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Structural and functional analyses of genes encoding VQ proteins in apple

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ABSTRACT

Recent studies with *Arabidopsis* and soybean have shown that a class of valine-glutamine (VQ) motif-containing proteins interacts with some WRKY transcription factors. However, little is known about the evolution, structures, and functions of those proteins in apple. Here, we examined their features and identified 49 apple VQ genes. Our evolutional analysis revealed that the proteins could be clustered into nine groups together with their homologues in 33 species. Historically, the main characteristics of proteins in Groups I, V, VI, VII, IX, and X were thought to have been generated before the monocot-dicot split, whereas those in Groups II, III + IV, and VIII were generated after that split. In the structural analysis, apple MdVQ proteins appeared to bind only with Group I and IIc MdWRKY proteins. Meanwhile, MdVQ1, MdVQ10, MdVQ15, and MdVQ36 interacted with multiple MdVQ proteins to form heterodimers but MdVQ15 formed a homodimer. The functional analysis indicated that overexpression of some apple *MdVQs* in *Arabidopsis* and tobacco plants effected their vegetative and reproductive growth. These results provide important information about the characteristics of apple MdVQ genes and can serve as a solid foundation for further studies about the role of WRKY-VQ interactions in regulating apple developmental and defense mechanisms.

1. Introduction

The WRKY transcription factors (TFs) constitute one of the largest families of transcriptional regulators in numerous land plants, where they function in a broad range of biological processes and in response to biotic and/or abiotic stresses [1–5]. The most striking feature of WRKY proteins is the highly conserved WRKY domain, which consists of approximately 60 amino acids at the N-terminus, followed by a $Cx_7Cx_{23}HxC$ or $Cx_{4\text{-}5}Cx_{22\text{-}23}HxH$ zinc-finger motif [1,3]. Based on the number of WRKY domains and the structure of their zinc-finger motifs, members of this gene family are divided into three groups. Group I proteins contain two WRKY domains (N-terminus $Cx_4Cx_{22}HxH$ and C-terminus $Cx_4Cx_{23}HxH$), Group II proteins have one WRKY domain ($Cx_{4-5}Cx_{23}HxH$), and Group III proteins contain one WRKY domain ($Cx_7Cx_{23}HxC$). Based on the primary amino acid sequence of the zinc-finger motif, Group II WRKY proteins are further divided into IIa through IIe subgroups [1,6].

Several WRKY proteins can physically interact with a class of valine-glutamine (VQ) motif-containing proteins to regulate various physiological processes [7–14]. These VQ motif-containing proteins, *i.e.*, VQ proteins, constitute a novel family of plant-specific transcriptional regulators, with members widely distributed among various species [11]. They are characterized by the presence of a conserved and single, short amino acid region, the FxxhVQxhTG motif, in all 34 *Arabidopsis* members of this family [8,10,15].

The VQ proteins perform diverse functions in plant growth and development. For example, Wang et al. [16] have shown that IKU1 (VQ14) interacts with MINI3 (WRKY10) to reduce the expression of *IKU2*, a leucine-rich repeat kinase gene, thereby affecting seed size. Cheng et al. [8] have indicated that the *vq8-1* mutant displays pale-green and stunted-growth phenotypes, while VQ10 links with WRKY25 and WRKY33 to reduce the mature plant size. Lei et al. [14] have revealed that VQ20 protein interacts with WRKY2 and WRKY34 TFs to regulate pollen development and function. Moreover, VQ proteins modulate diverse functions in biotic and abiotic stress responses. For example, Perruc et al. [17] have shown that *AtCaMBP25* (*VQ15*) negatively regulates tolerance to osmotic stress while Lai et al. [7] have demonstrated that resistance to the necrotrophic pathogen *Botrytis cinerea* is compromised in the *sib1*(*vq23*) and *sib2* (*vq16*) mutants but enhanced in *SIB1*-overexpressing transgenic plants. Hu et al. [9] have

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reported that VQ9 interacts with WRKY8 to repress the regulatory function of WRKY8 during the development of salt stress tolerance. Results obtained by Pecher et al. [10] have indicated that MVQ1 (VQ4) interacts with WRKYs to inhibit WRKY transcriptional activity. The MPK3/6 phosphorylates MVQ1 (VQ4) after elicitation of a pathogen-associated molecular pattern, inducing its degradation and allowing WRKY to facilitate transcription for defense-related genes. Wang et al. [18] have revealed that VQ12 and VQ29 proteins negatively mediate plant basal resistance against *B. cinerea*, while Jiang and Yu [12] have found that VQ23 (SIB1) and VQ16 (SIB2) interact with WRKY57 and WRKY33, leading to competitive regulation of JAZ1 (JASMONATE ZIM-DOMAIN1) and JAZ5 during *B. cinerea* infection.

Although significant advances have been made in VQ protein research, our understanding of the mechanisms and functions of action by these important transcriptional regulators is still very limited. Increasing numbers of family members have been identified and characterized based on the highly conserved VQ motif in *Arabidopsis* [8], rice (*Oryza sativa*) [19,20], moss (*Physcomitrella patens*) [11], grape (*Vitis vinifera*) [21], *Brassica rapa* ssp. *Pekinensis* [22], soybean (*Glycine max*) [13,23], maize (*Zea mays*) [24], poplar (*Populus trichocarpa*) [25] and Moso bamboo (*Phyllostachys edulis*) [26]. Although the VQ gene family is understood in considerable detail because it has been subjected to extensive genomic analysis; members of this family in apple (*Malus* × *domestica* Borkh.) have not been studied as thoroughly as those in other species. Therefore, we identified apple VQ genes and investigated their VQ domain, phylogenetic relationships, chromosomal locations, and structure. We also used yeast two-hybrid assays and bimolecular fluorescence complementation analysis to examine the interactions among and between MdVQ proteins and MdWRKY proteins. Here, overexpression of several apple *MdVQs* in *Arabidopsis* led to stunted or enhanced growth, serrated leaf margins, earlier flowering, and production of smaller siliques. Lines of tobacco (*Nicotiana benthamiana*) that over-expressed those genes showed stunted growth, shorter corollas, and smaller capsules. These results provide important information about the evolution, structure, and function of apple MdVQ genes and lay a solid foundation for further studies about the role of WRKY-VQ interactions in regulating developmental and defense mechanisms in that fruit crop.

2. Materials and methods

2.1. Plant materials and growth conditions

Plants of *Arabidopsis thaliana* 'Columbia' ('Col') wild type (WT) and transgenic lines were grown in chambers set at 24 °C, under a 10-h photoperiod. We also cultivated WT tobacco (*Nicotiana benthamiana*) and transgenic lines in a growth room at 25 °C, under a 14-h photoperiod.

2.2. Identification of apple VQ genes

We downloaded the database of the *Arabidopsis* VQ gene family from the TAIR website (http://www.arabidopsis.org/) [8]. As BlastP inquiry sequences (https://www.rosaceae.org/blast/protein/protein) for searching against predicted apple proteins, we chose 34 *Arabidopsis* VQ proteins. All of the apple VQ-motif sequences were identified by using the consensus protein sequences of the VQ motif Hidden Markov Model (HMM) profile (VQ, PF05678; http://pfam.xfam.org/family/PF05678) from the Pfam database (http://pfam.sanger.ac.uk/search). We then searched VQ sequences against the original apple v1.0 genome database (https://www.rosaceae.org/gb/gbrowse/malus_x_domestica/). The reliability of the candidate protein sequences was confirmed by ensuring that the VQ-motif was present in each candidate MdVQ protein. For this, we used the Pfam database and NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [27,28].

2.3. Sequence alignments, sequence logo, and phylogenetic analysis

The sequence logo of the VQ conserved domains for 49 MdVQ genes in apple was generated with the application WebLogo (http://weblogo.threeplusone.com) [29]. We performed multiple sequence alignments for the VQ conserved domains and protein sequences from those MdVQ genes by using DNAMAN 6.0.3.99 with its default parameters. A phylogenetic tree for the VQ gene family was constructed with MEGA 6.0 software (www.megasoftware.net) and the Neighbor-Joining (NJ) method, together with the VQ conserved domain protein sequences of 875 VQs from 33 plant species (Supplementary File 1). Related sequences were downloaded from the resource Plaza 3.0 (http://bioinformatics.psb.ugent.be/plaza/). We used the following parameters in the NJ method: bootstrap (1000 replicates), complete deletion, and amino:p-distance.

