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RESEARCH ARTICLE

Genome-wide identification of auxin response factor (*ARF*) family in kiwifruit (*Actinidia chinensis*) and analysis of their inducible involvements in abiotic stresses

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Abstract Auxin response factor (ARF) acts as a vital component of auxin signaling and participates in growth, development, and stress responses in plants. In the present study, we comprehensively analyzed kiwifruit's (*Actinidia chinensis*) *ARF* genes (*AcARFs*) and their involvement in abiotic stress response. We identified a total of 41 AcARFs encoding ARFs in the *A. chinensis* genome. *AcARF* genes were characterized by the classic ARF_resp and a B3 domain and primarily localized on the cytoplasm and nucleus. AcARFs were categorized into eight subgroups as per the phylogenetic analysis. Synteny analysis showed that 35 gene pairs in AcARF family underwent segmental and whole genome duplication events. Promoter cis-element prediction revealed that AcARFs might be involved in abiotic factors related to stress response, which was later assessed and validated by qRT-PCR based expression analysis. Additionally, AcARFs showed tissue-specific expression. These findings extend our understanding of the functional roles of AcARFs in stress responses. Taken together, the systematic annotation of the AcARF family genes provides a platform for the functional and evolutionary study, which might help in elucidating the precise roles of the AcARFs in stress responses.

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Introduction

Auxin is a plant hormone of utmost significance as it plays crucial roles in the course of plant growth and development, such as embryogenesis, flower and fruit development, apical dominance, tropic response, root architecture, and vascular development (Aloni et al. 2006; Esmon et al. 2006; Kazan 2013; Mattsson et al. 2003; Woodward and Bartel 2005). Auxin exerts its effects via the modulation of gene expression of a myriad of gene families, such as small auxin up RNA (SAUR), gretchen hagen 3 (GH3), indole-3-acetic acid (Aux/IAA) and auxin response factor (ARF) (Wang et al. 2018; Yuan et al. 2019; Zhou et al. 2019). ARFs function as transcription factors and are crucial components of the auxin signalling pathway. They modulate auxin-regulated genes (ARGs) by binding explicitly to the auxin response elements (AuxRE, 5' TGTCTC 3') in the promoter of ARGs (Berendzen et al. 2012). ARFs contain a conserved N-terminal DNA-binding domain (DBD) and a C-terminal dimerization domain (CTD) (Tiwari et al. 2003). The DBD of ARFs binds to AuxRE in ARG promoter regions. The C-terminal dimerization domain is identical to that of Aux/IAA proteins apportioned to domains III and IV; besides, it facilitates homodimerization or heterodimerization of the ARFs or numerous other Aux/IAA proteins (Ulmasov et al. 1999). ARFs also encompass a non-conserved middle region (MR), present between DBD and CTD. The ARFs mediated transcriptional activation or repression is based on the amino acid composition. The activator function is imparted

by the QSL-rich (glutamine (Q), serine (S), leucine (L) amino acids) and repressor activity by the S-rich (serine), SPL-rich (serine, proline, leucine), and SL / G-rich (serine, leucine and/or glycine) MR in ARFs (Ulmasov et al. 1999).

Multiple members of the ARF family have been identified and well-characterized in numerous plant species subsequent to the cloning of the *Arabidopsis thaliana*' first *ARF* gene, AtARF1. As per the genomic analyses, a total of 23 ARF members in *A.thaliana*, 39 in *Populus trichocarpa*, 25 in rice (*Oryza sativa*), 17 in *Eucalyptus grandis*, 22 in tomato (*Solanum lycopersicon*), 19 in sweet orange (*Citrus sinensis*), and 31 in maize and *Brassica rapa* have been identified so far (Bouzroud et al. 2018; Kalluri et al. 2007; Li et al. 2015a; Mun et al. 2012; Overvoorde et al. 2005; Wang et al. 2007; Xing et al. 2011; Yu et al. 2014). Besides, the functional significance of ARFs in various plant growth and development processes has been well elucidated. For instance, the functional role of AtARF3 and AtARF4 in leaf and floral organ patterning coincides (Pekker et al. 2005; Sessions et al. 1997), AtARF5 / MONOPTEROS (MP) participates in embryonic development and vascular tissue formation (Hardtke and Berleth 1998), AtARF7 and AtARF19 both control lateral root formation (Okushima et al. 2007; Wilmoth et al. 2005), and AtARF6 and AtARF8 both regulate flowering process (coordinating stamen development, petal expansion, anther dehiscence, and gynoecium maturation) (Nagpal et al. 2005). As per the recent studies, tomatoes have multiple ARF genes, such as *SlARF2*, *SlARF7*, *SlARF9*, which are involved in leaf morphogenesis, development of flowers and fruits and fruit ripening (de Jong et al. 2009; Hao et al. 2015; Wilmoth et al. 2005). As per the previous reports, as transcriptional regulators, ARFs directly contribute to auxin signal transposition (Guilfoyle and Hagen 2007; Ljung 2013) and are involved in other many stress responses and hormonal cross-talks (Guilfoyle and Hagen 2007; McAtee et al. 2013) in Arabidopsis (Kalve et al. 2020; Okushima et al. 2005), rice (Jain and Khurana 2009), citrus (Li et al. 2015a), strawberry (Wang et al. 2019), banana (Hu et al. 2015b), tomato (Bouzroud et al. 2018), and chickpea (Singh et al. 2017). In Arabidopsis, multiple ARF genes participate in abiotic stress responses (Ji and Jiang 2015; Kalve et al. 2020; Okushima et al. 2005). In tomatoes, the *SlARF* gene mediates salt, drought, and flooding stress response, and the *SlARF7* gene mediates cross-talks between auxin and GA signaling during the fruit and plant development in tomatoes (Bouzroud et al. 2018; de Jong et al. 2011; Kumar et al. 2011). Similarly, multiple *ARF* genes mediate abiotic stress responses against desiccation, salt, and cold stresses (Jain and Khurana 2009; Wang et al. 2007). In chickpea, *CaARF* genes significantly regulate abiotic stress (Singh et al. 2017).

Kiwifruit is a widely consumed and health-promoting fruit, and one of the most important fruit crops globally. The cultivated kiwifruit, Hongyang, is a heteroploid hybrid variety (2n = 2x = 58) derived from wild *A. chinensis* (Huang et al. 2013). Kiwifruits are frequently exposed to different abiotic stresses, such as drought, cold, salinity, and hormonal stress. In this study, we used the data from open-access databases for genome-wide identification and characterization of the ARF family of *A. chinensis*. We performed comparative phylogenetic analysis, gene structure and conservative motifs prediction, gene duplication events analysis, promoter cis-elements, and subcellular location prediction. Besides, we employed quantitative real-time PCR (qRT-PCR) to assess the expression profiles of the 12 *AcARF* genes in different stress conditions such as stress signalling induced by different phytohormones (IAA; abscisic acid, ABA; gibberellins, GA; salicylic acid, SA; jasmonic acid, JA), along with drought and salt stresses. The outcomes of our current study provide a platform for future research and highlight the importance of targeting *ARF* genes to obtain stress-resistant kiwifruit varieties.

Materials and methods

Identification and annotation of *ARF* genes in kiwifruit

The kiwifruit genome and proteome datasets were downloaded from Ensembl Plants (http://plants.ensembl.org/index.html). The *A. thaliana* ARF protein data set were downloaded from TAIR (https://www.arabidopsis.org/) (Swarbreck et al. 2008). The Hidden Markov Model (HMM) profiles of the ARF family were employed with default parameters and a cutoff value of 0.01 to identify the *ARF* genes from the *A. chinensis* genome. All candidate *ARF* genes containing ARF conserved domains were further examined via the Pfam (http://pfam.wustl.edu) and SMART (http://smart.embl-heidelberg.de) databases (Schultz et al. 1998). All the AcARF sequences were validated as a member of the ARF family by using the NCBI conserved domain database (http://www.omicsclass.com/article/310). Physical and chemical (molecular weight, MW, and the isoelectric point, pI) characterization of AcARF proteins was done using an online tool, ProtParam (http://web.expasy.org/protparam/) (Wilkins et al. 1999). The subcellular loci were predicted with WoLF PSORT (Horton et al. 2007).

