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Genome-Wide Analysis and Expression Profiling of Watermelon *VQ* Motif-Containing Genes Under Abiotic and Biotic Stresses

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Abstract: Valine-glutamine (VQ) motif-containing proteins play important roles in diverse plant developmental processes and signal transduction in response to biotic and abiotic stresses. However, no systematic investigation has been conducted on *VQ* genes in watermelon. In this study, we identified 31 watermelon *VQ* genes, which were classified into six subfamilies (I–VI). All of the deduced proteins contained a conserved FxxxVQxL/F/VTG motif. Eleven *ClVQs* were involved in segment duplication, which was the main factor in the expansion of the VQ family in watermelon. Numerous stress- and hormone-responsive *cis*-elements were detected in the putative promoter region of the *ClVQ* genes. Green fluorescent protein fusion proteins for ten selected ClVQs were localized in the nucleus, but three ClVQs also showed signals in cell membranes and the cell wall, thus confirming their predicted divergent functionality. Quantitative real-time PCR (qRT-PCR) analysis indicated that the majority of *ClVQ* genes were specifically or preferentially expressed in certain tissues or organs, especially in the male flower. Analyses of RNA-sequencing data under osmotic, cold, and drought stresses and *Cucumber green mottle mosaic virus* (CGMMV) infection revealed that the majority of *ClVQ* genes, especially those from subfamily IV, were responsive to these stresses. The results provide useful information for the functional characterization of watermelon *ClVQ* genes to unravel their biological roles.

Keywords: valine-glutamine (VQ); watermelon; identification; phylogeny; expression profiles; subcellular location; stresses

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1. Introduction

Valine-glutamine (VQ) motif-containing proteins, which incorporate a highly conserved FxxxVQxLTG motif and interact with WRKY transcription factors via the conserved V and Q residues, have been characterized in many plant species in recent years [1–4]. VQ proteins are integral to plant growth and developmental processes. A growing body of research suggests that these proteins also have significant roles in mediating responses to a variety of biotic and abiotic stresses. More than half of the identified *VQs* are responsive to biotic or abiotic stresses or phytohormones in *Arabidopsis*, rice, maize, and Chinese cabbage [1,2,5,6]. We summarized the biological functions of VQ proteins in different plants in response to various stresses in Table 1. In *Arabidopsis*, calmodulin (CaM)-binding proteins *CamBP25* (*AtVQ15*) and *AtVQ9* have negative effects in response to salt and osmotic stress [7,8]. The overexpression of *PeVQ28* from Moso bamboo and *StVQ31* from potato has been demonstrated to enhance salt tolerance through mechanisms involving osmotic and antioxidant cellular homeostasis, as well as the ABA-dependent signaling pathway [9,10]. Wheat *TaVQ4-D* has been shown to positively regulate drought tolerance

in transgenic plants [11]. Furthermore, certain VQ proteins are involved in defense responses against pathogens; specifically, *Arabidopsis VQ12* and *VQ29* have been found to negatively modulate basal defense against *Botrytis cinerea* [12]. In contrast, overexpressed *Arabidopsis VQ21* (*MKS1*) increases tolerance to *Pseudomonas syringae* in transgenic Petunia plants but leads to susceptibility to *B. cinerea* in *Arabidopsis* [13,14]. Meanwhile, the *vq21/mks1* knockout mutants exhibit increased susceptibility to strains of *P. syringae* and *Hyaloperonospora arabidopsidis* [15]. *Arabidopsis VQ28* acts as a negative regulator of plant non-host resistance (NHR) to *Phytophthora pathogens* through ABA, salicylic acid (SA), and jasmonate (JA) pathways [16]. *AtVQ23/SIB1* and *AtVQ16/SIB2* transcript levels are strongly induced by *B. cinerea* and *P. syringae* infection [17–19]. *JAV1/AtVQ22* modulates jasmonate (JA)-regulated plant immune responses [8]. The expression levels of rice *VQ* genes exhibit variation in response to *Xanthomonas oryzae*, *X. oryzae*, and *Magnaporthe oryzae* [20,21]. The overexpression of soybean *GmVQ35* and *GmVQ47* genes results in increased susceptibility of transgenic *Arabidopsis* to *Botrytis* infection [22]. Furthermore, the poplar *VQ1* gene imparts salt tolerance and pathogen resistance in transgenic *Arabidopsis* through modifications in hormonal signaling pathways [23].

Table 1. Biological functions of VQ proteins in different plants in response to various stresses.

Gene Name	Gene ID	Species	Functions	References
CamBP25 (AtVQ15) and *AtVQ9*	AT2G41010, AT1G78310	*Arabidopsis*	negatively effect in response to salt and osmotic stress	[7,8]
AtVQ12	AT2G22880	*Arabidopsis*	negatively modulates basal defense against *Botrytis cinerea*	[12]
AtVQ29	AT4G37710	*Arabidopsis*	negatively modulates basal defense against *Botrytis cinerea*	[12]
AtVQ21 (*MKS1*)	AT3G18690	*Arabidopsis*	increases tolerance to *Pseudomonas syringae* in transgenic petunia plants but leads to susceptibility to *B. cinerea* in *Arabidopsis*, its knockout mutants exhibit increased susceptibility to strains of *P. syringae* and *Hyaloperonospora arabidopsidis*	[13–15]
AtVQ28	AT4G20000	*Arabidopsis*	acts as a negative regulator of plant non-host resistance (NHR) to Phytophthora pathogens through ABA, salicylic acid (SA), and jasmonate (JA) pathways	[16]
AtVQ23/SIB1 and *AtVQ16/SIB2*	AT3G56710, AT2G41180	*Arabidopsis*	strongly induced by *B. cinerea* and *P. syringae* infection	[17–19]
JAV1/AtVQ22	AT3G22160	*Arabidopsis*	modulates jasmonate (JA)-regulated plant immune responses	[8]
StVQ31	PGSC0003DMG400028198	Potato	enhances salt tolerance via ABA-dependent signaling pathway	[10]

Table 1. *Cont.*

Gene Name	Gene ID	Species	Functions	References
PeVQ28	PH01007611G0010	Moso bamboo	enhances salt tolerance through mechanisms involving osmotic and antioxidant cellular homeostasis	[9]
TaVQ4-D	TraesCS1D02G340900	Wheat	positively regulates drought tolerance in transgenic plants	[11]
GmVQ35 and *GmVQ47*	Glyma08g15620, Glyma11g04970	Soybean	increase susceptibility of transgenic *Arabidopsis* to *Botrytis infection*	[22]
PtVQ1	Potri.001G029700	Poplar	imparts salt tolerance and pathogen resistance in transgenic *Arabidopsis* through modifications in hormonal signaling pathways	[23]

Watermelon represents a fruit crop of significant economic value and ranks among the top five most consumed fresh fruits globally. However, it is highly vulnerable to a range of adverse environmental conditions resulting in a reduction in its quality and yield. Therefore, the identification of resistance genes is of considerable importance for enhancing both the yield and quality of watermelon through molecular breeding techniques. *VQ* genes play a crucial role in the plant stress signaling network, and their identification and characterization have been conducted in various plant species [1,3–6,20,22,24–31]. Nevertheless, a comprehensive characterization of the *VQ* genes in watermelon remains lacking.

In this study, a comprehensive identification of 31 putative *VQ* elements in watermelon was conducted. These analyses encompassed their classification, chromosomal distribution, phylogenetic relationships, gene duplications, synteny relationships, conserved motifs, subcellular localizations, expression patterns, and so on. These studies offer a comprehensive analysis of *ClVQ* genes in watermelon, providing valuable insights for future functional investigations and the potential application of novel *ClVQ* candidate genes in crop improvement, particularly concerning stress resistance, growth, and development.

