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Genome-Wide Analysis of Snapdragon *WRKY* and *VQ* Gene Families and Their Expression in Response to Drought and Cold Stresses

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ABSTRACT

Snapdragon (*Antirrhinum majus*) is one of the most widely cultivated grass flowers in the world. *WRKY* transcription factors, *VQ* proteins and their interactions play crucial roles in plant response to abiotic stresses. However, little is known about *WRKY* and *VQ* gene families in snapdragon. In the present study, *WRKY* and *VQ* genes and their interactions were comprehensively analyzed in snapdragon using bioinformatics approaches, and their expression in response to drought and cold stresses was examined using real-time PCR. A total of 67 *AmWRKY* genes were identified in snapdragon, which were classified into different groups or subgroups based on phylogenetic analysis. Members in the same group or sub-groups exhibited similar exon-intron structure and conserved motifs distribution. Among these *WRKY* genes, 16 and 22 genes were found to differentially express under drought and cold stresses, respectively. A total of 32 *AmVQ* genes were identified, of which 10 and 18 were found to differentially express under drought and cold stresses, respectively. The *WRKY-VQ* or *WRKY-WRKY* interaction relationships were predicted for 11 cold-responsive genes, suggesting that they might exist in the same response pathway to cold stress. These results lay a foundation for further studies on roles of *WRKY* and *VQ* genes and their interactions in regulating abiotic responses in snapdragon.

KEYWORDS

Snapdragon; *WRKY* genes family; *VQ* genes family; gene expression; abiotic stress

1 Introduction

The *WRKY* gene family is one of the largest families of transcription factors, which have been identified throughout the plant kingdom. The name of the *WRKY* family is derived from the most prominent feature of these transcription factors, the *WRKY* core sequence, which generally is tryptophan-arginine-lysine-tyrosine-glycine-glutamine-lysine (*WRKYGQK*). Another defining feature of *WRKY* transcription factors is the zinc finger motif, which is either *C2H2* or *C2HC* type. The core sequence and the zinc finger motif form the *WRKY* domain [1,2]. According to the number of *WRKY* domains and the type of zinc finger motif they contain, *WRKY* transcription factors are usually classified into three main groups. Group I contains two *WRKY* domains and a *C2H2* zinc finger motif. Group II contains a single *WRKY* domain and a *C2H2* zinc finger motif, which is further divided into five subgroups based on their primary sequences, including *Ila*, *Ilb*, *Ilc*, *Ild* and *Ile* [1,2]. Group III contains a single *WRKY* domain and a *C2HC* zinc finger motif.



The WRKY transcription factors are essential regulatory components of plant response to abiotic stress. Numerous studies revealed that *WRKY* genes could rapidly differentially express under abiotic stresses, based on transcriptome analysis or real-time PCR [3–11]. Roles of *WRKY* genes in stress response have also been confirmed using mutant or transgenic plants. For instance, at least seven *WRKY* genes were validated to regulate abiotic stress response in *Arabidopsis*. Constitutive expressions of *AtWRKY25*, *AtWRKY26* or *AtWRKY33* enhance heat tolerance [12]. Loss function of *AtWRKY34* improves cold tolerance of pollen [13]. Activating expression of *AtWRKY30*, *AtWRKY53* or *AtWRKY57* improve drought tolerance [14–16]. Overexpression of *AtWRKY33* increases salt tolerance, while loss function of *AtWRKY53* decreases salt tolerance [17,18]. The WRKY transcription factors also play important roles in many other biologic processes in plant, such as flowering time, pollen development, fruit ripening, seed size, leaf senescence and secondary metabolite biosynthesis [19–25].

In general, WRKY transcription factors performed their biologic function by interacting with various proteins [26]. The valine-glutamine (VQ) protein is one of the most important WRKY interactors identified in the past few years. The VQ proteins contained a conserved VQ motif structure FxxhVQxhTG (F, Phenylalanine; x, any amino acid; h, hydrophobic residue; V, Valine; Q, Glutamine; T, Tryptophan, G, Glycine) [27]. Like WRKY, VQ proteins also have significant effects on abiotic stress response in plants. In *Arabidopsis*, at least two VQ genes were reported to be involved in abiotic response. *AtVQ9* was strongly induced by NaCl treatment. Mutation of *AtVQ9* enhanced *Arabidopsis* tolerance to salt stress, while over-expression of this gene increased sensitivity to salt stress [28]. *AtVQ15* was induced by dehydration, low temperature and high salinity. Knock-down of this gene enhances *Arabidopsis* tolerance to osmotic and salt stress. In contrast, over-expression of this gene showed increased sensitivity to these two abiotic stresses [28]. The role of VQ genes in plant response to abiotic stresses were also observed in other species, such as bamboo salt stress positive regulator *PeVQ28* [29] and tomato heat negative regulator *SIVQ6* [30].

It has been showed that VQ proteins could interact through their conserved V and Q residues with Group I and IIc WRKY transcription factors [31,32]). The two gene families jointly regulate plant development by forming complexes. For example, *Arabidopsis* *AtVQ9* negatively regulates salt tolerance by interacting with *AtWRKY8* [28]. *AtVQ15* regulates osmotic stress tolerance, which interacts with *AtWRKY25* and *AtWRKY51* [32,33]. Banana *MaWRKY26* interacts with *MaVQ5* to regulate expression of JA biosynthetic genes involved in cold resistance [34]. Several WRKY-VQ interactions were found to regulate *Arabidopsis* resistance to *Botrytis cinerea*, including *AtWRKY33/AtWRKY57-AtVQ16/AtVQ23*, *WRKY8-VQ10*, and *AtWRKY33-AtVQ23* [31,35,36]. In addition, co-expression between *WRKY* and *VQ* genes was observed in many plant species, such as rice, maize and melon [37–39].

Snapdragon (*Antirrhinum majus*) is a perennial herb of the Plantaginaceae. It originated in the Mediterranean coast, and has become one of the most widely cultivated grass flowers in the world. Snapdragon has a strong cold tolerance, which could keep blooming in early spring, late fall or even midwinter in some warm places. Snapdragon also has moderate drought tolerance, which could thrive in well-drained soils [40,41]. Several snapdragon genes have been found to enhance tolerance to cold or drought stresses [42,43]. The *WRKY* and *VQ* gene families have been systematically studied in many plant species [5,44,45], whereas there is little knowledge about *WRKY* genes in snapdragon. In this study, we performed a genome-wide analysis of *WRKY* and *VQ* genes in snapdragon, including gene classification, gene duplication investigation and protein-protein interaction prediction. The expression of these genes in response to cold and drought was further assayed using real-time PCR. Our study aimed to lay a foundation for further studies on the role of *WRKY* and *VQ* genes and their interactions in regulating snapdragon responses to abiotic stresses.