2.4. Intron-exon structures, genome distribution, and gene-duplication analysis

Gene distributions on chromosomes, and genome locations of the apple VQ genes were downloaded from the apple v1.0 genome database. Intron-exon distribution data for the 49 MdVQ genes were downloaded from Plaza 3.0, and were then mapped onto chromosomes by using MapInspect (www.plantbreeding.wur.nl/UK/software_mapinspect.html), a locational software for identifying chromosomal positions. Segmental- and tandem-duplication events were investigated according to the method of Tian et al. [30].

2.5. Yeast two-hybrid assays

Yeast (*Saccharomyces cerevisiae*) two-hybrid (Y2H) assays were performed according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA, USA). Because the full-length MdWRKY4/19/28/39/52/68/89/91/115 and MdVQ6/7/10/12/15/16/21/33/35/42/47 proteins exhibited strong self-activation, we used truncated fragments to test those interactions. As the N-terminus of most WRKY proteins with autoactivation, so the N-terminus deletion versions of MdWRKY4/19/28/39/52/68/89/91/115 (Supplementary Fig. S1) and MdVQ6/7/10/12/15/16/21/33/35/42/47 (Supplementary Fig. S2) were cloned into the Y2H bait vector pGBT9 (GAL4 DNA-binding domain). Because the N-terminus deletion versions of MdVQ6/7/12/16/21/33 still exhibited strong self-activation, we did not perform Y2H assays for them. Fusion constructs pGAD424-MdVQ, pGBT9-MdVQ, and pGBT9-MdWRKY were generated from the PCR-amplified coding sequences for *MdVQs* and *MdWRKYs* with gene-specific primers (Supplementary Table S1) and tested for *LacZ* reporter gene expression by assaying for β-galactosidase activity. Different combinations of these plasmids were co-transformed into the yeast strain 'Y2H Gold' by the lithium acetate method [31]. The cells were plated on a medium lacking Trp and Leu (SD/-Trp-Leu) and cultured at 30 °C. To screen for interactions, we transferred those colonies to a medium that was supplemented with X-α-gal but lacked Trp, Leu, His, and adenine (SD/-Trp-Leu-His-Ade), and used pGAD424-MdBHLH3/pGBT9-MdMYB9$^{\triangle C}$ as the positive control [32].

2.6. Bimolecular fluorescence complementation assays

Bimolecular fluorescence complementation (BiFC) assays were performed *in vivo* as described previously [33], with some modifications. Full-length cDNAs of MdVQ10 and MdVQ33 were recombined into pSPYNE-35S to form an N-terminus in-frame fusion with yellow fluorescence protein (YFP). Meanwhile, full-length cDNAs of MdVQ15 and MdWRKY52 were introduced into pSPYCE-35S to generate a C-terminus in-frame fusion with YFP. The primers are listed in Supplementary Table S1. Different combinations of these constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and then

Table 1
MdVQ genes in apple genome.

Gene name	Gene ID[a]	Chromosome location	Genomic length (bp)	Size (aa)	MW (Da)	PI
MdVQ1	MDP0000154438	chr5:24411684..24412664	981	326	34987.43	10.352
MdVQ2	MDP0000772371	chr11:27202239..27203222	984	327	35074.52	10.35
MdVQ3	MDP0000210996	chr14:5773661..5774894	1234	243	26152.45	7.639
MdVQ4	MDP0000148283	chr14:5773688..5774921	1234	243	26121.43	7.639
MdVQ5	MDP0000136351	chr10:18468977..18469636	660	219	24457.39	8.391
MdVQ6	MDP0000127852	chr1:19720882..19721559	678	225	24798.9	8.379
MdVQ7	MDP0000274905	chr5:9268043..9272371	4329	309	34765.83	9.553
MdVQ8	MDP0000624279	chr5:9271803..9272351	549	182	10739.76	9.964
MdVQ9	MDP0000566117	chr10:23907403..23918105	10703	444	49721.66	6.964
MdVQ10	MDP0000193206	chr11:29249228..29249974	747	248	26914.3	9.681
MdVQ11	MDP0000159948	chr14:27749489..27750277	789	262	28974.23	9.49
MdVQ12	MDP0000868063	chr14:14313663..14314598	936	311	34083.05	1.845
MdVQ13	MDP0000247951	unanchored:9276456..9281957	5502	1076	121961.89	9.258
MdVQ14	MDP0000295494	chr13:22565117..22570026	4910	469	50399.37	7.053
MdVQ15	MDP0000182830	chr1:4843548..4844804	1257	418	45261.36	6.943
MdVQ16	MDP0000478954	chr3:14934053..14934760	707	235	24833.98	7.685
MdVQ17	MDP0000856686	chr17:19445933..19446418	486	161	17897.29	9.695
MdVQ18	MDP0000340025	chr3:33375226..33375649	424	141	15932.1	6.69
MdVQ19	MDP0000881505	chr10:28361613..28362365	753	250	26902.8	9.597
MdVQ20	MDP0000805273	chr5:4465143..4465895	752	250	26794.67	9.564
MdVQ21	MDP0000893506	chr16:1106754..1107524	771	256	28571.59	7.259
MdVQ22	MDP0000176426	chr13:2350257..2351416	1160	291	32608.66	6.102
MdVQ23	MDP0000130738	chr13:2397540..2398325	786	261	29216.12	6.544
MdVQ24	MDP0000713750	chr14:29035374..29035940	567	188	20730.49	9.268
MdVQ25	MDP0000312336	chr14:6944125..6944649	525	174	19476.26	8.276
MdVQ26	MDP0000278259	chr12:27446491..27454777	8287	700	76548.29	6.301
MdVQ27	MDP0000152343	chr12:4314720..4315124	405	134	14995.24	9.552
MdVQ28	MDP0000833431	chr12:4290584..4290991	408	135	15150.31	9.783
MdVQ29	MDP0000179796	chr12:4318935..4319342	408	135	15150.31	9.783
MdVQ30	MDP0000182585	chr10:20105643..20105960	318	105	11823.34	9.187
MdVQ31	MDP0000346969	chr8:4614432..4614975	544	181	19723.05	8.193
MdVQ32	MDP0000264361	chr13:13681710..13682345	636	211	23505.9	9.784
MdVQ33	MDP0000885511	chr7:3452045..3452434	390	129	14696.42	6.341
MdVQ34	MDP0000514448	chr5:19258004..19258603	600	199	21202.02	8.782
MdVQ35	MDP0000183958	chr4:18845409..18846187	779	247	25863.98	6.594
MdVQ36	MDP0000227657	chr2:32172214..32172885	672	134	15190.12	7.332
MdVQ37	MDP0000248043	chr1:26787765..26788310	546	181	19682.08	8.738
MdVQ38	MDP0000148903	chr3:27382875..27383669	795	264	28839.93	9.502
MdVQ39	MDP0000216667	chr3:33776218..33777567	1350	449	47103.68	7.709
MdVQ40	MDP0000925584	chr9:11499527..11500117	591	196	22252.68	4.38
MdVQ41	MDP0000179253	chr9:15128158..15128661	504	167	18411.51	9.934
MdVQ42	MDP0000122563	chr11:28611623..28612399	777	258	28148.3	9.501
MdVQ43	MDP0000467241	chr15:29610577..29611920	1344	447	47083.52	7.187
MdVQ44	MDP0000564427	chr3:27385387..27386181	795	204	28839.93	9.502
MdVQ45	MDP0000261758	chr6:23012095..23015035	2941	371	40671.15	9.564
MdVQ46	MDP0000172223	chr17:11751984..11752562	579	192	21878.21	4.162
MdVQ47	MDP0000348190	unanchored:15635404..15636019	616	205	22582.08	8.123
MdVQ48	MDP0000284090	unanchored:33643566..33657170	13605	2064	233362.06	8.123
MdVQ49	MDP0000219970	chr9:11498559..11500162	1604	274	30979.08	6.175

[a] Gene ID in apple genome (https://www.rosaceae.org/gb/gbrowse/malus_x_domestica/).

infiltrated into tobacco leaves. Those infected tissues were analyzed 48 h after infiltration, under a confocal laser-scanning microscope (LSM510 META; Zeiss, Oberkochen, Germany). We used 4′,6-diamidino-2-phenylindole (DAPI) to mark the cell nucleus [33].

