horticulturae

MDPI

Article

Cucumber Auxin Response Factor *CsARF10a* Regulates Leaf Morphogenesis and Parthenocarpic Fruit Set in Tomato

Jian Xu [1,†], Pinyu Zhu [2,†], Xiefeng Yao [1], Yongjiao Meng [2], Lina Lou [1], Man Zhang [1], Guang Liu [1], Xingping Yang [1], Jinqiu Liu [1], Lingli Zhu [1], Qian Hou [1], Ji Li [2,*] and Jinhua Xu [1,*]

[1] Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China; xjian.1990@163.com (J.X.); 20100039@jaas.ac.cn (X.Y.); 20120053@jaas.ac.cn (L.L.); 20110041@jaas.ac.cn (M.Z.); 20070028@jaas.ac.cn (G.L.); 19830012@jaas.ac.cn (X.Y.); 20210092@jaas.ac.cn (J.L.); 20080992@jaas.ac.cn (L.Z.); 20090987@jaas.ac.cn (Q.H.)
[2] State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China; 18070156933@163.com (P.Z.); 2018204015@njau.edu.cn (Y.M.)
* Correspondence: liji1981@njau.edu.cn (J.L.); jinhuaxu@jaas.ac.cn (J.X.)
† These authors contributed equally to this work.

Abstract: Auxin response factors (ARFs) are pivotal transcription factors involved in many aspects of auxin-dependent developmental processes. While functions of ARFs have been extensively studied in Arabidopsis, their distinct role in cucumber remains unclear. In this study, a cucumber auxin response factor homolog, *CsARF10a*, was cloned and overexpressed in tomato plants. RT-qPCR analysis indicated that the expression abundance of *CsARF10a* was significantly decreased in cucumber leaves and female flowers, and the expression level of *CsARF10a* was relatively low in pollinated fruits and hormone-treated fruits compared with that in unpollinated fruits. Moreover, the overexpression of *CsARF10a* in tomato resulted in multiple phenotypic changes, including a wider leaf blade, delayed fruit ripening, and parthenocarpic fruit set in *CsARF10a*-OE lines. Taken together, our research shed light on the regulatory importance of *CsARF10a* in regulating various phenotype alterations and laid a solid foundation for further functional studies.

Keywords: ARFs; cucumber; *CsARF10a*; parthenocarpic fruit set

Citation: Xu, J.; Zhu, P.; Yao, X.; Meng, Y.; Lou, L.; Zhang, M.; Liu, G.; Yang, X.; Liu, J.; Zhu, L.; et al. Cucumber Auxin Response Factor *CsARF10a* Regulates Leaf Morphogenesis and Parthenocarpic Fruit Set in Tomato. *Horticulturae* **2024**, *10*, 79. https://doi.org/10.3390/horticulturae10010079

Academic Editor: Jose V. Die

Received: 27 December 2023
Revised: 7 January 2024
Accepted: 11 January 2024
Published: 12 January 2024

1. Introduction

Fruits are important sources of many nutrients for human health. Fruit development is an intricate process accompanied by a coordinated program of molecular, biochemical, and structural changes. Due to the fundamental importance of these processes, numerous studies have placed an emphasis on their regulation. One outcome of this research has been the discovery that phytohormones act as key regulators of the complicated processes involved [1]. Among various phytohormones, auxin, which has been demonstrated to have a significant impact on reproductive processes, is integral to the cell divisions that occur in response to fertilization and subsequent cell expansion [2–4]. Due to the complexity of auxin signaling during the fruit development process, the exact molecular mechanism via which auxin regulates fruit set remains to be further elucidated, although recent advances have shed light on its regulatory role [5–7].

The discovery of a series of auxin signaling components considerably improved the understanding of auxin responses in plants. In essence, Aux/IAAs inhibited auxin-dependent changes in gene expression through forming heterodimer complexes with ARF transcriptional regulators under conditions with low concentrations of auxin [8,9]. When auxin concentration increases, auxin binding to receptor TIR1/AFBs promotes the binding of Aux/IAAs to the $SCF^{TIR1/AFB}$ complex, leading to the ubiquitination and 26S proteasome-mediated degradation of Aux/IAA, thus activating the transcriptional response [10–15].

Given that auxin plays a crucial role in the regulation of the fruit initiation process, it can be expected that ARF transcriptional regulators may also regulate this process.

The ARF gene family was initially discovered in Arabidopsis and is extensively present in plant species as a kind of transcription factor [16]. A typical ARF protein is comprised of three modular domains: an N-terminal B3-derived DNA binding domain (DBD) that is unique to plants, an intermediate non-conserved domain named middle region (MR) that functions as a transcriptional activator or repressor, and a C-terminal Aux/IAA domain (CTD) that consists of two domains related to motifs III and IV of Aux/IAA proteins [17,18]. Nonetheless, not all ARF proteins had all the three domains. For example, 6 ARF protein members in *Oryza sativa* were found to have a lack of CTD domain [19].

To date, 23, 21, and 25 *ARF* gene members have been identified in Arabidopsis, tomato, and cucumber separately [20,21]. Loss-of-function studies revealed that ARF genes play a crucial function in many biological processes, including fruit set and development [22]. For instance, abnormal flower development was observed in an *arf3/eff* mutant of Arabidopsis [23]. Additionally, the phenomena of lager fruits and increased seed weight were discovered in an ARF8 mutant of *Fragaria ananassa* and an ARF18 mutant of *Brassica napus*, respectively [24,25]. Moreover, the silencing of *slARF4* in tomato could significantly elevate the starch content during the initial stages of fruit development [26], while the overexpression of *FaARF4* could promote flowering in woodland strawberry [27]. *slARF5* was verified to modulate fruit set and development in tomato through the mediation of gibberellin and auxin [28]. Silencing ARF2-ARF4 and ARF5 via microRNAs in Arabidopsis could cause abnormal pollen grain formation and seed abortion, which indicates their essential roles in regulating male and female gametophyte development [29]. *AtARF6* and *AtARF8* were discovered to have significant functions in regulating the differentiation of stamens and pistils [30]. *SlARF6A* was confirmed to regulate photosynthesis, sugar accumulation, and fruit development in tomato [31]. In addition, *SlARF7* and *SlARF8* were verified to have an inhibitory effect on the development of tomato [32,33]. As ARF10 is a crucial member of the ARF gene family, its functions have also been widely studied. The loss of *ARF10* and *ARF16* resulted in an abnormal differentiation of root cap cells and root growth defects in Arabidopsis [34,35]. The overexpression of *SlARF10* could indirectly inhibit cell elongation and seed formation, and induce parthenocarpic fruit formation in tomato [36]. Moreover, *SlARF10* was also engaged in mediating leaf water retention [37] and regulating the accumulation of chlorophyll and sugar during the fruit development process in tomato [38]. An expression analysis of subfamily genes of *CsARF10* between parthenocarpic and non-parthenocarpic cucumber fruits suggested that they may participate in the fruit development process [39]. However, the distinct mechanism of *CsARF10* in regulating fruit set and development still remains elusive. Thus, a functional study of the *CsARF10* gene would substantially enhance our comprehension of auxin signal pathway mechanisms in fruit development.

