



Research article

Identification of auxin response factor in the pan-genome and their expression pattern analysis in cucumber

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Abstract: Auxin response factors (ARFs) are important transcription factors that regulate the expression of early genes in response to auxin. Auxin is a crucial phytohormone that controls plant growth and development. Even though *ARF* genes have been identified in the cucumber (*Cucumis sativus*) genome version 3.0, the features of this gene family are unclear in the cucumber pan-genome and in the newly assembled genome v4.0. We identified a total of 18 CsARF genes, including two CsARF13s in Gy14. All ARF proteins, except ARF5 and 7, showed differences in protein length; all of them showed amino acid variation in the cucumber pan-genome, including 13 accessions. We also analyzed their organ-specific expression and their expression patterns in fruit development and in response to biotic stresses. Importantly, we found that the overexpression of CsARF12 significantly enhanced the resistance of cucumber cotyledons to gray mold infection. Therefore, this study lays a foundation for understanding the biological functions of CsARF genes and provides *CsARF12* as a candidate gene to improve the cucumber resistance to gray mold.

Keywords: cucumber; pan-genome; *ARF* gene; biotic stress response; gray mold

1. Introduction

Auxin is a crucial plant hormone that regulates key growth and developmental processes, including cell division, differentiation, and organogenesis. The physiological effects of auxin are triggered by molecular signaling pathways, including auxin response factors (ARFs), one of the two major families that control auxin signaling. At low auxin levels, ARF binds to the Aux/IAA protein to form a heterodimer, inhibiting auxin-mediated transcription; conversely, at higher auxin levels, Aux/IAA binds to the auxin receptor transport inhibitor reaction 1 (TIR1) to form a complex of TIR1-Aux/IAA, leading to the ubiquitination of Aux/IAA and its subsequent degradation. Then, the ARF protein is released to activate the transcription of early auxin-response genes [1,2].

Most ARF proteins contain three conserved domains: the DNA binding domain (DBD), the intermediate domain (MR), and Phox and Bem 1 (PB1) [3]. All ARFs have a conserved DBD at the N-terminal [4], which is functionally specific and requires additional n- and c-terminal amino acids for efficient binding to auxin response elements (AuxRE) [5,6]. Previous studies have identified TGTCGG as the highest-affinity ARF binding site, surpassing the original AuxRE, as demonstrated through protein binding microarrays [7,8]. In *Arabidopsis thaliana* and *Solanum lycopersicum*, most ARF proteins typically possess a single DBD [9]. Notable exceptions include AtARF23 and SlARF6B, which exhibit a truncated DBD structure [10]. In contrast, OsARF20 in rice contains two DBDs [11].

The middle region (MR) functions as either an activation (AD) or repression domain (RD), is adjacent to the DBD, and contains distinct amino acid sequences that help classify ARFs across plant species [6]. The length and composition of the MR amino acid sequence determine whether ARF plays an activation or inhibition role. Across species, ARF proteins exhibit distinct transcriptional regulatory roles: in *Arabidopsis thaliana*, AtARF5–AtARF8 and AtARF19 function as activators. Tomato (*Solanum lycopersicum*) activators include SlARF5, -6A, -7A, -7B, -8B, and 19 [10]. Rice (*Oryza sativa*) possesses nine activating ARFs: OsARF5, -6, -11, -12, -16, -17, -19, -21, and -25 [11]. Conversely, in sorghum (*Sorghum bicolor*), the most characterized SbARFs (SbARF1–6, -9–13, -16, -18, -20, -21, and -24) act as repressors [9]. The Phox and Bem 1 (PB1) is quite similar to the III and IV domains of Aux/IAA, facilitating protein–protein interaction through heterodimerization of ARF and Aux/IAA proteins or homodimerization of ARF proteins [5,12]. However, the presence of this conserved structural domain is not universal across all ARF family members. Several ARFs in each species lack this feature, including four ARFs in *Arabidopsis thaliana* (AtARF3, AtARF-13, AtARF-17, and AtARF-23) [4], five in tomato (*Solanum lycopersicum*; SlARF3, SlARF-6B, SlARF-16B, SlARF-17, and SlARF-24) [10], and five in sorghum (*Sorghum bicolor*; SbARF4, SbARF-5, SbARF-16, SbARF-20, and SbARF-21) [9].

ARFs are essential for all aspects of plant growth and development, as well as in the response to biotic and abiotic stresses. Several *ARF* genes have been reported to be key components in lateral root formation, such as *AtARF7*, *19* [13], *8* [14], *10*, and *16* [15], as well as *ZmARF4* [16] and *SbARF17* [9]; some in primary root formation, such as *AtARF6*, *8*, *17* [17], *OsARF1*, *11* [18], *12*, and *25* [19]; and some in the elongation of adventitious root, such as *OsARF12*, *25* [18], *16* [20], *AtARF7*, *19* [13], *TaARF4* [21], and *15* [22]. Many *ARF* genes regulate leaf morphogenesis and vein formation, such as *AtARF1* [23], *2*, *3*, *4*, *5* [24], *7*, *19* [13,25], *SlARF2* [26], *6A*, *8A*, *8B*, *24* [27], *OsARF11*, *16* [28], *19* [27], *TaARF15A* [22], and *SbARF18* [9]. Many *ARF* genes play roles in flower growth and fertility, including *AtARF3* [29], *5* [30], *6*, *8* [31], *17* [32], *SlARF1*, *9*, *10*, *11*, *15*, *16* [33], *2* [27], and *TaARF8* [21]. Some *ARF* genes are involved in fruit development, such as *AtARF2*, *8* [23], *SlARF2* [34], *4* [35],

5 [29], 7, 9 [36], 6, 8, 10, and 16 [37]. Also, some *ARFs* control seed development and germination, such as *AtARF2*, 8 [38], *ZmARF3*, 4, 6, 8, 10, 12, 13, 14, 15, 17, 18, 19, 21, 22, 25, 28, and 30 [39]. *ARF* genes also play significant roles in the response to biotic and abiotic stresses. For instance, *AtARF7*, 19 [40], *OsARF12* [41], 16 [42], and *ZmARF4*, 23 [27] are involved in the regulation of Pi stress; *AtARF2* [43] and *ZmARF2* [44] in mediating K⁺ stress; *OsARF10* [45] and *TaARF2* [46] in aluminum stress response; *OsARF11*, 15, and *ZmARF4* in salt stress response [47]; and *OsARF2*, 4, 10, 14, 16, 18, 19, 22, 23 [48], *SlARF1*, 4, 8A, 19, and 24 in drought stress response [49]. Moreover, several *ARF* genes, such as *OsARF5*, 11, 12, 16 [50], and 17 [46], play very important roles in antiviral immune response.

Based on the sequenced whole plant genomes, *ARF* families in many plant species have been systematically analyzed. There were 23 *AtARFs* in *Arabidopsis thaliana* [5], 22 *SlARFs* in *Solanum lycopersicum* [10], 25 *OsARFs* in *Oryza sativa* [51], 23 *TaARFs* in *Triticum aestivum* [22], 25 *SbARFs* in *Sorghum bicolor* [9], and 36 *ZmARFs* in *Zea mays* [52]. With the completion of *Cucumis sativus* genome v3.0 of *Chinese long 9930*, 17 *CsARFs* have been identified [53]; this has been updated to v4.0, in a nearly-complete cucumber reference genome [54]. Moreover, the first cucumber pan-genome has also been established [55]. Therefore, it is necessary to re-identify *CsARFs* in the new genome version and the cucumber pan-genome. Here, we found structural variations in *CsARF3*, a relocated chromosomal position for *CsARF5*, and widespread sequence divergence among *CsARFs* across 13 accessions. Functional studies implicated certain *CsARFs* in fruit development and pathogen responses, with transient overexpression of *CsARF12* enhancing gray mold resistance in cucumber cotyledons.

