

Article

# **Genome-Wide Identification of the** *Auxin Response Factor (ARF)* **Gene Family and Their Expression Analysis during Flower Development of** *Osmanthus fragrans*

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**Abstract:** Auxins have long been implicated in many aspects of plant growth and development. Auxin response factors (ARFs) are important proteins in auxin-mediated pathways and they play key roles in plant physiological and biochemical processes, including flower development. Endogenous indoleacetic acid (IAA) levels were measured and ARFs were studied in the flowers during the developmental stages in order to further elucidate the role of auxin in flower development of *Osmanthus fragrans*. A systematic analysis of *OfARFs* was conducted by carrying out a genome-wide search of *ARFs*. A total of 50 *ARF* genes (*OfARFs*) were detected and validated from the *Osmanthus fragrans* genome. Furthermore, a comprehensive overview of the *OfARFs* was undertaken, including phylogenetic relationship, gene structures, conserved domains, motifs, promoters, chromosome locations, gene duplications, and subcellular locations of the gene product. Finally, expression profiling, while using transcriptome sequencing from a previous study and quantitative real-time PCR (qRT-PCR), revealed that many *OfARF* genes have different expression levels in various tissues and flower developmental stages. By comparing the expression profiles among the flower developmental stages, and the relationship between *ARFs* and endogenous IAA levels, it can be supposed that *OfARFs* function in flower development of *O. fragrans* in an auxin-mediated pathway.

Keywords: auxin response factors; Osmanthus fragrans; auxin; flower development

# 1. Introduction

Flowering is a crucial developmental stage in the life cycle of plants, especially for ornamental flowering plants [1]. Previous studies have indicated that flowers react differently to the various endogenous phytohormone types and levels. For example, the levels of indole-3-acetic acid (IAA), gibberellic acid (GA<sub>3</sub>), and zeatin riboside (ZR) are downregulated, while the levels of abscisic acid (ABA) are upregulated during florescence and senescence in the petals of *Paeonia suffruticosa* [2]. Auxins, a class of phytohormone previously reported to be an essential factor in plant growth and development, play a pivotal role in the development process of flowers, especially in floral primordium initiation and



flower formation [3,4]. Great progress has been made in elucidating the mechanisms of auxin biology, including biosynthesis, polar transport, and signal transduction [5–7]. While considering the research basis of *O. fragrans*, exploring the auxin-mediated gene expression pathway, which is not studied in this species, should be beneficial for analyzing the function of auxin in regulating the flowering process.

Several gene families have been reported to be involved in the transcriptional regulation of auxin responses, including the auxin/indole-3-acetic acid (*Aux/IAA*), the Gretchen Hagen3 (*GH3*), the small auxin-up RNA (*SAUR*), and the auxin response factor (*ARF*) families. These early auxin-responsive genes are characterized by conserved promoter elements, including the TGA element (AACGAC), the auxin response element (AuxRE, TGTCTC), and the core element of the auxin response region (AuxRR-core, GGTCCAT) [8–10]. Auxin response factors (ARFs), which are important elements in the auxin-mediated gene transcription pathway, are transcription factors that regulate the auxin response by interacting with Aux/IAAs proteins [11]. A typical ARF protein contains three domains, including the conserved N-terminal B3-like DNA-binding domain (DBD), which recognizes the auxin response region in the promoter area, the C-terminal dimerization domain (CTD), which facilitates the protein-protein interactions among the ARFs, and the middle region (MR), which is located between DBD and CTD, acting as a transcriptional activator or repressor, depending on its amino acid composition [12].

The functions of *ARFs* have been well-studied in the model plant *Arabidopsis thaliana*. Generally, *AtARF1* and *AtARF2* influence flower initiation, stamen development, and flower senescence [13]. *AtARF4* controls flower patterning [14]. The mutation of *AtARF5* affects embryo, root, and shoot formation [15], whereas *AtARF6* and *AtARF8* control the maturation of the stamen and gynoecium, respectively [16]. *AtARF7* and *AtARF19* double mutations caused impaired hypocotyl and root development [17]. To sum up, the ARF proteins play key roles in the growth and development process of plants.

Osmanthus fragrans Lour., which is a popular evergreen woody tree, is well-known for its fragrant flowers [18]. It has been widely used as an ornamental landscape plant in China, as well as in South Korea, Thailand, India, and the USA [19–21]. However, the short flowering period of only 6–10 days severely affects its ornamental value [22]. Previous studies that are related to O. fragrans flowers have mainly focused on floral scent, floral color, and flowering period [23–25]. However, there have been only limited studies regarding the transcriptional pathway of flower development [26]. Therefore, the deciphering of the auxin-mediated transcriptional pathway in O. fragrans is urgently required, as this should help explain and solve the issue of the short flowering period. Recently, research on ARFs has been reported in many annual species, e.g., Zea mays [27], Capsicum annuum [28], Solanum lycopersicum [29], and some woody plants, including Prunus mume [30], Citrus sinensis [31], and Populus *trichocarpa* [32]. However, ARFs have not been reported from *O. fragrans*, which is one of the most important ornamental flowering species in the world. A chromosome-level reference genome of O. *fragrans* was constructed with the help of Nextomics Bioscience Institute, Wuhan, China in 2018. A total of over 118 Gb of polished reads was generated from HiSeq (45.1 Gb) and PacBio Sequel (73.35 Gb), giving 10× depth coverage for long reads. The combination of Illumina-short reads, PacBio-long reads, and Hi-C data produced the final chromosome quality genome of O. fragrans with a genome size of 727 Mb and a heterozygosity of 1.45%. Finally, the genome was annotated while using de novo and homology comparison, and further refined with transcriptome data. The recently published genome project of O. fragrans provides a powerful resource with which to investigate the expression and function of individual genes during O. fragrans flower development [33]. For most non-model organisms, the reference genome was sometimes not perfect, and there would be some incorrect annotations, such as start/stop errors. The genes were finally reconstructed and optimized based on the transcriptome sequencing information.