In a previous study, we characterized three auxin response factor genes in cucumber and designated them as *Cucumis sativus* ARF10a, ARF10b, and ARF10c (*CsARF10a*, *CsARF10b*, and *CsARF10c*) [39]. In this research, we present the isolation and functional investigation of *CsARF10a*. The phylogenetic relationship, gene structure, and RT-qPCR profiling in tissues and hormone induction, and a phenotypic analysis of transgenic plants, were investigated to understand the regulatory roles of *CsARF10a.* Overall, *CsARF10a* may function as an activator to stimulate the fruit set and development process.

2. Materials and Methods

2.1. Plant Materials and Treatment Methods

The cucumber inbred line '8419s-1' (non-parthenocarpic line) was used in this study, and all cucumber plants were cultivated in a greenhouse during the natural growing seasons (14 h photoperiod, 28/16 °C average day/night temperature) at Nanjing Agricultural University. When seedlings of '8419s-1' were in the three-leaf stage, leaves (n = 25 per group) were treated with 10 μM Gibberellic Acid (GA3, 10 μM), 10 μM 6-Benzylaminopurine (6-BA,

10 μM), and different concentrations of 1-naphthyl acetic acid (NAA, 5, 10, and 50 μM). Ovaries of '8419s-1' at 0 days post anthesis (dpa) were treated with pollen (pollination), N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU, 400 μM), 1-naphthyl acetic acid (NAA, 500 μM), Gibberellic Acid (GA3, 3000 μM), and Brassinosteroids (BRs, 0.2 μM). All treated samples of leaves and ovaries were gathered 6 h after treatment. For a further analysis of cucumber fruit set and the development process, female flowers of '8419s-1' were treated with bagging and pollination at 0 dpa, which represent the unpollinated fruit abortion process and pollinated fruit set process, respectively. Female flowers that had been treated were harvested at −1, 0, 2, 4, and 6 dpa. Samples of leaves and fruits without treatment were used as controls, and sampling was conducted on three independent instances. All samples were instantaneously frozen with liquid nitrogen and stored in an ultra-low temperature refrigerator (Thermo Fisher Scientific, Waltham, MA, USA) below −80 °C for RT-qPCR analysis.

The tomato cultivar 'Micro-Tom' was cultivated in an artificial climate incubator (16/8 h day/night cycle, 18/24 °C day/night temperature, and 75% relative humidity) at Nanjing Agricultural University. Leaf samples were harvested from the primary leaflets of the fifth leaf in each tomato plant, and all samples were immediately treated with liquid nitrogen and kept at −80 °C for RT-qPCR analysis.

2.2. Bioinformatics Analysis of CsARF10a

The homologs of auxin response factor genes were searched in the cucumber (Chinese Long) genome V2 database (http://www.cucurbitgenomics.org/, accessed on 28 June 2021) using the AtARF10 (AT2G28350.1) sequence. Three cucumber genes (Csa6G141390, Csa6G445210 and Csa6G405890) were revealed to have the highest similarity to AtARF10 via blast searches, and were designated *CsARF10a*, *CsARF10b*, and *CsARF10c*, respectively. The gene-specific primers (Forward: 5′-GGGTTTATTTTACATTTGGG-3′; Reverse: 5′-ACATTTCTTGGGTTCATTTT-3′) were designed to amplify the full-length cDNA of *CsARF10a* using primer premier 5.0 according to the CDS sequences of Csa6G141390, and the cDNA of cucumber ovaries was used as a template. The PCR amplification conditions were set as follows: pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 30 s; annealing at 58 °C for 30 s; extension at 72 °C for 150 s; 34 cycles; extension at 72 °C for 10 min; and storage at 10 °C. Then, the PCR products were directly sequenced by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing data of PCR products were submitted to GenBank (accession number NM_001288596.1). Alignments of the protein sequences of CsARF10a and AtARF10 were performed by DNAMAN7.0. And the sequence information of ARF proteins in cucumber, Arabidopsis, and tomato used in this study are listed in Table S1. A maximum likelihood (ML) phylogenetic tree was constructed with MEGA5.0 by aligning protein sequences of ARFs with 1000 bootstrap replicates.

2.3. RNA Extraction and RT-qPCR Analysis

All primers used for RT-qPCR are listed in Table 1. Based on the MIQE guidelines [40] and our previous studies [41], the extraction of total RNA was performed using TRIzol reagent (Invitrogen, Waltham, MA, USA). The DNase I (Fermentas, Waltham, MA, USA) was used to treat with RNA to eliminate any contaminating genomic DNA according to the manufacturer's instructions. Then, the first-strand cDNA was synthesized using the PrimeScript™ RT-PCR Kit (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was carried out using the SYBR® Premix Ex Taq™ Kit (TaKaRa, Tokyo, Japan) according to the manufacturers' protocols, and assays were performed with a CFX96 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The *Actin* genes of cucumber and tomato (*Cs-actin* and *Sl-actin*) were used as internal reference genes.

The quantification cycles (Cq) are recorded and listed in Table S2. And the relative normalized expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method [42]. Each sample was composed of three independent biological replicates, and data analysis was conducted based on data collected from three independent reactions.

Table 1. Sequence information of primers used in this study.

Primer Name	Forward	Reverse
CsARF10a-CDS	5′-GGGTTTATTTTACATTTGGG-3′	5′-ACATTTCTTGGGTTCATTTT-3′
CsActin	5′-TTCTGGTGATGGTGTGAGTC-3′	5′-GGCAGTGGTGGTGAACATG-3′
SlActin	5′-TGTCCCTATTTACGAGGGTTATGC-3′	5′-CAGTTAAATCACGACCAGCAAGAT-3′
CsARF10a-RT-qPCR	5′-CAATTCCCACTGTCGTCATC-3′	5′-GTATGCCTGGCTCCCTGTAT-3′

2.4. Transgenic Tomato Construction

Seeds of wild type (WT) tomato were surface-sterilized in 75% ethanol for 1 min at first, rinsed three times in sterile distilled water, then in 50% bleach solution for 12 min, then three to five times in sterile distilled water, and then sown on 1/2 Murashige and Skoog (MS) culture medium with vitamin R3 (0.5 mg L^{-1} pyridoxine, 0.25 mg L^{-1} nicotinic acid, and 0.5 mg L^{-1} thiamine) and 0.8% (*w*/*v*) agar, pH 5.9. Cotyledons from 10-day-old plants were used as explants for transformation.

