Genome-wide characterization of the auxin response factor (ARF) gene family of litchi (*Litchi chinensis* Sonn.): evolution, 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.

Keywords: Litchi, auxin, auxin response factors (ARFs), abscission, miRNA.

Introduction

Auxin, an essential plant hormone, 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 (Addicott and Lynch, 1955; Kepinski and Leyser, 2005; Li et al., 2016b;). 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), fruit trees 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., 2005a) 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 *ARF2/3/4* are targets of trans-acting siRNAs (tasiRNAs) generated from miR390-targeted TAS3 gene (trans-acting siRNA gene 3) (Allen et al., 2005; Axtell et al., 2006). These targeting relationships of miRNA on ARF genes are widely conserved in land plants (Xia, Xu & Meyers,

2017) and also in horticultural plants (Chen et al., 2018b). It has been reported that down-regulation of *ARF6* and *ARF8* by miR167 leads to floral development defects and female sterility in tomatoes (Liu et al., 2014b). Down-regulation of sly-miR160, increasing the expression of its targets *SlARF10/16/17*, regulates auxin-mediated ovary patterning as well as floral organ abscission and lateral organ lamina outgrowth (Damodharan, Zhao & Arazi, 2016). In *Arabidopsis* and tomato, the tasiRNA-mediated regulation of *ARF2* involved in controlling the onset of leaf senescence and floral organ abscission (Ellis et al., 2005b; Lim et al., 2010; Guan et al., 2014; Ren et al., 2017).

Litchi (*Litchi chinensis* sonn.), an important economic fruit trees in southern China, usually undergo serious fruitlet abscission before harvest, leading to a low yield. Many studies demonstrate *ARFs* play critical roles in regulating plant organ abscission (Ellis et al., 2005b; Kuang et al., 2012; Guan et al., 2014; Xie et al., 2015; Xu et al., 2015), while which *ARFs* are involved or more important than the other in the fruit abscission in litchi remains elusive. Here, we identified 39 ARF genes in litchi. Gene structure, phylogeny and targeting relationship with miRNAs were characterized. The expression of *LcARFs* was examined in diverse tissues and in the process of fruit abscission which was induced by three different treatments. Among them, *ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B* and *LcARF5A/B*, were found to be associated with litchi fruit abscission. Our results offer new knowledge and resources to study the function of plant ARF genes and their roles in the fruit abscission in litchi.

Materials & Methods

Plant materials and treatments

The young fruitlet used for RNA-seq in our study were collected from 9-year-old litchi trees (*Lithchi. chinensis* Sonn. cv. 'Feizixiao') in an orchard located at South China Agricultural University (Guangzhou, China). Three treatments have been performed 25 day after litchi 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 undeterminated 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).

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/).

RNA-seq analysis was carried on as described previously (Li et al., 2013). Based on the sequences of the identified *LcARFs*, a BLASTN search was conducted to find their gene

131 counterparts in previous data (Li et al., 2013). Finally, a global gene expression profiles were
132 visualized by heatmap via TBtools software (Chen et al., 2018a).
133 **Quantitative RT-PCR**
134 Total RNA of above seven tissue samples was extracted by column plant RNAout kit (TIANDZ,
135 China) according to the manufacturer's instructions. About 2 μg total RNA was applied to
136 synthesized first strand cDNA using reverse transcriptase AT311-03 (TRANSGEN BIOTECH,
137 China). PCR primers of *LcARFs* and reference genes *GAPDH* and *EF* (Zhong et al., 2011) were
138 designed by Primer Premier 5.0. qRT-PCR was performed according to the manufacturer's
139 specifications of THUNDERBIRD qPCR MIX QPS-201 (TOYOBO, China) on a LightCycler
140 480 (Roche, Switzerland). Each expression profile was independently verified in three biological
141 replicates. Relative expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak &
142 Schmittgen, 2001). Significance analysis was conducted in SPSS vision 22.0 and visulized by
143 SigmaPlot 12.5.
144

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 (LcARF16C), with PIs in the range of 5.28 (LcARF16C) to 8.64 (LcARF19A) (Table S1). 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 included LcARF5/6/7/8/19 (5A/B, 6A/B/C/D, 7A/B, 8A/B, 19A/B); Class II 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 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 just 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.

