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Aux/IAA gene family identification and analysis reveals roles in flower opening and abiotic stress response in *Osmanthus fragrans*

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Abstract

The *Aux/IAA* (*auxin/indole-3-acetic acid*) gene family plays a crucial role in regulating various aspects of plant growth, development, and abiotic tolerance in the auxin transduction pathway. However, limited information is available about the *Aux/IAA* family in *Osmanthus fragrans*. This study aims to comprehensively analyze the *Aux/IAA* gene family on a genome-wide scale. A total of 39 *OfIAA* genes containing four conserved domains were identified. These genes were unevenly distributed across 19 chromosomes and grouped into six clades based on phylogenetic analysis, showing conserved gene structure and motif composition. The expansion of *OfIAA* genes in the *O. fragrans* genome was partially due to segmental duplication events. Analysis of *cis*-regulatory elements (CREs) in the promoters of the *OfIAA* genes revealed the presence of many CREs related to different hormones and abiotic stresses. Through transcriptome and expression pattern analysis, we found that the majority of *OfIAA* genes were expressed in the stem tissue. Moreover, during the flower opening process, 18 *OfIAA* genes exhibited differential expression, while three and 11 *OfIAA* genes, respectively, showed altered expression patterns after salt and drought treatments. These differentially expressed genes are likely involved in the regulation of flower opening and abiotic stress response. This study provides new insights into the potential roles of *OfIAAs* and contributes to a better understanding of the regulatory mechanisms of flower opening and abiotic stress tolerance in *O. fragrans*.

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Introduction

Auxin, an important plant hormone, plays crucial roles in various aspects of plant growth and development processes, such as cell division, expansion, and differentiation[1], vascular tissue formation[2], apical dominance[3], and flower and fruit development[4,5]. During the initial stages of auxin signal transduction, specific gene families, including *Auxin/IAA*, *GH3* (*Gretchen Hagen3*), and *SAUR* (*small auxin up RNA*), exhibit high responsiveness to fluctuations in auxin levels. The *Auxin/IAA* gene family was initially identified by Walker & Key in soybean[6]. This family consists of members with four highly conserved domains: domains I, II, III, and IV. Each domain has a distinct function: domain I is responsible for transcriptional repression, domain II is essential for auxin signal transduction, and domains III and IV enable proteins to form homo- and heterodimers. Aux/IAA proteins regulate auxin-responsive genes by interacting with ARFs (Auxin Response Factors), rather than directly binding to AuxREs (auxin-responsive cis-elements). So far, the *Aux/IAA* gene family has been identified in various plant species. For example, there are 23 members in *Prunus persica*[7], 29 in *Arabidopsis*[8], 35 in *Populus euphratica*[9], 42 in *Malus domestica*[10], and 44 in *Musa nana*[11].

Several studies have reported the crucial roles of *Auxin/IAA* family genes in various aspects of plant growth and development. In *Arabidopsis*, *AtIAA6*, *9*, and *17* inhibit root initiation by interacting with *AtARF6* and *AtARF8*[12]. The TIR1/AFB-Aux/IAA module regulates hypocotyl growth[13]. Similarly, *PpIAA19*, which is mainly expressed in peach (*Prunus persica*) fruit, has been demonstrated to control lateral root number, stem elongation, parthenocarpy, and fruit shape[14]. In poplar *(Populus tomentosa),* PtIAA9 acts as a negative regulator of secondary xylem development and controls wood formation through the *PtIAA9-PtARF5* module[15]. Additionally, the Auxin/IAA gene family significantly influences cell proliferation, and cell expansion, and plays a vital role in flower development and the opening process[16]. In waterlily *(Nymphaeale)*, *Auxin/IAA* genes induce the constitutive flower opening and are involved in floral movement[17]. In *Rosa hybrida,* the suppression of *RhIAA14* and *RhIAA16* expression leads to reduced flower cell expansion and smaller petals[18,19]. Moreover, accumulating evidence suggests that the *Aux/IAA* gene family also participates in plant stress responses and defense. In *Arabidopsis*, the auxin-sensitive genes *AtIAA5*, *6*, and *19* have been found to enhance drought tolerance by regulating glucosinolate levels, which protect plants from herbivory and pathogen attack[20]. In *Medicago falcata*, *MfAIR12* (Auxin induced in root culture 12) contributes to cold tolerance by regulating the expression level of cold-responsive genes and ascorbate synthesis and redox state[21]. In rice, *OsIAA20* plays an important role in drought and salt stress responses through the ABA-dependent pathway[22]. On the other hand, *OsIAA6* enhances drought tolerance by regulating the expression of auxin biosynthesis genes[23]. Additionally, the overexpression of *MdIAA9* from cultivated apple

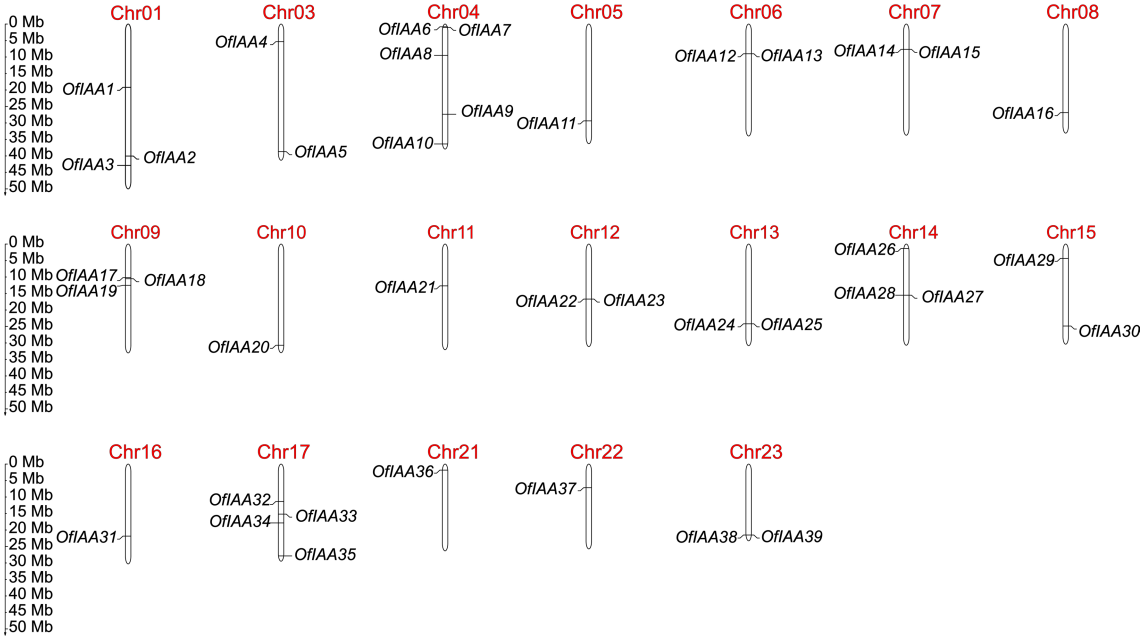

Fig. 1 Chromosomal distribution of *OfIAA* genes in *O. fragrans*.