The gene-specific primers (Forward: 5′-CAAACCGAAATTAGGGCAACA-3′; Reverse: 5′-TCGATCCTCGGTTTGGTG-3′) were used for the full-length cDNA sequence amplification of *CsARF10a*. The DNA fragment was inserted into the plp100-35S vector, then the recombinant vector (plp100-35S-*CsARF10a*) was transformed into the competent cells of *Escherichia coli* DH5α. Cells of DH5α were screened on Luria–Bertani (LB) culture medium with kanamycin (100 mg L^{-1}). Subsequently, positive clones were verified via PCR confirmation and sequencing in Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Ultimately, the plp100-35S-*CsARF10a* vector derived from positive clones was transferred to the strain of *Agrobacterium tumefaciens* C58 to generate transgenic plants according to Ren's methods [43]. Transformed tomato lines were selected on 1/2 MS culture medium containing 50 mg L^{-1} kanamycin. Additionally, the presence of T-DNA inserts in the transgenic tomato lines was further analyzed by RT-qPCR.

All strains used in this study were stored in the lab of cucurbit genetics and germplasm enhancement of Nanjing Agricultural University.

2.5. Phenotypical and Physiological Characterizations of Transgenic Tomato Plants

Seeds of T_2 generation transgenic tomatoes and WT plants were germinated in 8 cm Petri dishes at 25 °C temperature and 80% humidity in dark conditions, and were then grown under standard greenhouse conditions. In order to avoid the self-pollination of transgenic tomatoes and WT plants, flower buds were emasculated before the dehiscence of anthers. For the insurance of equivalent growth conditions of all tomato fruits, only five flowers were kept per plant. In addition, flower buds of transgenic tomatoes and WT plants were chosen to self-pollinate, producing seeds, and seed number per fruit was counted. The phenotypes affecting leaf growth and fruit development were observed on T_2 transgenic lines.

3. Results

3.1. CsARF10a Belongs to the Clade III Family of ARF

The *CsARF10a* gene contains a 2946-bp open reading frame encoding a putative amino acid. The sequence analysis of predicted amino acids showed that CsARF10a had B3-DNA and ARF domains, which indicate that CsARF10a has the typical conserved ARF domains (Figure 1). To investigate the evolutionary relationship among ARF proteins in different species, a phylogenic tree comprising 64 ARF sequences from cucumber, tomato, and Arabidopsis was generated using the maximum likelihood approach on the basis of amino acid sequences (Table S1). ARFs can be divided into four major clades: I, IIa, IIb, and III. A phylogenetic analysis indicated that CsARF10a belongs to the clade III family and has 58.34% sequence similarity to AtARF10 (Figures 1 and 2).

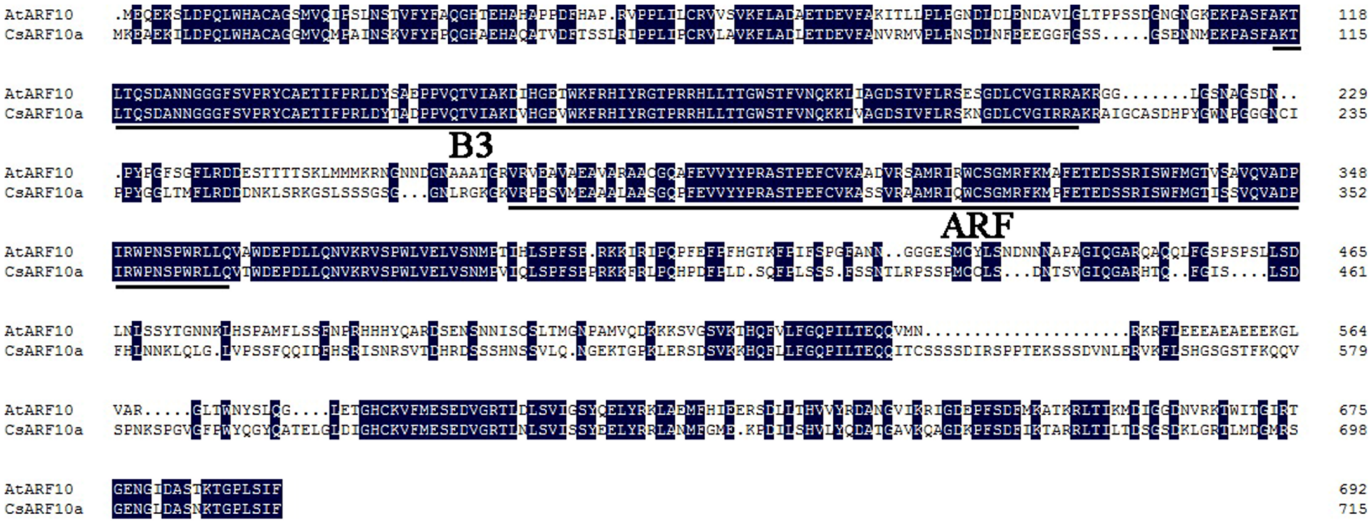

Figure 1. Sequence comparison of ARF10 proteins in cucumber and Arabidopsis. The positions of the amino acid residues are indicated by numbers on the right. Identical amino acid sequences are represented by shaded navy blue regions. The putative B3-DNA motif and conserved ARF domain are indicated by thick lines.

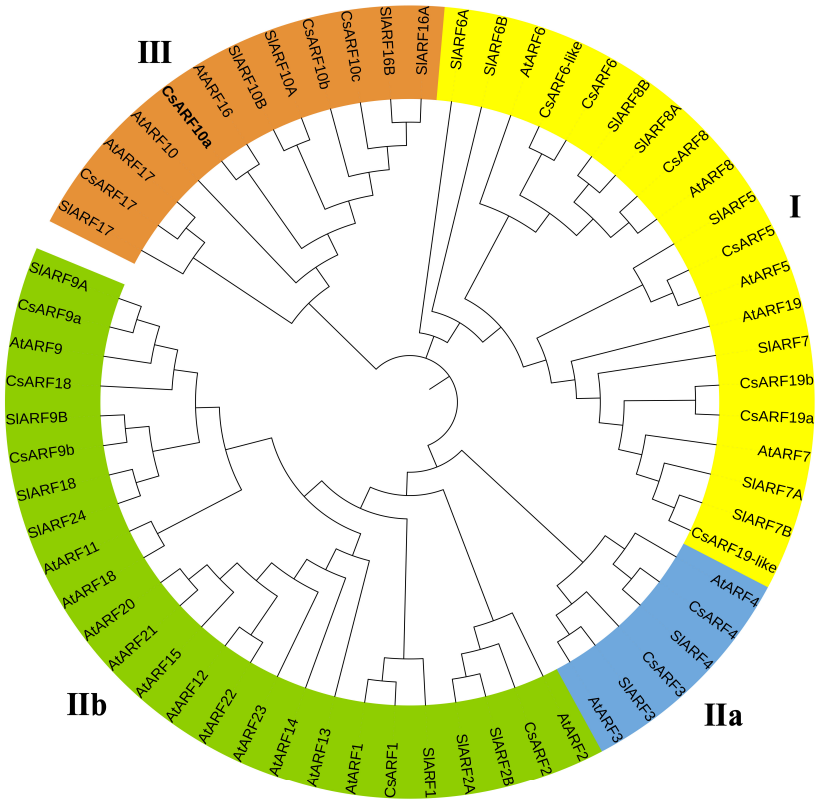

Figure 2. Evolutionary relationships among ARF protein family. The phylogenetic tree contains 64 ARF protein sequences from *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Cucumis sativus*. The tree was created using the maximum likelihood method via MEGA5.0 software. ARFs marked in yellow represent Clade I, ARFs marked in blue represent Clade IIa, ARFs marked in pale green represent Clade IIb, ARFs marked in orange represent Clade III.