2 Materials and Methods

2.1 Plant Materials and Treatments

A snapdragon variety, Shiyun, was used in this study. Each snapdragon plant was grown in a plot with a diameter of 15 cm and peat moss-based soil. They were firstly planted in a plant growth chamber (MLR-351, PHCbi) for about four weeks at 15°C with a 16/8 h light/dark photoperiod and a relative humidity of 80%. The soil moisture was monitored by a soil moisture meter (L99, Luge), and was maintained at about 70%. Then these plants were divided equally into three groups. The first group was set up as a control. It was kept in the growth chamber, which was grown under 15°C and well-watered conditions. The second group was set up as a drought treatment. It was kept in the same growth chamber, which was grown under 15°C but received no irrigation for one week. The third group was set up as cold treatment. This group was kept at 15°C in the same growth chamber for six days, and then was transferred to a low-temperature light incubator (PRXD-300, Chuanyi) with 0°C for one day. Leaves were collected from five-week-old plants growing under the control, drought stress (after one week treatment) and cold stress (after one day treatment) treatments.

2.2 Identification of WRKY and VQ Genes in Snapdragon

The snapdragon genome and annotation data (version 2) were downloaded from the Snapdragon Genome Database (<http://bioinfo.sibs.ac.cn/Am/index.php>). The WRKY protein sequences of *Arabidopsis* were downloaded from the TAIR (<http://www.arabidopsis.org/>), and used as query sequences. A BLASTP search was performed to identify AmWRKY protein using TBtools with an E-value threshold of 10^{-5} [46]. The HMM of the VQ motif (PF05678) was downloaded from the Pfam database (<http://pfam.sanger.ac.uk/>), and was used to search AmVQ proteins with HMM 3.2 software [47]. All potential *AmWRKY* and *AmVQ* genes were assessed for presence of the WRKY domain or VQ motif with the Web CD-search Tool (<http://www.ncbi.nlm.nih.gov/structure/cdd/>). Only the genes with the complete WRKY domain or VQ motif were accepted.

2.3 Sequence Alignment and Phylogenetic Analysis

Sequence alignment was performed by MEGA7 software using the Muscle method with default parameters [48]. Phylogenetic tree was constructed by MEGA7 using Maximum Likelihood method with the following parameters: 1000 bootstrap replicates, Jones-Taylor-Thomton model, partial deletion and 80% site coverage cutoff.

2.4 Analysis of Gene Structure, Conserved Motif, Gene Duplication and Cis-Acting Regulatory Element, and Prediction of Protein-Protein Interaction

Molecular weights and isoelectric points were analyzed using the ExPASy website (<http://web.expasy.org/protparam>). Conserved motifs were identified using the MEME program (<http://alternate.memesuite.org/tools/meme>) with the following parameters: minimum motif width = 6; maximum motif width = 50; maximum number of motif = 10; minimum sites per motif = 2; maximum sites per motif = 600 [49]. Gene duplication and synteny analysis were performed using the MCScanX software with the default parameters [50]. The *cis*-regulatory elements were analyzed using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare>). The protein-protein interactions were predicted by STRINGV11 online software with default score thresholds [51]. Only interactions predicted based on experimentally determined or from curated databases are presented.

2.5 RNA Extraction and Real-Time PCR

Total RNA was extracted using an RNeasy Plus Mini Kit (QIAGEN, German). First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan). Real-

time PCR was performed on an Applied Biosystems 7900HT using SYBR qPCR Mix kit (Toyobo, Japan). *Ubiquitin* was used as the endogenous control. Data were analyzed according to the $2^{-\Delta\Delta C_t}$ method [52]. Three independent biological replicates and three technical replicates per biological replicate were used. Differences in gene expression under abiotic stress and normal conditions were compared using *t*-tests, with a threshold of $P < 0.01$. Primers were designed using Primer Premier 5.0 software (Supplementary Table 1).

3 Results

3.1 Identification of WRKY Genes in Snapdragon

A total of 72 genes with WRKY domain were identified in snapdragon (Supplementary Table 2). Five of them were excluded from further study due to lack of the WRKYGQK sequence or zinc finger motif. The remaining 67 genes were designated from *AmWRKY1* to *AmWRKY67* based on their locations on the respective chromosome. The lengths of these *AmWRKY* proteins varied from 109 to 725 amino acids, the isoelectric points ranged from 4.90 to 10.13, and the molecular weights ranged from 12.9 to 78.3 kDa. Twelve of these 67 proteins contained two WRKY domains, and 45 of them contained one WRKY domain.

3.2 Phylogenetic Tree Analysis, Group Classification and Multiple Sequence Alignment of *AmWRKY* Genes

A phylogenetic tree was constructed based on the WRKY domain sequences of these *AmWRKY* proteins, and *Arabidopsis* WRKY domain sequences were included as references (Fig. 1). Sixty-six of 67 *AmWRKY* proteins were divided into three groups, according to the classification in *Arabidopsis* [1]. Twelve proteins harboring two WRKY domains were categorized into Group I, while 45 and nine proteins containing one WRKY domain were categorized into Group II and Group III, respectively. The remaining one protein, *AmWRKY65*, was not assigned to any group. The Group II was further divided into five subgroups. They were named IIa, IIb, IIc, IId and IIe, consisting of four, nine, eighteen, six and eight genes, respectively. This classification was consistent with the phylogenetic relationship base on full-length sequence of these *AmWRKY* proteins (Fig. 2a). Multiple sequence alignment was conducted for the WRKY domain of these proteins (Supplementary Fig. 1). All of them contained the conserved WRKYGQK sequence, except for *AmWRKY42* belonging to Group IIc, which carried a variant of WRKYGKK. Members in Groups I and III contained the C2H2-type (C-X4-5-C-X22-23-H-X-H) and C2HC-type (C-X7-C-X23-H-X-C) zinc finger motif, respectively. Members in Group II contained C2H2-type zinc finger motif, except for *AmWRKY8* and *AmWRKY54* belonging to Group IIe. *AmWRKY8* had a C-X5-C-X27-H-X-H type zinc finger motif, while *AmWRKY54* carried a 14 amino acids deletion at the N terminus of zinc finger motif.

3.3 Gene Structure, Motif Composition and Cis-Acting Regulatory Elements of *AmWRKY* Genes

The number of introns varied from two to seven for these *AmWRKY* genes (Fig. 2b). Most members of the same group had a similar intron number, except for Group IIb. All members of Groups IId, IIe and III contained two introns, most members of Groups I and IIa contained three or four introns, while most members of Group IIc contained one or two introns. In contrast, the intron number of Group IIb members was variable, including one, two, four, five or six introns.

Most members of the same group also shared similar exon-intron structures. An intron was located between two complete codons named phase 0, and after the first or second nucleotides in the codon defined as phases 1 and 2. All members of Groups IId, IIe and III were found in phase 0 at exon 1st and phase 2 at exon 2nd and 3rd (Fig. 2b). Members of Group IIa and IIb were only found in phase 0, except for *AmWRKY49* and *AmWRKY50*. Phases 0 and 1 were widely distributed in Groups I and IIc with phase 2 having the least distribution.