In this study, all of the potential *OfARFs* were identified on a genome-wide scale. Subsequently, the gene structures, domain distributions, and motif types, associated with each *OfARF*, were determined. The phylogenetic relationships among the identified *OfARFs* and between *OfARFs* and *AtARFs* 

were elucidated. Finally, the endogenous concentrations of IAA at different flowering periods were determined, and then correlated with the expression profile of *OfARF* genes in various *O. fragrans* tissues, including the flower, during the developmental process, with the goal of providing a sound basis for analysis of the auxin-mediated pathway in flowers and to further functional studies of *OfARF* genes.

# 2. Materials and Methods

# 2.1. Identification of ARF Genes in Osmanthus fragrans

A systematic identification of the ARF family members (*OfARFs*) in *O. fragrans* was conducted while using the full-length Arabidopsis ARF (AtARF) proteins previously achieved as initial BLAST queries out of the *O. fragrans* Genomic Database with the e-value of "10<sup>-3</sup>" [33,34]. Moreover, the ARF Hidden Markov Model (HMM), Pfam 06507, ARF (AUX\_RESP), was also employed to hmmsearch the ARFs from the *O. fragrans* genome, with the e-value of "10<sup>-3</sup>" [35]. Furthermore, the HMMSCAN (http://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) and NCBI Conserved Domain Search Service (CDSearch) (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) tools were used to further confirm the position and integrity of the detected conserved domains in individual *OfARFs* [36,37]. The sequences with broken B3, AUX\_RESP, or AUX/IAA domains were deleted. The number of amino acids, molecular weight (MW), and theoretical isoelectric point (pI) of the *OfARFs* were obtained from the online ProtParam tool (http://www.expasy.org/tools/protparam.html) [38].

## 2.2. Analysis of Gene Structures, Conserved Domains and Subcellular Localizations

The gene structure details of *OfARFs* were obtained from the *O. fragrans* genome database [33]. The conserved domains in all of the identified *OfARFs* were analyzed and confirmed while applying the Pfam tool (http://pfam.sanger.ac.uk/) [39]. The gene structure information and conserved domain distributions of *OfARFs* were then drawn while using TBtools [40]. The subcellular localization predictions of the *OfARFs* were performed on the database Subcellular Localization Predictor (CELLO v.2.5; http://cello.life.nctu.edu.tw) [41].

# 2.3. Chromosomal Localization, Gene Duplication and Evolution Analysis of OfARF Genes

The *OfARF* chromosomal locations were obtained from the *O. fragrans* Genomic Database [33], and then drafted, using MapInspect software (http://www.plantbreeding.wur.nl/uk/softwaremapinspect. html). The OfARF protein sequences were compared to themselves with the Blast Compare program in Tbtools, with the e-value of  $1 \times 10^{-5}$ , NumOfHits of 5, NumOfAligns of 5. Synteny analysis of *OfARFs* was conducted with the blast result and the *O. fragrans* gff file while using Quick MCScanX Wrapper program in TBtools. The graph of collinearity analysis was drawn by Amaizing Super Circos in TBtools software. Finally, the tool Circos were applied to locate *OfARFs*, and draft duplication relations of the 23 chromosomes in *O. fragrans* [42].

#### 2.4. Conserved Motifs and Promoter Cis-acting Regulatory DNA Elements Analysis

The 12 conserved protein motifs were obtained while using the MEME program (http://memesuite.org/tools/meme) and the optimum motif width was set to 6–200. The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was employed to analyze the cis-acting regulatory elements of *ARF* genes in *O. fragrans* [43].

#### 2.5. Phylogenetic Analysis of OfARF Genes

The *Arabidopsis* ARF (AtARF) protein sequences were downloaded from the online PlnTFDB as described in a previous report [34,44]. The OfARF proteins were sequence aligned by the DNAMAN (v8.0) software with its default settings. The sequences of *O. fragrans* and *A. thaliana* were aligned while using the Muscle program that was built in MEGAX software. The phylogenetic relationship tree was

constructed and drafted with MEGAX, using the neighbor-joining method [45]. The bootstrap was set with 1000 replicates. Additionally, two separate phylogenetic trees were constructed to explore the relationships among the *OfARFs* themselves and between *OfARFs* and *AtARFs*. The images of phylogenetic trees were drawn while using Fig Tree v1.4.2 (http://tree.bio.ed.ac.uk/). The gene structures of *OfARFs* were predicted using TBtools software with GFF3 file and ID numbers of *OfARFs*.

#### 2.6. Plant Material Collection, RNA extraction and cDNA Synthesis

In the present study, the flower samples were collected from a mature *O. fragrans* 'Rixianggui' on the campus of Nanjing Forestry University, Nanjing, Jiangsu Province, China. All of the samples were collected and immediately frozen in liquid nitrogen and then stored at −80 °C until further use. The flower developmental process in cultivar Rixianggui generally goes through the following stages of flower development: linggeng stage (S1), bud eye stage (S2), initial flowering stage (S3), full flowering stage (S4), and late flowering stage (S5). The total RNAs were extracted while using the TRNzol Universal Reagent (TIANGEN Biotech Co. Ltd., Beijing, China), according to the manufacturer's instructions [46]. The RNA quantity and quality were evaluated while using Agilent Bioanalyzer 2100 (Agilent Technologies, USA) and NanoDrop<sup>TM</sup> One UV-Vis spectrophotometer (Thermo Fisher Scientific, USA), and Qubit<sup>®</sup> 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA synthesis was completed while using TransScript one-step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing, China), following the manufacturer's guidelines [47].