3.2. Expression Analysis of *CsARF10a* Gene in Cucumber

The spatial–temporal transcriptional characteristics of the *CsARF10a* gene in cucumber were analyzed via RT-qPCR. Expression analysis of *CsARF10a* in different cucumber organs indicated that the *CsARF10a* gene displayed the highest abundance of mRNA in tissues as male flowers, while its mRNA abundance level was relatively low in leaves and female flower tissues (Figure 3a). The phytohormone responses of *CsARF10a* were investigated in exogenous hormone treatment experiments. Leaves of cucumber were treated with 10 μM GA3, 10 μM 6BA, 5 μM, 10 μM, and 50 μM NAA. A transcriptional analysis showed that 6BA, low, and medium concentrations of NAA could significantly induce the expression level of *CsARF10a* (Figure 3b).

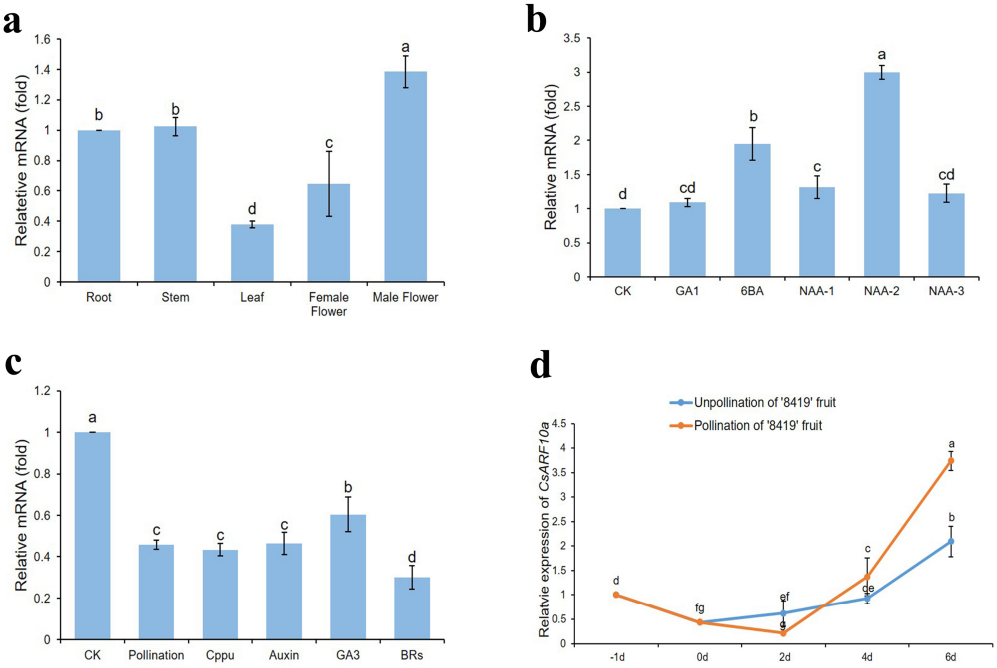

Figure 3. RT-qPCR analysis of *CsARF10a* transcription levels in cucumber. (**a**) Expression of *CsARF10a* in different cucumber organs (root, stem, leaf, female flower, and male flower). (**b**) Expression of *CsARF10a* in leaves of cucumber seedlings treated with 10 μM GA_3, 10 μM 6BA, 5, 10, and 50 μM NAA. (**c**) Expression of *CsARF10a* in cucumber ovaries under different treatments (pollination treatment, 400 μM CPPU, 500 μM NAA, 3000 μM GA_3, and 0.2 μM BRs). (**d**) Expression of *CsARF10a* in cucumber ovaries during early developmental stages of cucumber fruit. CK represents "Control". Expression data of *CsARF10a* gene in Root, CK, and -1d were normalized to 1. Three biological replicates were used for data analysis, and data are shown as mean ± SD. Difference among means of cucumber organs and treatments were evaluated by Student's *t*-test at probability level of 0.05, and histograms marked with the same letters represent that there are no significant differences.

To access the expression patterns of *CsARF10a* in cucumber ovaries under high concentrations of exogenous hormones treatment, ovaries (0 dpa) were treated with 500 μM NAA, 400 μM CPPU, 3000 μM GA3, and 0.2 μM BRs in this study. All these treatments could stimulate parthenocarpic fruit formation. Interestingly, the expression of *CsARF10a* was downregulated in pollinated fruit and hormone-induced parthenocarpic fruit (Figure 3c). Similar expression patterns were observed in '8419s-1' pollinated and non-pollinated fruits, where the expression level of *CsARF10a* decreased before pollination, and then increased once the fruit initiation process began (Figure 3d). All this evidence indicated that the *CsARF10a* gene might be involved in cucumber leaf morphogenesis and in the process of cucumber fruit development.

3.3. Functional Analysis of *CsARF10a* Gene

To investigate the physiological importance of the cucumber auxin response factor, homozygous transgenic tomato cultivar 'Micro-Tom' lines expressing *CsARF10a* were generated (designated as *CsARF10a*-OE). Three *CsARF10a*-OE lines (L2, L3, and L4) were obtained, and two of them were found to have a deficiency in growth point (Figure 4a). As *CsARF10a*-OE L4 was selected for further characterization, an RT-qPCR experiment was performed to analyze the expression level of *CsARF10a* in transgenic lines. It turns out that *CsARF10a* were expressed abundantly in *CsARF10a*-OE lines (Figure 4b). Interestingly, no significant difference in *SlARF10* expression was observed in the WT and *CsARF10a*-OE L4 lines, which indicated that all phenotypic differences between the WT and *CsARF10a*-OE L4 lines had no relevance to *SlARF10* expression (Figure 4c).

