Identification and Analysis of *Aux/IAA* Family in *Acer rubrum*

Wenpeng Zhu[1,2], Manyu Zhang[1,2], Jianyi Li[1,2], Hewen Zhao[1,2,3], Wei Ge[1,2,3] and Kezhong Zhang[1,2,3]

[1]Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing University of Agriculture, Beijing, People's Republic of China. [2]College of Landscape Architecture, Beijing University of Agriculture, Beijing, People's Republic of China. [3]Beijing Laboratory of Urban and Rural Ecological Environment, Beijing, People's Republic of China.

ABSTRACT: The phytohormone auxin are important in all aspects of plant growth and development. The *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) gene responds to auxin induction as auxin early response gene family. Despite the physiological importance of the *Aux/IAA* gene, a systematic analysis of the *Aux/IAA* gene in *Acer rubrum* has not been reported. This paper describes the characterization of *Acer rubrum Aux/IAA* genes at the transcriptomic level and *Acer yangbiense Aux/IAA* genes at the genomic level, with 17 *Acer rubrum AUX/IAA* genes *(ArAux/IAA)* and 23 *Acer yangbiense Aux/IAA (AyAux/IAA)* genes identified. Phylogenetic analysis shows that *AyAux/IAA* and *ArAux/IAA* family genes can be subdivided into 4 groups and show strong evolutionary conservatism. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to test the expression profile of *ArAux/IAA* genes in different tissues under indole-3-acetic acid (IAA) treatment. Most *ArAux/IAA* genes are responsive to exogenous auxin and have tissue-specific expression. Overall, these results will provide molecular-level insights into auxin metabolism, transport, and signaling in *Acer* species.

KEYWORDS: AUX/IAA, *Acer spp.*, phylogeny, gene expression, qRT-PCR

Introduction

Auxin play a key role in the regulation of plant physiological processes, including embryogenesis, vascularization, adventitious root development, establishment of apical dominance, and flower and fruit development.[1-3] Dynamic spatiotemporal changes in auxin hormone levels can precisely and rapidly trigger genetic reprogramming which requires auxin response genes, such as *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) family, the *auxin response factor* (*ARF*) family, the *auxin-responsive Gretchen Hagen3* (*GH3*) family and *small auxin upregulated RNA* (*SAUR*).[4-7] Numerous studies have shown that the promoters of these auxin-responsive genes contain a number of potential auxin-responsive elements (AuxREs), of which at least one conserved motif (TGTCTG) is present at high frequencies.[6] Among these genes, members of the *Aux/IAA* family have been identified as short-lived nuclear proteins that play a critical role in suppressing the expression levels of ARFs.[8,9] In the absence of auxin, AUX/IAA proteins can prevent ARFs from activating the promoters of auxin-responsive genes by DNA-binding to ARFs via domains III and IV. When auxin at high concentrations, these proteins bind to the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) and are ubiquitinated and subsequently degraded by the 26s protease[10-12] (Figure 1). In general, different TIR1/AFB-Aux/IAA protein combinations have different auxin hormone affinities and different levels of auxin in different tissues and developmental stages, resulting in different auxin corresponding effects.[13] Thus, plants can precisely regulate the growth and development of tissues at all stages by regulating the spatial and temporal levels of auxin and translating it into genetically programmed signals.[14]

Studies of the *Aux/IAA* gene family have been intensively investigated in *Arabidopsis thaliana*, and studies of *Aux/IAA* mutants in *Arabidopsis* have revealed that *Aux/IAA* exercises different functions during plant growth and development.[15-17] So far, 29 *Arabidopsis thaliana Aux/IAA* (*AtAux/IAA*) family proteins have been identified, along with an increasing number of candidate genes that may regulate AUX/IAA proteins.[18,19] *Acer rubrum* can be used as a foliage tree for both street trees and landscaping, and is widely used in parks, neighborhoods, and streets.[20] So far, most of the research on *A.rubrum* has been on physiological aspects, but relatively little has been done on the mechanisms of molecular regulation, especially on gene families in the auxin regulatory pathway. Notably, large numbers of *Aux/IAA* family members have also been found in other plants, including *Brassica rapa, Carica papaya*, Citrus, *Populus trichocarpa*.[21-24] However, no data on *Aux/IAA* gene family are available in *A.rubrum*.

As the genomes of species closely related to the *Acer rubrum* continue to be reported, such as citrus, longan, and *Acer yangbiense*,[25-27] this has made it possible to gather *Aux/IAA* family
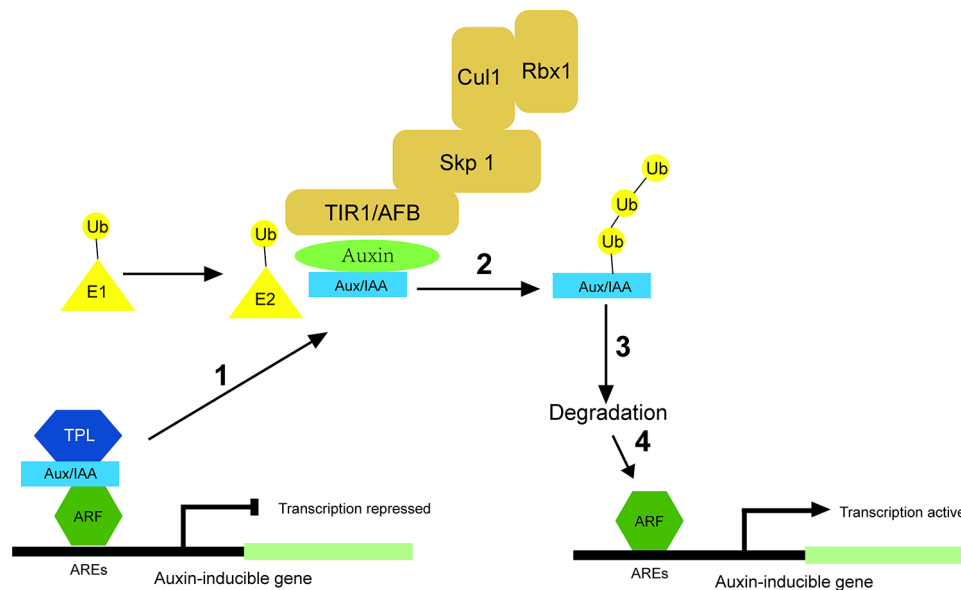
Figure1. The ARF protein family regulation of auxin-inducible genes transcription by forming dimers with auxin response elements (AREs) in the promoters of Auxin-inducible genes. In the absence of Auxin, the AUX/IAA transcriptional repressor recruits TOPLESS family (TPL) co-repressors by interacting with ARFs, which in turn recruit chromatin-modifying enzymes that inhibit downstream Auxin-inducible genes transcription.
The steps of the Auxin response pathway are indicated by numerical arrows. (1) In the presence of auxin, the Aux/IAA, and TIR1/AFB family F-box proteins bind together. (2) The F-box proteins are part of the SCF-type E3 ubiquitin protein ligase complex that transfers activated ubiquitin (Ub) from the E1/E2 enzyme system. (3) Polyubiquitylation of Aux/IAA leads to its degradation. (4) The dimer formed by ARF and AREs is released to activate Auxin-inducible genes transcription.

genetic information from the transcriptome level. From a taxonomic point of view, *A.yangbiense* is more suitable for *A.rubrum* transcriptome assembly than longan and citrus because it is in the same genus as *A.rubrum* and it is a wild species, so we used the *A.yangbiense* genome to assemble the *A.rubrum* transcriptome.[27] Since the *A.yangbiense* genome has high assembly quality, no recent whole-genome duplication (WGD) events and little chromosomal recombination, it is an important guide to identify the *AyAux/IAA* family for studying the evolution of *ArAux/IAA* family members. The aim of this study was to conduct a detailed study of the *Aux/IAA* gene family in *A.rubrum* and *A.yangbiense* based on the *A.rubrum* transcriptome and the *A.yangbiense* genome.

