Identification and characterization of ARF family members involved in Fig (*Ficus carica* L.) fruit development

Haomiao Wang[1], Hantang Huang[1], Yongkai Shang[1], Miaoyu Song[1], Huiqin Ma[1,2]

1 College of Horticulture, China Agricultural University, Beijing, China,
2 State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing, China

Corresponding Author:
Miaoyu Song[1], Huiqin Ma[1]
West Yuanmingyuan Road, Beijing, Country Email address:
Email address: songmiaoyu@cau.edu.cn; hqma@cau.edu.cn

Abstract: Auxin response factor (ARF) combine with AuxREs cis-acting elements in response to auxin to regulate plant development. To date, no comprehensive analysis of ARF genes expressed during fruit development has been conducted for common fig (*Ficus carica* L.). In this study, members of the FcARF gene family were screened, identified in the fig genome database and their features characterized using bioinformatics. Twenty FcARF genes were clustered into three classes, with almost similar highly conserved DBD (B3-like DNA binding domain), AUX/IAA (auxin/indole-3-acetic acid gene family) and MR domain structure among class members. Analysis of amino acid species in MR domain revealed 10 potential transcription activators and 10 transcription inhibitors, and 17 FcARF members were predicted to be located in the nucleus. DNA sequence analysis showed that the ARF gene family consisted of 4 - 25 exons, and the promoter region contained 16 cis-acting elements involved in stress response, hormone response and flavonoid biosynthesis. ARF genes were expressed in most tissues of fig, especially flower and peel. Transcriptomics analysis results showed that *FcARF2*, *FcARF11* and *FcARF12*, belonging to class-Ia, were stably and highly expressed in the early development stage of flower and peel of 'Purple peel' fig. However, their expression levels decreased after maturity. Expression of class-Ic member *FcARF3* conformed to the regularity of fig fruit development. These 4 potential transcription inhibitors may regulate fruit growth and development of 'Purple peel' fig. This study provides comprehensive information on the fig ARF gene family, including gene structure, chromosome position, phylogenetic relationship and expression pattern. Our work provides a foundation for further research on auxin-mediated fig fruit development.

39 **Key words:** *Ficus carica* **L.; Auxin response factor; Gene family; Expression analysis; Fruit**
40 **development**
41
42 **Introduction**
43 Auxin is an important plant hormone, which directly regulates cell growth, division and specific
44 differentiation, participating in embryogenesis, flower and fruit development, root formation and
45 vascular bundle development (Du et al. 2012). At the molecular level, auxin regulates plant
46 growth and development via a signal transduction pathway mediated by Aux/IAA proteins and
47 auxin response factor (ARF) (Liu et al. 2015; Woodward et al. 2005). ARFs typically consist of
48 three domains: an N-terminal B3-like DNA binding domain (DBD), a C-terminal dimerization
49 domain (CTD), which forms a heterodimer with Aux/IAA family genes, and the middle domain
50 (MR) with an activation (AD) or inhibition (RD) role (Guilfoyle & Hagen 2007; Kim et al. 1997;
51 Li et al. 2015). The DBD domain can identify Auxin Response Element (AuxRE, 'TGTCTC') in
52 auxin response gene promoter. The CTD of ARF protein contains two conserved motifs, motif
53 III and motif IV, which facilitate formation of homo- and hetero-dimerization among ARFs and
54 Aux/IAAs (Zhou et al. 2017). The MR domain either activates or inhibits transcription
55 depending on its amino acid composition (Ye et al. 2020). Therefore, these domains are
56 necessary for efficient regulatory function of ARF (Wang et al. 2007).
57 The ARF gene family has been identified in many plants. There are 23 ARFs in *Arabidopsis*
58 *thaliana (*Okushima et al. 2005*)*, 12 in *Fragaria vesca* (Wang et al. 2019), 19 in *Vitis vinifera*
59 (Wan et al. 2014), 19 in *Citrus sinensis* (Li et al. 2015), 19 in *Capsicum annuum* L. (Zhang et al.
60 2017), 22 in *Lycopersicon esculentum* (Kumar et al. 2011), 25 in *Oryza sativa L.* (Wang et al.
61 2007) and 37 in *Populus alba × P. glandulosa* (Yang et al. 2014). Based on evolutionary
62 relationship analysis, ARF genes in *Arabidopsis thaliana* cluster into three classes. Class I is the
63 largest with 15 members belonging to three classes. Members in class-Ia are mainly involved in
64 regulating flower and root development. For example, *AtARF1* and *AtARF2* promote flower
65 senescence and abscission (Ellis et al. 2005), whereas *AtARF11* promotes lateral root formation
66 (Feng et al. 2012). These genes share a high degree of similarity among their amino acid and
67 nucleotide sequences. In addition, *AtARF23* is a pseudogene (Okushima et al. 2005). Class-Ic has
68 two members, *AtARF3* and *AtARF4*, which regulate floral organogenesis and the timing and
69 pattern of development (Ellis et al. 2005; Harper et al. 2000; Hunter et al. 2006; Pekker et al.
70 2005; Sessions et al. 1997). Members of class-II are ARF^ClassA^ transcription activators, which
71 regulate auxin-mediated flower development, morphogenesis, lateral root formation and other
72 processes (Truskina et al. 2021). Class-III consists of three most structurally distinct members
73 that regulate pollen wall pattern formation, root cap formation or seed germination (Ellis et al.
74 2005; Guilfoyle & Hagen 2007).
75 As important plant growth regulators, ARF genes are expressed and play regulatory roles in
76 different tissues (**Table S1 and Table S2**), especially floral organs and fruit. *AtARF2* was found
77 to promote the development of stamens and participate in the senescence and abscission of floral
78 organs (Okushima et al. 2005). *AtARF4* regulates flower patterns and plays a significant role in
79 organ polarity development (Hunter et al. 2006; Liu et al. 2014; Pekker et al. 2005). *AtARF6/8*

80   and *SlARF6/8* can promote maturation of floral organs and regulate fruit and seed carpel
81   development (Goetz et al. 2006; Wu et al. 2006). In fruiting plants, *SlARF3* plays multiple roles
82   in tomato fruit development and participates in the formation of epidermal cells and trichomes
83   (Zhang et al. 2015). *SlARF9* regulates cell differentiation during early tomato fruit development
84   and *SlARF4* controls sugar metabolism during maturation (de Jong et al. 2015; Sagar et al. 2013).
85   Six CiARF genes (*CiARF1/2/6/7/12/18*) are highly expressed in fruit receptacle, where they
86   regulate the citrus pulp and peel development (Li et al. 2016). Seven PpARF genes
87   (*PpARF1/2/3/4/5/6/9*) perform similar functions in *Prunus persica* (Hou et al. 2021). Studies on
88   apples show that *MdARF5* induces ethylene synthesis by directly promoting the expression of
89   ethylene synthesis genes *MdACS3a*, *MdACS1* and *MdACO1*, which then promote apple fruit
90   ripening (Yue et al. 2020).
91   ARF proteins are negatively regulated by auxin/indole-3-acetic acid (Aux/IAA) proteins. The
92   potential for interaction between ARF and Aux/IAA proteins is high as their C-terminal domains
93   are homologous (Shen et al. 2010). Aux/IAA genes play a major role in regulating many auxin
94   processes in plants. Aux/IAA genes exist as a family in many plants and are associated with leaf
95   curl patterns, plant phototropism and tropism, plant height, and especially lateral root formation
96   (Reed 2001). In *Arabidopsis thaliana*, *axr5 / iaa1* mutant showed auxin resistance and loss of
97   root and stem geotropism and hypocotyl phototropism (Yang et al. 2004). Mutations in *AUXIN*
98   *RESISTANT1* (*AUX1*)/*LIKE-AUX1* (*LAX*) genes result in auxin-related developmental defects
99   and have been implicated in regulating root development, female gametophyte development and
100   embryo development in *Arabidopsis thaliana* (Swarup & Bhosale 2019). *AtIAA8* regulates lateral
101   root formation by interacting with auxin receptor *AtTIR1* and ARF transcription factors in the
102   nucleus (Arase et al. 2012). *MdARF1* regulates anthocyanin accumulation in fruit by interacting
103   with *MdIAA121* in apples (Wang et al., 2018).
104   As an important plant growth regulator, the role of ARF gene family in fruit ripening and
105   development has been investigated in many species but not in fig fruits. With its origins in the
106   Mediterranean coastal region, the common fig (*Ficus carica* L.) is one of the widely cultivated
107   ancient fruit trees in the world. The fig fruit (syconia), an aggregated fruit formed by the
108   expansion of female flower tissue and receptacle, has high antioxidant activity, great taste and
109   high nutritional value (Song et al. 2021). The fig fruit exhibits a typical double sigmoid growth
110   curve, including two rapid growth phase (I and III) separated by slower growth phase (II) (Song
111   et al. 2021). The fig has the advantages of short juvenile period, easy vegetative growth and
112   reproduction, and is gradually becoming a new model plant for studying fruit development
113   mechanisms (Wang et al. 2018). Presently, the genome-wide characterization of fig auxin signal
114   transduction-related components and their expression patterns has not been carried out. In this
115   study, genome-wide data of 'Horaishi' and 'Dottato' (Mori et al. 2017; Usai et al. 2020) and
116   'Purple Peel' fig transcriptome data at different developmental stages (NCBI acceptance No.
117   PRJNA723733) were used to predict ARF genes. In total, 20 ARF genes were identified in
118   common fig, and their features, including chromosome position, phylogenetic relationship, gene
119   structure and motif characteristics, characterized. In addition, we described the transcriptional
120   accumulation patterns of ARF genes in different tissues, including at different developmental