Analyses of miRNA targeting *LcARFs*

Twenty-two out of the 39 LcARF genes were found to be the targets of miRNA (Fig.3). All group members of Class III (*LcARF10A/B*, *16A/B/C/D* and *17A/B*) were found to be targeted by

Lc-miR160. *LcARF6A/B/C/D* and *8A/B* were members of Class II and all of them were collectively targeted by Lc-miR167. Two kinds of miRNA targeting patterns were observed in the class I. One group comprising *LcARF2E/2F/4B/4C/3A/3B* were targeted by tasiARFs. In litchi, *LcTAS3* are divided into two subgroups, long TAS3 genes(*LcTAS3_1* and *LcTAS3_2*) and short TAS3 genes (*LcTAS3_3* and *LcTAS3_4*), which trigger to produce two or one tasiARFs when cleaved by miR390 (Ma et al., 2018). Additionally, *LcARF2E/2F* incorporated one target site of tasiARF, while *LcARF3A/3B/4B/4C* contained two, which is in accordance with our previous study (Xia, Xu & Meyers, 2017). Interestingly, in the other group, a novel miRNA-ARF pathway was discovered, in which *LcARF9A/B* was targeted by the Lc-miRN43.

Expression of LcARF Genes in different organs and tissues

A large body of evidence support the importance of ARF genes in plant growth and development (Ellis et al., 2005b; Guilfoyle & Hagen, 2007; Lim et al., 2010; Li et al., 2016). To explore how LcARF genes function in the development of litchi, we examined their expression levels in various litchi organs/tissues by qRT-PCR. Seven organs/tissues samples, composing fruit-bearing shoots (FBS), male flower (MF), female flower (FF), sex undeterminated flower (USF), mature leaves (ML), young leaves (YL) and young fruits (YF) in 'FZX', were collected and tested. As shown in Fig. 4A, all 39 LcARF genes were expressed in all organs/tissues studied. Generally, most *LcARFs* show low expression in YL and YF but high in USF. *LcARFs* from different clades showed various expression patterns. *LcARFs* from class I and III were detected to be higher expressed in ML while those from class II were lowly accumulated, suggesting that these *LcARFs* in different classes may perform different functions in male flower development (Fig. 4A). Additionally, as is shown in Fig. 4B, ten *LcARFs* (*LcARF2B*, *3A*, *4A/B*, *5A/B*, *6D*, *9A/B*, *18B*) were significantly higher expressed in FF than MF, implying their potential function in ovule and ovary derived from female flowers. Remarkably, three *LcARFs* (*LcARF8A*, *LcARF10A/B*) were of significantly higher expression in MF than FF, indicated that they might play roles in male flower formation.

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

Auxin is a critical signal in the abscission of fruits in plants (Blanusa et al., 2005; Meir et al., 2010; Xie et al., 2013). To explore the role of *LcARFs* in fruitlet abscission in litchi, RNA-seq was carried on and transcription levels of LcARF genes in the abscission zone were investigated under three treatments (ETH, GPD, GPDD). Both the ETH and GPD treatments promote fruitlet abscission while GPDD delays the process (Peng et al., 2013, 2017; Li et al., 2015). As the results in Fig. 5, the transcript expression of most *LcARFs* were decreased after treated by ETH and GPD, but upsurge after GPDD treatment, which was corresponding to the process of

promoting or inhibiting of fruitlet shedding, respectively. It seemed that there was a negative correlation between *LcARFs* and the fruit abscission and among these *LcARFs*, those from two groups (group 2 and group 3) were particularly representative. Moreover, *LcARFs* from group 2 were more sensitive to GPDD treatment along with stronger expression than those from group 3. Thus, we could deduce that *LcARFs* from group 2, including *ARF2D/2E, 7A/7B, 9A/9B, 16A/16B*, played major roles to prevent litchi fruitlet abscission. In contrast, a few *LcARFs*, such as group 1 including *LcARF5A/5B*, showed opposite expression pattern, suggesting that they might function to accelerate the process of abscission. There were as well some LcARFs with no significant expression change and seemed to be unrelated to abscission.