#### 2.7. Expression Analysis of OfARF Genes

Transcriptome sequencing was applied to assess the expression levels of 50 OfARF genes in roots (young and mature), stems, leaves, and flowers at three developmental stages (initial, full, and late flowering stages). The TBtools software was used to generate the heatmap, while using transcriptome data obtained from a previous study [33]. Expression level was determined according to reads per kilobase of transcript per million mapped reads (RPKM). RPKM values of OfARF genes were normalized by log2 transform. The edgeR package (http://www.r-project.org/) was used to identify differentially expressed genes (DEGs) across initial flowering stage (S1), full flowering stage (S2), and full flowering stage (S3) flower samples. Identified DEGs were then used for enrichment analysis of GO functions. All DEGs were searching against GO terms in the Gene Ontology database (http://www.geneontology.org/). Numbers of the genes were calculated for every term. Enriched GO terms in DEGs when comparing to the genome background were defined by hypergeometric test. Furthermore, in order to verify the expression profiles of OfARF genes at different developmental stages of O. fragrans flowers, twelve OfARF genes from Class II, Class III, and Class IV were selected for quantitative real-time PCR (qRT-PCR) analysis. The genes were selected based on the RPKM values and the phylogenetic relationship of 50 Osmanthus fragrans auxin response factors (OfARFs) and 23 Arabidopsis thaliana auxin response factors (AtARFs). OfARF11a, 12, 13, 14a, and 14b were homologous genes of AtARF1 and AtARF2. OfARF10a and OfARF10b were homologs of AtARF4. OfARF21a, 22a, 22b, 24a, and 24b were homologs of AtARF6 and AtARF8. The gene-specific primers that were used in qRT-PCR were designed while using Primer Premier 5 software (Supplementary Table S1). The OfACT gene (forward, 5'-CCCAAGGCAAACAGAGAAAAAAT-3'; reverse, 5'-ACCCCATCACCAGAATCAAGAA-3'), which was the optimal reference gene for different floral developmental stages in O. fragrans, was used as an internal control in each qRT-PCR experiment [48]. The qRT-PCR was performed, while using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Realtime PCR Master Mix. The total reaction volume was 10 µL, which included 5 µL of SYBR Mix, 3 µL of ddH2O, 1 µL of cDNA sample, 0.4 µL of each gene-specific forward and reverse primer, and 0.2 µL of ROX Reference Dye. The PCR conditions were, as follows: polymerase activation for 3 min. at 94 °C, and then 20 s at 94 °C for 40 cycles, followed by 20 s at 62 °C, and finally 40 s at 72 °C. The amplification specificity of the primers was tested by the melting curve method. Three biological and three technical replicates were

conducted for each sample in the qRT-PCR system. The comparative cycle threshold (44Ct) and the  $2^{-\Delta\Delta CT}$  method were used to evaluate the relative expression level of all twelve genes. The error bars represent the standard error from the three biological replicates. Analysis of variance (ANOVA) was used to detect significant differences within the experiment, with pairwise multiple comparisons to identify significant differences between the samples being determined with Duncan's multiple range test at *p* < 0.05; analyses were carried out while using SPSS v.24 (IBM, Armonk, NY, USA) [46]. In the transcriptome analysis, there were three flowering stages (initial, full, and late flowering stages) (Figure S3). However, five flower stages, including linggeng stage (S1), bud eye stage (S2), initial flowering stage (S3), full flowering stage (S4), and late flowering stage (S5-1 & S5-2), were used in the qRT-PCR process in order to analyze the expressing profiles of *ARFs* in the whole flower development process (Figure S4).

#### 2.8. IAA Content Measurement

The flower samples were collected, and the surfaces of them were washed three times in deionized water. The samples were then blotted dry with a clean paper towel and weighed. After the addition of 500 pg of the  ${}^{13}C_{6}$ -IAA internal standard (MSD, ISOTOPES, Quebec, Canada), five independent biological replicates, each representing a 50-mg sample, were purified while using a ProElu C18 column (http://www.dikma.com.cn). The IAA concentrations were determined by separation and detection with FOCUS GC-DSQII single quadrupole GC–MS (Thermo Fisher Scientific Inc., USA) [49].

#### 3. Results

#### 3.1. Identification of the OfARF Gene Family

Two strategies were employed to comprehensively identify the ARFs in O. fragrans: BLASTP using the 23 full-length Arabidopsis ARF (AtARF) protein sequences previously identified as queries [34], and HMM search with the AUX\_RESP HMM model (PF06507) against the O. fragrans genome database. HMMSCAN (http://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) and NCBI Conserved Domain Search Service (CDSearch) (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) were applied to ensure the position and integrity of the detected conserved domains [38]. The sequences with broken domains (B3, AUX\_RESP, or AUX/IAA) were deleted. Finally, a total of 50 ARF genes were identified in O. fragrans, including 39 complete genes and 11 truncated genes. The entire collection of OfARFs that were identified in this study was named OfARF1 to OfARF29b, based on the homology relationship that was shown in the phylogenetic tree. The coding sequence (CDS) lengths of the OfARF genes ranged from 999 bp to 3327 bp, with deduced proteins of 332-1108 amino acids. The molecular weights (MW) of the 50 OfARF proteins were predicted to range from 37.46 to 122.67 kDa (Table 1). The theoretical isoelectric points (pI) were predicted to range from 5.22–9.05. Among them, seven were basic proteins, and the left forty-three were acidic proteins. The instability indexes were predicted to range from 41.46–68.19, which suggested that all of the proteins were unstable proteins. The grand averages of hydropathicity (GRAVY) values of them were all negative, which suggested that they were all hydrophilic proteins.