121 and maturation stages of female flowers and peel. Our study provides a good basis for further
122 exploring the role of *FcARFs* and characterizing their functions.
123
124 **Materials & methods**
125 **2.1 Plant materials**
126 The fig cultivar 'Purple Peel' plants were established at Shangzhuang Experimental Station of
127 China Agricultural University, Beijing, China. The roots, stems, leaves of 'Purple Peel', and fruits
128 at different developmental stages were collected for qRT-PCR analysis. Fruits were sampled at
129 six stages based on the characteristics of fruit development. Stage 1 represented phase I (the first
130 rapid growth period), stages 2, 3, and 4 were the early, middle, and late stages of phase II (slow
131 growth period), and stages 5 and 6 represented phase III (the second rapid growth period). At
132 each stage, 50 fruits were sampled. The peel and flower were separated onsite during sampling.
133 Fresh samples were quick-frozen with liquid nitrogen and stored at −80℃ until RNA extraction.
134 **2.2 Identification of ARF transcription factor family in fig**
135 Genome sequences of *cv*. Horaishi and *cv*. Dottato fig were downloaded from the National
136 Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/?term=Fi
137 cus+carica) (Mori et al. 2017; Usai et al. 2020). The sequences were blasted (E-value = -5) using
138 the hidden Markov model (HMM) files of B3, Auxin_resp and AUX_IAA domains in Pfam
139 (http://pfam.xfam.org/ search/sequence) with entry numbers PF02362, PF06507 and PF02309,
140 respectively (El-Gebali et al. 2019). Candidate genes containing known conserved domains were
141 retained, and further screened for ARF domains against three databases: Pfam, NCBI conserved
142 domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART
143 (http://smart.emblh eidelberg.de) (Letunic et al. 2021). ARF family genes in *Arabidopsis*
144 *thaliana* were downloaded from the Arabidopsis database (TAIR;https://www.Arabidopsis.org/).
145 Fig ARF homologs were compared with Arabidopsis ARFs using BLASTP with default
146 parameters to obtain annotation and grouping information. *FcARFs* sequences were analyzed
147 using bioinformatics methods, and the physicochemical parameters, MW, pI and GRAVY of
148 translated proteins were calculated using ExPASy (http://www.expasy.ch/tools/pi_tool.html)
149 (Guo et al. 2014).
150 **2.3 Multiple sequence alignment and phylogenetic analysis**
151 Using Clustal 2.0, multiple-sequence alignments of amino acid sequences of predicted ARF genes
152 in fig was carried out, with default parameters (Song et al. 2021). ClustalX 2.11 was used to
153 analyze phylogenetic relationships among ARF gene families in Arabidopsis, fig, apple, grape and
154 strawberry. (Liu et al. 2015; Luo et al. 2014; Sun et al. 2015a). The phylogenetic tree of *FcARFs*
155 was constructed using MEGA 7.0 via the Neighbor-Joining method with calibration test parameter
156 "Bootstrap = 1000 (1000 replicates)" (Tamura et al. 2011; Kumar et al. 2016). The analysis
157 involved 87 amino acid sequences and ARF gene families in five species including fig were
158 divided following the division of ARF gene families in *Arabidopsis thaliana*. Percentage
159 confidence values were shown on branches and the evolutionary distances used to infer the
160 phylogenetic tree via Poisson correction method (Zuckerkandl & Pauling 1965). Gaps were treated
161 as a complete deletion. There were a total of 1619 positions in the final dataset.

162 **2.4 Gene structure and conserved motif analysis**
163 *FcARFs* gene sequences were downloaded from fig genome
164 database(https://www.ncbi.nlm.nih.gov/gen-ome/?term=Ficus+carica), and the intron/exon
165 structure map of the fig ARFs was generated online using the Gene Structure Display Server
166 (GSDS: http://gsds.cbi.pku.edu.ch) (Guo et al. 2007). FcARFs amino acid sequences were
167 referenced to fig genome database protein RefSeq. Because ARF family genes contain highly
168 conserved domains, DNAMAN was used for multiple sequence alignment of FcARF protein
169 sequences. The length and sequence information of other conserved motifs except the ARF
170 conserved domain were obtained using the online MEME4.11. 2 tool
171 (http://meme.sdsc.edu/meme/itro.html) with the following parameters: number of repetitions
172 "any," highest motif number "15," motif length "6–100," and default values for the other
173 parameters.
174 **2.5 Chromosome mapping and collinearity analysis**
175 Tandem replication of ARF genes usually refers to adjacent homologous genes on the same
176 chromosome, whereas gene duplication events are regarded as repeated homologous genes on
177 different chromosomes (Liu et al. 2011). Whether *FcARF* gene is considered an important event
178 of tandem repeat between genes depends on its location on the chromosome. To study the
179 distribution of ARF genes on chromosomes, their chromosomal location and annotation
180 information were utilized. An openly available database and Map Draw software were used to
181 generate physical location information for homolinear analysis of *ARF* genes in fig. The genome
182 data of *Ficus hispida* and *Ficus microcarpa* were downloaded from the National Genomics Data
183 Center database (https://bigd.big.ac.cn/search/?dbId=gwh&q=PRJCA002187&page=1) (Zhang
184 et al. 2020). An interspecies collinearity analysis of ARFs in fig, *F. hispida*, and *F. macrocarpa*
185 was performed using MCscanX and TBtools (Song et al., 2021). The final map was generated
186 with Circos version 0.63 (http://circos.ca/). The non-synonymous replacement rate (Ka) and
187 synonymous replacement rate (Ks) of replicated gene pairs were calculated using
188 KaKs_Calculator 2.0 (Wang et al., 2010), with Ka/Ks ratio taken as environmental selection
189 pressure.
190 **2.6 Protein functional connection network**
191 Since fig species was not included in STRING database, the search species was set to
192 *Arabidopsis thaliana*. The interaction networks of 20 ARF proteins were analyzed using the
193 STRING protein interaction database (http://string-db.org/), which is an online protein-protein
194 interaction networks functional enrichment analysis prediction site (Szklarczyk et al. 2021).
195 Protein-protein interaction networks were built with an interaction score of -1 to 1. In total, 20
196 fig ARF proteins were aligned with AtARF proteins using Blastp alignment with E-value of 1e-0
197 (Rosas-Camacho 2009, Chen et al. 2015). The interaction network of FcARF protein was
198 constructed using homologous and *Arabidopsis thaliana* ARF protein, and genes of interest were
199 visualized using Cytoscape software (Doncheva et al. 2019).
200 **2.7 Expression analysis of *FcARFs* using RNA-seq and qRT-PCR**
201 TBtools was used to analyze the expression patterns of FcARFs in 'Purple-Peel' fig fruit based
202 on RNA-seq library (NCBI Accession No. PRJNA723733). Their peel and female flower tissue

