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Research article

Genome-wide analysis of the plant-specific VQ motif-containing proteins in tomato (*Solanum lycopersicum*) and characterization of SlVQ6 in thermotolerance

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A B S T R A C T

The VQ motif-containing (VQ) proteins are plant-specific proteins with a conserved "FxxhVQxhTG" amino acid sequence, which regulate plant growth and development. Little is known, however, about the function of VQ proteins in tomato (*Solanum lycopersicum*). Here, a total of 26 SlVQ proteins were confirmed and characterized using a comprehensive genome-wide analysis. The SlVQ proteins all contain the conserved motif with seven variations, which are classified into eight groups (I, II, IV-VI, VIII-X). Most of them were predicted to be localized in the nucleus. Besides, a network including SlVQ proteins interaction with WRKY transcription factors (SlWRKYs) and mitogen-activated protein kinases (SlMPKs) is proposed. In addition, among the *SlVQ* genes, *SlVQ6* was expressed in the range of organs and tissues with the highest levels and could response to different stresses. Ectopically overexpression of *SlVQ6* in Arabidopsis plants decreased high temperature tolerance. RNA sequencing analysis revealed that several stress-related genes, such as *HSP70-4*, *RD20*, *GolS1* and AT4g36010 were down-regulated in *SlVQ6* overexpressing plants compared to these in wild-type under normal growth conditions. This study provides critical information about *SlVQ* genes and their encoded proteins, as well as further research on SlVQ functions in tomato growth and development.

1. Introduction

Accumulating evidence shows that VQ motif-containing (VQ) proteins constitute a strictly plant-specific motif hallmarked by a conserved core sequence FxxhVQxhTG (h denotes hydrophobic residues and x represents any amino acid). Andreasson et al. (2005) found that this protein family shares the conserved VQ motif with unknown function (Protein Family PF05678). Since Morikawa et al. (2002) discovered the first VQ protein (Formerly known as SIB1, SIGMA FACTOR-BINDING PROTEIN1), known as AtVQ23 by now, more and more VQ proteins have been discovered.

VQ proteins regulate plant growth and development, seed development, responses to different stresses, and photomorphogenesis (Jing and Lin, 2015). *AtVQ14/IKU1* expressed in the early endosperm positively regulates endosperm development and seed growth (Wang et al., 2010). *AtVQ8* mutant shows pale-green and stunted growth, indicating that AtVQ8 regulates chloroplast development or photosystem assembly (Cheng et al., 2012). AtVQ29 negatively regulates photomorphogenesis (Li et al., 2014). AtVQ18 and AtVQ26 are involved in Arabidopsis germination and early seedling establishment (Pan et al., 2018). VQ proteins also play important roles in resistance to different stresses. VQ23 positively regulates the resistance to necrotrophic and biotrophic pathogens (Lai et al., 2011). *AtVQ4/MVQ1*-overexpressing plants decreases Flg22-induced resistance, acting as a negative regulator of PAMP-induced resistance to pathogens (Pecher et al., 2014). *VQ22/JAV1* functions as a negative regulator of JA-mediated plant response required for conferring resistance against necrotrophic pathogens and herbivorous insects (Hu et al., 2013a). Recently, a study

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further revealed that a RING-type E3 ubiquitin ligase (JUL1, JAV1-ASSOCIATED UBIQUITIN LIGASE 1) interacting with JAV1 to proteasomal degradation of JAV1, resulting in positive regulation of defense responses (Ali et al., 2019). Arabidopsis AtVQ15 (CAMBP25) and AtVQ9 act as a negative regulator of plant tolerance to osmotic and salinity stress, respectively (Perruc et al., 2004; Hu et al., 2013b).