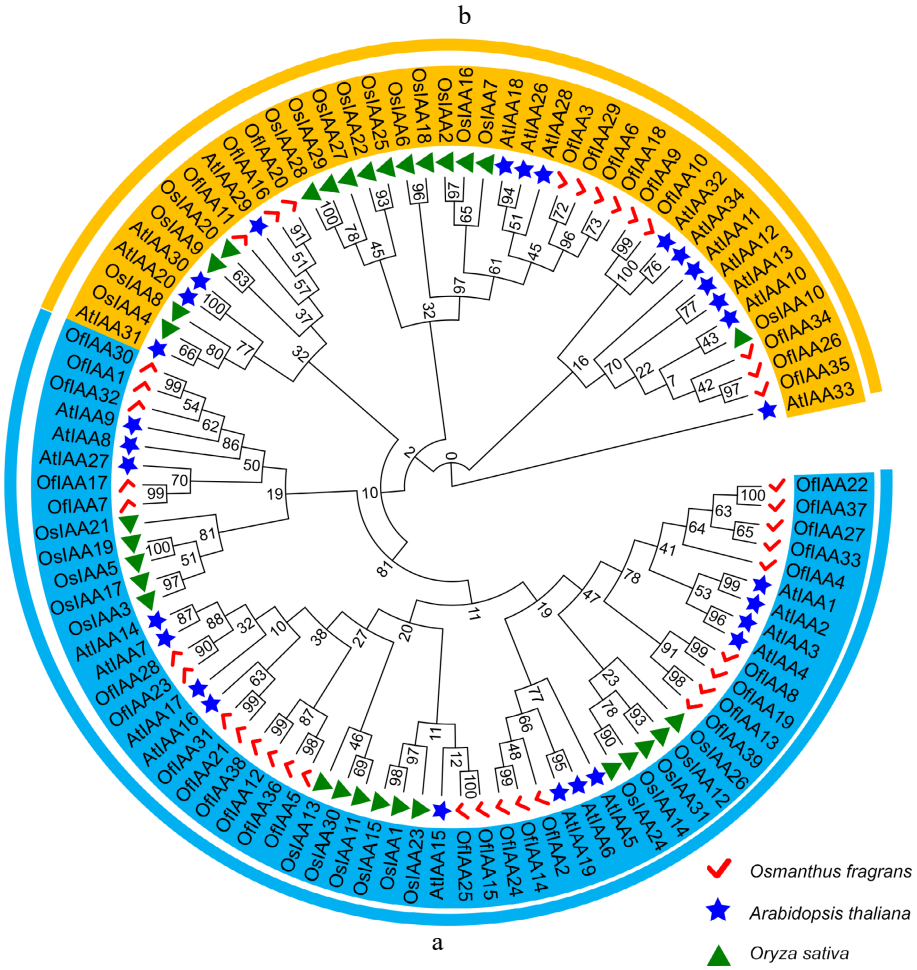

Fig. 2 Phylogenetic relationship of the IAAs among *O. fragrans*, *A. thaliana*, and *O. sativa*. The IAA proteins in *O. fragrans* are represented by the red ticks, the IAA proteins in *A. thaliana* are represented by the blue stars, the IAA proteins in *O. sativa* are represented by the green triangles. Two main groups (a) and (b) were displayed by colored arcs.

(*Malus* × *domestica*) significantly improved osmotic stress tolerance in transgenic tobacco (*Nicotiana tabacum* L.)[24].

Osmanthus fragrans, one of the ten most traditional flowers in China, is widely cultivated as a garden tree in many countries because of its remarkable ornamental value and delightful fragrance. Although genome-wide analysis of the *Aux/IAA* gene family has been performed in various species, the identification, characterization, and functional analysis of Aux/IAA family genes in *O. fragrans* have remained unexplored. In this study, a thorough genome-wide identification of *Aux/IAA* family members in the *O. fragrans* genome was conducted. Subsequently, their chromosomal locations, domains, phylogenetic relationships, gene duplications, gene structures, motifs, and cis-regulatory elements (CREs) were analyzed. Additionally, transcriptome sequencing and quantitative real-time PCR (qRT-PCR) were used to investigate the involvement of Aux/IAA family genes in regulating flower opening processes and responding to abiotic stress in *O. fragrans*. The aim of these results is to provide a comprehensive understanding of the *Auxin/IAA* gene family in *O. fragrans*.

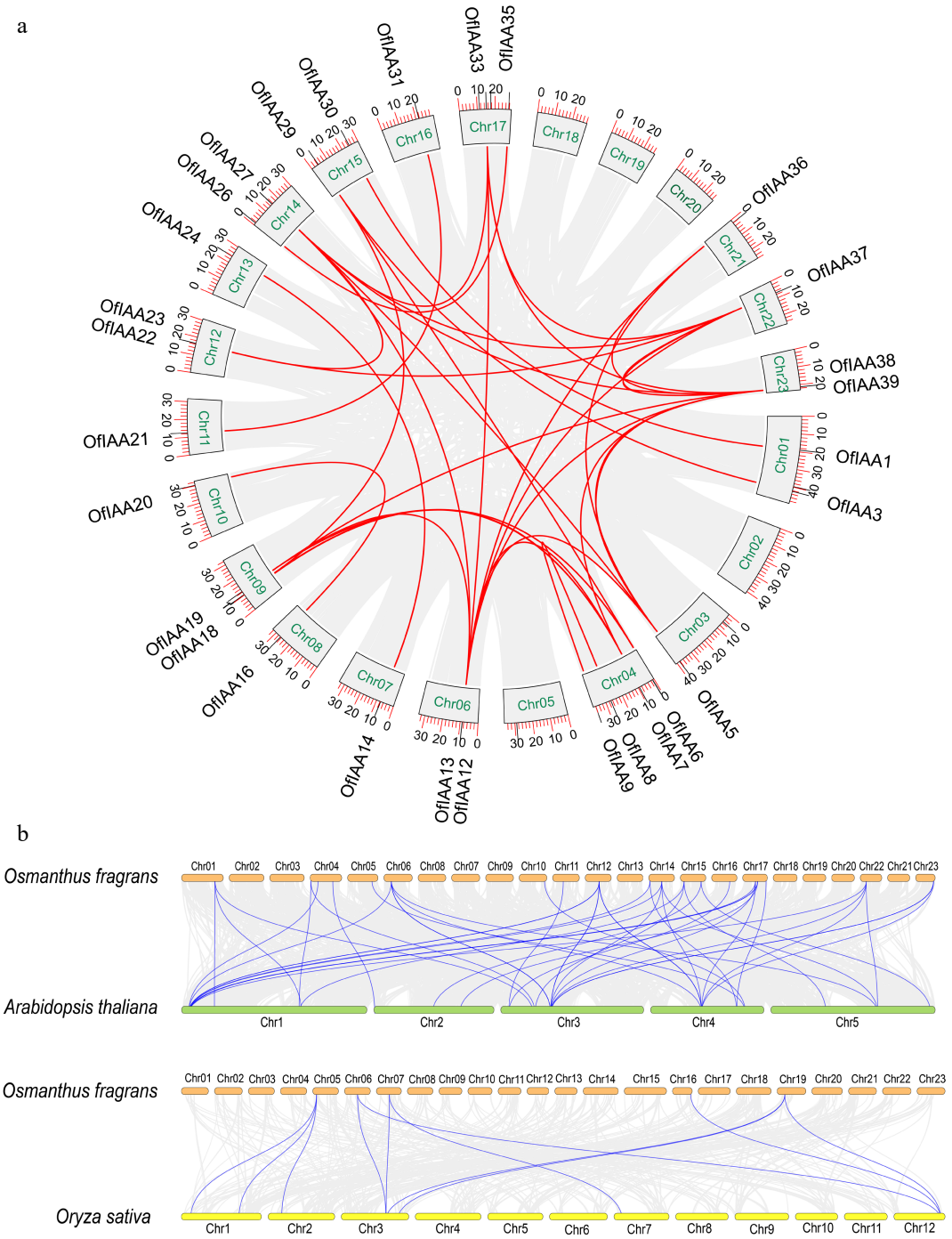

Fig. 3 Synteny analysis of the *IAA* genes. (a) Synteny analysis of the *OflAA* genes in *O. fragrans*. (b) Synteny analysis of the *OflAA* genes between *A. thaliana* and *O. sativa*.