2. Materials and methods

2.1. Gene identification and chromosomal locations

The Hidden Markov Model (HMM) file of the ARF domain (PF06507), downloaded from the Pfam protein family database (<http://pfam.sanger.ac.uk/>) (March 5, 2025), was used to identify *ARF* genes from the Cucumber Genome Database (V4.0) (<http://www.Cucumberdb.com/#/download>) (January 5, 2025) using HMMER 4.0. Based on the HMMER results, all *CsARF* genes retrieved from the cucumber genome were further validated using the Pfam (<http://pfam.xfam.org/search#tabview=tab1>) (March 15, 2025) and NCBI databases (<https://www.ncbi.nlm.nih.gov/>) (March 15, 2025) to confirm the presence of ARF domain sequences. Subsequently, each candidate gene was manually checked to ensure the integrity of the predicted ARF domains. The final identified genes were mapped to cucumber chromosomes based on their physical locations in the cucumber genome database. The subcellular localization of *CsARF* proteins was predicted using CELLO (<http://cello.life.ntcu.edu.tw/>) (March 23, 2025). Chromosomal positions of *ARF* genes were visualized with TBtools (v2.210) using the cucumber 9930 (v4.0) genome annotation file.

2.2. Analysis of *CsARF* gene and protein structure and cis-regulatory elements of their promoters in the pan-genome

Gene structures were visualized with TBtools (v2.210) based on annotation data. The MEME online tool (<https://meme-suite.org/meme/tools/meme>) (October 20, 2024) was used to analyze the motif composition of *CsARF* proteins. TBtools (Gene Structure View) was used to generate integrated

figures of phylogenetic clustering, conserved motifs, and gene structures of *CsARF* genes. To identify cis-regulatory elements in the promoter sequences of the *ARF* genes in the cucumber pan-genome, the promoter sequences of the *ARF* genes (2.0 kb upstream of the start codon) were obtained from the cucumber genome database. The promoter cis-regulatory elements were then analyzed on Plant CARE ([https:// bioinformatics. psb. ugent. be/webtools/plantcare/html/](https://bioinformatics.psb.ugent.be/webtools/plantcare/html/)) (December 5, 2024) and visualized using TBtools (v2.210).

2.3. *CsARF* gene classification and phylogenetic analysis

Cucumber *ARF* genes were clustered according to the Arabidopsis *ARF* gene classification scheme and the domain arrangements of CsARF and AtARF proteins. Amino acid sequence alignments were performed using ClustalX with default settings. A phylogenetic tree was constructed using the ClustalW website (<https://www.genome.jp/tools-bin/clustalw>) (December 15, 2024), visualized, and refined with Evolview ([http:// wwwevolgenius. info/ evolview](http://www.evolgenius.info/evolview)) (December 15, 2024).

2.4. Transcriptome analysis of the *ARF* genes in cucumber

Expression patterns of *CsARF* genes were analyzed using published RNA-seq data (SRA046916) from nine cucumber tissues [56]: roots, stems, leaves, female flowers, male flowers, ovaries, expanded unfertilized ovaries, expanded fertilized ovaries, and tendrils (one biological replicate per tissue). Published transcriptome data were used to analyze *CsARF* gene expression differences under biological stress (downy mildew: PRJNA321023/PRJNA285071 and gray mold: SRP009350) [57,58]. Heatmaps were generated using TBtools (v2.210).

2.5. Construction of recombinant plasmids and transient transformation of cucumber cotyledons

The coding sequences of four selected *ARF* genes (*ARF7*, *ARF8*, *ARF10*, and *ARF12*) were cloned into the expression vector pFGC5941 using NcoI and BamHI restriction sites. Primers for amplification of these genes are shown in Table S2. Recombinant plasmids were introduced into *Agrobacterium tumefaciens* (GV3101 strain) via the freeze-thaw method [59]. *Agrobacterium* cultures carrying plasmids were infiltrated into the cotyledons of 1-week-old cucumber seedlings. To assess disease resistance, gray mold was inoculated after 18 h post-infiltration of the constructs, and lesion areas were measured at 24 and 36 h post-infection. Lesion sizes were quantified using Digimizer software (version 5.4.4).

3. Results

3.1. Re-identification and chromosomal distribution of the *CsARF* genes in the cucumber pan-genome

Since a nearly-complete cucumber reference genome and the first pan-genome were recently established⁵⁴, we re-identified the *CsARF* gene family using a hidden Markov model (HMM) search with the ARF domain (PF06507). This analysis identified 17 *CsARF* genes in the updated *Chinese long 9930* genome, which were named according to their chromosomal locations (Table 1). Then, we identified *CsARFs* in the genomes of another 12 cucumber accessions: XTMC and Cu2 are East Asian

cultivars; Cuc80 is a Xishuangbanna cultivar; Cuc37, Gy14, and 9110gt are Eurasian cultivars; PI183967, Cuc64, W4, and W8 are Indian wild forms; and Hx14 and Hx117 are Indian cultivars. Only seven (9930, Cuc37, 9110gt, Hx14, Cuc64, W4, and PI183967) of the 13 accessions have all 17 *CsARFs*; the others lack at most one member. It is also worth noting that 18 *ARF* genes were identified in Gy14, with two *CsARF13* genes, namely *CsARF13a* (6G039530) and *CsARF13b* (UNG103910) (Table 2). These two proteins share a highly conserved amino acid sequence but have different protein lengths (Table 3).

Table 1. *CsARF* family members in *Chinese long 9930*.

Gene name	Sequence ID	Chromosomal site	Introns	Number of amino acids	Molecular weight	Theoretical pI	Subcellular location
<i>CsARF1</i>	CsaV4_1G000010	1	12	839	93229.91	6.17	Nucleus
<i>CsARF2</i>	CsaV4_1G002990	1	13	899	99671.25	6.01	Nucleus
<i>CsARF3</i>	CsaV4_2G000004	2	12	1097	121793.22	6.07	Nucleus
<i>CsARF4</i>	CsaV4_2G000690	2	13	1107	121881.68	6.1	Nucleus
<i>CsARF5</i>	CsaV4_2G002372	2	1	550	60285.43	6.24	Nucleus
<i>CsARF6</i>	CsaV4_3G004550	3	13	948	104475.8	5.31	Nucleus
<i>CsARF7</i>	CsaV4_4G000690	4	13	696	77936.11	7.06	Nucleus
<i>CsARF8</i>	CsaV4_5G000186	5	13	691	77024.4	5.99	Nucleus
<i>CsARF9</i>	CsaV4_5G001316	5	13	678	75394.74	6.02	Nucleus
<i>CsARF10</i>	CsaV4_5G001572	5	13	854	95099.62	5.91	Nucleus
<i>CsARF11</i>	CsaV4_6G001210	6	3	716	79038.52	8.75	Nucleus
<i>CsARF12</i>	CsaV4_6G001772	6	12	751	83259.58	6.67	Nucleus
<i>CsARF13</i>	CsaV4_6G002454	6	2	605	66714.09	6.64	Nucleus
<i>CsARF14</i>	CsaV4_6G002784	6	3	624	68205.75	6.45	Nucleus
<i>CsARF15</i>	CsaV4_6G003885	6	10	732	80321.08	7.09	Nucleus
<i>CsARF16</i>	CsaV4_6G004054	6	13	907	101064.55	6.18	Nucleus
<i>CsARF17</i>	CsaV4_7G001637	7	12	687	77264.29	5.81	Nucleus

Note: Comprehensive analysis of *CsARF* gene features in the v4.0 of *Chinese long 9930*.

