Genome-wide characterization of the auxin response factor (ARF) gene family of litchi (*Litchi chinensis* Sonn.): phylogenetic analysis, miRNA regulation and expression changes during fruit abscission

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Auxin response factors (ARFs) play fundamental roles in modulating various biological processes including fruit development and abscission via regulating the expression of auxin response genes. Currently, little is known about roles of ARFs in litchi (*Litchi chinensis* Sonn.), an economical important subtropical fruit tree whose production is suffering from fruit abscission. In this study, a genome-wide analysis of ARFs was conducted for litchi, thirty-nine ARF genes (*LcARFs*) were identified. Conserved domain analysis showed that all the LcARFs identified have the signature B3 DNA-binding (B3) and ARF (Aux_rep) domains, with only 23 members having the dimerization domain (Aux_IAA). Number of exons in LcARF genes ranges from 2 to 16, suggesting a large variation for the gene structure of *LcARFs*. Phylogenetic analysis showed that the 39 LcARFs could be divided into three main groups: class I, II, III. Totally, twenty-three *LcARFs* were found to be potential targets of small RNAs, with three conserved and one novel miRNA-*ARF* (miRN43-*ARF9*) regulatory pathways discovered in litchi. Expression patterns were used to evaluate candidate *LcARFs* involved in various developmental processes, especially in flower formation and organ abscission. The results revealed that most ARF genes likely acted as repressors in litchi fruit abscission, i.e. *ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B*, while a few *LcARFs*, such as *LcARF5A/B*, might positively involve in this process. These findings provide useful information and resources for further studies on the roles of ARF genes in litchi growth and development, especially in the process of fruit abscission.

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Abstract

Auxin response factors (ARFs) play fundamental roles in modulating various biological processes including fruit development and abscission via regulating the expression of auxin response genes. Currently, little is known about roles of ARFs in litchi (*Litchi chinensis* Sonn.), an economical important subtropical fruit tree whose production is suffering from fruit abscission. In this study, a genome-wide analysis of ARFs was conducted for litchi, thirty-nine ARF genes (*LcARFs*) were identified. Conserved domain analysis showed that all the LcARFs identified have the signature B3 DNA-binding (B3) and ARF (Aux_rep) domains, with only 23 members having the dimerization domain (Aux_IAA). Number of exons in LcARF genes ranges from 2 to 16, suggesting a large variation for the gene structure of *LcARFs*. Phylogenetic analysis showed that the 39 LcARFs could be divided into three main groups: class I, II, III. Totally, twenty-three *LcARFs* were found to be potential targets of small RNAs, with three conserved and one novel miRNA-*ARF* (miRN43-*ARF9*) regulatory pathways discovered in litchi. Expression patterns were used to evaluate candidate *LcARFs* involved in various developmental processes, especially in flower formation and organ abscission. The results revealed that most ARF genes likely acted as repressors in litchi fruit abscission, i.e. *ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B*, while a few *LcARFs*, such as *LcARF5A/B*, might positively involve in this process. These findings provide useful information and resources for further studies on the roles of ARF genes in litchi growth and development, especially in the process of fruit abscission.

Introduction

Auxin plays a central role in numerous aspects of plant developmental and physiological processes, including embryogenesis, apical dominance, vascular elongation, flowering, fruit development, and lateral root initiation (Woodward & Bartel, 2005; Fleming, 2006). Auxin response factors (ARFs) are a group of important transcription factors in the auxin signaling pathway, which can activate or repress the expression of early/primary auxin response genes by binding to the auxin response element (AuxRE) site in their promoter regions (Liscum & Reed, 2002; Guilfoyle & Hagen, 2007). A typical ARF is characterized by a highly conserved N-terminal B3-type DNA binding domain (DBD) that recognized the AuxRE motif, an activation domain (AD) or repression domain (RD), and a carboxy-terminal dimerization domain (CTD: domain III/IV), which is involved in protein-protein interactions by dimerizing with auxin/indole-3-acetic acid (Aux/IAA) family genes (Kim, Harter & Theologis, 1997; Guilfoyle & Hagen, 2007; Piya et al., 2014).

ARFs exert pivotal function in the regulation of plant growth and development through the auxin signaling pathway (Kepinski and Leyser, 2005; Li et al., 2016). Due to the significance of ARFs, genome-wide characterization of ARFs have been completed in many species such as model plants *Arabidopsis* (Hagen & Guilfoyle, 2002) and *Solanum lycopersicum* (Zouine et al., 2014), important crops such as soybean (Ha et al., 2013; Le et al., 2016) and maize (Xing et al., 2011; Wang et al., 2012), fruit trees like citrus (*Citrus sinensis*) (Xie et al., 2015) and apple (*Malus domestica*) (LUO et al., 2014), and so on. From embryogenesis to flowering, mutants in members of ARFs exhibit diverse phenotypes, which show their unique and redundant functions for plant development. For instance, in *Arabidopsis thaliana*, *arf1* and *arf2* mutations affect leaf senescence and floral organ abscission (Ellis et al., 2005) and the loss of *AtARF3* causes defectives in gynoecium patterning (Nemhauser, Feldman & Zambryski, 2000; Liu et al., 2014a). AtARF5 influences embryo, root and shoot development (Krogan et al., 2012; Crawford et al., 2015). AtARF9 acts in suspensor cells to mediate hypophysis specification (Rademacher et al., 2012), and AtARF10/16/17 play vital roles in negatively regulating seed germination and post-germination activities (Liu et al., 2007).