Results

Genome-wide identification of *OfAux/IAA* genes

A total of 44 potential Aux/IAA gene sequences were identified in the *O. fragrans* genome. Five sequences were excluded from further analysis due to the presence of open reading frame (ORF). Subsequently, 39 *OfAux/IAA* (*OfIAA*) genes with a typical Aux/IAA domain were obtained and designated as *OfIAA1* to *OfIAA39*. More details about these 39 *OfIAAs* are presented in Supplemental Table S1, including protein length, MW (molecular weight), pI (isoelectric point), instability index, aliphatic index, grand average of hydropathicity, and subcellular localization prediction. The identified OfIAAs have protein lengths ranging from 142 amino acids (OfIAA14) to 392 amino acids (OfIAA1), with MWs ranging from 15.5 to 42.5 kDa. The pI of the 39 *OfIAA* genes varies from 4.94 (*OfIAA14*) to 9.37 (*OfIAA20*). Additionally, chromosome mapping revealed that the 39 *OfIAA* genes are unevenly distributed across 19 chromosomes. Chromosome 4 contains the largest number of *OfIAA* genes (five members) (Fig. 1).

Phylogenetic classification and conserved domain analysis

Based on the phylogenetic analysis, the IAA proteins (39 members) were grouped into two major clades (clades A and B), similar to *Arabidopsis* and *Oryza sativa*. Clade A consisted of 27 *OfIAA* genes and clade B consisted of 12 (Fig. 2). Synteny analysis of the *OfIAA* genes was conducted and 34 pairs of segmental duplications were identified distributed across 19 chromosomes (Fig. 3a). The divergence time indicated that the duplications of the *OfIAA* genes commenced 75.90 million years ago (Mya) and continued until 0.56 Mya. All *OfIAA* genes evolved under purifying selection (Ka/Ks < 1) (Table 1). In addition, the synteny analyses revealed that 20 and five pairs of *OfIAA* homologous genes were identified in *Arabidopsis* and rice, respectively (Fig. 3b).

Multiple alignments of the amino acid sequences of OfIAA proteins revealed that 35 OfIAA proteins contained all four typical conserved domains I, II, III, and IV. However, OfIAA12, 18, 26, and 32 lacked one of these domains (Fig. 4). Specifically, OfIAA32 lacked domain I, OfIAA18 and 26 lacked domain II, and OfIAA12 lacked domain IV. Nuclear localization signals (NLS) were identified at the end of domain IV in 16 OfIAA proteins. Additionally, gene structure analysis showed that the number of exons ranged from two to five (Fig. 5). Furthermore, five different motifs were identified in the 39 OfIAA proteins, and the majority of OfIAA proteins contained all five motifs, with motif 1 being conserved in all the OfIAA proteins (Fig. 5).

Cis-elements analysis of the promoter

The 2,000 bp upstream promoter regions of 39 *OfIAA* genes were identified through CRE analysis (Fig. 6). A total of 11 types of CREs were identified, with the majority being associated with hormone responses, such as auxin, ABA (abscisic acid), GA (gibberellin), MeJA (methyl jasmonate), and SA (salicylic acid). In addition, a significant number of *OfIAA* promoters contained ABA-responsive elements (31 *OfIAA* genes), MeJA-responsive elements (26 *OfIAA* genes), and GA-responsive elements (23 *OfIAA* genes). Furthermore, the promoters of *OfIAA* genes related to defense and stress responsiveness, and low-temperature responsiveness were also identified.

Table 1. Ka/Ks analysis and estimated divergence time of *OfIAA* genes.

Duplicated gene pairs	Ka	Ks	Ka/Ks	Divergence time (Mya)
OfIAA1 & *OfIAA30*	0.07	0.25	0.29	8.46
OfIAA3 & *OfIAA29*	0.18	0.45	0.41	15.02
OfIAA5 & *OfIAA12*	0.22	0.92	0.24	30.57
OfIAA5 & *OfIAA28*	0.22	1.67	0.13	55.65
OfIAA5 & *OfIAA36*	0.04	0.22	0.21	7.27
OfIAA5 & *OfIAA38*	0.18	0.73	0.25	24.38
OfIAA9 & *OfIAA10*	0.01	0.02	0.69	0.56
OfIAA8 & *OfIAA13*	0.1	0.67	0.16	22.2
OfIAA8 & *OfIAA19*	0.07	0.12	0.62	3.89
OfIAA6 & *OfIAA18*	0.11	0.28	0.41	9.26
OfIAA7 & *OfIAA17*	0.08	0.24	0.35	8.16
OfIAA6 & *OfIAA29*	0.27	0.72	0.37	23.97
OfIAA8 & *OfIAA38*	0.36	2.28	0.16	75.9
OfIAA13 & *OfIAA19*	0.16	0.72	0.22	24.03
OfIAA13 & *OfIAA27*	0.2	1.65	0.12	55.07
OfIAA13 & *OfIAA33*	0.21	1.16	0.19	38.59
OfIAA12 & *OfIAA36*	0.21	0.76	0.28	25.36
OfIAA13 & *OfIAA37*	0.25	1.7	0.15	56.59
OfIAA12 & *OfIAA38*	0.1	0.2	0.48	6.59
OfIAA14 & *OfIAA24*	0.07	0.19	0.39	6.34
OfIAA15 & *OfIAA25*	0.09	0.28	0.3	9.44
OfIAA16 & *OfIAA20*	0.18	0.43	0.43	14.39
OfIAA18 & *OfIAA29*	0.3	0.97	0.31	32.19
OfIAA21 & *OfIAA31*	0.07	0.19	0.35	6.36
OfIAA22 & *OfIAA27*	0.14	0.52	0.27	17.18
OfIAA23 & *OfIAA28*	0.07	0.75	0.1	24.89
OfIAA23 & *OfIAA28*	0.07	0.75	0.1	24.89
OfIAA22 & *OfIAA37*	0.06	0.23	0.27	7.69
OfIAA26 & *OfIAA35*	0.07	0.24	0.3	8.15
OfIAA27 & *OfIAA33*	0.07	0.25	0.27	8.41
OfIAA27 & *OfIAA37*	0.12	0.72	0.17	24.12
OfIAA33 & *OfIAA37*	0.14	0.86	0.16	28.59
OfIAA36 & *OfIAA38*	0.17	0.67	0.26	22.23
OfIAA37 & *OfIAA39*	0.22	1.48	0.15	49.41

Ka, nonsynonymous; Ks, synonymous.

Expression patterns of *OfIAAs* in different tissues

To gain further insight into the expression patterns of the *OfIAA* genes in different tissues, transcriptome sequencing was performed. The expression profiles of the 39 *OfIAA* genes were examined in the six different tissues, including root, annual stem, perennial stem, young leaf, mature leaf, and flower (Fig. 7). The expression analysis revealed that the majority of the *OfIAA* genes exhibited broad expression patterns in all six tissues, except for *OfIAA4, 9,* and *10*. Of particular interest was the predominant expression of *OfIAA6* in the root, while *OfIAA7*, *16*, *20*, *28*, and *30* exhibited high expression levels in the annual stem. Additionally, *OfIAA29* and *32* had high expression levels in the perennial stem, and *OfIAA14* was overrepresented in the young leaf. Furthermore, the transcriptome analysis revealed that *OfIAA24* and *25* were predominantly expressed in the flower (Fig. 7).