Table 2. Identification of *CsARF* genes in the cucumber pan-genome of 13 accessions.

Gene name	9930	XTMC	Cu2	Cuc80	Cuc37	Gy14	9110gt	Hx14	Hx117	Cuc64	W4	W8	PI183967
<i>CsARF1</i>	1G000010	1G000100	1G000090	1G000100	1G000110	1G000110	1G000120	1G000650	1G000120	1G000110	1G000090	1G000100	1G00090
<i>CsARF2</i>	1G002990	1G038970	1G032340	-	1G036980	1G043350	1G035460	1G050460	1G053350	1G045860	1G042270	1G031660	1G24600
<i>CsARF3</i>	2G000004	2G000030	2G000030	2G001040	2G000030	2G000050	2G000040	2G000030	2G000030	2G000030	2G000030	-	2G00030
<i>CsARF4</i>	2G000690	2G007910	2G006960	2G008810	2G006880	2G013030	2G007990	2G011930	2G008870	2G006900	2G007970	2G007860	2G06610
<i>CsARF5</i>	2G002372	-	UNG260550	2G073040	2G080870	UNG001200	2G022530	2G031390	2G033460	2G064530	2G024940	2G027540	2G15960
<i>CsARF6</i>	3G004550	3G068420	3G056540	3G057920	3G061790	3G066700	3G053620	3G072080	3G072230	3G067320	3G052650	3G051260	3G43240
<i>CsARF7</i>	4G000690	4G008670	4G009760	4G006730	4G006750	4G009700	4G007690	4G007670	4G006710	4G011780	4G007750	4G007680	4G06550
<i>CsARF8</i>	5G000186	5G001820	-	5G001810	5G001880	5G002830	5G002960	5G004770	5G001840	5G004490	5G001810	5G006340	5G04540
<i>CsARF9</i>	5G001316	5G020590	5G025700	5G018730	5G019860	5G025160	5G021940	5G024310	5G030810	5G007140	5G016820	5G012050	5G01720
<i>CsARF10</i>	5G001572	5G028400	5G030430	5G042100	5G022970	5G029740	5G025560	5G028070	-	5G031390	5G021570	5G031750	5G13280
<i>CsARF11</i>	6G001210	6G019230	6G014970	6G019220	6G012070	6G018190	6G013210	6G018090	6G015130	6G012310	6G012190	6G016300	6G11520
<i>CsARF12</i>	6G001772	6G032330	6G022960	6G052700	6G018650	6G030140	6G023090	6G031970	6G029170	6G021300	6G019090	6G022280	6G16330
<i>CsARF13</i>	6G002454	6G043770	6G031310	6G075250	6G031670	6G039530/ UNG103910	6G031700	6G044250	6G041300	6G030860	6G028530	6G028860	6G21410
<i>CsARF14</i>	6G002784	6G051170	6G034580	6G078630	6G035980	6G044780	6G035060	6G051570	6G044680	6G034180	6G031820	6G032210	6G24420
<i>CsARF15</i>	6G003885	6G062170	6G046350	6G100520	6G046900	6G056660	6G047070	6G063450	6G055720	6G044960	6G043820	6G044060	6G35000
<i>CsARF16</i>	6G004054	6G066880	6G047970	6G102100	6G048550	6G058300	6G049760	6G066030	6G057390	6G046620	6G045500	6G045730	6G36590
<i>CsARF17</i>	7G001637	7G031550	7G024190	7G035430	7G037000	7G032190	7G023300	7G031550	7G031420	7G025840	7G021870	7G034900	7G13580

Note: For visibility purposes, gene IDs in the table omitted abbreviations that could represent cucumber varieties.

Table 3. Protein length of ARF in different cucumber accessions.

Protein number	9930	XTMC	Cu2	Cuc80	Cuc37	Gy14	9110gt	Hx14	Hx117	Cuc64	W4	W8	PI183967
CsARF1	839	839	839	839	839	839	813	839	839	839	839	839	839
CsARF2	899	899	899	-	899	899	899	899	899	899	899	899	816
CsARF3	1097	1097	1097	1097	1097	1097	1097	1097	1097	1097	1097	-	1002
CsARF4	1107	1127	1127	1127	1138	1138	1149	1127	1127	1127	1127	1127	1107
CsARF5	550	-	550	550	550	550	550	550	550	550	550	550	550
CsARF6	948	948	948	948	948	922	948	962	948	948	948	601	948
CsARF7	696	696	696	696	696	696	696	696	696	696	696	696	696
CsARF8	691	814	-	814	827	814	814	814	787	799	827	788	691
CsARF9	678	678	743	678	678	678	678	678	743	678	743	678	678
CsARF10	854	883	714	883	883	883	883	883	-	943	883	853	853
CsARF11	716	757	757	757	757	717	757	789	757	753	753	757	716
CsARF12	751	802	802	802	802	802	802	774	802	802	802	802	802
CsARF13	605	605	703	703	703	703/493	703	703	703	699	703	699	699
CsARF14	624	698	412	698	698	694	412	698	698	413	412	412	698
CsARF15	732	731	731	731	731	731	731	731	732	731	731	731	732
CsARF16	907	930	930	928	929	929	930	929	930	928	930	928	914
CsARF17	687	708	708	702	693	693	708	693	693	708	700	708	588

Note: Proteins that differ from 9930 in length are marked in red. - indicates that the *CsARF* gene was absent in this accession.

Subsequent analysis of the pan-genome annotation revealed a non-uniform distribution of *CsARF* genes across the seven cucumber chromosomes. Chromosome 6 contains the most *CsARFs*, whereas chromosomes 1 and 2 harbor two and three genes, respectively. Only one *CsARF* is located on each of chromosomes 3, 4, and 7. Notably, *CsARF5* and *CsARF13b* were found in unknown chromosomal locations in accession Gy14, and *CsARF5* was also unplaced in Cu2. In PI183967, *CsARF8* is positioned at the distal end of chromosome 5, potentially resulting from an evolutionary chromosomal inversion or translocation (Figure 1).

3.2. Characterization of *CsARF* genes and proteins in the pan-genome

To clarify the genetic diversity of *CsARF* genes, we investigated their protein length and amino acid variation among the 13 cucumber accessions (Table 3). The protein length of *CsARF5* and *CsARF7* is consistent among different accessions, except for the lack of *CsARF5* in XTMC. *CsARF1*, 2, and 3 differ in length in one accession. *CsARF9* and 15 have two protein lengths among the 13 accessions; *CsARF12* has three; *CsARF4*, 6, and 13 have four; *CsARF10*, 11, 14, and 16 have five; and *CsARF8* and 17 have six lengths (Table 3). In addition to variations in protein length, all *CsARFs* exhibit amino acid variations ranging from 1 to 18 among the 13 accessions (Table S1). *CsARF3* has the least number (1) of variants in the pan-genome, while *CsARF16* has the largest number (18) of amino acid variants (Table S1).