The most studied mechanism for VQ protein function so far is interaction with WRKY transcription factors. The interaction between AtVQ14 and AtWRKY10 determines endosperm growth and seed size (Wang et al., 2010). The AtVQ23-mediated resistance of Arabidopsis to *B. cinerea* is dependent on AtWRKY33 (Lai et al., 2011). AtVQ9 negatively modulates salt tolerance by suppressing AtWRKY8 (Hu et al., 2013b). Banana MaWRKY26 has been shown to physically interact with MaVQ5, which attenuates MaWRKY26-activated JA biosynthetic genes (Ye et al., 2016). Recently, AtVQ20 was reported to act as a vital partner of AtWRKY2/AtWRKY34 in the regulation of pollen development (Lei et al., 2017). Apple MdVQ proteins was shown to bind with Group I and IIc MdWRKYs (Dong et al., 2018). Another feature of VQ proteins is as a substrate for mitogen-activated protein kinases (MAPKs). AtVQ21/MKS1 is first discovered as a substrate of AtMPK4, and AtVQ21 and WRKY33 can be bridged by AtMPK4 to affect plant growth and disease resistance (Andreasson et al., 2005; Qiu et al., 2008; Gargul et al., 2015). Phosphorylation of 10 VQPs (MVQs) by AtMPK3 and AtMPK6 was reported by Pecher et al. (2014), and most of these AtMPK3/6-targeted VQ proteins interacting with WRKYs (proposed as WRKY-VQ-MPK modules) to regulate Arabidopsis immune responses. Furthermore, many other regulatory mechanisms of VQ proteins are increasingly being revealed. AtVQ29 interacts with PHYTOCHROME-INTERACTING FACTOR1 (PIF1) represses seedling deetiolation (Li et al., 2014). During seed germination, Arabidopsis AtVQ18 and AtVQ26 interact with transcription factor ABI5 to negatively regulate ABA response (Pan et al., 2018). JUL1 interacts with VQ22 and the VQ22/JUL1 system functions as an important coordinator of plant defense response (Ali et al., 2019).

Recently, based on the plant genome sequence databases, VQ proteins have been increasingly identified in diverse plants. There are 34, 40, 74, 29, and 61 members in Arabidopsis (Cheng et al., 2012), rice (Li et al., 2014), soybean (Wang et al., 2014), Chinese cabbage (Zhang et al., 2015), bamboo (Wang et al., 2017), and maize (Song et al., 2016). More recently, 49 MdVQ proteins were confirmed in apple (Dong et al., 2018) and 25 *CsVQ* genes were identified in tea (Guo et al., 2018) and the expression patterns of these gene were found in response to salt, drought, and high temperature stresses. However, the VQ protein family in tomato has remained largely unknown to date. In the present study, 26 SlVQ proteins were identified based on the tomato genome databases. Genome-wide analysis was carried out to study gene structure, conserved protein motif, phylogenetic relationship, and functional interaction network. In addition, *SlVQ6* has the highest levels in a variety of tissues. Ectopically overexpression of *SlVQ6* in Arabidopsis shows decreased high temperature (HT) tolerance. Simultaneously, to elucidate the molecular mechanism of SlVQ6-mediated negative regulation of HT stress, RNA sequencing (RNA-seq) based transcriptome analysis was performed. This study provides a solid foundation for further exploration on tomato SlVQ proteins, especially on SlVQ6.

2. Materials and methods

2.1. Characterization of SlVQ genes and encoded proteins

The tomato genome sequences were obtained from the *Solanum lycopersicum* iTAG2.4 database. A total of 26 *SlVQ* genes and encoded proteins were found and downloaded from the Phytozome database (http://www.phytozome.net) using the motif ID “PF05678” as the keyword for query in the tomato database. Then, the obtained SlVQ proteins were rechecked using the Interpro program.

The chromosomal location of *SlVQ* genes were mapped using Mapchart 2.2 (https://www.wur.nl/en/show/Mapchart.htm). The intron/exon structure analysis of the *SlVQ* genes was performed using the GSDS (http://gsds.cbi.pku.edu.cn). The physicochemical properties of each protein were obtained using ProtParam tool (https://web.expasy.org/protparam/). DeepLoc-1.0 predicted the subcellular localization of each SlVQ protein (Almagro Armenteros et al., 2017).