2.7. Pull-down assays

Full-length cDNAs of *MdVQ10* and *MdWRKY52* were recombined into the PGEX-4T-1 and PET-32a vector, respectively, before the recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) to express GST-MdVQ10 or HIS-MdWRKY52 protein. The primers are listed in Supplementary Table S1. We performed *in vitro* pull-down assays according to the instructions for the Pierce GST Spin Purification Kit (Thermo-Fisher Scientific, Waltham, MA, USA). After HIS-MdWRKY52 was incubated with GST-MdVQ10 or GST, the samples were detected by immuno-blotting with anti-GST and anti-HIS antibodies, respectively.

2.8. Vector construction and plant transformation

To construct the *MdVQ* overexpression (OE) vectors, we used RT-PCR to isolate the full-length cDNAs of *MdVQ6*, *MdVQ15*, *MdVQ25*, *MdVQ27*, and *MdVQ37* from 'Royal Gala' apple. All of the cDNAs were cloned into pRI 101-AN plant transformation vectors that were driven by the cauliflower mosaic virus (CaMV) 35S promoter. The primers are listed in Supplementary Table S1.

For *Arabidopsis* transformation, the five recombinant plasmids described above were introduced into the 'Col' ecotype *via* the *Agrobacterium tumefaciens* GV3101-mediated floral dip method. Seeds of the transgenic plants were individually harvested and screened with kanamycin monosulfate. Homozygous transgenic lines were used for further investigations.

For tobacco transformation, the recombinant plasmids for *MdVQ15*, *MdVQ25*, *MdVQ27*, and *MdVQ37* were introduced into leaf discs as mediated by *A. tumefaciens* strain LBA4404. Seeds of the transgenic plants were individually harvested and screened with kanamycin monosulfate. Homozygous transgenic lines were used in our

Fig. 1. Analysis of 49 apple VQ proteins.
(A) Multiple alignments of VQ domain sequences. Highly conserved residues in FxxxVQxhTG motif are shown in different colors. (B) Sequence logo of VQ domain in 49 apple VQ genes, generated by application WebLogo. Within each stack, heights of symbols indicate relative frequency of each amino acid at that position.

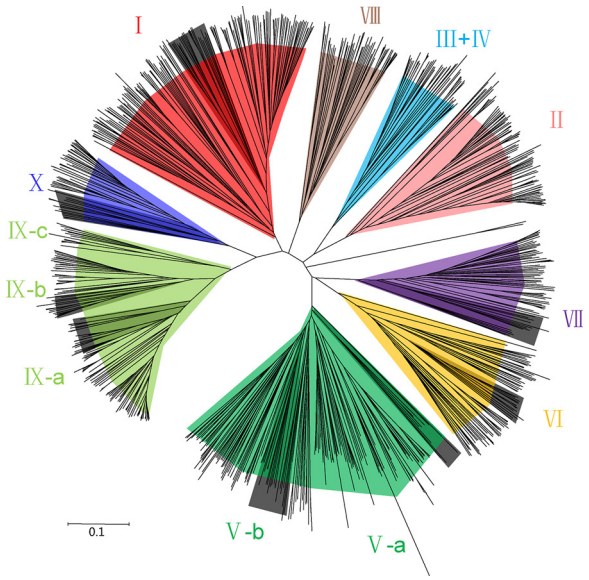

Fig. 2. Phylogenetic tree of 875 VQ genes from 33 plant species, clustered into 9 subgroups (I, II, III + IV, V, VI, VII, VIII, IX, and IX). Different color domains represent individual groups. Dark-gray domains indicate monocotyledon VQ proteins; other domains, dicotyledon VQ proteins.

examination.

2.9. RNA extraction and expression analysis

Total RNA was isolated from *Arabidopsis* and tobacco leaves with Trizol reagent (Thermo-Fisher Scientific) before cDNA synthesis was performed with a PrimeScript First-Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). We conducted the qRT-PCR assays in 20-μL reaction mixtures that contained 10 μL of SYBR® Premix Ex Taq™ (TaKaRa), and used a QuantStudio 5 instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration of cDNA was diluted to 1–10 ng μL^{-1}, and 1-μL aliquots were used for qRT-PCR. Relative changes in gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method [34], and the quantification results were normalized by *AtActin2* for transgenic *Arabidopsis* and *NtActin* for transgenic tobacco. At least three replicates per sample were used, and the primers are listed in Supplementary Table S1.

2.10. Statistical analysis

All data were examined with the IBM SPSS Statistics v. 20 (https://www.ibm.com/support/knowledgecenter/SSLVMB_20.0.0/com.ibm.spss.statistics_20.kc.doc/pv_welcome.html). One-way ANOVA and Duncan's tests were used to compare the results. Differences between treatments were considered statistically significant at $p < 0.05$.

3. Results

3.1. Identification and structural analysis of apple VQ gene family

We used 34 *Arabidopsis* VQ proteins and consensus protein sequences to reveal 49 VQ family genes in the apple genome that putatively encode VQ proteins. To verify these identities, we searched for the presence of the VQ motif in their amino acid sequences (Table 1 and Fig. 1A). All 49 proteins contained the conserved F(R/K/M) xΦVQxΦT(G/C) motif (where x represents any residue and Φ is a hydrophobic residue; Fig. 1B). Among the 49, 14 had the FMxΦVQxLTG motif, 13 had the FRxΦVQxLTG motif, 10 had the FKxΦVQxLTG motif, eight had FRxΦVQxFTG motif, and two each contained the FRxΦVQxVTG motif or the FRxΦVQxLTC motif (Fig. 1). All of these