Recently, small RNAs, especially miRNAs, have been emerging as critical regulators in almost all aspects of plant growth and development. Many members of the ARF family have been reported to be targets of miRNAs. In *Arabidopsis*, *AtARF6* and *AtARF8* are targets of miR167 (Wu, Tian & Reed, 2006), *AtARF10/16/17* are targets of miR160 (Liu et al., 2007, 2010), and

77   *ARF2/3/4* are targets of trans-acting siRNAs (tasiRNAs) generated from miR390-targeted TAS3

78   gene (trans-acting siRNA gene 3) (Allen et al., 2005; Axtell et al., 2006). These targeting

79   relationships of miRNA on ARF genes are widely conserved in land plants (Xia, Xu & Meyers,

80   2017) and also in horticultural plants (Chen et al., 2018b). It has been reported that down-

81   regulation of *ARF6* and *ARF8* by miR167 leads to floral development defects and female sterility

82   in tomatoes (Liu et al., 2014b). Down-regulation of sly-miR160, increasing the expression of its

83   targets *SlARF10/16/17*, regulates auxin-mediated ovary patterning as well as floral organ

84   abscission and lateral organ lamina outgrowth (Damodharan, Zhao & Arazi, 2016). In

85   *Arabidopsis* and tomato, the tasiRNA-mediated regulation of *ARF2* involved in controlling the

86   onset of leaf senescence and floral organ abscission (Ellis et al., 2005; Lim et al., 2010; Guan et

87   al., 2014; Ren et al., 2017).

88

89   Litchi (*Litchi chinensis* sonn.), an important economic fruit trees in southern China, usually

90   undergo serious fruit abscission before harvest, leading to a low yield. Many studies demonstrate

91   *ARFs* play critical roles in regulating plant organ abscission (Ellis et al., 2005; Kuang et al.,

92   2012; Guan et al., 2014; Xie et al., 2015; Xu et al., 2015), while which *ARFs* are involved or

93   more important than the other in the fruit abscission in litchi remains elusive. Here, we identified

94   39 ARF genes in litchi. Gene structure, phylogeny and targeting relationship with miRNAs were

95   characterized. The expression of *LcARFs* was examined in diverse tissues and in the process of

96   fruit abscission which was induced by three different treatments. Among them, *ARF2D/2E*,

97   *7A/7B*, *9A/9B*, *16A/16B* and *LcARF5A/B*, were found to be associated with litchi fruit abscission.

98   Our results offer new knowledge and resources to study the function of plant ARF genes and

99   their roles in the fruit abscission in litchi.

100

Materials & Methods

Plant materials and treatments

The young fruitlet used for RNA-seq in our study were collected from 9-year-old litchi trees (*Litchi. chinensis* Sonn. cv. 'Feizixiao') in an orchard located at South China Agricultural University (Guangzhou, China). Three treatments have been performed 25 day after anthesis. The three treatments included ethephon (ETH), girdling plus defoliation (GPD) and dipping in 20mg/L 2, 4-D for one minute after girdling plus defoliation (GPDD). Details can refer to Li et al (Li et al., 2015) and Peng et al (Peng et al., 2013). Samples of 'Feizixiao' (FZX) for qRT-PCR were obtained from the orchard of Guangzhou Fruit Research Institute (Guangdong, China). Tissues including fruit-bearing shoots (FBS), young leaves (YL), mature leaves (ML), female flower (FF), male flower (MF), sex undetermined flower (USF) and young fruit (YF) of 25 days after fertilization were collected from different directions of each tree. After separation, all tissues were quickly frozen in liquid nitrogen and stored at -80℃ before usage.

Identification and phylogenetic analyses of LcARFs

Amino acid sequences of 25 and 23 ARFs from rice (Wang et al., 2007) and *Arabidopsis* (Okushima et al., 2005; Wang et al., 2007) were downloaded from UniProt (http://www.uniprot.org/) and 22 ARFs from tomato (Zouine et al., 2014) were downloaded from Sol Genomics Network (https://solgenomics.net/). These ARFs were used as bait to identify potential ARFs in litchi genome and obtained by Blast analysis in TBtools (Chen et al., 2018a), with the E-value $1e^{-10}$. All sequences were then further validated by conserved domain search against the CDD (http://www.ncbi.nlm.nih.gov/cdd/) and PFAM (http://pfam.xfam.org/) databases. Based on the optimized alignment of amino acid sequences of LcARFs proteins, achieved by TrimAL 1.3 (http://phylemon2.bioinfo.cipf.es/index.html) after the initial sequence alignment in ClustalX 2.1, a maximum likelihood (ML) tree was constructed in MEGA 7.0 software with a bootstrap of 1000 replicates. Intron and exon distribution patterns and genome structure were analyzed and visualized in TBtools (Chen et al., 2018a). The detail of predicted protein sequences of LcARFs were shown in Data S1 and S2.