203   were collected at six stages of fruit development and significant differential expression was
204   determined with *p*<0.05 and |log2(fold change) |≥1.
205   Different tissues and fruits were sampled from 'Purple Peel' plant at six stages for qRT-PCR.
206   The peel and flower were separated during sampling. Fresh samples were quick-frozen with
207   liquid nitrogen and stored at −80℃ for RNA extraction. Total RNA was extracted using the
208   CTAB method and reversed transcribed using reverse transcription kit (TaKaRa, Dalian, China).
209   The specific primers for qRT-PCR are shown in **Table S5**. The design parameters for the primers
210   include amplicon length, 100-150 bp; primer length, 15-25 bp; melting temperature (Tm), 57-
211   61℃; GC base content, 40-60%; and excluding conserved domain (Li et al. 2019). Following a
212   previously published method (Song et al. 2021), all qRT-PCR reaction volumes were set at 10
213   μL. The cycle parameters were as follows: pre-denaturation at 94℃ for 15 s, annealing at 60℃
214   for 30 s and extension at 72℃ for 1 min, for 40 cycles. qRT-PCR reaction for each gene was
215   replicated three times. *Fc18S* and *FcActin* were used as an internal standard to calculate relative
216   fold differences based on comparative cycle threshold values. We calculated relative expression
217   levels using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. SPSS 26.0 software was used for significance
218   analysis.
219
220   **Results**
221   **3.1 ARF family members identified in fig**
222   Twenty ARF genes were identified in published fig genome and named *FcARF1* to *FcARF20*
223   following the equivalent classification for *Arabidopsis thaliana* (**Figure 1A and Table 1**).
224   Detailed information for each candidate gene, including gene name, gene ID, number of exons,
225   protein length, protein molecular weight (kDa), isoelectric point (pI), instability coefficient,
226   Aliphatic index, GRAVY and location in cells, is shown in **Table 1**. The length of amino acids
227   encoded by *FcARFs* ranged from 588 (*FcARF10*) to 1150 (*FcARF7*), averaging 782.95 amino
228   acids. The number of exons and predicted molecular weight ranged from 3 (*FcARF10*) to 17
229   (*FcARF18*) and 52.73 (*FcARF4*) to 127.06 KDa (*FcARF7*), respectively. The isoelectric point
230   (pI) ranged from 5.03 (*FcARF5*) to 9.52 (*FcARF11*), and acidic amino acids were enriched in the
231   protein molecules. In total, 80.95% of fig ARF family proteins had isoelectric points less than 7,
232   with most of them being weakly acidic, consistent with pl of ARF proteins in citrus and
233   strawberry (Li et al. 2015; Wang et al. 2019). On average, the total hydrophilicity of ARF family
234   proteins in fig was negative, ranging from -0.726 (*FcARF11*) to -0.322 (*FcARF17*), which
235   indicated that these proteins were hydrophilic. The instability index ranged from 42.05
236   (*FcARF17*) to 72.35 (*FcARF15*), suggesting that all these proteins were stable (II > 40). The
237   Aliphatic index ranged from 63.06 (*FcARF11*) to 84.46 (*FcARF18*). Twenty FcARF proteins
238   were localized in subcellular structures by online software Target P and CELLO v2.5. The
239   results showed that they were mostly distributed in the nucleus and cytoplasm.
240   **3.2 Phylogenetic analysis of FcARFs**
241   In total, 87 ARF proteins comprising 23 from *Arabidopsis thaliana*, 20 from fig, 12 from
242   strawberry, 19 from grape and 13 from apple were obtained from NCBI database to construct a
243   phylogenetic tree using Neighbor-Joining approach. FcARF proteins were divided into three

244   classes (**Figure 1**). Class-I was further divided into three classes, including class-Ia with the
245   following five members: *FcARF1*, *FcARF2*, *FcARF9*, *FcARF11* and *FcARF18*. Class-Ib
246   included three members (*FcARF12*, *FcARF14* and *FcARF20*) whereas class-Ic included two
247   (*FcARF3* and *FcARF4*). Class-II had six members and class-III had four. Compared with the
248   equivalent *Arabidopsis* class, the number of ARF members in fig class-Ib was less. These results
249   indicated that gene loss or gain occurred during the evolution of ARF family genes in fig.

250   **3.3 Analysis of ARF gene family structure**
251   Typical ARF protein has three conserved domains: N-terminal B3-like DNA binding domain
252   (DBD), C-terminal dimerization domain (CTD), and a middle region (MR) (Xing et al. 2011a).
253   **Figure 2** shows that all FcARFs contained MR as an intermediate domain. All proteins
254   contained a complete CTD structure except class-III members. Similarly, DBD structure existed
255   in all FcARFs proteins except *FcARF18*. The branches of the same phylogenetic tree always had
256   similar structures, but some genes in the same class exhibited different gene structures. Among
257   them, four FcARFs (*FcARF17*, *FcARF10*, *FcARF13* and *FcARF16*) had truncated CTD domains
258   distributed in class-III of the phylogenetic tree (**Figure 2A**). To exhaustively verify the accuracy
259   of FcARF conserved domains, the amino acid sequence alignment results of FcARFs and
260   identified the location of conserved domain were aligned using DNAMAN software (**Figure S1**).
261   The active MR domain has been found to be rich in glutamine (Q) whereas the inhibitory domain
262   is rich in proline (P), serine (S) and threonine (T) (Wu et al., 2011; Tiwari et al., 2003).
263   Considering these characteristics, we analyzed the amino acid sequence and composition of the
264   MR domain (**Figure S2**). Subsequently, we predicted 10 potential transcription activators
265   (*FcARF4*, *FcARF5*, *FcARF7*, *FcARF9*, *FcARF10*, *FcARF13*, *FcARF16*, *FcARF17*, *FcARF18*
266   and *FcARF19*) and 10 inhibitors (*FcARF1*, *FcARF2*, *FcARF3*, *FcARF6*, *FcARF8*, *FcARF11*,
267   *FcARF12*, *FcARF14*, *FcARF15* and *FcARF20*) (**Figure 2B**).

268   **3.4 Conservative FcARFs motif and cis-acting element analysis**
269   The exon-intron structures of genes in the same gene family are similar, reflecting the close
270   evolutionary relationship among all its members. The number of introns in *FcARF* ranged from 3
271   to 17 (**Figure 3A**). *FcARF18* gene in class-Ia had the most introns (17), whereas class-III
272   members contained the least, suggesting that members have experienced intron loss during
273   evolution. Conserved motifs 1-15 of *FcARFs* were analyzed (**Table S3, Figure S3**). Motif 2 and
274   motif 8 belonging to DBD and MR domains, respectively, were highly conserved in all FcARF
275   proteins. Our analysis of conserved motifs in the database revealed that conserved motifs in
276   FcARF are unstable, and their positions had changed except motif 2 and motif 8. In addition,
277   motifs 15 only existed in specific genes, which agrees with previously reported results (Wei et al.
278   2016). This indicates that these motifs may be essential for specific regulatory functions of these
279   genes.
280   We found 2 kb sequence upstream of the initiation codon of *FcARF* gene in PLACE database
281   that predicted many cis-acting elements larger than 4 bp. TGA-element and TGA-box were
282   associated with auxin response and transcriptional activation. There were at least 16 cis-acting
283   regulatory elements in the promoter region of *FcARF* family members in fig (**Figure 3B**). They
284   were involved in abiotic stress responses, including light response, anaerobic inducibility, anoxic

285 specific inducibility, defense and stress response, drought induction, low temperature responsive
286 and wound response. In addition, these cis-acting regulatory elements were involved in hormone
287 response, (abscisic acid response, auxin response, gibberellin response, MeJA response and
288 salicylic acid response), circadian control, flavonoid biosynthesis regulation, meristem
289 expression and other regulatory elements. Drought stress-related MYBHv1 binding sites were
290 found in the promoter regions of three ARF genes (*FcbARF2, FcARF14* and *FcARF18*).

291 **3.5 Chromosome localization and distribution of FcARFs**
292 Distributions of ARF transcription factors in fig linkage groups are shown in **Figure S4**. *FcARF1*
293 *~ FcARF20* were unevenly distributed in 12 chromosomes, apart from chromosomes 2 and 4.
294 Chromosomes 1, 6, 7, 10, 11 and 12 had one ARF gene each, which were *FcARF6, FcARF7,*
295 *FcARF5, FcARF9, FcARF14* and *FcARF20,* respectively. Chromosomes 3, 5 and 9 had 3 ARF
296 genes each, which were the most abundant. Tandem replication occurs when the distance
297 between genes is less than 100 kb and exists on the same chromosomes, only FcARF3 and
298 FcARF16 on Chr3 occurred. The FcARF gene family had 3 pairs of genes from fragment
299 replication (**Figure 4A**). During intraspecific evolution, the Ka/Ks values can explain positive
300 selection (Ka/Ks>1), neutral selection (Ka/Ks=1) and negative selection (Ka/Ks<1). Nineteen
301 pairs (43.18%) of ARF genes in fig species had Ka/Ks greater than 1, while 25 pairs (56.82%)
302 had Ka/Ks less than 1 **(Table S4)**, indicating the existence of 2 evolutionary modes of positive
303 and negative selection in fig, with negative selection being the main evolutionary mode.
304 Collinearity analysis identified 18 separate orthologous genes between *F. carica* and *F.*
305 *macrocarpa* as well as between *F. carica* and *F. hispida*, indicating that evolutionary distance
306 between fresh fig and two evergreen *Ficus* species was similar (**Figure 4B**). *FcARF20* did not
307 exhibit a collinear relationship with either of the two Ficus species, implying that there might be
308 a unique ARF in Fig evolution.