Phylogenic analysis of AcARFs and AtARFs

The full-length ARF proteins were aligned using Clustal X (Chenna et al. 2003) and visually adjusted using BioEdit V7.2 (Tippmann 2004). The Neighbor-Joining (NJ) phylogenetic trees (Silva et al. 2005) were constructed using Mega V7.0 with 1000 as the bootstrap replicates (Kumar et al. 2016).

In silico characterization of AcARFs

The chromosomal location of genes was retrieved from the *A. chinensis* genome browser by Scipio (Keller et al. 2008). The chromosomal location of the *AcARF* genes was visualized by MapChart V2.1 (Voorrips 2002). MCScan X with default parameters was employed to examine the gene duplication events in the *AcARF* genes (Wang et al. 2012). To study the relationship between the orthologous *ARF* genes obtained from kiwifruit and *A. thaliana*, a syntenic analysis map was constructed using DSP software. The *AcARF* gene structure was extracted from Ensembl Plants and visualized using GSDS V2.0 (http://gsds.cbi.pku.edu.cn) (Hu et al. 2015a). The Pfam (Finn et al. 2016) and MEME suite (Bailey et al. 2009) were used to reveal and visualize conserved motifs. The promoters (1500 bp before ATG) of the *AcARF* genes were obtained from the Ensembl Plants, and the cis-elements were predicted by PlantCARE (Lescot et al. 2002).

Plant materials

Kiwifruit 'Hongyang' (*A. chinensis*) plants were cultivated in Bairui Kiwifruit Research Institute located at Shaanxi, China (33° 42'N, 107° 39'E) from 2017 to 2019. Different tissues, including roots, stems, leaves (the fourth young leaves from the shoot), flowers, small green fruit (approximately 30 days after flower), and ripe fruit (approximately 120 days after flower) were collected from "Hongyang" trees (aged 5 years) during the 2020 growing season. The 2-year-old 'Hongyang' kiwifruit seedlings were used for hormonal treatments. All these seedlings were planted in 25 cm diameter pots in the greenhouse set to the temperature of 28 °C. The branch containing three to six pieces of leaves were treated with phytohormones. Leaves were sprayed with 100 $\mu M \cdot L^{-1}$ of phytohormones. The leaves sprayed with plain double distilled water (ddH_2O) were treated as the controls, and the leaf samples were collected at 0, 1, 6, and 12 h post-treatment (hpt). The salty stress was induced by irrigating each seedling with 2 L of 200 $mM \cdot L^{-1}$ NaCl solution, and seedlings irrigated with ddH_2O were treated as controls. The leaves were collected at 1, 6, 12, 24, and 48 hpt. Drought stress was induced by halting water supply to seedlings, and the leaves were collected at 48, 96, 144, and 168 hpt; these seedlings were re-watered after leaves collection, i.e., 48 hpt, in accordance with the previous study (Jing and Liu 2018). All the leaf samples were flash-frozen in liquid nitrogen and stored at − 80 °C for RNA extraction. For each treatment, six different leaves were sampled from six different seedlings.

RNA extraction and qRT-PCR

Total RNAs were extracted from around 100–200 mg of frozen leaf samples using a TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Genomic DBA contamination was removed by using the Turbo DNA-free^{TM} kit (Ambion). The total RNA was reverse transcribed into cDNA by the Invitrogen reverse transcription kit (SuperScript III Reverse Transcriptase). The primers were designed using NCBI's primer design tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supplementary Table 5). qRT-PCR was performed using ABI Quant Studio tm 6 Flex Real-Time PCR System with SYBR-Green PCR Master Mix. The reaction mixture (20 µL) contained 10 µL SYBR Green Master Mix (ROX), 1 µL forward specific primer (10 µM), 1 µL reverse specific primer (10 µM), 1 µL cDNA (30 $ng \cdot \mu L^{-1}$) template, and 7 µL ddH_2O. DNA amplification was performed by using the following thermocycling program: 95 °C for 3 min, 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 71 cycles with an increasing temperature gradient of 0.5 °C per cycle from 60 to 95 °C for 30 s. *ACT2* gene (GenBank: EF063571) was used as an internal standard to calculate the relative fold change as per the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001) with three biological and technical replicates. P value < 0.05 was treated as statistically significant.

Results

ARF annotation in kiwifruit genome

To identify potential *ARF* genes in kiwifruit genome, 23 Arabidopsis ARF protein sequences were queried against the annotated *A. chinensis* genome in Ensembl Plants database (http://plants.ensembl.org/index.html). After manual curations, a total of 41 *A. chinensis* ARFs were identified and named as per their respective chromosomal locations (Table 1). In instances where one chromosome contained two or more than two *AcARF* genes, chromosome number was used to name the genes, such as AcARFxa and xb, where x represents chromosome number. The number of amino acids (aa) in AcARF proteins

Table 1 *ARF* genes annotated in kiwifruit genome

Proposed name	Gene locus ID	Protein ID	Chromosome	Genome location	Strand	CDS (bp)	Deduced polypeptide			Exon No	Subcellular Loci
							Length (aa)	MW (kDa)	pI		
AcARF1a	CEY00_Acc00874	PSS36364	1	13,865,023–13,873,850	R	3285	1094	120.88	6.67	14	N
AcARF1b	CEY00_Acc01134	PSS36616	1	16,930,409–16,944,951	F	2499	832	92.95	6.16	14	N
AcARF4	CEY00_Acc04567	PSS31271	4	1,583,350–1,590,708	F	2814	937	104.07	5.16	14	N
AcARF5	CEY00_Acc05310	PSS30109	5	4,333,617–4,349,879	R	2517	838	93.37	6.44	14	N
AcARF6a	CEY00_Acc06528	PSS28912	6	6,395,758–6,403,508	F	2727	908	100.25	6.61	14	N
AcARF6b	CEY00_Acc06668	PSS29108	6	11,930,675–11,940,050	F	2025	674	74.75	5.98	14	N
AcARF7	CEY00_Acc07566	PSS26273	7	3,577,668–3,582,300	R	2091	696	78.28	6.35	14	N
AcARF8a	CEY00_Acc08485	PSS23661	8	2,728,297–2,733,191	F	2082	693	76.58	6.89	3	N
AcARF8b	CEY00_Acc08534	PSS23710	8	3,506,669–3,511,682	F	2088	695	76.63	6.85	3	N
AcARF9a	CEY00_Acc09765	PSS20727	9	49,366–58,115	F	3309	1102	121.97	6.44	14	N
AcARF9b	CEY00_Acc10474	PSS21430	9	10,532,186–10,548,890	F	2475	824	92.31	6.41	14	N
AcARF10	CEY00_Acc11482	PSS19432	10	12,006,365–12,015,037	F	1818	605	66.04	6.40	2	C
AcARF11	CEY00_Acc12325	PSS17537	11	6,086,355–6,093,271	R	1854	617	66.68	6.51	2	N
AcARF12	CEY00_Acc12933	PSS15442	12	326,217–333,996	F	3285	1094	122.13	6.83	13	N
AcARF13	CEY00_Acc15218	PSS14651	13	18,376,711–18,383,003	R	2148	715	79.58	6.12	14	N
AcARF14	CEY00_Acc15563	PSS11289	14	3,093,711–3,098,427	R	2082	693	76.61	7.12	14	N
AcARF18a	CEY00_Acc20673	PSS04814	18	17,050,049–17,058,335	R	2187	728	79.76	7.48	10	N
AcARF18b	CEY00_Acc20780	PSS04920	18	18,316,935–18,325,018	R	2568	855	94.71	6.47	14	N
AcARF19a	CEY00_Acc21018	PSS02616	19	4,800,434–4,808,599	F	2745	914	101.27	6.75	14	N
AcARF19b	CEY00_Acc21307	PSS02927	19	12,175,957–12,185,263	R	1986	661	73.57	5.89	14	N
AcARF20a	CEY00_Acc22947	PSS01591	20	9,529,472–9,543,768	R	3342	1114	123.24	6.83	14	N
AcARF20b	CEY00_Acc23407	PSS02049	20	17,645,064–17,652,229	R	3225	1074	119.55	6.40	13	N
AcARF21a	CEY00_Acc24112	PSS00154	21	10,640,749–10,650,658	F	1764	587	63.87	6.04	2	N
AcARF21b	CEY00_Acc24323	PSS00355	21	14,345,431–14,360,209	R	2520	839	93.43	6.32	14	N
AcARF21c	CEY00_Acc23580	PSR99607	21	1,675,774–1,682,646	F	2823	940	104.44	5.53	14	N
AcARF22a	CEY00_Acc24765	PSR98167	22	8,326,523–8,330,541	F	2124	707	78.15	6.80	3	N
AcARF22b	CEY00_Acc25125	PSR98607	22	15,181,826–15,189,564	R	2190	729	79.57	7.24	10	N
AcARF22c	CEY00_Acc25233	PSR98710	22	16,436,617–16,444,355	R	2568	855	95.29	6.59	14	N
AcARF23a	CEY00_Acc25537	PSR94783	23	1,055,888–1,063,561	F	2124	707	78.90	6.88	14	N
AcARF23b	CEY00_Acc22108	PSR95973	23	16,703,082–16,708,820	R	2505	834	93.16	6.71	14	N
AcARF23c	CEY00_Acc26853	PSR96798	23	25,767,051–25,772,916	R	2076	691	77.32	7.22	12	N
AcARF24	CEY00_Acc27607	PSR92998	24	10,114,460–10,118,587	F	2013	670	73.80	7.33	4	N
AcARF25	CEY00_Acc28770	PSR91426	25	13,477,559–13,482,498	R	2046	681	76.07	6.24	14	N
AcARF26a	CEY00_Acc29618	PSR89417	26	9,438,365–9,443,808	F	2541	846	94.13	6.25	14	N
AcARF26b	CEY00_Acc30158	PSR89961	26	16,439,919–16,444,404	R	2115	704	78.05	7.42	4	N