Genes of the *Aux/IAA* family of *A.rubrum* and *A.yangbiense* were analyzed, including total number of genes, gene structure, phylogenetic relationships, chromosome localization, conserved sequences, and protein domains. Coanalysis with the *AyAux/IAA* family helps to uncover specific information about the *ArAUX/IAA* genes. In addition, we examined the expression pattern of *ArAUX/IAA* in different tissues under IAA treatment. The results of this paper provide new data for future studies of auxin signaling in *A.rubrum*.

Materials and Methods
Aux/IAA *gene family identification in* A.rubrum

To identify *Ay Aux/IAA* gene family, all *A.rubrum* RNA-Seq clean data of were retrieved from National Center for Biotechnology Information (NCBI) SRA Database (SRR5232063, SRR5234825, SRR5234856) and mapped to the *A.yangbiense* genome (http://gigadb.org/dataset/100610) with HISAT2 for obtained protein sequences.[27,28]

The protein sequences of the twenty-nine *Arabidopsis Aux/IAAs* (https://www.arabidopsis.org/) were selected to search against the transcript of *A.rubrum* by Tblastn method, *AtAux/IAAs* (https://www.arabidopsis.org/) were selected to search against the transcript of *A.rubrum* by Tblastn method, Parameter selection, matrix is BLOSUM62, expect is less than 1e-005, gap-existence is 11, gap-existension is 1, Filter is Low-complexity.[14,19,29] The hidden Markov model (HMM) profile of *Aux /IAA* domain (PF02309) was downloaded from Pfam (http://pfam.xfam.org/).[30] The PF02309 based domains in *A.rubrum* proteins were identified using HMMER software with E-value cut off 1.0. The filtered *A.rubrum* genes obtained from the HMM search then subjected to a Pfam bath search to confirm the gene families. SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) web server was used to examine the conserved domains of the identified *A.rubrum* genes. Finally, the candidate *ArAux/IAA* gene with the UniProt Knowledge Base (UniProtKB-http://www.uniprot.org/help/uniprotkb) to verify its homologues with other species.

Obtained *ArA Aux /IAA* genes were named according to their *Arabidopsis* homologs. The protein characteristics, including molecular weight (MW), isoelectric points (pIs), and lengths were online predicted by ProtParam tool.[31] Subcellular localization was examined by CELLO software (http://cello.life.nctu.edu.tw/). Identification of *Aux/IAA* family genes at the genome-wide using the same method for the *A.yangbiense*.

Gene structure and conserved motif analysis

Phylogenetic analysis was carried out with MEGA7 software, the neighbor-joining (NJ) tree was constructed with bootstrap values tested for 1000 trails, models with the lowest BIC scores is JTT + G + I to describe the substitution pattern the best. This analysis involved 95 amino acid sequences. All positions with less than 80% site coverage were eliminated, that is, fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position.[31,32] MEME was used to find the conserved motif of *Ar* Aux/IAA proteins.[33] The candidate Aux/IAA proteins were further examined to confirm the presence of Aux/IAA repeats using Pfam and SMART software.[34] The image data were displayed in Tbtools.[35]

GO functional identification

The *Ar* Aux/IAA protein sequences were compared against the NCBI database using BLASTP. The Gene Ontology (GO) terms of functional annotation were analyzed using Blast2GO software (https://www.blast2go.com/) with default parameters. The annotations of GO terms were investigated using Gene Ontology Consortium (http://geneontology.org/).

Plant materials and RNA extraction

The plant material for this experiment was obtained from the branches of 3-year-old *A.rubrum* from the teaching nursery of Beijing University of Agriculture (40.09º N, 116.30º E). Branches of similar growth were treated by immersion in water and IAA solution (300 mg/L) for 1 hour, with 3 biological replicates for each group. The phloem, mature leaves, and young leaves were taken from the treated branches for RNA extraction. RNA samples were extracted with the Tiangen RNA prep Pure Plant Kit (Tiangen Biomarket, Beijing, China) according to the manufacturer's protocol using the Tianroot RNA extraction method, and then stored at –80°C for backup.

Expression analysis

For qRT-PCR expression analysis, RNA samples were reverse transcribed by using the TRAN Reverse Transcription System (Transgen Biotech, Beijing, China). Each qRT-PCR reaction contained a final volume of 20 μL including 1 μL cDNA template, 0.5 μL gene-specific primers (10 μM), 10 μL TransStart Tip Green qPCR Supermix (Transgen Biotech, Beijing, China), and 8 μL ddH2O. MN026864, an actin gene used in *Acer palmatum* research. It was selected as a reference gene to standardize the expression levels of *ArAux/IAA* target genes.[36] The PCR primers were designed outside the conserved region to produce amplification products with 100 to 200 bp. All primer sequences were listed in detail in (Table 1). The PCR parameters applied here were as follows: 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 15 s at 60°C. Finally, melting curve analysis was performed to verify the specificity of the primers. The relative expression level of the *A.rubrum* gene was determined by the $2^{-\triangle\triangle Ct}$ method. Values represent the mean calculated from 3 biological replicates and 3 technical replicates. Significance analysis of *ArAux/IAA* expression in different treatments using *t*-test and 1-way ANOVA for *ArAux/IAA* expression in different tissues. Heatmap representation was illustrated using expression value in GraphPad Prism 8.

