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Global Analysis of the WOX Transcription Factor Family in *Akebia trifoliata*

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Abstract: *Akebia trifoliata* is an economically important self-incompatible fruit tree in the family Lardizabalaceae. Asexual propagation is the main strategy used to maintain excellent agronomic traits. However, the generation of adventitious roots during asexual propagation is very difficult. To study the important role of the WUSCHEL-related homeobox (WOX) transcription factor in fruit and adventitious root growth and development, we characterized this transcription factor family in the whole genome of *A. trifoliata*. A total of 10 *AktWOXs* were identified, with the following characteristics: length (657~11328 bp), exon number (2~5), isoelectric point (5.65~9.03), amino acid number (176~361 AA), and molecular weight (20.500~40.173 kDa). 10 *AktWOXs* were classified into modern (6), intermediate (2), and ancient clades (2) and that all *AktWOXs* had undergone strong purifying selection during evolution. The expression profile of *AktWOXs* during *A. trifoliata* fruit development and adventitious root formation indicated that *AktWOXs* are widely involved in the development of the three fruit tissues, flesh, seeds and rind, and play an important role in the regulation of adventitious root development. Overall, this is the first study to identify and characterize the *WOX* family in *A. trifoliata* and will be helpful for further research on *A. trifoliata* fruit development and adventitious root formation.

Keywords: *Akebia trifoliata*; WUSCHEL-related homeobox; transcription factor; adventitious roots

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1. Introduction

Akebia trifoliata (Thunb.) Koidz. (2n=2x=32) belongs to the flowering plant family Lardizabalaceae **Error! Reference source not found.**. As the third generation of emerging fruit, the flesh of *A. trifoliata*, which is deeply loved by people, not only has a delicate texture and sweet taste but also contains many free essential amino acids[2]. Therefore, the artificial cultivation of *A. trifoliata* has been rapidly increasing in Southwest China and the middle and lower reaches of the Yangtze River in recent years. However, *A. trifoliata* breeding techniques are not yet able to meet the needs of farmers because *A. trifoliata* is a cross-pollinated plant and good maternal traits can only be maintained through asexual reproduction [3].

To date, research on asexual breeding methods for *A. trifoliata* has mainly focused on tissue culture breeding and cutting breeding. In the exploration of tissue culture, Wu et al. (2015) established and optimized an efficient callus culture system using leaves as explants and established a method for the rapid propagation of stems with leaf buds, with induction and rooting rates of more than 80% in the optimized medium [4]. This method can shorten the seedling cycle of seedlings. However, since the formation of endogenous toxins and adventitious roots of *A. trifoliata* is hard to achieve, it is still difficult to establish a complete tissue culture system of *A. trifoliata*[5]. The method of culturing cuttings of *A. trifoliata* has also attracted the attention of researchers. Studies have shown that fine river sand + nutrient soil is the preferred medium for cuttings of *A. trifoliata*[6]. In addition, plant growth regulators are also used to promote the growth of *A. trifoliata* roots. There is a report showing that ABT2 rooting- powder treatment can effectively promote the rooting of *A. trifoliata* cuttings [7]. In China, the *A. trifoliata* cutting system has slowly begun to mature. The unstable roots produced by cuttings and tissue culture are liable to fall off and do not easily survive field transplantation. The root system plays a crucial role in the growth and development of the whole plant. Therefore, the study of the formation and development process of adventitious roots helps us to obtain high-quality saplings of *A. trifoliata*.

The formation of adventitious roots is one of the key steps of plant asexual propagation [8], and the WUSCHEL-related homeobox (WOX) transcription factors widely present in plant genomes have been shown to be involved in the regulation of adventitious root formation [9]. For example, in *Arabidopsis thaliana*, *WOX11* and *WOX12* respond to auxin induction and then activate the expression of *WOX5* and *WOX7* to change the cell fate from root invasive cells to root primordium cells and achieve adventitious root regeneration [9,10]; in *Oryza sativa*, *OsWOX3A* leads to an increase in plant lateral root number, indicating that *OsWOX3A* may be involved in the regulation of GA-IAA crosstalk in rice root development 11; in the gymnosperms *Picea-Abies* and *Populus nigra*, *PsWOX3* is expressed in a few cells on the peripheral surface of the shoot apical meristem, and *PaWOX3* is highly expressed in the root tip [12,13]. Overexpression of *MdWOX11* promotes adventive root primordium formation in apple, while interference of *MdWOX11* inhibits adventive root primordium production 1414. Therefore, some members of the WOX transcription factor family play important roles in the growth and development of adventitious roots.

At present, genome-wide identification of the WOX transcription factor family has been completed in many plants. The WOX family is a group of plant-specific transcription factors and belongs to the homeobox (HB) transcription factor family. The typical homeodomain (HD) of the HB superfamily has 60-66 amino acid residues that fold into a "helix−loop−helix−turn−helix" spatial structure, where a combination of the second and third helices forms a "helix−turn−helix" that can bind to specific DNA sequences 15. *WUSCHEL (WUS)* is the most primitive gene of the WOX transcription factor family. In 2004, Haecker et al. identified 14 other members with similar structures by using homology search methods for *A. thaliana WUS* genes 16. According to phylogenetic tree analysis, it can be divided into three clades: the first clade is the modern/WUS clade (*WUS, AtWOX1-AtWOX7*), which exists in higher plants; the second clade is the intermediate clade (*AtWOX8, AtWOX9, AtWOX11* and *AtWOX12*), which originates from tracheophytes; and the third clade is the ancient clade (*AtWOX10, AtWOX13* and *AtWOX14*), which originated from phycophyta [17,18]. The ancient origin of the WOX transcription factor and other evolutionary branches derived from plant evolution suggest that this gene family is essential for plant survival.

In the present study, we comprehensively identified the *WOX* genes from the *A. trifoliata* genome. We first determined the *AktWOX* gene structures, motif compositions, and chromosomal distributions. Furthermore, we analyzed the phylogenetic relationships and evolutionary patterns of the *AktWOXs*. In addition, the expression patterns of *AktWOXs* in different *A. trifoliata* tissues and under different AR formation conditions were determined. Our results provide insights for further understanding *WOX* family genes in *A. trifoliata*, clarify their evolutionary history, and facilitate their application in gene transformation for improving plants.

2. Results

2.1. Systemic Characterization of the WOX Gene Family in A. trifoliata

A total of 10 *WOX* genes were identified from the *A. trifoliata* genome through HMM analysis. They were sequentially named *AktWOX1–9* and *AktWUS* (chromosome 2) (Table 1) according to their positions on the chromosome 19. The 10 *AktWOXs* had a wide range in gene length (from 657 bp to 11328 bp) and exon number (from 2 to 5). In terms of protein properties, the 10 *AktWOXs* had obvious differences in amino acid length (from 176 to 361), molecular weight (from 20.500 to 40.173), and isoelectric point (from 5.65 to 9.03). Subcellular localization analysis showed that these proteins were spatially located in the nucleus but had no obvious signal peptide signature.