Table 1 continued

Proposed name	Gene locus ID	Protein ID	Chromosome	Genome location	Strand	CDS (bp)	Deduced polypeptide			Exon No	Subcellular Loci
							Length (aa)	MW (kDa)	pI		
AcARF26c	CEY00_Acc30159	PSR89962	26	16,447,935–16,451,988	R	1890	629	69.61	7.44	2	N
AcARF26d	CEY00_Acc30181	PSR89984	26	16,706,738–16,714,589	F	2388	795	88.20	6.08	12	N
AcARF28a	CEY00_Acc32215	PSR86591	28	11,475,181–11,478,826	F	1347	448	49.72	8.10	4	C
AcARF28b	CEY00_Acc32235	PSR86611	28	11,709,582–11,718,194	F	2379	792	87.98	6.00	12	N
AcARF29	CEY00_Acc33209	PSR85224	29	13,156,570–13,166,125	R	3348	1115	123.53	6.78	14	N
AcARF30	CEY00_Acc33626	PSR82863	ps1sf1593	6027–10,001	R	2109	702	77.51	6.84	3	N

All AcARFs are listed. pI: isoelectric point; aa: amino acid; Chr: chromosome; MW: molecular weight. In column strand, R presents reverse stand and F presents forward stand. In column subcellular loci, N represents nucleus, while C represents Cytoplasm

ranged from 448 (AcARF28a) to 1115 (AcARF29) with 49.72 to 123.53 kDa MWs, respectively. The PI values ranged from 5.53 (AcARF21c) to 8.1 (AcARF28a), which indicates that different AcARF proteins might work in different microenvironments. All the AcARF proteins were located in the nucleus except AcARF10 and AcARF28a proteins, which were located in the cytosol (Table 1).

Alignment and phylogeny of the AcARFs

All the 41 predicted AcARFs proteins demonstrated characteristic ARF family structure, including a highly conserved B3-like DNA-binding and ARF_resp domain in the N-terminal region as per the sequence alignment and Pfam based protein motif analysis of the predicted proteins (Supplementary Fig. 1). ARFs can function as activators or repressors depending upon the amino acid composition of the MR. Also, according to the previous functional studies on Arabidopsis, ARF proteins containing glutamine (Q)-rich MR function as activators and rest of the ARF proteins function as repressors. Out of the 41 AcARF proteins, AcARF5, 12, 20b, and 29 showed Q-rich MR, which suggests that these proteins might function as transcriptional activators. In contrast, the other 37 AcARF proteins might function as repressors (Supplementary Fig. 1).

To characterize the evolutionary relationships between kiwifruit and Arabidopsis ARF family members, we carried out an NJ phylogenetic analysis of all the proteins of 41 AcARFs and 23 AtARFs (Fig. 1). As per the phylogenetic analysis, the ARFs were categorized into eight major groups (group 1–8) with well-supported bootstrap values. However, we found only one organism-specific group (group 4) from the phylogenetic analysis. In the common clades, we observed that IAAs were distributed unequally between two organisms. For instance, group 1 contained 11 and 3 members from kiwifruit and Arabidopsis, respectively, and group 2 contained 3 and 11 members from kiwifruit and Arabidopsis, respectively. The Arabidopsis ARF family was overrepresented in this class due to seven genes with tandem duplication, which encodes AtARF12-15 and AtARF20-22 proteins whose orthologous proteins were not found in kiwifruit. All transcriptional activators of *AcARF* genes were clustered in groups 6 and 7.

Chromosomal distribution of AcARF genes

The 41 *AcARF* genes were distributed across 23 chromosomes as per the in silico chromosomal mapping of the gene loci with 1–4 *AcARF* genes per chromosome (Fig. 2). These genes were primarily located on chromosomes 1, 4–14, 18–26, 28, and 29, whereas none of the *AcARF* genes were located on chromosomes 2, 3, 15–17, and 27.

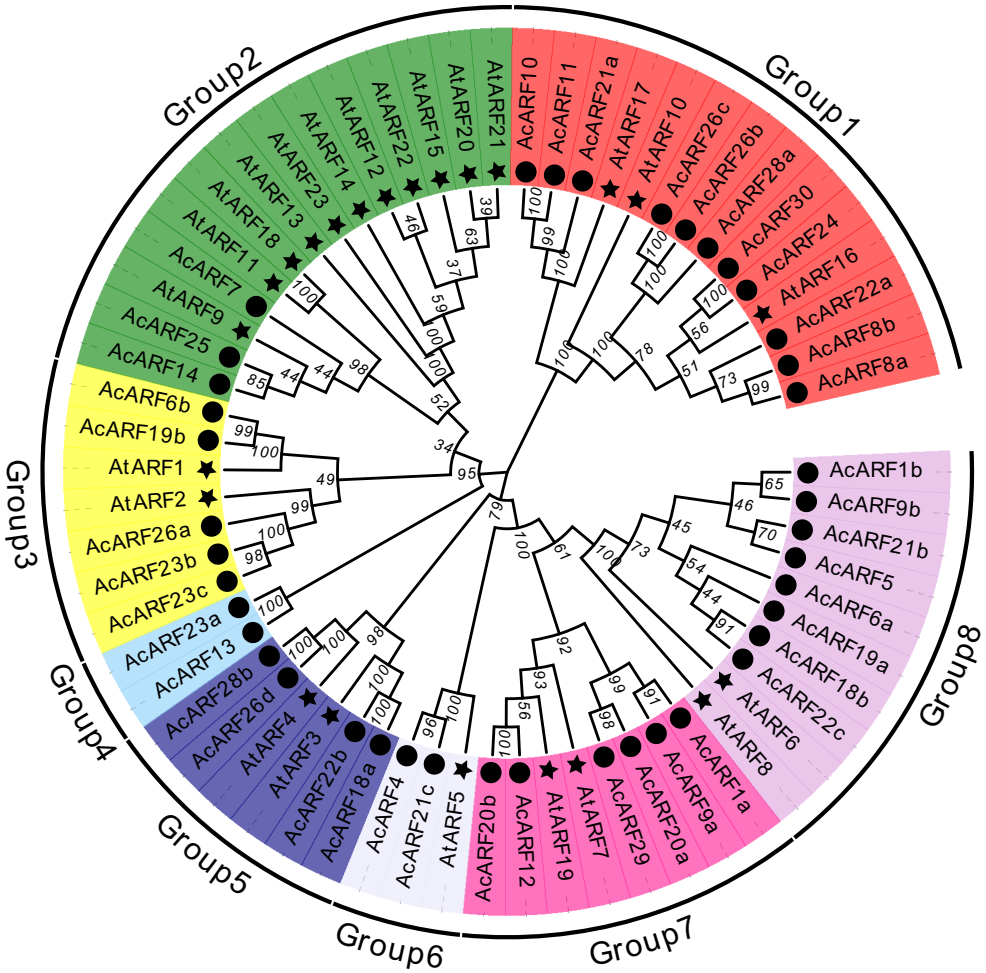

Fig. 1 Phylogenetic analysis of *A. thaliana* and *A. chinensis* ARFs. MEGA V7.0 software was employed to construct the NJ phylogenetic tree with 1000 bootstrap replicates based on the ARF amino acid sequences of *A. chinensis* and *A. thaliana*. Different colors represent different AcARFs and AtARFs groups. Black colored dots and stars indicate *A. chinensis* and *A. thaliana*, respectively. Branches are drawn to scale; the length of the branch represents the number of substitutions per site