309 **3.6 Protein interaction network of FcARFs**
310 The ARF protein interacts with other proteins, such as IAA and AUX1 proteins, to regulate
311 downstream gene expressions and exert biological functions. Correlation analysis of ARF
312 proteins and interacting proteins in figs, as well as the gene expression correlation network (Song
313 et al 2022), was analyzed based on gene expressions in the developmental stages of fig fruits
314 (**Figure 5B**). A protein interaction network was constructed by STRING, which included 5 ARF
315 proteins and 9 other proteins (5 IAA proteins). Expressions of the five FcARFs were positively
316 correlated with each other, co-expression scores were all above 0.7, with FcIAA1, FcBZR1 and
317 FcAUX1 being negatively correlated with expressions of the five FcARFs.
318 In **Figure 5A**, thickness of lines represents protein interaction strengths. IAA8 interacted with
319 five FcARFs, and their expressions were positively correlated, including FcARF15-IAA8. In
320 *Arabidopsis*, interactions of AtARF15 and AtIAA8 proteins respond to early auxin responses and
321 cooperatively regulates lateral root growth, development as well as morphogenesis (Arase et al.
322 2012). There was a strong interaction between ARF6-PIF4, with an expression correlation
323 coefficient of 0.808, implying a positive correlation. Direct inhibition of auxin-responsive gene
324 expressions mediated by interactions of AtPIF4 and AtARF6 in *Arabidopsis* constitutes a new
325 layer in regulatory mechanisms of photoinhibition of auxin-induced hypocotyl elongation (Oh et

326 al. 2014). IAA1 was predicted to interact with FcARF8 and FcARF19, and expression
327 correlation indices were -0.582 and -0.571, respectively, implying negative correlations. This is
328 consistent with the prediction that IAA interacts with ARF and negatively regulates its
329 expressions. In the regulatory network composed of fig ARFs, there were strong positive
330 correlations among FcARFs levels, indicating functional complementarity and redundancy
331 among them. The expression levels and interaction coefficients predict that FcIAA8 is a key
332 positive cooperative factor in the network, while IAA1 is a negative regulatory cooperative
333 factor.

334 **3.7 Expressions of *FcARFs* in different tissues**
335 Expression patterns of 20 FcARFs in fig's root (RO), stem (ST), old leaf (OL), young leaf (YL),
336 flower (FL) and peel (PE) were analyzed by qRT-PCR (**Figure 6**). Twenty *FcARFs* showed
337 tissue and organ expression specificity. In class-I, *FcARF1/12/14/18/20* were highly expressed in
338 female flowers and peel, the major fruit components. *FcARF 2/3/4/9/11* were highly expressed in
339 the stem. In class-II, *FcARF 5/8/15* were highly expressed in female flowers and peel while
340 *FcARF 6/7/19* were highly expressed in the stem. In class-III, all FcARFs (*FcARF 10/13/16/17*)
341 were highly expressed in female flowers and peel. These findings imply that expressions of these
342 *FcARF* genes are high in reproductive organs, but low in vegetative organs, such as leaves
343 (**Figure 6**).

344 **3.8 Expressions of *FcARFs* during fig fruit ripening**
345 Expressions patterns of *FcARFs* in 'Purple peel' fig peel and flower tissues at different growth
346 stages were constructed by TBtools (**Figure 7**). Based on FPKM values, they were divided into 4
347 groups, among which 4 members greater than 100 came from 3 classes, respectively. Class-I had
348 4 members (*FcARF2*, *FcARF3*, *FcARF11*, and *FcARF12*) expressed in early stages of flower and
349 peel development. The 50-100 group consists of 3 members (*FcARF1, FcARF8* and FcARF18)
350 from class-Ia and class-II. Expression patterns of *FcARF1* and *FcARF18* were consistent, being
351 highest in early stages of female flower development. The rest of the members with FPKM
352 values between 10 and 50.
353 During the 6 growth stages of female flowers and peel, *FcARF3* from class-Ic was stably
354 expressed, with FPKM values > 50. In addition, expressions of *FcARF3* in fast-growing phase I
355 and III were higher than those in slow-growing phase II. Expressions of class-Ia members
356 *FcARF2/9/11* were highest in the early developmental stages of fruits and were decreased after
357 maturity. *FcARF11* levels were highest at P1, P2 and P4, while *FcARF9* levels were highest at
358 P3. In this study, *FcARF* gene expressions in peel and flower tissues of fig fruit at 6 different
359 stages of ripening levels were evaluated by qRT-PCR (**Figure S5 and S6**). Expression profiles
360 of *FcARF* in female flowers and peel tissues obtained from transcription sequencing data should
361 be verified.
362
363 **Discussion**
364 To establish the significance of ARF genes in the auxin signal transduction pathway in fig, we
365 evaluated the main structural characteristics of the ARF gene family in fig. Using genome
366 sequencing results, we isolated and identified 20 fig ARF genes.

Phylogenetic tree and protein structure predictions of ARF protein function

Phylogenetic trees have been used to predict the functions of different genes (Pandey et al. 2018). **Figure 1** shows that fig is highly homologous to *Arabidopsis thaliana*, a model plant, with 6 sister pairs. Phylogenetic tree analysis of ARF proteins in *Arabidopsis* and fig can predict the functions of FcARFs by clarifying their phylogenetic relationships. For instance, *FcARF3* and *FcARF4* of fig belong to class-Ic with *AtARF3* and *AtARF4*, which may be involved in regulation of flower formation, development and patterns. Class-Ia, which consists of *FcARF1*, *FcARF2*, *FcARF9*, and *FcARF11* may have a role in regulating leaf senescence, floral organ shedding and auxin homeostasis. *FcARF10*, *FcARF13*, *FcARF16* and *FcARF17* belong to class-III with *AtARF10*, *AtARF16* and *AtARF17*, which may be involved in regulation of pollen tube wall formation, root cap formation and seed germination.

Apart from *FcARF18,* which does not have the DBD domain, other FcARFs (*FcARF1 ~ 17, 19,20*) have DBD and MR domains that determine their functions based on amino acid compositions (Sun et al. 2015b; Wang et al. 2007; Okushima et al. 2005). Through domain comparisons (**Figure S3** and **Table S3**), it can be found that *FcARF18* lacks motif 2 on the DBD domain, which inhibits its ability to recognize and bind DNA by forming a helix-turn-helix domain (Tiwari et al. 2003 Roosjen et al. 2018). In addition, most plant ARF gene families have an ARF protein without the CTD domain. All class-III FcARFs have truncated CTD domains, which is similar in *Arabidopsis thaliana* and other species (**Figure 2A**). Responses and signal transduction of plant cells to auxin are mainly mediated by the TIR1/AFB/-Aux/IAA-ARF signaling pathway in the nucleus. ARF participates in the auxin signaling pathway by forming a heterodimer with CTD regions of the Aux/IAA protein. The C-terminal domains of proteins are homologous. Without a complete CTD domain, FcARF10/13/16/17 may not participate in the auxin signaling pathway or perform its independent functions (Liu et al. 2011).

The ARF genes are transcriptional activators or inhibitors. The activation domain (AD) in glutamine (Q) and leucine (L) in the middle region of the ARF protein can act as a transcription activator, while the repression domain (RD) rich in proline (P), serine (S) and threonine (T) can act as a transcription inhibitor (Tiwari et al. 2003; Zouine et al. 2014). In this study, the intermediate region of transcription factors in class-Ia and class-II was found to be rich in glutamine (Q), and it is postulated that class-II members have transcriptional activation functions. *AtARF5*, *AtARF6*, *AtARF7*, *AtARF8* and *AtARF19* in *Arabidopsis thaliana*, which are homologous to fig class-II, are ARF[ClassA] transcription activators. Middle regions of classes-I and -III are rich in proline (P), serine (S) and threonine (T). Therefore, members of class-Ia and class-II inhibit transcription. Our findings should be verified by studies of regulatory mechanisms (Roosjen et al. 2018).