2. Materials and Methods

2.1. Identification of VQs in Watermelon and Cucumber

Protein sequences of the *VQ* gene, comprising 34 members in *Arabidopsis*, 40 in rice, 61 in maize, 74 in soybean, and 51 in grapevine, were obtained from the Phytozome portal. (http://phytozome.jgi.doe.gov/pz/portal.html, accessed on 14 December 2024). These sequences were then used as queries to carry out BLASTP searches with E-value of 1×10^{-5} as the threshold. These sequences were then used as queries to identify the *VQ* genes in putative watermelon and cucumber VQ proteins as reported in He et al. [32]. ExPASy (http://web.expasy.org/compute_pi/, accessed on 14 December 2024) and WoLF PSORT website (https://wolfpsort.hgc.jp/, accessed on 14 December 2024) were used to predict molecular weights, isoelectric points (PIs), and subcellular localizations of putative VQ proteins of watermelon, respectively.

2.2. Phylogenetic Analysis, Motif Analysis, and Gene Structure Construction

Multiple-sequence alignment of predicted peptide sequences of the conserved VQ domain was carried out using Clustal X v1.81 with default parameters [33]. Phylogenetic analysis based on full-length protein sequences was performed with MEGA 5.0 software us-

ing the neighbor-joining (NJ) method with 1000 bootstrap replicates [34]. The predicted peptide sequences of conserved domains in VQ proteins were confirmed using the Conserved Domains Database (NCBI CDD; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 14 December 2024) and Simple Modular Architecture Research Tool (SMART) database (http://smart.emblheidelberg.de/, accessed on 14 December 2024). The similarity of VQs from *Arabidopsis*, watermelon, and cucumber was calculated using DNAStar software (Madison, WI, USA). Motif analysis and annotation of conserved motifs in VQ proteins were conducted using MEME (http://meme-suite.org/tools/meme, accessed on 14 December 2024) with a 5 maximum numbers of motifs. Intron/exon distribution of all watermelon VQ genes was analyzed using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/, accessed on 14 December 2024).

2.3. Chromosomal Localization, Evolutionary, and Gene Ontology (GO) Enrichment Analyses

The chromosomal locations of all *VQ* genes were obtained from CuGenDB (http://cucurbitgenomics.org/, accessed on 14 December 2024). The segmental duplication, tandem duplication, synonymous (*Ks*) and non-synonymous (*Ka*) substitution rates, synteny analyses, and divergence time of each duplicated gene pair were examined following the methodologies outlined by He [32]. Furthermore, the *VQ* genes in watermelon were subjected to Gene Ontology (GO) enrichment analysis using AgBase (https://agbase.arizona.edu/, accessed on 14 December 2024).

2.4. Analysis of cis-Acting Elements in VQ Putative Promoter Regions in Watermelon

To identify the *cis*-elements in promoter sequences of *VQ* genes in watermelon, the 1.5-kb upstream regions were obtained from the Cucurbit Genomics Database and analyzed using the online tools of the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html, accessed on 14 December 2024). The obtained results were subsequently manually screened for *cis*-elements associated with stress- or hormone-responsiveness, based on the website annotations, and are summarized.

2.5. Expression Analysis of Watermelon VQ Genes and Watermelon Plant Growth and Treatments

The watermelon advanced inbred line '97103' was used for expression analyses. The plants were grown in temperature-controlled greenhouses under day/night temperatures of 28/22 ± 1 °C, light intensity of 200 μmol m^{-2} s^{-1}, and a 16 h light/8 h dark photoperiod. Roots, stems, leaves, male flowers, female flowers, and fruit (diameter about 3 cm) were sampled during the flowering period. All materials were frozen at −80 °C until RNA isolation. Three biological and three technical replicates for each sample were analyzed. The expression levels of *ClVQ* genes in fruit across various developmental stages, as well as in response to diverse abiotic and biotic stresses, were assessed utilizing RNA-Seq data available from the Cucurbit Genomics Database. Fruit at different stages, biotic and abiotic stress-inducible expression patterns of the detected *ClVQs* were analyzed and used to generate the heatmap with Multiple Array Viewer [35].

2.6. RNA Isolation and qRT–PCR

High-quality RNA was extracted from samples using TRIzol™ reagent (Invitrogen, Germany) by following the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg total RNA using the PrimeScript™ RT Reagent Kit (Takara, Japan). Specific primers used in the qRT-PCR analysis were designed using Primer 5 software, and each primer was searched in the watermelon database to ensure its specificity (Table S6). The qRT-PCR reactions were performed following the protocol established by He et al. (2018) [32]. The qRT-PCR reactions (reaction volume, 20 μL) were performed on a CFX96 Real-Time System machine (Bio-RAD, USA), programmed to heat for 30 s at 95 °C, followed by

40 cycles of 5 s at 95 °C and 45 s at 55 °C, and finally, 1 cycle of 1 min at 95 °C, 30 s at 50 °C, and 30 s at 95 °C. Three biological and three technical replicates for each sample were analyzed using the SYBR® Premix Ex Taq™ kit (TOYOBO, Japan). Watermelon *β-actin* (*Cla007792*) was selected as an internal control. The relative gene expression level was calculated using the $2^{-\triangle\triangle Ct}$ method.

2.7. Subcellular Localization Analyses

The CDS sequences of ten *ClVQ* genes representing all 6 VQ subfamilies, namely *ClVQ02*, *ClVQ05*, *ClVQ07*, *ClVQ11*, *ClVQ16*, *ClVQ18*, *ClVQ22*, *ClVQ24*, *ClVQ25*, and *ClVQ26*, were amplified using gene-specific primers and cloned into the pFGC-eGFP plasmid via the *Xba*I and *BamH*I restriction sites (Table S6). These plasmids were transformed into *Agrobacteriumt tumefaciens* GV3101 and transiently expressed in tobacco leaf cells as reported previously by He et al., 2018. [32]. Images were acquired at 48 h using a Leica DMLE camera (Leica, Wetzlar, Germany).

3. Results

3.1. Identification and Characterization of VQ Genes in Watermelon and Cucumber

In total, 389 VQ protein sequences from *Arabidopsis*, rice, maize, soybean, Chinese cabbage, and grapevine were downloaded and then used as queries in searches using the Protein Basic Local Alignment Search Tool (BLASTP) with an E-value of 1×10^{-5} as the threshold in the Cucurbit Genomics Database. A total of 35 putative hits were identified in the watermelon genome database. Thirty-two sequences were annotated as watermelon *VQ* genes after searches with HMMER 3.0 using the global HMM profile of the VQ characteristic domain. Redundant sequences identified by these two methods were omitted to obtain unique putative *VQ* genes, and the remaining hits were further filtered using the CDD and SMART databases based on the presence of structural characteristics and the conserved VQ domain. Ultimately, 31 *ClVQ* genes were identified and named *ClVQ1* to *ClVQ31,* consistent with the linear order along a chromosome.

The amino acid sequences of all ClVQ proteins ranged from 80 to 405 amino acids in length, and the molecular weights ranged from 9.2 to 42.48 kDa (Table S1). All of the ClVQs had a highly conserved VQ domain at the N-terminal end, as identified by SMART, which contains a conserved short VQ motif (Figure S1). Five motifs (motifs 1–5) were identified by the MEME tool, of which motif 1 corresponded to the VQ motif (Figure 1C). Of the 31 ClVQ proteins identified, 23 contained the conserved VQ motif FxxxVQxLTG, and the remaining 8 proteins contained variant motifs. It is worth noting that the VQ motifs of all ClVQs from subfamily I were variants of which five ClVQs contained the motif FxxxVQxFTG and one ClVQ contained FxxxVQxVTG (Figure 2), which were probably associated with the functional variation of ClVQs in subfamily I. In addition, the core amino acids of ClVQ07 were LTA instead of LTG, which was similar to maize ZmVQ06 [5].

The watermelon ClVQs were predicted to be located in the nucleus (17 ClVQs), chloroplast (11 ClVQs), and cytoplasm (3 ClVQs) (Table S1). It is worth mentioning that all *ClVQ* genes lacked an intron except *ClVQ09* and *ClVQ29*, which each contained one intron (Figure 1B). The intron-free phenomenon was detected previously in certain species, such as *Arabidopsis*, Chinese cabbage, and maize [5,6,36], whereas all *VQs* in grapevine and Moso bamboo contain introns [4,26]. The number of *VQ* genes in diverse plant species varies from 18 (grapevine) to 74 (soybean). The 31 *VQs* identified in watermelon are presently fewer than the number detected in all other plant species except Moso bamboo (29), tea (25), and grapevine (18).