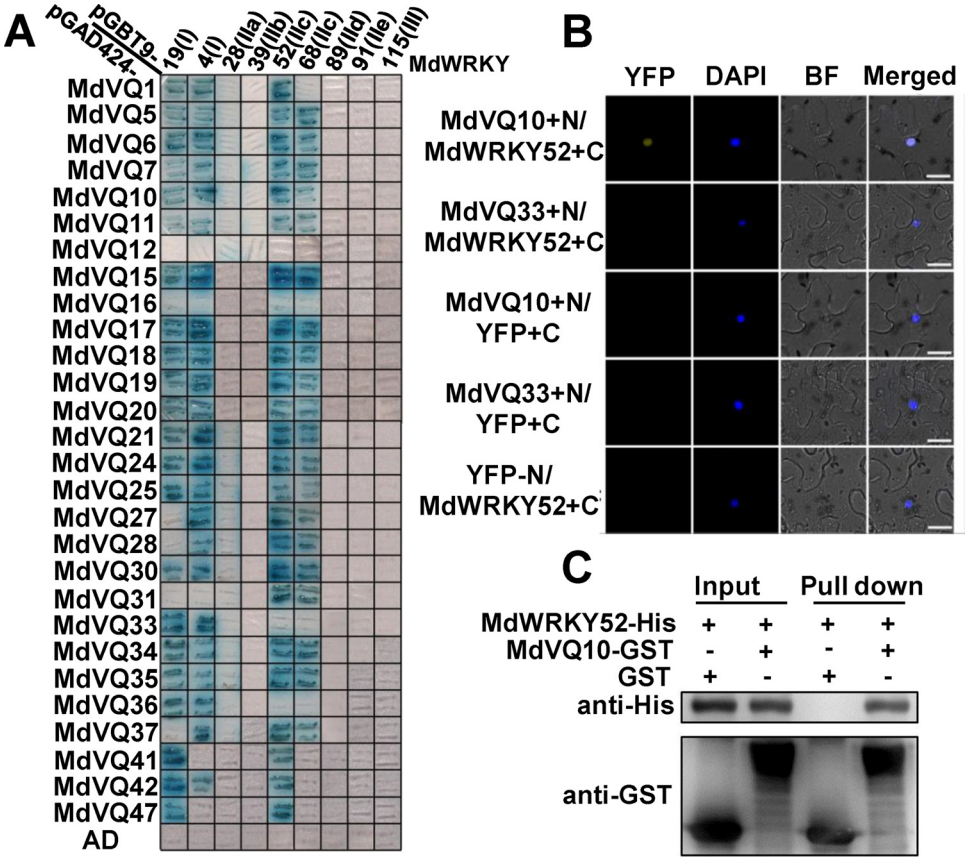

Fig. 3. Physical interactions between MdVQ proteins and MdWRKY proteins.
(A) Yeast two-hybrid assay, based on pGAD424-MdVQ fusion prey vectors co-transformed with pGBT9-MdWRKY domain fusion bait vectors into yeast cells. Positive interactions were indicated by ability of cells to grow on synthetic dropout medium with additive x-α-gal but lacking Leu, Trp, His, and Ade. Empty AD prey vector plus pGBT-MdWRKY fusion bait vectors were used as negative controls. For details regarding negative/positive controls and different subgroups of MdWRKY proteins, refer to Fig S3. (B) Assay of bimolecular fluorescence complementation (BiFC), showing fluorescence in nuclear compartments of tobacco leaf epidermal cells that resulted from complementation of N-terminus part of YFP fused to MdVQ10 or MdVQ33 (MdVQ10 + N and MdVQ33 + N) with C-terminus part of YFP fused to MdWRKY52 (MdWRKY52 + C). No signal was observed from negative controls. Scale bar: 20 μm. (C) Pull-down assay. MdVQ10-GST or purified GST was incubated with MdWRKY52-His protein and purified using GST purification kit. Resultant protein samples were immunoblotted with anti-HIS or anti-GST antibodies. Negative control was GST.

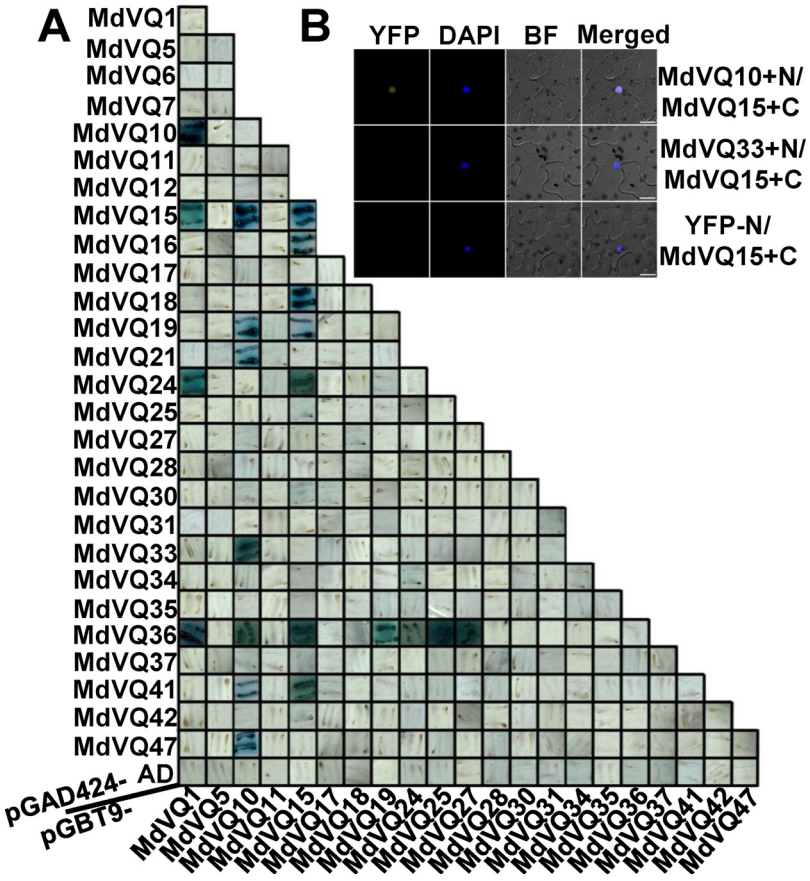

Fig. 4. Physical interactions among MdVQ proteins.
(A) Yeast two-hybrid assay. pGAD424-MdVQ fusion prey vectors were co-transformed with pGBT9-MdVQ fusion bait vectors into yeast cells. Positive interactions were indicated by ability of cells to grow on synthetic dropout medium with additive x-α-gal but lacking Leu, Trp, His, and Ade. Empty AD prey vector plus pGBT-MdVQ fusion bait vectors were used as negative controls. For details regarding negative controls, refer to Fig S4. (B) Assay of bimolecular fluorescence complementation (BiFC) assay, showing fluorescence in nuclear compartments of tobacco leaf epidermal cells that resulted from complementation of N-terminus part of YFP fused to MdVQ10 or MdVQ33 (MdVQ10 + N and MdVQ33 + N) with C-terminus part of YFP fused to MdVQ15 (MdVQ15 + C). No signal was observed from negative controls. Scale bar: 20 μm.

slightly variable VQ motifs were also found in the VQ proteins from *Arabidopsis*, rice, grapevine, soybean, poplar, maize, and Moso bamboo, indicating that they are conserved across plant species. Similar to those other species, the majority of the apple VQ genes (39 of 49) were intronless and encoded relatively small proteins, with 36 of them being < 300 amino acids in size (Table 1 and Supplementary Fig. S1). Thus, the VQ protein family is substantially larger in apple than in plants such as rice (39 members), *Arabidopsis* (34), Moso bamboo (29), and grapevine (18). This is probably because all domesticated apple genotypes are highly heterozygous due to a relatively recent (approximately 60 to 65 million years ago) genome-wide duplication that resulted in the transition of nine ancestral chromosomes to 17 chromosomes [35]. Indeed, alignment of the amino acid residues, phylogenetic tree construction, and our chromosome localization revealed that most of the apple VQ proteins have at least one close homologue (Supplementary Figs. S3 and S4). Among them, some are tandem repeats (*i.e.*, MdVQ3/MdVQ4, MdVQ7/MdVQ8, MdVQ22/MdVQ23, MdVQ38/MdVQ44, MdVQ40/MdVQ49, and MdVQ27/MdVQ28/MdVQ29), while others are segmental repeats (MdVQ19/MdVQ20, MdVQ26/MdVQ35, and MdVQ33/MdVQ36). In addition, we linked multiple pairs to at least 15 potential chromosomal segmental duplications, as indicated with grey areas in Supplementary Fig. S2. These observations suggested that many close VQ homologs in apple resulted from duplication of chromosome regions.

3.2. Phylogenetic tree for plant VQ genes

Our comprehensive phylogenetic tree was constructed based on the conserved domain protein sequences of 875 plant VQs from 33 species (Table S1). In examining the evolutionary relationships among those proteins, we assigned them to nine groups according to the structural features of their VQ conserved domain (Fig. 2). Proteins in Groups V and IX were further classified into two subgroups, a and b. Whereas Groups I, V, VI, VII, IX, and X contained both monocots and dicots, Groups II, III + IV, and VIII comprised only dicots, suggesting that the main characteristics of VQ proteins in Groups I, V, VI, VII, IX, and X were generated before the monocot-dicot split while those of VQ proteins in the other groups appeared after that split had occurred. For the 35 species investigated here, Group VIII members contained the fewest VQ proteins (47) while Group V had the most (174) (Fig. 2 and Supplementary File 1).