Evolution and Gene Structures of the ARF Family

Gene replication, including tandem repeat and large fragment replication, is the most important mechanism of gene recombination and amplification (Vision et al. 2000). Tandem repeat refers to two adjacent genes on the same chromosome, while large fragment replication events occur on different chromosomes, which is an important way of gene expansion and species evolution (Liu et al. 2011; Xing et al. 2011b). Compared to other plants, there are 20 ARF genes in fig, which

408 are less than those in other plants, such as 23 in *Arabidopsis thaliana*, 25 in rice and 39 in poplar.
409 Combined with collinearity analysis, there are probably no large-scale gene repetition events in
410 fig evolution. Collateral gene pairs (*FcARF13* and *FcARF10*) were located on chromosome 1,
411 while *FcARF19*, *FcARF20*, *FcARF13* and *FcARF17* were located on chromosomes 9, 11, 13 and
412 3, respectively. The former belongs to tandem replication, while the latter belongs to large
413 fragment replication. In this study, 3 (15%) of 20 FcARFs were established to be involved in
414 tandem repeat or large fragment replication events, while 33% of MdARFs in apples were
415 associated with gene replication (Luo et al. 2014). Even though there are few gene replication
416 events in fig, we postulated that gene replication is the main mode of ARF gene family
417 amplification, implying that different types of ARFs gene family members have different
418 evolutionary paths and rates.
419 FcARFs are mainly involved in plant hormone responses, growth regulation, light response and
420 environmental stress responses. As trans-acting factors, hormones can bind hormone response
421 elements in promoter sequences, thereby regulating the transcriptional activities of target genes,
422 and can participate in plant growth as well as environmental stress processes, such as salt and
423 temperature as signal transduction factors (Gunes et al. 2007). We found that the ARF gene
424 family has various hormone-responsive elements, hat hormones induce ARF gene family
425 expressions in fig, participating in plant growth and development.
426 **FcARFs play important roles in fig fruit development**
427 Expression levels of ARF family members in different parts of fig were analyzed to elucidate the
428 function of *FcARFs*. There were spatial expressions of *FcARFs* in roots, stems, old leaves, young
429 leaves, female flowers and peel at the transcription level. Most ARF genes were highly expressed
430 in female flowers and peels, followed by stems and roots, and least expressed in old leaves,
431 indicating that ARF family members have important roles in fig fruit growth and development.
432 Besides, *AtARF1* is involved in floral organ abscission (Ellis et al. 2005), and its homologous
433 gene (*FcARF1*) is highly expressed in female flower tissues, implying a role in flower growth.
434 Moreover, *FcARF*s (*FcARF 1/2/3/4/5/6/7/8/9/11/12/14/15/18/19/20*) exhibited tissue-specific
435 transcript accumulation patterns. It has been reported that *AtARF11* is highly expressed in the
436 roots and promotes lateral root formation (**Table S1**). *FcARF11* has a high homology with
437 *AtARF11* and has high transcript accumulations in stem and roots (**Figure 6**), indicating that the
438 roles of ARF during root development in fig are similar to those in *Arabidopsis thaliana*.
439 ARF7/ARF19 mutants in *Arabidopsis* exhibit strong auxin-related phenotypes, including during
440 severe damage to root formation (Okushima et al. 2007; Okushima et al. 2005). AtARF19 are
441 homologous and have high transcript expressions in roots. It has been postulated that *FcARF19*
442 has important roles in root formation and fig development.
443 Fruit development and ripening is a coordinated process of reproductive organ cell division,
444 differentiation and expansion that is regulated by transcriptional regulatory networks (Uchiumi
445 & Okamoto 2010). Auxin promotes the growth of female flowers as well as receptacles and
446 causes fruit enlargement that plays a key role in fruit ripening (Flaishman et al. 2008; Kumar et
447 al. 2011). In this study, all *FcARFs* were expressed from the first stage (phase I) to sixth stage
448 (end of phase III) of fruit development. The fruits of figs went through a fast-slow-fast

449 developmental period. Sensing and transmitting signals have been shown to reveal changes in
450 auxin expression patterns (Estrada-Johnson et al. 2017). *AtARF2* has an important role in
451 regulating flower formation as well as senescence of *Arabidopsis thaliana* (Ellis et al. 2005). Its
452 homologous gene (*FcARF2*) was highly expressed in the early flower and peel development
453 stages, which may regulate fig flower morphologies. *FcARF11* and *FcARF12* from class-Ia were
454 abundantly expressed during the early stages of fig fruit female flower as well as peel
455 development and were predicted to function as transcriptional repressors (**Figure 2B**). It has not
456 been established whether FcARF inhibition acts synergistically with other TFs.
457 *AtARF3* has a major role in nutrition and reproductive development, regulation of gene and
458 embryonic development, and determining the morphological patterns of pistil (Harper et al.
459 2000; Sessions et al. 1997; Xiao et al. 2004). Its homologous gene (*FcARF3*) is highly expressed
460 during whole growth periods of fig fruits, and suppressed in middle slow growth periods, in
461 tandem with the fig fruit development curve (Song et al. 2021). Moreover, it may be involved in
462 various reproductive and developmental processes, such as female flower development in figs.
463 The fig fruit development candidates obtained from fig ARF gene family analysis is the basis for
464 the next step in fruit development, such as expansion mechanisms caused by female flower
465 development.
466 **Conclusions**
467 The ARF genes are mainly expressed in fruits and flowers and are involved in development and
468 morphogenesis. The common fig fruit contains enlarged female flowers that are closely
469 correlated with development and maturity. Twenty FcARFs containing similar conserved motif
470 structures were identified from fig genome databases, and were divided into three classes (I, II
471 and III) by phylogenetic analyses. They were differentially expressed in tissues, with
472 *FcARF1/5/8/10/12/13/1415/16/17/18/20* being highly expressed in female flowers and peels,
473 indicating their possible importance in fruit development. Further analysis of the *FcARFs*
474 expression levers in female flower and peel during common fig *cv*. Purple Peel fruit
475 development. *FcARF2*, *FcARF11* and *FcARF12* are class-Ia members that are mainly involved in
476 regulating flower development. They were highly expressed in early stages of female flower
477 development. Expression patterns of *FcARF3* were in line with fig fruit development curve,
478 implying that it may be involved in the rate-limiting process of fig fruit development as a
479 repressor. This study forms the basis for further functional verification of the fig ARF gene
480 family.
481 **Acknowledgments**
482 Not applicable.
483
484 Table S1 (Chen et al. 2021; de Jong et al. 2015; Diao et al. 2020; Ellis et al. 2005; Ge et al. 2016;
485 Goetz et al. 2006; Kelley et al. 2012; Krogan et al. 2014; Kumar et al. 2011; Li et al. 2016; Lim
486 et al. 2010; Liu et al. 2015; Luo et al. 2018; Okushima et al. 2005; Pekker et al. 2005; Sagar et al.
487 2013; Sessions et al. 1997; Shen et al. 2010; Song et al. 2015; Wan et al. 2014; Wang et al. 2021;
488 Wang et al. 2005; Wang et al. 2010; Wang et al. 2018; Yang et al. 2013; Ye et al. 2016; Zhang et
489 al. 2020; Zhang et al. 2021; Zhang et al. 2015)

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Figure 1

Figure 1. Phylogenetic analysis of auxin response factors from *Ficus carica* L., *Arabidopsis thaliana, Fragaria vesca, Vitis vinifera* and *Malus domestica*.

The phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0. Bootstrap values from 1,000 replicates were indicated at each branch. Gene groups were indicated with different colors and were classified into four groups: class-I, class-II and class-III. class-I includes three subclasses: class-Ia class-Ib and class-Ic.

Figure 2

Figure 2. Analysis of the sequence and structure of the ARF gene family in Fig.

(A) Analysis of protein domains and gene structures. The B3 DNA binding domain (DBD), middle region (MR), and AUX/IAA (CTD) family domain were shown in green, purple and pink, respectively. The orange circle represents a transcription activator, and the blue circle represents a transcription inhibitor. (B)Distribution of the conserved motifs of FcARFs. Sequence alignment of the conserved B3 DNA binding domain (DBD), middle region (MR), and AUX/IAA (CTD) were performed by CLUSTALW.