Results

Identification of the Aux/IAA Gene family members in A.rubrum and A.yangbiense

An HMM-based search for *AUX/IAA* gene families in *A.rubrum* and *A.yangbiense* with Pfam accession PF02309 resulted in the identification of 70 genes and 68 with *Aux/IAA* domains. After careful verification, 32 and 40 genes with B3 and *ARF* domains were identified, confirming the deletion of the *ARF* gene family. A search of all identified genes in the UniProt database using megaBLAST further confirmed the identification and revealed that most of the top hits were Aux/IAA proteins from the *Arabidopsis* genomes. The domains of candidate genes were tested by SMART and InterProScan web servers. After manual screening and removal of variable splicing, 17 *A.rubrum* and 23 *A.yangbiense* genes were obtained. Identified genes were named from *ArAux/IAA*1 to *ArAux/IAA*17 and *AyAux/IAA*1 to *AyAux/IAA*23 based on the similarity of their domains to *Arabidopsis*. Information of these genes, including gene name, loucus Id, protein length, basic parameters of the deduced peptide and CELLO localization are detailed in Table 2. The sizes of the predicted ArAux/IAA proteins varied remarkably from 113 amino acids (*ArAux/IAA*12) to 379 amino acids (*ArAux/IAA*04) with an average of 258 amino acids and their predicted PI and molecular masses ranged from 4.77 (*ArAux/IAA*12) to 9.72 (*ArAux/IAA*17) and 12761.40 to 40855.23 Da, respectively. Subcellular localization predictions showed that all ArAux/IAA proteins were locate the nucleus. This prediction is consistent with the report that Aux/IAA proteins can inhibit the activation of downstream genes by ARFs in the absence of auxin. In the nature of these proteins, the *ArAux/IAA* family of encoded proteins appears to share many similarities with the *AyAux/IAA* family, predicted AyAux/IAA protein sizes ranged from 107 amino acids (*AyAux/IAA*4) to 378 amino acids (*AyAux/IAA*02), with a mean value of 250 differing little from the predicted *ArAux/IAA* protein size and little difference in predicted Mass (Da) and PI values.

Conservative domain, gene structure and chromosomal distribution analysis of ArAux/IAA and AyAux/IAA genes

Through HISAT2 splicing, we have a preliminary understanding of the possible chromosomal location and intron structure

Table 1. Specific primers used for RT-PCR in this study.

CODE	GENE NAMES		PRIMER SEQUENCES (5′→3′)	LENGTH OF AMPLIFIED FRAGMENT (BP)
1	*ArAux/IAA01*	F	TTAACTTGGAAGCGACAGAGC	144
		R	GTGTGGTGTTGGAATCGTCTC	
2	*ArAux/IAA02*	F	ACTGGATGCTAGTTGGAGATGT	104
		R	TCCACAAGAAGTCAAGCCTCT	
3	*ArAux/IAA03*	F	AACTCATTGGCAACCACTTCG	158
		R	TCAAGAGCAGAAGACAGTTCCT	
4	*ArAux/IAA04*	F	ACTGCTGCTTCTAACAACAACA	173
		R	CCATCCATGCTAACCTTGACAA	
5	*ArAux/IAA05*	F	GTGGTCTCCTCCTCTTCTTCTT	101
		R	GCTACAGAATCGGCACTCCT	
6	*ArAux/IAA06*	F	CTCCACCATCTCCTTCTTCTCA	197
		R	CACCACCACCACCAAGTAGA	
7	*ArAux/IAA07*	F	AACACCACCTTGACCACCAT	122
		R	AAAGTTCTCCAGGGCACATCT	
8	*ArAux/IAA08*	F	GCCAAGATGTTCAGTTCCTTCA	143
		R	ATCCAGTCACCATCCTTGTCTT	
9	*ArAux/IAA09*	F	CACCACTCGCTCGTCTTCT	190
		R	CACCACCACCTCCATTACCA	
10	*ArAux/IAA10*	F	CCAGCCAAGCCTCCTTCTAA	119
		R	ATGTTGTTGTTGCCACTCTCC	
11	*ArAux/IAA11*	F	TTACGAGGACAAGGATGGTGAT	147
		R	GTGCTTGAGTTCTTGGTTGGT	
12	*ArAux/IAA12*	F	ACGAGGACAACGAAGGAGAC	199
		R	AGCCAGACTCAGAAATCATTGC	
13	*ArAux/IAA13*	F	GGTGGTGCTTGTTTGTTGGA	115
		R	GGCTCTCATTCGTCTGGACT	
14	*ArAux/IAA14*	F	TGGAATGGTGAACAAGCAAGAG	105
		R	AGCATCCTGTCTCCTTCGTTAT	
15	*ArAux/IAA15*	F	TATGGAACTCTCATCGGCTCTT	112
		R	ATCCTTCAGACGACTCTCACTC	
16	*ArAux/IAA16*	F	CAATGTTCTGAGCCGAGATGG	102
		R	ATCCAGTCACCGTCCTTGTC	
17	*ArAux/IAA17*	F	CCAGCGTATCAGCCTCCATA	148
		R	GTGACCAGGAACAGCATTAGAG	

of the *ArAux/IAA* gene. Through comparing the cDNA sequences with the *A.yangbiense* genomic DNA sequences, Intronic, and exonic regions were identified for each *ArAux/IAA* gene validated by *A.yangbiense* gene gff file, the number and position of introns and exonic were revealed (Figure 2A).

For better analysis of the similarities and differences between *ArAux/IAA* and *AyAux/IAA*, the chromosomal location and intron structure of *AyAux/IAA* are also shown in Figure 2B. The *AyAux/IAA* and *ArAux/IAA* genes were distributed in chro1, 2, 3, 4, 5, 6, and 10 and most of them with 3 to 5 exons was conserved with single domain Aux/IAA. However, 6 genes *ArAux/IAA02, ArAux/IAA12, ArAux/IAA17, AyAux/IAA03, AyAux/IAA13*, and *AyAux/IAA23* were found had less exons and shorted sequences length than the average genes.

Table 2. Gene name, loucus ID, sequence length, molecular weight, theoretical isoelectric points (pI) and CELLO localization of *ArAux/IAAs* and *AyAux/IAAs.*

