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Identification of Aux/IAA Gene Family in Pecan (*Carya illinoinensis*) and Its Expression in Graft Healing

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Abstract Aux/IAA (Auxin/Indole- 3- Acetic Acid) family, one of the early response gene family of auxin signals, is involved in regulating numerous aspects of plant growth and development. As an important economic plant, the identification of *Aux/IAA* family genes in pecan is of great significance. In this study, a total of 37 *Aux/IAA* family genes were identified in the pecan genome using bioinformatics method, subcellular localization revealed that all its members are located in the nucleus. Based on the phylogenic relationship with *Arabidopsis Aux/IAAs* gene, the 37 pecan *Aux/IAA* gene were categorized into two major groups (A and B). Multiple sequence alignment analysis showed that there were four typical conserved domains (I-IV) in Aux/IAA proteins of pecan. The cis-acting elements analysis revealed that all pecan Aux/IAAs had cis-acting elements in response to hormones in the promoter regions. According to the expression patterns of pecan *Aux/IAA* genes during graft healing, four genes with high expression were identified, which may play important roles in the formation of graft union in pecan. This study will be helpful to elucidate the function of pecan Aux/IAAs in the process of graft healing and find candidate genes.

Keywords *Carya illinoinensis*; Aux/IAA; Grafting; Expression patterns

Auxin plays an important role in the regulation of plant growth and development, including cell division, cell elongation and differentiation, programmed cell death, plant apical dominance, fruit formation and rooting. Auxin plays a role by regulating the expression of multiple auxin responsive genes. These auxin responsive genes can be roughly divided into three gene family: Aux/IAA (auxin/indole-3-etic acid), GH3 (Gretchen hagen 3) and SAUR (small auxin up RNA) (Xie et al., 2010; Reed, 2001). *Aux/IAA* belongs to the primary/early auxin response gene, which responds rapidly under auxin treatment and plays an important role in the early stage of auxin signal transduction (Chapman and Estelle, 2009). When the concentration of auxin is low, Aux/IAA inhibits the function of ARF (Auxin response factor) by combining with the transcription factor ARF. When the concentration of auxin is high, the SCF^{TIR1} protein complex (Auxin transport inhibitor response 1-SKP1-Cullin-F-box complex) can detect auxin through the auxin concentration dependent ubiquitin mediated pathway and reduce the expression level of *Aux/IAA*, so Aux/IAA cannot inhibit ARF, and ARF downstream genes can be activated (Liu and Yuan, 2009).

The typical Aux/IAA protein has four highly conserved domains (I-IV), which are the basis for these proteins to exercise specific functions (Hagen and Guilfoyle, 2002). Domain I at the N-terminal of the protein contains the conserved leucine repeat sequence LxLxLx, which has an epistasis on the transcriptional activation of ARF as a transcription inhibitor (Tiwari et al., 2001). The conserved GWPPV sequence in domain II interacts with the F-box protein TIR1 to regulate the degradation of Aux/IAA proteins. The half-life of Aux/IAA varies from 10 minutes to a few hours, mainly depending on the properties of domain II. Some Aux/IAA proteins carrying mutations in the II domain have a long half-life and are insensitive to auxin (Kepinski and Leyser, 2005). The domains III and IV at the C-terminal of Aux/IAA protein share homology with ARF, mediating the aggregation of Aux/IAA with ARF to form homologous or heterologous dimers and inhibiting the function of ARF (Piya et al.,

2014). Aux/IAA that lacks at least one conservative region is considered atypical. The emergence of atypical Aux/IAA seems to be an ancient evolutionary event of great significance for plants to adapt to different environments. It can be seen that atypical Aux/IAA exists in many plants Aux/IAA gene family (Li et al., 2017).

The mechanisms of Aux/IAA in various biological processes of plant growth and development have been well summarized (Reed, 2001; Halliday et al., 2009). In addition to regulating plant growth and development, Aux/IAA can also participate in responding to different environmental stresses (Halliday et al., 2009). For example, a dominant *shy2/iaa3* mutant was isolated from the screening of inhibitors of phytochrome chromophore mutant hy2, indicating that Aux/IAA was involved in light signal transduction (Tian et al., 2002). *SbIAA1* has different expression levels in both root and leaf tissues under salt stress and drought conditions in sorghum, so this gene may have a positive effect on sorghum's salt and drought resistance (Cakir et al., 2013).