Gene duplication and synteny analyses of the AcARF genes

To determine potential gene duplication within the kiwifruit genome, the duplication events including segmental and whole genome duplications throughout the evolutionary process of *ARF* gene family were analyzed. A total of 35 duplicated *AcARF* gene pairs were observed in *A. chinensis* genome (Fig. 3 and Supplementary Table 1). Out of 35 duplicated gene pairs, 33 were located on different chromosomes, which might be due to whole-genome duplication events, whereas two pairs (*AcARF23b* & *AcARF23c*, *AcARF8a* & *AcARF8b*) were located on the same chromosomes, which might be due to segmental duplication events. To gain a detailed understanding of the evolutionary constraints on *ARF* gene family, the Ka/Ks ratios of the *AcARF* gene pairs were analyzed (Table 2). The outcomes showed Ka/Ks < 1 for 15 duplicated *AcARF* gene pairs, which suggests that the kiwifruit *ARF* gene family had undergone selective pressure during evolution. Thus, we hypothesized that these gene duplication events in kiwifruit led to an increase in the *AcARF* gene family members with higher functional diversity. To further infer the phylogenetic mechanisms of *A. chinensis ARF* genes family, we constructed a comparative syntenic map of *A. chinensis* and *A. thaliana* (Fig. 4). It showed that a total of 18 *AcARF* genes have a syntenic relationship with *ARF* genes of *Arabidopsis* (Supplementary Table 2). It suggests that these genes may have played a crucial role in the evolution of the *ARF* gene family.

Conserved motifs and exon-intronic structures

Phylogeny and conserved motif analysis of AcARFs were performed to identify conserved regions (Fig. 5a). A total of 41 AcARFs with 8–15 conserved motifs were detected

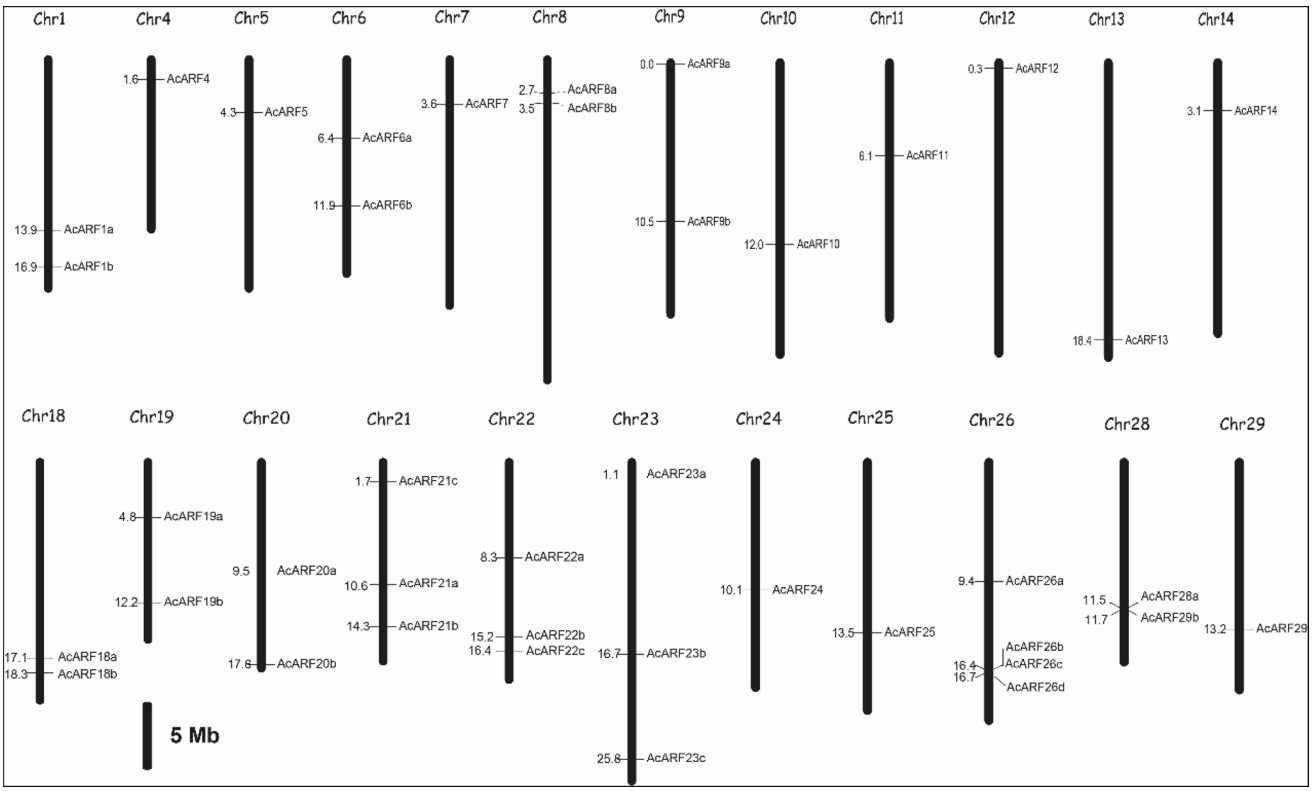

Fig. 2 Genomic localization of AcARF genes.The numbers at the head of each chromosome represent chromosome serial numbers. 41 AcARF genes were unevenly located on the chromosomes, and these genes were mapped based on the kiwifruit genome database using MapChart V2.2. The length of chromosomes is on the scale (Mb). The ARFs gene location is mentioned on the left while name of the right side

through this analysis (Fig. 5b and Supplementary Table 3). As per the conserved domains (Supplementary Fig. 1), motif 1 and 3 were identified as B3 conserved domain, motif 6 and motif 7 as Auxin_resp domain, and motif 10 as PB1 domain. Moreover, all the 41 AcARFs contain motifs 1, 3, 6, and 7. And, most of the AcARFs (30 / 41) proteins contain PB1 (AUX_IAA) domain except AcARF8b, 10, 11, 18a, 21a, 22a, 22b, 23c, 26b, 26c, and 28a. The structural analysis demonstrated that AcARFs contain 2–14 exons and 1–13 introns (Fig. 5c). Notably, the number of introns between the *AcARF* genes was different; however, the genes present in one cluster showed highly identical distributions of exons and introns. Motif composition, arrangements, and gene structures were in line with the phylogenetic tree (Fig. 5a). Furthermore, an in-depth structural analysis of exon–intron can unravel the evolution of the *AcARFs* gene family.

Putative cis-regulatory elements in the promoters of AcARFs

The interaction between cis-elements and the corresponding *trans*-acting factors modulates gene regulation. PlantCARE was used to predict the cis-regulatory elements in the promoter region of the *AcARF* genes to unravel the AcARFs mediated gene regulation. We found that promoter sequences of all the 41 *AcARF* genes contain several light-responsive elements. It indicates that AcARFs play a crucial role in kiwifruit morphogenesis. Besides, we found cis-regulatory elements related to hormonal stress (due to JA, SA, ABA, and GA), stress-responsive elements (anaerobic induction, defense, drought, and low temperature) in the promoter regions of the majority of the *AcARF* genes (Table 3). However, few *AcARF* genes showed tissue-specific elements (endosperm, meristem, and cell cycle), and circadian control elements.