GENE	LOUCUS ID	LENGTH (AA)	PI	MASS (DA)	CELLO LOCALIZATION
ArAux/IAA01	MSTRG.6632.1	194	6.62	21527.32	Nuclear
ArAux/IAA02	MSTRG.859.1	184	5.96	20599.36	Nuclear
ArAux/IAA03	MSTRG.20621.1	361	6.96	39368.32	Nuclear
ArAux/IAA04	MSTRG.13561.1	379	7.53	40855.23	Nuclear
ArAux/IAA05	MSTRG.10030.1	277	6.31	29665.93	Nuclear
ArAux/IAA06	MSTRG.1050.1	293	9.1	31702.41	Nuclear
ArAux/IAA07	MSTRG.11555.1	298	8.72	31466.4	Nuclear
ArAux/IAA08	MSTRG.860.1	256	8.14	28129.19	Nuclear
ArAux/IAA09	MSTRG.6630.1	247	7.65	26921.59	Nuclear
ArAux/IAA10	MSTRG.9882.1	249	8.77	27537.56	Nuclear
ArAux/IAA11	MSTRG.3926.1	220	5.7	23816.92	Nuclear
ArAux/IAA12	MSTRG.12318.1	113	4.77	12761.4	Nuclear*
ArAux/IAA13	MSTRG.3923.1	186	5.35	20981.81	Nuclear
ArAux/IAA14	MSTRG.26400.1	314	9.36	34327.51	Nuclear
ArAux/IAA15	MSTRG.3740.2	353	8.53	37802.52	Nuclear
ArAux/IAA16	MSTRG.11773.1	282	8.73	30691.68	Nuclear
ArAux/IAA17	MSTRG.11318.1	173	9.72	19524.07	Nuclear
AyAux/IAA01	AcyanUnG0000400.1	361	6.96	39368.32	Nuclear*
AyAux/IAA02	Acyan10G0083700.1	378	6.13	40616.93	Nuclear
AyAux/IAA03	Acyan09G0169100.1	184	6.59	20608.21	Nuclear
AyAux/IAA04	Acyan06G0085500.1	107	6.27	12198.22	Nuclear
AyAux/IAA05	Acyan05G0135400.1	159	5.21	18060.98	Nuclear
AyAux/IAA06	Acyan05G0077200.1	158	5.21	17963.86	Nuclear
AyAux/IAA07	Acyan05G0069200.1	184	5.96	20599.36	Nuclear
AyAux/IAA08	Acyan05G0056600.1	236	5.79	26873.15	Nuclear
AyAux/IAA09	Acyan04G0158000.1	353	8.53	37802.52	Nuclear
AyAux/IAA10	Acyan04G0141800.1	282	8.73	30691.68	Nuclear
AyAux/IAA11	Acyan04G0141700.1	242	9.21	26000.4	Nuclear
AyAux/IAA12	Acyan04G0082500.1	337	9.15	36940.51	Nuclear
AyAux/IAA13	Acyan04G0082200.1	337	9.15	36940.51	Nuclear
AyAux/IAA14	Acyan03G0094100.1	186	5.35	20981.81	Nuclear
AyAux/IAA15	Acyan02G0109700.1	193	6.44	21889.91	Nuclear
AyAux/IAA16	Acyan02G0109600.1	259	6.84	28468.15	Nuclear
AyAux/IAA17	Acyan02G0084100.1	220	5.7	23816.92	Nuclear
AyAux/IAA18	Acyan02G0083400.1	201	8.32	22382.72	Nuclear
AyAux/IAA19	Acyan02G0068900.1	255	7.66	27647.3	Nuclear
AyAux/IAA20	Acyan01G0104800.1	264	8.77	29453.85	Nuclear
AyAux/IAA21	Acyan01G0078300.1	256	8.14	28129.19	Nuclear
AyAux/IAA22	Acyan01G0078200.1	298	8.72	31466.4	Nuclear
AyAux/IAA23	Acyan01G0010900.1	293	9.1	31702.41	Nuclear
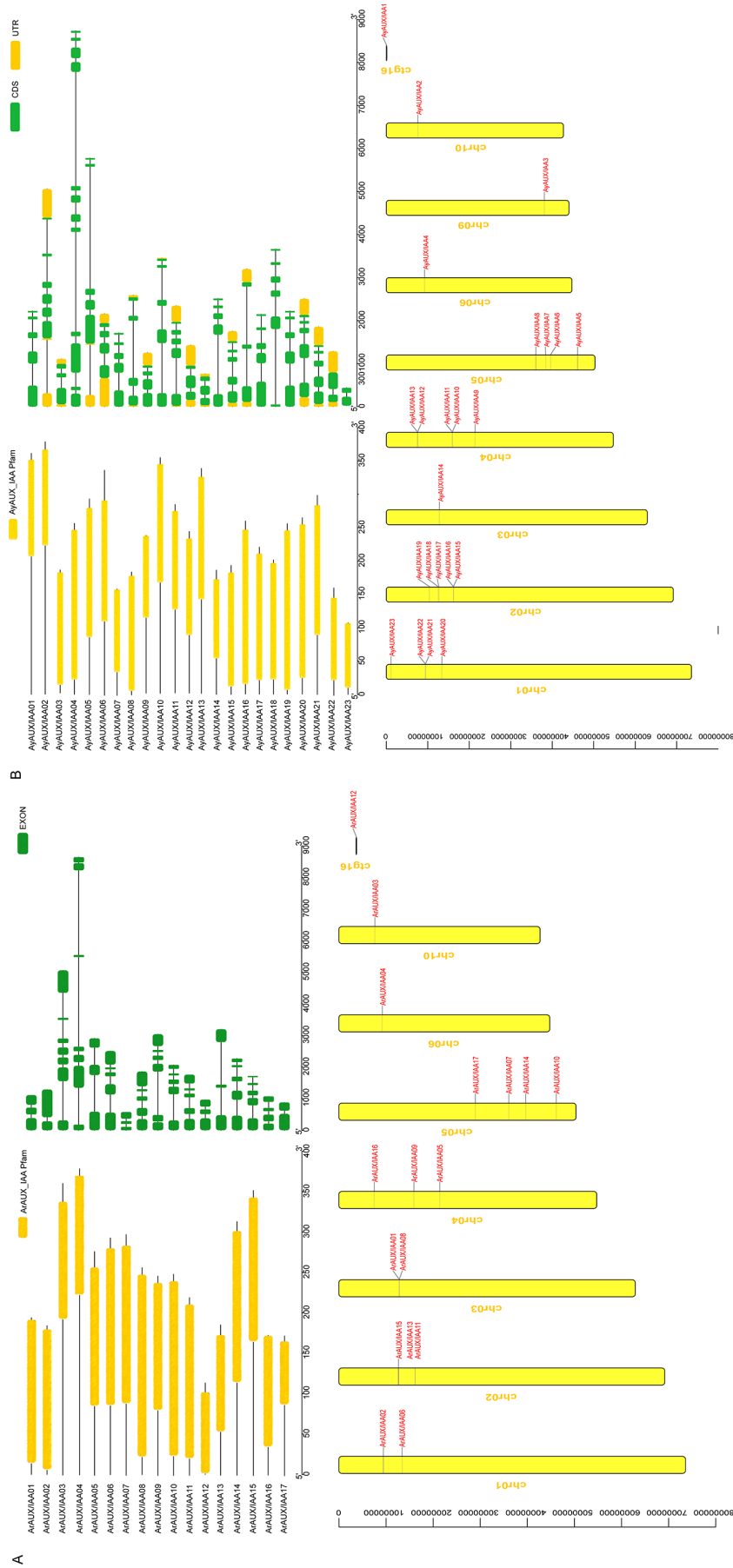
Figure 2. Conservative domain, gene structure and chromosomal distribution of *ArAux/IAA* (A) and *AyAux/IAA* (B) genes.
The pfam are shown in yellow, exon and CDS are shown in green, Chromosome numbers are listed next to the chromosome (yellow) and gene names are shown in red.

Because *ArAux/IAA* was identified at the transcriptomic level, the number of members was less than that of *A.yangbiense*, but the chromosomal location and intron structure were consistent with that of *A.yangbiense*.