3.3. Interactions of MdVQ proteins with MdWRKY proteins

Previous studies have shown that multiple VQ proteins interact with WRKY TFs in *Arabidopsis* [2,11]. To determine whether this was also true for apple, we performed systematic, extensive Y2H assays. Fully expanded leaves of 'Royal Gala' were used to isolate RNA. In all, 28 *MdVQ* genes were cloned from that gene family in apple before their corresponding genes were fused with the yeast GAL4 activation domain (AD) in the Y2H prey vector pGAD424. We also cloned nine apple *MdWRKY* genes from different groups based on their conserved WRKY domain. Because the full-length MdWRKY proteins showed strong transcriptional activation, we fused such proteins with deleted activation domains in apple Group I (MdWRKY4 and MdWRKY19), Group IIa (MdWRKY28), Group IIb (MdWRKY39), Group IIc (MdWRKY52 and MdWRKY68), Group IId (MdWRKY89), Group IIe (MdWRKY91), and Group III (MdWRKY115) to the GAL4 DNA-binding domain (BD) in the Y2H bait vector pGBT9 (Supplementary Fig. S3). The fused pGAD424-MdVQ and pGBT9-MdWRKY recombinant vectors were then co-transformed into yeast cells and tested for *LacZ* reporter gene expression by assaying for β-galactosidase activity. As shown in Fig. 3A, 26 of the 28 MdVQ proteins interacted with the Group I and Group IIc MdWRKY proteins while none interacted with the WRKY domains of Group IIa,

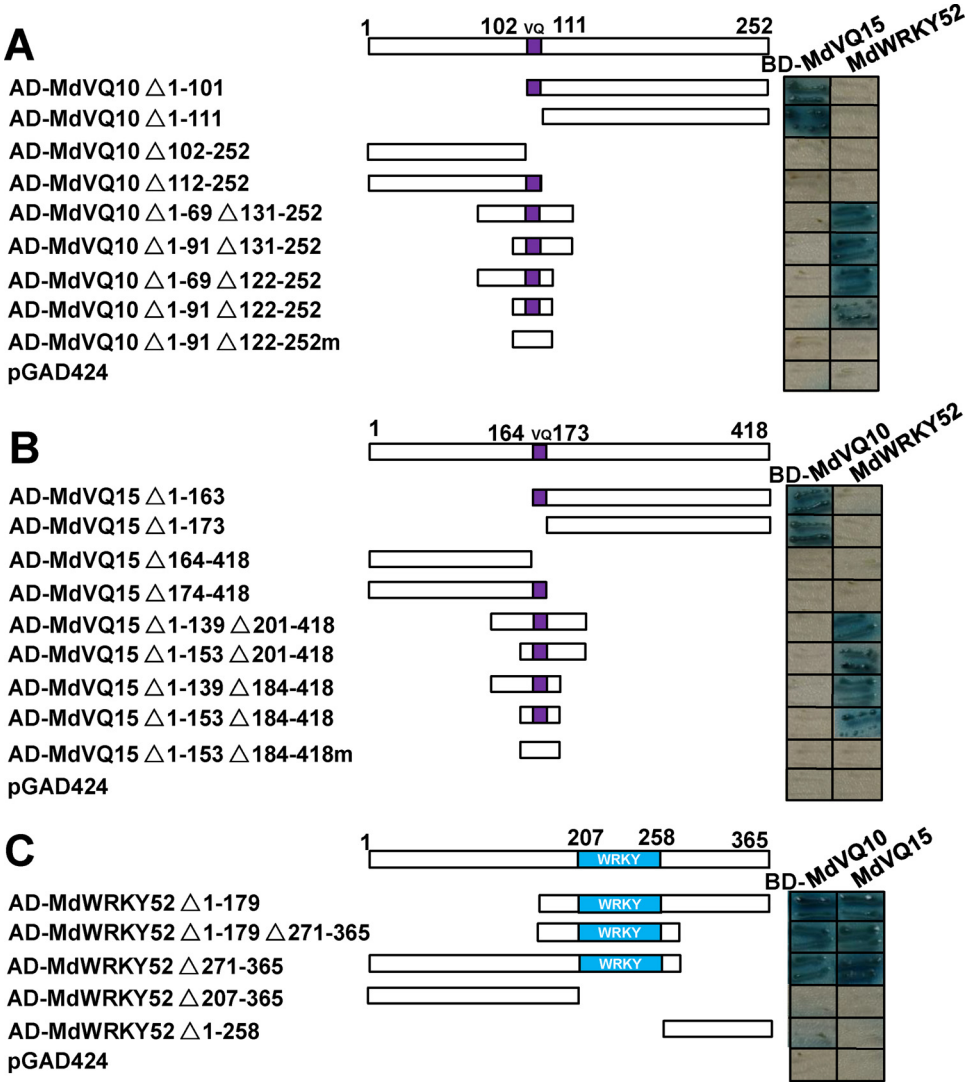

Fig. 5. Mapping of interactions among MdVQ10, MdVQ15, and MdWRKY52.
According to domains of MdVQ10 (N-terminus domain, VQ motif, and C-terminus domain), MdVQ15 (N-terminus domain, VQ motif, and C-terminus domain), and MdWRKY52 (N-terminus, WRKY, and C-terminus domain), truncated MdVQ10 (A), MdVQ15 (B), and MdWRKY52 (C) with specific deletions or mutations, were inserted into pGAD424 prey vector and used to assess interactions. pGAD424 fusion prey vectors were co-transformed with pGBT9 fusion bait vectors into yeast cells. Positive interactions were indicated by ability of cells to grow on synthetic dropout medium with additive x-α-gal but lacking Leu, Trp, His, and Ade. Empty AD prey vector plus pGBT-MdVQ fusion bait vectors were used as negative controls.

IIb, IId, IIe or III MdWRKY proteins. Furthermore, MdVQ12 and MdVQ16 proteins failed to interact with any of the tested Group I or Group IIc MdWRKY proteins (Fig. 3A). Thus, MdVQ proteins exhibited slight preferences in their binding specificity to MdWRKY proteins. These results indicated that MdVQ proteins appeared to interact only with Group I and IIc MdWRKY proteins. For further verification of these interactions revealed in the Y2H assays, we conducted *in vivo* BiFC assay and *in vitro* pull-down analyses. In the former, full-length MdVQ10 and MdVQ12 proteins were fused to the N-terminus region of YFP (MdVQ10 + N and MdVQ12 + N), while MdWRKY52 protein was fused to the C-terminus region of YFP (MdWRKY52 + C). When MdVQ10 + N was co-infiltrated with MdWRKY52 + C in tobacco leaves, DAPI-staining enabled us to detect a YFP signal in the nuclei (Fig. 3B). No fluorescence was observed in any of the negative controls (MdVQ12 + N/MdWRKY52 + C, MdVQ10 + N/YFP + C, MdVQ12 + N/YFP + C, or YFP-N/MdWRKY52 + C) (Fig. 3B). These results demonstrated that MdVQ10 interacts with MdWRKY52 in the cell nuclei. We also confirmed the interaction of MdVQ12 with MdWRKY52 through pull-down assays and found that the GST-fused MdVQ12 could retain MdWRKY52-His but GST-alone could not (Fig. 3C). These findings indicated that MdVQ proteins bind only to Group I and IIc MdWRKY proteins.

3.4. MdVQ interactions

To determine whether apple VQ proteins can interact with themselves or other types of proteins, we used systematic, extensive Y2H assays and introduced the full-length coding sequences of 17 apple VQ proteins into the pGBT9 fusion bait vector. Because the full-length MdVQ6/7/10/12/15/16/21/33/35/42/47 proteins showed strong transcriptional activation, we cloned the MdVQ6/7/10/12/15/16/21/33/35/42/47 proteins, with deleted N-terminus domains, into the pGBT9 fusion bait vector (Supplementary Fig. S4). From this, we discovered that the truncated pGBT9-MdVQ6/7/12/16/21/33 fusion bait vectors still exhibited strong self-activation (Supplementary Fig. S4). Therefore, we conducted Y2H analysis of these vector interactions with all pGAD424-MdVQ fusion prey vectors. Details for testing the self-activation of pGBT9-MdVQ are presented in Fig. S4. The fused pGAD424-MdVQ and pGBT9-MdVQ recombinant vectors were then co-transformed into yeast cells and *LacZ* reporter gene expression was examined by assaying for β-galactosidase activity. As shown in Fig. 4A, MdVQ10, MdVQ15, and MdVQ36 interacted with multiple MdVQ proteins to form a heterodimer while MdVQ15 interacted only with itself to form a homodimer in yeast. For further verification of these Y2H assays, we used BiFC procedures and fused the full-length MdVQ15 protein to the C-terminus region of YFP (MdVQ15 + C). When MdVQ10 + N was co-infiltrated with MdVQ15 + C in tobacco leaves, DAPI-staining revealed a YFP signal in the nuclei, while no fluorescence was observed in any of