The secondary structure of 10 *AktWOX* proteins was predicted and analyzed (Table S1). The α-helical structure and β-folded structure are ordered structures of proteins that have high stability, and random curling is a disordered structure of proteins. The results showed that 10 *AktWOX* proteins were mainly randomly curled, accounting for 56.28% to 71.75% of the secondary structure, followed by α helices. This indicated that the protein secondary structure of the *AktWOX* family genes was unstable as a whole. The instability coefficient of the proteins in this family was greater than 40, and the hydrophilicity value was less than 0, indicating that they were poorly stable and hydrophilic proteins.

Table 1. Characteristics of the identified *WOX* gene family members from the *A. trifoliata* genome.

WOX genes	Gene length	Chromosome location			Exon	Cell location	Putative protein					
							Length AA	MW (kDa)	PI	Instability index	Hydrophilic	Signal peptide
AktWOX1	**2840**	**chr1**	**25213082**	**25215922**	**5**	Nucleus	361	40.173	6.8	57.89	-0.593	0.0009
AktWOX2	5392	chr2	896118	901510	3	Nucleus	279	31.869	6.02	59.79	-0.946	0.0005
AktWOX3	2204	chr2	7184275	7186479	3	Nucleus	212	24.462	9.03	52.78	-1.06	0.0023
AktWOX4	11328	chr3	50099349	50110677	3	Nucleus	307	35.238	5.65	65.16	-0.927	0.0003
AktWOX5	796	chr6	6820221	6821017	2	Nucleus	232	26.506	8.42	51.72	-0.919	0.0006
AktWOX6	1120	chr8	2341031	2342151	2	Nucleus	198	23.316	8.75	61.48	-0.891	0.0003
AktWOX7	1950	chr9	5460894	5462844	4	Nucleus	316	36.673	8.44	75.25	-0.949	0.015
AktWOX8	2212	chr15	429281	431493	3	Nucleus	265	29.411	5.85	60.72	-0.317	0.0007
AktWOX9	657	chr15	27705270	27705927	2	Nucleus	176	20.500	8.85	53.15	-0.903	0.0044
AktWUS	1622	chr2	2976634	2978256	3	Nucleus	267	29.505	6.83	60.26	-0.788	0.0012

AA, amino acids; PI, isoelectric point; MW, molecular weight. "Instability index" >40 means unstable; "hydrophilicity" <0 is hydrophilic, and >0 is hydrophobic.

2.2. Phylogenetic Analysis of AktWOX

A phylogenetic tree of the WOX protein family was constructed based on the amino acid sequences of 39 WOX proteins from *A. trifoliata* (10), *O. sativa* (14) and *A. thaliana* (15). According to the evolutionary tree, the 39 WOX proteins were divided into three main branches, and the 10 AktWOXs of *A. trifoliata* were unevenly distributed into the three branches (Figure 1). Among them, six WOX members were assigned to the modern clade, including *AktWUS*, *AktWOX9*, *AktWOX6*, *AktWOX3*, *AktWOX7*, and *AktWOX5*. *AktWOX1* and *AktWOX8* were assigned to intermediate clades, and *AktWOX2* and *AktWOX4* were assigned to ancient clades.

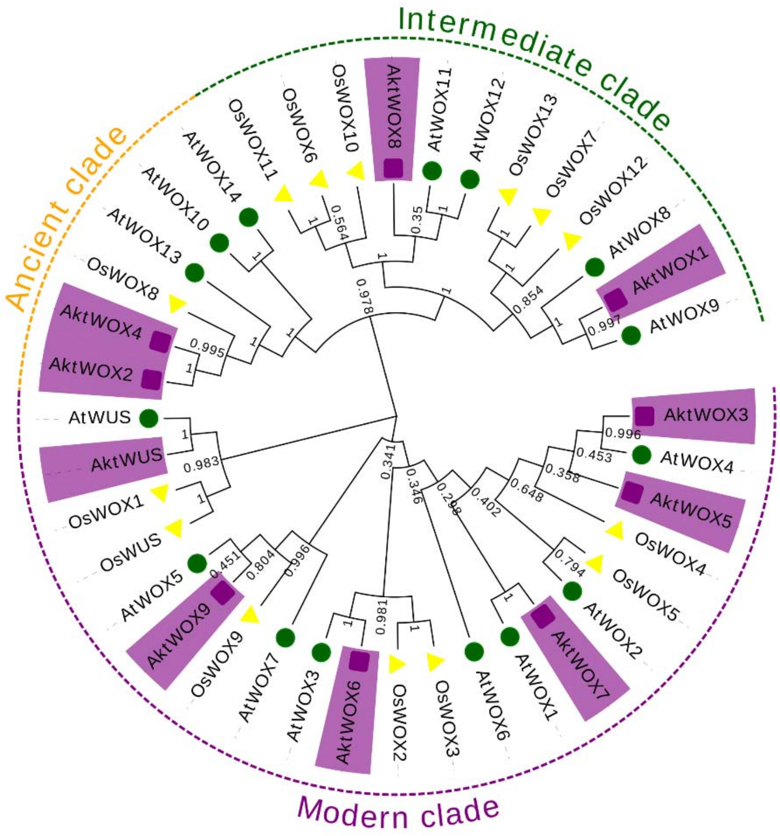

Figure 1. Phylogenetic tree analysis of *WOX* genes in *A. trifolium* and other species. *At*: *A. thaliana* (green); *Os*: *O. sativa* (yellow); *Akt*: *A. trifoliata* (purple).

2.3. Gene Structure and Conserved Motifs of AktWOXs

Domain analysis showed that 10 AktWOX protein sequences had conserved HD and WUS-box domains (Figure S1). Further motif analysis showed that the 10 AktWOX proteins contained 10 relatively conserved motifs (Table S2). Motif 1 and motif 2 were found in all 10 Akt*WOXs* and contained a highly conserved "helix-ring-helix-corner-helix" HD domain (Figure S2). Motif 5 was the WUS-box motif (Figure S3) and existed in *AktWUS*, *AktWOX9*, *AktWOX6*, *AktWOX3*, *AktWOX7*, and *AktWOX5* (modern evolution branch) (Figure S2). Motif 7 existed only in *AktWOX1* and *AktWOX8* (intermediate clades), while motif 4 and motif 8 existed only in *AktWOX2* and *AktWOX4* (ancient clades).