Expression analyses of the ARF Genes in *Actinidia chinensis* organs

We investigated the spatial-specific expression pattern of the 41 AcARFs in six different organs, including roots, stems, leaves, flowers, small green fruit (SF) and ripe fruits (RF), to investigate the physiological functions of AcARFs (Fig. 6). All the AcARFs were detected in different organs and showed tissue-specific expression patterns in *Actinidia chinensis*. Most *AcARFs* showed higher stem and flower specific expression compared with other organs, except

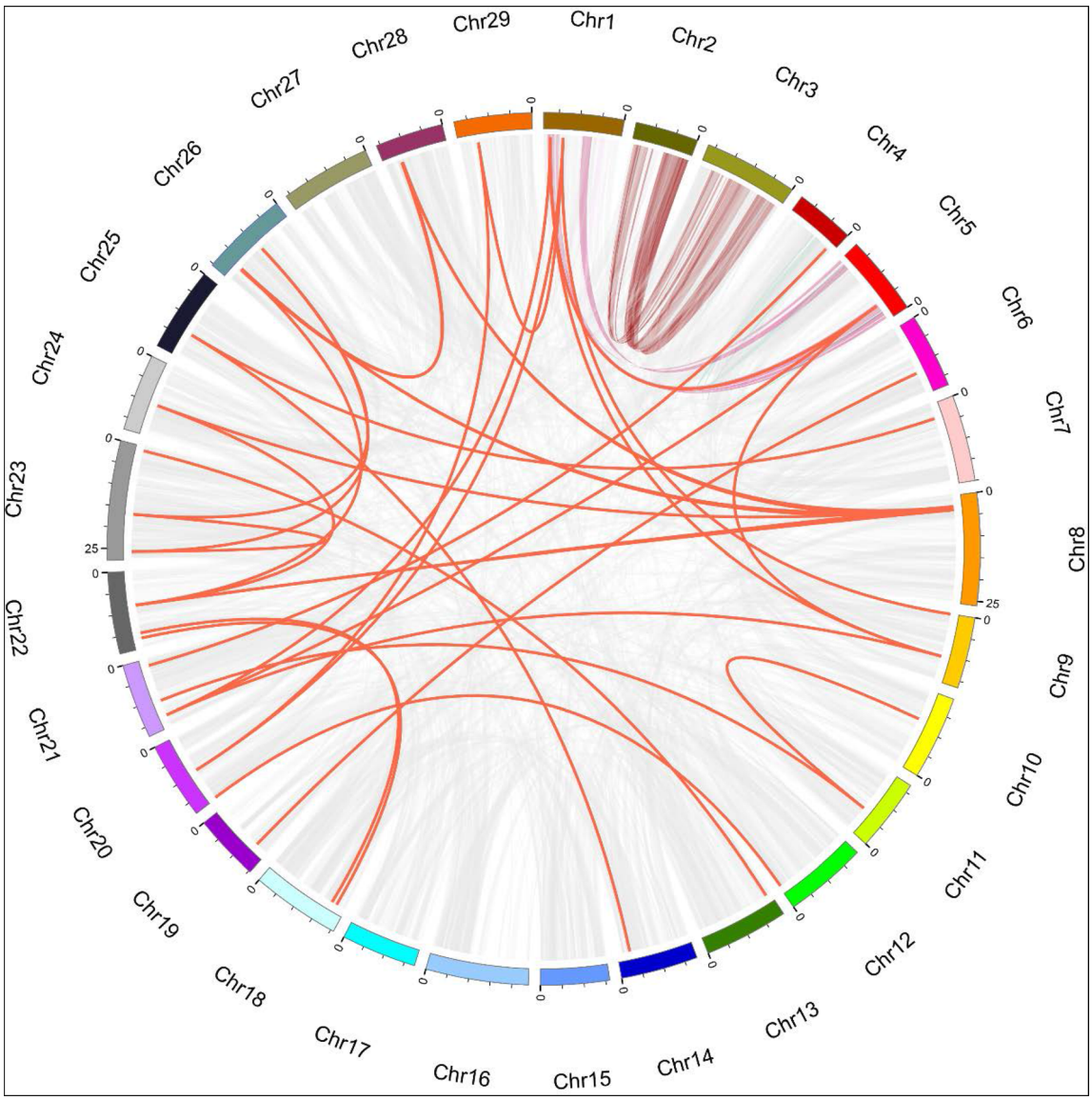

Fig. 3 Duplicated gene pairs in AcARF family. Circos was employed to demonstrate the syntenic relationships between AcARF genes. Colored bars represent the kiwifruit chromosomes. Pink lines linked the duplicated genes. The length of chromosomes is on the scale (Mb)

AcARF7 and *AcARF29*, whose expression levels were upregulated in leaf. *AcARF10* showed root-specific expression. *AcARF23a* and *AcARF23b* showed SF-specific expression, while *AcARF26d* showed RF-specific expression. Transcriptional analysis of the AcARFs showed tissue-specific expression in *Actinidia chinensis*, suggesting that *AcARF* genes might play distinct roles in different organs during kiwi fruit development.

The AcARFs expression patterns under various environmental and hormonal stress

To test the responsiveness of *AcAR*s to exogenous hormonal stimuli, we choose 12 ARF genes from different clusters and no collinear relationship between them as far as possible for expression profiling. So, we analyzed expression patterns of *AcARF* genes such as *AcARF1a*, *AcARF4*, *AcARF5*, *AcARF6a*, *AcARF7*, *AcARF10*, *AcARF18b*, *AcARF19a*, *AcARF23a*, *AcARF26a*, *AcARF28a*, *AcARF28b* in the kiwifruit leaves, 1, 6 and 12 h after IAA, ABA, GA, SA, MeJA treatment using qRT-PCR (Fig. 7 and Supplementary Fig. 2). The outcome of this analysis demonstrated that most of these *AcARF* genes were responsive to auxin except for *AcARF1a* and *AcARF23a*. Post-IAA treatment, *AcARF4, AcARF5*, *AcARF6a*, *AcARF10*, *AcARF19a*, *AcARF28a*, *AcARF28b* genes were down-regulated at different time-points, while *AcARF7*, *AcARF26a* and *AcARF18b* were up-regulated.

Table 2 Ka, Ks, and Ka/Ks calculation and divergent time of the duplicated *AcARF* gene pairs

Duplicated Gene Pairs	Ka	Ks	Ka/Ks	P-Value	D-Time (MYA)	Duplication Type
AcARF1a&AcARF9a	0.0387122	0.110494	0.350357	3.59E−12	3.68	WGD
AcARF1b&AcARF9b	0.0317694	0.0846606	0.375256	5.99 E−07	2.82	WGD
AcARF1b&AcARF21b	0.074508	0.41973	0.177514	4.80 E−61	13.99	WGD
AcARF1b&AcARF5	0.0750642	0.422097	0.177836	1.26 E−60	14.07	WGD
AcARF5&AcARF9b	0.0850916	0.402738	0.211283	4.73 E−51	13.42	WGD
AcARF13&AcARF23a	0.0504375	0.136242	0.370205	4.63 E−09	4.54	WGD
AcARF18a&AcARF22b	0.0419214	0.176157	0.237978	1.57 E−19	5.87	WGD
AcARF18b&AcARF22c	0.0411156	0.115764	0.355169	1.69 E−10	3.86	WGD
AcARF19b&AcARF6b	0.0274147	0.106419	0.257612	5.51 E−11	3.55	WGD
AcARF20a&AcARF29	0.0414775	0.118124	0.351135	2.03 E−13	3.94	WGD
AcARF21b&AcARF5	0.0209461	0.0947506	0.221066	1.95 E−13	3.16	WGD
AcARF21b&AcARF9b	0.0846204	0.402476	0.210249	1.68 E−50	13.42	WGD
AcARF21c&AcARF4	0.0399383	0.141847	0.281559	3.14 E−17	4.73	WGD
AcARF26d&AcARF28b	0.0338359	0.140912	0.240121	2.78 E−17	4.70	WGD
AcARF8a&AcARF8b	0.0323226	0.185864	0.173904	3.78 E−25	6.20	SD

The Ka/Ks Calculator V2.0 was used to determine Ka/Ks. D-Time: divergence time (million years ago); WGD: whole genome duplication; SD: segmental duplication