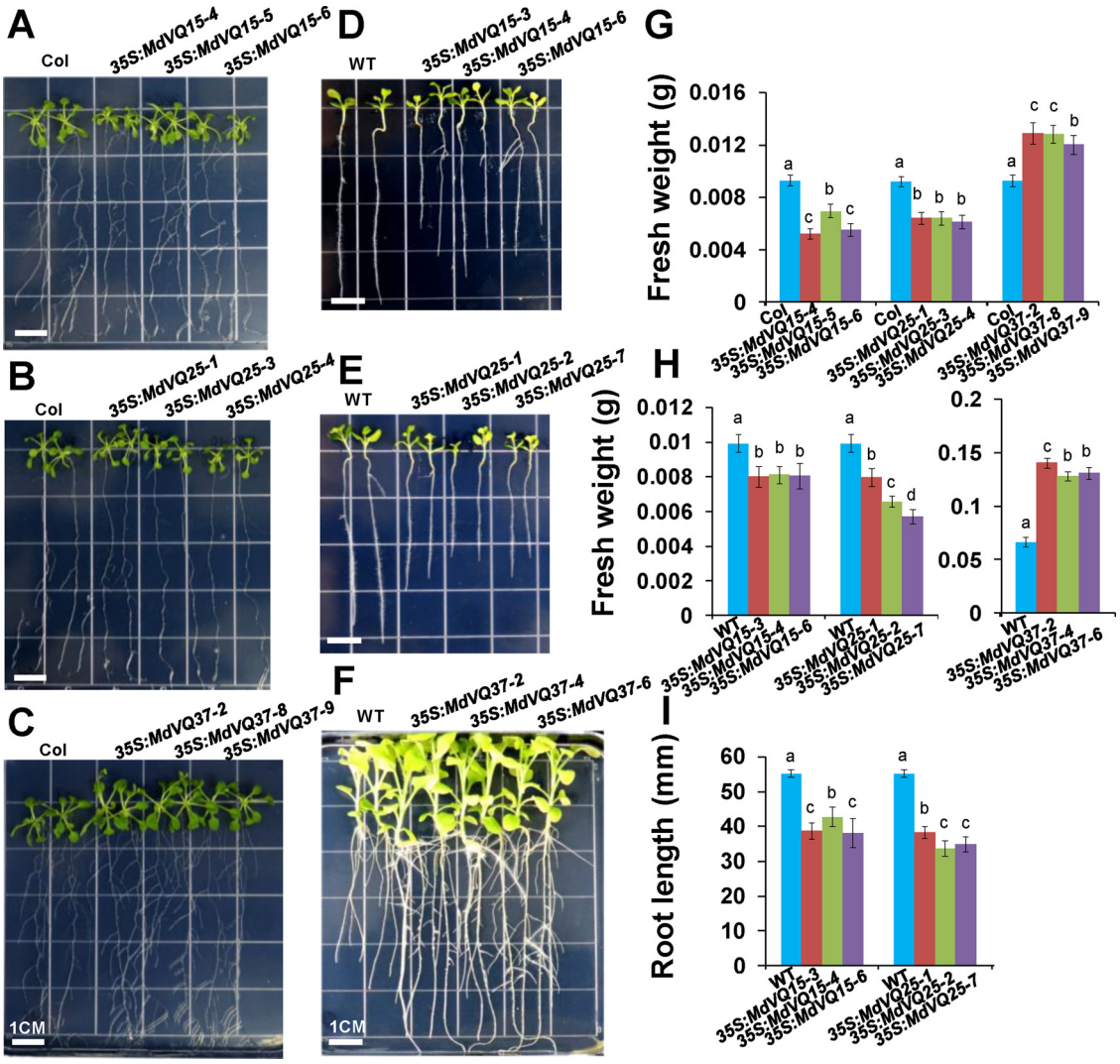

Fig. 6. Phenotypic analysis of transgenic seedlings.
(A–C) Seeds of 'Col-0' WT *Arabidopsis* and OE lines for *MdVQ15* (A), *MdVQ25* (B), and *MdVQ37* (C) were germinated in MS media. Pictures were taken 20 d after germination. (D–F) Seeds of wild-type (WT) tobacco and OE lines for *MdVQ15* (D), *MdVQ25* (E), and *MdVQ37* (F) were germinated in MS media. Pictures were taken 15 d (D, E) or 35 d (F) after germination. (G) Fresh weights from 20 *Arabidopsis* seedlings of 'Col-0' and OE lines measured 20 d after germination. (H) Fresh weights from 20 tobacco seedlings of WT and *MdVQ15*-OE and *MdVQ25*-OE lines measured 15 d after germination, plus 20 tobacco seedlings of WT and *MdVQ37*-OE line measured 35 d after germination. (I) Root lengths from 20 tobacco seedlings of WT and *MdVQ*-OE lines measured 15 d after germination. Error bars represent SD based on 3 independent replicates. For G, H, and I, bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's tests.

the negative controls (MdVQ33 + N/MdVQ15 + C or YFP-N/MdVQ15 + C) (Fig. 4B). These results showed that MdVQ10 interacts with MdVQ15 in the nuclei.

3.5. Identification of interaction regions among MdVQ10, MdVQ15, and MdWRKY52

To determine which regions of MdVQ10, MdVQ15 and, MdWRKY52 proteins are required for their interactions, we separated MdVQ10 and MdVQ15 into their N-terminus domain, VQ motif, and C-terminus domain, and divided MdWRKY52 into its N-terminus, WRKY, and C-terminus domains (Fig. 5A–C). We also mutated the amino acids (MVQQ) in the VQ motif of MdVQ10 and the amino acids (MVQE) in the VQ motif of MdVQ15 to EDLE. Finally, we inserted nine truncated MdVQ10, nine truncated MdVQ15, and five truncated MdWRKY52, with specific deletions or mutations, into the pGAD424 prey vector (Fig. 5A–C). The fused pGAD424 and pGBT9 recombinant vectors were then co-transformed into yeast cells and tested for *LacZ* reporter gene expression through assays of β-galactosidase activity. The Y2H assay showed that deleting the N-terminus residues and the VQ motifs of MdVQ10 and MdVQ15 did not aff ;ect their physical interactions (Fig. 5A, B). However, deletion of the C-terminus of MdVQ10 or MdVQ15 eliminated those interactions. These results demonstrated that the C-terminus domains of MdVQ10 and MdVQ15 are critical for their physical interactions.

The Y2H assays also showed that deleting the N- or C-terminus residues of MdVQ10 and MdVQ15 did not aff ;ect their physical interactions with MdWRKY52. That is, the VQ motif, containing only 30 amino acid residues in MdVQ10 (△1-91△122-252) and MdVQ15 (△1-153△184-418), was still capable of interacting with MdWRKY52 (Fig. 5A, B). However, mutations of the VQ motif of MdVQ10 (△1-91△122-252) or MdVQ15(△1-153△184-418) prevented those interactions. Therefore, the interaction of MdVQ10 and MdVQ15 with MdWRKY52 required not only the VQ motif but also several amino acid residues flanking the VQ motif.

The Y2H assays also indicated that deleting the N- or C-terminus

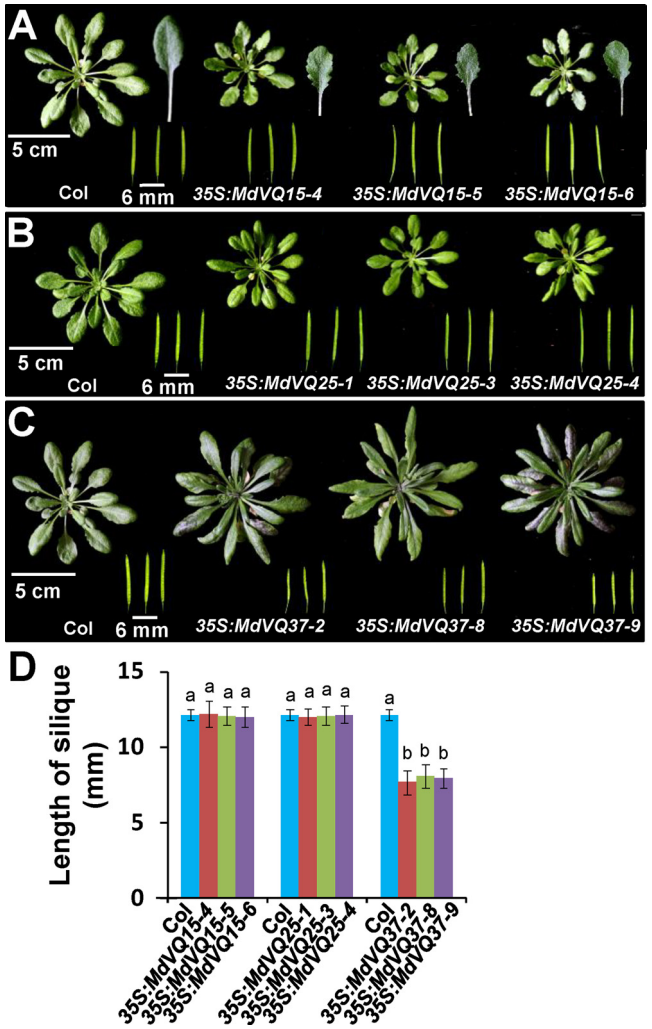

Fig. 7. Phenotypic analyses of *Arabidopsis* leaves and siliques from 'Col-0' and *MdVQ15*- (A), *MdVQ25*- (B), and *MdVQ37*- (C) OE lines. Seeds were germinated in peat and vermiculite (2:1, v:v). Pictures were taken 6 weeks after germination. (D) Lengths measured from 7th or 8th silique sampled from top of WT and OE lines (30 siliques each). For D, bars not labeled with same letters indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's test.