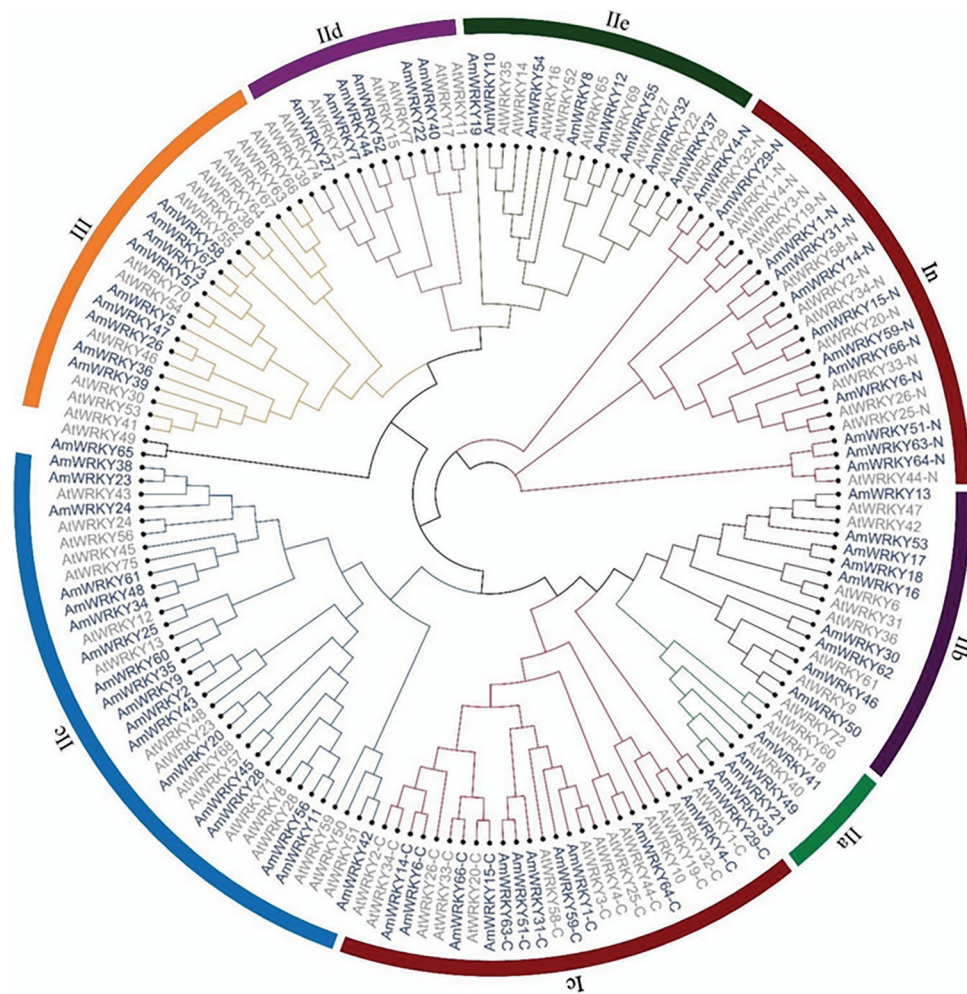


Figure 1: Phylogenetic tree of WRKY proteins among snapdragon and *Arabidopsis*. The phylogenetic tree of AmWRKY proteins was constructed using MEGA7.0 by the maximum likelihood method with 1,000 bootstrap replicates. AtWRKY proteins were used as references to classify AmWRKY proteins

The conserved motifs of AmWRKY proteins were further identified. As shown in Fig. 2c and Supplementary Fig. 2, the motifs 1, 2, 3, and 7 contained the WRKY core sequence and zinc finger motif. The remaining motifs exhibited a similar distribution in the same group. All members of Group I contained motifs 4 and 10. All members of Group IIa contained motifs 5 and 8. All members of Group IIb contained motifs 6 and 9. All members of Group IIc contained motif 4.

The potential *cis*-regulatory elements were investigated in the promoter region of *AmWRKY* genes. Five types of stress-related and six types of hormone-related elements were identified (Supplementary Table 3). Among the stress-related elements, anoxic-responsive element (ARE and GC-motif) was the most abundant, which was found in promoters of 55 of 67 *AmWRKY* genes. The remaining four types, including drought-responsive element (MBS), low temperature-responsive element (LTR), defense- and stress-responsive element (TC-rich repeats) and wound-responsive element (WUN-motif), were found in 30, 28, 23 and 3 *AmWRKY* genes, respectively. The hormone-related elements included abscisic acid (ABA)-responsive element (ABRE), gibberellin-responsive elements (GARE-motif, P-box and TATC-box), methyl jasmonate (MeJA)-responsive element (CGTCA-motif), ethylene-responsive element (ERE), auxin-

responsive elements (AuxRR-core and TGA-element) and salicylic acid (SA)-responsive element (TCA-element), which were found in 60, 56, 52, 45, 31 and 28 *AmWRKY* genes, respectively.