To evaluate the functional-structural divergence of tomato SlVQ proteins, the distribution of the SlVQ conserved motifs in tomato was analyzed by MEME with 10 maximum number of motifs (http://meme-suite.org/). Phosphorylation analysis of SlVQ6 was completed using the online plant-specific software P3DB (https://www.p3db.org/).

2.2. Sequence alignment and phylogenetic method

The alignments of full-length sequences of 26 SlVQ proteins from tomato were performed using ClustalX 2 and was visualized using Genedoc software. Thirty-four Arabidopsis VQ protein sequences (*Arabidopsis thaliana* TAIR10) and 39 rice VQ protein sequences (*Oryza sativa* v7_JGI) were downloaded from Phytozome database. The alignments of full-length sequences of VQ proteins from tomato, Arabidopsis, and rice were performed using ClustalX 2 and using the neighbor-joining method to construct the phylogenetic tree.

2.3. Analysis of SlVQ proteins-mediated interaction networks

The homologous AtVQ proteins interacting network were integrated using the Arabidopsis Interactions Viewer at BAR (http://bar.utoronto.ca/interactions/cgi-bin/arabidopsis_interactions_viewer.cgi). Only interactions from published data sets were shown. Query protein-protein interactions was from BAR PPI or from BioGrid, IntAct and BAR. Homologous proteins in tomato are listed. The prediction of main interaction between SlVQ proteins and SlWRKY proteins were forecasted based on PAIR (public.synergylab.cn) and BAR website. The interaction network was drawn by omicshare tool (http://www.omicshare.com/tools/Home/Soft/cytoscape).

2.4. Expression profile of SlVQ6

RNA-seq data of the expression of *SlVQ* genes in different tissues of tomato is from TomExpress database (http://gbf.toulouse.inra.fr/tomexpress). The expression data was visualized using heat maps and graphs. Besides, RNA-seq expression data of *SlVQ6* were obtained for different organs of cultivar M82 from the tomato eFP browser at bar.utoronto.ca. Tissue-specific expression of *SlVQ6* was further detected with qRT-PCR. Tissues from root, stem, leave, flower, green fruit, and red fruit of different stages of tomato plants (*Solanum lycopersicum* ‘OFSN’) were collected. In the days post-anthesis, the flowering stage and fruit development were recorded. The flower, green fruit and red fruit samples were taken from 0, 25 and 40 days after flowering.

For different environmental stresses, five-week-old tomato seedlings were subjected to drought, salt, and HT treatments. For salt and drought, the plants were immersed in 100 mM NaCl or 200 mM mannitol solution for 1, 3, 6, 12, and 24 h. For HT stress, the seedlings were treated under 42 °C conditions for 1, 3, 6, and 12 h. The leaves were harvested at indicated times, frozen in liquid nitrogen, and stored at −70 °C for further RNA extraction.

2.5. Generation of transgenic arabidopsis plants overexpressing SlVQ6

To generate *SlVQ6* overexpressing plants, the full-length ORF was amplified with specific primers and was inserted into binary vector pBI121 driven by the CaMV 35S promoter. The constructs were then transformed into *Arabidopsis thaliana* (Col-0 ecotype) by the floral-dip method. T1 seeds were sown on sterile media containing 50 μg ml^{-1} kanamycin to screen the transformants. Based on the 3:1 segregation of