residues of MdWRKY52 had no influence on its physical interactions with MdVQ10 and MdVQ15 (Fig. 5C). However, deleting the WRKY domain of MdWRKY52 eliminated those interactions (Fig. 5C). Therefore, the WRKY domain of MdWRKY52 is essential for its interaction with MdVQ10 and MdVQ15.

3.6. Functional analysis of MdVQ genes in Arabidopsis and tobacco

To characterize the biological functions of *MdVQ*s in planta, we randomly generated transgenic *Arabidopsis* and tobacco plants that constitutively over-expressed those genes. Our PCR-screening of kanamycin-resistant transgenic lines was performed using *Arabidopsis* and tobacco genomic DNAs as templates, with primers specific to *MdVQ6*, *MdVQ15*, *MdVQ25*, *MdVQ27*, and *MdVQ37* (Supplementary Figs. S5 and S6). Transformants, as identified *via* qRT-PCR, exhibited elevated levels of transcripts. From these, we selected three transgenic lines with high *MdVQ* expression. Seeds from transgenic *Arabidopsis* and tobacco were germinated on Murashige and Skoog (MS) agar media. After 20 d of growth on the MS media, the *MdVQ15*-OE and *MdVQ25*-OE *Arabidopsis* seedlings were smaller than the 'Col-0' WT seedlings (Fig. 6A, B) and their fresh weights were much lower (Fig. 6G). In contrast, the *MdVQ37*-OE *Arabidopsis* seedlings were much larger than the WT, and they had stronger roots and flowered earlier (Fig. 6C). After 15 d of growth, the *MdVQ15*-OE and *MdVQ25*-OE tobacco seedlings were smaller than the WT (Fig. 6 D, E), and values were lower for their fresh weights and root lengths (Fig. 6H, I). By comparison, 35-day-old *MdVQ37*-OE tobacco seedlings were larger than the WT, and they had stronger roots and higher fresh weights (Fig. 6F). Finally, early development was not affected by overexpression of *MdVQ6* in *Arabidopsis* seedlings or by overexpression of *MdVQ27* in either *Arabidopsis* or tobacco (Supplementary Fig. S7).

The phenotypes of transgenic *Arabidopsis* and tobacco were examined after 6 and 11 weeks, respectively, of growth under normal conditions. The *MdVQ15*-OE and *MdVQ25*-OE *Arabidopsis* seedlings were smaller than the WT (Fig. 7A, B) and the leaves of plants over-expressing *MdVQ15* had obvious serrated margins (Fig. 7A). The *MdVQ37*-OE *Arabidopsis* plants were larger, flowered earlier, and had smaller siliques than the WT (Fig. 7C, D). Similar contrasts in phenotypes were observed between the transgenic and WT tobacco plants. Those over-expressing *MdVQ15* and *MdVQ25* had significantly shorter stems and corollas (Fig. 8A, B, D, E, G, H), while *MdVQ37*-OE seedlings had more branches, shorter corollas, and smaller capsules than the WT (Fig. 8C, F, H, I). In contrast, growth was not affected for *MdVQ6*-OE and *MdVQ27*-OE *Arabidopsis* plants or for *MdVQ27*-OE tobacco plants when compared with their WT counterparts (Supplementary Figs. S8 and S9). Therefore, we concluded from all of these results that expression of several apple MdVQ genes can influence various developmental processes.

4. Discussion

The plant-specific proteins encoded by VQ genes make up large families, and all members contain a conserved VQ motif [11]. Apple is one of the most economically important woody plants and the most widely cultivated fruit trees in the world. Recent sequencing of the genome for *Malus domestica* has provided a good platform for genome-wide analyses of all putative gene families [35–37]. Details about the VQ gene family have been reported for various species [8,11,13,19–26], but not for apple. Our investigation revealed 49 genes belonging to this family. Because a higher-quality apple GDDH13 v1.1 reference genome has now been published [37], we used it to match the IDs of MdVQ genes with those presented in the original v1.0 genome. In all, we discovered that 28 MdVQ gene IDs in the v1.0 apple genome failed to match with the GDDH13 v1.1 genome, including those for cloned *MdVQ*s. Daccord et al. [37] have stated that the number of genes has been overestimated in the v1.0 genome because of the assembly and subsequent annotation of both haplotypes and the fragmentation of the original genome. Therefore, this 'GDDH13' gene prediction has decreased the estimated number of annotated genes in apple from 63,541 to 42,140. Along with reducing the amount of predicted repeat and fragmental genes, we believe that the latest version also removes many existing genes that are of smaller length, *e.g.*, intronless MdVQ genes.

Based on phylogenetic analyses and structural features of full-length sequences or the VQ domain, this gene family can be divided into seven or 10 groups [10,19,21,25,26]. However, previous phylogenetic investigations have covered only a limited number of species along with *Arabidopsis* VQ proteins. Therefore, it has been difficult to conduct a thorough examination of classifications for this gene family. Our comprehensive study of the evolution and classification of plant VQ proteins comprised conserved VQ domain sequences from 33 species, including 25 dicots and eight monocots. Based on previous genomics research results, we arranged the 875 plant VQ proteins into nine groups and found that VQ members in Groups II, III + IV, and VIII do not contain monocots while Group I, V, VI, VII, IX, and X include both monocotyledon and dicotyledon species. This means that members in the first three groups were generated after the monocot-dicot split, and it suggests that those proteins have special biological roles when compared