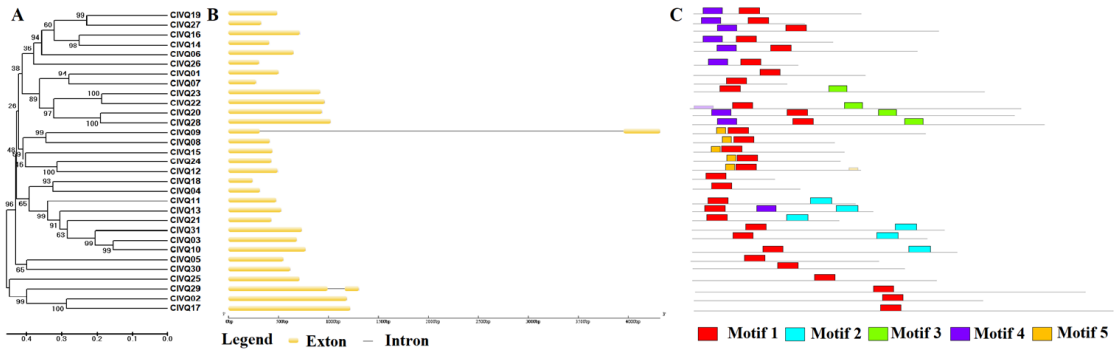

Figure 1. Phylogenetic relationships, gene structure, and conserved motifs of all VQs identified in watermelon. (**A**) Unrooted phylogenetic tree generated based on the deduced amino acid sequences using the neighbor-joining method implemented in MEGA 5. Bootstrap support values from 1000 replicates are provided for each branch. (**B**) Gene structure analyzed using the Gene Structure Display Server. Yellow boxes indicate exons, and lines indicate introns. (**C**) Motif analysis performed using MEME 4.0 software. Different-colored boxes represent different motifs in the corresponding position of each ClVQ protein.

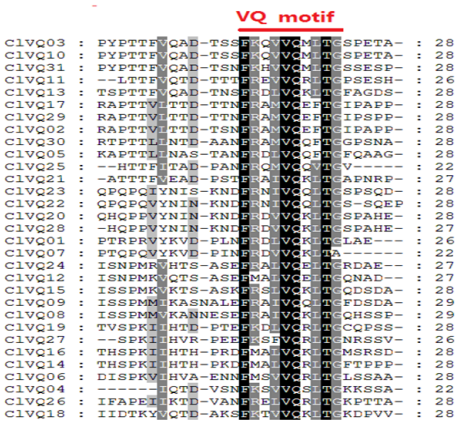

Figure 2. Amino acid sequence alignment of the VQ domain from watermelon VQs. Sequences were aligned using Clustal X. Conserved motifs are marked.

Table 2. Summary of the number of *VQ* genes in diverse plant species.

Species	The Number of VQs	Genome Size (Mb)	Reference
Arabidopsis thaliana	34	125	[1]
Oryza sativa	39	373	[2]
Zea mays	61	230	[5]
Brassica rapa	57	284	[6]
Phyllostachys edulis	29	2021	[26]
Glycine max	74	975	[3]
Camellia sinensis	25	4000	[34]
Vitis vinifera	18	487	[4]
Populus trichocarpa	51	480	[25]
Cucumis sativus	32	367	This work
Citrullus lanatus	31	425	This work

3.2. Phylogenetic Relationship of Plant VQ Members

To further analyze the phylogenetic relationships among VQ proteins, the amino acid sequences of 328 VQ proteins from *Arabidopsis*, rice, maize, soybean, Chinese cabbage, cucumber, and watermelon were compiled into a multiple alignment and subjected to phylogenetic analysis. On the basis of the phylogenetic tree, the VQ proteins could

be classified into six distinct subfamilies (Figure 3). Subfamily IV contained the highest number of VQ family members, whereas subfamily II comprised the fewest VQ proteins. Watermelon VQs were distributed among all of the six subfamilies. In detail, six ClVQ proteins were placed in subfamily I, one ClVQ protein in subfamily II, four ClVQ proteins in subfamily III, eight ClVQ proteins in subfamily IV, seven ClVQ proteins in subfamily V, and five ClVQ proteins in subfamily VI. Within a subfamily, ClVQs were usually most closely related to cucumber VQs. In turn, the VQs of watermelon and cucumber showed one-to-many relationships with soybean VQs. The phylogenetic analysis indicated that the VQ proteins of the five dicotyledon species (*Arabidopsis*, soybean, Chinese cabbage, watermelon, and cucumber) showed closer relationships than with the VQs of rice and maize, which was consistent with the general evolutionary relationships between dicotyledon and monocotyledon angiosperms.

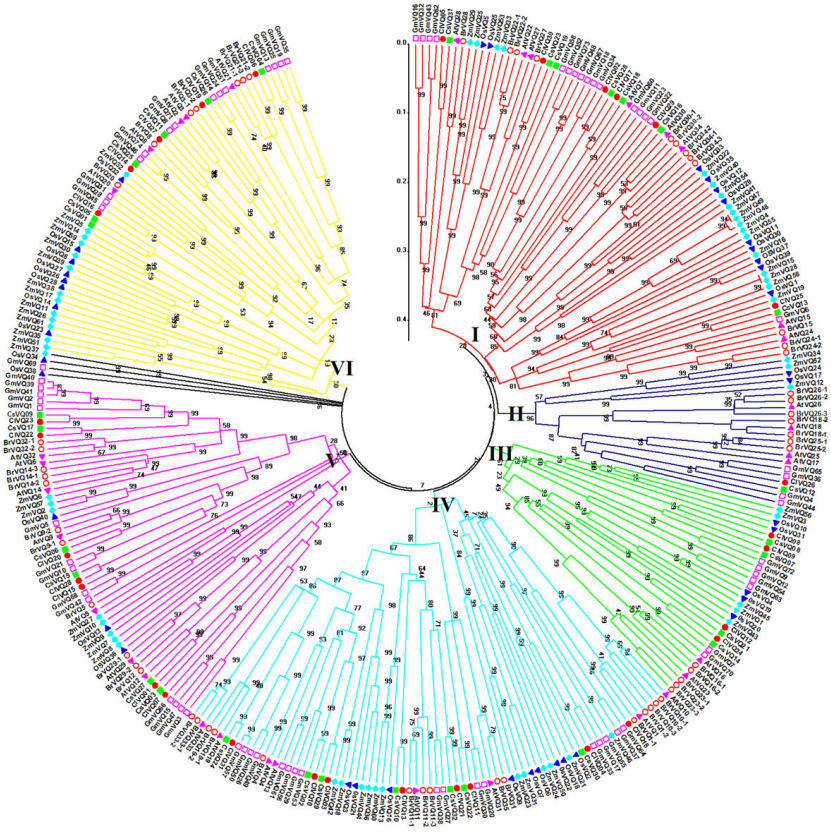

Figure 3. Phylogenetic relationships among VQ proteins of *Arabidopsis*, rice, maize, soybean, Chinese cabbage, watermelon, and cucumber. Phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA 5.0. Bootstrap support values from 1000 replicates are provided for each branch. The bar represents relative divergence of the sequences examined. Different subgroups of VQ are highlighted in different colors.

3.3. Genomic Distribution and Evolutionary Analysis of Watermelon VQs

All *VQ* genes of watermelon were non-randomly located on the 11 watermelon chromosomes (Figure 4). Chromosome (Chr) 10 carried the most *ClVQ* genes, and a single *ClVQ* was mapped to each of Chr 03 and Chr 06, respectively. Only one tandem duplication pair involving 2 *ClVQ* genes was identified (Figure 4 and Table 3). The duplicated gene pair (*ClVQ08* and *ClVQ09*) was mapped to Chr 02 and showed 65.9% amino acid sequence similarity to each other (Figure 4 and Table S2). The *Ks* of the duplicated pair was 9.0346 with a corresponding divergence time of 694.97 million years ago (Mya). Five gene pairs involving 9 *ClVQ* genes were the result of segment duplication (Table 3). *ClVQ03* on Chr 01 was mapped to a duplicated region shared with *ClVQ31* on Chr 11. *ClVQ14* on Chr

05 was duplicated with *ClVQ16* on Chr 06. The putative duplicated gene pair of *ClVQ20* and *ClVQ28* were located on Chr 08 and Chr 11. *ClVQ22* on Chr 09 and *ClVQ23* on Chr10 also represented a pair of duplicated genes. The *Ks* values of the segment-duplicated gene pairs ranged from 1.4489 to 49.2582 and the corresponding divergence times ranged from 111.45 to 3789.09 Mya. The *Ka*/*Ks* values of all tandem and segment duplicates were less than 1, indicating that they have undergone purifying selection.