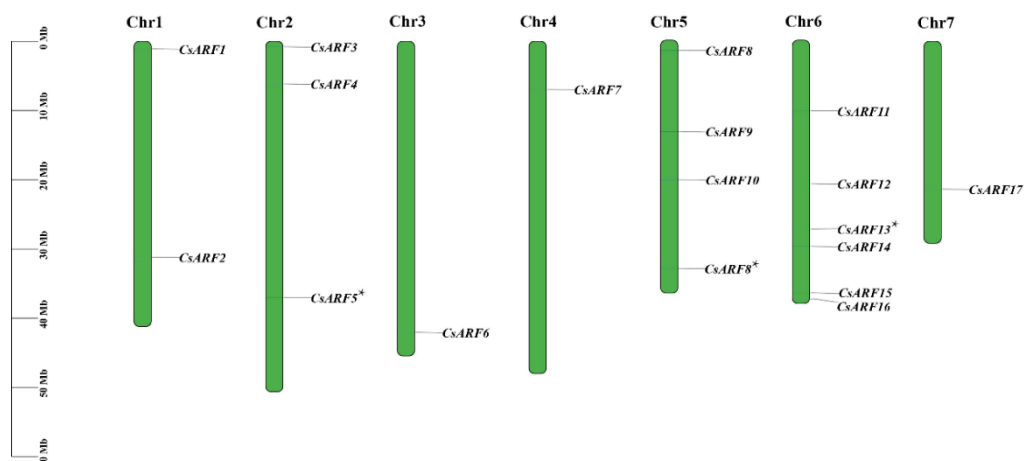


Figure 1. Chromosomal map of *CsARF* genes in the pan-genome. *CsARF8* * means that the *CsARF8* in PI183967 is located at the rear end of chromosome 5. *CsARF5* * and *CsARF13* * mean that their location on chromosomes is unknown yet in Gy14 and Cu2.

To explore the differences in *CsARF* gene structure, we analyzed the gene structures of those CsARFs that exhibited differences in protein length among different accessions, such as *CsARF8*, *CsARF10*, *CsARF11*, *CsARF13*, *CsARF14*, *CsARF16*, and *CsARF17*. *CsARF11* has the same protein length, gene structure, and conserved domains in XTMC, Cu2, Cuc80, 9110gt, Hx117, and W8, and the same protein length and conserved domains but different gene structure in 9930 and PI183967 (Figure 2A). *CsARF13* has the same protein length, gene structure, and conserved domains in 9930 and XTMC, and the same protein length, gene structure, and conserved domains in XTMC, Cu2, Cuc80, Cuc37, Gy14, 9110gt, Hx14, Hx117, and W4. However, in the Gy14, ARF13b has a shorter protein length due to the base mutation, lacking 8 motifs (Figure 2B). *CsARF14* has three kinds of the same protein length and conserved domains: one is in Cu2, W4, 9110gt, W8 and Cuc64, another in Gy14, Cuc37, Hx14, Hx117, Cuc80, and XTMC, and a unique kind in 9930. *CsARF14* has four kinds of gene structures: one has no intron in Cu2, W4, 9110gt, W8, and Cuc64, one other has two introns in Gy14, Cuc37, Hx14, Hx117, Cuc80, and XTMC, the third has three introns in 9930, and the fourth has three introns with 5' and 3' UTRs in PI183967 (Figure 2C). The gene and protein structure and conserved domains for other genes are detailed in Figure S1.

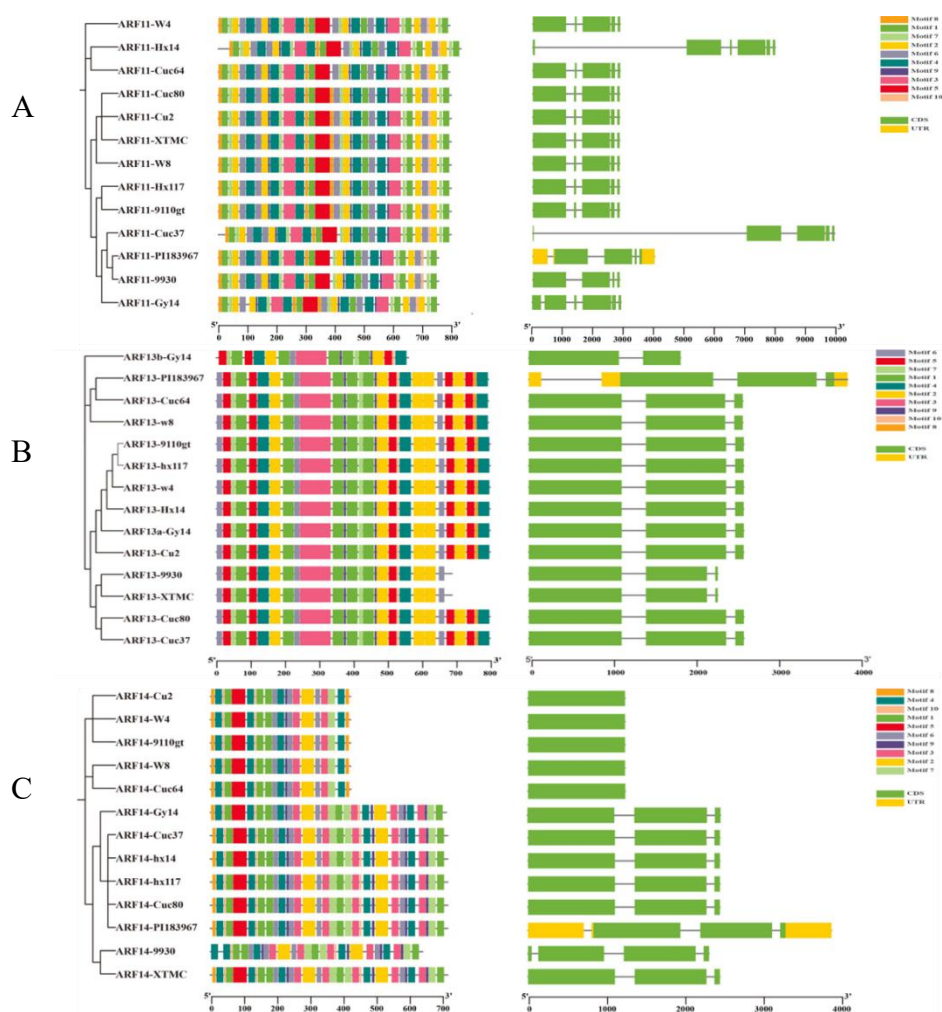


Figure 2. Phylogenetic tree based on protein and gene structure of the *CsARF* genes. (A) *ARF11*. (B) *ARF13*. (C) *ARF14*. Right panel: Gene structure; green box indicates the CDS region, gray line indicates introns, and yellow box indicates UTR region. Middle panel: Protein structure; the colored boxes depict the different patterns. Left panel: Phylogenetic tree. CDS: coding sequence; UTR: non-coding region.

In an effort to identify potential regulatory mechanisms governing *CsARFs*, the 2.0-kb promoter sequences of *CsARF* genes from the pan-genome were analyzed for cis-regulatory elements using the PlantCARE online tool. Each *CsARF* promoter in the pan-genome contains at least one cis-acting element responsive to hormone (jasmonic acid, salicylic acid, abscisic acid, gibberellin, and auxin) and to stress (drought, low temperature, damage, salt stress, and anaerobic induction). Most *CsARF* promoters contain at least one cis-acting element involved in meristem (endosperm, palisade mesophyll cell) and light response. These findings suggest that *CsARF* genes are likely involved in regulating plant growth and development, as well as in abiotic stress responses and hormone signal transduction (Figure 3 and Figure S2).