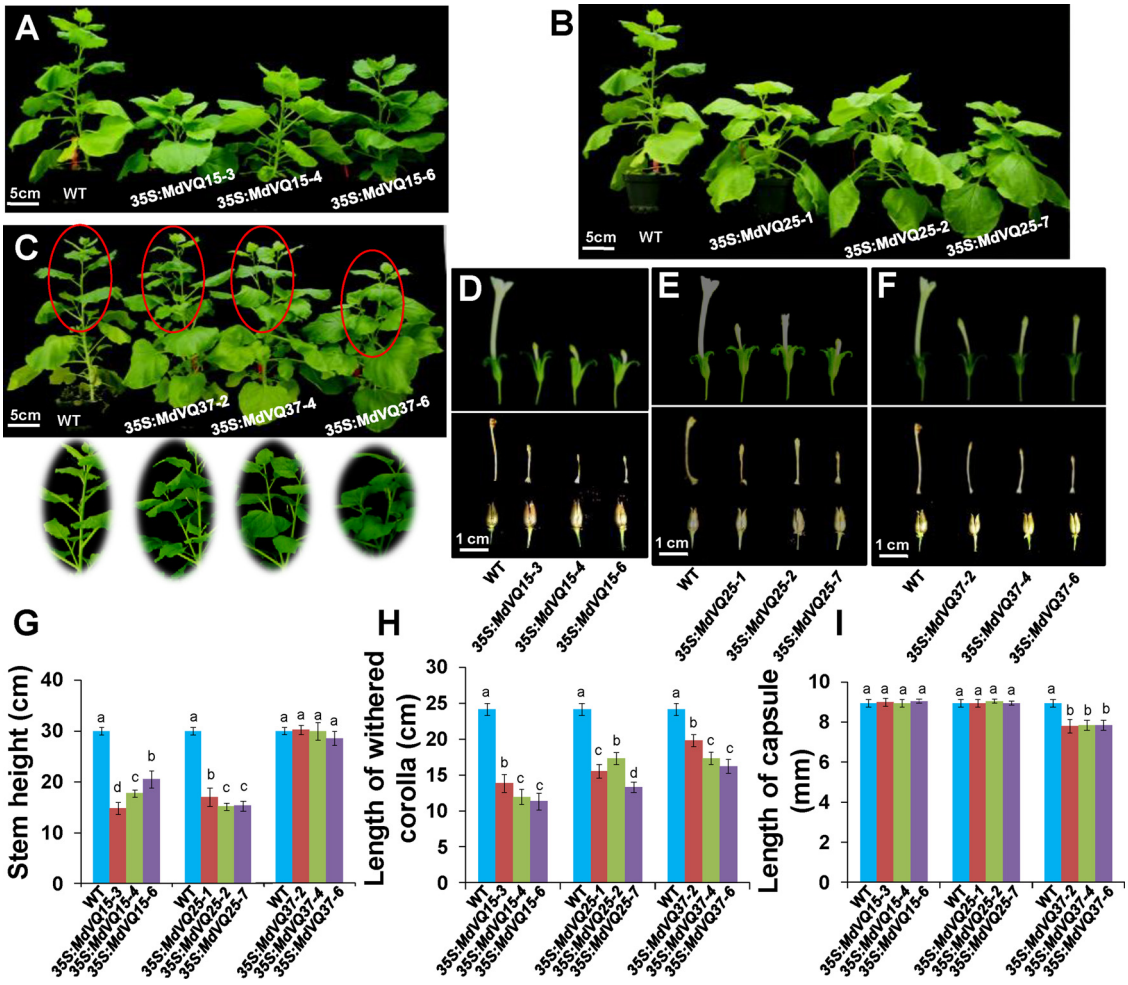

Fig. 8. Phenotypic analyses of tobacco stems and flowers from WT and *MdVQ15*- (A), *MdVQ25*- (B), and *MdVQ37*- (C) OE lines. Seeds were geminated in peat and vermiculite (2:1, v:v). Pictures were taken 11 weeks after germination. D–F, comparisons of withered corollas and mature capsules. (G) Heights of stems sampled from WT and OE lines (10 stems each). (H) Lengths of corollas from WT and OE lines (20 corollas each). (I) Lengths of capsules from WT and OE lines (20 capsules each). For G, H, and I, bars not labeled with same letters in each panel indicate values are significantly different at $p < 0.05$, based on one-way ANOVA and Duncan's test.

with VQ members in Groups I, V, VI, VII, IX, and X.

Results from Y2H assays with multiple *Arabidopsis* and soybean VQ proteins have shown that they can bind to the C-terminus WRKY domains of Group I WRKY proteins and the single WRKY domain of Group IIc WRKY proteins [8,10,13]. The combinatorial complexity of these VQ-WRKY interactions can cause VQ transcriptional regulators to affect the transcriptional function of their interacting WRKY proteins, possibly allowing for sophisticated transcriptional regulation of downstream targets and, consequently, diverse physiological, abiotic, and biotic responses [11]. Several reports support those proposals. For example, VQ9 protein and its interacting partner Group IIc protein WRKY8 might modulate salt stress responses partly by altering the expression of downstream target gene *RD29A* of the WRKY8 factor [9]. Furthermore, VQ14 (IKU1) protein and Group I protein WRKY10 (MIN3) form a complex that regulates the downstream gene *IKU2* (a leucine-rich repeat kinase) to affect the development of seed endosperm [10]; VQ16 (SIB2) and VQ23 (SIB1) interact with Group I protein WRKY33, enhancing the transcriptional activation of the WRKY33 factor to function in plant defenses against necrotrophic pathogens [7]; and VQ16 (SIB2) and VQ23 (SIB1) interact with Group I protein WRKY33 and Group IIc protein WRKY57 to affect WRKY33 and WRKY57, which competitively regulate the downstream genes *JAZ1* and *JAZ5* to compromise *B. cinerea* resistance [12]. The VQ20 protein interacts with Group I WRKY2 and WRKY34 and affects the transcriptional functions of WRKY2 and WRKY34 to modulate pollen development and function [14]. All of these reports indicate that AtVQ proteins could negatively or positively alter the expression of target genes for interacting WRKY proteins by affecting their transcription-regulating activities and DNA-binding.

In this study, we systematically demonstrated through Y2H, BiFC, and pull-down assays that at least 20 MdVQ proteins interact with Group I and Group IIc WRKYs. Further analysis showed that the interaction of MdVQ and MdWRKY is required for the VQ motif and WRKY domain. Therefore, these results suggest that the mechanism for VQ-WRKY interactions to regulate plant development and biotic/abiotic stress responses is also present in apple. This VQ-WRKY partnership becomes even more complicated with additional signaling components. Both VQ29 and VQ12 physically interact with themselves *via* their C-terminus fragments to form homodimers and a heterodimer so they can negatively modulate basal defenses against *B. cinerea* [18]. Moreover, VQ29 protein strongly interacts with WRKY25 and WRKY33 *via* the VQ motif, based on the high activities of β-galactosidase that have been detected [8], and WRKY33 positively regulates the response of plants against infections by *B. cinerea* [38]. The VQ proteins can affect DNA-binding or other properties of interacting WRKY proteins [2,11]. Therefore, it is possible that WRKY33 functions antagonistically with its interacting partner VQ29 to modulate *B. cinerea* resistance, probably because the interaction of VQ12 with VQ29 *via* the C-terminus

fragment competitively affects the DNA-binding activity of WRKY33 or the expression of downstream target genes. Such an intricate network that consists of a large protein complex can provide more precise, effective, and differential regulatory mechanisms for plants in response to environmental stimuli. Nevertheless, future studies are needed to analyze these mechanisms in modulating defense responses. Our findings indicated that MdVQ10, MdVQ15, and MdVQ36 interact with multiple MdVQ proteins to form heterodimers, and MdVQ15 interacts with itself *via* the C-terminus fragment to form a homodimer. These results suggest that apple also has this large protein complex that modulates its defense responses.

Transgenic lines that express antisense *AtCaMBP25*(*VQ15*) and *vq9* mutants appear to be more tolerant of osmotic and NaCl stresses [9,17]. The *vq8-1* mutant and *VQ17*-OE, *VQ18*-OE, or *VQ22*-OE plants of *Arabidopsis* display phenotypes of stunted growth throughout their life cycles [8]. Zhou et al. [13] have revealed that *GsVQ22* inhibits the growth of wild soybean and that this negative eff ;ect is intensified by cold treatment. Overexpression of *VQ29* substantially delays *Arabidopsis* flowering [8]. Flowering is promoted but seed set is reduced in transgenic *Arabidopsis* with elevated transcript levels for soybean *GmVQ43*, *GmVQ62*, and *GmVQ37* [13]. Moreover, VQ5, VQ20, VQ21, and VQ22 negatively regulate plant basal defenses against *B. cinerea* infections [8,39,40], while SIB1 (VQ23) and SIB2 (VQ16) positively mediate plant defenses against that pathogen [7]. Several other VQ proteins are involved in plant resistance against the biotrophic *Pseudomonas syringae* and/or attacks by herbivorous insects [8,10,40,41]. We found here that transgenic *Arabidopsis* and tobacco plants with elevated transcript levels for apple *MdVQ15* and *MdVQ25* had stunted growth, and that overexpression of those genes led to shorter corollas in tobacco. Growth was stimulated for transgenic *Arabidopsis* and tobacco plants with elevated transcript levels for apple *MdVQ37*, and development of their siliques and capsules was affected. Therefore, such multiple roles for VQ proteins in plant growth, development, and defenses may indicate that the signal transduction for those processes requires tight regulation and fine-tuning. It is also possible that these proteins play broad roles in maintaining an appropriate balance in the functioning of diff ;erent signaling pathways, thereby resulting in proper plant growth and development under adverse conditions. However, knowledge is still limited about the exact complex and differential molecular mechanisms that underlie their involvement. Further studies are required to identify putative interacting VQ proteins and elucidate the signaling pathways in which they participate.

Author contributions

F.M., Z.Z. and Q.D. conceived and designed the experiments. Q.D., S.Z., D.D., Y.T., Y.W. and K.M. performed the experiments. F.M. and Q.D. analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.04.029.

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