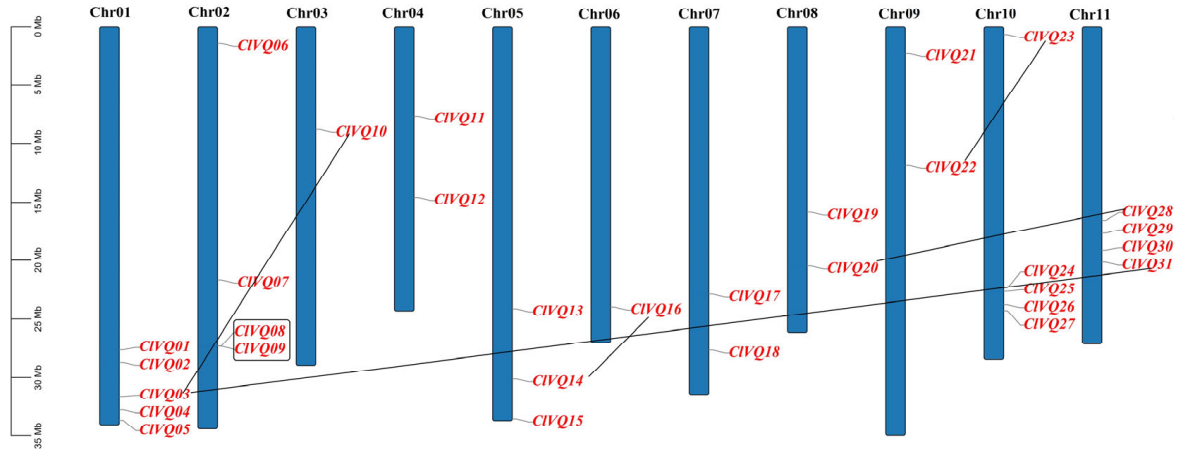

Figure 4. Chromosomal distribution of *VQ* genes in watermelon. Chromosome number is indicated at the top of each chromosome. Black box highlights pairs of tandemly duplicated genes. Duplicated gene pairs are linked by a black line.

Table 3. *Ka*/*Ks* calculation and divergent time of the duplicated watermelon *VQ* gene pairs.

Duplicated Gene Pairs	*Ks*	*Ka*	*Ka/Ks*	Duplicated Type	Purify Selection	Time * (MYA)
ClVQ08/ClVQ09	9.0346	0.8071	0.0893	Tandem	Yes	694.97
ClVQ03/ClVQ10	1.4489	0.3047	0.2103	Segmental	Yes	111.45
ClVQ03/ClVQ31	6.4362	0.4161	0.0647	Segmental	Yes	495.09
ClVQ14/ClVQ16	49.2582	0.5760	0.0117	Segmental	Yes	3789.09
ClVQ20/ClVQ28	1.9273	0.3427	0.1778	Segmental	Yes	148.25
ClVQ22/ClVQ23	2.3609	0.3732	0.1581	Segmental	Yes	181.61

* Abbreviation: MYA, million years ago.

We assessed the syntenic relationships of *VQ* genes from the watermelon and cucumber genomes, and a total of 39 syntenic gene pairs involving 28 *ClVQs*, which were located on all watermelon chromosomes except for Chr 04, were identified between watermelon and cucumber (Figure 5). Five genes (*ClVQ01* to *ClVQ05*) on watermelon Chr 01 showed the highest number of syntenic genes (eight *CsVQ* genes) in the cucumber genome.

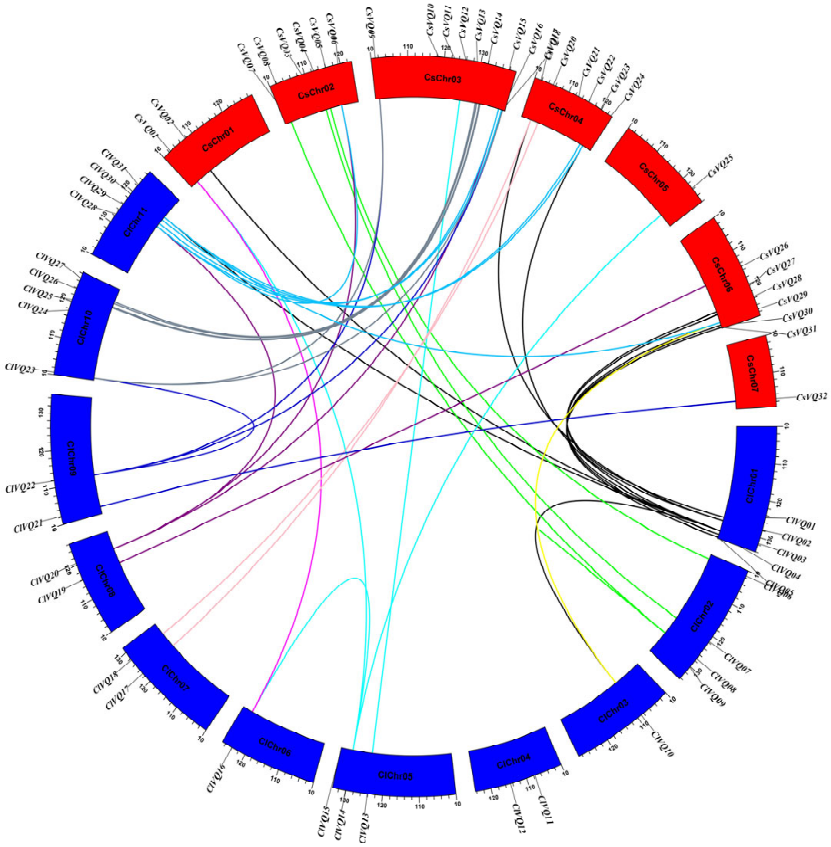

Figure 5. Relationship of syntenic *VQ* genes in chromosomal synteny regions distributed in watermelon and cucumber. Chromosomes of watermelon and cucumber are represented by blue and red arcs according to their own sizes. Different-colored lines link representative syntenic *VQ* genes in watermelon and cucumber.

3.4. Analysis of Putative cis-Acting Elements in ClVQ Promoters

We identified and analyzed *cis*-acting elements in the putative promoter region of the *VQ* genes in watermelon (Table S3). Numerous hormone- and stress-responsive elements were found in the *ClVQ* promoters. Fifty-four methyl jasmonate (MeJA)-responsive and 35 abscisic-acid-responsive elements were identified in the *ClVQ* promoters. Thirteen drought- and seven low-temperature-responsive elements were detected in the *ClVQ* promoters. Almost all WRKY proteins were observed to recognize and bind with the W-box motif. Numerous W-box motifs were detected in the promoters of all watermelon *ClVQs* except *ClVQ08* and *ClVQ14*. The promoters of *Arabidopsis* and soybean VQ genes are indicated to show strong enrichment of W-box motifs [1,22]. As many as 10 W-box motifs were detected in the *ClVQ21* promoter region. The average frequency of W-box motifs in the 1.5-kb promoter of the 31 *ClVQ* genes was 6.125, which is much higher than the frequency observed in *Arabidopsis* (3.8) [1].