At present, the Aux/IAA gene family of several plant species has been identified. For example, there are 29 members of the Aux/IAA gene family in Arabidopsis (Reed, 2001), 31 in rice (Jain et al., 2006), 26 in sorghum (Cakir et al., 2013), 36 in barley (Shi et al., 2020), 26 in potato (Audran-Delalande et al., 2012), 25 in tomato (Wu et al., 2012), etc. At present, research on the function of the *Aux/IAA* gene in pecan is still limited. Pecan (*Carya illinoinensis* ((Wangenh.) K. Koch)), commonly known as Bigenguo, is an important nut tree species belonging to the genus Carya, which is native to the United States and Mexico. The seeds of pecan are rich in unsaturated fat acid, protein, vitamin B and rich in bioactive substances, with high nutritional value. At present, pecan is widely planted and has important economic value. In the United States, the annual production of pecan exceeds 1.3×108 kg, valued at over 600 million USD (Huang et al., 2019). Grafting is an asexual reproduction technology, which is widely used in the commercial cultivation of pecan, and can shorten the young period and increase the yield of nuts. Plant hormones, especially auxin, plays an important role in the process of graft survival (Huang et al., 2019). So it is of great significance to study the expression pattern of Aux/IAA gene family in the grafting of pecan. In this study, 37 members of the Aux/IAA gene family were identified in pecan. They were divided into two groups through phylogenetic analysis, and further analyze its gene structure, sequence characteristics, expression level, etc.

In this study, the whole genome identification and bioinformatics analysis of the Aux/IAA gene family of pecan were carried out, and the expression patterns of these genes in the process of graft healing were analyzed to provide a theoretical basis for screening key candidate genes.

1 Result and Analysis

1.1 Identification of Aux/IAA gene family in pecan

Based on the HMM model, 58 candidate Aux/IAA proteins were retrieved from the genome of pecan. The structural domains of the candidate proteins were analyzed by using Pfam database, and 21 genes containing the ARF domain were removed. Finally, 37 Aux/IAA protein sequences were obtained. It was found that the protein length ranges from 155 (CIL1193S0081) to 447 (CIL0948S0109) by analyzing the physicochemical properties of Aux/IAA family members; The relative molecular weight ranges from 17 201.51 (CIL1193S0081) to 49 914.75 (CIL0948S0109) Da; The isoelectric point is between 4.75 (CIL1619S0013) and 9.77 (CIL0202S0016). All Aux/IAA proteins are predicted to be localized in the nucleus (Table 1).

1.2 Phylogenetic analysis of Aux/IAA gene family in pecan

A phylogenetic tree was constructed by combining 37 *Aux/IAA* genes in pecan identified in this study with 29 Arabidopsis *Aux/IAA* genes (Figure 1). Based on previous research results (Remington et al., 2004; Jain et al., 2006), these 66 *Aux/IAA* genes were divided into two groups: A and B. According to this phylogenetic tree, the classification of Arabidopsis is consistent with previous studies. 18 pecan *Aux/IAA* genes are located in group A and 19 genes are located in group B. Groups A and B are further divided into 7 and 6 subgroups (A1~A7; B1~B6). Each subgroup contains the *Aux/IAA* genes of pecan and Arabidopsis, indicating that the *Aux/IAA* differentiation time is earlier than the species differentiation time. There are 28 pairs of sisters pairs in the phylogenetic tree, most of which are paralogous proteins, 24 pairs in total (15 pairs of pecan, 9 pairs of Arabidopsis), and 4 pairs of

orthologous proteins. Direct homologous proteins only appear in subgroups A3, A7, and B4, B6, while other subgroups only contain collateral homologous proteins. In the B1 subgroup, six pecan *Aux/IAA* genes were clustered together. Further analysis revealed that the gene structures and conserved motifs of these six genes were similar, and there were relatively significant differences compared to other pecan *Aux/IAA* members.