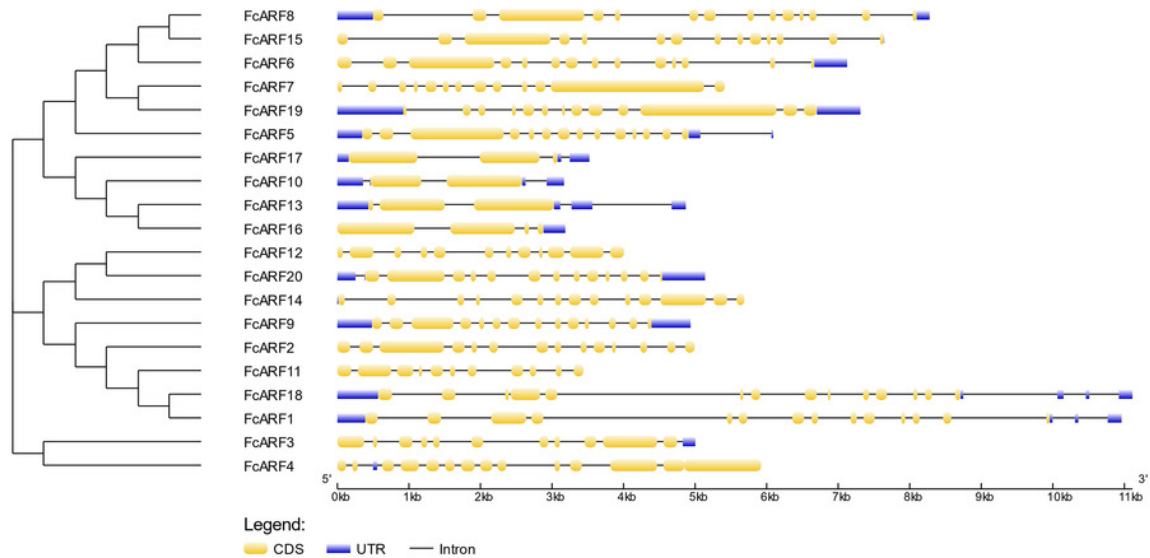
A

B

Figure 3

Figure 3 Analysis of gene structures and cis-acting elements for FcARF genes.

(A) Analysis of exon-intron structures for FcARF genes. Blue rectangles, yellow rectangles and blank lines were indicated upstream/downstream, exons and introns, respectively. Thelengthsofexonsandintronsare indicatedbythescalebar. (B) The distribution of cis-elements in the 2000 bp upstream promoter regions of FcARF genes were analyzed. The *cis*-elements were predicted at PlantCARE (https:// bioin forma tics. psb. ugent. be/ webto ols/ plant care/ html/). Different*cis*-elements were presented with different shapes and colors.

A

B

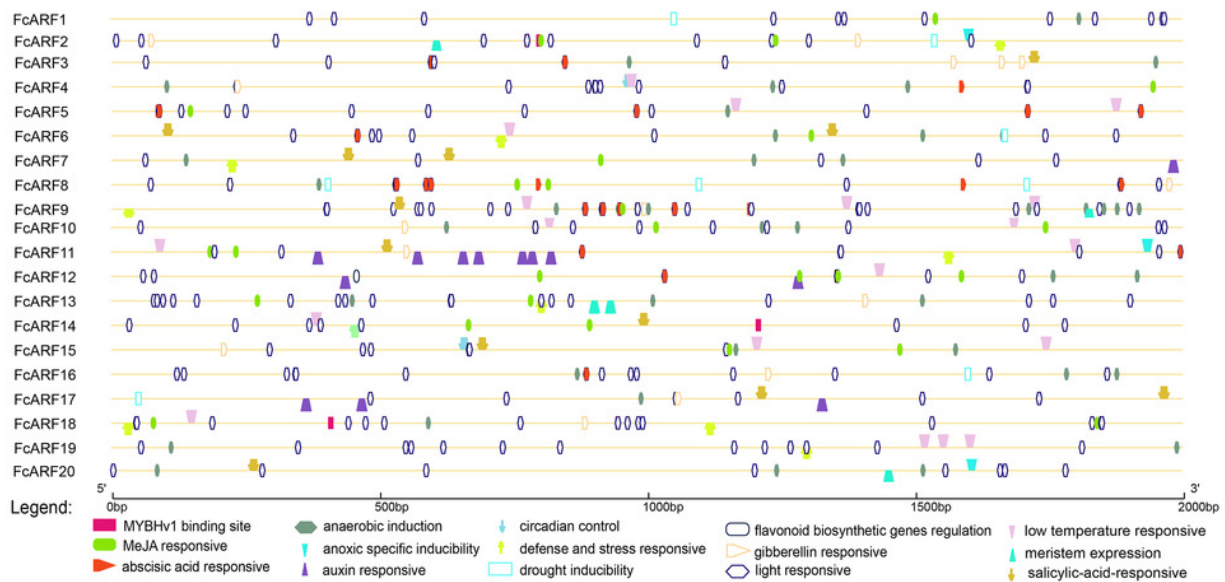



Figure 4

Figure 4. Chromosomal localization and collinearity analysis of the FcARF gene family.

(A) Collinearity analysis of FcARF genes, the circle plot was created with the MCScanX tool. Identified collared genes are linked by colored lines. (B) Collinearity relationship of ARF genes among *Ficus carica* (Fc), *Ficus hispida* (Fh), and *Ficus microcarpa* (Fm). Identified colinear genes are linked by blue lines.

A

B

Fm-genome Chr01 Chr02 Chr03 Chr04 Chr05 Chr06 Chr07 Chr08 Chr09 Chr10 Chr11 Chr12 Chr13

Fc-genome Chr01 Chr02 Chr03 Chr04 Chr05 Chr06 Chr07 Chr08 Chr09 Chr10 Chr11 Chr12 Chr13

Fh-genome Chr01 Chr02 Chr03 Chr04 Chr05 Chr06 Chr07 Chr08 Chr09 Chr10 Chr11 Chr12 Chr13 Chr14

Figure 5

Figure 5. Correlation analysis network among auxinsignaling-relatedpredicted proteins .

(A) The FcARFs and other predicted proteins interaction network were analyzed in the STRING database. Green color indicated FcARFs and the gray color indicated other auxinsignaling-related predicted proteins. Athickerlineindicates astrongerinteractionbetweenthetwo proteins. (B) The auxinsignaling-relatedpredicted genes expression correlation analysis was performed by Pearson algorithm, based on these gene's expression of the female flower and peel during the fig fruit development, the data in the cells were indicated the correlations scores .