LcARF targets of miRNAs and RNA-seq analysis

Most of the *ARF*-targeting miRNA/tasiRNAs were identified in our previous study (Ma et al., 2018). To further validate the targeting relationship, target sites were verified by psRNATarget (http://plantgrn.noble.org/psRNATarget) (Dai & Zhao, 2011) and miRBase (http://www.mirbase.org/).

135   RNA-seq analysis was carried on as described previously (Li et al., 2013). These RNA-seq data

136   was normalized using TPM method (Wagner, Kin & Lynch, 2012). Based on the sequences of

137   the identified *LcARFs*, a BLASTN search was conducted to find their gene counterparts in

138   previous data (Li et al., 2013). Finally, a global gene expression profiles were visualized by

139   heatmap via TBtools software (Chen et al., 2018a).

140

141   **Quantitative RT-PCR**

142   Total RNA of above seven tissue samples was extracted by column plant RNAout kit (TIANDZ,

143   China) according to the manufacturer's instructions. About 2 μg total RNA was applied to

144   synthesized first strand cDNA using reverse transcriptase AT311-03 (TRANSGEN BIOTECH,

145   China). PCR primers (Table S1) of *LcARFs* and reference genes *GAPDH* and *EF* (Zhong et al.,

146   2011) were designed by Primer Premier 5.0. qRT-PCR was performed according to the

147   manufacturer's specifications of THUNDERBIRD qPCR MIX QPS-201 (TOYOBO, China) on a

148   LightCycler 480 (Roche, Switzerland). Each expression profile was independently verified in

149   three biological replicates. Relative expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$

150   method (Livak & Schmittgen, 2001). Significance analysis was conducted in SPSS vision 22.0

151   and visulized by SigmaPlot 12.5.

152

Results

Identification and phylogenetic analysis of LcARF genes

To identify all ARF members in litchi, protein sequences of ARFs in rice, *Arabidopsis* and tomato were used as queries in BLASTP to search against litchi annotated gene database. Sixty-eight potential ARF genes were identified. After redundant result elimination and further conserved domain validation, 39 LcARFs were obtained ultimately. Among them, 23 protein sequences contained B3, ARF and the Aux/IAA domains. Length of these litchi ARF proteins ranged from 260 (LcARF1B) to 1200 (LcARF4A) and the relative molecular mass of them varied from 29522.5 (LcARF1B) to 144936.9 Da (LcARF16C), with PIs in the range of 5.28 (LcARF16C) to 8.64 (LcARF19A) (Table S2). According to the subcellular localization predictor (CELLO v.2.5; http://cello.life.nctu.edu.tw) prediction, most LcARFs were predicted to be located in the nucleus.

A phylogenetic tree was generated using the maximum likelihood method based on alignment of litchi ARFs with their orthologs from rice, *Arabidopsis* and tomato (Fig. 1). All 109 ARFs fell into three broad groups: class I, II and III, which contained 53, 34 and 22 members, respectively. Obviously, most ARFs of the four species were clustered together in the first two classes. Litchi ARFs were named according to their positions with orthologs from the other three species in the tree. Thus, the 39 LcARFs could also be assigned to three separate clusters as well. Class I contained LcARF1/2/3/4/9/18 (1A/B/C/D, 2A/B/C/D/E/F, 3A/B, 4A/B/C, 9A/B, 18A/B); Class II included LcARF5/6/7/8/19 (5A/B, 6A/B/C/D, 7A/B, 8A/B, 19A/B); Class III included LcARF10/16/17 (10A/B, 16A/B/C/D, 17A/B).

Gene structure and conserved domains of litchi ARFs

To better understand the structure evolution of LcARF genes, their gene structure (intron/exon number and positions) and functional domains were analyzed. As shown in Fig. 2, 23 *LcARFs* harbored the typical ARF protein structure which composed of a highly conserved DNA-binding domain (DBD) in the N-terminal region with a plant specific B3-type subdomain, an Auxin-resp subdomain, and an AUX_IAA dimerization subdomains. The remaining 16 *LcARFs* contained only B3 and Auxin_resp subdomains. Gene structure analysis revealed that the exon number of class III (2-4 exons) was significantly less than the other two groups (8-17 exons for class II and 10-16 exons for class I). These results provided additional evidence confirming the phylogenic relationships among LcARFs.

187   **Analyses of miRNA targeting *LcARFs***

188   Twenty-two out of the 39 LcARF genes were found to be the targets of miRNA (Fig. 3). All

189   group members of class III (*LcARF10A/B*, *16A/B/C/D* and *17A/B*) were found to be targeted by

190   Lc-miR160. *LcARF6A/B/C/D* and *8A/B* were members of class II and all of them were

191   collectively targeted by Lc-miR167. Two kinds of miRNA targeting patterns were observed in

192   the class I. One group comprising *LcARF2E/2F/4B/4C/3A/3B* were targeted by tasiARFs. In

193   litchi, *LcTAS3* are divided into two subgroups, long TAS3 genes(*LcTAS3_1* and *LcTAS3_2*) and

194   short TAS3 genes (*LcTAS3_3* and *LcTAS3_4*), which trigger to produce two or one tasiARFs

195   when cleaved by miR390 (Ma et al., 2018). Additionally, *LcARF2E/2F* incorporated one target

196   site of tasiARF, while *LcARF3A/3B/4B/4C* contained two, which is in accordance with our

197   previous study (Xia, Xu & Meyers, 2017). Interestingly, in the other group, a novel miRNA-*ARF*

198   pathway was discovered, in which *LcARF9A/B* was targeted by the Lc-miRN43.