The analysis of its exon and intron structure revealed that the *AktWOX* gene contained 2-4 CDSs (coding DNA sequences); *AktWOX3, AktWOX5, AktWOX6* and *AktWOX9* contained 2 CDSs; *AktWOX7* had 4 CDSs; and *AktWUS, AktWOX3* and 4 members of the middle branch and ancient branch contained 3 CDSs (Figure 2c).

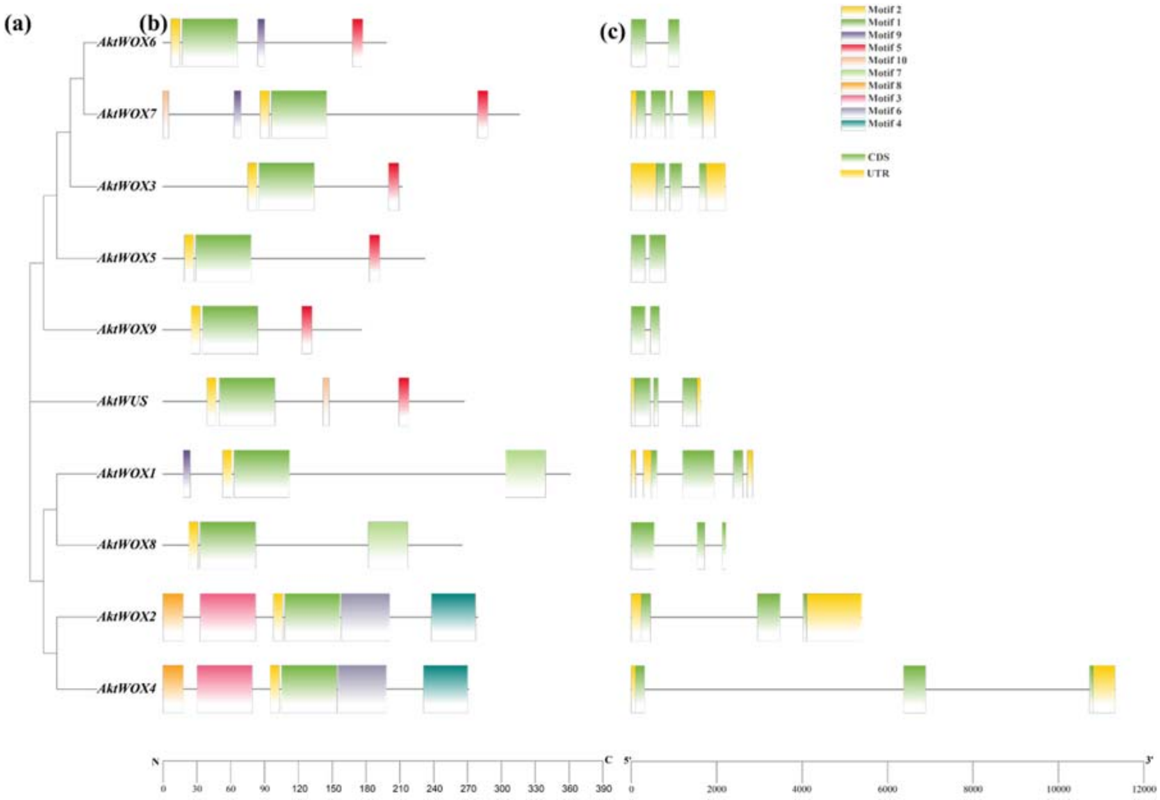

Figure 2. Gene and protein structure analyses of the AktWOX family. (a) Phylogenetic tree of AktWOXs. (b) Motifs of AktWOX proteins. (c) Exon–intron structures of AktWOXs.

2.4. Chromosomal Location and Evolutionary Analyses of AktWOXs

Chromosomal location analysis showed that 10 *AktWOXs* were distributed on 7 chromosomes of *A. trifoliata* (Figure 3), and two *AktWOXs* were located on chromosome 15. The remaining five *AktWOXs* are found on chromosomes 1, 3, 6, 8, and 9.

In terms of evolution, intraspecies collinearity analysis showed that dispersed and segmental or whole-genome duplication (WGD) events were the main sources of *AktWOX* expansion (Figure 3), but the majority (8; 80%) *AktWOXs* were derived by dispersed replication, and the minority (2; 20%) *AktWOXs* were derived by WGD events.

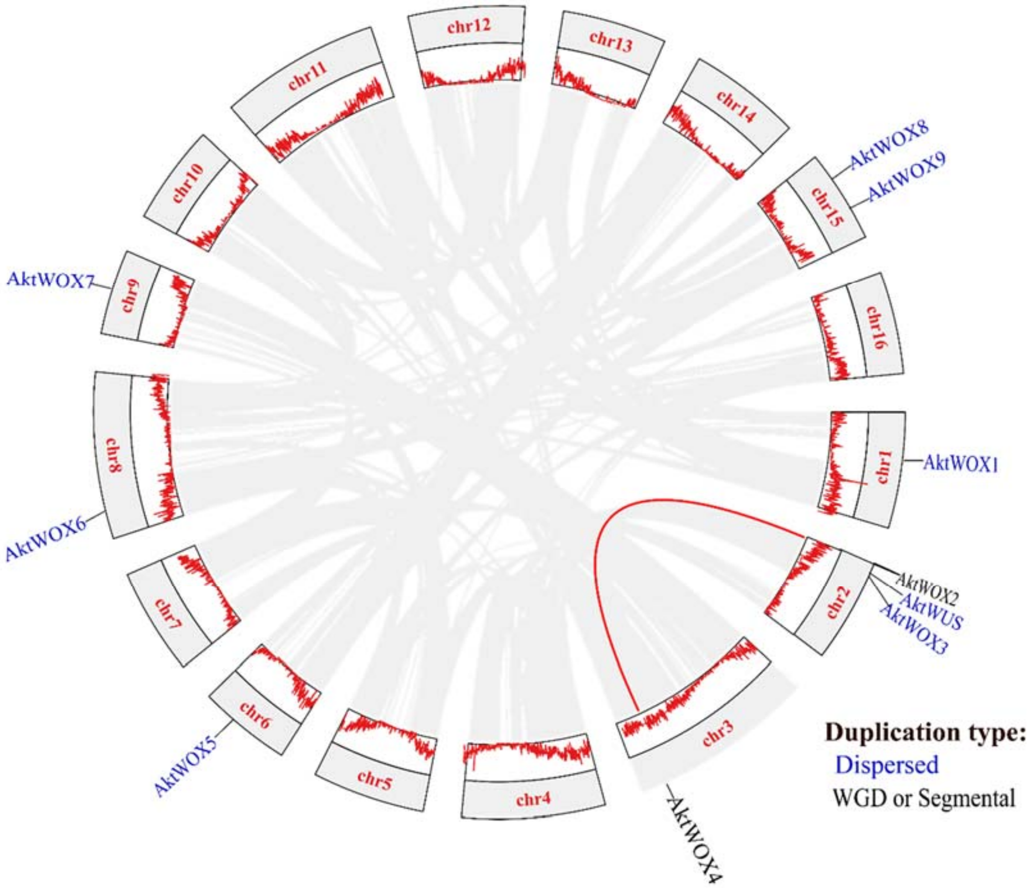

Figure 3. Collinearity and gene duplication events and gene clusters of *AktWOXs*. The red line indicates the *AktWOX* collinear gene pair; the two gene duplication types (dispersed, WGD or segmental) are represented in blue and black, respectively.