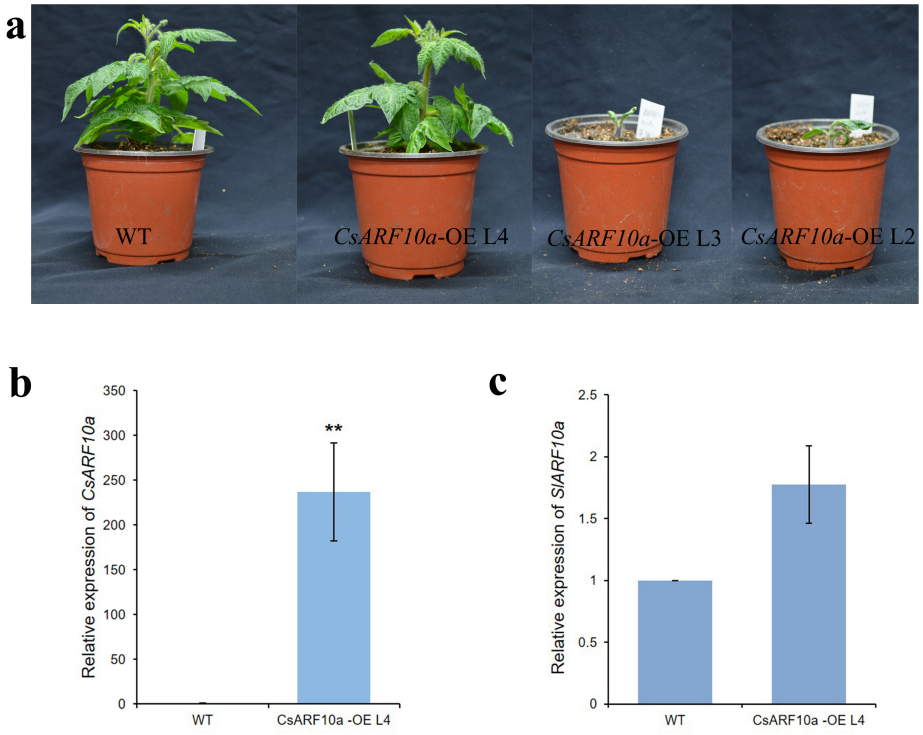

Figure 4. RT-qPCR detection of positive transgenic tomato lines and expression analysis of SlARF10a in transgenic tomato lines. (**a**) Generation of *CsARF10a*-OE tomato plants. Two lines exhibited developmental defects of growing point. (**b**) Expression analysis of *CsARF10a* in transgenic tomato plants. *CsARF10a*-OE L4 exhibited a relatively high amount of *CsARF10a* transcript accumulation. (**c**) Expression analysis of *SlARF10a* in *CsARF10a*-OE L4. The data of gene expression in WT were normalized to 1, respectively. Error bars represent SD. Significant differences are calculated with respect to expression in WT. Asterisks indicate that there are significant differences between WT and *CsARF10a*-OE L4 (*t* test, ** $p < 0.01$).

Leaf form change was the most logical alteration in the transgenic plants. The CsARF10a-OE L4 exhibited wider leaf blades compared with WT (Figure 5a), which indicates that CsARF10a transcript accumulation is positively correlated with blade outgrowth. Interestingly, this is consistent with previous research results, which found that slARF10 protein acts as a positive regulator in leaf morphology [41]. Moreover, orange fruits and ripening fruits were observed in WT plants, while the fruits of CsARF10a-OE L4 still remained a mature green color at 86 days after sowing (Figure 5b). The outcome of this phenomena implied that overexpressing CsARF10a could delay the maturation process of fruit. Although the transcription analysis showed that expression levels of CsARF10a were downregulated during both the parthenocarpic and pollinated fruit set processes, emasculation experiments in transgenic plants suggested that the overexpression of CsARF10a

could induce parthenocarpic fruit formation in tomato (Figure 5c). Meanwhile, the overexpression of CsARF10a could result in a reduced seed number, so the self-pollination experiment showed that the seed number of CsARF10a-OE L4 was significantly reduced compared with WT (Figure 5d).

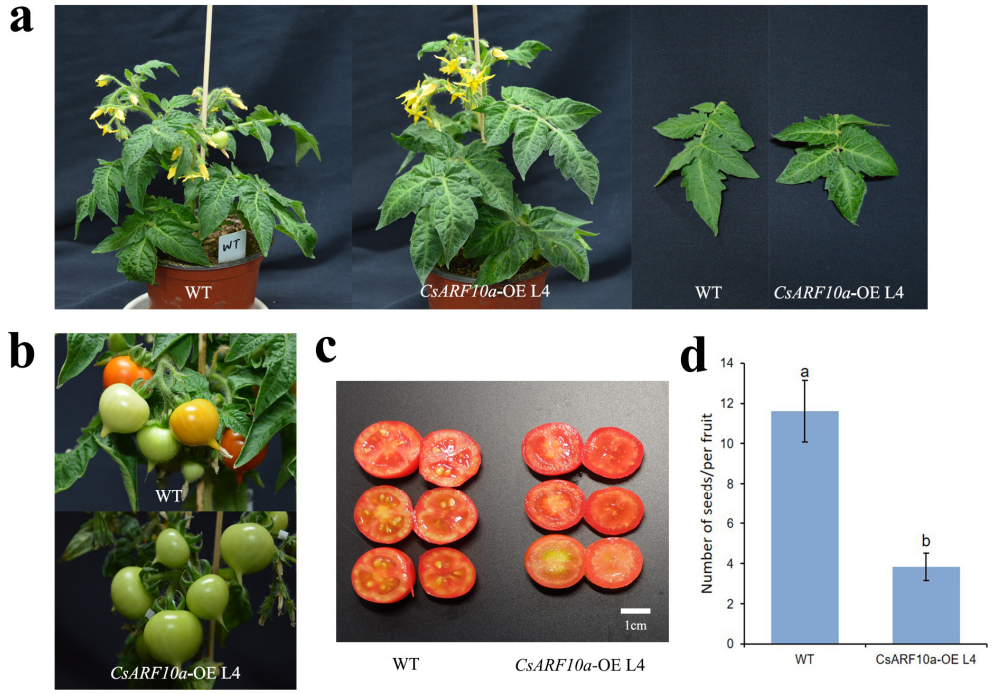

Figure 5. Altered phenotype of leaves, fruits, and seeds in *CsARF10a*-OE tomato plants. (**a**) Leaves of *CsARF10a*-OE plants exhibited wider leaf blades compared with the WT lines. (**b**) Fruit ripening was delayed in *CsARF10a*-OE lines compared with the WT lines. (**c**) Seeded fruits in WT lines and parthenocarpic fruits in transgenic lines. Scale bar = 1 cm. (**d**) Seed number in each fruit of WT plants and *CsARF10a-OE* plants. Error bars represent SD; differences among means of different genotypes were evaluated by Student's *t*-test at a probability level of 0.05, and histograms marked with different letters above bars represent significant differences.

4. Discussion

Auxin signaling is known to play a pivotal role in diverse aspects of plant growth and development. Generally, auxin regulates physiological processes of plants via a typical TIR1/AFB-Aux/IAA-ARF pathway. Thus, ARFs are critical transcription factors that respond to auxin signaling by regulating the expression of auxin response genes. As an ideal model plant for the *Cucurbitaceae* species, cucumber exhibits a variety of characteristics that may be regulated by various auxin-related genes. However, few components of auxin-mediated developmental signal transduction pathways have been studied in cucumber. Here, we characterized the cucumber homolog AtARF10, and constructed transgenic plants with increased *CsARF10a* expression to investigate the role of auxin response factors in plant development.