199

200   **Expression of LcARF Genes in different organs and tissues**

201   A large body of evidence support the importance of ARF genes in plant growth and development

202   (Ellis et al., 2005; Guilfoyle & Hagen, 2007; Lim et al., 2010; Li et al., 2016). To explore how

203   LcARF genes function in the development of litchi, we examined their expression levels in

204   various litchi organs/tissues by qRT-PCR. Seven organs/tissues samples, composing fruit-

205   bearing shoots (FBS), male flower (MF), female flower (FF), sex undetermined flower (USF),

206   mature leaves (ML), young leaves (YL) and young fruits (YF) in 'FZX', were collected and

207   tested. As shown in Fig. 4A, expression of all *ARFs* was detected at least in several tissues.

208   Generally, most *LcARFs* show low expression in YL and YF but high in USF. *LcARFs* from

209   different clades showed various expression patterns. *LcARFs* from class I and III were detected

210   to be higher expressed in ML while those from class II were lowly accumulated, suggesting that

211   these *LcARFs* in different classes may perform different functions in mature leaves development

212   (Fig. 4A). Additionally, as is shown in Fig. 4B, ten *LcARFs* (*LcARF2B*, *3A*, *4A/B*, *5A/B*, *6D*,

213   *9A/B*, *18B*) were significantly higher expressed in FF than MF, implying their potential function

214   in ovule and ovary derived from female flowers. Remarkably, three *LcARFs* (*LcARF8A*,

215   *LcARF10A/B*) were of significantly higher expression in MF than FF, indicated that they might

216   play roles in male flower formation. Additionally, a table for LcARFs and their potential

217   function was conclued in Data S3 according to early study which may be useful for further

218   functional validation.

219

220   **Expression profiles of LcARF genes in response to ETH, GPD, and GPDD treatment**

221   Auxin is a critical signal in the abscission of fruits in plants (Blanusa et al., 2005; Meir et al.,

222   2010; Xie et al., 2013). To explore the role of *LcARFs* in fruitlet abscission in litchi, RNA-seq

223   was carried on and transcription levels of LcARF genes in the abscission zone were investigated

224   under three treatments (ETH, GPD, GPDD). Both the ETH and GPD treatments promote fruitlet

225   abscission while GPDD delays the process (Peng et al., 2013; Li et al., 2015). As the results in

226   Fig. 5, the transcript expression of most *LcARFs* were decreased after treated by ETH and GPD,

227   but upsurge after GPDD treatment, which was corresponding to the process of promoting or

228   inhibiting of fruitlet shedding, respectively. It seemed that there was a negative correlation

229   between *LcARFs* and the fruit abscission and among these *LcARFs*, those from two groups

230   (group 2 and group 3) were particularly representative. Moreover, *LcARFs* from group 3 were

231   more sensitive to GPDD treatment along with stronger expression than those from group 2. Thus,

232   we could deduce that *LcARFs* from group 3, including *ARF2D/2E, 7A/7B, 9A/9B, 16A/16B*,

233   played major roles on the litchi fruitlet abscission. In contrast, a few *LcARFs*, such as group 1

234   including *LcARF5A/5B*, showed opposite expression pattern, suggesting that they might function

235   to accelerate the process of abscission. There were as well some LcARFs with no significant

236   expression change and seemed to be unrelated to fruitlet abscission.

237

238 **Discussions**

239 Litchi is an important tropic fruit trees and massive fruit abscission before harvest usually leads

240 to low and even no production. Auxin is proposed to be one of the endogenous hormones playing

241 significant roles in the regulation of fruit abscission in litchi (Yuan, 1988; Stern & Gazit, 2000).

242 In this study, 39 LcARF genes were identified, which was more than the other model plants,

243 such as *Arabidopsis* (23) (Okushima et al., 2005; Wang et al., 2007), rice (25) (Wang et al.,

244 2007) and tomato (22) (Zouine et al., 2014), implying extensive duplication and diversification

245 of the ARF gene family in litchi. Analysis of conserved motifs revealed that all LcARFs had a

246 typical DBD domain required for efficient binding to AuxRE and a Auxin_resp (Fig. 2) (Hagen

247 & Guilfoyle, 2002; Ha et al., 2013). However, only 23 of 39 LcARFs contain AUX_IAA

248 domain, which can mediate the dimerization of ARFs or ARF and Aux/IAA protein (Guilfoyle &

249 Hagen, 2007). Lack of the AUX_IAA domain for dimerization makes it interesting to address

250 questions like how these ARFs function and whether they need dimerization with other proteins.