3.5. Expression Pattern of ClVQ Genes in Various Tissues and During Fruit Development

We performed qRT-PCR analysis to detect the expression patterns of the 31 *ClVQ* genes in different watermelon organs and tissues (Figure 6A; Table S5). Ten *ClVQ* genes showed no detectable expression, and the remaining twenty-one *ClVQ* genes were expressed in either 1 or multiple organs/tissues. The majority of the detectable *ClVQ* genes were relatively highly expressed in reproductive organs. In detail, 12 of the *ClVQ* genes were most highly expressed in the male flower, of which *ClVQ05*, *ClVQ21*, and *ClVQ28* were specifically expressed in the male flower. Three genes, namely *ClVQ01*, *ClVQ22*, and *ClVQ26*, were expressed most highly in the fruit. Only *ClVQ29* was expressed most highly

in the female flower. In contrast, the majority of *ClVQ* genes showed low transcript levels in vegetative tissues. Nine *ClVQ* genes showed the lowest expression level in the stem. The majority of *ClVQ* genes were weakly expressed in the leaf, but *ClVQ02*, *ClVQ08*, and *ClVQ12* were most highly expressed in the leaf and *ClVQ12* was specifically expressed in leaves. *ClVQ03* and *ClVQ10* showed the highest expression level in the root, and *ClVQ07* and *ClVQ09* were most highly expressed in the root after that in the male flower.

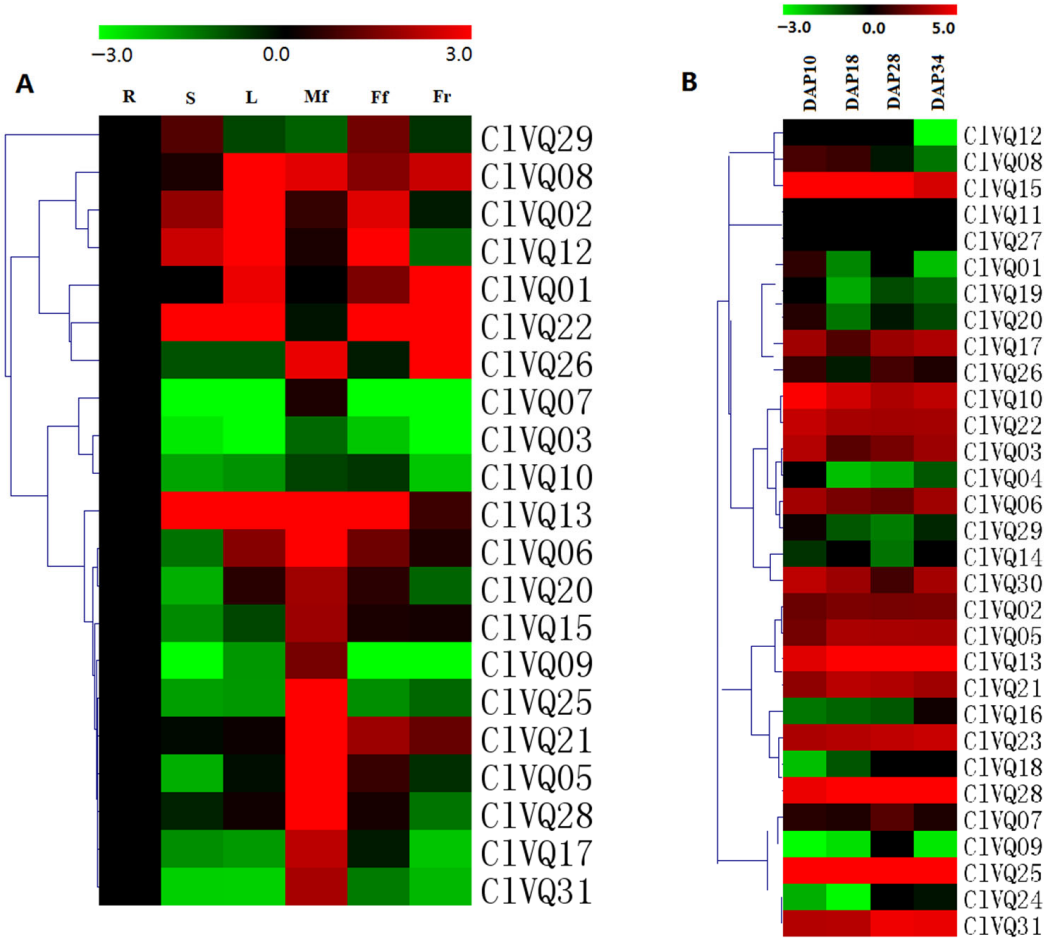

Figure 6. Expression analysis of *ClVQ* genes in different tissues of watermelon and at different stages of watermelon fruit development. (**A**) Expression analysis of the *ClVQ* genes in different tissues of watermelon detected by quantitative real-time PCR. (**B**) Transcriptome profiles of *ClVQ* genes in fruit at four stages: 10, 18, 26, and 34 days after pollination (DAP) during watermelon fruit development.

Among the 31 *ClVQ* genes, no transcripts of eight genes consisting of *ClVQ09/11/12/14/18/19/24/27* were detectable in the fruit at any developmental stage in the RNA-sequencing (RNA-seq) data (Figure 6B). The Reads Per Kilobase per Million mapped reads (RPKM) values of *ClVQ10/13/15/25/28/31* were relatively high in the fruit. For the majority of *ClVQ* genes, such as *ClVQ01*, *ClVQ03*, *ClVQ06*, *ClVQ08*, *ClVQ10*, *ClVQ15*, *ClVQ22*, and *ClVQ30*, the transcript level was highest at an early stage of fruit development (10 days after pollination). Only *ClVQ28* and *ClVQ31* showed a transcript level higher at an advanced stage of fruit development.

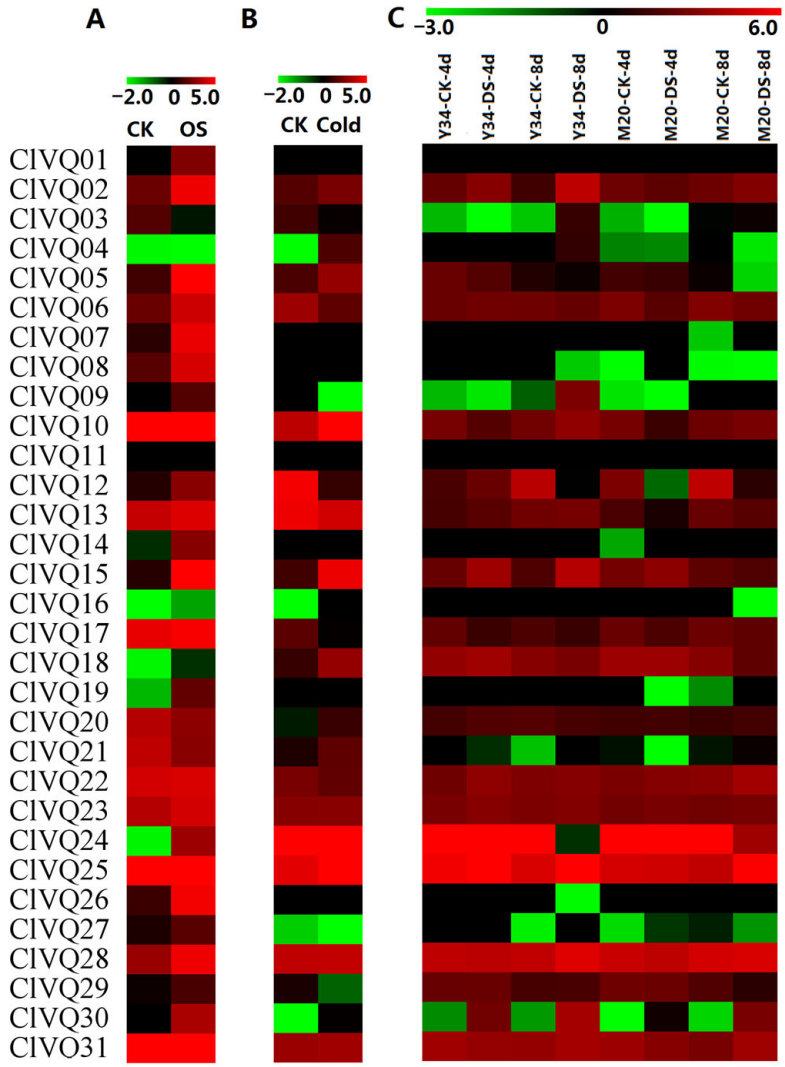

Figure 7. Transcriptome profiles of watermelon *VQs* in response to abiotic stresses. (**A**) The digital expression profiles of watermelon *VQs* in response to osmotic stress. Roots from watermelon 'M08' with four true leaves were collected under osmotic stress. (**B**) The expression patterns of *ClVQ* genes in response to cold stress in watermelon. Cold stress was imposed by placing watermelon in cold chamber at 4 °C and leaves were collected. (**C**) The digital expression profiles of *ClVQ* genes in leaves of two watermelon cultivars ('Y34' and 'M20') subjected to drought stress (for 4 d and 8 d) and without drought stress (control, 4 d, and 8d).