Discussions

Litchi is an important tropic fruit trees and massive fruit abscission before harvest usually leads to low and even no production. Auxin is proposed to be one of the endogenous hormones playing significant roles in the regulation of fruit abscission in litchi (Yuan, 1988; Stern & Gazit, 2000). In this study, 39 LcARF genes were identified and this number was larger than other model plants, such as *Arabidopsis* (23) (Okushima et al., 2005; Wang et al., 2007), rice (25) (Wang et al., 2007) and tomato (22) (Zouine et al., 2014), implying extensive duplication and diversification of the ARF gene family in litchi. Analysis of conserved motifs revealed that all LcARFs had a typical DBD domain required for efficient binding to AuxRE and a Auxin_resp (Fig.2) (Hagen & Guilfoyle, 2002; Ha et al., 2013). However, just 23 of 39 LcARFs contain AUX_IAA domain, which can mediate the dimerization of ARFs or ARF and Aux/IAA protein (Guilfoyle & Hagen, 2007). Lack of the AUX_IAA domain for dimerization makes it interesting to address questions like how these ARFs function and whether they need dimerization with other proteins. In plants, ARFs can function as transcription repressors (ADs) or activators (RDs), according to the amino acid composition of Auxin_resp domain (Guilfoyle & Hagen, 2007). In *Arabidopsis thaliana*, ARF ADs and RDs were proposed to contain biased amino acid sequences, which ARF ADs were enriched in glutamine (Q), while RDs were enriched in serine (S), serine and proline (SP), serine glycine (SG) (Guilfoyle & Hagen, 2001; Tiwari, Hagen & Guilfoyle, 2003). Intriguingly, no ARFs in litchi were enriched in Q, but with SPL and SP/SG enrichment; then none of LcARF proteins seem to be activator (reviewed in (Guilfoyle & Hagen, 2007)). Further experiments are needed to verify this observation.

Much evidence demonstrates that miRNAs play essential roles in post-transcriptional gene regulation in plants (Jones-Rhoades, Bartel & Bartel, 2006; Li & Zhang, 2016). It has been found that several ARF genes are regulated by a few miRNAs. In our work, 22 out of 39 LcARF genes were found to be targets of miRNAs (Fig. 3) and *LcARFs* from different classes were displayed different miRNA targeting pattern. Members of Class III (*LcARF10A/B*, *16A/B/C/D* and *17A/B*) were found to be targeted by Lc-miR160, which might affect flower development of litchi, as down-regulation of *ARF10/16/17* by miRNA160 is reported to regulate floral organ abscission in tomato (Damodharan, Zhao & Arazi, 2016). Consistent with *Arabidopsis*, *LcARF6/8* were collectively targeted by Lc-miR167. Interestingly, even though there has been reported *LcARF8B* to be targeted by Lc-miR167 (Ma et al., 2018), *LcARF8A* was a novel target by Lc-miR167. Notably, in another group, a novel miRNA-*ARF* pathway was discovered in litchi, in which LcARF9A/B was targeted by Lc-miRN43. In fact, miRN43 is an innovative miRNA as well, for it unable to find any ortholog in the database of miRase, which may provide a new idea for us to

study the specific functions of litchi.

ARF genes have been reported to be involved in plant organ abscission. Overexpression of *SlARF2* in tomato results in flower organ senescence (Ren et al., 2017) and SlARF1, 2, 7, 11 and 19 showed overlapping functions in tomato abscission (Guan et al., 2014). Similar roles of ARFs in abscission were observed in *Arabidopsis* (Ellis et al., 2005a). Our previous studies show that the treatment of girdling plus defoliation in litchi could reduce the transcript level of auxin response factor (*LcARF1*) mRNA, along with the increase of fruitlet abscission (Kuang et al., 2012). Here in our RNA-seq survey of ARF genes expression, we found that most of the ARF genes show opposite correlation with the fruit abscission, i.e., ARF genes were down-regulated by abscission induced treatments (ETH and GPD), but up-regulated by GPDD, in which the abscission of GPD was inhibited by the addition of 2, 4-D, suggesting that the majority of LcARF genes are negatively involved in litchi fruit abscission, especially *LcARFs* from group 2 （*ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B*），with more prominent expression after GPDD treatment. By contrast, a few *LcARFs*, such *LcARF5A/5B*, show positive correlation with fruit abscission, indicating that they might serve as contributing factors to fruit shedding.

Conclusions

In this study, a total of 39 ARF genes were identified from the litchi genome. Comprehensive analyses, including phylogenetic relationship, exon-intron structure, conserved domain, and potential targets for small RNAs, revealed that the ARF gene family was expanded in litchi with species-specific features. A novel miRNA-*ARF* (miRN43-*ARF9*) regulatory pathway was discovered, which is likely specific in litchi. Expression profiles in various organs and under different abscission-related treatments (ETH, GPD and GPDD) uncovered the expression diversity of these litchi ARF genes. Some ARF genes, including *ARF2D/2E*, *7A/7B*, *9A/9B*, *16A/16B* and *5A/5B*, likely play predominant roles in the process of litchi fruit abscission. These findings provide new knowledge and resources for further functional characterization of ARF genes in litchi.

Additional Information and Declarations

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

RX, CL, and JL contributed to designing the experiments.

YZ and CL performed the experiments, collected, and analyzed the data.

YZ, ZZ, CC, RX and JL contributed to data interpretation and preparation of the manuscript.

All authors reviewed the manuscript.

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Data Availability

The following information was supplied regarding data availability:

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/).

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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.