251 In plants, ARFs can function as transcription activators (ADs) or repressors (RDs), according to

252 the amino acid composition of Auxin_resp domain (Guilfoyle & Hagen, 2007). In *Arabidopsis*

253 *thaliana*, ARF ADs and RDs were proposed to contain biased amino acid sequences, which ARF

254 ADs were enriched in glutamine (Q), while RDs were enriched in serine (S), serine and proline

255 (SP), serine glycine (SG) (Guilfoyle & Hagen, 2001; Tiwari, Hagen & Guilfoyle, 2003).

256 Intriguingly, no ARFs in litchi were enriched in Q, but with SPL and SP/SG enrichment; then

257 none of LcARF proteins seems to be an activator (reviewed in (Guilfoyle & Hagen, 2007)).

258 Further experiments are needed to verify this observation.

259

260 Much evidence demonstrates that miRNAs play essential roles in post-transcriptional gene

261 regulation in plants (Jones-Rhoades, Bartel & Bartel, 2006; Li & Zhang, 2016). It has been found

262 that several ARF genes are regulated by a few miRNAs. In our work, 22 out of 39 LcARF genes

263 were found to be targets of miRNAs (Fig. 3) and *LcARFs* from different classes were displayed

264 different miRNA targeting pattern. Members of class III (*LcARF10A/B*, *16A/B/C/D* and *17A/B*)

265 were found to be targeted by Lc-miR160, which might affect flower development of litchi, as

266 down-regulation of *ARF10/16/17* by miRNA160 is reported to regulate floral organ abscission in

267 tomato (Damodharan, Zhao & Arazi, 2016). Consistent with *Arabidopsis*, *LcARF6/8* were

268 collectively targeted by Lc-miR167. Interestingly, even though there has been reported *LcARF8B*

269 to be targeted by Lc-miR167 (Ma et al., 2018), *LcARF8A* was a novel target by Lc-miR167.

270 Notably, in another group, a novel miRNA-*ARF* pathway was discovered in litchi, in which

271 *LcARF9A/B* was targeted by Lc-miRN43. In fact, miRN43 is an innovative miRNA as well, for it

272   is unable to find any ortholog in the database of miRase, which may provide a new idea for us to

273   study the specific functions of litchi.

274

275   The expression profiles can help us screen for candidate *LcARF* genes with potentially distinct

276   functions. Most *LcARFs* were higher accumulated in mature leaves than young leaves including

277   *LcARF2,* and its Arabidopsis homolog AtARF2 has been reported to regulate leaf senescence

278   with high expression in mature leaves (Ellis et al., 2005). Thus we can deduce that LcARF2 is

279   likely to involve in leaf senescence in litchi. It has been reported that Arabidopsis ARF genes

280   regulate flower formation, especially gametophyte development, which are critical for sexual

281   reproduction. AtARF2–AtARF4 and AtARF5 play significant roles in regulating both female

282   and male gametophyte development in Arabidopsis (Liu et al., 2017) and ARF6 and ARF8

283   regulate both stamen and gynoecium maturation (Nagpal, 2005; Ru et al., 2006; Wu, Tian &

284   Reed, 2006; Liu et al., 2014b). Thus their litchi homologs LcARF2B, 3A, 4A/B, 5A/B, 6D and

285   8A may be important for female and male gametophyte development in litchi, along with

286   significantly higher accumulation in female or male flower. Moreover, *LcARF4*, *5* and *9* were

287   prominent higher expressed in female flower than male flower, implying their potential function

288   in ovule and ovary development.

289

290   ARF genes have been reported to be involved in plant organ abscission. Overexpression of

291   *SlARF2* in tomato results in flower organ senescence (Ren et al., 2017) and SlARF1, 2, 7, 11 and

292   19 showed overlapping functions in tomato abscission (Guan et al., 2014). Similar roles of ARFs

293   in abscission were observed in *Arabidopsis* (Ellis et al., 2005). Our previous studies show that

294   the treatment of girdling plus defoliation (GPD) in litchi could reduce the transcript level of

295   auxin response factor (*LcARF1*) mRNA, along with the increase of fruitlet abscission (Kuang et

296   al., 2012). Here in our RNA-seq survey of ARF genes expression, we found that most of the

297   ARF genes show opposite correlation with the fruit abscission, i.e., ARF genes were down-

298   regulated by abscission induced treatments (ETH and GPD), but up-regulated by GPDD, in

299   which the abscission of GPD was inhibited by the addition of 2, 4-D, suggesting that the majority

300   of LcARF genes are negatively involved in litchi fruit abscission, especially *LcARFs* from group

301   3 (*ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B*), with more prominent expression after GPDD treatment.

302   By contrast, a few *LcARFs*, such *LcARF5A/5B*, show positive correlation with fruit abscission,

303   indicating that they might serve as contributing factors to fruit shedding.

