Identification and Analysis of Aux/IAA Family in Acer rubrum

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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 (AuxRE), of which at least one conserved motif (TGTCGTG) is present at high frequencies.8 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.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 protease10-12 (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 napus, 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
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. 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, and ArAux/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-extension 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 Ar 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. MEME was used to find the conserved motif of Ar Aux/IAA proteins. The candidate Aux/IAA proteins were further examined to confirm the presence of Aux/IAA repeats using Pfam and SMART software. The image data were displayed in Tbtools.

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 20µL including 1µL 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. 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 Arrubum gene was determined by the 2−ΔΔCt 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 Arrubum and 23 A. yangbiense genes were obtained. Identified genes were named from ArAux/IAA1 to ArAux/IAA17 and AyAux/IAA1 to AyAux/IAA23 based on the similarity of their domains to Arabidopsis. Information of these genes, including gene name, locus 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/IAA12) to 379 amino acids (ArAux/IAA04) with an average of 258 amino acids and their predicted PI and molecular masses ranged from 4.77 (ArAux/IAA12) to 9.72 (ArAux/IAA17) 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/IAA4) to 378 amino acids (AyAux/IAA02), 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
of the *ArAux/IAA* gene. Through comparing the cDNA sequences with the *Ayangbiense* genomic DNA sequences, Intronic, and exonic regions were identified for each *ArAux/IAA* gene validated by the *Ayangbiense* 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 shorter sequences length than the average genes.

### 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: TTAACCTTGGAAGCGACAGAGC                                      | 144                              |
|      |                | R: GTTGTTGTTGGAATCGCTTC                                      |                                  |
| 2    | *ArAux/IAA02*  | F: ACTGGATGCTAGTTGGAGATGT                                      | 104                              |
|      |                | R: TCCACAAGAAATCGACTCTC                                      |                                  |
| 3    | *ArAux/IAA03*  | F: AAATCTTGGCAACCCACTTCG                                      | 158                              |
|      |                | R: TCAAGAGCAGAACACGAGTTC                                      |                                  |
| 4    | *ArAux/IAA04*  | F: ACTGCTGCTTCTCTAACAACACAA                                    | 173                              |
|      |                | R: CCATCCATGCTACCTGTTAGAC                                     |                                  |
| 5    | *ArAux/IAA05*  | F: GTGGTCTCCTCCTCCTCTCTCCTCTCT                                 | 101                              |
|      |                | R: GCTACAGAATCGGACTCTC                                        |                                  |
| 6    | *ArAux/IAA06*  | F: CTCACCACATCCTCCTCCTCCTC                                    | 197                              |
|      |                | R: CACCCAACCCACCCAGATG                                        |                                  |
| 7    | *ArAux/IAA07*  | F: AACACCACCTTGGACCCACAT                                       | 122                              |
|      |                | R: AAAGTTCTCCAGGAGCACAC                                        |                                  |
| 8    | *ArAux/IAA08*  | F: GCCAAGATGGTTCAGTTCTCCTC                                     | 143                              |
|      |                | R: ATCCAGTCCATCCTTCATTC                                        |                                  |
| 9    | *ArAux/IAA09*  | F: CACCACCTACCGCTGGTCTT                                        | 190                              |
|      |                | R: CACCCAACCCACCTACCC                                          |                                  |
| 10   | *ArAux/IAA10*  | F: CCAGCCAACGCTGCTCCTAA                                        | 119                              |
|      |                | R: ATGTGGTTGCTGCCACTCTCC                                       |                                  |
| 11   | *ArAux/IAA11*  | F: TTACGAGGACAAGGATGGGTG                                        | 147                              |
|      |                | R: GTGCTAGTTGCTGGTGGT                                          |                                  |
| 12   | *ArAux/IAA12*  | F: ACGAGGACAACGAGAGAGG                                         | 199                              |
|      |                | R: AGCCAGACTCAGAAATATTG                                        |                                  |
| 13   | *ArAux/IAA13*  | F: GGCTGGTCTGTTGGGTGGA                                        | 115                              |
|      |                | R: GGTCTCATTCTGCTGGGACT                                       |                                  |
| 14   | *ArAux/IAA14*  | F: TGGAATGGTGGACAAAGCAAGG                                     | 105                              |
|      |                | R: AGCATCCGTGCTCTGGTTAGT                                       |                                  |
| 15   | *ArAux/IAA15*  | F: TATGGAACTCCTCATGGCCTCTCTC                                   | 112                              |
|      |                | R: ATCCCTACGAGCTGACCTC                                          |                                  |
| 16   | *ArAux/IAA16*  | F: CAATGTTCTGAGACGGAGATG                                       | 102                              |
|      |                | R: ATCCAGGTCACGCCTGGTGGG                                       |                                  |
| 17   | *ArAux/IAA17*  | F: CCAGCGATACGCGCTCCTCATA                                     | 148                              |
|      |                | R: GTGAGCCAAGAAGGATGGG                                         |                                  |
Table 2. Gene name, locus 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 *AyAux/IAA*, but the chromosomal location and intron structure were consistent with that of *AyAux/IAA*.