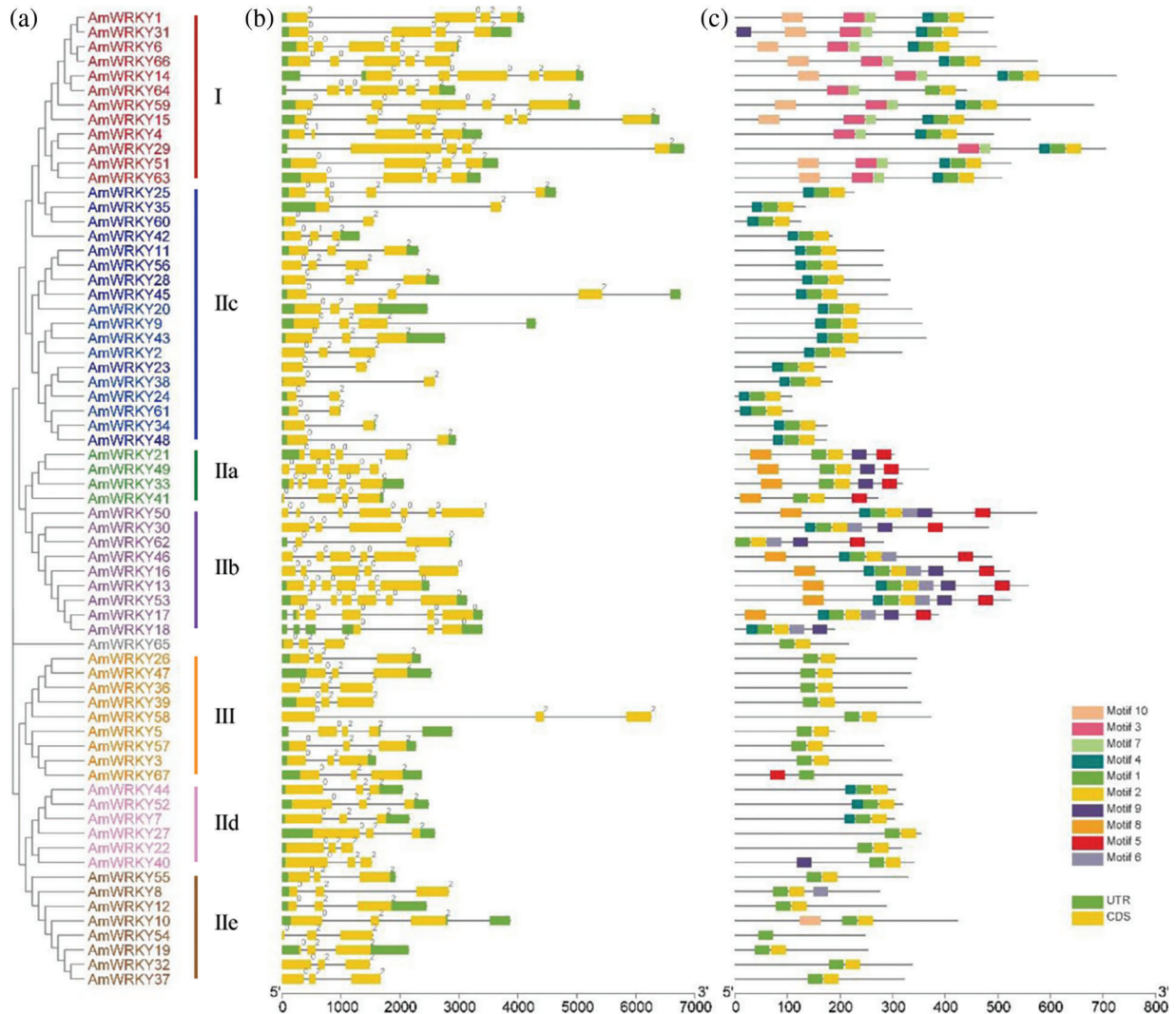


Figure 2: Structure characterization of *AmWRKY* genes. (a) Phylogenetic tree of *AmWRKY* proteins. (b) The exon-intron structures of *AmWRKY* genes. The introns phases 0, 1 and 2 were indicated by numbers 0, 1 and 2, respectively. (c) The conserved motifs of *AmWRKY* proteins

3.4 Chromosome Location and Gene Duplication of *AmWRKY* Genes

The 67 *AmWRKY* genes were distributed across all the eight snapdragon chromosomes, but their distribution across the respective chromosomes was uneven (Fig. 3). Chromosome 2 contained the highest number of 18, followed by chromosomes 4 and 11. Chromosome 5 contained the lowest number of three. The number of *WRKY* genes on the remaining five chromosomes ranged from five to nine. In general, a chromosomal region within 200-kb containing multiple genes is defined as a gene cluster [53]. According to this criterion, three gene clusters were found, which were distributed on chromosomes 4 and 7 (Fig. 3).

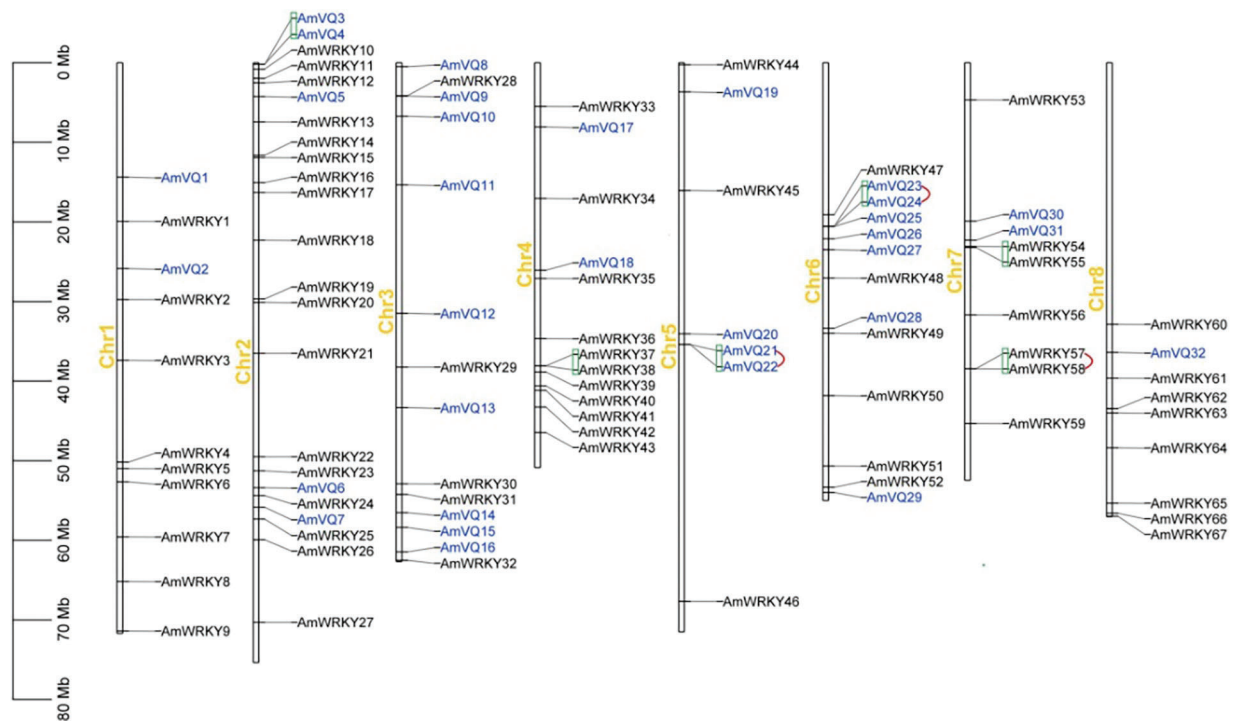


Figure 3: Locations of *AmWRKY* and *AmVQ* genes on snapdragon chromosomes. Gene clusters were marked in green line, and tandem duplication genes were marked in red line

Segmental and tandem duplication were investigated for these *AmWRKY* genes. A total of 74 segmental duplication events involving 44 *AmWRKY* genes were found, which were distributed on all eight chromosomes (Fig. 4a). In contrast, only one tandem repeat gene pair *AmWRKY57/AmWRKY58* was identified, which was distributed on chromosome 7 (Fig. 3). The collinear relationship between snapdragon and *Arabidopsis* was further analyzed. A total of 34 orthologous *WRKY* gene pairs were identified, which involved in 34 *AmWRKY* genes and 29 *AtWRKY* genes (Supplementary Table 4).

3.5 Identification of VQ Genes in Snapdragon

A total of 32 *AmVQ* genes were identified, which were designated from *AmVQ1* to *AmVQ32* based on their locations on the respective chromosome (Supplementary Table 5). For these genes, the protein lengths varied from 102 to 396 amino acids, the isoelectric points ranged from 4.58 to 11.51, and the molecular weights ranged from 11.30 to 42.40 kDa. All of these VQ proteins contained the conserved FxxhVQxhTG motif, except for *AmVQ24* which carried a variant of FxxhVQxhTC (Supplementary Fig. 3).

A phylogenetic tree was constructed based on the VQ-motif sequences of these *AmVQ* proteins (Fig. 5). They were cluster into three groups, according to the classification in *Arabidopsis*. Number of members ranged from one to five in these groups. All of *AmVQ* genes had no introns, except for *AmVQ25* and *AmVQ26* that contained two and one introns, respectively (Supplementary Fig. 4b). The conserved motifs exhibited a district distribution in different group, apart from that of the motif 1 corresponding to the VQ-motif presented in each of *AmVQ* protein (Supplementary Fig. 4c). For example, all members of Group I contained motifs 4, all members of Group IV contained motifs 1 and 2, and all members of Group VI contained motifs 3, 6 and 9. The *cis*-regulatory elements in the promoter were also investigated. Among the 32 *AmVQ* genes, 29 were found to carry at least one type of stress-related element, and 31 were found to carry at least one type of hormone-related element (Supplementary Table 6).