Analysis of *OfIAAs* expression in flower opening processes

In *O. fragrans*, the scent release is significantly influenced by the flower opening process, with the transformation from the S1 to S2 stages playing a particularly crucial role. To investigate the potential involvement of *OfIAA* genes in this process, a comprehensive analysis of their expression profiles was

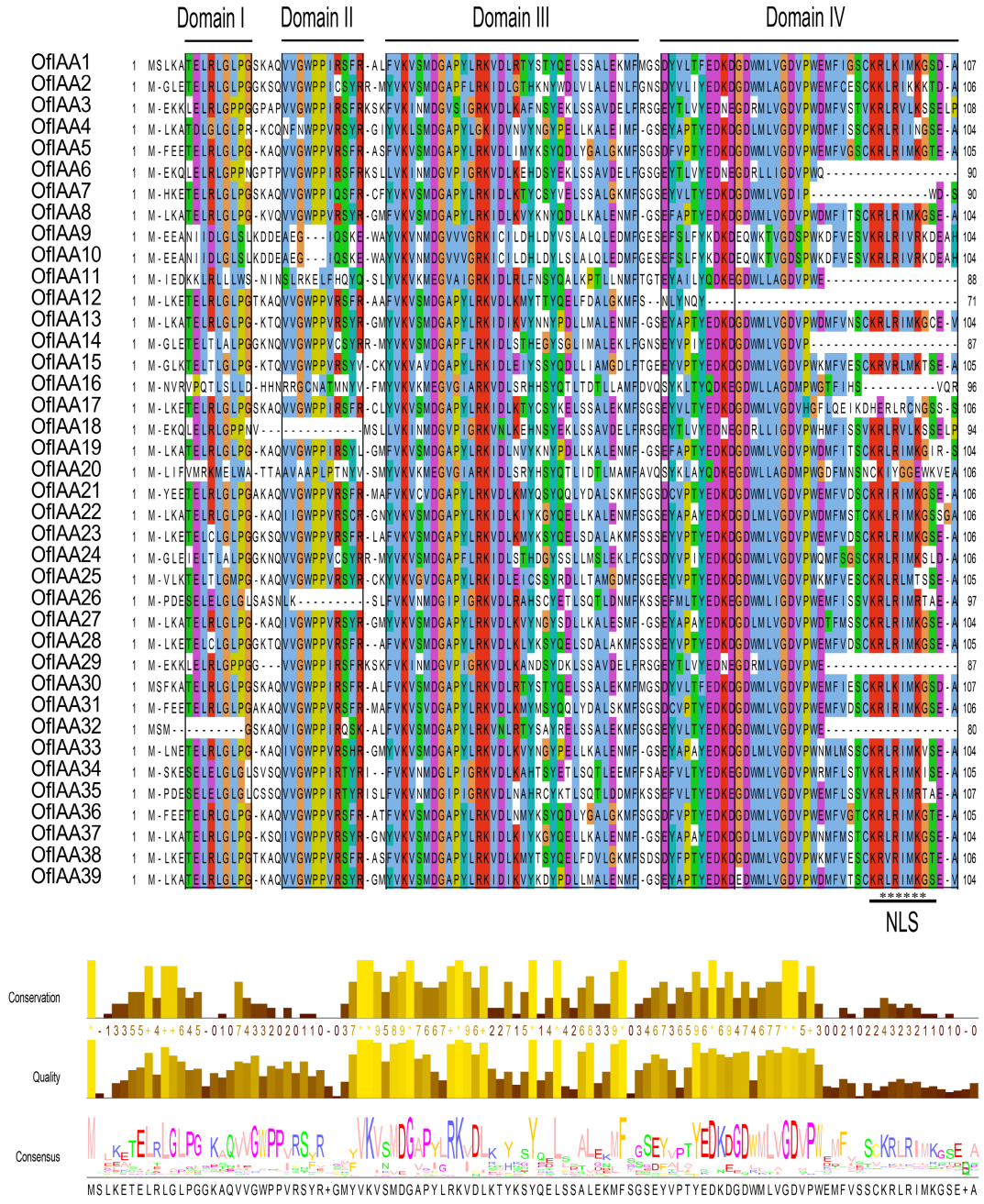

Fig. 4 Multiple sequence alignment of the OfIAA proteins. The conserved domains (I, II, III, and IV) of the *OfIAA* gene family are underlined. Nuclear localization signals (NLS) are indicated with black asterisks. Bits indicate amino acid conservation at each position.

conducted *via* RNA-seq in flower buds at the S1 and S2 stages (Table 2). Through analysis, a total of 18 DEGs (differentially expressed genes) were identified from the S1 to S2 stage (Table 2). Among these DEGs, 16 genes were upregulated, including *OfIAA2*, *8*, *11*, *12*, *13*, *14*, *15*, *20*, *25*, *27*, *28*, *33*, *36*, *37*, *38*, and *39*, while two genes, namely OfIAA6 and 29, were downregulated. Subsequently, the expression patterns of all DEGs were validated in flower buds at the S1 and S2 stage using qRT–PCR (Fig. 8). The qRT-PCR results were consistent with the expression trends observed in the transcriptomic data. These results suggest that these differentially expressed *OfIAA* genes may have a potential role in regulating the flowering process.

Analysis of *OfIAAs* expression under different abiotic stresses

Salinity and drought stress have a profound impact on the growth, development, and natural distribution of *O. fragrans*. To investigate the potential role of *OfIAA* genes in the responding to salt and drought stress, we analyzed their expression patterns under these conditions. The transcriptomic results revealed that the expression of the majority of *OfIAA* genes were significantly altered in response to salt and drought stress. However, the expression of *OfIAA9*, *10*, *20*, *24*, and *27* was nearly absent after treatments (Fig. 9a). Based on the DEG thresholds, three DEGs (*OfIAA18*, *22,* and *23*) showed downregulation after