In this study, RT-qPCR experiments were conducted to detect the transcripts level of *CsARF10a* in cucumber. The expression levels of *CsARF10a* transcripts were significantly lower in pollination fruit and hormone-induced parthenocarpic fruit than in abortion fruit (Figure 3c). Furthermore, the expression patterns of *CsARF10a* were observed in '8419s-1' fruits during the abortion and fruit set processes. The experimental results implied that the expression level of *CsARF10a* decreased before pollination, then increased once the fruit initiation process began, and that *CsARF10a* transcripts attained the minimum level at 0 days post anthesis (Figure 3d). Based on the

results of transcription level analysis, it is speculated that *CsARF10a* may be involved in the process of cucumber fruit set and development as a repressor.

Previous evolution studies of the ARF gene family have indicated that the main ARFs are classified into three main groups in land plants, namely Clade A, Clade B, and Clade C. Clade C, which includes ARF10, ARF16, and ARF17, mainly consists of repressors [44]. In Arabidopsis, genetic and phenotypic analyses of loss-of-function mutants were conducted to reveal distinct functions of individual ARFs [22,45]. No phenotypic defects were found in *arf10* or *arf16* single mutants [46], while the absence of lateral root formation was observed in the *arf10/arf16* double mutants [34,47,48]. The overexpression of ARF10 in *Arabidopsis* results in developmental defects such as twisted siliques, curled stems, serrated leaves, contorted flowers, and even seedling lethality [49]. In tomato, the upregulation of *SlARF10A* can severely inhibit leaflet blade outgrowth [36,50]. Conversely, the silencing of *SlARF10A* causes extra blade outgrowth and ectopic blade formation [51]. All this evidence seems to indicate that *CsARF10a* might also act as a negative regulator of the plant growth process. To explore the distinct function of *CsARF10a*, overexpressed *CsARF10a* was transformed into tomato plants. Interestingly, the observation of phenotypes demonstrated that *CsARF10a* can promote leaf growth and parthenocarpic fruit formation (Figure 5a,c), suggesting that *CsARF10a* is important for leaf architecture and fruit development as an activator, which means that ARF10 might have pleotropic functions in various species. As the number of genes in the subfamily of ARF10 varies among species, the functional redundancy and functional differentiation of *CsARF10* could be one explanation for the inconsistence of ARF10 functions among cucumber and other species. In addition, there is a possibility that the neofunctionalization of *CsARF10a* might happen during the evolutionary process of cucumber, which could lead to the changes in gene function in *CsARF10a*.

Despite the paradoxical results following qRT-RCR and *CsARF10a*-overexpression experiments, *CsARF10a* might have a completely different role in the process of fruit set and process of fruit development, respectively. Given that there is a growing body of evidence on the post-transcriptional regulation of ARF10 transcript abundance via miR160 in various species [49,51–55], we propose a hypothesis that the cleavage regulation of *CsARF10a* via *CsmiR160* exists in the early developmental stages of cucumber fruits (-1dpa-0dpa) and is essential in the cucumber fruit set process, which needs to be further verified in vivo.

5. Conclusions

In the present study, an auxin response factor gene, *CsARF10a*, was identified and cloned from the cucumber genome. An expression analysis via RT-qPCR suggested that *CsARF10a* participated in the regulation of the pollination-induced and hormone-treatment-induced fruit set of cucumber. The overexpression of *CsARF10a* in various tomato-induced phenotypic changes, including leaf shape alteration and parthenocarpic fruit formation, indicates that *CsARF10a* might positively regulate cucumber fruit set and development. These results can help us to better understand the role of auxin response factors in cucumber, while a further investigation of downstream genes and specific molecular partners that interact with *CsARF10a* is still needed to elucidate the exact mechanisms via which auxin signaling components regulate cucumber fruit set and development.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10010079/s1, Table S1: List of auxin response factor proteins used for the construction of the phylogenetic tree in this study; Table S2: Quantification cycles of RT-qPCR experiments conducted in this study.

Author Contributions: Conceptualization, J.X. (Jian Xu), P.Z., J.L. (Ji Li) and J.X. (Jinhua Xu); data curation, J.L. (Jinqiu Liu), L.Z. and Q.H.; formal analysis, J.X. (Jian Xu), P.Z., X.Y. (Xiefeng Yao), Y.M., X.Y. (Xingping Yang), J.L. (Ji Li) and J.X. (Jinhua Xu); investigation, J.X. (Jian Xu), P.Z., X.Y. (Xiefeng Yao), Y.M., L.L., M.Z. and G.L.; validation, J.X. (Jian Xu), P.Z., X.Y. (Xiefeng Yao), Y.M., X.Y. (Xingping Yang), J.L. (Ji Li) and J.X. (Jinhua Xu); writing—original draft, J.X. (Jian Xu), P.Z., X.Y. (Xiefeng Yao), Y.M., L.L., M.Z., G.L., X.Y. (Xingping Yang), J.L. (Ji Li) and J.X. (Jinhua Xu); writing—review and editing, J.X. (Jian Xu), P.Z., X.Y. (Xiefeng Yao), Y.M., L.L., M.Z., G.L., X.Y. (Xingping Yang), J.L. (Jinqiu

Liu), L.Z., Q.H., J.L. (Ji Li) and J.X. (Jinhua Xu). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Natural Science Foundation of Jiangsu Province (BK20190263), the National Industrial Technology System for Watermelon and Melon (CARS-25), and the Earmarked Fund for Jiangsu Agricultural Industry Technology Systems (JATS [2022] 424).