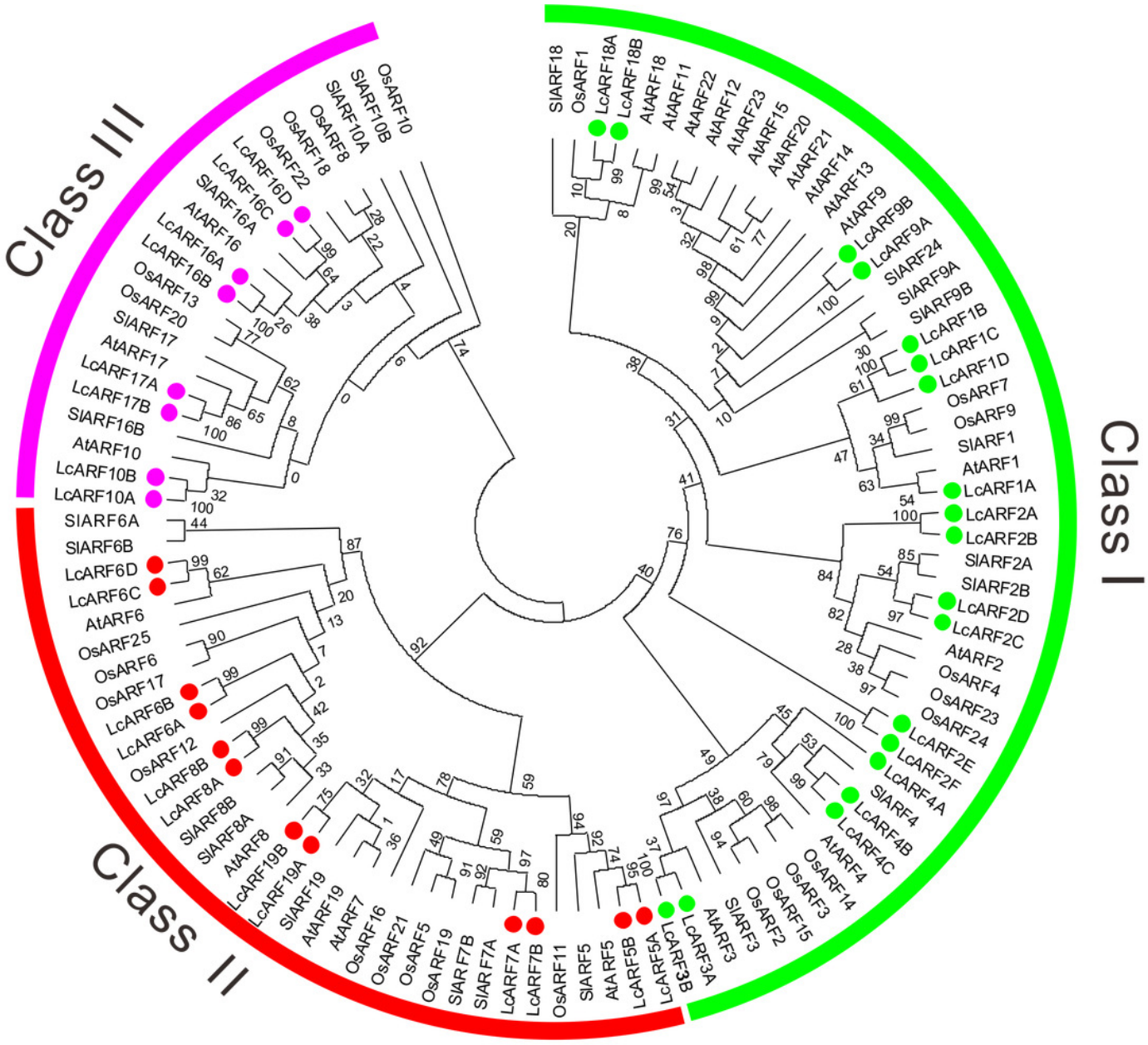


Figure 2

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.