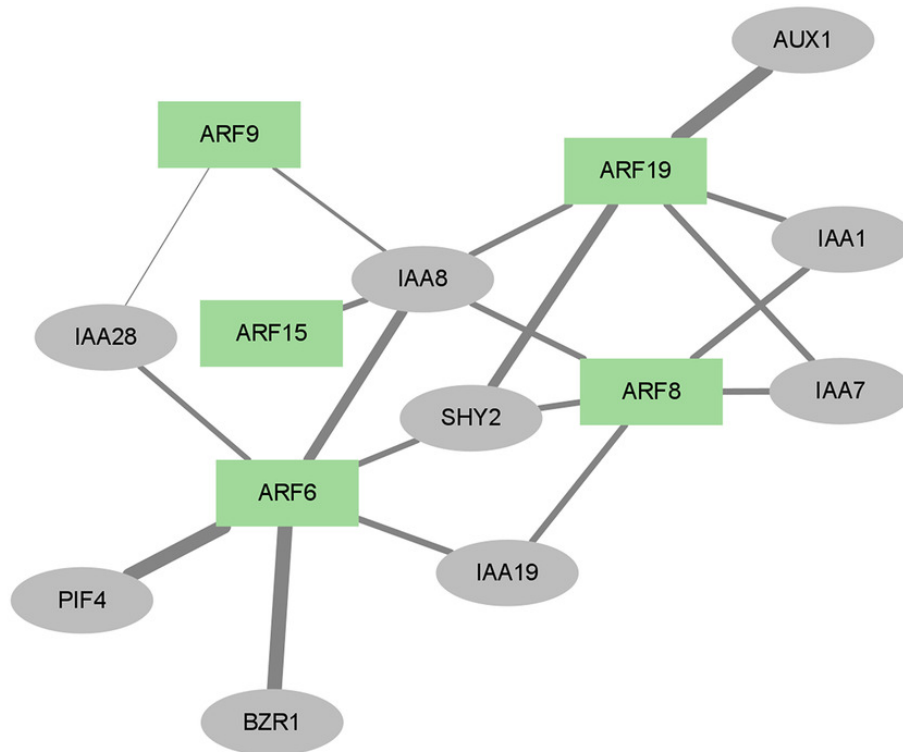
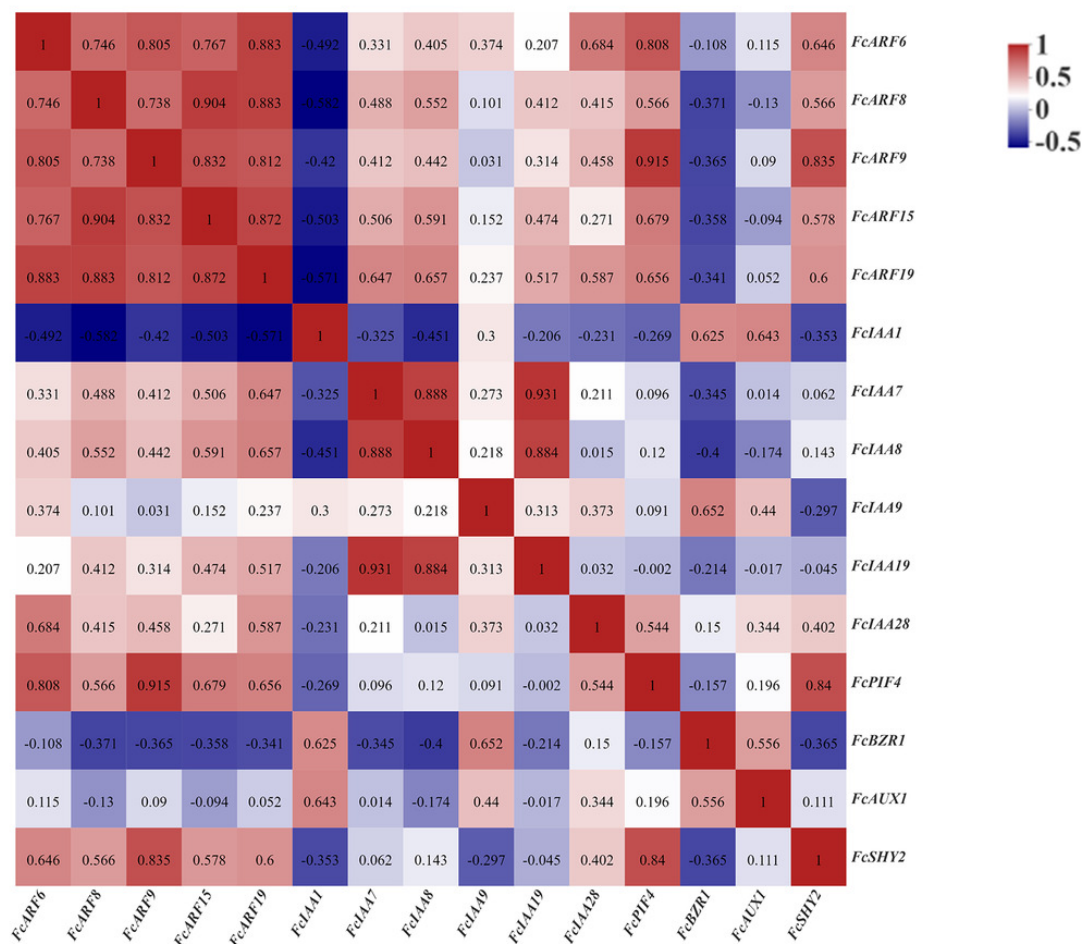



Figure 6

Figure 6. Tissue-specific transcript expression patterns of 20 ARF genes in the 'Purple Peel' fig.

Different plant tissues. RO: Root; ST: Stem; OL: Old Leaf; YF: Young Leaf; FL: Flower; PE: Peel.

Relative transcript levels are calculated by qRT-PCR with *β-actin* as a standard. Data are

means ± SD of three separate measurements. The qRT-PCR data were analyzed by relative

quantification using $2^{-\Delta\Delta Ct}$. Different letters above the bars indicate statistically significant

differences (*P*=0.05).

Relative expression level

FcARF1, *FcARF2*, *FcARF3*, *FcARF4*, *FcARF5*, *FcARF6*, *FcARF7*, *FcARF8*, *FcARF9*, *FcARF10*, *FcARF11*, *FcARF12*, *FcARF13*, *FcARF14*, *FcARF15*, *FcARF16*, *FcARF17*, *FcARF18*, *FcARF19*, *FcARF20*

RO ST OL YL FL PE

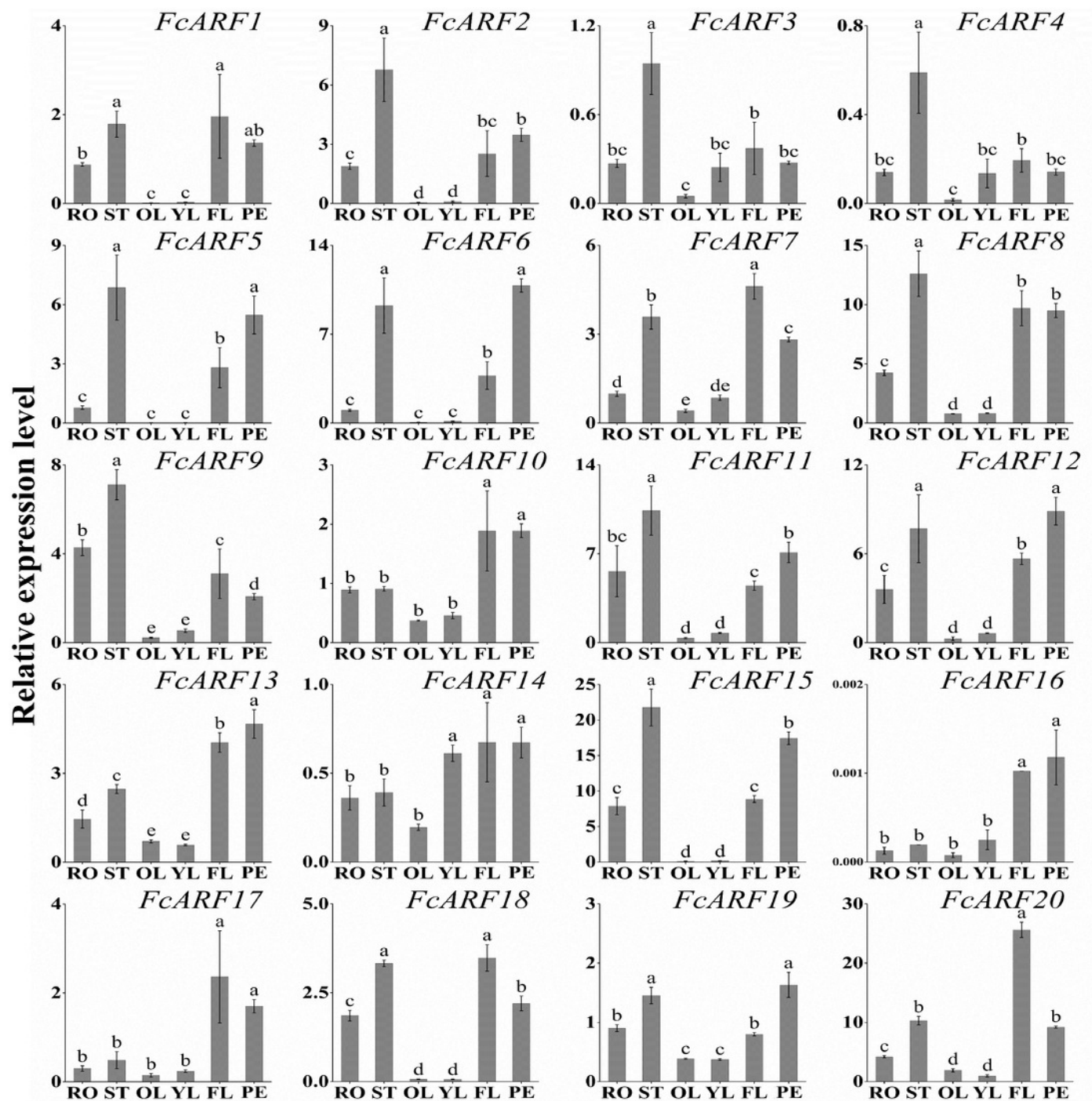



Figure 7

Figure 7: Transcript expression pattern of 20 ARF genes in the 'Purple Peel' fig during different fruit development stages.