304

305   **Conclusions**

306   In this study, a total of 39 ARF genes were identified from the litchi genome. Comprehensive

307   analyses, including phylogenetic relationship, exon-intron structure, conserved domain, and

308   potential targets for small RNAs, revealed that the ARF gene family was expanded in litchi with

309   species-specific features. A novel miRNA-*ARF* (miRN43-*ARF9*) regulatory pathway was

310   discovered, which is likely specific in litchi. Expression profiles in various organs and under

311   different abscission-related treatments (ETH, GPD and GPDD) uncovered the expression

312   diversity of these ARF genes in litchi. Some ARF genes, including *ARF2D/2E*, *7A/7B*, *9A/9B*,

313   *16A/16B* and *5A/5B*, likely play predominant roles in the process of litchi fruit abscission. These

314   findings provide new knowledge and resources for further functional characterization of ARF

315   genes in litchi.

316

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Figure 1

Phylogenetic analysis of ARFs from litchi, rice, *Arabidopsis* and tomato.

The phylogenetic tree was generated using the Maximum Likelihood method with the JTT matrix-based model (Kumar, Stecher & Tamura, 2016) and the bootstrap test was carried out with 1000 bootstrap replicates. Numbers on the nodes indicate the credibility values of each clay. Three subgroups were shown as Class I, II, and III.

Figure 2

Phylogenetic relationship, exon-intron structure, conserved domains analyses of LcARFs.

(A) Phylogenetic relationship among the litchi ARF proteins. The unrooted tree was generated using the maximum likelihood method by JTT matrix-based model. The reliability was assessed using 1000 bootstrap replicates. Three clusters are labeled as Class I, Class II and Class III. **(B) Exon- intron structure and conserved domains of LcARFs.** Information of exon, intron, and functional domain was obtained from model gene annotation and results of NCBI CDD search and visualized by TBtools. B3: B3 DNA-binding domain; Auxin-resp: ARF domain; AUX_IAA: C-terminal dimerization domain. Lengths of exons and introns and domains of each LcARF protein were exhibited proportionally.

A

B

Class III

Class I

Class II

	Intron
	CDS
	B3
	Auxin_resp
	AUX_IAA
	UTR
∨	5kb Intron

5'   3'
0kb   2.5kb   5kb   7.5kb   10kb   12.5kb   15kb
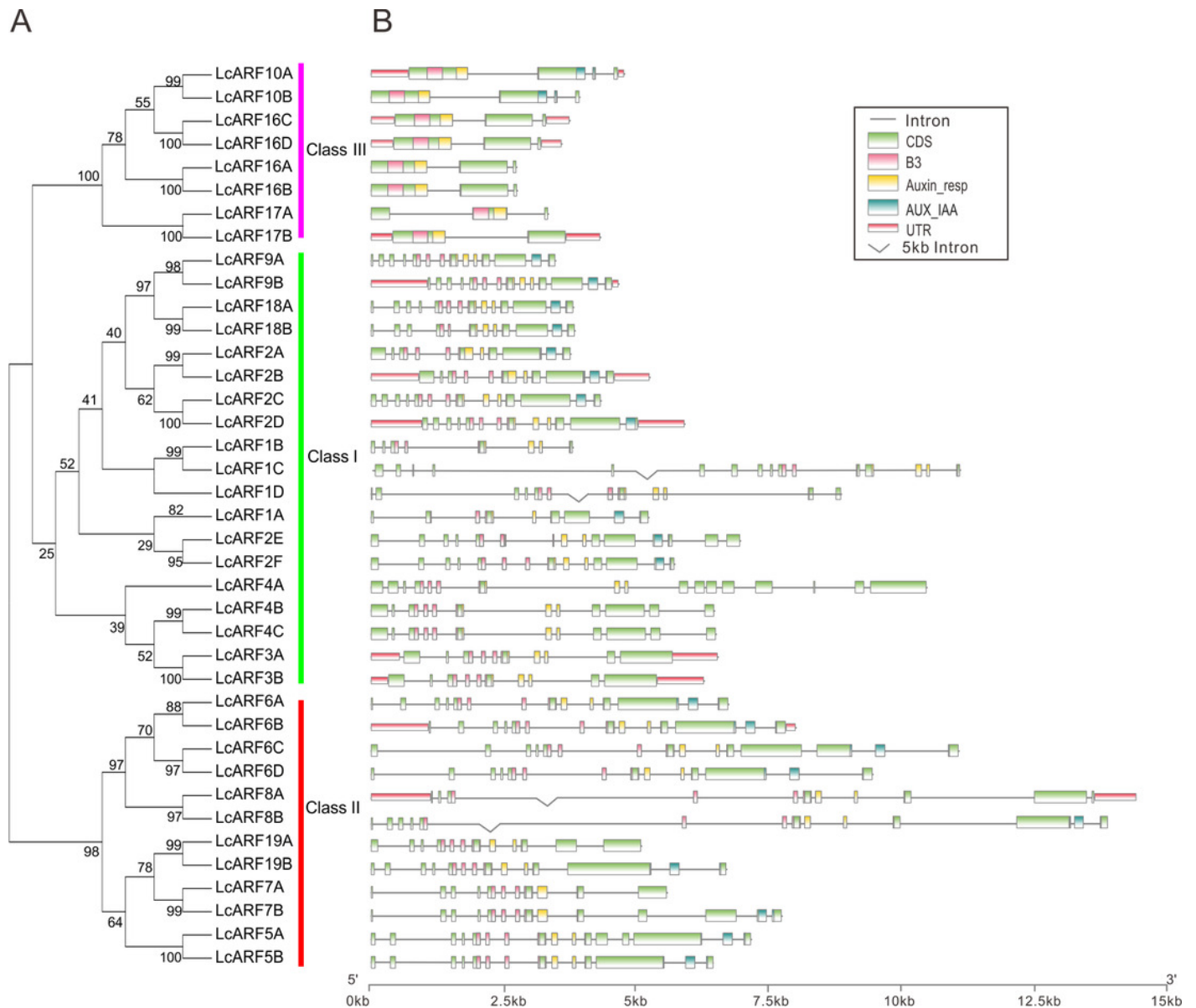
Figure 3