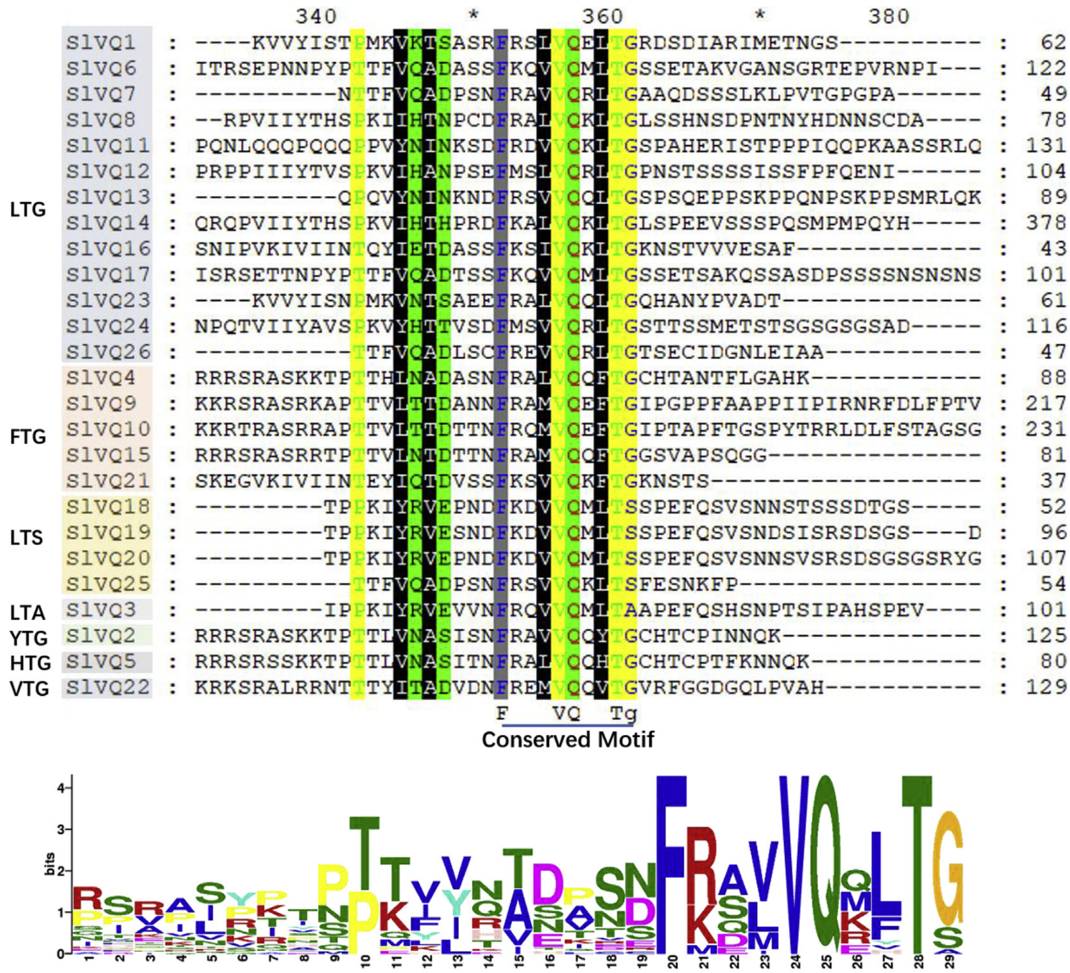

Fig. 1. Multiple sequence alignment of SlVQ proteins in tomato. Sequences were aligned using GeneDoc software. The FxxxVQxLTG motif is clearly highly conserved and shown below.

kanamycin resistance, T2 lines containing a single transgene were selected, and homozygous T3 seeds were harvested for further analysis. For phenotype of *SlVQ6*-overexpressing plants under HT stress, the hypocotyl elongation method was carried out. The plates with seeds on 1/2 MS medium were covered with foil, placed at 4 °C for 2 days, and then put in a vertical position at 23 °C for 2 days. Wrapped plates were treated with 45 °C for 120 min, followed by a vertical position at 23 °C for another 8 days. For seedling test, the 20 d old seedlings of WT and *SlVQ6*-overexpressing lines on 1/2 MS media were subjected to 45 °C for 150 min and then recovered at 22 °C for 10 d. Experiments were repeated three times and the representative images were shown.

2.6. RNA isolation and qRT-PCR

Quantitative real-time PCR (qRT-PCR) was applied to evaluate the transcript levels of *SlVQ6*. Total RNA was isolated from different tissues using RNAprep pure Plant Kit (TIANGEN). cDNA templates were synthesized by using SHiScript® Q-RT SuperMix for qPCR. The 10 μl qRT-PCR solutions contained 5 μl of SYBR-Green Mix (BioRad), 0.25 μM forward and 0.25 μM reverse primer, and 50 ng of cDNA template. qRT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems). The tomato reference gene is *Ubiquitin* (*Ubq*) gene and the Arabidopsis reference gene is *ACTIN*. Three biological replicates were used. The relative expression levels of amplified products were calculated according to the relative quantization method ($2^{-\Delta\Delta CT}$). All primers used in this study are shown in Table S1.