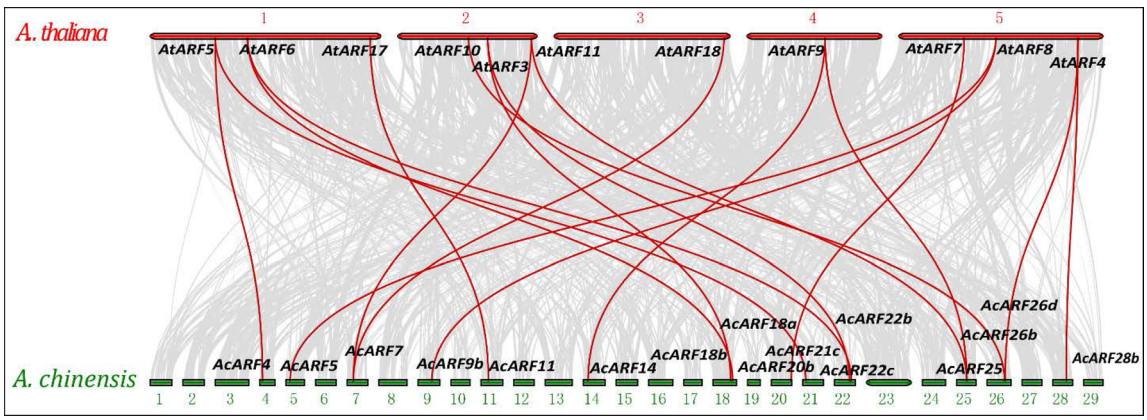

Fig. 4 The collinear correlation of the Aux/IAA between *A. thaliana* and *A. chinensis*. The green color represents 29 *A. chinensis* chromosomes, and the red color represents 5 *A. thaliana* chromosomes. Gray lines in the background indicate the collinear blocks between *A. thaliana* and *A. chinensis*, whereas the red lines indicate the syntenic ARF gene pairs between *A. thaliana* and *A. chinensis*

Interestingly, the *AcARF7* and *AcARF26a* were up-regulated by IAA, but they were down-regulated by ABA treatment. In contrast, the *AcARF4* gene was up-regulated by ABA at all the time-points and down-regulated by IA. *AcARF1a* gene was overexpressed 6 h post ABA treatment, and later its expression level decreased to the baseline level. The transcript levels of most of the genes such as *AcARF6a*, *AcARF7*, *AcARF23a* and *AcARF28b* increased significantly post GA treatment, whereas *AcARF26a* and *AcARF28a* gene expression was first down-regulated and later reverted to normal. SA treatment induced the down-regulation of *AcARF1a* and *AcARF10* genes at all time points. JA treatment induced the upregulation of *AcARF6a* and *AcARF28a* after 6 h post treatment, while *AcARF18b* was up-regulated at all time points. The diverse *AcARF* gene expression pattern in response to different hormonal treatments indicates the complexity of auxin-regulated gene expression.

To explore the expression patterns of *AcARF* genes in kiwifruit under drought and salt stress, the expression pattern of 12 *AcARF* genes was investigated through qRT-PCR (Fig. 8 and Supplementary Figs. 3 and 4). The outcome of this analysis showed that drought stress induces the upregulation of *AcARF1a*, *AcARF5*, *AcARF7*,

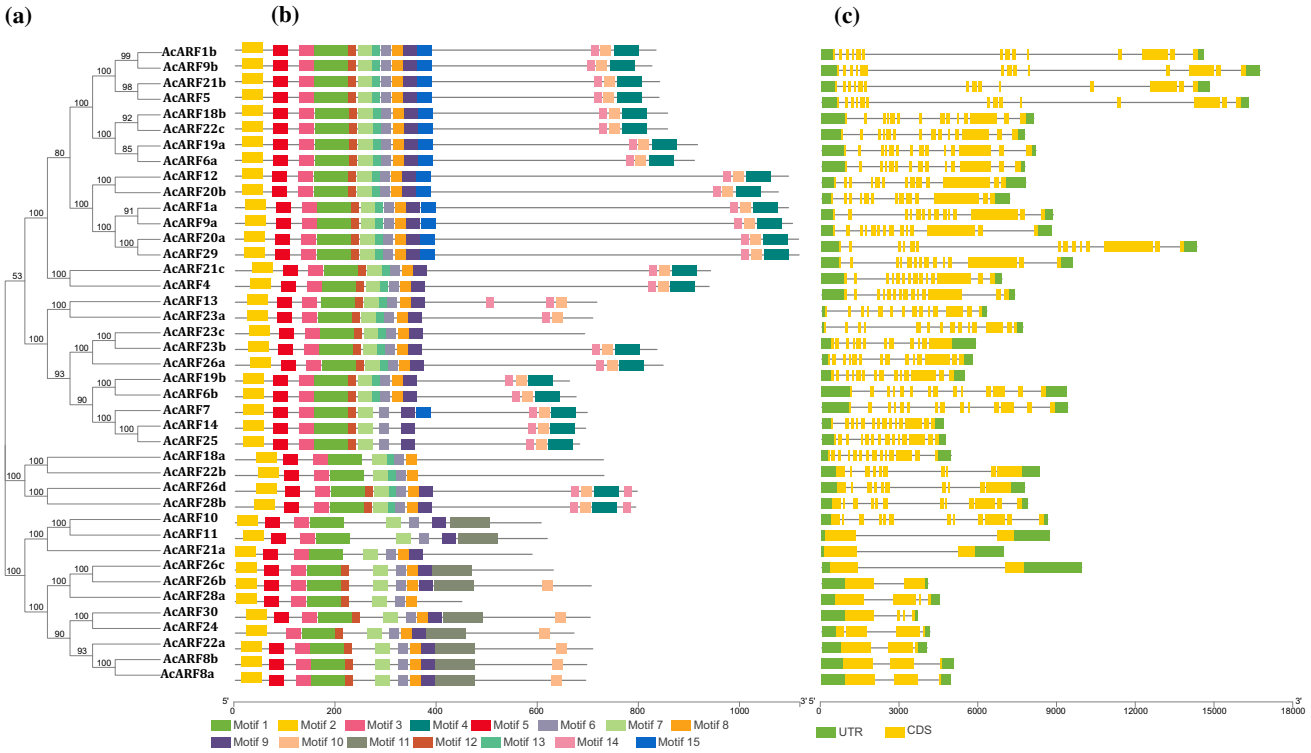

Fig. 5 Phylogeny, motifs, and exon-intronic structures of AcARFs. (**a**) Unrooted NJ phylogeny of AcARFs. MEGA V7.0 was employed to construct the phylogenetic tree based on full-length AcARFs amino acid sequences (bootstrap = 1000, Poisson model) with a length corresponding to the number of substitutions per site. (**b**) MEME was used to determine the conserved motifs of AcARFs. Motifs and their positions (1–15) are represented by the colored boxes at the bottom. The scale bar denotes the length of the amino acid sequence (Supplementary Table 1). (**c**) GSDS was used to depict the structure of AcARF genes. Exons (yellow box); UTR (green); scale bar represents the length of the respective DNA sequences

AcARF19a, *AcARF26a*, *AcARF28a*, and *AcARF28b* genes. As demonstrated in Fig. 8 and Supplementary Fig. 3, *AcARF1a*, *AcARF19a*, *AcARF26a*, *AcARF28a*, and *AcARF28b* genes were up-regulated throughout different time points, while the transcription levels of *AcARF5* and *AcARF7* increased only after 168 h post-drought stress induction. Besides, once the water supply was restored, the expression level of these genes reverted to normal. Under salt stress, the *AcARF26a* gene was up-regulated across all the time-points, while *AcARF4*, *AcARF5*, *AcARF23a*, and *AcARF28a* genes were up-regulated 12 h, 24 h and 48 h post salt stress induction (Fig. 8 and Supplementary Fig. 4).

Discussion

Auxin plays a crucial role in the growth and development of plants. ARFs are vital components of the auxin signaling pathway; thus, they can regulate the transcription of auxin-responsive genes involved in the majority of plant-growth stages, development, and stress responses (Tiryaki 2009). Thus, to elucidate the role of kiwifruit' ARFs in specific auxin responses, we used *A. chinensis* genome and performed a genome-wide comprehensive survey of *ARF* gene family in kiwifruit. In this study, 41 kiwifruit *ARF* genes were identified and designated as per their respective chromosomal location (Fig. 2). The number of *AcARF* genes in kiwifruit was more as compared to other species, such as Arabidopsis (23), tomato (22), and rice (25) due to a higher number of gene duplication events (Supplementary Tables 4). Synteny analysis showed that 35 *AcARF* gene pairs underwent gene duplication events via the segment and whole genome duplication mechanism (Fig. 3), and 15 duplicated *AcARF* gene pairs had Ka/Ks < 1. Comparative genomic analysis of the kiwifruit and Arabidopsis *AcARF* genes validated gene duplication events (whole genome duplication) in at least 18 orthologous *AcARF* genes (Fig. 4). Segmental and whole-genome duplication events are vital for the gene family's expansion (Li et al. 2015b). The phylogenetic analysis of kiwifruit and Arabidopsis showed that kiwifruit AcARFs have orthologs in Arabidopsis except for two specific genes, AcARF13 and AcARF23a, in Group 4 (Fig. 1).