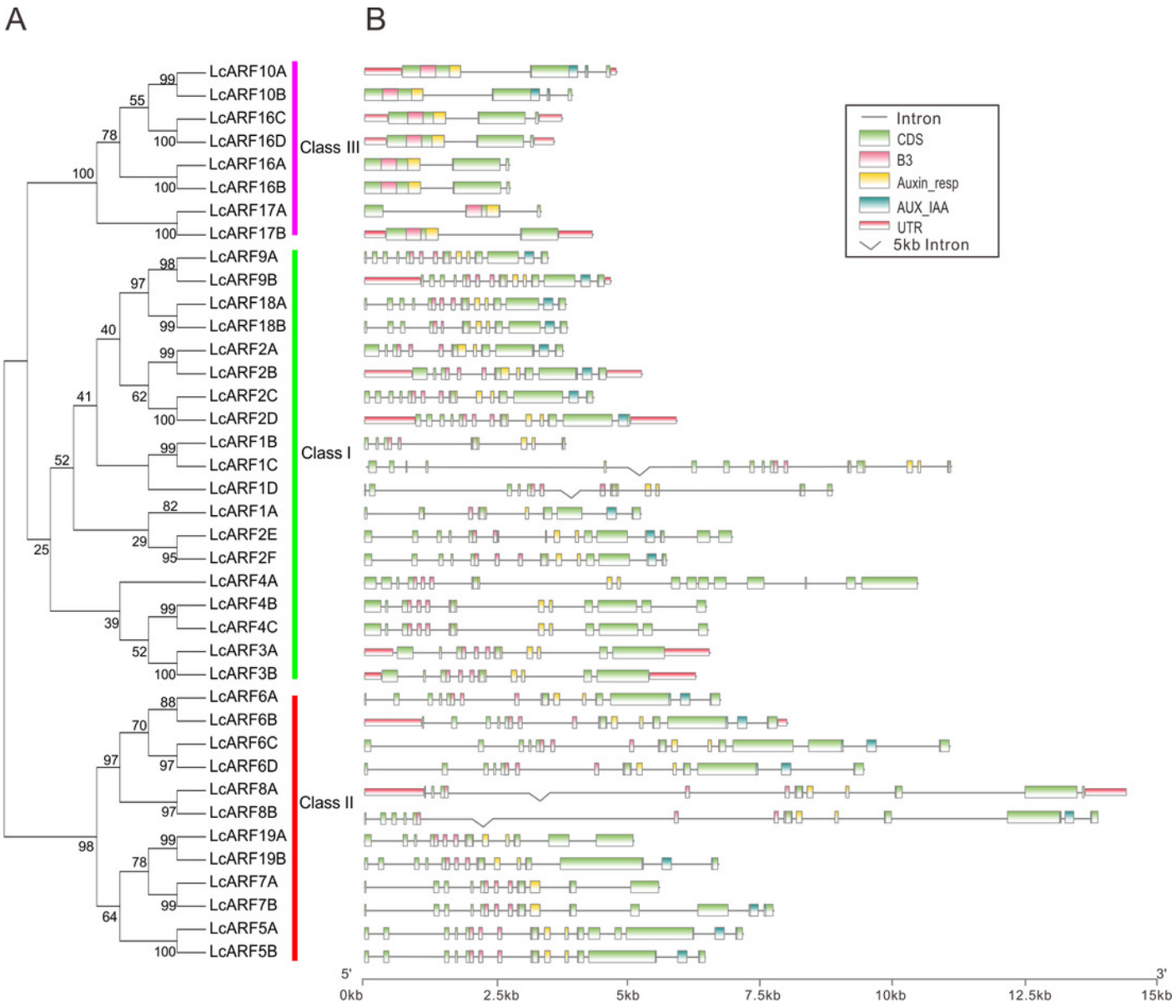