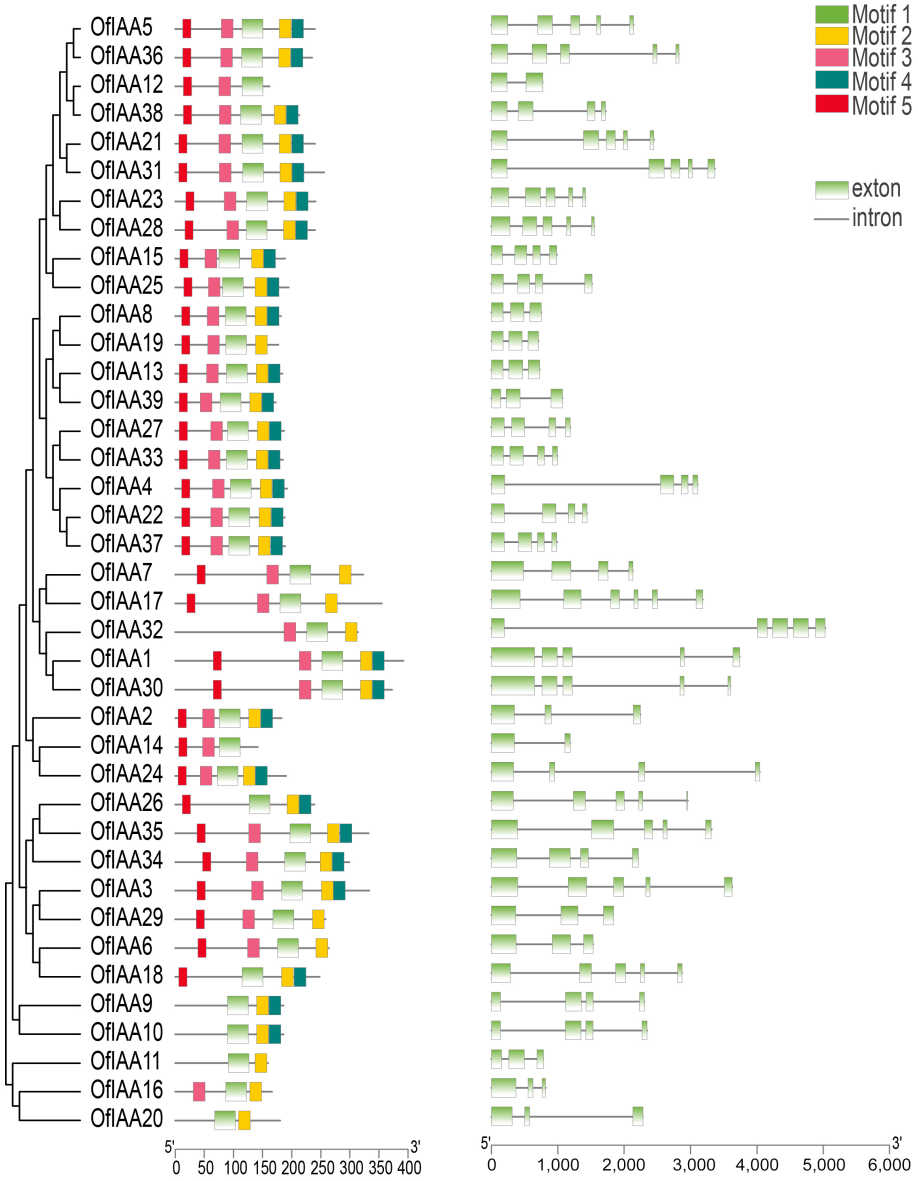

Fig. 5 Motif and gene structure analysis of OfIAAs.

salt stress, while 11 DEGs were identified under drought stress, including ten downregulated genes (*OfIAA13*, *15*, 18, *21*, *22*, *23*, *28*, *29*, *31*, and *36*) and one upregulated gene (*OfIAA5*) (Table 3). The spatiotemporal expression of all the DEGs was further verified during 24-h after salt and drought treatments, respectively. In particular, *OfIAA18*, *22*, and *23* showed a decreasing trend within 12 h after salt treatment, but their expression levels increased after 12 h (Fig. 9a). In the context of the drought treatment, *OfIAA5* and *28* exhibited a decreasing trend within 12 h, followed by an increase at 24 h (Fig. 9c). Furthermore, *OfIAA15*, *18*, *21*, *22*, *23*, *29*, *31*, and *36* exhibited a general decline over the 24-h period following the drought treatment (Fig. 9b). *OfIAA13* showed an increasing trend from 0 to 12 h, but was downregulated at 24 h after the drought treatment (Fig. 9c). These results indicate that the differentially expressed *OfIAA* genes are involved in the response to salt and drought stress in *O. fragrans*.

Discussion

Auxin, an essential plant hormone, plays a crucial role in plant growth, development, and physiological processes. In the context of auxin signaling, the *Auxin/IAA* gene serves as a key regulator of downstream responses. Identification of the *Aux/IAA* gene family provides a valuable foundation for further research into auxin signaling and regulatory mechanisms. With the advent of molecular biology techniques and genome sequencing, comprehensive analyses of the Auxin/IAA gene family have been conducted in various species, such as *Arabidopsis*[8], rice[25], maize (*Zea mays*)[26], and *Populus*[9]. However, limited information is available on the *Aux/flAA* gene family in *O. fragrans*. Therefore, it is imperative to conduct a comprehensive investigation of this gene family in *O. fragrans* to gain a better understanding of its potential functions in the flower opening processes and responses to abiotic stress.

In this study, a genome-wide identification and comprehensive analysis of the *OfIAA* gene family in *O. fragrans* was

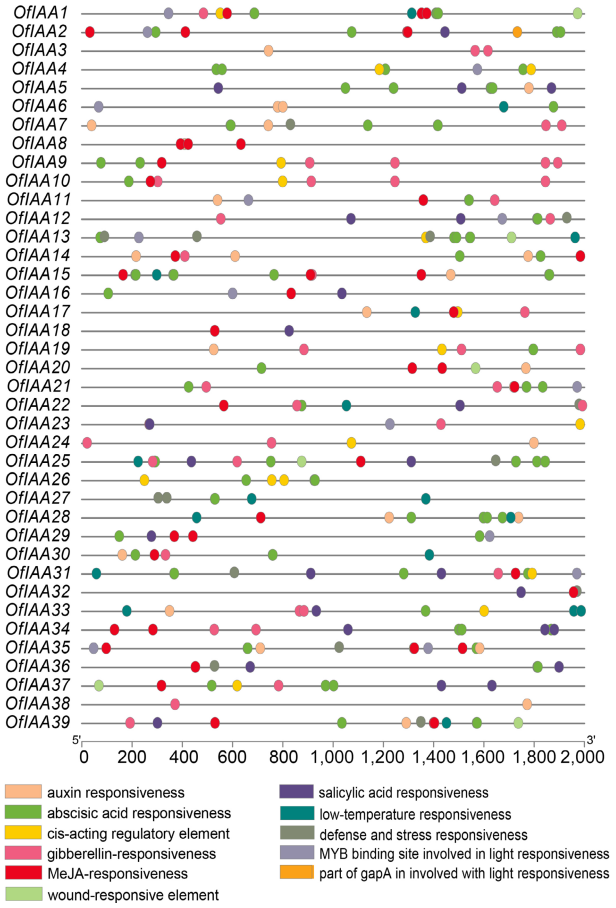

Fig. 6 *Cis*-elements distribution of *OfIAA* genes.

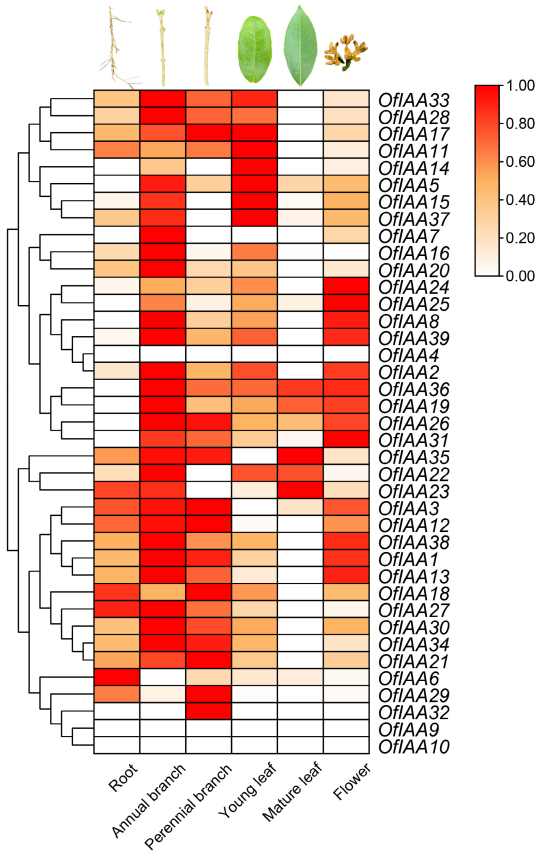

Fig. 7 Expression analysis of *OfIAAs* in different tissues.

Table 2. Identification of DEGs in the flower opening processes of *O. fragrans*.