Table 1 Basic information of Aux/IAA family genes in pecan

Gene ID	Isoelectric point	Molecular weight (kD)	Protein length(aa)	Subcellular localization
CIL1101S0049	9.18	24 217.31	218	Nucleus
CIL0203S0027	6.48	39 645.71	365	Nucleus
CIL0948S0109	5.21	49 914.75	447	Nucleus
CIL1193S0081	7.93	17 201.51	155	Nucleus
CIL1332S0001	7.73	27 342.26	241	Nucleus
CIL0385S0027	5.11	25 501.06	233	Nucleus
CIL0967S0141	6.75	28 915.42	257	Nucleus
CIL0967S0140	6.63	21 933.9	195	Nucleus
CIL1134S0054	7.01	32 138.98	302	Nucleus
CIL0899S0160	8.16	33 981.44	320	Nucleus
CIL0202S0015	5.42	23 180.27	213	Nucleus
CIL0202S0016	9.77	25 830.53	230	Nucleus
CIL1153S0084	5.93	27 515.72	242	Nucleus
CIL1153S0069	6.63	22 056.99	196	Nucleus
CIL0912S0030	8.55	33 568.8	318	Nucleus
CIL0979S0163	8.71	40 033.94	370	Nucleus
CIL0976S0134	6.85	36 052.89	334	Nucleus
CIL0990S0019	5.65	23 875.92	218	Nucleus
CIL1173S0085	8.72	26 136.84	236	Nucleus
CIL1293S0053	8.3	33 327.04	315	Nucleus
CIL0344S0014	9.01	39 305.19	359	Nucleus
CIL1034S0041	8.36	26 693.66	236	Nucleus
CIL1034S0042	5.55	23 249.07	207	Nucleus
CIL0970S0074	5.98	23 812.68	214	Nucleus
CIL0970S0075	6.66	25 698.61	228	Nucleus
CIL1396S0028	5.37	21 833.47	194	Nucleus
CIL1358S0014	5.89	26 853.94	235	Nucleus
CIL1141S0030	4.81	29 993.31	267	Nucleus
CIL1184S0075	5.88	25 399.15	224	Nucleus
CIL1184S0074	6.76	22 020.37	195	Nucleus
CIL1485S0008	6.63	22 315.49	200	Nucleus
CIL1268S0028	8.89	40 282.02	366	Nucleus
CIL1268S0077	7.71	22 313.67	212	Nucleus
CIL1294S0084	5.17	18 985.33	170	Nucleus
CIL1130S0027	8.95	20 874.81	190	Nucleus
CIL1619S0013	4.75	21 983.33	195	Nucleus
CIL1320S0049	7.62	33 826.14	320	Nucleus

1.3 Analysis of conservative domains in the Aux/IAA family of pecan

Through multiple sequence alignment, it is found that most of the Aux/IAA protein sequences of pecan contain four conservative domains: Domain I, Domain II, Domain III and Domain IV (Figure 2). There are 21 pecan Aux/IAA proteins containing 4 conserved domains.

1.4 Analysis of gene structure and conserved motifs of Aux/IAA family in pecan

Previous studies have shown that the diversity of gene structure is the main driving force for the evolution of many gene family (Singh and Jain, 2015). Further analysis of the exons/introns of the Aux/IAA gene in pecan was

conducted to investigate its structural diversity. The results showed that all genes contained introns, ranging from 1 to 6 in number. Most genes (16) contain 3 introns, while *CIL1193S0081*, *CIL1268S0077*, and *CIL0202S0016* only contain 1 intron. *CIL0203S0027* has the highest number of introns, with 6 (Figure 3A). According to the phylogenetic tree results, genes in the same subgroup have similar gene structures, such as *CIL0967S0140*, *CIL1153S0069*, *CIL1034S0042*, and *CIL0970S0074*.

Five motifs were found in the Aux/IAA protein of most pecans. According to MEME analysis, the four conserved motifs correspond to the four conserved domains of Aux/IAA protein (Figure 3B). Motif 1, 2, 3, and 4 correspond to domain IV, III, II, and I, respectively (Figure 3C). There are 21 pecan Aux/IAA proteins containing all 4 conserved motifs, most of which belong to Group B. And the other 16 proteins lack at least one conserved motif, belonging to atypical Aux/IAAs. Among them, 6 proteins are missing motif 1, 1 protein is missing motif 2, 6 proteins are missing motif 3, and 11 proteins are missing motif 4. Specifically, 8 proteins are missing two motifs simultaneously, of which 6 are missing both motif3 and motif4, all of which belong to Group A. The conservative motif analysis is consistent with the previous conservative structural domain analysis results.

