delta\Delta CT}$method.

3. Results

3.1 Cloning and sequence analysis of CcPILS Gene

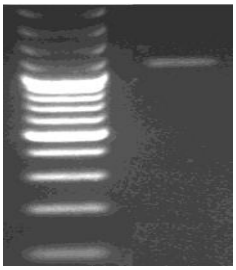

Fig.1 The PCR results of CcPILS in C.*carthayensis*

Full-length cDNA of CcPILS was cloned from the leaves of hickory by RACE technique with an open reading frame of 1263 bp, which is the same size as expected (Fig. 1). The predicted molecular weight of the protein was 46.22 kDa and the theoretical isoelectric point (PI) was 5.38. The protein was composed of 20 kinds of amino acids, among which the leucine content was the highest (14.0%), and the histidine content is the lowest (0.5%). The CcPILS protein belonged to the labile protein due to its instability index was 41.99. The aliphatic amino acid index of the protein reached 120.64, and the hydrophilic average coefficient was 0.601. ProtScale program predicted the protein hydrophobic region both in the N, C ends of a hydrophobic region, which was separated by the middle of the hydrophilic region. Therefore, the protein was predicated as a hydrophobic and unstable protein. TMpred analysis indicated that CcPILS protein had five transmembrane domains in the N-terminal and C-terminal regions, and the transmembrane region corresponded to the hydrophobic region. Predict protein analysis showed that the protein was localized in the endoplasmic reticulum membrane and belonged to the auxin efflux carrier.

3.2 Analysis of CcPILS protein homology and construction of phylogenetic tree

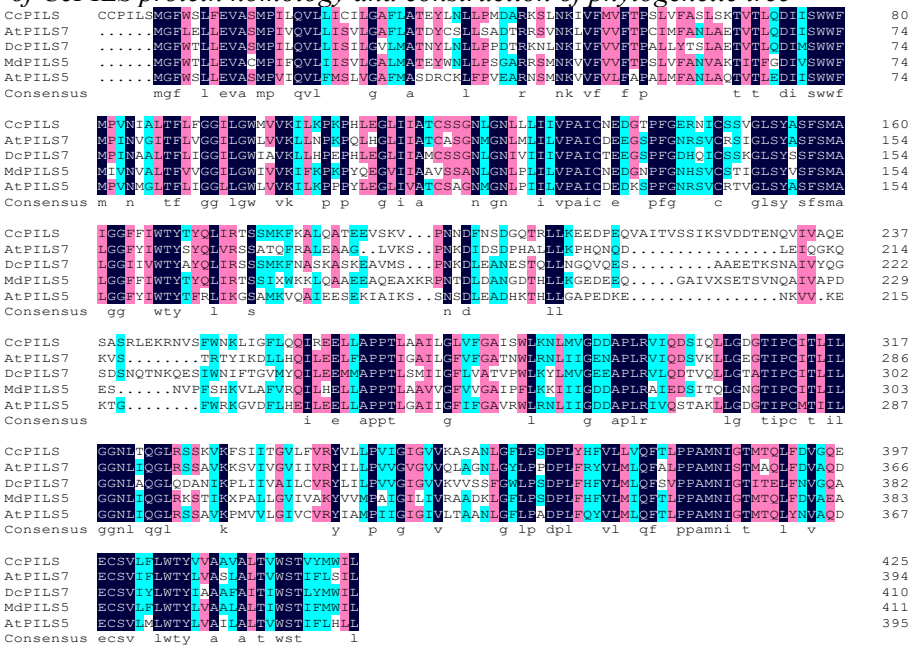

Fig.2 Comparison of the amino sequences of PILS

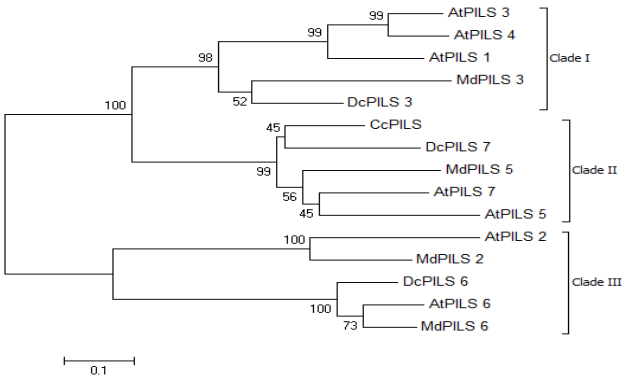

Fig.3 Phylogenetic tree based on the amino acid of CcPILS and the other PILS genes alignment

To evaluate the homology and the genetic distance of CcPILS protein, we aligned the amino-acid sequence and constructed the phylogenetic tree using the CcPILS, 8 AtPILS, 3 DcPILS and 3 MdPILS. The result showed that the amino acid sequences of CcPILS shared high homology with those of other plants, The consistency was 58.9%, 61.9%, 61.3% and 64.1%, separately (Fig. 2). As shown in Fig.3, CcPILS cluster in the same branch with AtPIL5, AtPIL7, DcPILS7 and MdPILS5.