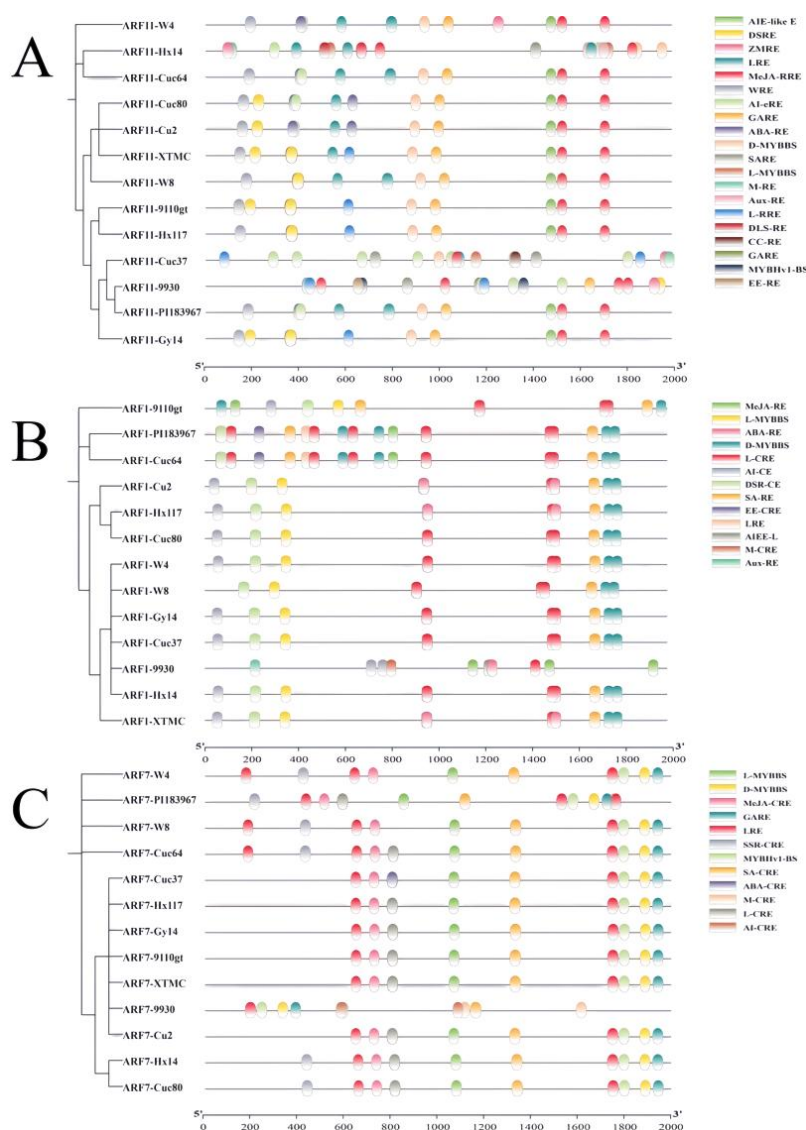


Figure 3. The cis-acting elements of *ARF* genes. (A) *ARF1*. (B) *ARF7*. (C) *ARF11*. MeJA-CRE, cis-acting regulatory element involved in the MeJA-responsiveness; ABA-CRE, cis-acting element involved in the abscisic acid responsiveness; SA-CRE, cis-acting element involved in salicylic acid responsiveness; GARE, gibberellin-responsive element; Aux-RE, auxin-responsive element; L-MYBBS, MYB binding site involved in light responsiveness; LRE, light responsive element; L-CRE, cis-acting regulatory element involved in light responsiveness; D-MYBBS, MYB binding site involved in drought-inducibility; AI-CRE, cis-acting regulatory element essential for the anaerobic induction; DSR-CRE, cis-acting element involved in defense and stress responsiveness; DLS-CRE, cis-acting element involved in dehydration, low temperature, and salt stress; W-RE, wound-responsive element; SSR-CRE, cis-acting regulatory element involved in seed-specific regulation; M-CRE, cis-acting regulatory element related to meristem expression; EE-CRE, cis-regulatory element involved in endosperm expression; MYBHV1-BS, MYBHV1 binding site; ZMRE, cis-acting regulatory element involved in zein metabolism regulation; CC-CRE, cis-acting regulatory element involved in circadian control; AIE-like E, enhancer-like element involved in anoxic specific inducibility.

For instance, in the *CsARF1* promoter, 13 cis-regulatory elements were identified, including hormone response (MeJA-CRE, ABA-CRE, SA-CRE, GARE), environmental stress response (D-MYBBS, DSR-CRE, DLS-CRE, AI-CRE), light signal response (L-MYBBS, LRE, L-CRE), tissue-specific and development-related types (EE-CRE, M-CRE), and special induction types (AIE-like E), with 11 occurring only in specific accessions (Figure 3A). In the *CsARF7* promoter, 12 cis-regulatory elements were identified, including hormone response (MeJA-CRE, ABA-CRE, SA-CRE, GARE), environmental stress response (L-MYBBS, LRE, L-CRE, D-MYBBS, AI-CRE), tissue-specific (SS-CRE, M-CRE), and special binding site types (MYBHv1-BS), with 6 occurring only in specific accessions (Figure 3B). In the *CsARF11* promoter, 20 cis-regulatory elements were identified, including hormone response (MeJA-CRE, ABA-CRE, SA-CRE, Aux-RE, GA-CRE), environmental stress response (D-MYBBS, DSR-CRE, DLS-CRE, AI-CRE, W-RE), light signal response (L-MYBBS, LRE, L-CRE), tissue-specific and development-related types (EE-CRE, M-CRE, SS-CRE), metabolic regulation (ZMRE), biological clock regulation (CC-CRE), special binding site types (MYBHv1-BS), and special induction types (AIE-like E), with 17 occurring only in specific accessions (Figure 3C). The presence of different cis-regulatory elements among *CsARF* promoters in the pan-genome suggests that they may have different biological functions.

Concurrently, notable alterations were also identified at the protein level, namely, for ARF17, the insertions of two fragments between the amino acid residues 182 and 183 and between amino acid residues 212 and 213 (Figure 4). This may cause changes in protein function or affect the interaction of proteins with gene regulatory elements, contributing to the evolution of this gene to some extent.

ARF protein has two conserved domains: the Auxin_resp and B3 domains. The Auxin_resp domain of CsARF is completely conserved in the cucumber pan-genome, but multiple amino acid substitutions were observed in the B3 domain in several CsARFs (Figure 5), which might provide an opportunity for their neofunctionalization.

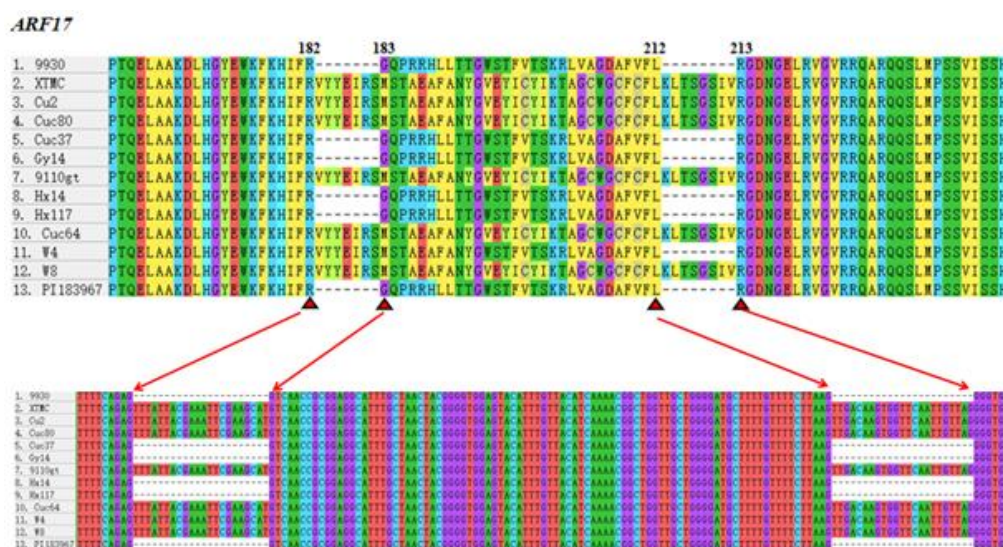


Figure 4. Specific variation of *CsARF17* in the pan-genome. The inserted amino acid fragment—corresponding coding sequence is indicated by the red arrow.