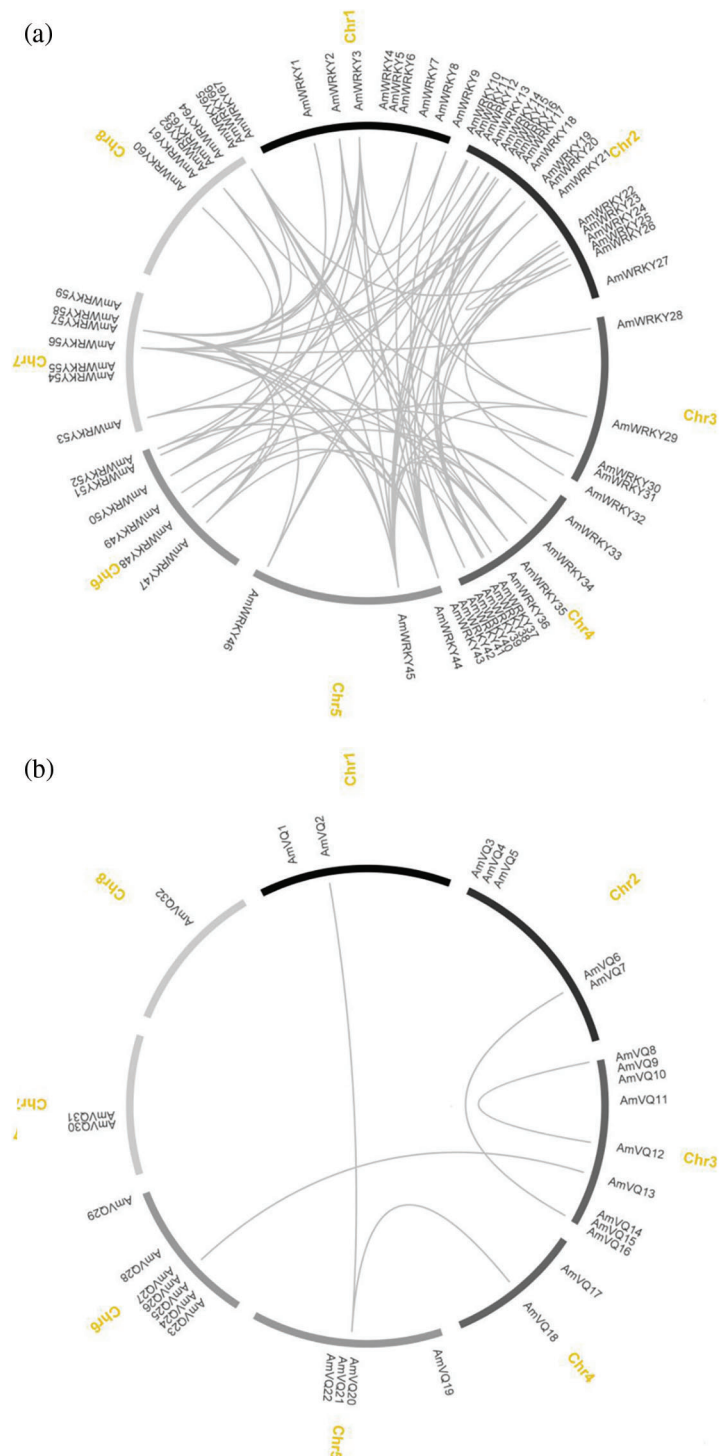


Figure 4: Segmental duplications of *AmWRKY* (a) and *AmVQ* genes (b). The gray lines indicated segmentally duplicated gene pairs

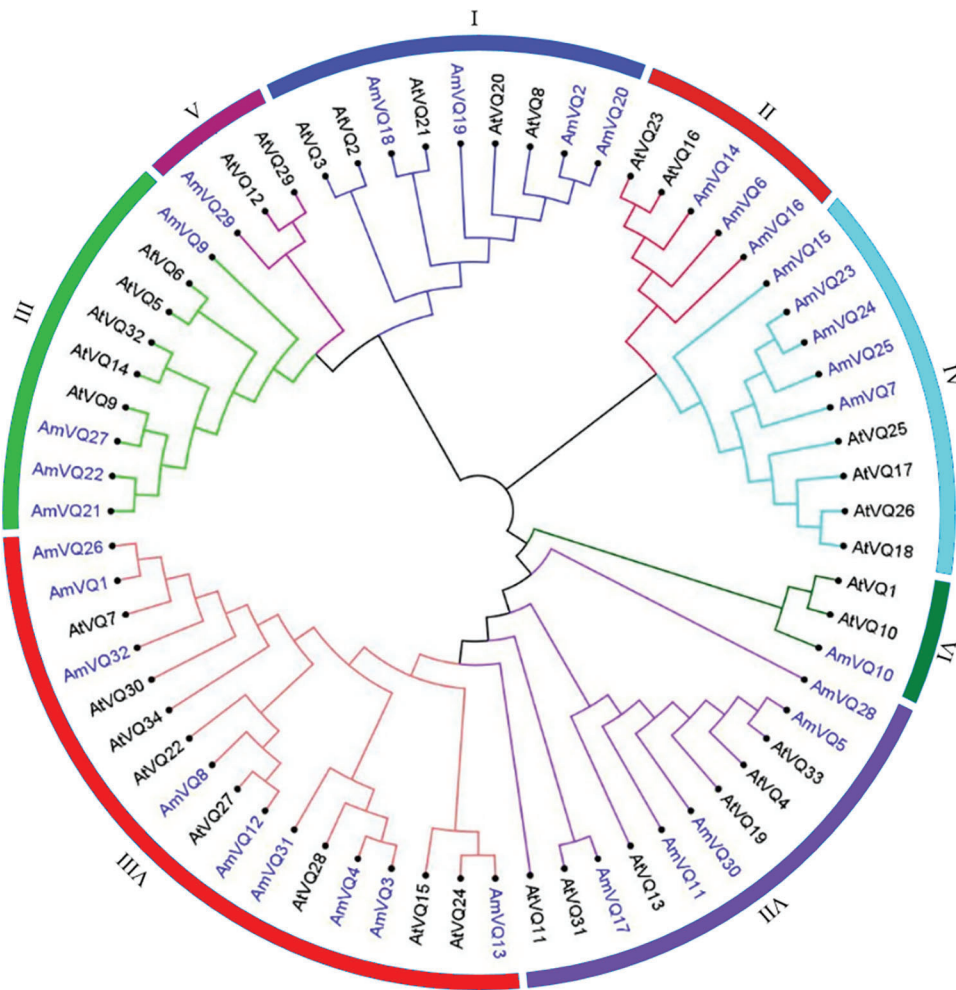


Figure 5: Phylogenetic tree of VQ proteins among snapdragon and *Arabidopsis*. The phylogenetic tree of AmVQ proteins was constructed using MEGA7.0 by the maximum likelihood method with 1,000 bootstrap replicates. AtVQ proteins were used as references to classify AmVQ proteins

The 32 *AmVQ* genes were distributed across all the eight snapdragon chromosomes, and their distribution across the respective chromosomes was also uneven (Fig. 3). Chromosome 3 contained the highest number of nine, followed by chromosomes 6, 2 and 5. Chromosomes 1, 4, 7 and 8 only contained one or two *AmVQ* genes. Three gene clusters were found, which were distributed on chromosomes 2, 5 and 6 (Fig. 3). Two of the clusters consisted of tandem repeat gene pair, including *AmVQ21/AmVQ22* and *AmVQ57/AmVQ58*. A total of five segmental duplication events involving nine *AmVQ* genes were found, which were distributed on chromosomes 1, 2, 3, 4 and 6 (Fig. 4b). The orthologous *VQ* gene pairs between snapdragon and *Arabidopsis* were also analyzed. Ten orthologous gene pairs were identified, which involved in seven *AmVQ* genes and ten *AtVQ* genes (Supplementary Table 4).