To further understand the gene duplication mechanism of the *WOX* gene family in *A. trifoliata*, a comparative map was generated with the dicotyledonous plants *A. thaliana*, *Liriodendron tulipifera, Populus x canescens, Solanum lycopersicum, Glycine max, Solanum tuberosum* and *Amborella trichopoda* and the monocotyledonous plants *O. sativa, Zea mays,* and *Andropogon gerardi*. They were analyzed with the Chlorophyta plant *Chlamydomonas reinhardtii* (Figure 4). The number of homologs between *A. trifoliata* and *A. thaliana* was 8, *L. tulipifera* was 12, *Populus x canescens* was 18, *S. lycopersicum* was 7, *G. max* was 19, *S. tuberosum* and *A. trichopoda* were 6 and 6, the monocotyledonous plant *O. sativa* was 3, *Z. mays* was 5, *A. gerardi* was 14, and the chlorophyta plant *C. reinhardtii* did not contain any homologs, indicating a strong direct homology between the *A. trifoliata WOXs* and the dicotyledons members, which showed a high degree of evolutionary divergence compared with the monocotyledons.

Determining the Ka/Ks ratio can effectively improve the understanding of the evolutionary constraints of the *WOX* gene family. The Ka/Ks values of all 45 homologous *AktWOX* pairs were much lower than 1 and varied from 0.01 to 0.33 (Table S3), indicating that the *AktWOXs* could have experienced strong purifying selection during their evolutionary history.

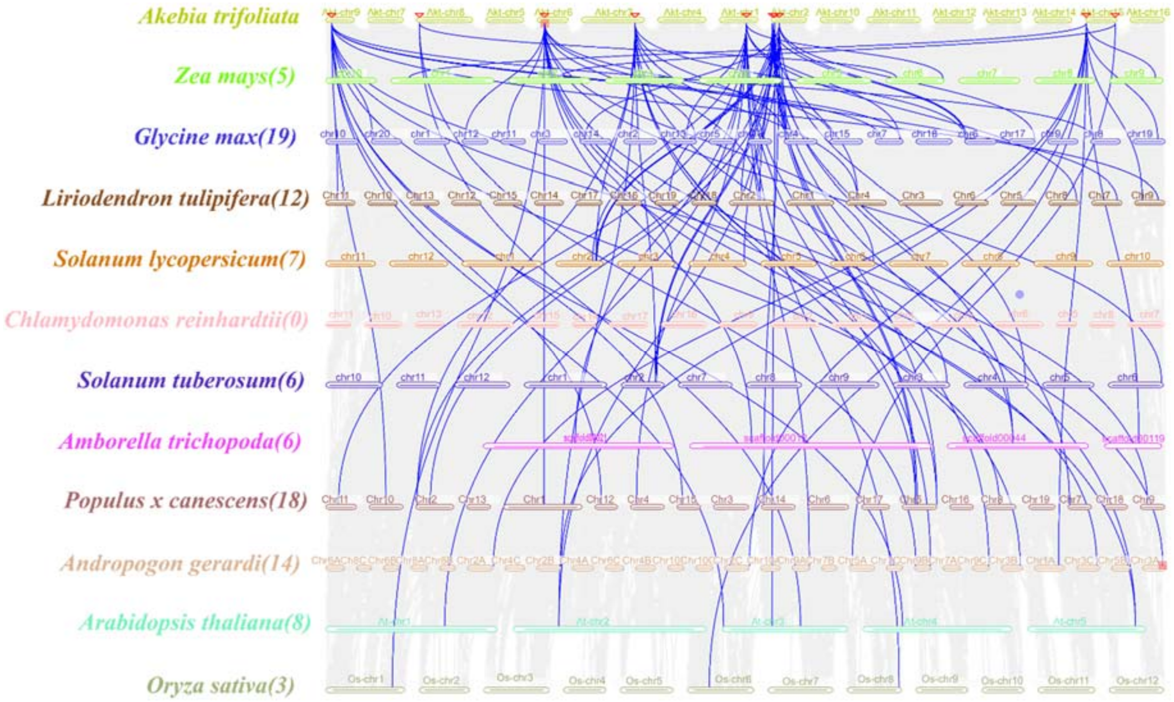

Figure 4. Collinearity analysis between *WOX* genes of *A. trifoliata* and *WOX* genes of other species. Different species names and chromosomes are represented by different colors. The blue line indicates the homologous *WOX* gene pairs between other species and *AktWOXs*, and the number in parentheses after the species name indicates the number of collinear pairs between the *WOX* genes of the other species and *AktWOXs*.

2.5. Identification of Cis-Acting Elements of the AktWOX Gene Family

The cis-element analysis results of the upstream sequence of *AktWOXs* are shown in Figure 5. The types of *AktWOX* cis-elements included hormone-responsive elements and environment-responsive elements, and each element had 5 and 7 subtypes, respectively.

There were three cis-acting elements related to stress resistance: defense and stress response elements (13), low-temperature induction response elements (8), light responsiveness elements (131), anaerobic induction elements (22), zein metabolism regulation elements (9), elements involved in endosperm expression (9) and elements involved in flavonoid biosynthesis genes (2). Cis-acting elements related to hormone regulation mainly included auxin (7), gibberellin (11), abscisic acid (33), MeJA responsiveness elements (42) and salicylic acid response elements (8). Cis-acting elements related to substance synthesis: elements involved in metabolic regulation of zein, elements involved in endosperm expression, and MYB-binding site elements involved in flavonoid biosynthesis genes.

Both the type and the number of cis-acting elements also widely varied among members of the *AktWOXs* (Table S5). We found that every *AktWOX* had a light-responsive element with numbers ranging from 6 to 19, and *AktWOX5* and *AktWUS* had the most (51) and least (17) cis-acting elements, respectively. The number of cis-acting element subtypes varied from 6 (*AktWUS*, *AktWOX8* and *AktWOX9*) to 9 (*AktWOX4*, *AktWOX6* and *AktWOX7*), and the *AktWOX3* genes contained seven cis-acting element subtypes, and the *AktWOX1*, *AktWOX2* and *AktWOX5* genes contained eight cis-acting element subtypes.