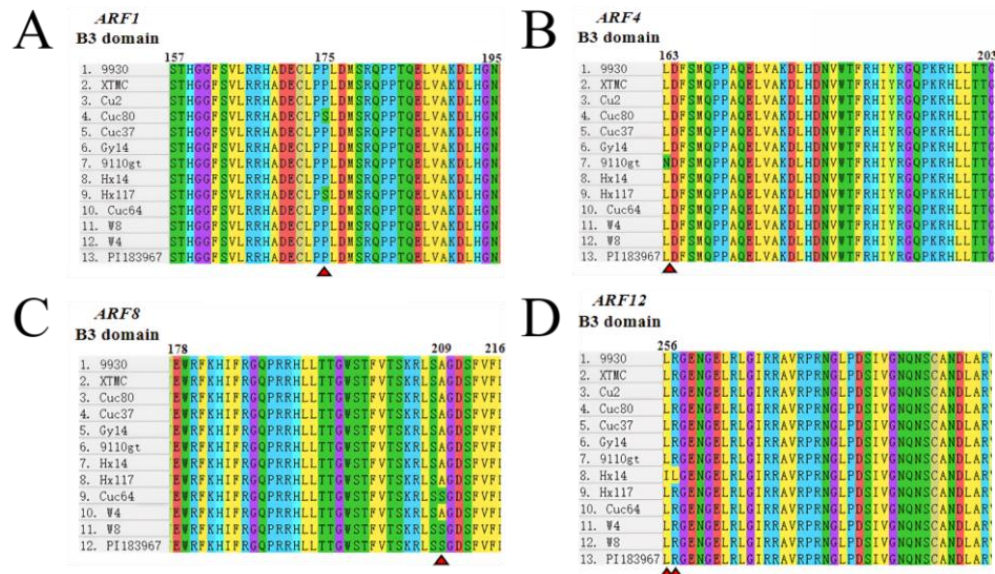


Figure 5. Conserved domains of the *ARF* genes in the pan-genome are compared. (A) *ARF1*. (B) *ARF4*. (C) *ARF8*. (D) *ARF12*. The sites of single amino acid differences are indicated by red triangles.

Outside the two conserved domains, CsARFs exhibit a wide range of amino acid–level variations, including changes in individual amino acids with similar or different properties, and changes in deletion/insertion of protein sequence fragments (Figure S3-6). From a genetic evolution perspective, the variation in amino acids with different natures and the insertion/deletion of protein fragments directly affect the structure and function of the proteins. These variations are the material basis of biological evolution, closely related to the neofunction of genes. Other amino acid variations are listed in Table S1.

3.3. Phylogenetic analysis of total *ARF* proteins in cucumber along with *Arabidopsis* and variation in *Cucurbitaceae* crops

A phylogenetic tree was constructed based on multiple alignments of cucumber and *Arabidopsis* *ARF* domain amino acid sequences (Figure S7). According to the classification of the *ARF* gene family in *Arabidopsis*, cucumber *ARF* proteins were categorized into four large groups (Ia, IIa, IIb, and III). Of the 17 *ARF* proteins, five were assigned to group Ia, six to group IIa, two to group IIb, and four to group III. Group Ib was only composed of the *AtARF* proteins.

To explore the evolutionary process of CsARFs in the pan-genome, we constructed a phylogenetic tree (Figure 6). It is clear that CsARF2, CsARF3, CsARF5, and CsARF12 are more conserved and may play important roles in the growth and development of cucumber, while other *ARFs* are highly variable and active during evolution and may have been affected by environmental selection pressure.

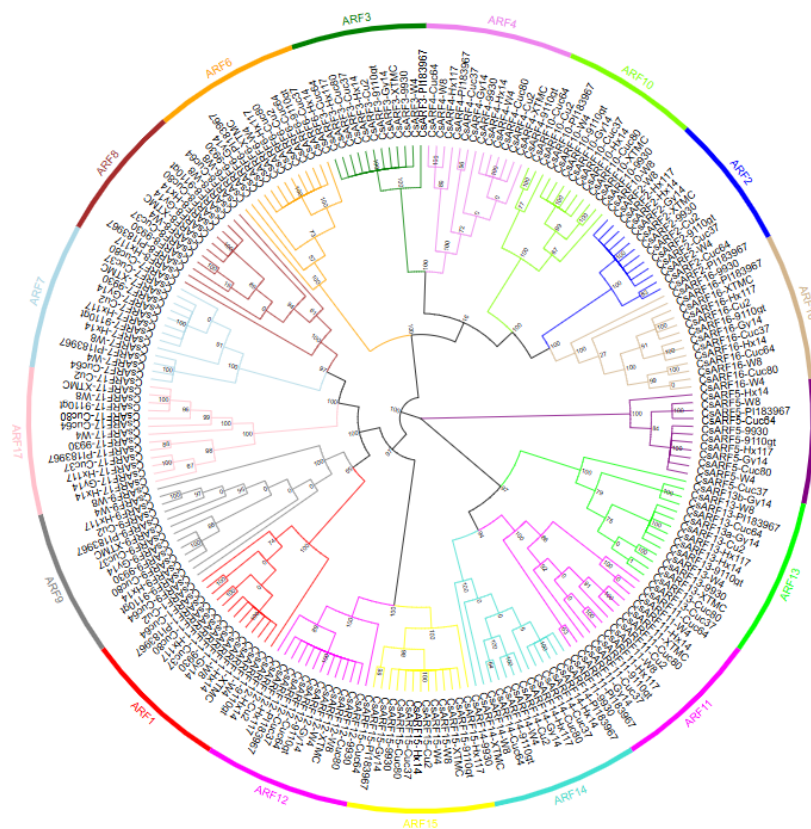


Figure 6. Phylogenetic tree of CsARF proteins in the pan-genome. Arcs of different colors represent different ARFs. CsARF13a-Gy14: 6G039530; CsARF13b-Gy14: UNG103910.

In the phylogenetic tree of *ARF* genes across the pan-genome (Figure 6), the branches of *ARF9*, *11*, and *17* are more distantly related, exhibiting highly differentiated characteristics; in stark contrast, the branches of *ARF2*, *3*, *5*, and *12* are closely clustered with shorter branch lengths, suggesting that these four genes have lower levels of sequence variation and are more closely related. Based on the conservation of ARF2, ARF3, ARF5, and ARF12 in the pan-genome, we explored their conservation in cucumber (9930 v4.0), *Arabidopsis*, watermelon (97103 v2.5), and melon (DHL92 v4) (Figure 7). Notably, ARF2 lacks both the B3 domain and the Auxin_resp domain in the watermelon (Cla97C03G057760.1), and ARF3 has an insertion of 74 amino acids (Figure 7). However, the B3 domain and the Auxin_resp domain of ARF5 and ARF12 are highly conserved in the four species (Figure 7).