Gene	Expression		FoldChange	Padj value
	S1	S2		
OfIAA2	0.00	22.82	7.00	6.69×10^{-7}
OfIAA6	1,121.83	435.01	−1.37	3.97×10^{-29}
OfIAA8	28.68	789.65	4.79	1.93×10^{-99}
OfIAA11	5.05	24.49	2.26	1.20×10^{-3}
OfIAA12	13.62	145.78	3.42	2.75×10^{-21}
OfIAA13	18.45	60.19	1.70	8.14×10^{-5}
OfIAA14	0.32	7.61	4.45	9.80×10^{-3}
OfIAA19	92.29	490.40	2.41	1.30×10^{-41}
OfIAA20	0.30	6.22	4.15	4.90×10^{-2}
OfIAA25	25.79	131.80	2.36	1.76×10^{-15}
OfIAA27	4.82	23.02	2.25	5.20×10^{-3}
OfIAA28	3.73	173.53	5.60	5.41×10^{-28}
OfIAA29	1,136.52	235.71	−2.27	4.41×10^{-60}
OfIAA33	4.68	33.32	2.86	9.99×10^{-5}
OfIAA36	44.20	92.87	1.06	7.90×10^{-4}
OfIAA37	101.89	292.73	1.52	1.54×10^{-15}
OfIAA38	110.89	292.85	1.40	1.45×10^{-13}
OfIAA39	24.51	134.72	2.44	2.23×10^{-11}

performed. A total of 39 *OfIAA* genes were identified in *O. fragrans*, which is more than the number found in *Arabidopsis* (29) and *Populus* (35) (Supplemental Table S1). The *OfIAA* gene family exhibited significant variation in protein length, MW, and pI consistent with findings in *Arabidopsis* and rice[8,25] (Supplemental Table S1). Upon analysis of the chromosomal distribution, we observed a non-uniform distribution of the *OfIAA* genes across the 19 chromosomes (Fig. 1). Notably, *OfIAAs* located on the same chromosome had similar gene structures and gene motifs. A typical Aux/IAA protein consists of four conserved domains[27]. The present analysis revealed that 35 OfAux/IAA proteins had all four domains, while the remaining proteins lacked one domain (Fig. 2). For instance, OfIAA32 lacked domain I, indicating that it cannot repress *via* interaction with the TPL (TOPLESS) corepressor, thereby losing its repressor function in auxin signaling[28]. Similarly, the absence of domain II in OfIAA18 and 26 result in reduced protein stability and an inability to participate in the TIR1 (transport inhibitor response 1) degradation pathway, making them resistant to degradation at elevated auxin levels[29]. Moreover, the absence of domain IV in OfIAA12 indicates that it cannot form homo- and heterodimers with ARF proteins[30]. Gene duplications, particularly segmental duplications, play a key role in the expansion of gene families[31]. A total of 34 pairs of segmental duplications were found among the *OfIAA* genes, with no tandem duplications observed (Fig. 3, Table 1). It was hypothesized that segmental duplications may have contributed to the expansion of the Aux/IAA gene family in *O. fragrans*. Phylogenetic analysis classified the 39 OfIAA proteins into two main groups, consistent with *Arabidopsis* and rice[32]. Notably, *OfIAA* genes within the same clade displayed conserved intron-exon structures (Fig. 5), suggesting potential functional similarities. Five types of motifs in the *OfAux/IAA* gene family were also identified (Fig. 5), which is consistent with *Hordeum vulgare*[33] and pepper[34]. In addition, the promoter regions of the *OfIAA* genes

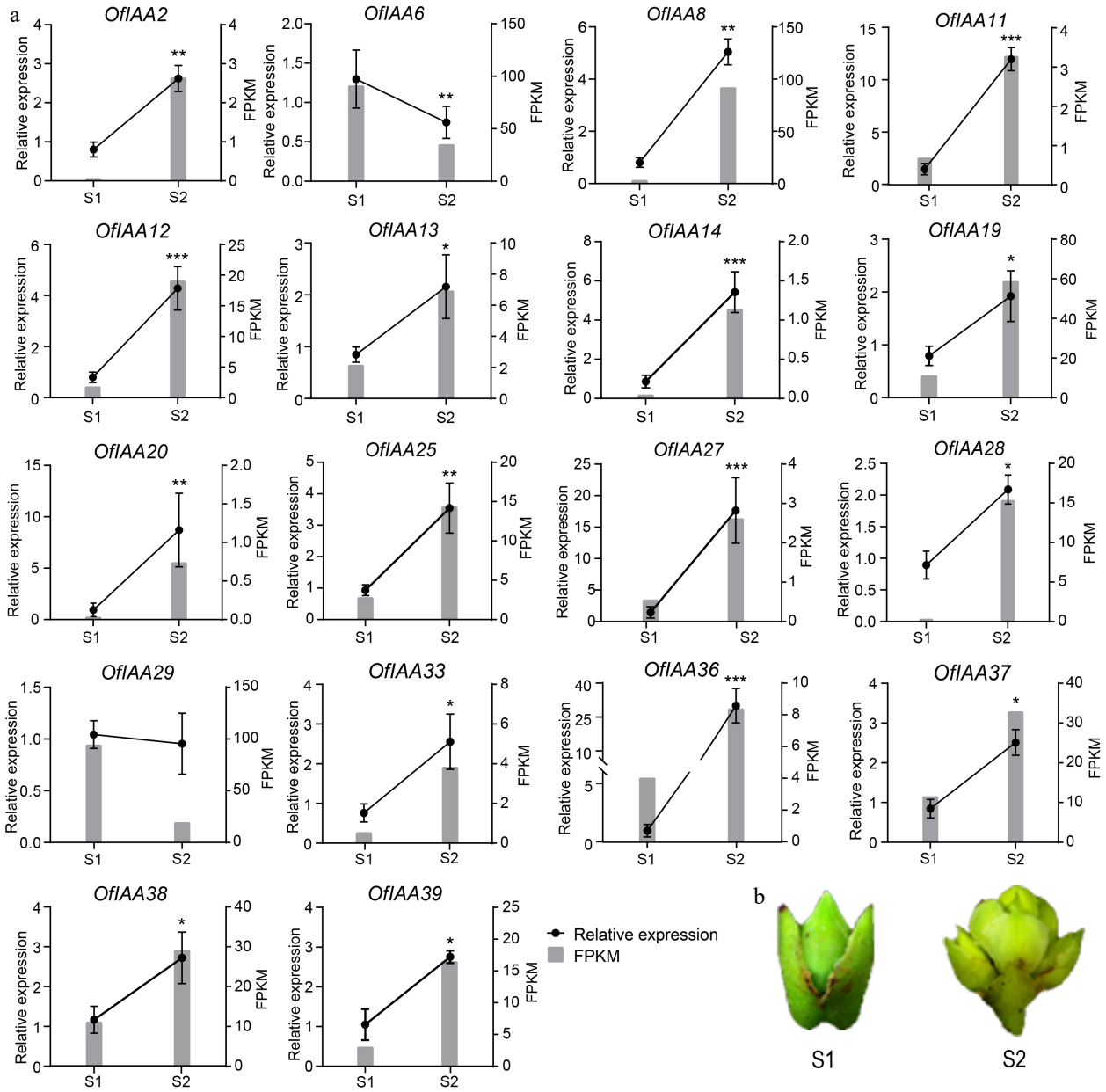

Fig. 8 Expression analysis of the differently expressed *OfIAA* genes during flower opening processes in *O. fragrans*. (a) FPKM and qRT–PCR analysis of the differentially expressed *OfIAA* genes at stage S1 and S2. Error bars represent the standard error of three replicates. (b) Phenotypes of flower buds at S1 and S2 stage. Significance was assessed by Duncan's multiple-range test (DMRT) at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

contained numerous hormone-responsive and stress-responsive elements (Fig. 6). These elements are involved in regulating the expression of the *OfIAA* genes in response to hormone signals and stress conditions in *O. fragrans*.