**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 *Arabidopsis, A. yangbiense*, 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 citrus37 (Figure 3B). Group A contained the most members with 15 *Arubrum* 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/IAA22*, 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* were 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/IAA* 3 and *ArAux/IAA* 4 showed high expression in mature leaves and *ArAux/IAA* 1 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/IAA* tested, except (*ArAux/IAA* 8-10, *ArAux/IAA* 13-17) showed that their transcript accumulation was positively regulated by auxin. The data indicated that transcript accumulation of 9 *ArAux/IAA* genes was significantly enhanced. Interestingly, there were genes whose expression accumulation is upregulated differently depending on the tissue, for example, *ArAux/IAA* 3, *ArAux/IAA* 4, and *ArAux/IAA* 7 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/IAA* is influenced by unexplored factors in addition to being induced by auxin and thus involved in plant developmental processes.

**Discussion**

*Aux/IAA* function in various biological processes in plants including growth and development processes, via bind with ARF5 and prevent activation of auxin-responsive genes.10,38 Functional analysis and expression profiling of *Aux/IAA* helps
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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.\textsuperscript{19,23,24} The identification of members of the \textit{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 \textit{AyAux/IAA} genes at the genome level of the \textit{A.yangbiense}, which is traditionally taxonomically closely related to the \textit{A.rubrum}, we found that both the molecular structure and the phylogeny are highly related to \textit{ArAUX/IAA}. The AUX/IAA
family of genes is slightly contracted in A. yangbiense compared to Arabidopsis (29 genes) and Citrus (26 genes). 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 ArAux/IAA (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. 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. 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. 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. 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. Domain I has been identified as an inhibitory domain with the ethylene response factor (ERF)-related amphiphilic repression (EAR) motif “LxLxL,” which recruits a TOPELESS (TPL) co-repressor. 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. Domain III contains a 2-sided βαα-fold that is similar in structure and function to the DNA recognition motif. Recent studies have confirmed the role of the βαα-fold in homo- and heterodimerization with Aux/IAA or with ARF proteins.
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. 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/IAA22), and also Aux/IAA lacked domain2 (AtAux/IAA22, CitAux/IAA23). The clade ArA has the least number of members and is missing Domain1 and 2. The close association of AtAux/IAA18, AtAux/IAA26, and AtAux/IAA28 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 Aux/IAA proteins, such as the 3 Aux/IAA proteins in tomato (StAux/IAA13, StAux/IAA16, StAux/IAA20) and the 5 Aux/IAA proteins in potato (StAux/IAA13, StAux/IAA15, StAux/IAA16, StAux/IAA18, and StAux/IAA20) were all found to be deficient in domains I and II.49,51 70.6% of ArAux/IAA (12) and 73.9% of AyAux/IAA (17) had complete conserved signature domains. In

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.
contrast to ArAux/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 Ar.rubrum. The absence of this domain may allow for a more diverse role for Aux/IAA in the auxin 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,22 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 undergone a WGD event after the core eudicot common hexaploidization. Additionally, 11 homologous pairs (ArAux/IAA9/AyAux/ IAA19, ArAux/IAA10/AyAux/IAA20, ArAux/IAA48/AyAux/ IAA21, ArAux/IAA44/AyAux/IAA22, ArAux/IAA15/AyAux/ IAA9, ArAux/IAA33/AyAux/IAA1, ArAux/IAA3/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 Ar.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.25,33,34 This study found that some of the ArAux/IAA expression patterns were different in mature and young leaves, and that the expression of ArAux/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 last 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 absence 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,19,25,51 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 A. yangbiense and A. 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 auxin 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 A. rubrum species.

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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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