In this study, protein domain analysis revealed that kiwifruit AcARFs contain highly conserved DNA binding

Table 3 Putative cis-regulatory elements in the promoters of AcARFs. The promoters (1500 bp prior to the start codon, ATG) of the *AcARF* genes were obtained from Ensembl Plants. The cis-elements were predicted by PlantCARE

Promoter	Light responsive	Circadian control	Zein metabolism regulation	Hormone response					Stress response				Tissure specific		
				Abscisic acid	Auxin	Gibberellin	MeJA	Salicylic acid	Anaerobic induction	Defense	Drought	Low temperature	Meristem	Endosperm	Cell cycle
AcARF1a	+			+				+	+		+			+	
AcARF1b	+		+	+	+	+	+				+	+	+		
AcARF4	+			+					+				+		
AcARF5	+				+				+						
AcARF6a	+					+	+								+
AcARF6b	+				+	+					+				
AcARF7	+			+	+	+	+		+		+				
AcARF8a	+	+		+		+	+		+		+		+	+	
AcARF8b	+			+		+		+		+	+				
AcARF9a	+					+			+		+	+		+	
AcARF9b	+			+	+		+		+	+		+		+	
AcARF10	+				+			+						+	
AcARF11	+						+	+						+	
AcARF12	+	+		+			+		+			+			
AcARF13	+					+			+						
AcARF14	+			+	+	+	+	+	+			+	+		
AcARF18a	+	+							+			+			
AcARF18b	+			+	+		+		+	+					
AcARF19a	+			+	+				+		+		+		
AcARF19b	+				+		+				+				
AcARF20a	+			+	+				+		+	+	+		
AcARF20b	+						+		+					+	
AcARF21a	+		+	+		+	+						+		+
AcARF21b	+	+	+	+			+								
AcARF21c	+		+	+			+		+	+		+			
AcARF22a	+			+	+				+						
AcARF22b	+						+	+	+						
AcARF22c	+	+		+	+		+			+					+
AcARF23a	+			+		+		+	+						
AcARF23b	+	+		+		+	+	+					+		
AcARF23c	+			+					+			+	+		
AcARF24	+	+		+	+		+	+		+					

Table 3 continued

Promoter	Light responsive	Circadian control	Zein metabolism regulation	Hormone response					Stress response				Tissure specific			
				Abscisic acid	Auxin	Gibberellin	MeJA	Salicylic acid	Anaerobic induction	Defense	Drought	Low temperature	Meristem	Endosperm	Cell cycle
AcARF25	+		+	+	+		+		+	+		+			
AcARF26a	+			+	+	+	+					+	+		
AcARF26b	+				+	+	+	+	+		+			+	
AcARF26c	+			+	+	+		+	+						
AcARF26d	+				+		+	+	+				+		
AcARF28a	+			+		+	+	+	+				+		
AcARF28b	+				+	+			+	+	+			+	
AcARF29	+										+				
AcARF30	+	+		+	+		+	+	+			+			

domain along with plant-specific B3-type and an ARF subdomain, which binds explicitly to auxin response elements (AuxRE: TGTCTC) in the promoter region of auxin response genes (Fig. 5). ARFs were categorized into two groups: transcriptional activators and repressors, as per the amino acid composition of MR. The average activator/repressor ratio of AcARFs was 0.1, which was less than that of Arabidopsis (0.59), rice (0.56), tomato (0.27). The CTD showed the presence of two motifs, domains III and IV, which are also found in Aux/IAA. These motifs facilitate the formation of homodimers and heterodimers between ARFs and Aux/IAAs. The percentage of CTD-truncated AcARF (13.67%) was similar to that of Arabidopsis (17.39%), but it was lower than other species, such as tomato (28.57%), rice (24%), and sweet orange (21.05%). The AcARFs without CTD suggest that some auxin-responsive genes in kiwifruit are regulated in an auxin independent manner. Also, the cis-element analysis confirmed that most of the *ARF* gene promoter sequences contain hormonal stress response-related cis-regulatory elements, tissue-specific, and stress response elements (Table 3).

Expression patterns of AcARFs were analysed in different organs using qRT-PCR to study their physiological functions (Fig. 6). Most AcARFs showed higher expression levels in the leaf and stem, which indicated their differential roles during kiwifruit development. *AcARF23a* and *AcARF23b* showed preferential expression in small fruit, suggesting their important roles during early fruit development. Also, we observed higher transcription levels of *AcARF26b* from the small fruit to the ripe fruit stage and higher expression throughout the fruit ripening. This study showed AcARFs have organ-specific expression patterns, and the function of them also needs to be further studied.

Kiwifruits are recurrently exposed to abiotic stresses, such as drought, cold, salinity, defense, and hormonal stimulation during the fruiting and various developmental stages. Previous studies have reported that ARF as transcriptional regulators are directly involved in Auxin signal transposition (Ljung 2013; Guilfoyle and Hagen 2007), and stress response, and hormonal cross-talks (Guilfoyle and Hagen 2007; McAtee et al. 2013). In the current study, we selected 12 *AcARF* candidate genes to determine their response in stresses induced by five distinct phytohormones along with salt and drought stress. In the present study, we found that all the 12 candidate *ARF* genes were responsive to exogenous hormone and abiotic stresses in a time-dependent manner. According to the cis-elements, we found that some ARF-induced expression results confirmed the results of promoter element analysis, for example, AcARF1a, 5, 7,10, 28b, induced by exogenous hormones. However, some ARF-induced expression patterns were in line with promoter element analysis; for