#### 3.2. Phylogenetic Analysis and Gene Structure of OfARFs

A phylogenetic tree was constructed by aligning all the 50 OfARF and 23 AtARF protein sequences, while using the neighbor-joining method, to detect phylogenetic relationships. It was revealed that the 50 OfARF proteins were broadly distributed into four classes, namely Class I, Class II, Class III, and Class IV, with well-supported bootstrap values (Figure 1). Among all of the OfARFs, Class IV had the largest number (16) of OfARFs. In contrast, there were only four members in Class II, with Class I and Class III each containing fifteen members (Figure 2A). The exon-intron components of the genes were obtained by comparing the cDNA sequences with the corresponding genomic DNA sequences to compare the structure of the different *OfARF* genes (Figure 2B). The number of exons varied from 2 to

15 (Table 1). Generally, the members that shared similar gene structures tended to be distributed in the same subfamilies.

Gene Name	Gene ID	Exon No. <sup>a</sup>	Len <sup>b</sup> (aa)	MW <sup>c</sup> (kDa)	pI <sup>d</sup>	Instability Index	GRAVY <sup>e</sup>	Conserved Domain	Homologous
OfARF1a	Contig151.75	4	702	77.19	7.29	48.43	-0.305	B3, ARF, Aux/IAA	AtARF16
OfARF1b	Contig73.170	6	760	83.87	7.82	46.64	-0.289	B3, ARF,	AtARF16
OfARF2	Contig328.11	4	685	74.8	5.81	40.99	-0.305	B3, ARF, Aux/IAA	AtARF16
OfARF3a	Contig281.61	4	687	75.86	7.91	50.00	-0.322	B3, ARF, Aux/IAA	AtARF16
OfARF3b	Contig592.12	5	646	71.52	6.55	46.00	-0.248	B3, ARF, Aux/IAA	AtARF16
OfARF4a	Contig37.40	3	674	74.86	6.43	47.95	-0.370	B3, ARF, Aux/IAA	AtARF16
OfARF4b	Contig295.42	2	601	66.52	6.36	42.06	-0.428	B3, ARF	AtARF16
OfARF5a	Contig275.68	4	704	77.28	6.48	44.24	-0.283	B3, ARF, Aux/IAA	AtARF16
OfARF5b	Contig10.361	3	690	76.3	6.69	47.23	-0.332	B3, ARF, Aux/IAA	AtARF16
OfARF6a	Contig86.172	3	681	75.78	6.02	48.21	-0.374	B3, ARF, Aux/IAA	AtARF16
OfARF6b	Contig12.215	2	677	75.43	7.25	43.42	-0.407	B3, ARF,	AtARF10
OfARF7a	Contig84.135	2	552	60.82	5.25	47.13	-0.355	B3, ARF	AtARF17
OfARF7b	Contig77.21	2	552	60.85	5.95	46.36	-0.281	B3, ARF	AtARF17
OfARF8a	Contig38.67	2	372	42.26	6.8	41.46	-0.257	B3, ARF	AtARF17
OfARF8b	Contig38.68	2	478	53.89	6.06	42.24	-0.343	B3, ARF	AtARF17
OfARF9a	Contig26.229	11	692	75.99	6.33	60.34	-0.451	B3, ARF	AtARF3
OfARF9b	Contig172.11	11	726	79.71	6.92	48.75	-0.367	B3, ARF	AtARF3
OfARF10a	Contig151.50	13	753	84.46	6.05	51.77	-0.432	B3, AKF, Aux/IAA	AtARF4
OfARF10b	Contig73.186	12	787	87.64	5.97	48.99	-0.409	Aux/IAA	AtARF4
OfARF11a	Contig324.17	14	659	73.98	5.84	65.97	-0.551	B3, AKF, Aux/IAA	AtARF1
OfARF11b	Contig97.3	14	653	73.33	5.76	64.99	-0.525	Aux/IAA	AtARF1
OfARF12	Contig194.92	14	665	74.53	6.08	61.36	-0.520	Aux/IAA	AtARF1
OfARF13	Contig76.252	14	842	94.36	6.28	56.97	-0.568	Aux/IAA	AtARF2
OfARF14a	Contig6.38	14	828	92.59	6.06	61.49	-0.549	Aux/IAA	AtARF2
OfARF14b	Contig2.130	14	838	93.5	6.02	64.87	-0.609	Aux/IAA	AtARF2
OfARF15	Contig105.88	10	332	37.46	7.26	55.57	-0.436	B3, ARF	AtARF9
OfARF16a	Contig317.63	14	658	74.16	6.7	57.20	-0.550	B3, ARF, A11x/IAA	AtARF9
OfARF16b	Contig153.78	12	411	46.67	6.77	60.92	-0.372	B3, ARF	AtARF9
OfARF17a	Contig54.9	14	666	74.97	6.67	56.90	-0.414	B3, ARF, Aux/IAA	AtARF9
OfARF17b	Contig21.49	14	634	71.54	6.33	49.95	-0.476	B3, ARF, Aux/IAA	AtARF9
OfARF18	Contig354.37	15	720	81.77	9.05	50.43	-0.551	B3, ARF, Aux/IAA	AtARF18
OfARF19	Contig389.17	14	685	76.95	6.06	52.79	-0.436	B3, ARF, Aux/IAA	AtARF18
OfARF20a	Contig267.9	14	680	76.3	6.26	55.17	-0.516	B3, ARF, Aux/IAA	AtARF18
OfARF20b	Contig390.3	14	686	77	6.19	55.97	-0.514	B3, ARF, Aux/IAA	AtARF18
OfARF21a	Contig314.6	14	893	98.79	6.11	67.35	-0.362	вз, AKF, Aux/IAA	AtARF6
OfARF21b	Contig236.41	14	893	98.5	5.74	65.08	-0.339	B3, ARF, Aux/IAA	AtARF6
OfARF22a	Contig109.86	14	890	98.99	6.2	67.24	-0.454	вз, ARF, Aux/IAA	AtARF6

**Table 1.** *Osmanthus fragrans* auxin response factor (*OfARF*) genes encoding auxin response factor (ARF) proteins, along with the gene and protein molecular details.