3.6 Expression of *AmWRKY* and *AmVQ* Genes in Response to Drought and Cold Stresses

Expression levels of *AmWRKY* and *AmVQ* genes under drought and low temperature treatments were investigated using real-time PCR. Sixteen *AmWRKY* genes showed no detectable expression (Supplementary Table 1). Among the remaining 51 genes, 16 responded to drought and 22 responded to cold (Fig. 6). Compared with normal condition, eight *AmWRKY* genes were up-regulated and the other

eight *AmWRKY* genes were down-regulated under the drought treatment. Under the low temperature treatment, eight *AmWRKY* genes were up-regulated and the remaining 14 genes were down-regulated.

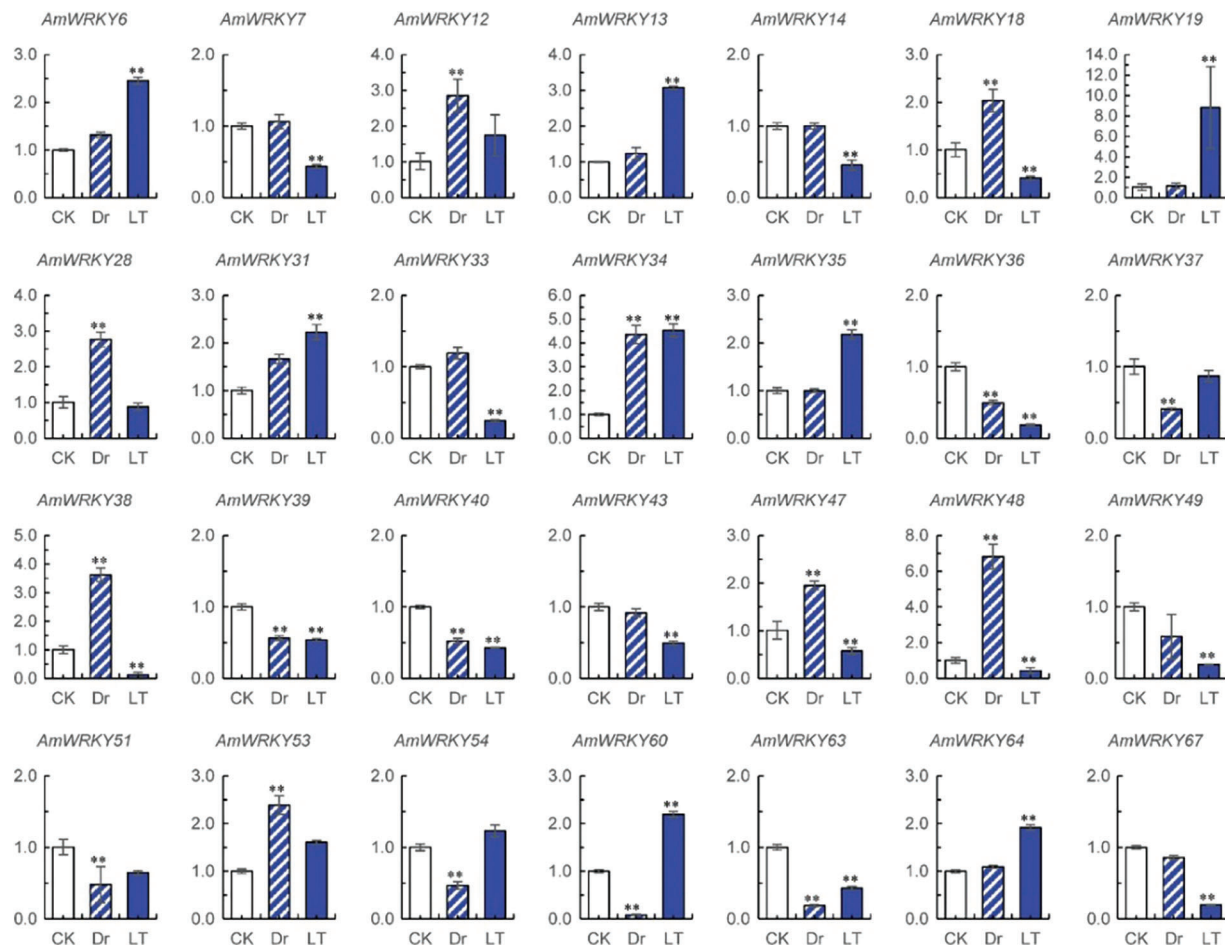


Figure 6: Expressions of *AmWRKY* genes under drought (Dr) and low temperature (LT) treatments. Values are means \pm SD ($n = 3$). Significance was determined using t-test for comparison of gene expression under normal (CK) and abiotic conditions (Dr or LT) (** $P < 0.01$)

Ten of these *AmWRKY* genes simultaneously responded to drought and cold stresses. Five of them exhibited an opposite response pattern to different stresses. *AmWRKY18*, *AmWRKY38*, *AmWRKY47* and *AmWRKY48* were up-regulated under drought but down-regulated under cold, while *AmWRKY60* was down-regulated under drought but up-regulated under cold. The remaining five genes exhibited a same response pattern to the different stresses. *AmWRKY34* was up-regulated under both drought and cold stresses, while *AmWRKY36*, *AmWRKY39*, *AmWRKY40* and *AmWRKY63* were down-regulated under both stresses.

For the 32 *AmVQ* genes, two showed no detectable expression (Supplementary Table 1). Among the remaining 30 genes, 10 were found to respond to drought stress and 18 were found to respond to cold stress (Fig. 7). In the drought treatment, six *AmVQ* genes were up-regulated and the other four genes were down-regulated. In the low temperature treatment, nine *AmVQ* genes were up-regulated and the other nine genes were down-regulated. Eight of these *AmVQ* genes simultaneously responded to drought and cold stresses, all of which showed a same response pattern to both stresses. *AmVQ10*, *AmVQ15* and

AmVQ31 were up-regulated under both stresses, while *AmVQ12*, *AmVQ14*, *AmVQ16*, *AmVQ19* and *AmVQ29* were down-regulated under both stresses.