3.3 Spatiotemporal Expression of CcPILS Gene in the graft process

Expression patterns of CcPILS in different tissues of *C. carthayensis* showed that the relative expression level of CcPILS was the highest in roots, followed by leaves, fruits and male inflorescence (Fig. 4). In addition, the expression levels of CcPILS gene in the graft process of hickory were different at different stages. As shown in the Fig. 5, the relative expression levels of CcPILS were increased and reached the peak at 3 DAG in both rootstock and scion, compared to that of control. The expression level of CcPILS in rootstock was nearly 3 times higher than that in 0 DAG and correspondingly in scion 1.6 times higher. After 7 DAG, its expression level was still induced in rootstock, but inhibited in scion, compared to that of control. The relative expression levels were decreased in both rootstock and scion after 14 DAG, compared to that of control.

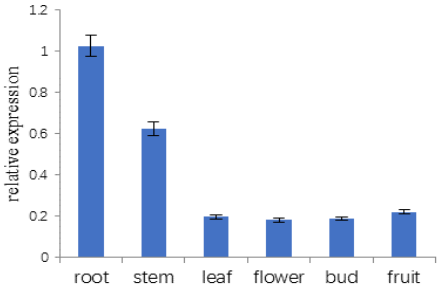

Fig.4 Expression patterns of CcPILS by real-time PT-PCR analysis in different tissues of *C. carthayensis*

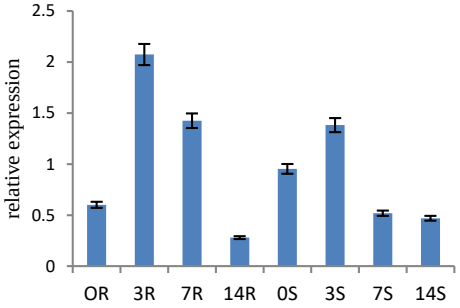

Fig.5 Expression patterns of CcPILS by real-time PT-PCR analysis during grafting of *C. carthayensis*

4. Discussion

Auxin is widely distributed in various organs of higher plants, with a short-distance unidirectional polar transport mode [17]. In this study, the full-length CcPILS gene of hickory was cloned. The results showed that CcPILS had two hydrophobic regions with 3-5 transmembrane domains in each hydrophobic region. There was a hydrophilic ring in the middle of the protein, and the hydrophilic loop was located in the cytoplasm. The intracellular and subcellular locations were similar to PIN5 and PIN8 of Arabidopsis, which were localized in the endoplasmic reticulum and were responsible for the transport of auxin from the cytoplasm to the endoplasmic reticulum, and thus participating in the polar transport of auxin [19-20]. Unlike the PIN family proteins, PILS proteins are also present in lower plants such as algae, suggesting that the PILS family proteins play an important role in biological functions compared to the early appearance of PIN family in evolution [21]. Some research has shown the function of AtPILS5 and AtPILS7 are cellular auxin homeostasis by regulating auxin metabolism [21].

CcPILS protein located in the endoplasmic reticulum and clustered in the same branch with AtPILS5 and AtPILS7, suggesting CcPILS and AtPILS5 and AtPILS7 has a similar function, responsible for auxin from the cytoplasm to the endoplasmic reticulum Transport and thus participate in the auxin-mediated regulation process. Grafting is an important horticultural technique in the asexual reproduction of hickory, and endogenous auxin plays an important role in the process of callus differentiation and formation of vascular bridge [15]. CcPILS, which belongs to Clade III subfamily, may play an important role in this process. CcPILS protein is a high instability index, which belongs to the unstable protein, provides the conditions for rapid regulation of the quantity of CcPILS on the endoplasmic reticulum membrane and the regulation of auxin transport.

The auxin content in 4-6 days to reach the peak in the grafted hickory [15], so auxin efflux carrier gene CcPILS expression was the highest in 3 DAG. Callus between scion and rootstock has formed in 7 DAG [17]. Auxin in the scion efflux slowly to the rootstock, so CcPILS expression in the rootstock was higher than that in the scion. The expression of CcPILS in the scion and rootstock decreased, and researched to the normal level after 14 DAG because of the disappearance of auxin in the grafted hickory.