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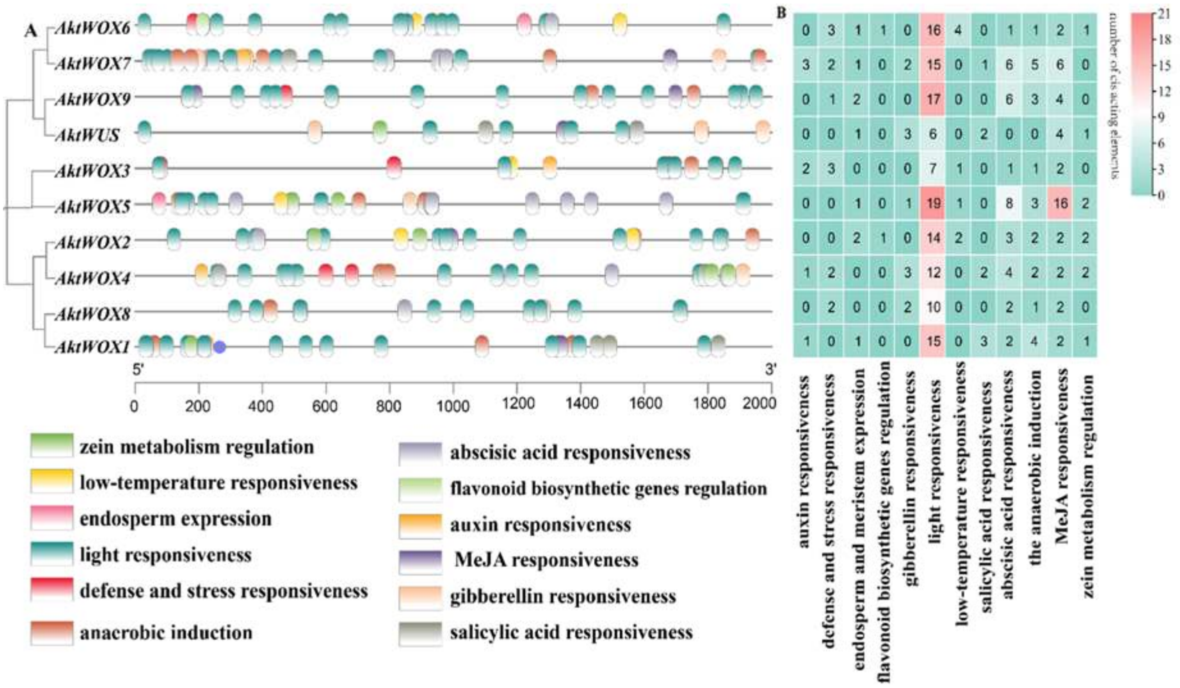

	auxin responsiveness	defense and stress responsiveness	endosperm and meristem expression	flavonoid biosynthetic genes regulation	gibberellin responsiveness	light responsiveness	low-temperature responsiveness	salicylic acid responsiveness	abscisic acid responsiveness	the anaerobic induction	MeJA responsiveness	zein metabolism regulation
AktWOX6	0	3	1	1	0	16	4	0	1	1	2	1
AktWOX7	3	2	1	0	2	15	0	1	6	5	6	0
AktWOX9	0	1	2	0	0	17	0	0	6	3	4	0
AktWUS	0	0	1	0	3	6	0	2	0	0	4	1
AktWOX3	2	3	0	0	0	7	1	0	1	1	2	0
AktWOX5	0	0	1	0	1	19	1	0	8	3	16	2
AktWOX2	0	0	2	1	0	14	2	0	3	2	2	2
AktWOX4	1	2	0	0	3	12	0	2	4	2	2	2
AktWOX8	0	2	0	0	2	10	0	0	2	1	2	0
AktWOX1	1	0	1	0	0	15	0	3	2	4	2	1

Figure 5. Prediction of cis-elements in the *WOX* promoter of *A. trifoliata.* (A) The distribution of cis-acting elements in the 2000 bp region upstream of the transcription start site of *AktWOXs*; (B) the number of cis-acting elements of the two functional categories in *AktWOXs*, respectively indicated by different colors and numbers, cyan-white–red represents the increasing number of cis-acting elements.

2.6. GO Enrichment Analysis of AktWOX Genes

The 10 *AktWOX* genes were divided into three categories, molecular functions (MFs), cellular components (CCs), and biological processes (BPs), by GO enrichment analysis, with 9, 12, and 183 subcategories (Table S7), respectively (Figure 6). Eight *AktWOX* genes were involved in MFs, such as transcriptional regulatory activity and DNA-binding transcription factor activity; four were involved in CCs; and seven were involved in BPs, such as RNA biosynthesis, the regulation of cell metabolism, the regulation of biosynthesis, nucleic acid metabolism, and transcription regulation.

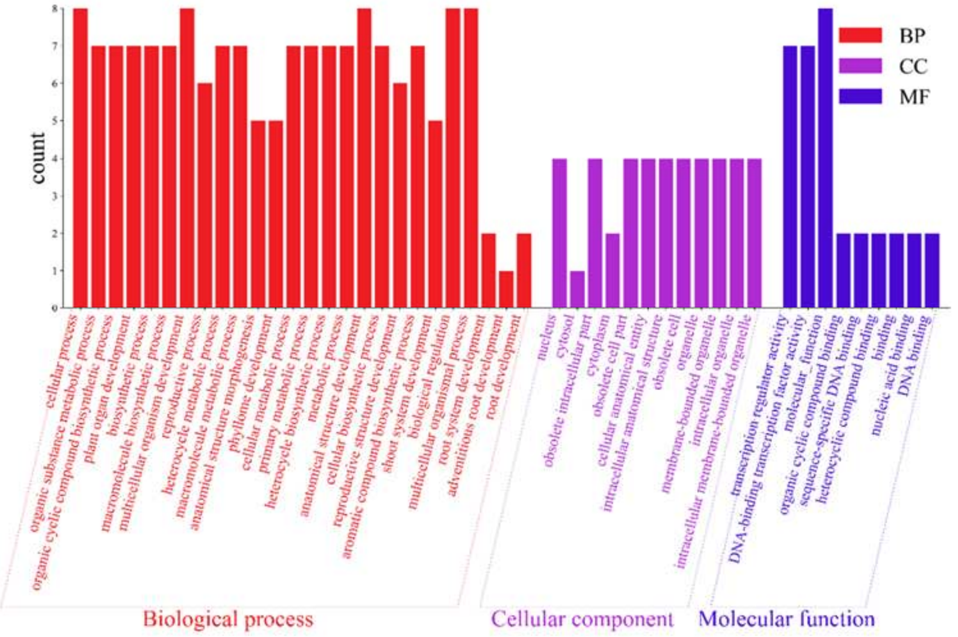

Figure 6. GO function analysis histogram.