LcARFs targeted by some LcmiRNAs.

miR160 targets *LcARF10/16/17*, miR167 targets *LcARF6/8* and miRN43 targets *LcARF9*. Additionally, the *LcTAS3* was targeted by miR390 and then triggered the production of tasiARF which target *LcARF2E/2F/4B/4C/3A/3B*.
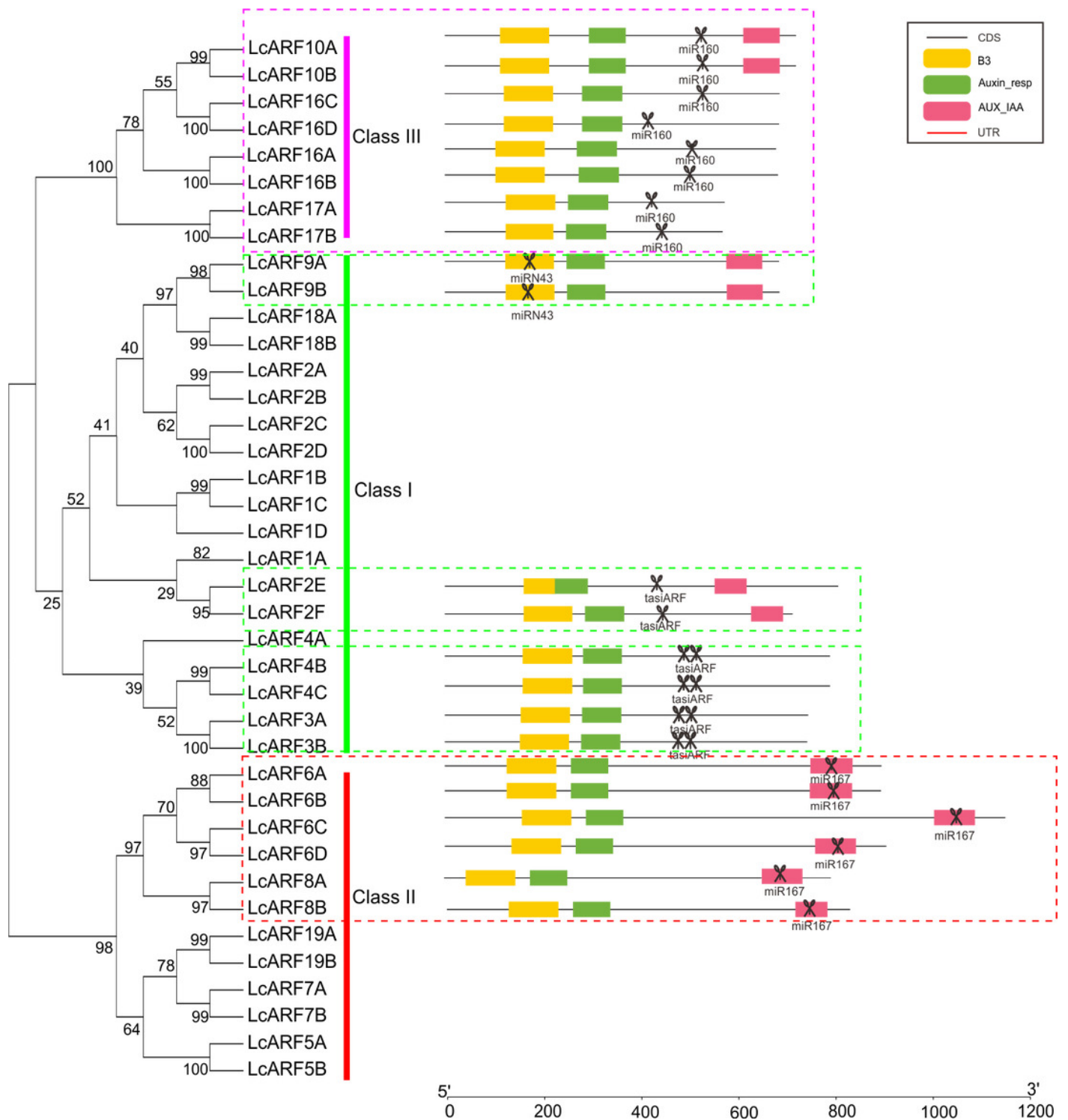
Figure 4

Expression Profiles of LcARF Genes in various tissues of 'FZX' by qRT-PCR.