Data Availability Statement: Data are contained within the article and supplementary materials.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. McAtee, P.; Karim, S.; Schaffer, R.; David, K. A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening. *Front. Plant Sci.* **2013**, *4*, 79. [CrossRef]
2. Mapelli, S.; Frova, C.; Torti, G.; Soressi, G.P. Relationship between set, development and activities of growth-regulators in tomato fruits. *Plant Cell Physiol.* **1978**, *19*, 1281–1288.
3. Friml, J. Auxin transport—Shaping the plant. *Curr. Opin. Plant Biol.* **2003**, *6*, 7–12. [CrossRef] [PubMed]
4. Sundberg, E.; Ostergaard, L. Distinct and dynamic auxin activities during reproductive development. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a001628. [CrossRef] [PubMed]
5. Pattison, R.J.; Csukasi, F.; Catalá, C. Mechanisms regulating auxin action during fruit development. *Physiol. Plant.* **2014**, *151*, 62–72. [CrossRef]
6. Godoy, F.; Kühn, N.; Muñoz, M.; Marchandon, G.; Gouthu, S.; Deluc, L.; Delrot, S.; Lauvergeat, V.; Arce-Johnson, P. The role of auxin during early berry development in grapevine as revealed by transcript profiling from pollination to fruit set. *Hortic. Res.* **2021**, *8*, 140. [CrossRef]
7. Zhang, Y.; Yu, J.; Xu, X.; Wang, R.; Liu, Y.; Huang, S.; Wei, H.; Wei, Z. Molecular mechanisms of diverse auxin responses during plant growth and development. *Int. J. Mol. Sci.* **2022**, *23*, 12495. [CrossRef]
8. Hagen, G.; Guilfoyle, T. Auxin-responsive gene expression: Genes, promoters and regulatory factors. *Plant Mol. Biol.* **2002**, *49*, 373–385. [CrossRef]
9. Szemenyei, H.; Hannon, M.; Long, J.A. TOPLESS mediates auxin-dependent transcriptional repression during embryogenesis. *Science* **2008**, *319*, 1384–1386. [CrossRef]
10. Gray, W.M.; Kepinski, S.; Rouse, D.; Leyser, O.; Estelle, M. Auxin regulates SCF-dependent degradation of AUX/IAA proteins. *Nature* **2001**, *414*, 271–276. [CrossRef]
11. Rogg, L.E.; Bartel, B. Auxin signaling: Derepression through regulated proteolysis. *Dev. Cell* **2001**, *1*, 595–604. [CrossRef] [PubMed]
12. Kepinski, S.; Leyser, O. Ubiquitination and auxin signaling: A degrading story. *Plant Cell* **2002**, *14*, S81–S95. [CrossRef] [PubMed]
13. Dharmasiri, N.; Dharmasiri, S.; Estelle, M. The F-box protein TIR1 is an auxin receptor. *Nature* **2005**, *435*, 441–445. [CrossRef]
14. Kepinski, S.; Leyser, O. The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* **2005**, *435*, 446–451. [CrossRef]
15. Weijers, D.; Friml, J. SnapShot: Auxin signaling and transport. *Cell* **2009**, *136*, 1172–1172.e1. [CrossRef]
16. Li, S.B.; Xie, Z.Z.; Hu, C.G.; Zhang, J.Z. A review of auxin response factors (ARFs) in plants. Front. *Plant Sci.* **2016**, *7*, 47. [CrossRef]
17. Guilfoyle, T.J.; Hagen, G. Auxin response factors. *J. Plant Growth Regul.* **2001**, *20*, 281–291. [CrossRef]
18. Tiwari, S.B.; Hagen, G.; Guilfoyle, T. The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* **2003**, *15*, 533–543. [CrossRef] [PubMed]
19. Wang, D.K.; Pei, K.M.; Fu, Y.P.; Sun, Z.X.; Li, S.J.; Liu, H.Q.; Tang, K.; Han, B.; Tao, Y.Z. Genome-wide analysis of *the auxin response factors* (ARF) gene family in rice (*Oryza sativa*). *Gene* **2007**, *394*, 13–24. [CrossRef]
20. Remington, D.L.; Vision, T.J.; Guilfoyle, T.J.; Reed, J.W. Contrasting modes of diversification in the *Aux/IAA* and *ARF* gene families. *Plant Physiol.* **2004**, *135*, 1738–1752. [CrossRef]
21. Wang, L.; Hua, D.P.; He, J.N.; Duan, Y.; Chen, Z.Z.; Hong, X.H.; Gong, Z.Z. *Auxin response factor2* (*ARF2*) and its regulated homeodomain gene *HB33* mediate abscisic acid response in *Arabidopsis*. *PLoS Genet.* **2011**, *7*, e1002172. [CrossRef] [PubMed]
22. Guilfoyle, T.J.; Hagen, G. Auxin response factors. *Curr. Opin. Plant Biol.* **2007**, *10*, 453–460. [CrossRef]
23. Nemhauser, J.L.; Feldman, L.J.; Zambryski, P.C. Auxin and ETTIN in *Arabidopsis* gynoecium morphogenesis. *Development* **2000**, *127*, 3877–3888. [CrossRef]
24. Zhou, J.H.; Sittmann, J.; Guo, L.; Xiao, Y.W.; Huang, X.L.; Pulapaka, A.; Liu, Z.C. Gibberellin and auxin signaling genes and repress accessory fruit initiation in diploid strawberry. *Plant Physiol.* **2021**, *185*, 1059–1075. [CrossRef] [PubMed]
25. Liu, J.; Hua, W.; Hu, Z.Y.; Yang, H.L.; Zhang, L.; Li, R.J.; Deng, L.B.; Sun, X.C.; Wang, X.F.; Wang, H.Z. Natural variation in gene simultaneously affects seed weight and silique length in polyploid rapeseed. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E5123–E5132. [CrossRef]
26. Sagar, M.; Chervin, C.; Mila, I.; Hao, Y.W.; Roustan, J.P.; Benichou, M.; Gibon, Y.; Biais, B.; Maury, P.; Latché, A.; et al. SlARF4, an auxin response factor involved in the control of sugar metabolism during tomato fruit development. *Plant Physiol.* **2013**, *161*, 1362–1374. [CrossRef]