2.7. *AktWOXs Expression in Fruit and Root of A. trifoliata*

The 10 *AktWOXs* were grouped into four groups based on their relative expression levels: (A) they were expressed at different developmental stages and in different tissues, and their expression levels were higher in the overall expression level (*AktWOX2*, *AktWOX4*); (B) they were tissue-specific, expressed almost exclusively at the seed growth stage (*AktWUS*, *AktWOX8*), at the third flesh growth stage (*AktWOX7*), and in the rind and flesh of the fruit (*AktWOX3*); and (C) they showed low or no expression throughout the developmental stages (*AktWOX5*, *AktWOX6*, *AktWOX9*, *AktWOX1*). It has been speculated that genes in group A may positively regulate fruit growth and development in *A. trifoliata*, while genes in group C may play a minor role in the growth and development of the flesh, seed, and rind and may even have a negative regulatory effect.

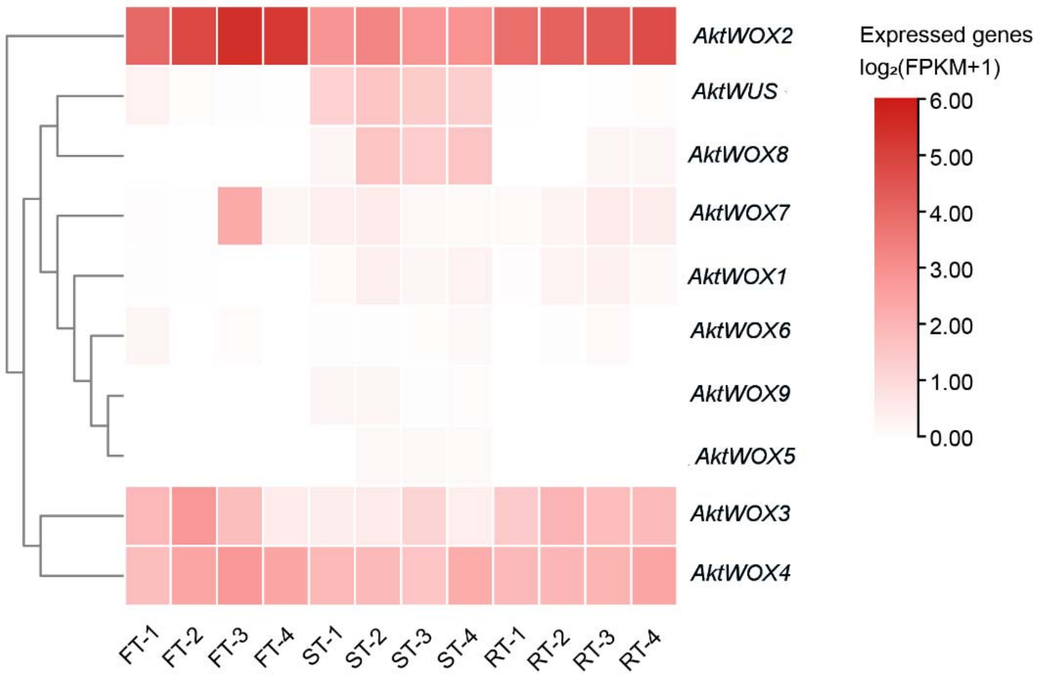

Figure 7. Heatmap of the expression patterns of *WOX* genes in different tissues. FT: flesh, ST: seed, RT: rind.

Sequence homology alignment revealed that six AktWOX genes (AktWOX1, AktWOX2, AktWOX3, AktWOX4, AktWOX8, and AktWOX9) were homologous to AtWOX4, AtWOX5, AtWOX7, AtWOX9, AtWOX11, AtWOX12, AtWOX13, and AtWOX14 in Arabidopsis 20. We further examined the expression of these six genes during the growth of adventitious roots of A. trifoliata. These genes are reportedly related to root growth and development.

The RT–qPCR results showed that the expression of the *WOX* genes during adventitious root formation of *AktWOX1*, *AktWOX2*, *AktWOX8* and *AktWOX9* increased to the highest values at 42 d of development, and then their expression gradually decreased to below the initial levels. Their expression increased at 28 d and decreased slightly with increasing development time but was still above the initial level. *AktWOX2*, *AktWOX3* and *AktWOX4* expression patterns were similar throughout adventitious root formation, with their expression decreasing at the beginning of development, being rapidly upregulated at 35 d and continuing until 42 d, after which their expression again decreased.

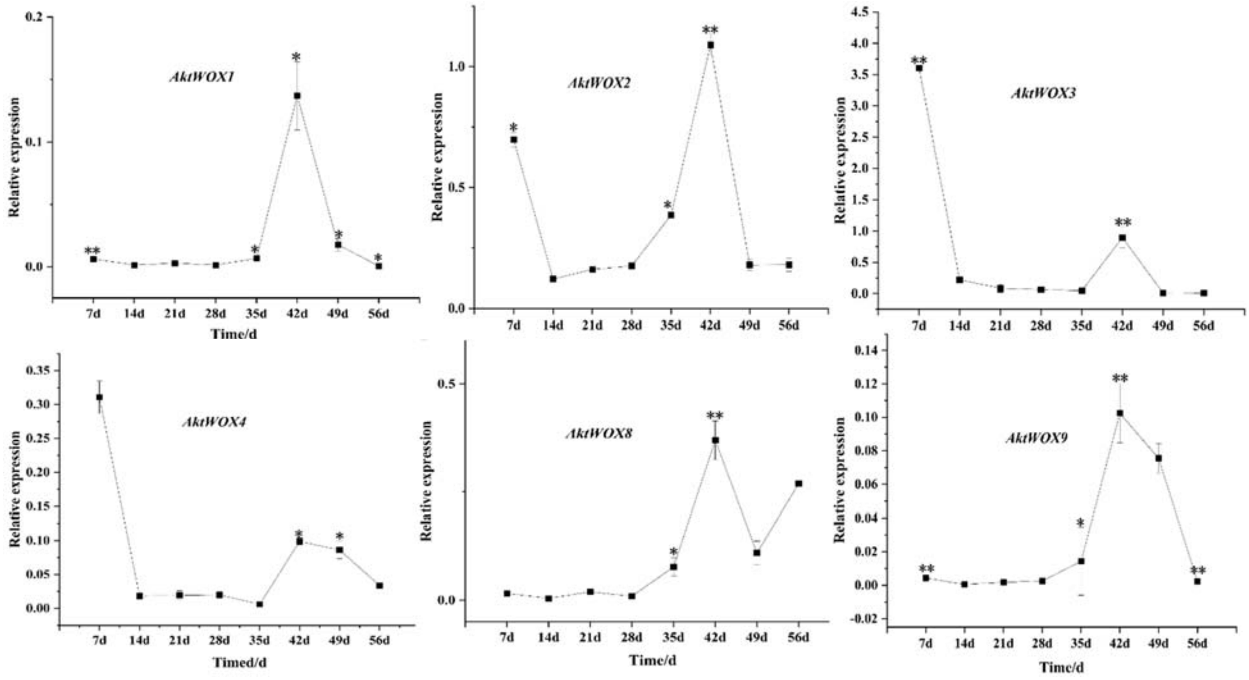

Figure 8. qRT−PCR analysis of the expression of some AktWOXs. Transcript levels of *AktWOXs* were calculated using the $2^{-\Delta\Delta Ct}$ method. * represents the significance between peak expression (**, $p < 0.01$).