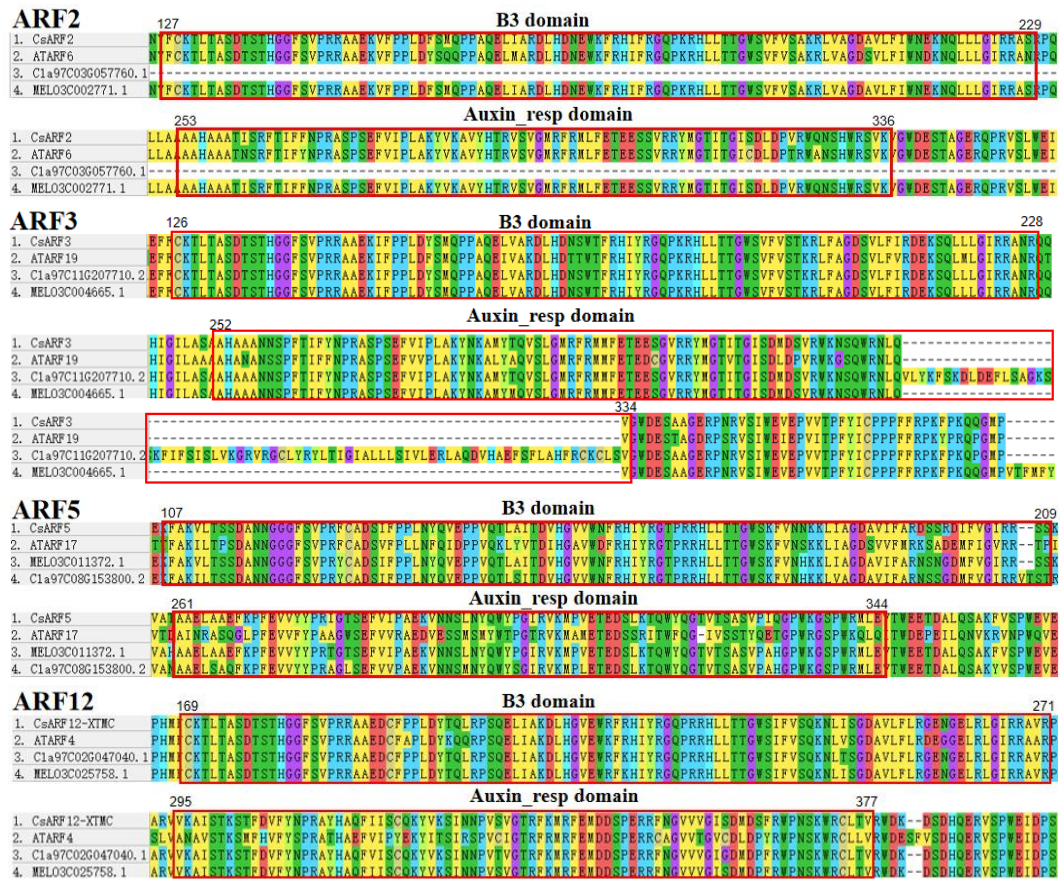


Figure 7. Sequence alignment of the conserved proteins of ARF2, 3, 5, and 12 in cucumber, *Arabidopsis*, watermelon, and melon. ARF in watermelon: Cl_a97C03G057760.1, Cl_a97C11G207710.2, Cl_a97C08G153800.2, Cl_a97C02G047040.1; ARF in melon: MELO3C002771.1, MELO3C004665.1, MELO3C011372.1, MELO3C025758.1. Conserved protein sequences have been marked with red boxes.

3.4. *CsARF* expression profiles in different organs

In this study, the expression patterns of the *CsARF* genes in *Chinese long 9930* in nine different tissues were analyzed with the previously published data [58]. We found that *CsARF4* and *CsARF15* are specifically expressed in roots, implying that they may be key factors regulating root growth and development. *CsARF11* is specifically expressed in male flowers, suggesting that it may regulate male flower development and morphology. *CsARF9* is specifically expressed in expanded unfertilized ovaries. These results demonstrate that certain *ARF* genes may play critical roles in plant growth and morphological development (Figure 8).

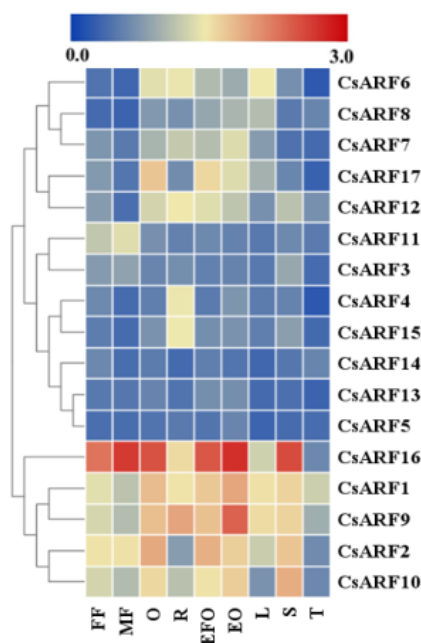


Figure 8. Heat map of tissue-specific expression of *CsARF* genes. From blue to red, the expression level increases. S, stem; FF, female flowers; MF, male flowers; L, leaves; T, tendril; R, root; EO, expanded unfertilized ovary; O, ovary; EFO, expanded fertilized ovary.

3.5. Expression patterns of *CsARFs* in response to downy mildew and gray mold

To assess the responses of *CsARFs* to downy mildew and gray mold, we analyzed the specific expression of the *CsARF* genes in *Chinese long 9930* under these stresses [57, 58]. *CsARF3*, *CsARF7*, *CsARF8*, and *CsARF9* are highly expressed 2 days post-infection (dpi) of downy mildew, while *CsARF10*, *CsARF14*, and *CsARF16* are upregulated at 6 dpi (Figure 9). These temporal expression patterns suggest that *CsARF* genes are likely regulated in a stage-specific manner, suggesting their potential contribution to phased resistance responses in cucumber.

Analysis of public transcriptome data [58] from gray mold-inoculated cucumber leaves revealed that 11 out of 17 *CsARF* genes had differential expression ($|\text{Log}_2\text{FC}| \geq 1$) compared to the control group at 96 h post-inoculation. Notably, *CsARF5* exhibited upregulation following inoculation, indicating its positive induction by gray mold stress. In contrast, the majority of other *CsARF* genes displayed downregulation patterns (Figure 10). These findings suggest that *CsARF* genes may play significant roles in cucumber's response to gray mold infection. To explore whether the expression of *CsARF* genes changes in response to gray mold infection, we selected four genes (*ARF7*, *ARF8*, *ARF10*, and *ARF12*) to test their possible function in the resistance of cucumber to gray mold by transient overexpression in cotyledons.

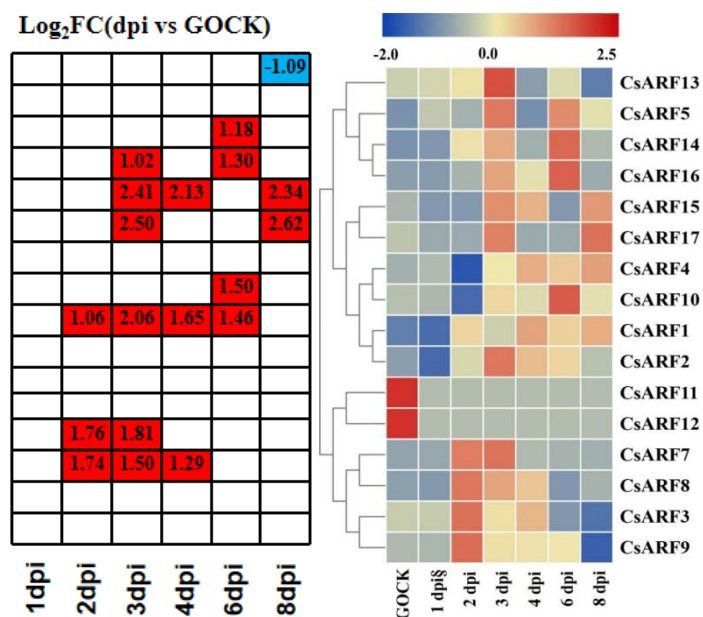


Figure 9. Heat map of *CsARF* gene expression under downy mildew stress. The transcription of *CsARF* genes was determined for 1–8 days, with no inoculation as the control (GOCK). Numbers in the table and fold-change in red indicate upregulated genes, and those in blue indicate downregulated genes. FC: fold-change; dpi: days post inoculation.