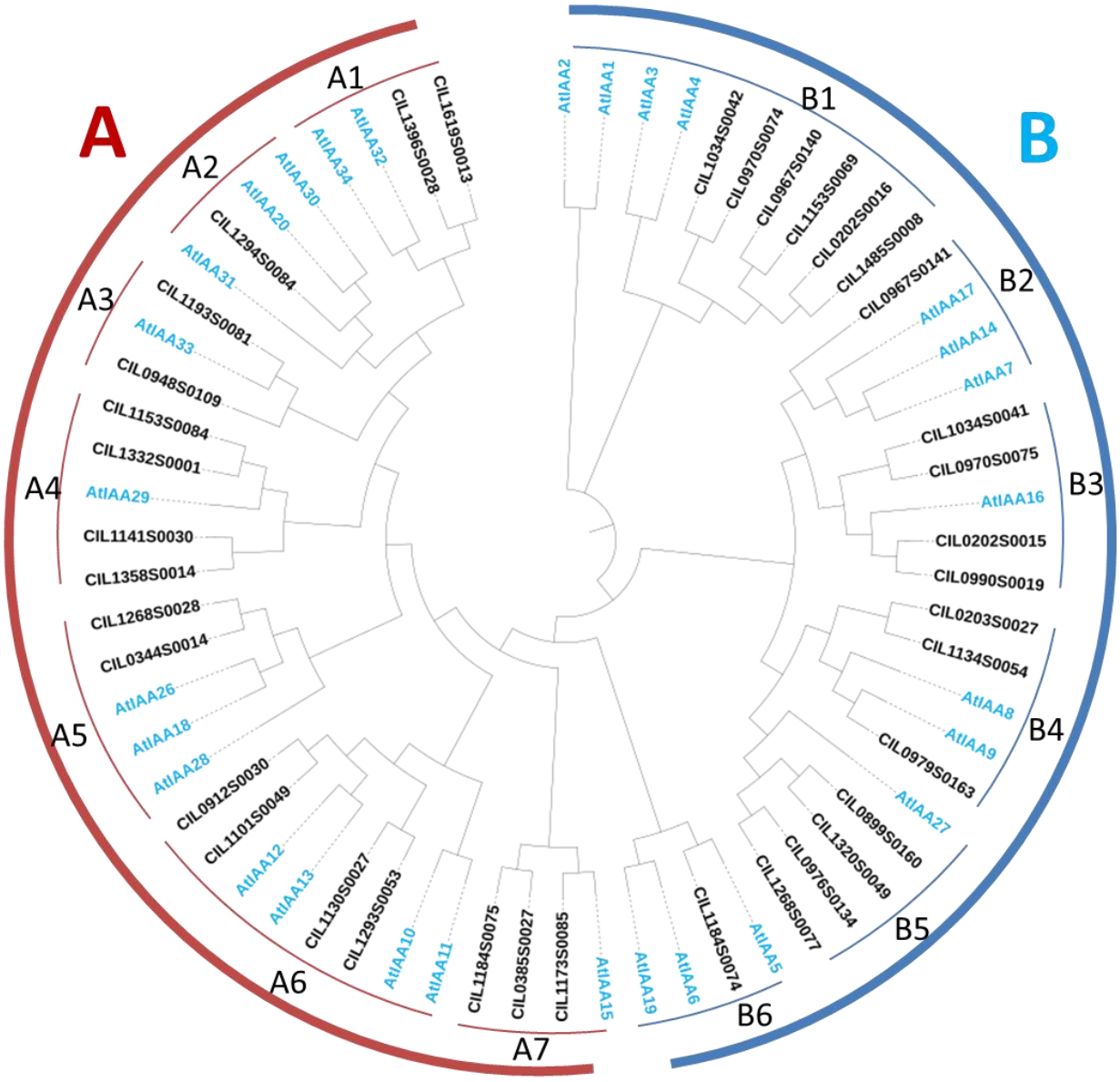

Figure 1 Phylogenetic tree of Aux/IAA family in pecan

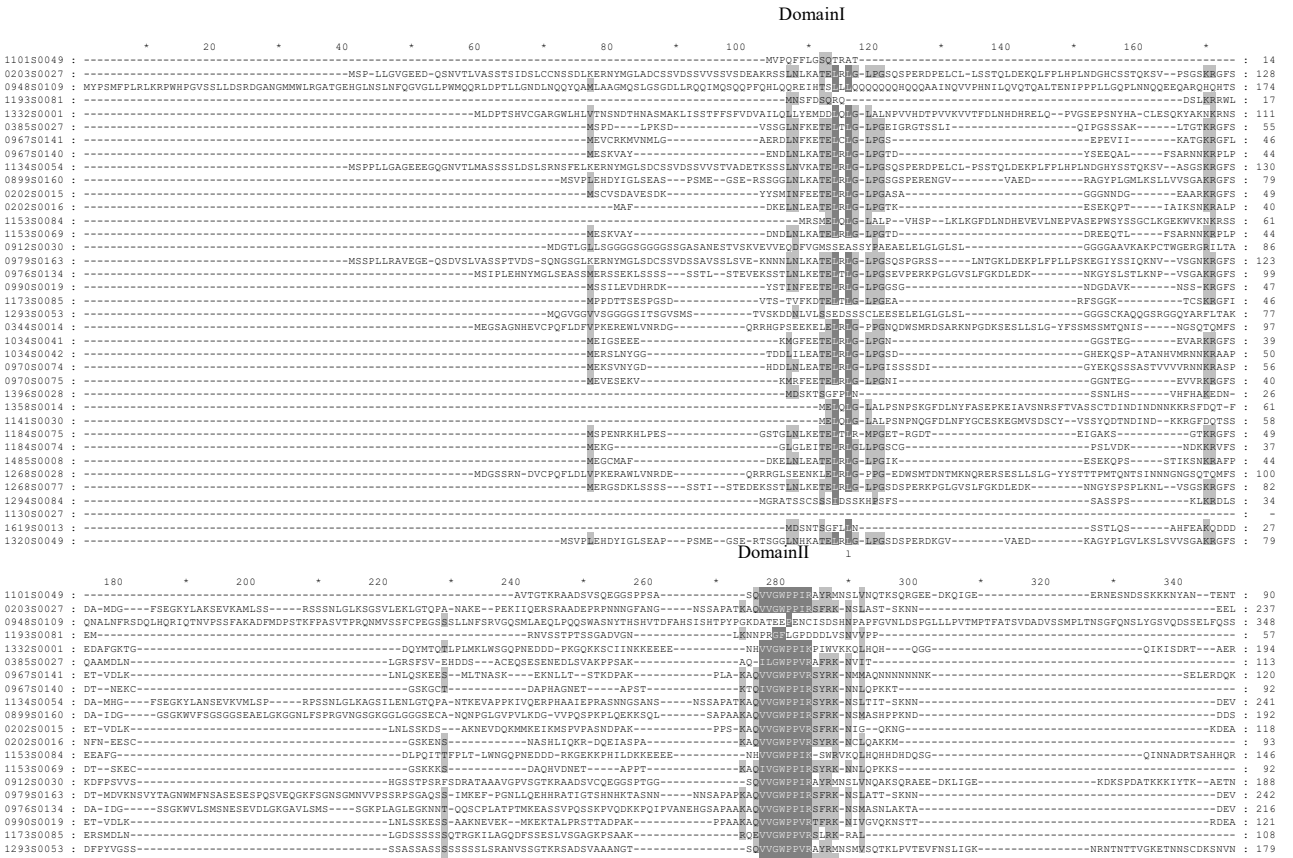

Figure 2 Multiple sequence alignment of the Aux/IAA peptides in pecan

1.5 Analysis of cis-acting elements in the Aux/IAA family of pecan

The cis-acting elements of the Aux/IAA family of response hormones in pecan can be divided into five categories: response to auxin, response to salicylic acid, response to abscisic acid, response to methyl jasmonic acid and

response to gibberellin. Each pecan Aux/IAA member contains varying amounts of hormone responsive elements. The cis-acting elements responding to auxin are AuxRR-core and TGA-element. Among them, 10 *Aux/IAA* genes (*CIL0385S0027*, *CIL1293S0053*, *CIL1332S0001*, *CIL1332S0001*, *CIL1358S0014*, *CIL1396S0028*, *CIL1485S0008*, *CIL0976S0134*, *CIL1101S0049*, *CIL1134S0054*) have elements that respond to auxin (Figure 4).