Gene Name	Gene ID	Exon No. <sup>a</sup>	Len <sup>b</sup> (aa)	MW <sup>c</sup> (kDa)	pI <sup>d</sup>	Instability Index	GRAVY <sup>e</sup>	Conserved Domain	Homologous
OfARF22b	Contig154.52	14	898	99.9	6.07	68.19	-0.462	B3, ARF, Aux/IAA	AtARF6
OfARF23	Contig72.89	15	763	86.2	6.16	61.71	-0.456	B3, ARF, Aux/IAA	AtARF8
OfARF24a	Contig189.73	14	838	93.16	6.04	57.06	-0.445	B3, ARF, Aux/IAA	AtARF8
OfARF24b	Contig165.100	14	839	93.65	6.04	57.64	-0.478	B3, ARF, Aux/IAA	AtARF8
OfARF25a	Contig158.77	14	935	103.58	5.35	56.60	-0.440	B3, ARF, Aux/IAA	AtARF5
OfARF25b	Contig16.261	15	809	90.34	5.22	56.51	-0.292	ARF, Aux/IAA	AtARF5
OfARF26a	Contig183.139	13	1026	113.47	6.16	61.78	-0.567	B3, ARF, Aux/IAA	AtARF7
OfARF26b	Contig259.21	13	1028	113.72	6.16	62.17	-0.573	B3, ARF, Aux/IAA	AtARF7
OfARF27	Contig248.99	11	390	43.46	8.26	64.10	-0.282	B3, ARF	AtARF7
OfARF28a	Contig312.37	14	1091	120.3	6.07	56.21	-0.516	B3, ARF, Aux/IAA	AtARF7
OfARF28b	Contig116.35	14	1078	119.52	6.17	56.52	-0.512	B3, ARF, Aux/IAA	AtARF7
OfARF29a	Contig132.116	15	964	106.71	6.44	58.78	-0.555	B3, ARF, Aux/IAA	AtARF7
OfARF29b	Contig8.81	14	1108	122.67	6.3	57.79	-0.553	B3, ARF, Aux/IAA	AtARF7

Table 1. Cont.

<sup>a</sup>: Nuber of exons; <sup>b</sup>: length of proteins; <sup>c</sup>: molecular weight; <sup>d</sup>: the theoretical isoelectric points; <sup>e</sup>: the grand average of hydropathicity.



**Figure 1.** Phylogenetic relationship of 50 *Osmanthus fragrans* auxin response factors (*OfARFs*) and 23 *Arabidopsis thaliana* auxin response factors (*AtARFs*).





**Figure 2.** Phylogenetic relationship, gene structure, and conserved motif analysis of the *Osmanthus fragrans* auxin response factor (*OfARF*) gene family. (**A**) Phylogenetic analysis of OfARF proteins. A total of 50 OfARF proteins from *O. fragrans* were used to construct the neighbor-joining tree. (**B**) Gene structure analysis of *OfARFs*. The lengths of coding sequences (CDSs) and introns of each *OfARF* gene are displayed proportionally. The black blocks represent CDSs and black lines represent introns. (**C**) Conserved motif analysis of OfARF proteins. The B3, AUX\_RESP, and AUX\_IAA domains are marked in brown, orange, and green, respectively.

### 3.3. Analysis of Conserved Domains of Osmanthus fragrans Auxin Response Factor (OfARF) Proteins

The domains of OfARF protein sequences were aligned and analyzed while using the online Pfam tool (http://pfam.sanger.ac.uk/) and SMART database (http://smart.embl-heidelberg.de/). Three conserved domains, namely B3, AUX\_RESP, and AUX\_IAA, were identified and drafted into four classes according to the phylogenetic tree (Figure 2C). All of the OfARF proteins, except OfARF7a–OfARF9b, OfARF15, OfARF16b, and OfARF17, contained a C-terminal Aux/IAA domain, which was consistent with AtARF3, 13, and 17. However, when compared with *A. thaliana*, OfARF25b showed an absence of DNA-binding domains (DBDs), which indicated that there were some differences between *A. thaliana* and *O. fragrans* ARF genes.

# 3.4. Motif Characterization and Promoter Analysis of Osmanthus fragrans Auxin Response Factor (OfARF) Proteins

The online software MEME was employed to investigate the motif distributions in all 50 OfARF proteins, with 12 individual motifs identified, to further study the characteristic regions of OfARF proteins. Among all the motifs, motifs 2, 3, 6, and 7 were found in all 50 OfARF proteins. As predicted, those with common motif compositions came mostly from the same subfamily from the phylogenetic

tree, which suggests functional similarities among the close homologs (Figure 3). By comparing with the conserved domain distribution, it was concluded that motif 4 and 5 belong to B3 domain, motif 7 and motif 8 belong to Auxin\_resp domain, and motif 11 and 12 belong to Aux/IAA domain.



Figure 3. Motif distribution of the Osmanthus fragrans auxin response proteins (OfARFs).