Phylogeny and motif distribution of ArAux/IAA and AyAux/IAA proteins

Because there are different families and genera in the selected species, we selected amino acid sequences for phylogenetic tree construction, taking into account the low selection pressure on nucleic acids and the large number of mutations in the evolutionary process. Ninety-five amino acid sequences were aligned using Clustalx, including Aux/IAA encoded proteins of *A.rubrum, A.yangbiense, Arabidopsis*, and citrus and a phylogenetic tree was constructed using MEGA software (Figure 3A). The generated trees were divided into 4 groups, named ArA, ArB, ArC, and ArD according to their phylogeny. Both phylogenetic trees and conservative motif matched well with previously studied plants *Arabidopsis* and citrus[19,23] (Figure 3B). Group A contained the most members with 15 *A.rubrum* and 17 *A.yangbiense Aux/IAA* and all had conserved sequences of motifs 1, 2, 3, 4, and 5, and most contained motifs 6, 7, and 8. The B group contained 2 *AyAux/IAAs* and 3 *ArAUX/IAAs*, and excluding *AyAux/IAA*22, all other members of the Sapindales members had motifs of 1, 2, 3, and 10 and showed high conservatism. Whereas more *AyAux/IAA* (4) were classified into Group C than *ArAux/IAA* (1), and up to 6 members of the same Sapindales citrus were also classified into Group C. From the results of the phylogenetic tree, it is possible that there are Group C *ArAux/IAA* members that were not identified. motifs 1 and 3 were high frequency in group C, and there was also less variety in motifs than in the A and B groups. Group D has the lowest number of members and the lowest number of motifs of the 4 groups. Overall, groups A and B possess more intact proteins, while C and D truncated proteins predominate, although all members possess conserved domains of *Aux/IAA*, which can also be demonstrated on motifs.

Gene Ontology functional analysis of A.rubrum and A.yangbiense Aux/IAAs

Molecular Function (MF), Biological Processes (BP), and Cellular Components (CC) are the 3 main GO categories that help elucidate gene signatures and various functions of proteins.[37] The GO annotations of Aux/IAAs for 17 *A.rubrum* and 23 *A.yangbiense* were further investigated using Blast2GO software (Figure 4). Seventeen *ArAux/IAAs* are annotated to BP and CC, and 11 are annotated to MF, MF results showed 11 genes with DNA–binding transcription factor activity (GO:0003700) and transcription regulator activity (GO:0140110) related, indicating the *ArAux/IAAs* bind to *ARF* to regulate genes in nucleus (Figure 4A). It's worth noting that All genes are involved in the nucleus in CC

(GO:0005634), suggesting that *Aux/IAA* are localized in the nucleus, consistent with the prediction of subcellular localization (Table 1). Mover, the response to chemical (GO:0042221), response to endogenous stimulus (GO:0009719) and signal transduction (GO:0007165) were found in the BP category, suggesting these *ArAux/IAA* is closely related to auxin regulatory pathways and auxin hormone correspondence (Figure 4A). The go annotation results of *AyAux/IAA* are similar to those of *ArAux/IAA* (Figure 4B). Interestingly, on MF, *AyAux/IAA*, like *ArAux/IAA*, is only partially annotated to transcription regulator activity (*ArAUX/IAA* has 11, *AyAux/IAA* has 17), and these *Aux/IAA* in Phylogeny also exhibits proximity to similar characteristics (Figure 3).

qRT-PCR Quantification of ArAux/IAA genes in different tissues in response to IAA treatment

To understand the spatial pattern of *ArAux/IAA* gene expression, transcript accumulation in young leaves (YL), mature leaves (ML) and phloem (P) was assessed under IAA (300 mg/L) treatment and clear water (CK) treatment. The expression pattern was studied by qRT-PCR for 17 expressed *ArAux/IAA* genes. The results showed that the genes *ArAux/IAA*1-7, *ArAux/IAA*11, and *ArAux/IAA*12 responded positively to IAA treatment in different tissues (Figure 5).

For most *ArAux/IAA* genes, the highest expression levels are found in phloem, which is known to give the tissue an important role in the polar transport of auxin. Some *ArAux/IAA* genes showed significant preferential expression in specific tissues, such as *ArAux/IAA3* and *ArAux/IAA4* showed high expression in mature leaves and *ArAux/IAA1* showed the highest expression in the phloem. Tissue-preferential expression of *Aux/IAA* genes may indicate distinct roles in specific plant tissues and developmental processes.

The first *Aux/IAA* gene was identified because of its rapid response to growth hormone induction. All of the *ArAux/IAAs* tested, except (*ArAux/IAA 8-10, ArAux/IAA13-17*) showed that their transcript accumulation was positively regulated by auxin. The data indicated that transcript accumulation of 9 *ArAux/IAAs* genes was significantly enhanced. Interestingly, there were genes whose expression accumulation is upregulated differently depending on the tissue, for example, *ArAux/IAA3, ArAux/IAA4*, and *ArAux/IAA7* genes are sensitive to auxin in mature leaves and phloem, while young leaves do not respond. These data suggest in addition to being major molecular in the auxin response, the expression of some *Aux/IAAs* is influenced by unexplored factors in addition to being induced by auxin and thus involved in plant developmental processes.