3. Discussion

3.1. The AktWOX gene structure is extremely conserved during evolution

In plants, the WOX family is an extremely important gene family, and the proteins it encodes are involved in the growth and development of almost every organ of angiosperms 21. As an increasing number of plant genomes are sequenced and released, many *WOX* genes in angiosperms have been systematically identified and studied. Enriching the number of reported *WOX* genes in basal dicots will further improve our understanding of the evolution of the *WOX* gene family 20. In this study, we identified 10 *WOX* genes from the genome of the basal dicot *A. trifoliata*. We found that although the *AktWOX* genes have wide differences at the DNA sequence level, mainly reflected in the number of introns and sequence length, the physical and chemical properties of the proteins they encode are extremely similar, including similar protein lengths and molecular weights and the instability and hydrophilicity of the protein structure (Table 1). Moreover, these characteristics of *WOX* genes in *A. trifoliata* are very similar to those of monocots, including wheat 15 and four Euphorbiaceae plants 22, as well as core dicots, including *A. thaliana* 16 and *G. max* 23. This indicates that *WOX* genes are highly conserved during evolution, and the gain and loss of introns/exons are the driving forces for the evolution of this gene family.

From an evolutionary perspective, genes duplicated by different mechanisms, such as WGDs, and tandem and dispersed duplications, are primary raw materials for new gene origins and evolution and ultimately result in functional novelty and specialization **Error! Reference source not found.**. Some studies have shown that following WGD events, genes encoding TFs are preferentially retained 24. Two WGD events occurred in *A. trifoliata* approximately 85 and 140 million years ago (ϴ event), respectively. The former is a specific WGD event in *A. trifoliata*, and the latter occurred during the early stages of dicotyledonous plant differentiation (ϴ event) 26. In this study, 8 (80%) of 10 identified *AktWOXs* were found to be derived from dispersed duplication, 2 *AktWOXs* were found to be derived from WGD (Figure 3), which suggested that dispersal was the major force of AktWOX origin, and the *AktWOX* gene family was involved in only one WGD event. We reconstructed the evolution of twelve species over time (Figure S4) and showed that the *AktWOX* gene family was involved in a specific genome-wide duplication event in *A. trifoliata*. In addition, the fact that all Ka/Ks values of the homologous *AktWOX* pairs were much lower than 1 (Table S4) further suggested that all *AktWOXs* experienced strong purifying selection during their evolutionary history. The Ka/Ks value of two combinations between *AktWUS* and both *AkWOX2* and *AktWOX4* was very close to 0.004, while the combination (*AktWUS* and *AktWOX8*) with the largest Ka/Ks value was also related to *AktWUS* (Table S3), which indicated that *AktWUS* could be an ancestral gene of the *AktWOX* family. This evolutionary evidence further demonstrates that *AktWOXs* are highly conserved.

3.2. AktWOX Gene Family Members May Have Greatly Diverged Functions

Many reports have confirmed that WOX transcription factors play important roles in regulating plant growth and development, including embryonic development, maintenance of meristematic stem cells, seed formation, regeneration of isolated tissues and organs, and response to abiotic stress. For instance, *WOX* genes play different roles in the development of *O. sativa* roots, stems, and leaves 27. The *OsWOX6* gene plays a major role in the regulation of seed development, especially for the growth and development of seeds under water-deficient conditions 28. *WOX* genes are widely involved in the growth and development of different plant organs as well as physiological and biochemical processes, but their protein structure and gene number are very conserved, which indicates that this gene family has extensive functional differentiation. In this study, sequence analysis of the *AktWOX* promoter results showed that *AktWOXs* not only play important roles in the response to light signals and resistance to stress but also play a role in endosperm or seed development and meristem formation. GO enrichment analysis indicated seven *AktWOXs* widely involved in various growth development and tissue metabolism processes in biological processes of A. trifoliata. The results indicate that the *AktWOX* gene family is functionally diverse.

In addition, our transcriptomic data analysis revealed that some *AktWOXs* in the whole fruit, such as *AktWOX1*, *AktWOX5*, *AktWOX6* and *AktWOX9*, exhibited very low expression, while some *AktWOXs*, such as *AktWOX2* and *AktWOX4*, exhibited high expression. In addition, *AktWUS, AktWOX2, AktWOX4,* and *AktWOX8* had seed-specific expression patterns, while *AktWUS, AktWOX7*, and *AktWOX8* had developmental stage-specific patterns. These results indicate that *AktWOXs* may be functionally diverse. The HD and WUS-box domains are two conserved domains of the WOX family 16. At present, research on the WUS-box domain is mainly based on the *WUS* gene. In the process of WUS participating in maintaining the characteristics of stem cells in the plant stem meristem, the WUS-box mainly exerts inhibitory activity and maintains the dynamic balance of stem cell proliferation regulation 29. Studies have shown that the WUS box plays an important role in the maintenance of stem cell characteristics 29. Therefore, the modern branch of *AktWOXs* may be involved in regulating the development of stem cells. This is further evidence that members of the *AktWOX* gene family may have wide functional differences.

3.3. The AktWOX Gene may be Involved in adventitious root Regulation

The *WOX* gene family is widely involved in the formation of adventitious roots. In A. thaliana, *AtWOX4*, *AtWOX5*, *AtWOX7*, *AtWOX9*, *AtWOX11*, *AtWOX12*, *AtWOX13*, and *AtWOX14* are associated with root growth and development 20, while there are no homologous genes for At*WOX7*, *AtWOX12*, and *AtWOX14* in *AktWOX*. De novo root organogenesis from tissue explants requires consecutive cell fate transition steps to finally form an adventitious root. The first step of cell fate transition is priming, which results in the formation of adventitious root founder cells. The second step of cell fate transition is initiation, which results in the formation of the dome-shaped root primordium via cell division. The expression levels of *AtWOX11/12* decrease and those of *AtWOX5/7* increase as the root founder cells transition into the root primordium 9. In the formation of adventive roots of *A. trifoliata*, *AktWOX2* and *AktWOX8* were highly expressed in the late stage, and *AktWOX3* and *AktWOX4* were highly expressed in the early stage. This result, which is similar to that of Hu et al. (2016), may indicate that *AktWOX3* and *AktWOX4* are related to the initiation of adventitia root cells in A. trifoliata, while *AktWOX2* and *AktWOX8* may be related to the initiation of adventitious root cells.