Previous studies indicated that AtARF1, 2, 3, 4, and 9 act as transcriptional repressors [13,14], which have middle regions rich in threonine, serine, and proline residues, whereas AtARF5, 6, 7, 8, and 19 were transcriptional activators [15–17], with middle regions that were rich in glutamine residues. The detailed sequence analysis of all 50 OfARF proteins identified threonine-, serine-, and proline-rich middle regions in OfARF9a, 9b, 10a, 10b, 11a, 11b, 12, 13, 14a, 14b, 20, and 21, indicating that they are likely to be repressors (Figure 4). The glutamine-rich middle regions were detected in OfARF25a, 25b, 21a, 21b, 23, 24a, 24b, 26a, 26b, and 27, indicating that they are probably activators of the *O. fragrans* flower development process.



**Figure 4.** Multiple sequence alignment of the *Osmanthus fragrans* auxin response factor (OfARF) proteins obtained with DNAMAN 8.0. Conserved domains of OfARF proteins are marked by the overline.

Putative promoter sequences (2000-bp upstream of the 5'UTR (untranslated region) of *OfARFs* were isolated from the *O. fragrans* genome sequence. Several auxin response elements were detected through searching with the PlantCARE database. Three auxin response elements (two TGA-boxes and one AuxRR element) were found in the promoter region of the *OfARF4a* gene, and three auxin response elements (two TGA-elements and one AuxRR element) were detected in *OfARF4a*. Two TGA-elements were detected in *OfARF2* and *OfARF24b*, whereas two AuxRRs were discovered in *OfARF7b*. Only one

TGA-element was detected in each of 10 other *OfARF* genes, and one AuxRR element was present in *OfARF23* (Figure 5). However, no auxin response elements were detected in 34 *OfARF* genes.



Figure 5. Promoter analysis of Osmanthus fragrans auxin response factor genes (OfARFs).

The locations of 50 *OfARF* genes were demonstrated on *O. fragrans* chromosomes by using the BLASTN search. All of the genes were distributed over 20 out of the 23 *O. fragrans* chromosomes, with none being found on chromosomes 02, 15, and 20. Each of the 20 chromosomes carried one to five *OfARF* genes. The largest number of *OfARF* genes was located on chromosome 03 (five), followed by chromosome 12 and 13 (four), with three genes being distributed on chromosomes 01, 03, 05, 10, 11, 14, 17, and 18 (Figure 6). A collinearity analysis was performed while using MCScanX software to reveal the duplication events that occurred within the *OfARF* genes. Thirty-three fragment duplication gene pairs were found among the 50 *OfARFs*. Fragment duplication genes were the most abundant on chromosome 03, followed by chromosomes 07 and 13, whereas there were no pairs of fragment duplication genes on chromosomes 09 and 23 (Figure 7).

#### 3.5. Subcellular Localization Analysis of OfARFs

The online tool CELLO V2.5 was utilized to predict the subcellular localization of *OfARFs* (http//cello.life.nctu.edu.tw/). To detect the subcellular localization, the full-length coding sequences of OfARFs were employed. Only OfARF3a, OfARF7b, and OfARF8a were predicted to be located in the chloroplast, while all of the remaining 47 proteins were predicted to be located in the nucleus (Supplementary Table S2). The prediction results were consistent with the function of being transcription factors as expected, which should regulate the expressions of the downstream genes.

#### 3.6. Expression of OfARF Genes in Different Tissues and Various Flower Development Stages of O. fragrans

The spatial-specific expression profiles of the 41 *OfARF* genes were determined in different tissues (nine genes not expressed), namely roots, stems, leaves (young and mature), and flowers based on data from the sequencing of transcriptomes that were performed in the previous study (Figure 8). The expression of most *OfARF* genes occurred in almost all tissues, which indicated that they might have various functions in different aspects of plant physical and biological events.



**Figure 6.** Distribution of auxin response factor genes (*OfARFs*) on *Osmanthus fragrans* chromosomes. The chromosome number is indicated at the left of each chromosome. The scale is in bases Chr01–Chr23 are abbreviations for chromosome numbers 01–23, and blue rectangles refer to gene pairs of tandem duplications.



Figure 7. Gene duplication analysis among 50 Osmanthus fragrans auxin response factor genes (OfARFs).



**Figure 8.** Heatmap of *Osmanthus fragrans* auxin response factor (*OfARF*) gene expression in three flower developmental stages and various tissues. Levels of down expression (green) or up expression (red) are shown on a log2 scale from the lowest to the highest expression of each *OfARF* gene. Reads per kilobase of transcript per million mapped reads (RPKM) values of *OfARF* genes were normalized by log<sub>2</sub> transform and the heatmap was constructed by TBtools software.

After differentially expressed genes (DEGs) analysis, 28 DEGs were found in S1 vs. S2 with 12 (OfARF1a, 5a, 5b, 14b, 18, 21a, 22a, 22b, 26b, 27, 28a, 28b) upregulated and 16 (OfARF1b, 3a, 6a, 7b, 9b, 10a, 10b, 11b, 13, 14a, 15, 17a, 24a, 24b, 25b, 29b) downregulated, 33 DEGs were found in S2 vs. S3