The analysis of transcriptomic expression of the *OfIAA* genes in different tissues revealed that the *OfIAA* genes are widely expressed in stem tissue. Moreover, certain *OfIAA* genes show unique preferential expression patterns (Fig. 7). Among the OfIAA genes, 16 *OfIAA* genes (*OfIAA2*, *6*, *7*, *14*, *16*, *17*, *20*, *21*, *24*, *25*, *28*, *30*, *32*, *33*, *34*, and *35*) showed the highest expression in different tissues of *O. fragrans* (Fig. 7), indicating the different functions of the *OfIAA* gene family in these tissues. *Aux/IAA* gene play important roles in regulating plant growth and development, particularly in the flowering processes. For example, in rose (*Rosa hybrida*), repressing the specific ethylene-repressed *RhIAA14* gene causes restricted cell expansion, leading to reduced flower size and limited petal expansion[18]. Similarly, petal abscission is delayed when *RhIAA4-1* is silenced[35], while downregulation of *RhIAA16* promotes petal abscission[19]. In *O. fragrans*, the functions of *OfIAAs* in flower opening processes remain unexplored. The present study identified 18 differentially expressed *OfIAA* genes (16 upregulated and two downregulated) from the S1 stage to the S2 stage during flower opening (Fig. 8). These DEGs suggest a possible involvement in the regulation of flower opening in *O. fragrans*. Furthermore, the *Aux/IAA* gene family has been proven to be involved in the abiotic stress response and improved abiotic stress tolerance. Overexpression of *TaIAA15-1A* in bread wheat

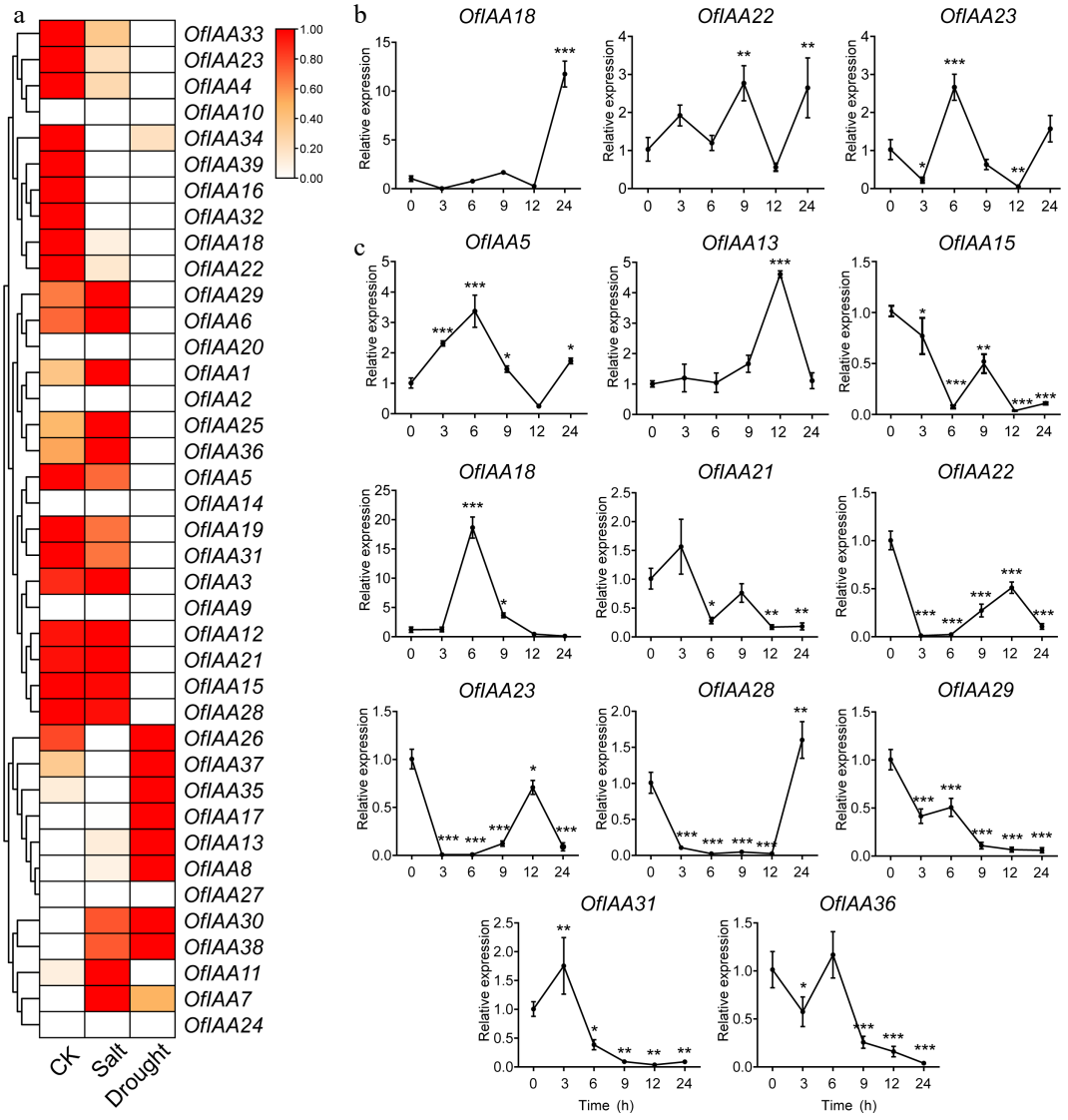

Fig. 9 Expression profiles of the differentially expressed *OflAA* genes after (a) salt and (b) drought treatments by qRT–PCR in *O. fragrans*. Error bars represent the standard error for three replicates. Significance was assessed by Duncan's multiple-range test (DMRT) at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Table 3. Identification of DEGs under salt and drought treatments in *O. fragrans*.

Stress	Gene	Expression		FoldChange	Padj value
		CK	Treatment		
Salt	*OflAA18*	1.24	0.28	−2.18	2.2×10^{-3}
	OflAA22	4.047	0.67	−2.59	3.6×10^{-5}
	OflAA23	6.47	1.92	−1.75	1.5×10^{-4}
Drought	*OflAA5*	35.73	12.14	−1.54	1.6×10^{-17}
	OflAA13	0.38	6.59	4.15	8.1×10^{-12}
	OflAA15	2.87	0.41	−2.80	2.3×10^{-4}
	OflAA18	1.24	0.23	−2.41	6.4×10^{-3}
	OflAA21	79.82	29.78	−1.41	3.3×10^{-26}
	OflAA22	4.05	0.20	−4.30	4.1×10^{-9}
	OflAA23	6.47	1.06	−2.59	2.9×10^{-7}
	OflAA28	2.07	0.71	−1.53	3.7×10^{-2}
	OflAA29	13.73	6.75	−1.01	4.9×10^{-5}
	OflAA31	60.42	15.61	−1.94	1.9×10^{-26}
	OflAA36	52.94	16.89	−1.63	3.1×10^{-17}

(*Triticum aestivum*) improves drought tolerance by regulating the expression of genes involved in auxin, ABA, phenolamide, and antioxidant signaling pathways[36]. In grapevine (*Vitis vinifera*), *VvIAA18* significantly enhances salt tolerance by upregulating downstream genes, as demonstrated in transgenic tobacco plants and *Escherichia*[37]. Three DEGs (*OflAA18*, *22*, and *23*) were identified after salt treatment (Fig. 9), indicating that salt stress inhibits auxin signaling. Drought treatment significantly induced 11 DEGs, suggesting their potential role in regulating drought tolerance in *O. fragrans*. The responses of *OflAA* genes to both salt and drought suggest their involvement in abiotic stress adaptation in *O. fragrans*. These results highlight the multiple roles of *OflAA* genes in regulating flower opening and abiotic stress response in *O. fragrans*.