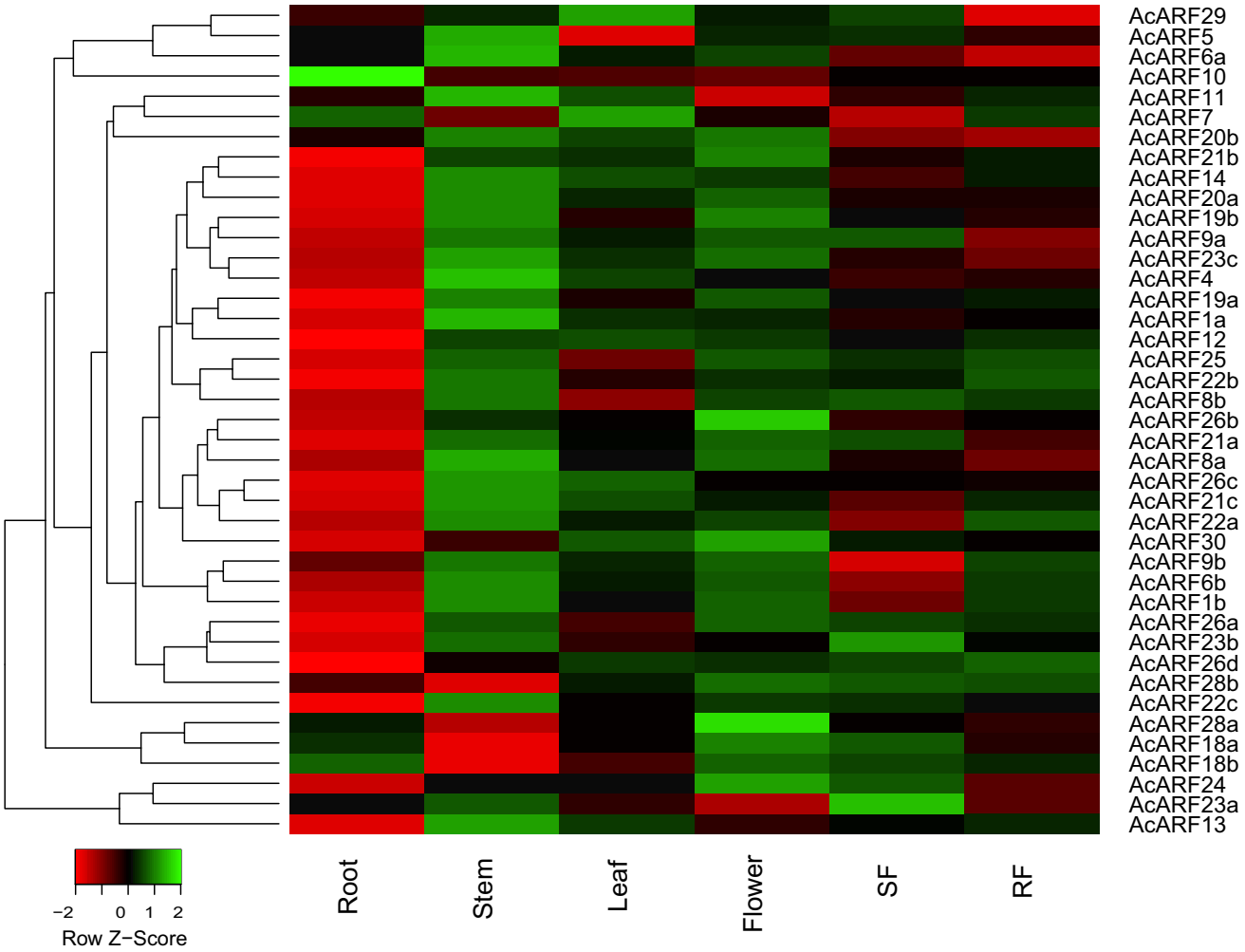

Fig. 6 Heatmap of AcARFs' transcriptional profiles in different organs. The log2 values of the relative expression data were used for the heatmap visualization. The relative expressions of individual AcARFs were normalized to *ACT 2* gene in different tissues, including root, stem, leaf, flower, small green fruit (SF, approximately 30 days after flower), and ripe fruit (RF, approximately 120 days after flower). Green blocks and red blocks represent downregulated and upregulated transcription levels, respectively

instance, AcARF4, 28a, lacking IAA promoter response elements were significantly down-regulated by IAA; 18b, 19a, 23a promoters containing ABA elements were not affected by the ABA treatment. The differential expression patterns of 12 *AcARF* genes indicated that all these genes respond to abiotic stress, which suggests their distinct functions during the kiwi fruit development as far as the stress response is concerned. The current study provides a foundation for an in-depth investigation of ARF functions, specifically the ARF-mediated hormonal cross-talks during the fruit growth, development, and stress responses.

Conclusions

This is the first study of which we are aware to have comprehensively profiled and annotated ARF expression profiles in kiwifruit in response to different stresses. Herein, we were able to identify a total of 41 AcARFs in the kiwifruit genome, which allowed us to explore the functional significance and evolutionary development of this gene family. Notably, the AcARFs expression patterns suggested organ-specific expression patterns and the complex inducible involvements of these genes in abiotic stresses and signaling. These findings present new prospects for a detailed study of the precise functions of AcARFs. It highlights the correlations between the AcARFs expression and stress response; besides, the data could facilitate the screening of ARFs for in-depth

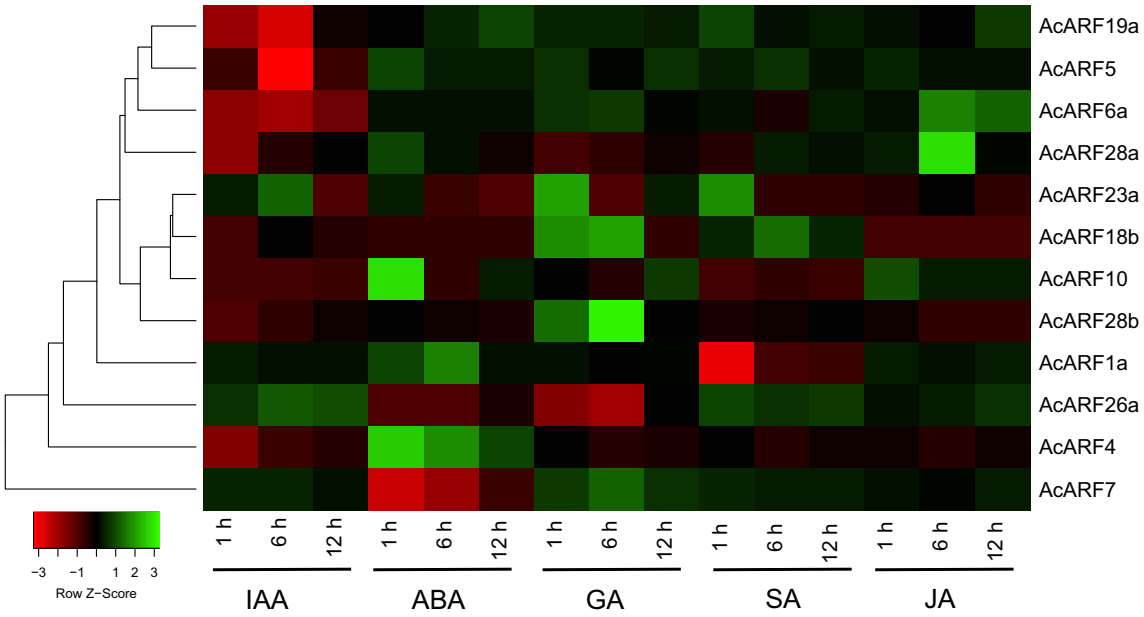

Fig. 7 Heatmap showing differential expression patterns of 12 AcARFs under hormonal stress. Transcription levels at 0 h (untreated plant) were taken as an internal control (mean value = 0) to determine the relative mRNA abundance of each gene. The *ACT 2* gene was used as an internal standard. The experiments were performed in triplicates, and the mean expression values from three independent experiments were used for the heatmap construction

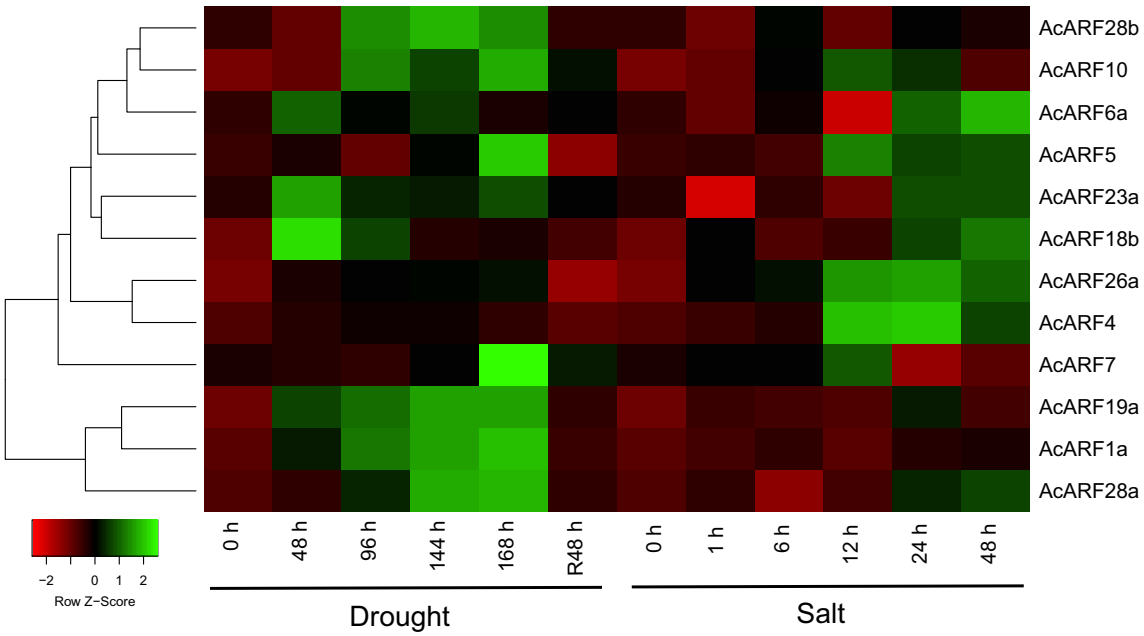

Fig. 8 Heatmap showing differential expression of 12 AcARFs under drought and salt stresses. The annotation is the same as Fig. 6

functional characterization and genetic improvement of kiwifruit's agronomic traits.

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Author contributions Conceptualization, QL and LS; methodology, MX, YL, JD, YW and LS; software, LS; formal analysis, LS; resources, LS; data curation, LS. and QL; writing—original draft preparation, LS; writing—review and editing, QL; funding acquisition, LS and QL; All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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