2.7. RNA-seq

For RNA-seq, two separate cDNA libraries were prepared from 21-day old WT seedlings and *SlVQ6*-overexpressing seedlings under normal conditions. There were three replicates for each library and 6 samples in all. Briefly, total RNA was extracted from samples using the RNeasy Mini Kit (Qiagen, Germany) and the extracted RNA was then quantified and assessed for integrity using the NanoDrop (Thermo, USA). The poly-A containing mRNA mRNA was purified using Dyna beads OligodT (Invitrogen Dynal). The cleaved RNA fragments were transcribed into cDNA using reverse transcriptase and random N6 primers. The synthetic double-stranded DNA ends are flattened and phosphorylated at the 5′ end, and the 3′ end forms a sticky end protruding “A", and then a bubble having a convex “T" at the 3′ end is attached. The ligation product was amplified by specific primers and the PCR product is thermally denatured into a single strand. The single stranded DNA is circularized with a bridge primer to obtain the single-stranded circular DNA library. The deep sequencing was conducted at the Huada Genomics Institute (Wuhan, China) using the BGISEQ-500 platform (Zhu et al., 2018).

After extensive preprocessing and quality control, these raw reads were filtered by the software SOAPnuke v1.5.2. For gene-level analyses, HISAT2 (v2.0.4) was used to align reads to Arabidopsis genome. Bowtie2 was used to compare clean reads to reference sequences to get statistical gene alignment rates, and then RSEM was used to calculate gene and transcript expression levels. The DEseq2 algorithm was used for differential gene detection (Fold Change ≥ 2.00, fold change

= < 0.5, and Adjusted P value ≤ 0.05). For GO and KEGG enrichment analysis, all DEGs were mapped to GO terms in the GO database and KEGG database using the Goseq R package and the KOBAS software, respectively.

3. Result

3.1. Identification of SlVQ family members in tomato

To identify the systematic VQ protein family in tomato, the VQ conserved motif (PF05678) was used as a key word to search against the *Solanum lycopersicum* iTAG2.4 from Phytozome database. In total, 26 genes encoding VQ proteins were identified and named SlVQ1 to SlVQ26 based on their physical location (Table S2). These *SlVQ* genes were distributed on 10 chromosomes (Fig. S1). *SlVQ1* and *SlVQ24* were located on chromosome 1 and 12, respectively. The highest numbers of *SlVQ* genes were found on chromosome 2. The information of locus ID, location coordinates, CDS, the number of amino acids, isoelectric point for each protein and the localization prediction were analyzed (Table S2). The CDS ranges from 246 bp to 2031 bp and protein varied from 81 to 676 amino acids, with the average of 236.7 amino acids. The molecular weight of SlVQ proteins ranges from 9.06 to 70.95 kDa with isoelectric point range of 4.67–11.87. Most proteins were predicted to be localized in the nucleus. Multiple sequence alignment further confirmed the structural features of VQ motif of SlVQ proteins. All proteins contained the typical conserved motif FxxhVQxhTG (Fig. 1). However, 26 SlVQ proteins contained seven variations of the conserved motif and the main type was FxxxVQxLTG, accounting for half.

3.2. Phylogenetic tree of SlVQ proteins

To explore the evolutionary relationship of the 26 SlVQ proteins, we constructed a phylogenetic tree with 26, 34, and 40 VQ motif-containing proteins of tomato, Arabidopsis, and rice, respectively. The construction of phylogenetic tree by MEGA 6.0 was based on the neighbor-joining (NJ) method (Fig. 2). SlVQ proteins in tomato could be divided into eight subgroups (I, II, IV-VI, VIII-X) according to the classification of Arabidopsis in the previous studies based on the whole sequence similarities (Pecher et al., 2014). The group IX and I included the most with six SlVQ proteins in IX and five SlVQ proteins in I. There was only one VQ protein in subgroup X. Protein sequences in the same group mean they have similar origin and evolutionary relationships (Guo et al., 2018). Moreover, the evolutionary relationship indicates that the SlVQ proteins have a close affinity with the Arabidopsis VQ proteins and have a distant affinity with the rice VQ proteins in the same group. This is because tomato and Arabidopsis are a dicotyledon, while rice is a monocotyledon.