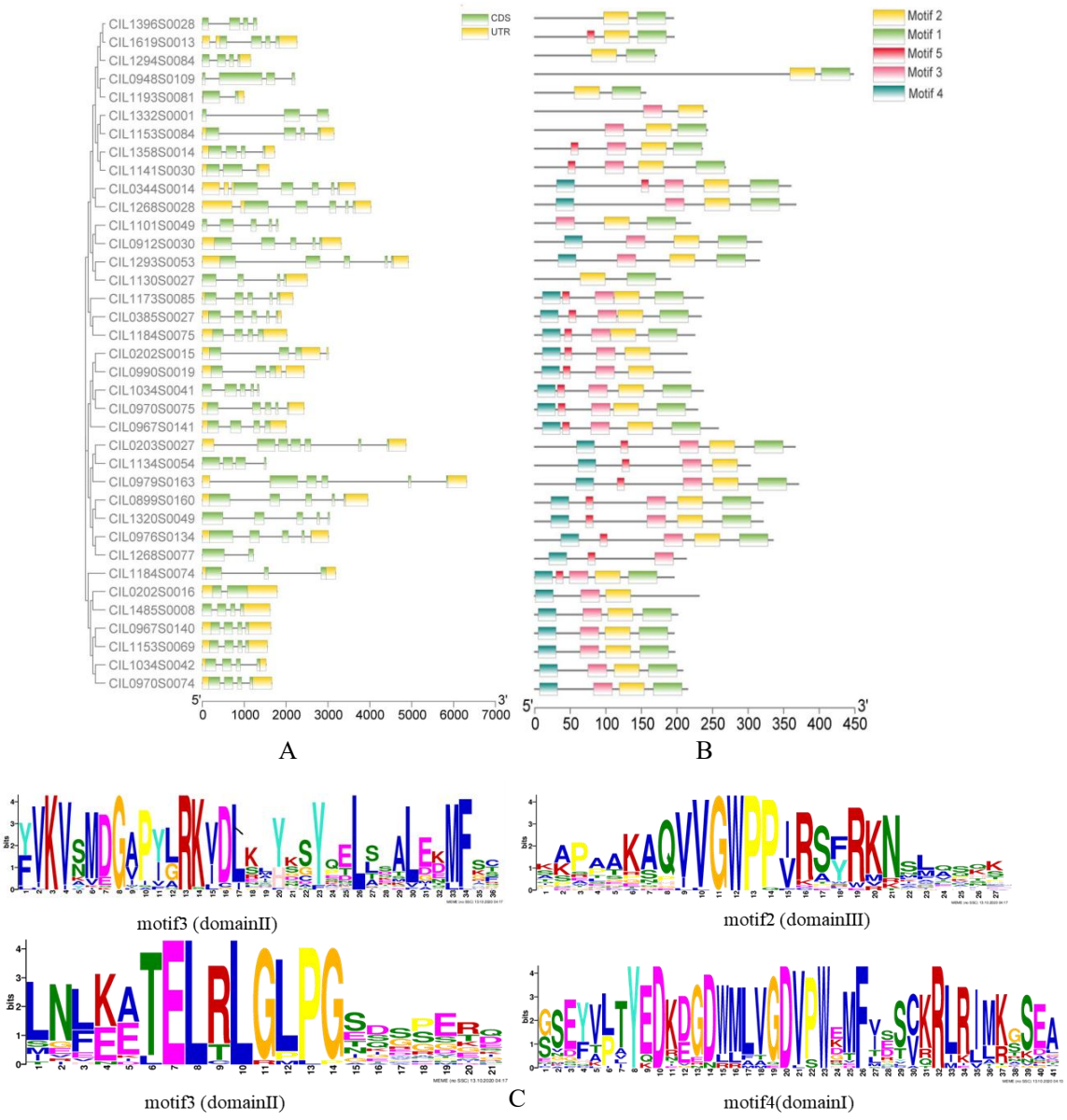

Figure 3 Gene structure and conserved motifs of Aux/IAA family in pecan

Note: A: Gene structure of Aux/IAA family in pecan; B: Conserved motifs of Aux/IAA family in pecan; C: Conserved motif sequences of Aux/IAA family in pecan

1.6 Expression patterns of Aux/IAA family in grafting healing of pecan

In order to explore the potential function of Aux/IAA gene in the grafting process of pecan, we analyzed the expression pattern of these 37 Aux/IAA genes at 0 ,8, 15 and 30 d after grafting by using the transcriptome data from the grafting healing process (Figure 5). 8 and 15 d after grafting are the initial stage of grafting callus formation and the stage of a large number of callus formation, respectively, while 30 d after grafting are in the stage of vascular tissue formation. It was found that about 1/3 of pecan Aux/IAA genes have low expression levels in different samples, indicating that these genes may not be involved in the response to grafting healing process, while the remaining 2/3 genes exhibit different expression patterns. Among them, we found that four genes (CIL0990S0019, CIL1320S0049, CIL0203S0027, and CIL0979S0163) had high expression levels in the process

of graft healing, and CIL0990S0019, CIL0203S0027, and CIL1320S0049 all reached the highest expression levels on the 8th day, indicating that these genes may play an important role in the process of callus formation and vascular tissue regeneration, especially in the early stage of callus formation.