3.6. Expression Patterns of ClVQ Genes in Response to Various Abiotic and Biotic Stresses

To detect the response patterns of *ClVQ* genes in response to various abiotic and biotic stresses, the expression levels of each *ClVQ* gene were estimated based on RNA-Seq data (Figure 7). The results indicated that the majority of watermelon *VQs* may be generally regulated by various stresses.

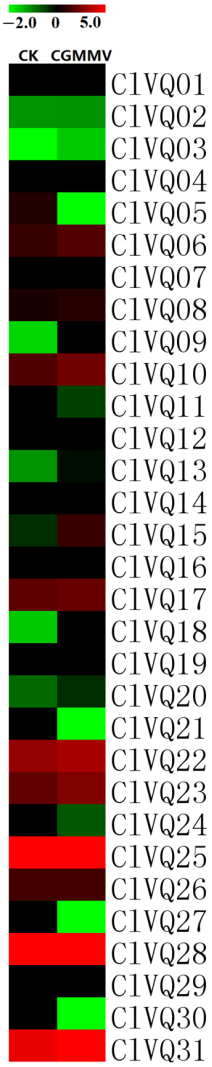

Figure 8. Transcriptome profiles of *ClVQ* genes in watermelon fruit in response to *Cucumber green mottle mosaic virus* (CGMMV) infection.

In response to osmotic stress, the majority of *ClVQs* in the root were distinctly induced (Figure 7A). *ClVQ03*, *ClVQ10*, and *ClVQ21* were repressed, and eight *ClVQ* genes comprising *ClVQ04*, *ClVQ11*, *ClVQ13*, *ClVQ17*, *ClVQ20*, *ClVQ22*, *ClVQ23*, and *ClVQ31* showed no response to osmotic stress treatment. The transcript level of *ClVQ* genes in the roots of two watermelon genotypes, a drought-tolerant cultivar ('M20') and a drought-sensitive cultivar ('Y34'), were analyzed at 4 and 8 d after drought treatment (Figure 7C). The results indicated that *ClVQ* genes exhibited diverse response patterns to drought treatment. After drought treatment for 4 d, three *ClVQ* genes, namely *ClVQ15*, *ClVQ25*, and *ClVQ30*, were distinctly induced, whereas *ClVQ03* and *ClVQ24* were repressed in the drought-sensitive cultivar ('Y34'). In the drought-tolerant cultivar ('M20'), nine *ClVQ* genes were markedly repressed and only *ClVQ27* and *ClVQ30* showed dramatically increased expression. After drought treatment for 8 d, the number of *ClVQ* genes responsive to drought treatment had increased in 'Y34', of which seven genes consisting of *ClVQ01/02/09/15/21/25/30* were induced and two genes (*ClVQ12* and *ClVQ24*) were repressed. In contrast, in 'M20', the number of *ClVQ* genes responsive to drought treatment declined. *ClVQ25* and *ClVQ30* were induced, whereas six *ClVQs* consisting of *ClVQ05/07/12/19/24/27* were repressed by drought treatment at 8 d. *ClVQ25* and *ClVQ30* were both induced in 'Y34' and 'M20'. *ClVQ12* and *ClVQ24* were repressed in 'Y34' and 'M20'. The transcripts of 21 *ClVQ* genes were detected in response to cold stress (Figure 7B). In detail, nine *ClVQ* genes were

distinctly induced, especially *ClVQ04*, *ClVQ15*, and *ClVQ30*, which were induced more than 10-fold. Six genes were repressed by cold treatment, of which the transcript levels of *ClVQ12* and *ClVQ16* decreased by more than 10-fold.

The expression pattern and roles of *VQ* genes in response to plant virus infection have not been reported previously. Here, we detected the response patterns of *ClVQ* genes in response to *Cucumber green mottle mosaic virus* (CGMMV) infection in watermelon (Figure 8). The RNA-seq data from watermelon fruit revealed that 23 *ClVQ* genes were detectable and 11 *ClVQ* genes were obviously responsive to CGMMV infection. In detail, eight *ClVQ* genes comprising *ClVQ03*, *ClVQ13*, *ClVQ31*, *ClVQ28*, *ClVQ15*, *ClVQ11*, *ClVQ21*, and *ClVQ24* were distinctly induced. *ClVQ05*, *ClVQ09*, and *ClVQ18* were repressed by CGMMV infection.

3.7. Subcellular Localization Analysis of ClVQ Proteins in Watermelon

The subcellular localization of ten ClVQ proteins randomly selected from all six VQ subfamilies, namely ClVQ02, ClVQ05, ClVQ07, ClVQ11, ClVQ16, ClVQ18, ClVQ22, ClVQ24, ClVQ25, and ClVQ26, were analyzed by transient expression of the green fluorescent protein (GFP) fusion proteins in tobacco leaf epidermal cells (Figure 9). All detected ten ClVQ proteins were localized in the nucleus. In detail, six ClVQs, comprising ClVQ02, ClVQ07, ClVQ11, ClVQ16, ClVQ22, and ClVQ25, were predicted to be localized in the nucleus. Among them, the fluorescence signal of ClVQ02, ClVQ16, ClVQ22, and ClVQ25 was detected only in the nucleus. It is worth mentioning that the fluorescence signal of ClVQ02-GFP was dotted in the nucleus. Weak signals for ClVQ07 and ClVQ11 were also detected in the cell membrane and cell walls in addition to the nucleus. Additionally, ClVQ05, ClVQ24, and ClVQ26 were predicted to be localized in the chloroplast, but the fluorescence signal for each protein was detected in the nucleus, and in addition, weak signal for ClVQ05 was detected in the cell membrane or cell wall. ClVQ18 was predicted to be localized in the cytoplasm, but fluorescence was detected specifically in the nucleus.