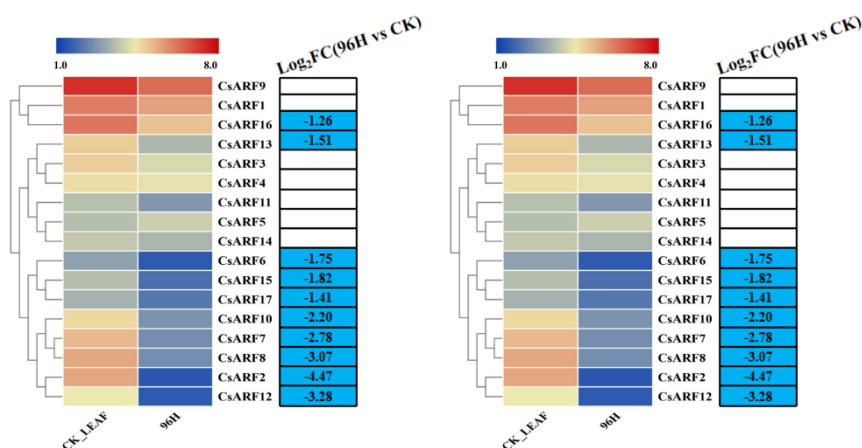


Figure 10. Heat map of *CsARF* gene expression under gray mold stress. The transcription of *CsARF* genes was determined at 96 hpi, with no inoculation as the control (CK). Numbers in the table and fold-change in red indicate upregulated genes, and those in blue indicate downregulated genes. FC: fold-change; hpi: hours post-inoculation.

We investigated the potential roles of these four genes in cucumber's response to gray mold stress. Using a transient expression system, we transformed these genes (driven by the cauliflower mosaic virus 35S promoter) and the empty vector as the control into cucumber cotyledons. Subsequently, these transiently transfected cucumber seedlings were grown under standard conditions for approximately 18 h, after which they were inoculated with gray mold to observe their responses. The results show

that among the four tested genes, the cotyledons with overexpressing *CsARF12* exhibited the mildest disease symptoms, with the smallest necrotic spots. These findings strongly suggest that *CsARF12* may play a beneficial role in cucumber's defense mechanisms against gray mold, particularly as a key factor in promoting cucumber resistance to gray mold (Figure 11). Therefore, further studies of this gene can help us better understand the interaction mechanism between cucumber and gray mold and provide important clues for the development of new disease resistance strategies.

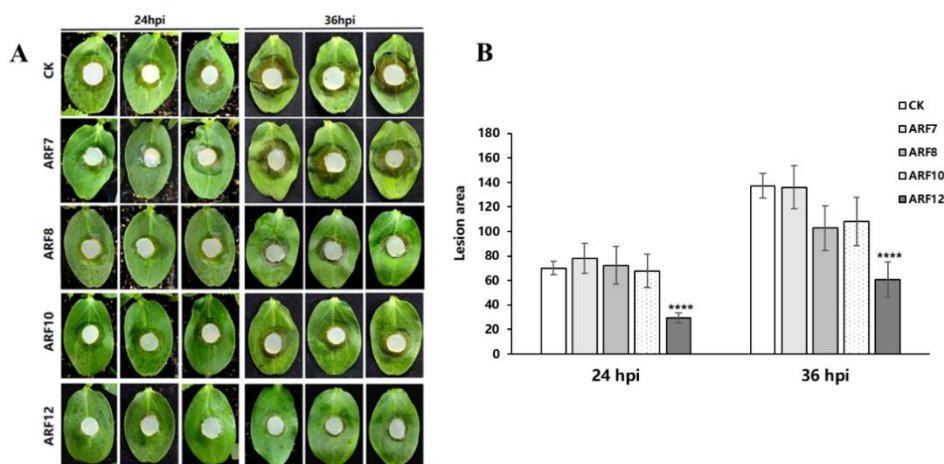


Figure 11. The overexpression of *CsARF12* increases the resistance of cucumber cotyledons to gray mold. (A) Symptoms on cucumber cotyledons carrying empty vector or *CsARF* genes inoculated with gray mold. (B) Quantitative statistics of lesion areas for (A). Error bars indicate standard deviation; hpi, hours post-inoculation; **** $P < 0.00001$.

4. Discussion and conclusions

The *ARF* gene family is crucial for plant growth and development, as the ARF protein plays an indispensable role in the auxin signal transduction pathway.

In 2013, the cucumber *ARF* gene family was initially identified in the first sequenced genome (v1.0) of *Chinese long 9930* [60], which has since been excluded from the Cucurbitaceae genome database due to its lower quality. A re-identification was performed in 2023 based on the updated v3.0 genome [53]. The cucumber genome of *Chinese long 9930* has now been updated to v4.0 [54], and our analysis revealed persistent errors in the v3.0 version. For instance, *CsARF5* (CsaV3_1G023020) was mistakenly mapped on chromosome 1 in the genome v3.0, whereas its correct position in v4.0 is on chromosome 2 (CsaV4_2G002372) (Table 1). Additionally, both the protein and gene structure of *CsARF3* differ between the v3.0 (CsaV3_2G001050) and v4.0 (CsaV4_2G000004) (Figure S8). Therefore, it is necessary to identify the *CsARF* gene family using the improved v4.0 genome.

Botrytis cinerea (causing gray mold) is a fungal plant pathogen of major economic importance, causing substantial crop losses both before and after harvest. The complex interaction between *B. cinerea* and its hosts has made it a key focus of research. Resistance mechanisms have been well characterized in some species: in *Arabidopsis thaliana*, through the cytokinin oxidase gene (*CKX5*), the WRKY33-dependent pathway, and the jasmonic acid catabolism mediated by JAO/JOX [61–63]; and in tomato, via MADS-box proteins and the interaction between B-cell lymphoma 2 (Bcl2) and

SIBAP1 [64,65]. In cucumbers, gray mold is a widespread and persistent disease. While the CsWRKY10-mediated defense response in gray mold has been previously reported [66], the role of the *ARF* gene family in gray mold resistance remains unknown.

Analysis of transcriptome data under gray mold stress revealed altered expression of several *ARF* genes (Figure 10). Functional validation showed that *ARF12* significantly enhances resistance in cucumber cotyledons (Figure 11). To our knowledge, this is the first report demonstrating the involvement of *ARF* genes in gray mold resistance, expanding our understanding of plant–pathogen interaction mechanisms and providing a new perspective for the mining of gray mold resistance gene resources. Further validation across different crops and pathogen populations will be essential to leverage these findings for developing resistant varieties and improving crop yield and quality.

In this study, we identified and analyzed the *ARF* family in cucumber, detecting 18 *ARF* genes in the cucumber pan-genome. We analyzed their chromosomal locations, conserved motifs, gene structures, and evolutionary relationships. Furthermore, several *CsARF* genes were found to respond to downy mildew and gray mold stresses, with functional assays confirming that *CsARF12* positively regulates gray mold resistance in cucumber cotyledons. In conclusion, our study provides a basis for future studies of *CsARF* genes as well as new gene sources of resistance for breeding programs.

Author contributions

Conceptualization, Z.R., L.W. and C.C.; methodology, Q.Z. and K.W.; validation, Q.Z.; software, Q.Z., K.W., B.Y. and X.G.; formal analysis, Q.Z. and K.W. and L.Z.; investigation, Q.Z. and K.W.; data curation, Q.Z. and K.W.; writing—original draft preparation, Q.Z.; writing—review and editing, Z.R.; supervision, L.W. and Z.R.; project administration, Z.R., L.W. and C.C.; funding acquisition, Z.R. and L.W. All authors have read and agreed to the published version of the manuscript.

Use of Generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no competing financial interest.

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