A) Expression of all *LcARFs* in different tissues. The heatmap was generated based on the relative expression values of 39 *LcARFs* obtained by qRT-PCR in seven different tissues and organs. Red and blue were represented relatively high and lower expression ($\log_2$ ratio), respectively. Every sample has three biological replicates. YF: young fruit (25 days after fertilization), FF: female flower, MF: male flower, USF: undeterminated sex flowers, ML: mature leaves, YL: young leaves, FBS: fruit-bearing shoots. **B) Relative abundance of *LcARFs* significantly expressed in FF.** Data from three independent biological replicates are shown with standard deviation (SD). Asterisks on the top of bars indicate significant differences as determined by Student's t-test (*P, 0.05).
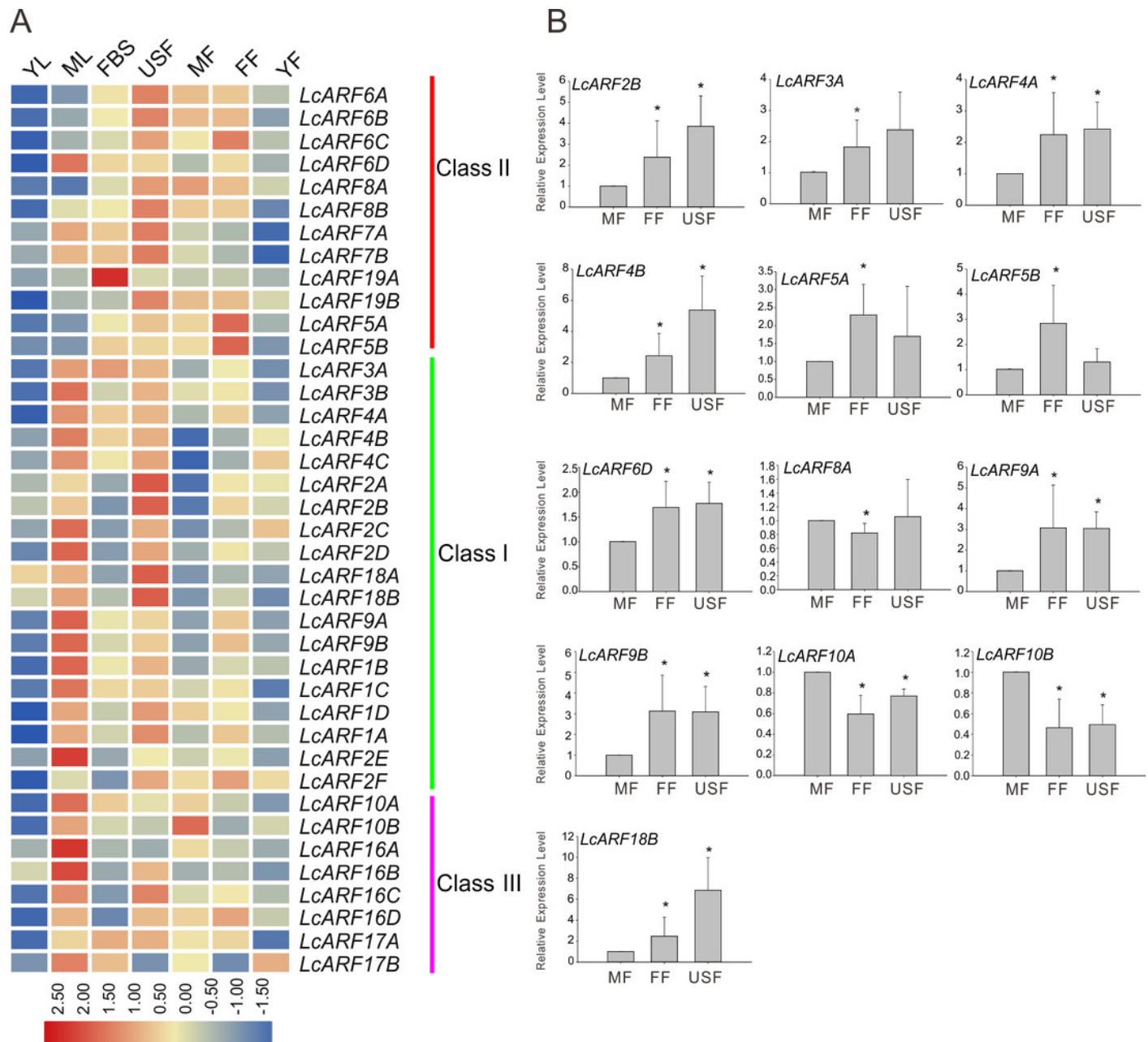
Figure 5

Expression Profiles of LcARF Genes in Response to ETH, GPD, and GPDD treatments.

Fruit-bearing shoots of 'FZX' litchi were obtained at 25 days after anthesis and then carried on ETH, GPD, and GPDD treatment from 0, 1, 2 and 3 days, respectively. CK: control; ETH: treated by ethylene; GPD: girdling plus defoliation; GPDD: dipping in 2, 4-D after GPD treatment. The heatmap was created based on the TPM values of *LcARFs* from the transcriptome data. In the heatmap, red and blue were represented higher and lower expression ($\log_2$ ratio), respectively. Heatmap and hierarchical clustering were performed by average linage (default) method.