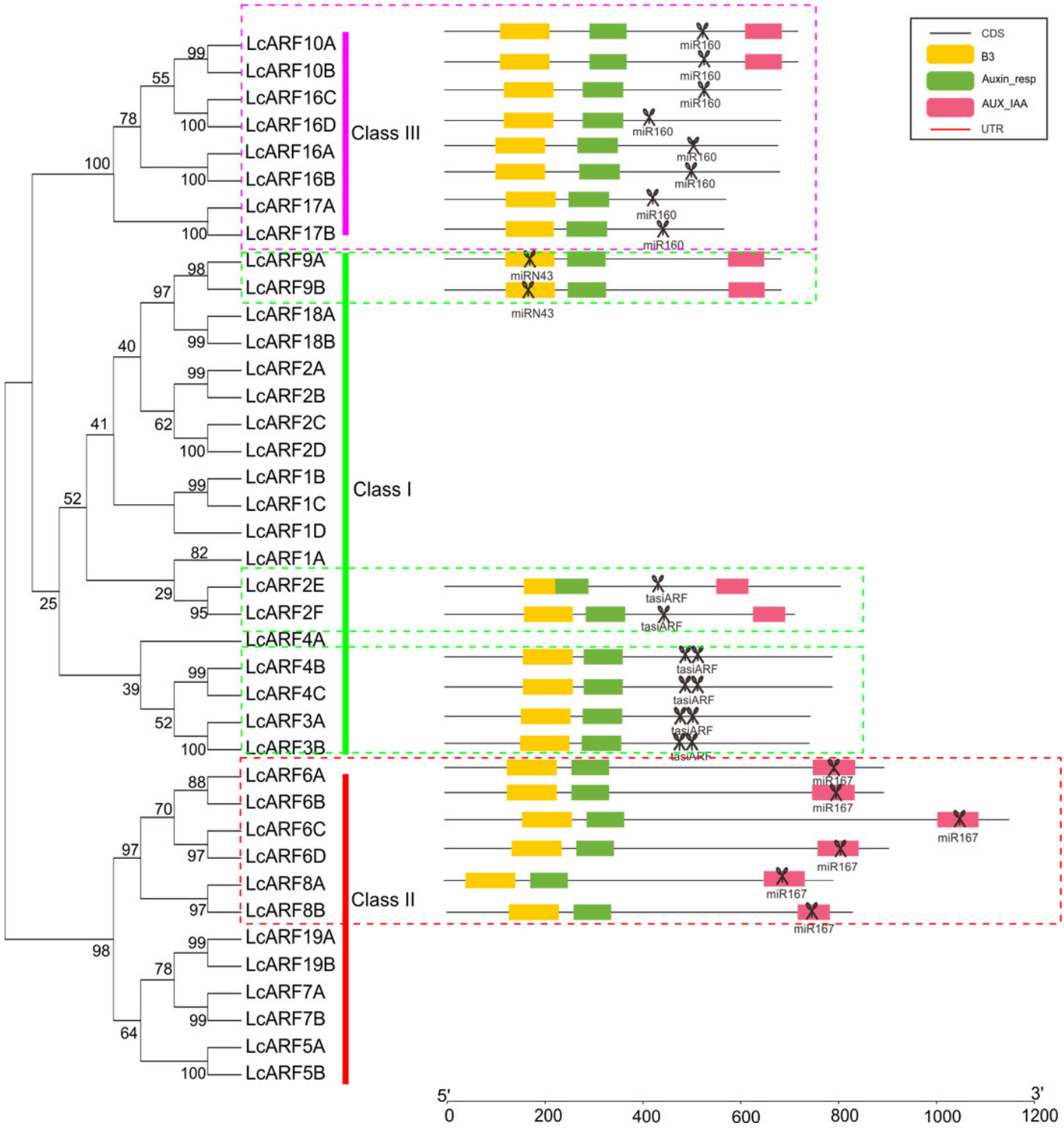