3.3. Protein conserved motif and gene structure

To further study the features of SlVQ proteins, the different motifs were discovered using the MEME online tool (Fig. 3). Ten conserved motifs were predicted. The motif 1 represents the VQ-containing motif distributed in all SlVQ proteins with seven variations of the conserved motifs. The motif 2 and motif 6 were distributed across SlVQ18, 19 and 20 proteins, which belonged to the same group IV. The motif 3 and 5 were shown in SlVQ6, 7, 17, 25, and 26, which was classified to the same group I. The same motif in the same group suggests the similar biological function. Besides, the motif 4 was distributed across seven SlVQ proteins, which belonged to three different group. In addition to VQ motif, there were different motifs in each SlVQ proteins indicating the structural basis of functional diversity. The gene structure of *SlVQ* genes were also analyzed using the GSDS. The results showed that the most *SlVQ* genes (92.3%, 24/26) had no introns (Fig. S2) and only two genes (*SlVQ3*, *SlVQ14*) contained one and three introns, respectively.

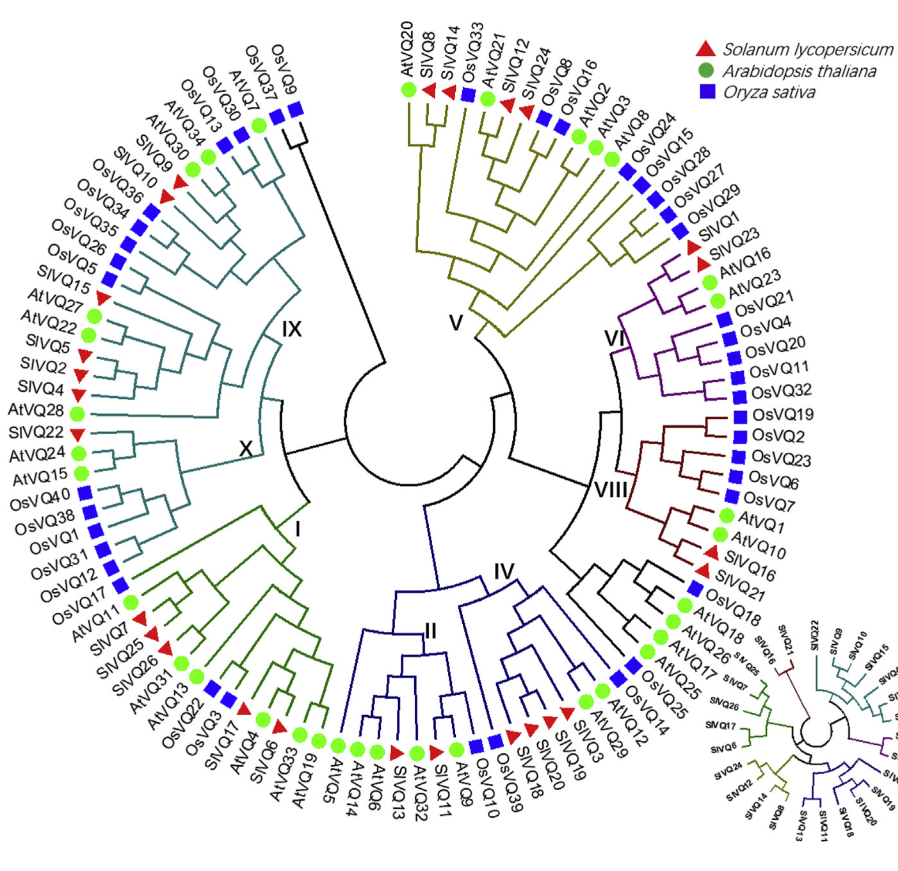