Discussion

Aux/IAAs function in various biological processes in plants including growth and development processes, via bind with *ARFs* and prevent activation of auxin-responsive genes.[10,38] Functional analysis and expression profiling of *AUX/IAA* helps
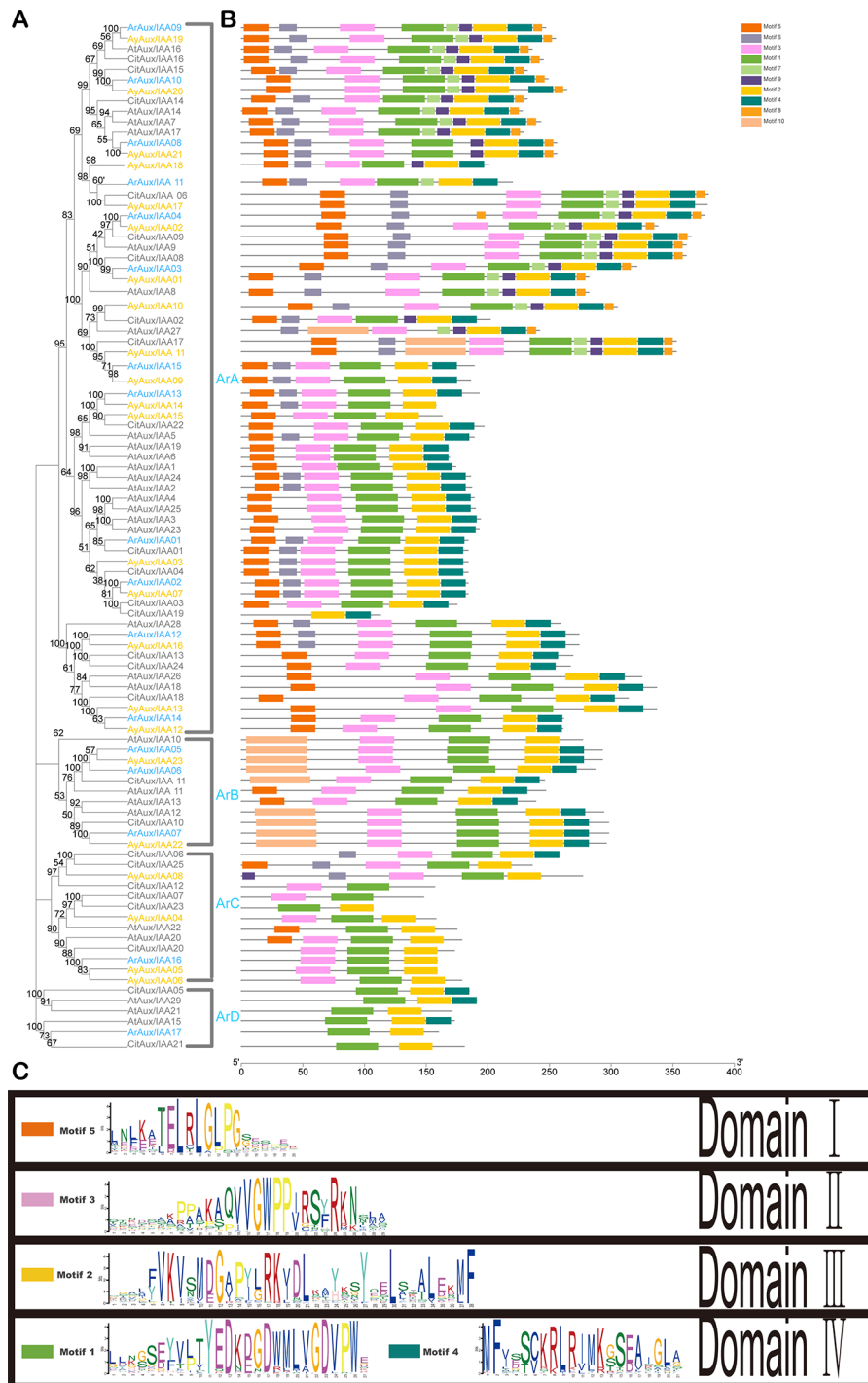
Figure 3. Neighboring trees (NJ) and representative conserved motif patterns of Aux/IAA proteins of *A.rubrum, A.yangbiense*, citrus, and *Arabidopsis*: (A) a phylogenetic tree was constructed for 95 full-length Aux/IAA proteins from 5 plant species, including *A.rubrum* (Ar), *A.yangbiense* (Ay), citrus (Cit), *Arabidopsis* (At), (B) distribution of Aux/IAA proteins of 10 motifs in 4 species, and (C) 5 motifs representing 4 domains I, II, III, and IV were mapped on all Aux/IAA proteins by different colors.

to reveal how auxin regulates plant growth and development in vivo and responds to environmental changes in spatio-temporal specific ways. With the advent of genome sequencing technology, the *AUX/IAA* gene family has been identified by whole-genome analysis in more than 30 species of plants, including 29 genes from *Arabidopsis*, 35 from *Populus trichocarpa*, 26 from Citrus.[19,23,24] The identification of members of the

ArAUX/IAAs described here provides new insights into the changes that have occurred during the evolution of the *AUX/IAA* gene. And based on this, through the identification of *AyAux/IAA* genes at the genome level of the *A.yangbiense*, which is traditionally taxonomically closely related to the *A.rubrum*, we found that both the molecular structure and the phylogeny are highly related to *ArAUX/IAA*. The *AUX/IAA*
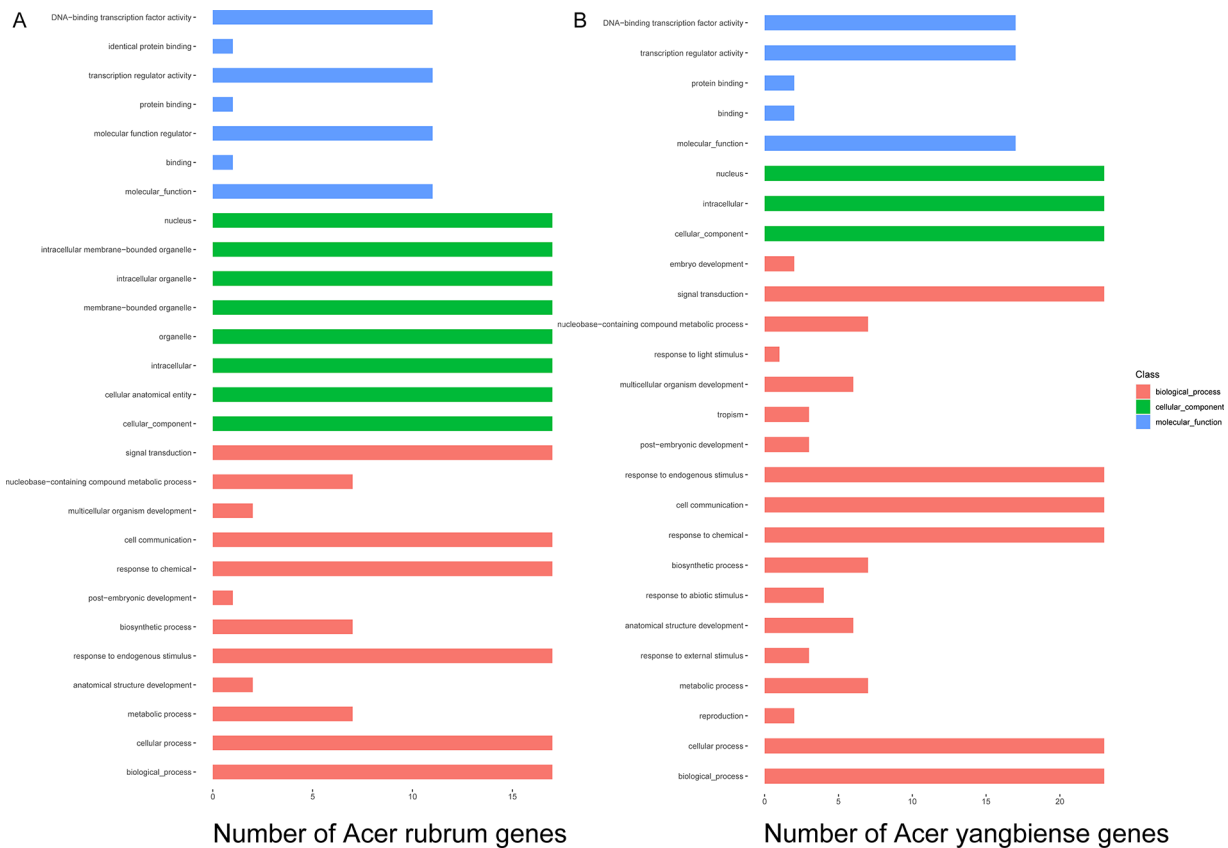
Figure 4. Gene Ontology (GO) analysis of *Aux/IAA* genes in *A.rubrum* (A) and *A.yangbiense* (B).
CC: MF: molecular function (blue); cellular component (green); BP: biological process (red).