with 14 (OfARF2, 4a, 4b, 5b, 7b, 12, 13, 14a, 16a, 24b, 25b, 27, 28a, 28b) upregulated and 19 (OfARF1a, 5a, 6a, 6b, 7a, 9b, 10a, 10b, 11a, 14b, 15, 16b, 17a, 18, 22b, 24a, 25a, 29a, 29b) downregulated, and 35 DEGs were found in S1 vs. S3 with 15 (OfARF2, 4a, 4b, 5b, 7b, 12, 13, 14a, 21a, 22a, 25b, 26b, 27, 28a, 28b) upregulated and 20 (OfARF1a, 1b, 3a, 6a, 6b, 7a, 9a, 9b, 10a, 10b, 11a, 15, 16b, 17a, 17b, 24a, 24b, 25a, 29a, 29b) downregulated, respectively (Figure S1). These identified DEGs were then used for enrichment analysis of GO functions. In S1 vs. S2, the most enriched term in "biological process" was "response to stimulus" (Counts = 27), the most enriched terms in "cellular component" was "cell", "cellular anatomical entity", and "intracellular" (Counts = 26), and the most enriched term in "molecular function" was "binding" (Counts = 26). In S2 vs. S3, the most abundant term in "biological process" was "cellular process" (Counts = 29), the most abundant term in "cellular component" was "cellular anatomical entity", (Counts = 28), and the most abundant term in "molecular function" was "binding" (Counts = 29). In S1 vs. S3, the most enriched term in "biological process" was "cellular process" (Counts = 30), the most enriched term in "cellular component" was "cellular anatomical entity" (Counts = 30), and the most enriched term in "molecular function" was "binding" (Counts = 29). Totally, the most enriched term in "molecular function" of the three groups was "binding", which is in accordance with the functions of the ARFs that functions by binding to the downstream genes (Figure S2).

The expression profile of *ARF* genes in flower development, as previously reported, as well as the role of auxin in controlling flower development, prompted us to analyze the expression of *OfARF* genes during the *O. fragrans* flower development process [2–4]. The transcript abundance level of individual *OfARF* genes was determined at five developmental stages of *O. fragrans* flowers by qRT-PCR to investigate the expression profiles. The results indicated that the expression of all twelve genes tested was down-regulated from stage S1 to stage S3, and it was markedly up-regulated from stage S3 to stage S5. Similar expression patterns for all twelve *OfARF* genes indicate that they play important roles during flower developmental processes in *O. fragrans* (Figure 9).



**Figure 9.** Expression profiles of 12 *Osmanthus fragrans* auxin response factor genes (*OfARFs*) by qPCR. The flower development process in cultivar Rixianggui was marked by stages S1 (linggeng stage), S2 (bud eye stage), S3 (initial flowering stage), S4 (full flowering stage), and S5 (late flowering stage). Changes in the expression levels during different flower developmental stages depicted above are relative to RNA accumulation levels. The error bars represent the standard error from three biological replications. Significant differences detected with Duncan's multiple range test at *p* < 0.05 are indicated by different lowercase letters above the error bars.

After comparison, some differences would easily been seen between the expression profiles in the heatmap and qRT-PCR results. This is perhaps due to the different flower stage distributions between the transcriptome analysis and the qRT-PCR. In the transcriptome analysis, there were three flowering stages (initial flower, full flower, and late flower) (Figure 3). However, there were five flower stages: Linggeng stage (S1), bud eye stage (S2), initial flowering stage (S3), full flowering stage (S4), and Late lowering stage (S5-1 & S5-2) (Figure 4). The initial stages in the transcriptome analysis should be equivalent to the stages of S1, S2, and S3 in qRT-PCR, and the full flower stages should be equivalent to the stage of S4 in qRT-PCR, and late flowering stages should be equivalent with the stage of S5-1 in the qRT-PCR. For example, the expression profiles of *OfARF10a*, *10b* in the heatmap showed a slow decline from the initial flowering stage to the full flowering stage, but a sharp decline from the full flowering stage to the full flowering stage, but a sharp decline from the full flowering stage) were higher than the levels in the initial flowering stage, and there should be a sharp increase of expression level during the later time of the late flowering stage.

#### 3.7. Endogenous Indoleacetic Acid (IAA) Concentration Measurement

Endogenous IAA levels were measured to examine the role of auxin concentration in different stages of flower development. The data showed that the endogenous IAA levels were higher in the flowers at the early stages of development than in the flowers at later stages. Specifically, the endogenous IAA level declined from S1 to stage S3, and then rose significantly from S3 to S5 (Figure 10).



**Figure 10.** The indoleacetic acid (IAA) concentrations at different stages of *Osmanthus* S1 (linggeng stage), S2 (bud eye stage), S3 (initial flowering stage), S4 (full flowering stage), and S5 (late flowering stage). The error bars represent the standard error from three biological replications. Significant differences detected with Duncan's multiple range test at p < 0.05 are indicated by different lowercase letters above the error bars.

#### 4. Discussions

Auxins play an important role in the development of plant reproductive organs, especially the flowers, as has been previously reported. These organs react differently to the diverse levels of auxins. It was hypothesized that the endogenous IAA concentration affected flower development, so the endogenous IAA concentration in the flowers was measured at different development stages in this study [4]. The *O. fragrans ARFs* were identified and characterized in order to investigate the putative functional genes involved during the development stages of flowering.

The auxin-mediated pathway is mainly comprised of the products of two gene families, the ARFs and the Aux/IAAs. As an important element in the auxin-signaling pathway, ARFs bind directly to

downstream target genes and regulate their expression during flower development, and they are also involved in the reproduction of various plant species [50]. The most widely accepted model is that Aux/IAA inhibits transcriptional activities of ARF proteins by dimerizing with ARFs and by recruiting additional proteins that can inhibit gene expression. When auxin concentration increases in the cell, the ubiquitin-proteasome pathway degrades Aux/IAA proteins. Thus, the ARF protein is available to homodimerize with itself or to heterodimerize with another ARF protein to regulate transcription [51]. Identification and analysis of *OfARFs*, as achieved in the current study, should allow us to reveal the mechanisms operating in auxin-mediated flower development of *O. fragrans*.