27. Dong, X.X.; Li, Y.J.; Guan, Y.H.; Wang, S.X.; Luo, H.; Li, X.M.; Li, H.; Zhang, Z.H. Auxin-induced AUXIN RESPONSE FACTOR4 activates APETALA1 and FRUITFULL to promote flowering in woodland strawberry. *Hortic. Res.* **2021**, *8*, 115. [CrossRef] [PubMed]
28. Liu, S.Y.; Zhang, Y.W.; Feng, Q.S.; Qin, L.; Pan, C.T.; Lamin-Samu, A.T.; Lu, G. Tomato AUXIN RESPONSE FACTOR 5 regulates fruit set and development via the mediation of auxin and gibberellin signaling. *Sci. Rep.* **2018**, *8*, 2971. [CrossRef]
29. Liu, Z.N.; Miao, L.M.; Huo, R.X.; Song, X.Y.; Johnson, C.; Kong, L.J.; Sundaresan, V.; Yu, X.L. ARF2–ARF4 and ARF5 are essential for female and male gametophyte development in Arabidopsis. *Plant Cell Physiol.* **2018**, *59*, 179–189. [CrossRef]
30. Nagpal, P.; Ellis, C.M.; Weber, H.; Ploense, S.E.; Barkawi, L.S.; Guilfoyle, T.J.; Hagen, G.; Alonso, J.M.; Cohen, J.D.; Farmer, E.E.; et al. Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. *Development* **2005**, *132*, 4107–4118. [CrossRef]
31. Yuan, Y.J.; Xu, X.; Gong, Z.H.; Tang, Y.W.; Wu, M.B.; Yan, F.; Zhang, X.L.; Zhang, Q.; Yang, F.Q.; Hu, X.W.; et al. Auxin response factor 6A regulates photosynthesis, sugar accumulation, and fruit development in tomato. *Hortic. Res.* **2019**, *6*, 85. [CrossRef] [PubMed]
32. Goetz, M.; Hooper, L.C.; Johnson, S.D.; Rodrigues, J.C.M.; Vivian-Smith, A.; Koltunow, A.M. Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in *Arabidopsis* and tomato. *Plant Physiol.* **2007**, *145*, 351–366. [CrossRef]
33. de Jong, M.; Wolters-Arts, M.; Feron, R.; Mariani, C.; Vriezen, W.H. The auxin response factor 7 (ARF7) regulates auxin signaling during tomato fruit set and development. *Plant J.* **2009**, *57*, 160–170. [CrossRef] [PubMed]
34. Wang, J.W.; Wang, L.J.; Mao, Y.B.; Cai, W.J.; Xue, H.W.; Chen, X.Y. Control of root cap formation by microRNA-targeted auxin response factors in *Arabidopsis*. *Plant Cell* **2005**, *17*, 2204–2216. [CrossRef]
35. Liu, X.D.; Huang, J.; Wang, Y.; Khanna, K.; Xie, Z.X.; Owen, H.A.; Zhao, D.Z. The role of floral organs in carpels, an *Arabidopsis* loss-of-function mutation in MicroRNA160a, in organogenesis and the mechanism regulating its expression. *Plant J.* **2010**, *62*, 416–428. [CrossRef]
36. Hendelman, A.; Buxdorf, K.; Stav, R.; Kravchik, M.; Arazi, T. Inhibition of lamina outgrowth following *Solanum lycopersicum* AUXIN RESPONSE FACTOR 10 (*SlARF10*) derepression. *Plant Mol. Biol.* **2012**, *78*, 561–576. [CrossRef]
37. Liu, X.; Dong, X.F.; Liu, Z.H.; Shi, Z.H.; Jiang, Y.; Qi, M.F.; Xu, T.; Li, T.L. Repression of ARF10 by microRNA160 plays an important role in the mediation of leaf water loss. *Plant Mol. Biol.* **2016**, *92*, 313–336. [CrossRef] [PubMed]
38. Yuan, Y.J.; Mei, L.H.; Wu, M.B.; Wei, W.; Shan, W.; Gong, Z.H.; Zhang, Q.; Yang, F.Q.; Yan, F.; Zhang, Q.; et al. *SIARF10*, an auxin response factor, is involved in chlorophyll and sugar accumulation during tomato fruit development. *J. Exp. Bot.* **2018**, *69*, 5507–5518. [CrossRef]
39. Guo, Q.W.; Li, J.; Cui, L.; Zhang, T.L.; Kere, G.M.; Chen, J.F. Cloning and expression analysis of cucumber *CsARF10* genes subfamily. *Hortic. Plant J.* **2013**, *40*, 1071–1080.
40. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef]
41. Xu, J.; Li, J.; Cui, L.; Zhang, T.; Wu, Z.; Zhu, P.Y.; Meng, Y.J.; Zhang, K.J.; Yu, X.Q.; Lou, Q.F.; et al. New insights into the roles of cucumber TIR1 homologs and miR393 in regulating fruit/seed set development and leaf morphogenesis. *BMC Plant Biol.* **2017**, *17*, 130. [CrossRef] [PubMed]
42. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
43. Ren, Z.X.; Li, Z.G.; Miao, Q.; Yang, Y.W.; Deng, W.; Hao, Y.W. The auxin receptor homologue in *Solanum lycopersicum* stimulates tomato fruit set and leaf morphogenesis. *J. Exp. Bot.* **2011**, *62*, 2815–2826. [CrossRef]
44. Finet, C.; Berne-Dedieu, A.; Scutt, C.P.; Marlétaz, F. Evolution of the ARF gene family in land plants: Old domains, new tricks. *Mol. Biol. Evol.* **2013**, *30*, 45–56. [CrossRef]
45. Rademacher, E.H.; Lokerse, A.S.; Schlereth, A.; Llavata-Peris, C.I.; Bayer, M.; Kientz, M.; Rios, A.F.; Borst, J.W.; Lukowitz, W.; Jürgens, G.; et al. Different auxin response machineries control distinct cell fates in the early plant embryo. *Dev. Cell* **2012**, *22*, 211–222. [CrossRef] [PubMed]
46. Okushima, Y.; Overvoorde, P.J.; Arima, K.; Alonso, J.M.; Chan, A.; Chang, C.; Ecker, J.R.; Hughes, B.; Lui, A.; Nguyen, D.; et al. Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: Unique and overlapping functions of ARF7 and ARF19. *Plant Cell* **2005**, *17*, 444–463. [CrossRef] [PubMed]
47. Bustos-Sanmamed, P.; Bazin, J.; Hartmann, C.; Crespi, M.; Lelandais-Brière, C. Small RNA pathways and diversity in model legumes: Lessons from genomics. *Front. Plant Sci.* **2013**, *4*, 236. [CrossRef]
48. Turner, M.; Nizampatnam, N.R.; Baron, M.; Coppin, S.; Damodaran, S.; Adhikari, S.; Arunachalam, S.P.; Yu, O.; Subramanian, S. Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. *Plant Physiol.* **2013**, *162*, 2042–2055. [CrossRef]
49. Liu, P.P.; Montgomery, T.A.; Fahlgren, N.; Kasschau, K.D.; Nonogaki, H.; Carrington, J.C. Repression of AUXIN response FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *Plant J.* **2007**, *52*, 133–146. [CrossRef]
50. Damodharan, S.; Zhao, D.Z.; Arazi, T. A common miRNA160-based mechanism regulates ovary patterning, floral organ abscission and lamina outgrowth in tomato. *Plant J.* **2016**, *86*, 458–471. [CrossRef]

51. Ben-Gera, H.; Dafna, A.; Alvarez, J.P.; Bar, M.; Mauerer, M.; Ori, N. Auxin-mediated lamina growth in tomato leaves is restricted by two parallel mechanisms. *Plant J.* **2016**, *86*, 443–457. [CrossRef] [PubMed]
52. Tian, J.X.; Chen, J.H.; Li, B.L.; Zhang, D.Q. Association genetics in *Populus* reveals the interactions between *Pto-miR160a* and its target *Pto-ARF16*. *Mol. Genet. Genom.* **2016**, *291*, 1069–1082. [CrossRef] [PubMed]
53. Lin, Y.L.; Lai, Z.X.; Tian, Q.L.; Lin, L.X.; Lai, R.L.; Yang, M.M.; Zhang, D.M.; Chen, Y.K.; Zhang, Z.H. Endogenous target mimics down-regulate miR160 mediation of *ARF10*, *-16*, and *-17* cleavage during somatic embryogenesis in *Dimocarpus Longan Lour*. *Front. Plant Sci.* **2015**, *6*, 956. [CrossRef] [PubMed]
54. Damodharan, S.; Corem, S.; Gupta, S.K.; Arazi, T. Tuning of *SlARF10A* dosage by *sly-miR160a* is critical for auxin-mediated compound leaf and flower development. *Plant J.* **2018**, *96*, 855–868. [CrossRef]
55. Dai, X.H.; Lu, Q.; Wang, J.; Wang, L.L.; Xiang, F.N.; Liu, Z.H. *MiR160* and its target genes *ARF10*, *ARF16* and *ARF17* modulate hypocotyl elongation in a light, BRZ, or PAC-dependent manner in *Arabidopsis*: miR160 promotes hypocotyl elongation. *Plant Sci.* **2021**, *303*, 110686. [CrossRef]