family of genes is slightly contracted in *A.yangbiense* compared to *Arabidopsis* (29 genes) and Citrus (26 genes).[39] Consistent evolutionary trends for ArAux/IAA, AyAux/IAA, and CitAux/IAA, based on the phylogenetic results, suggesting that the *Aux/IAA* of these 3 plants originated from a single ancestor. The number of gene members showed that *AyAux/IAA* and *ArAux/IAA* members were less than AtAux/IAA. The evolutionary tree analysis revealed that the number of Aux/IAA members had a specific preference in evolution, with the 4 species in clade ArA having the most *Aux/IAA* members and the highest proportion; in clade ArB, the degree of member expansion was not significant; in clade ArC, compared to *AtAux/IAA* (2 genes) and *ArAu/IxAA* (1 gene), *AyAux/IAA* (4 genes) and *CitAux/IAA* (6 genes) members were expanded to a small extent; in clade ArD, no *AyAux/IAA* members were found.[21,24] Few *Aux/IAA* genes were identified in lower plants, such as *Marchantia polymorpha* (1 gene), and *Physcomitrella patens* (3 genes), *Selaginella moellendorffii* (9 genes), compared to higher plants.[39-41] Thus, the amplification of *Aux/IAA* gene members in higher plants, on the 1 hand, creates functional redundancy and, on the other hand, appears to be associated with other new functions in order to adapt to changes in the environment.[14,42] *A.yangbiense*, the first *Acer* species to have a high quality genome, has a similar whole-genome duplication (WGD) to that of grapes, with no recent WGD events, and is an endangered species only found in Yangbi County in Yunnan Province

in Southwest China, this unique geography has created species isolation, allowing limited genetic exchange.[27,43] This also explains why there are fewer *Aux/IAA* members for *A.rubrum* compared to *Arabidopsis* and citrus. Although the genome of the *A.yangbiense* provides a high quality reference for the Acer, we are currently unable to identify the full *ArAux/IAA* family of genes through the transcriptome because of the spatio-temporally specific expression of genes. However, the 17 *ArAux/IAAs* identified were analyzed for evolutionary tree, motif, and expression, and the results showed some representativeness.

The conserved sequences and structural domains of the *Aux/IAA* genes involved in the construction of the tree were investigated (Figure 3B, C). The diversity of differentiated branches is also reflected in the variation of conserved structures of Aux/IAA proteins. Most Aux/IAA proteins possess 4 conserved signature domains: Domain I, II, III, and IV.[6] Domain I has been identified as an inhibitory domain with the ethylene response factor (ERF)-related amphiphilic repression (EAR) motif "LxLxL," which recruits a TOPLESS (TPL) co-repressor.[44] Domain II is an auxin degron with a conserved "GWPPV" motif, which can interact directly with SCFTIR1 and is closely related to TIR1-mediated ubiquitination.[45,46] Domain III contains a 2-sided βαα-fold that is similar in structure and function to the DNA recognition motif.[47] Recent studies have confirmed the role of the βαα-fold in homo- and heterodimerization with Aux/IAA or with ARF proteins.[6,48]
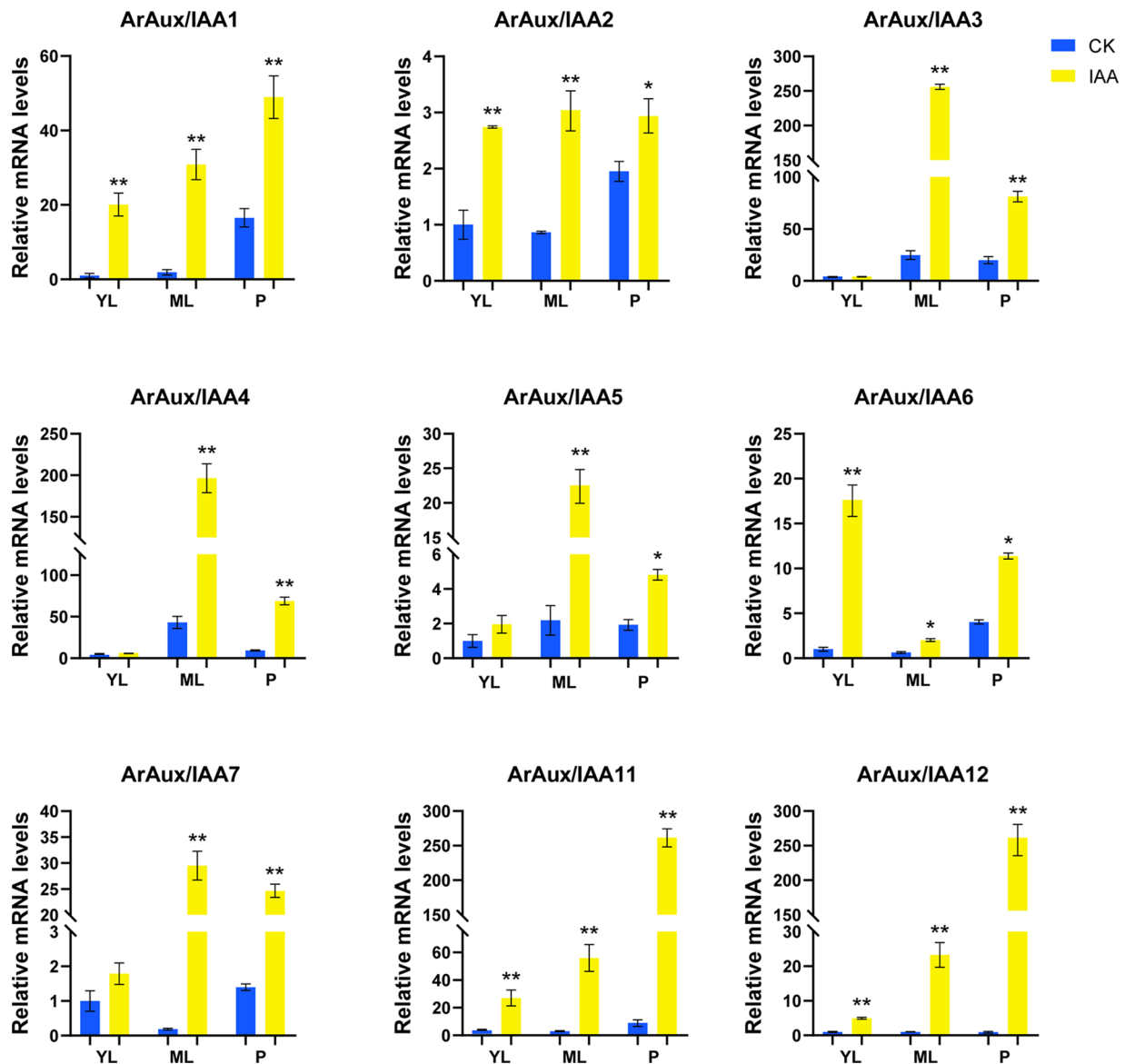
Figure 5. Expression profiles of *ArAux/IAA* in new leaves (YL), mature leaves (ML), and phloem (P). Clear water treatment (CK) is shown in blue, IAA treatment (IAA) in yellow, genes that were not significantly different did not show.
The expression patterns were revealed by qRT-PCR. Biological triplicates were averaged and analyzed statistically using a *t*-test (Differences between treatments,*$P < .05$, **$P < .01$). Bars indicate the SD of the 3 experimental repetitions.