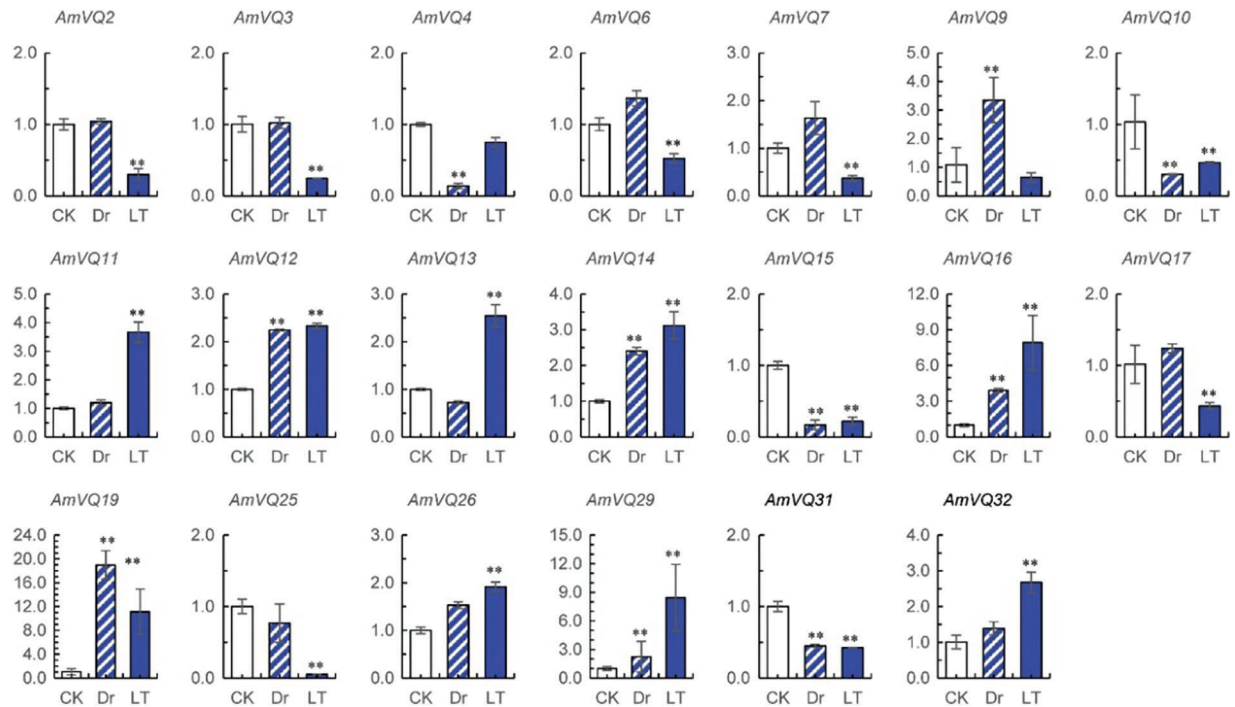


Figure 7: Expressions of *AmVQ* genes under drought (Dr) and low temperature (LT) treatments. Values are means \pm SD ($n = 3$). Significance was determined using the t-test for comparison of gene expression under normal (CK) and abiotic conditions (Dr or LT) (** $P < 0.01$)

3.7 Protein-Protein Interaction of *AmWRKY* and *AmVQ* Proteins

Protein-protein interactions between *AmWRKY* and *AmVQ* proteins were predicted based on the *Arabidopsis* orthologs (Fig. 8). Three *AmWRKY* proteins belonging to Groups I or IIc were predicted to interact with four *AmVQ* proteins. *AmWRKY6* (AtWRKY25 ortholog) was presumed to interact with four *AmVQ* proteins, including *AmVQ7* (AtVQ8/18 ortholog), *AmVQ11* (AtVQ19/33 ortholog), *AmVQ15* (AtVQ25 ortholog) and *AmVQ28* (AtVQ11 ortholog). *AmWRKY14* (AtWRKY34 ortholog) and *AmWRKY43* (AtWRKY24 ortholog) were also presumed to interact with *AmVQ7* (AtVQ8/18 ortholog).

Interactions among *AmWRKY* proteins were also identified. Two members of Group IIa, *AmWRKY33* (AtWRKY18 ortholog) and *AmWRKY49* (AtWRKY40 ortholog), were presumed to interact with each other. Two members of Group IIe, *AmWRKY32* (AtWRKY29 ortholog) and *AmWRKY37* (AtWRKY22 ortholog), were presumed to interact with each other. *AmWRKY36* (AtWRKY30 ortholog) was predicted to interact with *AmWRKY26* (AtWRKY53 ortholog), *AmWRKY57* (AtWRKY54 ortholog) and *AmWRKY67* (AtWRKY70 ortholog), all of which belonging to the Group III.

4 Discussion

The *WRKY* gene family is one of the largest families of transcription factors. The *VQ* protein is the most important *WRKY*-interacting protein identified in the past few years [26]. The *WRKY* and *VQ* genes and their interactions are crucial for regulating life processes in plants [26,27,54]. In this study, we performed a genome-wide analysis of *WRKY* and *VQ* genes in snapdragon, including gene classification, gene

duplication investigation, gene expression response to abiotic stresses and protein-protein interaction prediction. Our work lays a foundation for elucidating the structure, evolution and functional roles of *WRKY* and *VQ* genes in snapdragon, which generates new knowledge on essential and critical components of abiotic stress tolerance in this plant species.

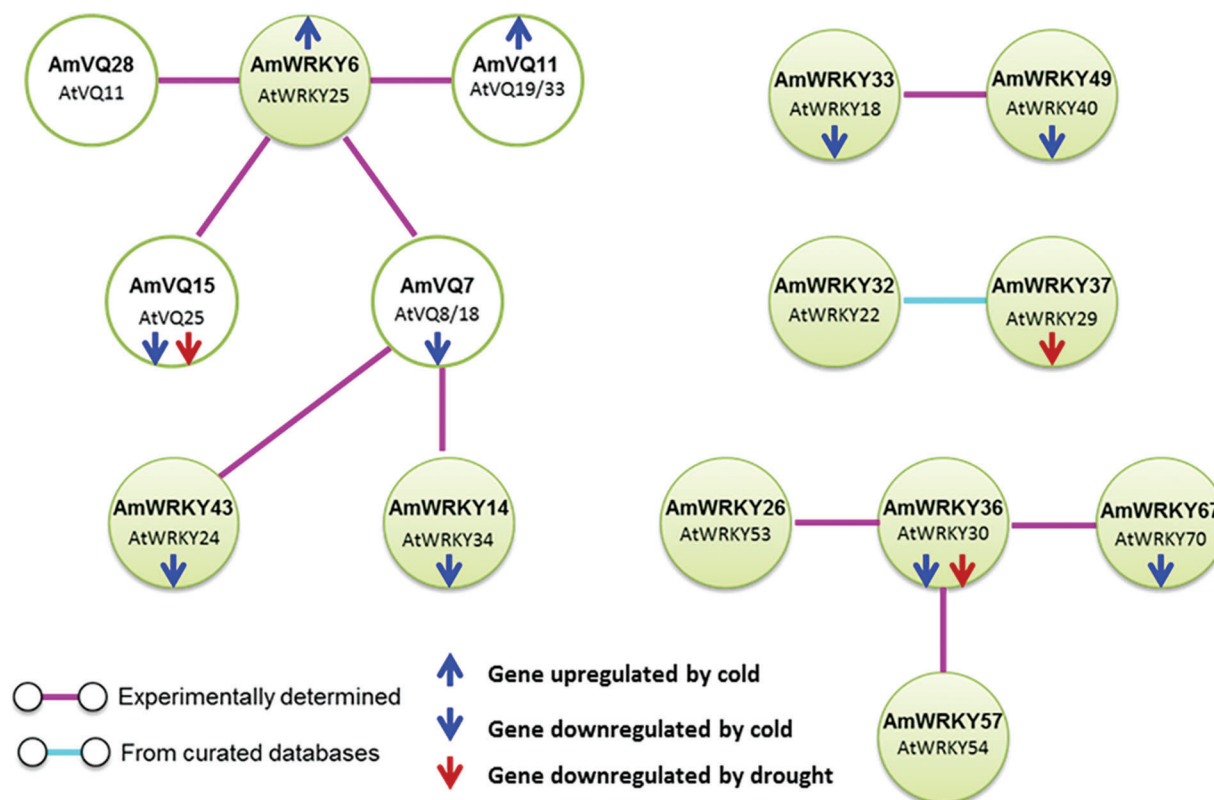


Figure 8: Protein-protein interactions of WRKY and VQ proteins predicted in snapdragon