Fig. 2. Phylogenetic analysis of SlVQ proteins from tomato, Arabidopsis and rice. All sequences were downloaded from the phytozome v12.1 (https://phytozome.jgi.doe.gov/). The VQs of Arabidopsis and rice were named according to Cheng et al. (2012) and Li et al. (2014), respectively. The phylogenetic tree of the 26 tomato SlVQs, 34 Arabidopsis AtVQs and 40 rice OsVQs were made by MEGA 6 using to the neighbor-joining method. Based on the clustering of the VQ proteins, tomato SlVQs were clustered into six subgroups (I, II, IV-VI, VIII-X). Proteins from tomato, Arabidopsis, and rice are denoted by red triangles, green circles and blue squares, respectively. The Phylogenetic tree of only SlVQs was in the lower right corner. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

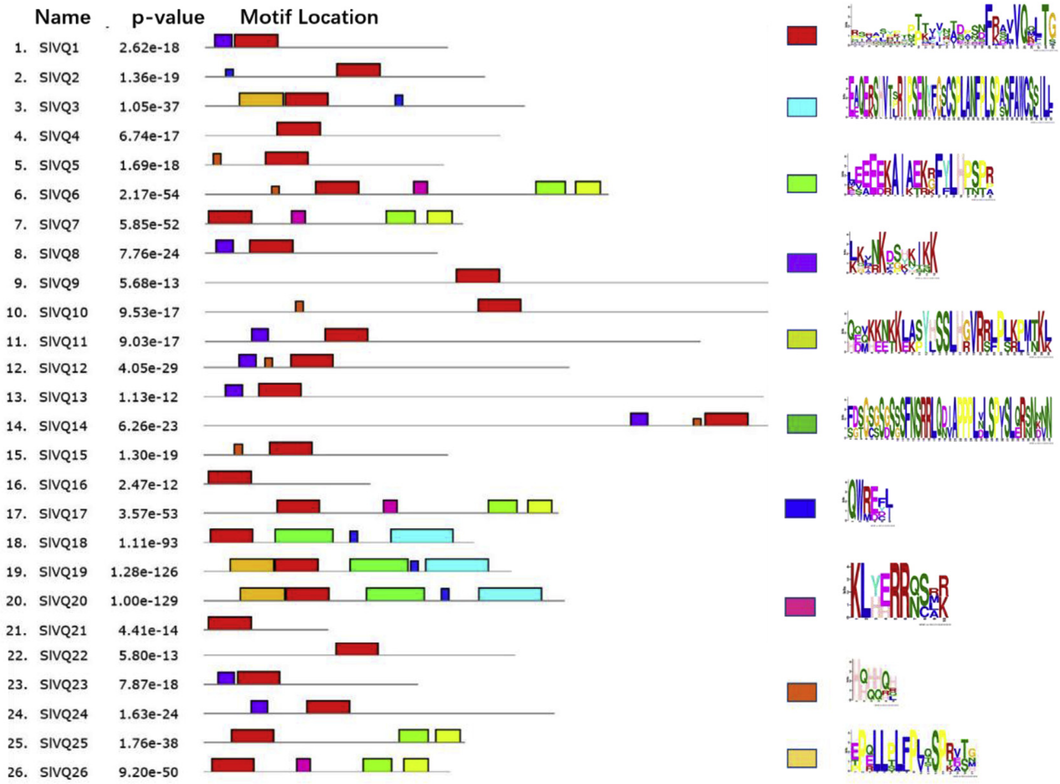

Fig. 3. Conserved motif analysis of the SlVQ proteins in tomato. Distribution of the SlVQ conserved motifs in tomato was analyzed by MEME with 10 Maximum Number of Motifs (http://meme-suite.org/). Each specific motif is marked by a different colored box, and the names are included in the center of each box.