4. Materials and Methods

4.1. Identification and Physicochemical Characterization of AktWOX Sequences

To identify *WOX* genes in the A. trifoliata genome, hidden Markov model (HMM) ID PF00046 files downloaded from the Pfam database (http://smart.embl.de/smart/batch.pl, accessed on 28 December 2021) were used to filter protein sequences of A. trifoliata with the 10e-5 e-value parameter 30. After obtaining the *A. trifoliata WOX* genes, the AktWOX protein sequence was submitted to the conserved domain database (CDD: https: //www.ncbi.nlm.nih.gov/Structure/bwrpsb/BWRPSB. Cgi) for structural domain filtering to determine the final implant AktWOX transcription factor family members 31. Gene position on chromosomes and collinearity mapping using TBtools software 30.

The ExPASy's ProtParam online tool (http://www.ExPASy.org/tools/protparam.html/) was used to predict the physical and chemical properties of the AktWOX transcription factors 32. We used SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/) 33 to predict the secondary structure of the WOX gene in A. trifoliata. DataProtComp9.0 (http://linux1.softberry.com/berry.phtml?topic=protcomppl&group=Programs&subgroup=proloc) and SignalIP5.0 (http://www.cbs.dtu.dk/services/SignalP/) were used for subcellular localization and signal peptide prediction 34.

4.2. Sequence Characteristic Analysis, Phylogenetic Analyses, GO Enrichment Analysis and Collinearity of AktWOXs

Multiple alignments of the full-length protein sequences were executed by using ClustalW (https://www.genome.jp/tools-bin/clustalw, accessed on 20 May 2023). A phylogenetic tree was constructed using MEGA 11 software (version 11.0.10) via the maximum likelihood (ML) method with 1000 bootstrap replicates [35–38]. The GFF3 file of the A. trifoliata genomic annotation was used to analyze the gene sequence characteristics. The Gene Structure Display Server (http://gsds.gao-lab.org/, accessed on 20 May 2023) was used to count the number and location of exons/introns of the AktWOXs. The conserved motifs of the A. trifoliata proteins were analyzed by MEME Suite (https://meme-suite.org/meme/tools/meme, accessed on 20 May 2023) 39, where the maximum motif number was set to 10 and the other settings were set to their default values. The above results were subsequently visualized by TBtools 30 software (version 1.0876). To display the evolutionary selection pressure between collinear gene pairs 40, the Ka/Ks ratio was calculated by TBtools 30 software (version 1.0876). The reference genome sequences of *A. thaliana, L. tulipifera, Populus x canescens, S. lycopersicum, G. max, S. tuberosum* and *A. trichopoda*, the monocotyledonous plants *O. sativa, Z. mays*, and *A. gerardi*, and the Chlorophyta plant *C. reinhardtii* (accession on 9 August 2023). We downloaded data from the NCBI database and used them to perform a collinearity analysis with the sequence of *A. trifoliata*. The PlantCARE online website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 26 May 2022) was then used to analyze the cis-acting elements in the 2000 bp promoter region upstream of *A. trifoliata* 42. Timetree5 (http://timetree.org/) was used to reconstruct the evolution of twelve species over time 43. The Metascape (Metascape.org) web-based portal was used for comprehensive gene annotation and analysis resources 44. A bubble chart was plotted using the Bioinformatics (www.bioinformatics.com.cn) free online platform for bioinformatics-related data analysis 44.

4.3. Expression analysis of AktWOXs in fruit development

The transcriptomic data of *A. trifoliata* were downloaded from the NCBI database under BioProject ID PRJNA671772 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA671772; accessed on 25 April 2023). The A. trifoliata transcriptomic data contained data on three tissue types (fruit flesh, seeds, and rind) at four different stages (young, enlargement, coloring, and mature stages), and there were data for three biological replicates (young stage, SAMN16551934-36, enlargement stage; SAMN16551937-39, coloring stage; SAMN16551940-42, mature stage). FPKM values calculated by HISAT2 and DESeq2 were used to estimate gene expression levels 45. TBtools (version 1.0876) software was used to construct a heatmap of AktWOX expression 30.

4.4. AktWOX expression during adventitious root formation

The cuttings used for the experimental treatment were obtained from the same tree cuttings and were exposed to the same cultivation conditions. The cuttings were transplanted in the germplasm nursery of the Sichuan Agricultural University Chongzhou Research Station (30◦430 N, 103◦650 E); the RNA of 2 cm stem base and root mixed samples at 7, 14, 21, 28, 35, 42, 49 and 56 d during the cutting period of *AktWOX* Shusen 1 was extracted. Total RNA was extracted with an M5 Plant RNeasy Complex Mini Kit (Polysaccharides and Polyphenolics-rich) (JUHEMAI, Beijing, China). The integrity and purity of the RNA were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Austin, TX, USA), respectively. Then, the RNA of the samples was reverse transcribed into cDNA using an EasyScript One-Step gDNA Removal and cDNA Synthesis Supermix Kit (TransGen Biotech, Beijing, China).

The primer pairs for the *AktWOXs* and *GAPDH* genes were designed using Primer 3.0, and the primer sequences and related details are listed in Table S3. The amount of cDNA was 1 μmol as the amplification substrate, and the reaction was carried out as follows: 92°C for 30 s, followed by 45 cycles of 5 s at 92°C and 30 s at 53°C. To determine the expression patterns of the AktWOXs, RT–qPCR was conducted on a Thermal Cycler CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) together with PerfectStart Green qPCR SuperMix (TransGen Biotech, Beijing, China). Each sample included three technical replicates. The 2−ΔΔCt method was used to calculate the expression level of genes. Statistical analysis was performed with SPSS (version 20.0.0) and Origin 2018 software (version 9.5.1).

5. Conclusions

In this study, we identified 10 candidate *AktWOXs* that were unevenly distributed on 7 high-quality assembled chromosomes of the *A. trifoliata* genome. All 10 *AktWOXs* were classified into 3 groups, and in terms of evolution, they were mainly produced by dispersal events and underwent strong purifying selection. Many *AktWOXs* exhibited tissue and developmental stage-specific expression patterns. We further identified four genes, namely, *AktWOX2*, *AktWOX3*, *AktWOX4*, and *AktWOX8*, that could be involved in the response to adventitious root formation conditions. In addition, this study provides important information concerning the *WOX* genes of *A. trifoliata* and provides a theoretical reference for their functions in adventitious root formation.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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