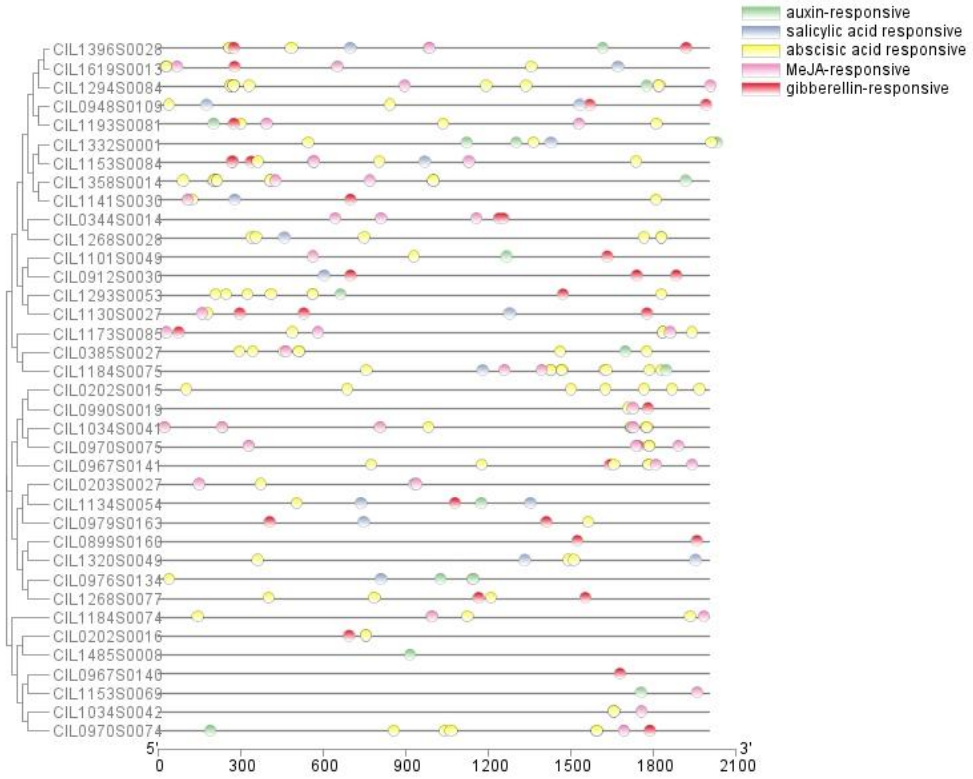

Figure 4 *Cis*-acting elements in response to hormones of Aux/IAA family in pecan

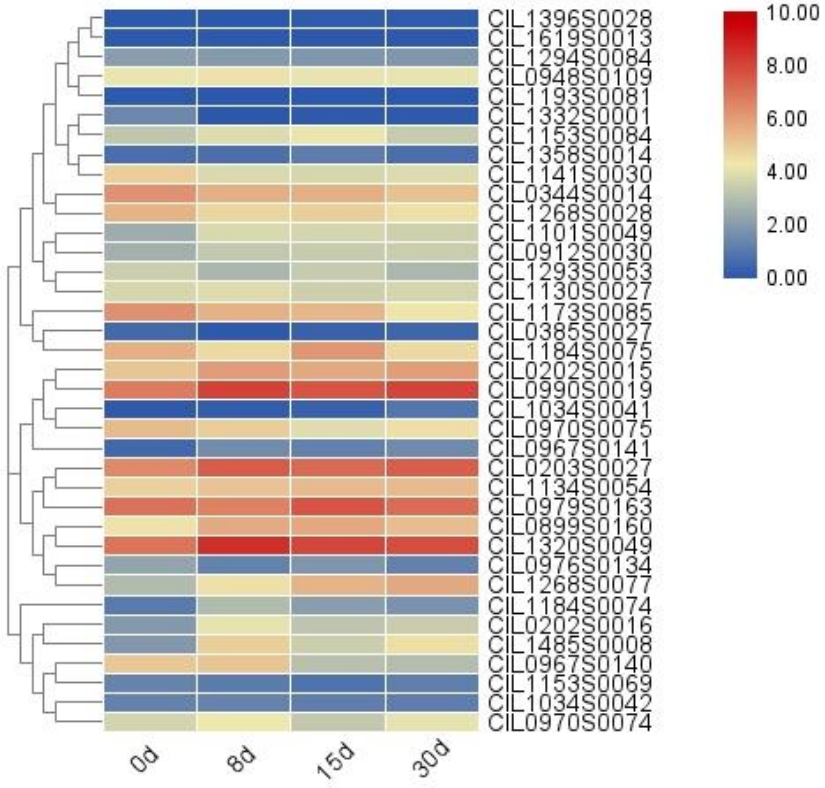

Figure 5 Expression patterns of *Aux/IAA* genes in pecan during grafting healing process

2 Discussion

The vigorous development of plant genome sequencing has laid the foundation for gene function research. In recent years, the genome sequencing of pecan has been completed, which greatly promoting the development of gene function research in pecan. Auxin is a key endogenous hormone regulating plant growth and development, and *Aux/IAA* gene plays an important role in auxin signal transduction. The Aux/IAA family genes have been identified in many plants. Grafting is an asexual reproduction technology, which is widely used in the commercial cultivation of pecan, and can shorten the juvenile stage and increase the yield of nuts. Plant hormones, especially auxin, play an important role in graft survival. Therefore, the identification and analysis of Aux/IAA family genes in pecan and the study of their expression patterns in pecan grafting will help to further explore the function of *Aux/IAA* genes.