A
B
LcARF10A
LcARF10B
LcARF16C
LcARF16D
LcARF16A
LcARF16B
LcARF17A
LcARF17B
Class III
LcARF9A
LcARF9B
LcARF18A
LcARF18B
LcARF2A
LcARF2B
LcARF2C
LcARF2D
LcARF1B
LcARF1C
LcARF1D
LcARF1A
LcARF2E
LcARF2F
LcARF4A
LcARF4B
LcARF4C
LcARF3A
LcARF3B
Class I
LcARF6A
LcARF6B
LcARF6C
LcARF6D
LcARF8A
LcARF8B
LcARF19A
LcARF19B
LcARF7A
LcARF7B
LcARF5A
LcARF5B
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*.


CDS
B3
Auxin_resp
AUX_IAA
UTR
LcARF10A
LcARF10B
LcARF16C
LcARF16D
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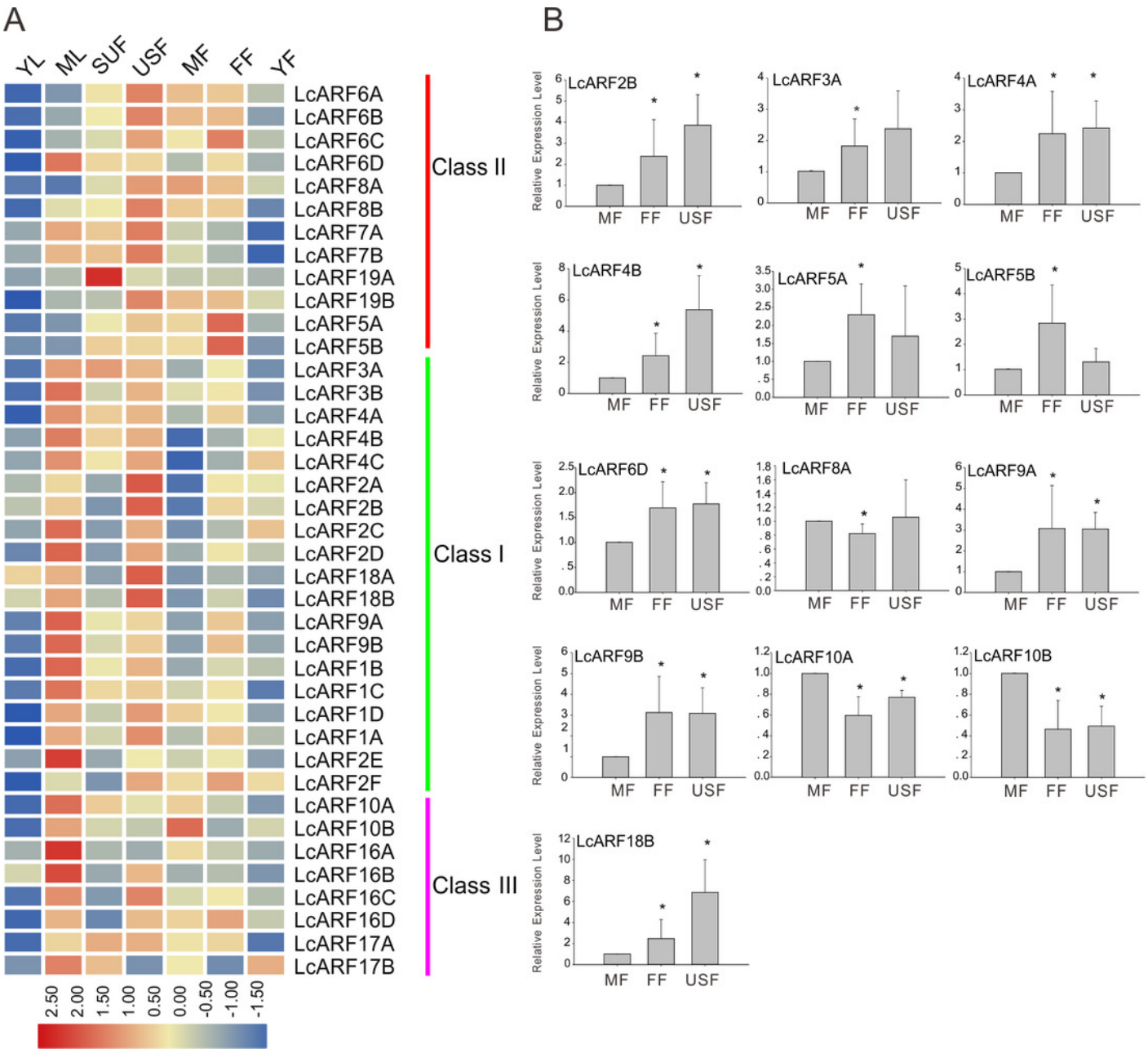
LcARF16B
LcARF17A
LcARF17B
Class III
LcARF9A
LcARF9B
LcARF18A
LcARF18B
LcARF2A
LcARF2B
LcARF2C
LcARF2D
LcARF1B
LcARF1C
LcARF1D
LcARF1A
LcARF2E
LcARF2F
LcARF4A
LcARF4B
LcARF4C
LcARF3A
LcARF3B
Class I
LcARF6A
LcARF6B
LcARF6C
LcARF6D
LcARF8A
LcARF8B
Class II
LcARF19A
LcARF19B
LcARF7A
LcARF7B
LcARF5A
LcARF5B
miR160
miRN43
tasiARF
miR167
5'
3'
0
200
400
600
800
1000
1200

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 represent the means and standard errors of three independent biological samples. Asterisks on the top of bars indicate significant differences as determined by Student’s t-test (*P, 0.05).