Fruit developmental stages. Stage 1: represented phase I (the first rapid growth period);

stages 2, 3, and 4: were the early, middle, and late stages of phase II (slow growth period);

stages 5 and 6: represented phase III (the second rapid growth period).

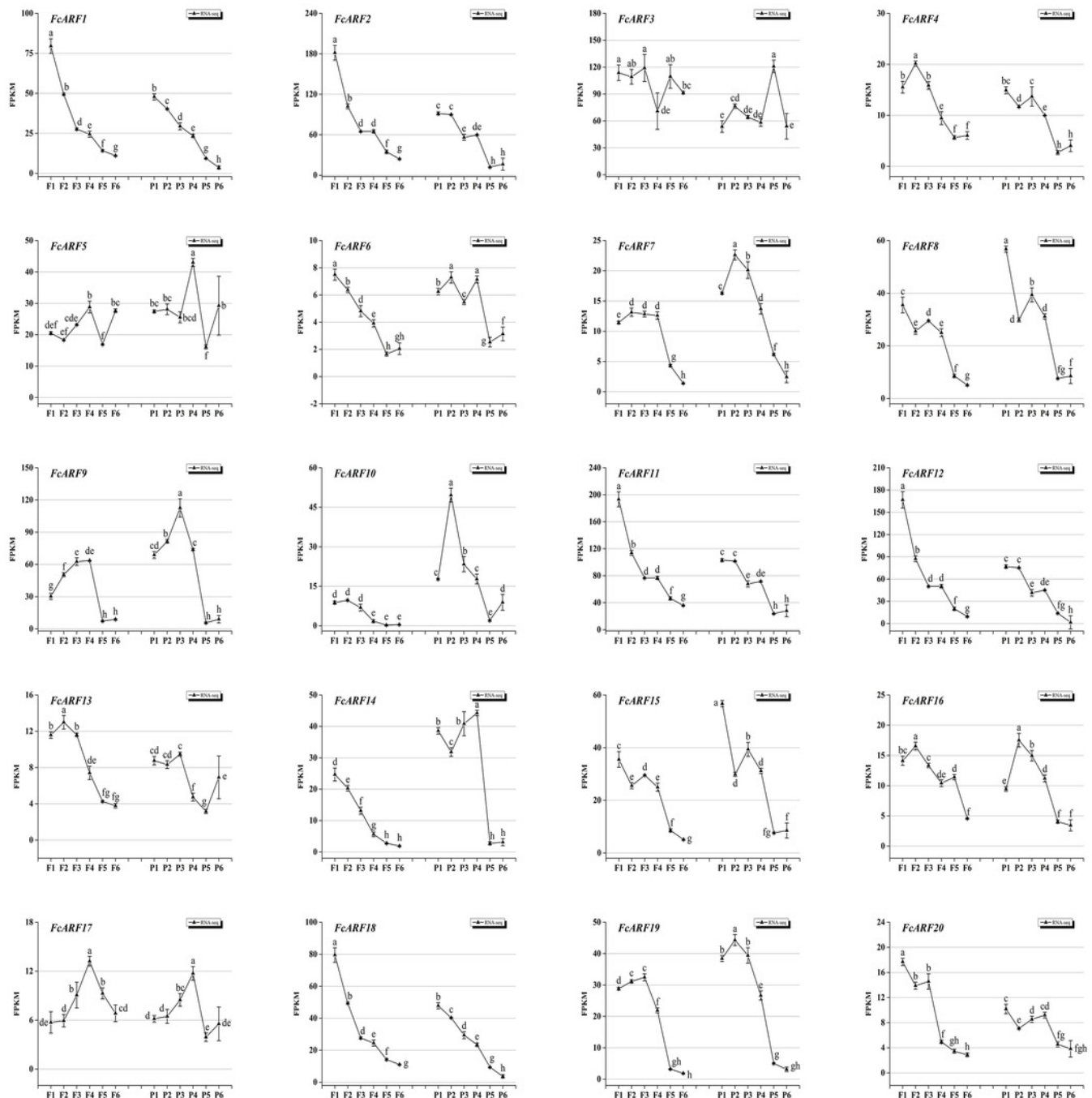



Table 1 FcARF genes encoding ARF proteins along with their molecular details.

Table 1 FcARF genes encoding ARF proteins along with their molecular details.

Gene name	Gene ID	Exon No.	Length (AA)[a]	Mw (k Da)[b]	Atomic Formula	pI[c]	Instabiliy index	Aliphatic index	GRAVY	Localization
FcARF1	FCD_00029581	16	897	99.27	$C_{3279}H_{5126}N_{928}O_{1019}S_{34}$	6.04	71.85	73.03	-0.463	Nuclear
FcARF2	FCD_00016093	14	843	94.02	$C_{4116}H_{6423}N_{1191}O_{1271}S_{34}$	6.72	55.25	64.06	-0.654	Nuclear
FcARF3	FCD_00001359	12	813	89.96	$C_{3946}H_{6167}N_{1111}O_{1229}S_{35}$	6.55	56.15	71.5	-0.465	Nuclear
FcARF4	FCD_00032414	11	768	52.73	$C_{2335}H_{3623}N_{647}O_{714}S_{17}$	7.21	57.64	69.49	-0.510	Nuclear
FcARF5	FCD_00003481	15	942	103.77	$C_{4544}H_{7139}N_{1241}O_{1448}S_{45}$	5.03	58.47	78.33	-0.370	Nuclear
FcARF6	FCD_00013415	14	987	99.27	$C_{4360}H_{6798}N_{1234}O_{1347}S_{38}$	6.04	71.85	73.03	-0.463	Nuclear
FcARF7	FCD_00017652	13	1150	127.056	$C_{5531}H_{8723}N_{1595}O_{1737}S_{53}$	6.57	63.56	73.5	-0.511	Nuclear
FcARF8	FCD_00034325	14	899	99.39	$C_{4367}H_{6781}N_{1229}O_{1362}S_{34}$	5.86	71.46	72.45	-0.491	Nuclear
FcARF9	FCD_00028996	14	687	76.74	$C_{3366}H_{5311}N_{949}O_{1054}S_{25}$	6.22	52.53	72.63	-0.523	Nuclear
FcARF10	FCD_00021037	3	588	65.32	$C_{2901}H_{4478}N_{806}O_{871}S_{23}$	6.95	43.7	70.1	-0.475	Nuclear
FcARF11	FCD_00022972	11	595	67.15	$C_{2969}H_{4638}N_{870}O_{878}S_{1}$	9.52	60.21	63.06	-0.726	Nuclear
FcARF12	FCD_00032300	12	689	78.08	$C_{3453}H_{5432}N_{1012}O_{1020}S_{20}$	9.42	54.24	72.12	-0.655	Nuclearn
FcARF13	FCD_00005247	5	699	76.48	$C_{3378}H_{5233}N_{945}O_{1025}$	6.36	50.71	71.43	-0.390	Cytoplasmic

					S_{31}					
FcARF14	FCD_00007016	14	721	81.01	$C_{3572}H_{5504}N_{1000}O_{1081}S_{39}$	6.35	60.08	66.81	-0.519	Nuclear
FcARF15	FCD_00016691	14	902	99.73	$C_{4381}H_{6807}N_{1233}O_{1368}S_{34}$	5.83	72.35	72.64	-0.493	Nuclear
FcARF16	FCD_00001287	4	708	77.28	$C_{3399}H_{5310}N_{972}O_{1031}S_{31}$	7.62	44.78	72.57	-0.366	Cytoplasmic
FcARF17	FCD_00001289	4	620	67.06	$C_{2960}H_{4639}N_{819}O_{930}S_{15}$	5.90	42.05	79.69	-0.322	Nuclear
FcARF18	FCD_00035227	17	617	69.43	$C_{3054}H_{4812}N_{862}O_{921}S_{34}$	6.04	56.32	84.46	-0.326	Cytoplasmic
FcARF19	FCD_00010593	13	1115	124.55	$C_{5423}H_{8466}N_{1576}O_{1712}S_{44}$	6.03	70.65	68.66	-0.704	Nuclear
FcARF20	FCD_00013930	14	719	80.78	$C_{3576}H_{5568}N_{1014}O_{1074}S_{26}$	6.28	51.39	75.26	-0.554	Nuclear

3   [a] Length of open reading frame in base pairs

4   [b] Molecular weight (Kilodaltons)

5   [c] Isoelectric point