In the current study, the recently developed reference genome database of *O. fragrans* was employed to identify the members of the *OfARF* gene family [33]. The number of *OfARF* genes (n = 50) was greater than that of *AtARF* genes (n = 23), *Oryza sativa ARF* genes (n = 25), and *Populus trichocarpan ARF* genes (n = 39). Protein domain analysis provided useful information on the biological function of ARFs. A typical ARF contains three domains, namely DBD, MR, and CTD. Aux/IAAs bind to CTDs and form heterodimers. The presence of numerous *OfARFs* without CTDs indicated that some auxin-response genes in *O. fragrans* might be regulated in an auxin-independent way.

A phylogenetic tree was built to analyze the relationship of ARFs between O. fragrans and A. thaliana. The results showed that six sister gene pairs with high bootstrap values ( $\geq$ 99%) were identified between O. fragrans and Arabidopsis, which suggested that OfARFs were highly homologous to AtARFs. Many AtARFs have been already been reported, which reveals useful information on the respective biological functions in O. fragrans. In Arabidopsis, transcription factors AtARF1 and AtARF2 have been reported to function in flower initiation and senescence. Of ARF11a, 12, 13, 14a, and 14b, which were homologous genes of AtARF1 and AtARF2, were highly expressed in floral development stages S1 and S5, indicating that they play key roles in early flower formation and floral senescence, whereas *AtARF4* was previously reported to be involved in flower patterning [14]. *OfARF10a, OfARF10b,* which are both homologs of *AtARF4*, showed high expression levels in the early flower developmental stages. AtARF8 regulates a complex process by promoting petal growth, filament elongation, and flower bud opening [16]. The expression of OfARF24a and 24b, homologs of AtARF8, showed the greatest expression levels at the initial flowering stage, indicating a putative function in flower initiation. In Solanum lycopersicum, SIARF1-10 and 14-17 have been reported to function in regulation of cell division and enlargement during flower development [29]. In Carica papaya L., CpARF1 and CpARF2 have been reported to participate in flower bud formation. Furthermore, CpARF1, CpARF2, and CpARF4-related auxin expression regulation was decreased, while CpARF6-mediated auxin expression regulation was activated in the mature flowers [52].

It is well known that auxins may play important roles in the early flower development stages [4]. However, there are still no reports that endogenous IAA plays roles in O. fragrans flower development. The developmental pathways that are regulated by ARF proteins have been reported in a previous study [51,53]. In the absence of auxin, ARF proteins bind to Aux/IAA proteins that inhibit transcription by chromatin remodeling. Auxins induce Aux/IAA turnover, freeing ARF proteins to activate transcription. In the current study, the endogenous IAA concentrations decreased from S1 to S3, and then increased from S3 to S5, which suggested that a high level of endogenous IAA contributes to both the initiation and senescence of flowers. ARF activity is inhibited at lower auxin levels, which results in the repression of auxin-responsive genes, so that the ARF is released and can regulate the transcription of its target auxin response genes when the auxin levels are higher, as has been previously reported [4]. The transcript abundance level of *OfARF* genes was measured at five developmental stages of *O. fragrans* flowers. The results indicated that all twelve selected genes were down-regulated from S1 to S3 and they were markedly upregulated from S3 to S5. Similar expression patterns for all twelve Of ARF genes indicated that they might play important roles during flower developmental processes in O. fragrans. The relationship between the IAA levels and ARF gene expression profiles during flower development showed a significant positive correlation, which is in accordance with the

earlier hypothesis of a positive link between IAA concentration, flower development, and *ARF* gene expression (Supplementary Table S3).

### 5. Conclusions

This is the first study on the genome-wide identification and analysis of the ARF family members in O. fragrans. The comprehensive analysis of the ARF gene family in O. fragrans has revealed structural characterization, molecular evolution, expression profiling, and functional properties of the OfARFs, especially the functions that are related to flower development. Studies of the phylogenetic relationship and chromosomal distribution of the OfARF genes have provided valuable insights into the evolutionary aspects of the O. fragrans genome. Expression analysis showed that all twelve selected genes might play important roles during flower developmental processes in O. fragrans. The relationship between the IAA levels and ARF gene expression profiles during flower development showed a significant positive correlation, especially OfARF11a, 12, 13, and 14a, which could be taken as the target candidate genes for the future transgenic improvement. The results of an expanded expression analysis of all identified OfARF genes relative to endogenous IAA concentration will help to focus molecular genetic studies, leading to a better understanding of the functions of the OfARF genes in O. fragrans and future practical applications of this knowledge, such as extending the flowering period of O. fragrans. The comprehensive identification and subsequent characterization of OfARF genes that are described above would provide new insight into the potential role of some ARF genes in mediating O. fragrans flower development via the auxin-mediated pathway.

**Supplementary Materials:** The following materials are available online at http://www.mdpi.com/1999-4907/11/ 2/245/s1. Figure S1: differentially expressed genes (DEGs) across S1, S2, and S3 flower samples; Figure S2: A: Level2 GO terms of differentially expressed genes (DEGs) across S1 and S2 flower samples; B: Level2 GO terms of differentially expressed genes (DEGs) across S2 and S3 flower samples; C: Level2 GO terms of differentially expressed genes (DEGs) across S1 and S3 flower samples; Figure S3: Flower stages in the heatmap; Figure S4: Flower stages in the qRT-PCR process; Table S1: Specific primers used for qPCR; Table S2: Subcellular locations predicted for OfARF proteins; Table S3: Correlation analysis between *ARF* gene expression profiles and IAA concentrations.

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