A
YL
ML
SUF
USF
MF
FF
YF
LcARF6A
LcARF6B
LcARF6C
LcARF6D
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LcARF8B
LcARF7A
LcARF7B
LcARF19A
LcARF19B
LcARF5A
LcARF5B
LcARF3A
LcARF3B
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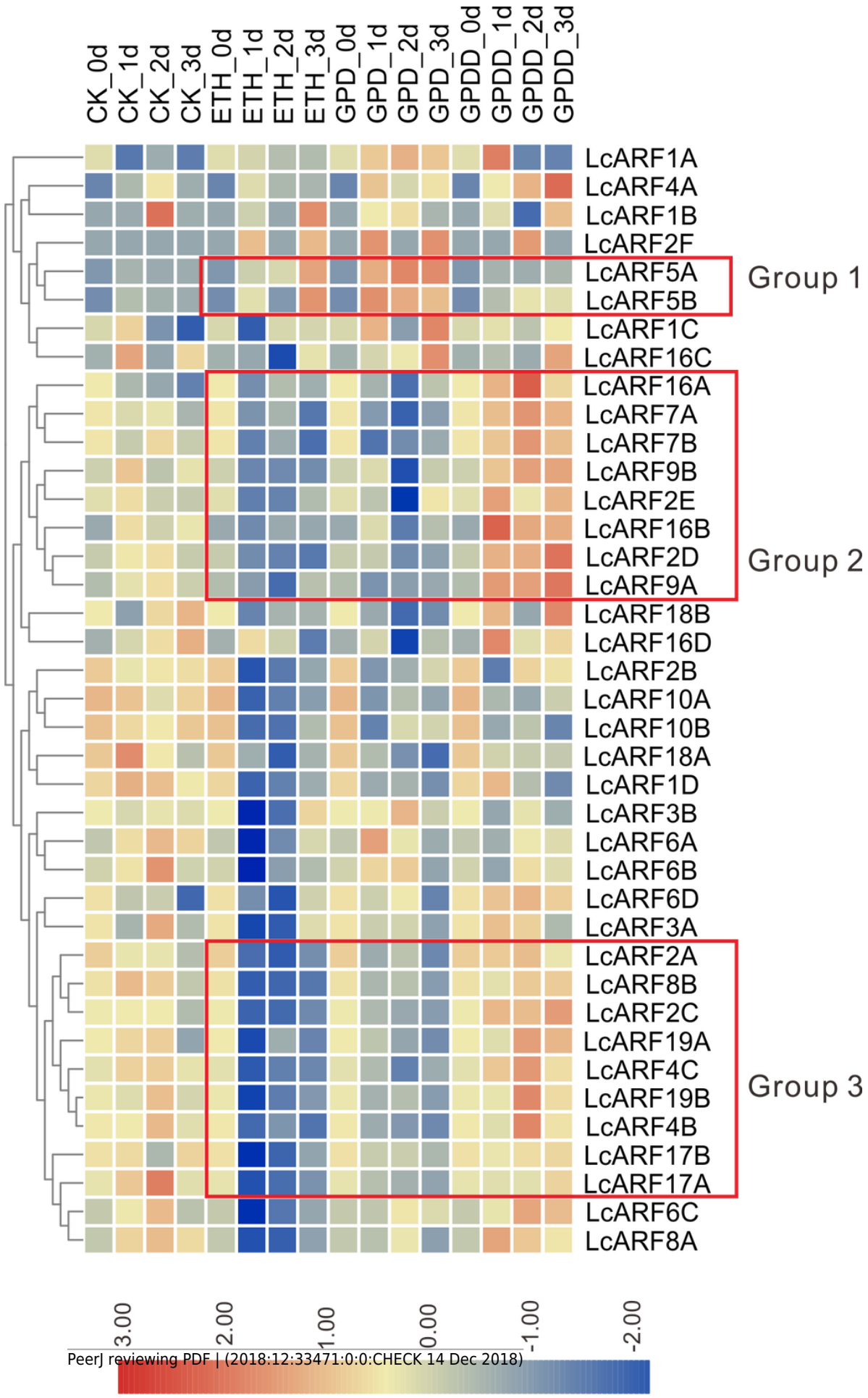
LcARF2C
LcARF2D
LcARF18A
LcARF18B
LcARF9A
LcARF9B
LcARF1B
LcARF1C
LcARF1D
LcARF1A
LcARF2E
LcARF2F
LcARF10A
LcARF10B
LcARF16A
LcARF16B
LcARF16C
LcARF16D
LcARF17A
LcARF17B
Class II
Class I
Class III
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
B
Relative Expression Level
LcARF2B
LcARF3A
LcARF4A
LcARF4B
LcARF5A
LcARF5B
LcARF6D
LcARF8A
LcARF9A
LcARF9B
LcARF10A
LcARF10B
LcARF18B
MF
FF
USF

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 creates based on the RPKM values of *LcARFs* from the transcriptome data. In the heatmap, red and blue were represented relatively high and lower expression (log_2 ratio), respectively. Heatmap and hierarchical clustering were performed by average linage (default) method.


CK_0d
CK_1d
CK_2d
CK_3d
ETH_0d
ETH_1d
ETH_2d
ETH_3d
GPD_0d
GPD_1d
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GPD_3d
GPDD_0d
GPDD_1d
GPDD_2d
GPDD_3d
LcARF1A
LcARF4A
LcARF1B
LcARF2F
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LcARF5B
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LcARF16C
LcARF16A
LcARF7A
LcARF7B
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LcARF3A
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LcARF8B
LcARF2C
LcARF19A
LcARF4C
LcARF19B
LcARF4B
LcARF17B
LcARF17A
LcARF6C
LcARF8A
Group 1
Group 2
Group 3
3.00
2.00
1.00
0.00
-1.00
-2.00