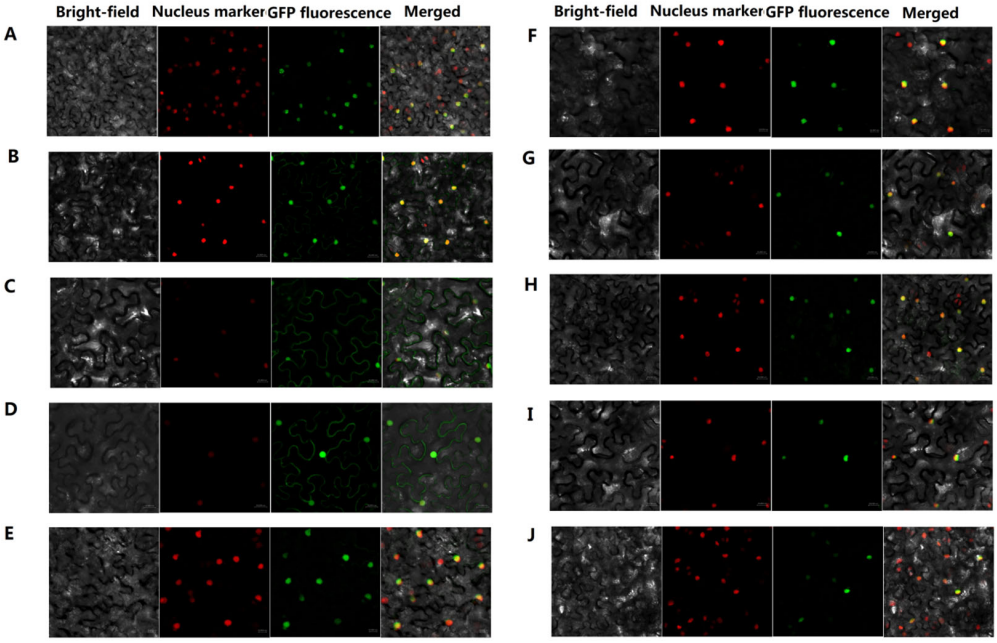

Figure 9. Subcellular localization of ClVQ proteins. Green fluorescent protein (GFP) fusion proteins were transiently expressed in tobacco leaf epidermal cells. After 48 h of incubation, the GFP signal was detected with a fluorescence microscope. Bright-field, endoplasmic nucleus marker, fluorescence, and merged images of p35S::*ClVQ02*-GFP (**A**); p35S::*ClVQ05*-GFP (**B**); p35S::*ClVQ07*-GFP (**C**); p35S::*ClVQ11*-GFP (**D**); p35S::*ClVQ16*-GFP (**E**); p35S::*ClVQ18*-GFP (**F**); p35S::*ClVQ22*-GFP (**G**); p35S::*ClVQ24*-GFP (**H**); p35S::*ClVQ25*-GFP (**I**); and p35S::*ClVQ26*-GFP (**J**).

3.8. Gene Ontology Enrichment Analysis of ClVQ Genes

To further explore their functions, the *ClVQ* genes were subjected to GO enrichment analysis. The enriched GO terms were grouped into three categories: molecular function, cellular component, and biological process (Figure 10 and Table S4). The significantly enriched GO terms for molecular function were protein binding (GO:0005515) and binding (GO:0005488). The ClVQ proteins were mainly classified in the categories integral to the membrane, intrinsic to the membrane, membrane part, and nucleus. The enriched terms for the biological process were classified mainly into 26 categories, such as response to stress (GO:0006950), regulation of cellular process (GO:0050794), regulation of biological process (GO:0050789), and biological regulation (GO:0065007). Thus, the GO enrichment analysis indicated that *ClVQs* played important roles in plant development processes and various stress responses.

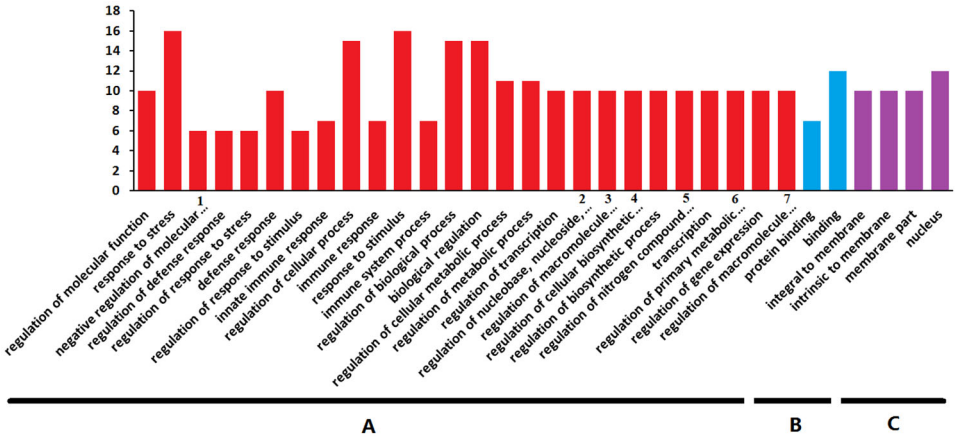

Figure 10. Gene Ontology (GO) analysis of *VQ* genes in watermelon. The *ClVQ* genes were categorized into three groups: molecular function (A); biological process (B); and cell component (C). Note: 1, negative regulation of molecular function; 2, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; 3, regulation of macromolecule biosynthetic process; 4, regulation of cellular biosynthetic process; 5, regulation of nitrogen compound metabolic process; 6, regulation of primary metabolic process; 7, regulation of macromolecule metabolic process.

4. Discussion

The *VQ* family genes have been identified in diverse plant species, including *Arabidopsis*, rice, maize, soybean, Chinese cabbage, poplar, grapevine, and so on. The genomes of these species encode variable numbers of *VQs* ranging from 18 (grapevine) to 74 (soybean) (Table 2). The watermelon genome contains 31 *VQs*, which is fewer than that of other species studied except Moso bamboo, tea, and grapevine. However, the genome of watermelon (425 Mb) is smaller than that of these three species. The present results indicated that *VQ* family size and genome size are not correlated but probably result from whole-genome duplication events. Only one pair of watermelon VQs (*ClVQ08* and *ClVQ09*) was associated with tandem duplication, and 5 gene pairs involving nine *ClVQ* genes were located on segmental duplication chromosomal fragments. This result revealed the important contribution of segmental duplication to watermelon *VQ* family expansion. However, it was observed that 50.0% to 91.4% of *VQs* are located on segmentally duplicated regions in some species, including *Arabidopsis* (59.4%), rice (50.0%), maize (72.6%), soybean (91.0%), and Chinese cabbage (91.4%) [1–3,5,6]. Thus, the percentage is much higher than that of watermelon (29.0%). All of the above-mentioned six duplicated gene pairs in watermelon are distributed in 4 subfamilies comprising III, IV, V, and VI. The estimated divergence times of the pairs *ClVQ20*/*ClVQ28* and *ClVQ22*/*ClVQ23* of subfamily V were duplicated at 148.25 and 181.61 Mya, respectively. The estimated divergence time of the

pair *ClVQ03*/*ClVQ10* was 111.45 Mya. These three duplicated gene pairs were probably duplicated during the diversification of angiosperms. The gene pair *ClVQ03/ClVQ31* of subfamily IV was estimated to have diverged about 495.06 Mya, which corresponds to the period of algal differentiation. However, *ClVQ14/ClVQ16* of subfamily VI was the most ancient duplicated gene pair with an estimated divergence time of about 3789.09 Mya. In addition, the tandem duplication gene pair of *ClVQ08* and *ClVQ09* were estimated to have diverged about 649.91 Mya. These results indicated that the *ClVQ* genes might have been segmentally duplicated in multiple historic periods. Additionally, the watermelon *VQ* gene family preserved evidence for ancient gene duplication, which confirmed that recent whole-genome duplication was absent and only a small number of segmental duplication events have occurred in the watermelon genome [37].

We selected 10 watermelon *ClVQ* proteins representing all 6 subfamilies to analyze their subcellular localizations via green fluorescent protein (GFP) fusion proteins. All ClVQ proteins showed GFP signals in the nucleus, supporting previous findings that VQ proteins regulate DNA binding and transcription-regulating activities of WRKY transcription factors in the nucleus [1]. Among the identified ClVQ proteins, seven were exclusively localized within the nucleus. In contrast, the remaining three proteins, designated as ClVQ05, ClVQ07, and ClVQ11, were additionally observed in association with cell membranes and the cell wall. In addition, the results revealed that ClVQs classified in the same subfamily usually differed in subcellular localization, which was indicative of divergent roles for these ClVQs in the same subfamily. In detail, ClVQ02, ClVQ05, and ClVQ25, which belong to subfamily I, were localized in various subcellular compartments. ClVQ02 was dotted in the nucleus, but the GFP signal for ClVQ05 and ClVQ25 was evenly distributed in the nucleus, and ClVQ05-eGFP showed a weaker signal in cell membranes and the cell wall. ClVQ11 and ClVQ18 are members of subfamily IV. In contrast to the predicted results, ClVQ11-eGFP was localized in the nucleus, cell membranes, and cell wall, whereas ClVQ18-eGFP was localized in the nucleus. These results are the first report of the localization of plant VQ proteins to cell membranes and the cell wall. This membrane-associated transcription factor has also been studied in *Arabidopsis* such as AtNAC089 [38]. The similar localization of proteins probably not only facilitates a rapid transcriptional response to external stimuli in plants but also enables the transfer from the endoplasmic reticulum to designated cellular sites under stress conditions.