A total of 67 *AmWRKY* genes were identified in this study (Supplementary Table 2). These genes were classified into the different groups or subgroups based on the phylogenetic tree, of which Group IIc possessed the most members (Fig. 1). This was similar to many other dicot plant species [6,7,9,11,55,56], suggesting that this subgroup was more active in the evolution of dicots. The WRKYGQK core sequence is a typical signature of WRKY proteins. Overwhelming majority of *AmWRKY* proteins had the highly conserved WRKYGQK core sequence. However, *AmWRKY42* belonging to Group IIc was found to carry the WRKYGKK variant (Supplementary Fig. 2). This was consistent with that the WRKYGKK variant was frequently occurred in Group IIc of plant *WRKY* genes [4,7,9,55,57–59]. It has been showed that variation of the WRKYGQK core sequence might affect the binding ability of the WRKY transcription factor to the W-box motif [60,61]. In *Arabidopsis*, three WRKY proteins carrying the variant WRKYGKK domain were identified, including *AtWRKY50*, *AtWRKY51* and *AtWRKY59*. These three genes were clustered into a same clade with *AmWRKY42*, based on the WRKY domain sequence (Fig. 1). Among these three *Arabidopsis* genes, *AtWRKY50* was reported to lack W-box binding activity [62], while *AtWRKY59* was found to activate downstream genes mainly through a newly discovered WT-box motif rather than the W-box motif [63]. Therefore, *AmWRKY42* might have different DNA binding specificity. A total of 32 *AmVQ* genes were found in this study (Supplementary Table 5). All of them contained the conserved FxxhVQxhTG motif, except for *AmVQ24* which carried a variant of FxxhVQxhTC

(Supplementary Fig. 3). The FxxhVQxhTG motif mediates protein localization and the interaction between VQ proteins and other regulators [64]. Thus, AmVQ24 may possess a different biological function.

Tandem duplication and segmental duplication play important roles in the amplification and evolution of the plant gene family. In the present study, 74 segmental duplication events involving 44 *AmWRKY* genes were identified (Fig. 4), whereas only one tandem duplication event was found with two *AmWRKY* genes (Fig. 3). Therefore, segmental duplication events might be the main driving force in the evolution of *WRKY* gene family in snapdragon. For *AmVQ* genes, five segmental duplication events and two tandem duplication events were found, implying that both duplication events contributed to the evolution of *VQ* gene family in snapdragon.

Gene expression reprogramming is one of the vital strategies for plants to adapt to diverse environmental stresses. In the present study, 28 *AmWRKY* genes and 21 *AmVQ* genes were found to respond to drought and cold stresses (Figs. 6 and 7), indicating they might participate in the regulation of snapdragon abiotic stress tolerances. Two of these *WRKY* genes, *AmWRKY14* and *AmWRKY36*, were found to be the orthologs of *Arabidopsis* abiotic stress regulators *AtWRKY34* and *AtWRKY30*, respectively (Supplementary Table 4). *AtWRKY34* and *AtWRKY30* were up-regulated under cold and drought stresses, respectively [13,14]. However, *AmWRKY14* and *AmWRKY36* were repressed by cold and drought in the present study, respectively (Fig. 6). These two *AmWRKY* genes might have different functioning ways compared with their orthologs in *Arabidopsis*. Among the *AmVQ* genes, eight were found to simultaneously respond to drought and cold stresses, all of which showed similar expression patterns under the two different stresses (Fig. 7). A similar phenomenon was also observed for rice *VQ* genes involved in drought and salinity responses [65], and for *Eucalyptus grandis* *VQ* genes involved in cold and heat responses [66]. These results suggested that *VQ* genes may exist in the same response pathway to different abiotic stresses.

Accumulated evidences suggest that *WRKY-VQ* interaction plays important roles in plant response to abiotic stresses. For instance, *Arabidopsis AtWRKY8* and *AtVQ9* could be strongly induced by salt stress, which antagonistically regulates salinity stress tolerance by forming a physical complex [28]. Banana *MaWRKY26* and *MaVQ5* could be rapidly induced by cold stress, and they positively regulate cold stress tolerance by physical interacting with each other [34]. In the present study, *WRKY-VQ* interactions were predicted between three *AmWRKY* transcription factors and four *AmVQ* proteins (Fig. 7). All these *AmWRKY* genes and three *AmVQ* genes, including *AmWRKY6*, *AmWRKY14*, *AmWRKY43*, *AmVQ7*, *AmVQ11* and *AmVQ15* exhibited differential expression under cold stresses (Figs. 6–8). These *AmWRKY* and *AmVQ* genes might regulate snapdragon cold response by forming a complicated network. *AmWRKY6*, *AmWRKY14* and *AmWRKY43* belonged to either Group I or Group IIc. This was consistent with the previous observation that *VQ* proteins interact only with Group I and IIc *WRKY* transcription factors, but failed to interact with Group IIa, IIb, IIc, IIe, and III *WRKY* transcription factors [32,67].

Cooperation among *WRKY* proteins is another area of interest in the research community. *WRKY* transcription factors regulate downstream gene expression by binding conserved W-box *cis*-acting elements of the downstream gene. Some *WRKY* genes also have W-box elements in their promoters. Thus, these *WRKY* transcription factors could perform self-regulation or cross-regulation by binding with their own W-box elements [5]. Cooperation among *WRKY* proteins could also be achieved by protein-protein interactions. The *WRKY-WRKY* interactions have been previously observed within members of Group IIa, Group IIb and Group III, or between members of these groups in plants [26]. In the present study, a total of 41 *AmWRKY* genes were found to carry the W-box *cis*-acting elements in their 2-kb promoter region (Supplementary Tab. 3). The *WRKY-WRKY* interaction was also predicted within members of Group IIa and Group IIb (Fig. 8). These results indicated that the cooperation among *WRKY* transcription factors may also exist in snapdragon. Three *WRKY* genes with W-box elements, *AmWRKY36*, *AmWRKY67* and *AmWRKY49* were predicted to participate in *WRKY-WRKY* interactions.

All of them were found to be downregulated under cold stress. These three genes could be good targets for studying the cooperation of *WRKY* genes in snapdragon response to abiotic stresses.

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