5. Conclusion

CcPILS, a key auxin efflux carrier, encoded 294 amino acids and localized at endoplasmic reticulum membrane. The gene contained a central hydrophilic loop. The CcPILS belonged to Clade III sub-family. Real Time RT-PCR analysis showed that the gene expressions were weakly induced in bud, inflorescence, fruit, leaf and stem, while strongly in root. The expression levels were strongly induced and reached a peak at the third day of grafting in scion and rootstock compared to that of control. CcPILS may be involved in regulating auxin signal transduction during hickory graft process.

References

[1] Friml J, Vieten A, Sauer M, et al. Efflux-dependent auxin gradients establish the apical–basal axis of Arabidopsis. Nature, 2003, 426(6963):147-53.
[2] Dubrovsky J G, Sauer M, Napsucialymendivil S, et al. Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proceedings of the National Academy of Sciences of the United States of America, 2008, 105(25):8790-4.
[3] Zhao Y. Auxin biosynthesis and its role in plant development. Annual Review of Plant Biology, 2010, 61(1):49-64.
[4] Reinhardt D, Pesce E R, Stieger P, et al. Regulation of phyllotaxis by polar auxin transport. Nature, 2003, 426(6964):255-60.
[5] Barbez E, Kubeš M, Rolčík J, et al. A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. Nature, 2012, 485(7396):119-22.
[6] Feraru E, Vosolsobě S, Feraru M I, et al. Evolution and structural diversification of PILS putative auxin carriers in plants. Frontiers in Plant Science, 2012, 3(4):227.
[7] Huang J, Zhang T, Zhang Q, et al. The mechanism of high contents of oil and oleic acid revealed by transcriptomic and lipidomic analysis during embryogenesis in *Carya cathayensis* Sarg. BMC

genomics 2016, 17:113.
[8] Huang Y, Zhou Q, Huang J, et al. Transcriptional profiling by DDRT-PCR analysis reveals gene expression during seed development in *Carya cathayensis* Sarg. Plant Physiology and Biochemistry 2015, 91:28-35.
[9] Shen C, Xu Y, Huang J, et al. Molecular characterization and expression analysis of the critical floral genes in hickory (*Carya cathayensis* Sarg.). Plant physiology and biochemistry 2014, 83:142-150.
[10] Li J, Zeng Y, Shen D, et al. Development of SSR Markers in Hickory (*Carya cathayensis* Sarg.) and Their Transferability to Other Species of Carya. Current genomics 2014, 15(5):357-379.
[11] Wang Z, Huang J, Huang Y, et al. Deep sequencing of microRNAs from hickory reveals an extensive degradation and 3' end modification. Plant Biotechnology Reports 2013, 8(2):203-209.
[12] Wang Z, Huang J, Huang Y, et al. Discovery and profiling of novel and conserved microRNAs during flower development in *Carya cathayensis* via deep sequencing. Planta 2012, 236(2):613-621.
[13] Wang Z, Huang J, Huang Y, et al. Cloning and Characterization of a Homologue of the FLORICAULA/LEAFY Gene in Hickory (*Carya cathayensis* Sarg). Plant Molecular Biology Reporter 2011, 30(3):794-805.
[14] Jin S, Huang J, Li X, et al. Effects of potassium supply on limitations of photosynthesis by mesophyll diffusion conductance in *Carya cathayensis*. Tree physiology 2011, 31(10):1142-1151.
[15] Zheng B, Chu H, Jin S, et al. cDNA-AFLP analysis of gene expression in hickory (*Carya cathayensis*) during graft process. Tree physiology 2010, 30(2):297-303.
[16] Sima X, Jiang B, Fang J, et al. Identification by deep sequencing and profiling of conserved and novel hickory microRNAs involved in the graft process. Plant Biotechnology Reports 2015, 9(3):115-124.
[17] Qiu L, Jiang B, Fang J, et al. Analysis of transcriptome in hickory (*Carya cathayensis*), and uncover the dynamics in the hormonal signaling pathway during graft process. BMC Genomics, 2016, 17(1):935.
[18] Huang Y, Liu L, Huang J, et al. Use of transcriptome sequencing to understand the pistillate flowering in hickory (*Carya cathayensis*, Sarg.). BMC Genomics, 2013, 14(1):299-299.
[19] Mravec J, Skůpa P, Bailly A, et al. Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature, 2009, 459(7250):1136-40.
[20] Ding Z, Wang B, Moreno I, et al. ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in Arabidopsis. Nature Communications, 2012, 3(1):10-10.
[21] Barbez E. A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. 2012, 485(7396):119-122.