Conclusions

A comprehensive genome-wide analysis was conducted to identify 39 *OflAA* genes in *O. fragrans*. The analysis of gene

characterization revealed the evolutionary conservation of the *OfIAA* gene family. Additionally, synteny analysis suggested that segmental duplication had a significant role in the evolution of the *OfIAA* gene family. Through transcriptomic and qRT–PCR analyses, it was found that almost all the *OfIAA*s were expressed in the stem tissue. Furthermore, 18 DEGs were identified, which contributed to the regulation of the flower opening in *O. fragrans*. Moreover, three and 11 DEGs were obtained after salt and drought treatments, respectively. The expression patterns of differentially expressed *OfIAA* genes suggested their essential role in responding to abiotic stress tolerance.

Materials and methods

Plant material and treatments

The potted material of *O. fragrans* 'Yanhonggui' is maintained in the Osmanthus Germplasm Resource Garden of Zhejiang Agriculture and Forestry University in Hangzhou, China. All the materials were kept in a greenhouse under natural conditions. To investigate the tissue-specific expression patterns, samples were collected from different plant tissues, including the root, annual stem, perennial stem, young leaf, mature leaf, and flower. During flower opening, flower buds were collected at two different developmental stages. The S1 stage involved the buds in the globular form with the inner bracts visibly covering the inflorescence. The S2 stage involved the buds when the inflorescence had burst through the bracts, and the florets were closely crowded. According to Dong et al.[38], uniform branch cuttings of *O. fragrans* 'Yanhonggui', each 20 cm long, were used for salt and drought treatment. All the cuttings were subjected to 200 mmol/L D-mannitol and 200 mmol/L NaCl, respectively, in a growth chamber under controlled conditions (temperature of 23 °C, 14/10-h light/dark cycle, and relative humidity of 60%). Three biological replicates were performed for each treatment. The third or fourth fully expanded leaf from the top was collected from each treatment at 0, 3, 6, 9, 12, and 24 h. All samples were immediately frozen in liquid nitrogen and stored at −80°C.

Genome-wide identification of the *AUX/IAA* gene family

The nucleotide and protein sequences of *O. fragrans* were downloaded from the *O. fragrans* genome database in NCBI (www.ncbi.nlm.nih.gov/genome/?term=Osmanthus+fragrans). The Hidden Markov Model (HMM) for the *Aux/IAA* gene family (PF02309) was downloaded from the pfam database (www.ebi.ac.uk/interpro) and utilized with the hmmbuild tool (www.hmmer.org) to identify potential *Aux/IAA* genes in the genome of *O. fragrans*. The amino acid sequences of OfIAA proteins were analyzed using the online tool Expasy (www.expasy.org) to calculate the length of amino acid, molecular weight (MW), and isoelectric point (pI). Additionally, the Protein Parameter Calculator in TBtools (https://github.com/CJ-Chen/TBtools) was employed to determine the instability index, aliphatic index, and grand average of hydropathicity for the OfIAA proteins. The subcellular localization prediction of OfIAA proteins was performed using WoLF PSORT (https://wolfpsort.hgc.jp/).

Phylogenetic tree construction, gene structure, and motif analysis

The AUX/IAA protein sequences of *Arabidopsis thaliana* and rice (*Oryza sativa*) were obtained from the NCBI (National Center for Biotechnology Information) database (Supplemental Tables S2 & S3). Subsequently, the Aux/IAA protein sequences of *O. fragrans*, *Arabidopsis*, and rice were aligned using the MEGA11 software[39], and a phylogenetic tree was generated by the neighbor-joining method with a bootstrap value of 1,000. In addition, the exon-intron distribution of the *OfAux/IAA* genes were analyzed using Gene Structure Display Sever 2.0 (http://gsds.cbi.pku.edu.cn/). The motifs of the OfAux/IAA proteins were predicted by the Multiple Expectation Maximization for Motif Elicitation (MEME) online program (https://meme-suite.org/meme/tools/meme).

Promoter region analysis of *OfAux/IAA* genes

To investigate cis-regulatory elements (CREs) in promoter sequences of *OfAux/IAA* genes, 2,000 bp genomic regions upstream of the translational start codons were analyzed using the Plantcare program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The putative CREs in the promoter sequences of each *OfAux/IAA* gene were visualized in TBtools software[40].

Chromosomal location, sequence, and synteny analysis

The chromosome information of *OfAux/IAA* genes was analyzed by TBtools software[40], including chromosome length, numbers, and the start and end sites of genes. The domains of the predicted OfAux/IAA proteins were visualized using DNAMAN version 7 software. The pattern of gene duplication of *OfAux/IAAs* was assessed by MCScanX v1.0 software. The DnaSP v5.0 soft was used to analyze the synonymous (Ks) and nonsynonymous (Ka) substitution ratios of the gene pairs. The divergence time was calculated using the formula T = Ks/2r, where Ks represents the synonymous substitutions per site and r represents the rate of divergence of nuclear genes in plants. For dicotyledonous plants, the rate of divergence (r) was considered to be 1.5×10^{-8} synonymous substitutions per site per year. Synteny analyses of *OfAux/IAA* genes, *OfAux/IAAs* and *AtAux/IAAs*, and *OfAux/IAAs* and *OsAux/IAAs* were conducted using the Quick MCScanX Wrapper program in TBtools.

RNA isolation and RNA-sequencing

A total of 0.5 g of material was used for total RNA extraction using a FastPure Universal Plant Total RNA Isolation Kit (Vazyme, Nangjing, China) according to the manufacturer's instructions. For library construction and RNA sequencing, 3.0 μg of RNA from each sample was utilized. The RNA sequencing method employed was previously described by Ye et al.[37]. Three biological replicates were performed for RNA sequencing. Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) was employed to calculate the expression, the differentially expressed genes (DEGs) were determined by the threshold of $|\log2(\text{ratio treatment/CK})| \geq 1$, along with Padj: (p.adjust) < 0.001. The RNA sequencing data were accessed at the SRA (Sequence Read Archive) database (www.ncbi.nlm.nih.gov/sra) under Bioproject number PRJNA961323. TBtools software was used to generate the heatmap to visualize the expression of *OfIAA* genes.

cDNA synthesis and quantitative real-time PCR (qRT–PCR)

cDNA synthesis was carried out using ToloScript All-in-one RT EasyMix for qPCR (TOLOBIO, Shanghai, China) according to the manufacturer's recommendations. qRT–PCR analysis was

performed using the LightCycler480II System (Roche, Basel, Switzerland) as described by Yang et al.[41]. Three biological repetitions were performed. The relative expression level of genes was calculated by the $2^{-\Delta\Delta C_T}$ method. The sequence-specific primers are listed in Supplemental Table S4.

Statistical analysis

Data treatment and analysis were performed by Excel 2022 (Microsoft, Redmond, USA). IBM SPSS 20 software (IBM, Armonk, USA) was utilized to determine significant differences. Significance was assessed by Duncan's multiple-range test (DMRT) at $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

Author contributions

The authors confirm contribution to the paper as follows: study design and supervision: Zhao H, Dong B; participating in the entire thesis writing and data analysis: Cao S, Dong B; assisting in the bioinformatics analysis of the gene family: Ye Y, Wang Y; participated in the experiment process: Zheng Z, Zhong S, Xiao Z; providing guidance and manuscript revision: Fang Q, Deng J. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The plant materials are preserved in the Osmanthus Germplasm Resource Garden of Zhejiang Agriculture and Forestry University (Hangzhou, China). The raw reads files have been accessed on NCBI Sequence Read Archive (SRA) under the BioProject number of PRJNA961323.

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Conflict of interest

The authors declare that they have no conflict of interest.

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