VQ genes are involved in diverse plant growth and development processes. In watermelon, the majority of detected *ClVQ* genes were preferentially expressed in the male flower. In addition, one and three *ClVQ* genes were most highly expressed in the female flower and the fruit, respectively. These expression patterns are consistent with findings in *Arabidopsis*, where a significant proportion of *VQ* genes are preferentially expressed in reproductive tissues [1]. In contrast, the majority of *VQ* genes in rice and tea demonstrate preferential expression in vegetative tissues [2,39]. The temporal and spatial expression patterns of some *ClVQ* genes are conserved. *Arabidopsis VQ20* is specifically expressed in male flowers and regulates male sterility [40]. Its homologous gene, *ClVQ06*, exhibits the highest expression levels in male flowers, indicating a potential role in male sterility in watermelon. The *Arabidopsis* genes *MKS1/AtVQ21* and *VQ29*, along with soybean genes *GmVQ43* and *GmVQ62*, have been implicated in the regulation of flowering time. Correspondingly, the homologous genes *ClVQ05*, *ClVQ06*, and *ClVQ07* in watermelon are predominantly expressed in male flowers, suggesting their involvement in flowering processes [1,13]. Furthermore, *Arabidopsis IKU1* (*AtVQ14*) and soybean genes *GmVQ06* and *GmVQ53* are active in seed development [3,41]. QRT-PCR results reveal that the homologous genes in watermelon, *ClVQ22*, *ClVQ25*, and *ClVQ31*, are highly expressed during fruit development, implying similar roles in seed and fruit development. These findings

suggest that certain homologous *VQ* genes may exhibit conserved expression patterns and functions in watermelon.

Many *VQs* have been functionally characterized and play roles in response to infection by diverse pathogens. *Arabidopsis MKS1/AtVQ21* functions as a regulator of pathogen defense responses [42,43]. *Arabidopsis VQ12* and *VQ29* negatively mediate basal resistance to *Botrytis cinerea* [12], and *VQ16* and *VQ23* interact with *WRKY33* to modulate plant resistance against necrotrophic pathogens [19]. However, the role of *VQs* in response to virus infection has not been studied previously. In this study, we analyzed the expression pattern of watermelon *VQs* in response to CGMMV infection based on RNA-seq data. Among all of the watermelon *ClVQs*, eleven *ClVQ* genes showed a distinct response to CGMMV infection. Eight *ClVQ* genes among them were induced, ranging from 2.10-fold (*ClVQ03*) to 3.14-fold (*ClVQ13*). Additionally, the remaining three *ClVQ* genes, including *ClVQ05*, *ClVQ09*, and *ClVQ18*, were repressed by CGMMV infection. It is well known that salicylic acid (SA) and jasmonate (JA) are two critical signaling molecules in the disease defense response. Additionally, *VQs* are involved in SA- and JA-mediated defense responses [1,2,6,12]. *AtVQ21* in *Arabidopsis* is crucial for SA- and JA-mediated defense against various pathogens [15,41]. *AtVQ23 (SIB1)* and *AtVQ16 (SIB2)* interact with *WRKY* genes, leading to competitive regulation of *JAZ1* and *JAZ5* during *B. cinerea* infection [44]. *AtVQ22/JAV1* controls JA-regulated resistance to necrotrophic pathogens and herbivorous insects [8]. In this study, we identified 54 MeJA-responsive elements (CGTCA- and TGACG-motifs) in the promoter regions of all watermelon *ClVQ* genes except *ClVQ10*, *ClVQ15*, *ClVQ21*, *ClVQ22*, *ClVQ26*, and *ClVQ29*. Additionally, 18 SA-responsive motifs (TCA-elements) were found in the promoters of 12 *ClVQ* genes that typically do not respond to CGMMV infection. These findings suggest that *ClVQ* genes involved in the JA signaling pathway likely play broader roles in responding to CGMMV infection in watermelon.

The majority of *ClVQ* genes were induced in response to osmotic stress, except *ClVQ03*, *ClVQ10*, and *ClVQ21* from subfamily IV, which were repressed by osmotic stress. In contrast, fewer *ClVQ* genes were responsive to drought stress. After drought treatment for 4 d, three *ClVQ* genes were induced and two genes were repressed in drought-sensitive 'Y34,' whereas in drought-tolerant 'M20' nine *ClVQ* genes were repressed and two genes were induced. *ClVQ02* and *ClVQ15* were distinctly induced in 'Y34' but were not drought-responsive in 'M20.' Inversely, *ClVQ05*, *ClVQ10*, *ClVQ13*, and *ClVQ27* were distinctly responsive to drought in 'M20' but were not drought-responsive in 'Y34.' This difference in expression patterns of certain ClVQs in drought-tolerant and -sensitive cultivars of watermelon may reflect the plant's drought resistance. Meanwhile, some *ClVQ* genes showed similar expression patterns in response to drought stress in the two cultivars. For example, *ClVQ25* and *ClVQ30*, which both belong to subfamily I, were induced by drought in the two cultivars. *ClVQ24* was repressed by drought treatment for 4 and 8 d in the two cultivars. *ClVQ03*, *ClVQ09*, and *ClVQ21* were repressed at 4 d but induced at 8 d in 'Y34' and 'M20.' Additionally, *ClVQs* from subfamily IV exhibited unique expression patterns in response to the tested stresses. Subfamily IV, which contained the most *ClVQs*, was responsive to cold, drought, and CGMMV infection. In detail, *ClVQ03* and *ClVQ21* were distinctly affected by CGMMV infection, cold, drought, and osmotic stresses. All subfamily IV members were responsive to CGMMV infection except *ClVQ04* and *ClVQ10* and were responsive to drought stress except *ClVQ04* and *ClVQ18* in 'M20' and 'Y34.' In response to osmotic stress, almost all watermelon VQs were induced, but only the subfamily IV members *ClVQ03*, *ClVQ10*, and *ClVQ21* were repressed. It is necessary to explore the roles of the subfamily genes in future studies, and the aforementioned results will help in screening candidate *ClVQ* genes responsive to various stresses.

5. Conclusions

By implementing an integrative approach, we identified 31 VQ genes in watermelon in a genome-wide analysis. The present results provide insights into gene structure, conserved domains, phylogenetic relationships, chromosome distribution, and expression profiles of the VQ gene family in watermelon. Gene duplication events among watermelon VQs have occurred rarely and preserved evidence for ancient gene duplication. The majority of *ClVQ* genes were preferentially expressed in the male flower and probably play important roles in flower development. The temporal and spatial expression patterns of selected *ClVQ* genes are conserved compared with those of homologous genes in other species, which implies that the functions of ClVQs are conserved in watermelon. The present results include the first report of expression patterns of plant VQ genes in response to virus infection. Half of the watermelon VQs were distinctly affected by CGMMV infection and are probably involved in the MeJA signal-response pathway. Watermelon VQs show a variety of expression patterns in response to cold, drought, osmotic stresses, and CGMMV infection. Notably, ClVQs from subfamily IV were generally responsive to these four stresses. All of the ten tested ClVQs representing the 6 subfamilies were localized in the nucleus, but three ClVQs were also localized in cell membranes and the cell wall. Membrane- and wall-localized VQs have not been reported previously, and thus the corresponding functions of the proteins require further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11010081/s1, Figure S1: Domain structures of VQ proteins in watermelon. Domain structures were analyzed using the SMART tool and are drawn in accordance with their original location and size; Table S1: Putative members of the *VQ* gene family in *Citrullus lanatus* and *Cucumis sativus* genome sequences; Table S2: Similarity of amino acid sequences of the VQ proteins from *Arabidopsis*, cucumber, and watermelon; Table S3: Summary of *cis*-elements in promoter regions of the *VQ* genes in watermelon; Table S4: GO enrichment analyses of ClVQ proteins in watermelon; Table S5: Expression data for *ClVQs* in watermelon in this study; Table S6: Gene-specific primers used for subcellular localization and qRT-PCR analysis of watermelon VQ gene expression.

Author Contributions: Conceptualization, Y.H.; software, X.X. and Y.H.; validation, J.S. and X.X.; data curation, Y.H., J.S. and W.S.; writing—original draft preparation, Y.H.; writing—review and editing, Y.H. and W.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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

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