Domain IV contains a conserved motif "GDVP" between β4 and α2, the motif contributes to the electrostatic interaction of proteins.[48,49] The Domain III and Domain IV together form type I/II Phox and Bem1p (PB1) domains.[5] The clade ArA has the largest number of *Aux/IAA* members and also the most intact structural domain, and the structural integrity suggest that these proteins are involved in the *Aux/IAA* functional model that is degraded under the regulation of auxin. The clade ArB has 3 complete structural domains and a longer conserved sequence (motif10) (Except *AtAux/IAA11, AtAux/IAA13*). Most of clade ArC *Aux/IAA* genes lacked domain1 (except *CitAux/IAA25, AtAux/IAA20, AtAux/IAA 22*), and also *Aux/IAA* lacked domain2 (*AtAux/IAA 22, CitAux/IAA23*). The clade ArA has the least number of members and is missing Domain1 and 2. The close association of *AtAux/IAA18, AtAux/*

IAA 26, and *AtAux/IAA 28* in the clade ArD with the bryophyte Aux/IAA implies that the formation of the clade ArA may be traced back to plant origins, and that the evolution of additional members of *A.rubrum* and *A.yangbiense* over a long period of evolution has led to the expansion of clade ArD membership, and that this trend is present in all 4 species involved in the composition of the phylogenetic tree.[50] Whereas group D lacks Domains 1 and 2 (motifs 5 and 3), such incomplete Domains are common in the evolution of A*ux*/IAA proteins, such as the 3 A*ux*/IAA proteins in tomato (*SIAux/IAA*13, *SIAux/IAA*16, *SIAux/IAA*20) and the 5 Aux/IAA proteins in potato (*StAux/IAA13, StAux/IAA15, StAux/IAA16, StAux/IAA18*, and *StAux/IAA*20) were all found to be deficient in domains I and II.[39,51] 70.6% of *ArAux/IAA* (12) and 73.9% of *AyAux/IAA* (17) had complete conserved signature domains. In

contrast to *AyAux/IAA*, the simultaneous absence of Domain I and Domain II was found in *ArAux/IAA17* that did not show differential expression under IAA treatment. This lack of specific response of *Aux/IAA* to IAA because of a Domain deficiency has also been found in citrus (*CitAux/IAA 24, 25*)[23] and *ArAux/IAA17* may have a specific function in the *A.rubrum*. The absence of this domain may allow for a more diverse role for *Aux/IAA* in the axuin signaling pathway and the response of *Aux/IAA* to changes in the environment in which the plant is located.

Using the YN model to calculate the Ks values,[52] the dot plot shows that the longer syntenic blocks between *A.yangbiense* and grape are nearly 1:1, indicating that *A.yangbiense* has a similar evolutionary history to grape and has not undergoing a WGD event after the core eudicot common hexaploidization.[27] Additionally, 11 homologous pairs(*ArAux/IAA9/AyAux/IAA19, ArAux/IAA10/AyAUX/IAA20, ArAUX/IAA8/AyAUX/IAA21, ArAUX/IAA4/AyAUX/IAA2, ArAUX/IAA15/AyAUX/IAA9, ArAUX/IAA3/AyAUX/IAA1, ArAUX/IAA13/AyAUX/IAA14, ArAUX/IAA2/AyAUX/IAA7, ArAUX/IAA14/AyAUX/IAA12, ArAUX/IAA5/AyAUX/IAA23*, and *ArAUX/IAA7/AyAUX/IAA22*) were found which had close evolutionary relationships similar gene structures. More than half of the homologous pairs and overlapping chromosomal distribution mean that the evolutionary history of *A.rubrum* and *A.yangbiense* is similar. The presence of these homologous pairs in different species suggests that they evolved from a common ancestor and are highly conserved in evolution. To our knowledge, few functional analyses of *AUX/IAA* in the genus Acer have been performed. Therefore, this gene family deserves continued future research to investigate the potential specific functions of the *AUX/IAA* family that diverged during evolution in the *Acer*.

The expression pattern of *ArAUX/IAA* in young leaves, mature leaves, and phloem under IAA treatment was analyzed. In general, there is tissue-specific expression of *AUX/IAA* in other species studied, and there is an increase in expression with IAA treatment.[23,53,54] This study found that some of the *ArAUX//IAA* expression patterns were different in mature and young leaves, and that the expression of *ArAXU/IAA* in mature leaves was significantly higher than that in young leaves under IAA treatment. The *ArAUX/IAA* gene exhibits a different expression pattern in response to exogenous auxin. Interestingly, 9 *ArAUX/IAA*, 4 *ArAUX/IAA*, and 8 *ArAUX/IAA* genes in the bast were significantly increased in expression with IAA treatment, while the expression of the remaining *ArAUX/IAA* did not change significantly. The dynamics of the *ArAUX/IAA* family of genes under IAA treatment suggests that different *ArAUX/IAA* genes are involved in the changes in auxin regulation. Notably, *ArAux/IAA16* with absence of Domain 1 and *ArAux/IAA17* with adsence of Domain 1 and 2 were consistently expressed at low levels under Auxin. This atypical *Aux/IAA* whose expression pattern is also highly restricted in other species,[19,23,51] and this atypical *ArAUX/IAA* gene may have a

specific function in auxin-mediated plant development that needs to be investigated subsequently

Conclusions

In this study, 17 *ArAux/IAA* and 23 *AyAux/IAA* were identified in *Acer yangbiense* and *Acer rubrum*. Most *ArAux/IAA* genes can be expressed in at least 1 tissue, and some genes show tissue specificity and increased expression under IAA treatment. We found 2 *ArAux/IAAs* that responded to exogenous IAA treatment in mature leaves but not in young leaves, which likely implies that these 2 genes are specifically involved in the auxin regulation. The next step of identification and functional analysis of the auxion responsive elements of these *Aux/IAA* gene promoters will facilitate the explain of their transcriptional changes and their biological functions. In addition, our work reveals that *AyAux/IAA* and *ArAux/IAA* are highly conserved in evolution, with 11 pairs of genes that are highly consistent in conserved sequence and chromosomal localization, and these genes are also highly inbred with *CitAux/IAA* by phylogeny analysis. Although *AyAux/IAA* and *ArAux/IAA* require further analysis, the results on gene structure and gene expression levels studied here will help to accelerate a deeper study of *Aux/IAA* function in *Acer* species.

Author contributions

Kezhong Zhang, Wei Ge and Hewen Zhao designed the study; Manyu Zhang and Jianyi Li collected and prepared the materials; Wenpeng Zhu conducted the experiments and data analysis. Wenpeng Zhu wrote the manuscript; Wei Ge revised the manuscript. All authors read and approved the final draft.

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