We identified a total of 37 pecan Aux/IAA family genes in this study, which were not significantly different from the number of members of this family in Arabidopsis and rice (Remington et al., 2004; Jain et al., 2006). According to phylogenetic analysis, these 37 family members can be divided into two groups: A and B, and 13 subgroups. Each subgroup contains pecan and Arabidopsis *Aux/IAA* genes. The phylogenetic tree includes 28 pairs of sisters pairs, most of which are paralogous proteins. More than half of these genes (57%) are typical Aux/IAA proteins, containing four conserved domains, and most of them belong to Group B, while the rest are atypical Aux/IAA proteins. Through gene structure analysis, it was found that all genes contain introns, ranging from 1 to 6, and genes in the same subgroup have similar gene structures. Through *cis*-acting element analysis, it is found that all members have acting elements that respond to hormones in varying numbers (1~10), of which 10 genes contain elements that respond to auxin. Based on the expression heatmap of the Aux/IAA family of pecan in grafting healing, we identified four highly expressed genes that may play an important role in the formation of grafting callus. These findings will help screen for key candidate genes and further analyze the function of Aux/IAAs in the grafting healing process of pecan.

3 Materials and Methods

3.1 Identification of Aux/IAA gene family in pecan

All protein sequences of pecan were downloaded from GigaDB database（http://gigadb.org/dataset/100571）; The HMM model file for Aux/IAA domains (Pfam02309) was downloaded from Pfam database (http://pfam.xfam.org/); we used HMMER 3.0 software (www.hmmer.org) to preliminarily identify 58 members of the Aux/IAA family in pecan. Since some ARF gene family proteins share a conservative C-terminal with Aux/IAA family proteins, the 58 initially discovered pecan Aux/IAA proteins include some ARF proteins with B3 DNA-binding domain (Pfam02362) and AUX/RESP domain (Pfam06507). We weeded out these proteins and retained 37 of them for further research.

3.2 Analysis of sequence characteristics

Using Cell-PLoc 2.0 website (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) to predict the subcellular localization information of Aux/IAA protein in pecan; The relative molecular weight, isoelectric point, protein length and other sequence characteristics of the protein were analyzed by using ExPASy website (https://web.expasy.org/protparam/).

3.3 Phylogenetic analysis

We downloaded the Arabidopsis Aux/IAA protein sequence from TAIR (https://www.arabidopsis.org/), and built a phylogenetic tree by using Aux/IAA protein sequence file from pecan and Arabidopsis in MEGA7.0 file. The multi sequence comparison method was ClustalW, and the neighbor-joining method (NJ) was used to construct the tree. The parameters were set as follows: the test method is Bootstrap method, the test frequency was set to 1 000 times, and the gap processing selected Pairwise Deletion method. The phylogenetic tree was visualized by using the online tool iTOL (https://itol.embl.de/).

3.4 Multiple sequence alignment

MEGA software ClustalX procedure was used to conduct multiple sequence alignment for the Aux/IAA protein of pecan, and the GENEDOC software was used to visualize the conservative domain.

3.5 Gene structure and conserved motif analysis

The whole genome sequence file and GFF annotation file of pecan were downloaded from the GigaDB database (http://gigadb.org/dataset/100571). Gene structure maps were drawn by TBtools software (https://github.com/CJ-Chen/TBtools). Conservative motifs were analyzed by using online software MEME (http://meme-suite.org/tools/meme).

3.6 Analysis of cis-acting elements

In order to investigate the possible cis-acting elements in the Aux/IAA gene promoter of pecan, a 2 000 bp upstream region of the Aux/IAA gene was extracted from the genome sequence of pecan. Predicting cis-acting elements of *Aux/IAA* genes using Plant CARE databases (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Select elements related to hormone response and use TBtools software for visualization.

3.7 Transcriptome analysis

Three biological samples were respectively collected from the grafting site of pecan at 0, 8, 15, and 30 d after grafting for transcriptome analysis. Calculate the transcriptional abundance of Aux/IAA genes in pecan by using FPKM values. The raw data of RNA-seq can be obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/) through PRJNA411951. Converting FPKM value+1 to log2 value, and use TBtools software to draw a heat map of Aux/IAA gene expression in pecan.

Authors' contributions

FPH is the executor of this experimental study; FPH completed data analysis and wrote the first draft of the paper; ZKK participated in the analysis of experimental results and the revision of the paper; TPP and LYR participated in the experimental design. PFR is the project leader, who guides experimental design, data analysis, paper writing, and revision. All authors read and approved the final manuscript.

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