3.4. Interaction network of SlVQ proteins

An interaction network of SlVQ proteins was constructed through STRING and Interactions Viewer to improve the understanding of the functional proteome-wide regulation network (Fig. 4). First, the tomato SlVQ proteins interaction network was constructed in STRING11.0 using tomato match (Fig. 4A). Nine SlVQ proteins have been shown to interact with 16 proteins including seven WRKYs (SlWRKY18, 20, 28, 30, 31, 33, 71), three MAPKs (SlMPK5, 6, 7), SIG1, and csn5. To further explore the network, the AtVQ proteins interaction network was constructed using Arabidopsis Interactions Viewer. The query protein-protein interactions from BioGrid, IntAct and BAR PPI were shown in

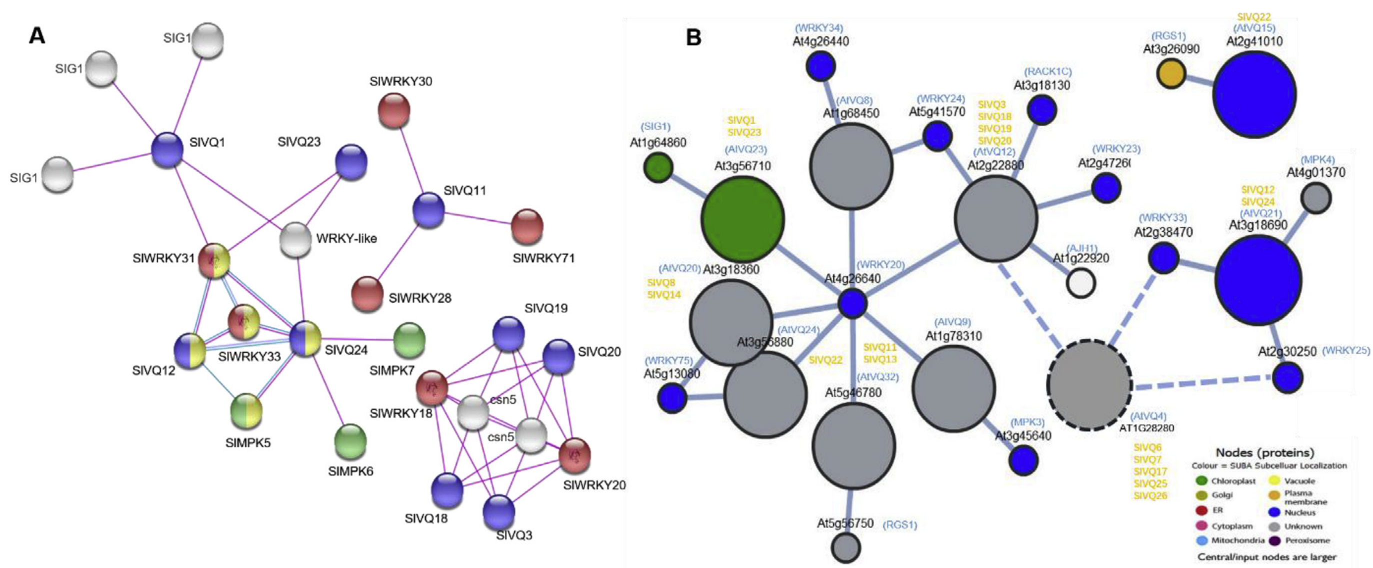

Fig. 4. Putative interaction network of SlVQ proteins in tomato. A, The SlVQs interacting network were integrated using the STRING 11.0. Basic Settings:active interaction sources: Experiments, Databases and Gene Fusion; max number of interactors: 100; display simplifications: hide disconnected nodes in the network; The confidence parameters were set at a 0.40 threshold. Red node: WRKY; Blue node: SlVQ proteins; Green node, MAPK; Yellow node: MAPK signaling pathway in plant. B, The AtVQs interacting network were integrated using the Arabidopsis Interactions Viewer at BAR. Only interactions from published data sets were shown. Query protein-protein interactions was from BAR PPI. Homologous genes in tomato and Arabidopsis are shown in yellow. Dotted lines indicate protein interactions are confirmed in bar.utoronto.ca/interactions2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

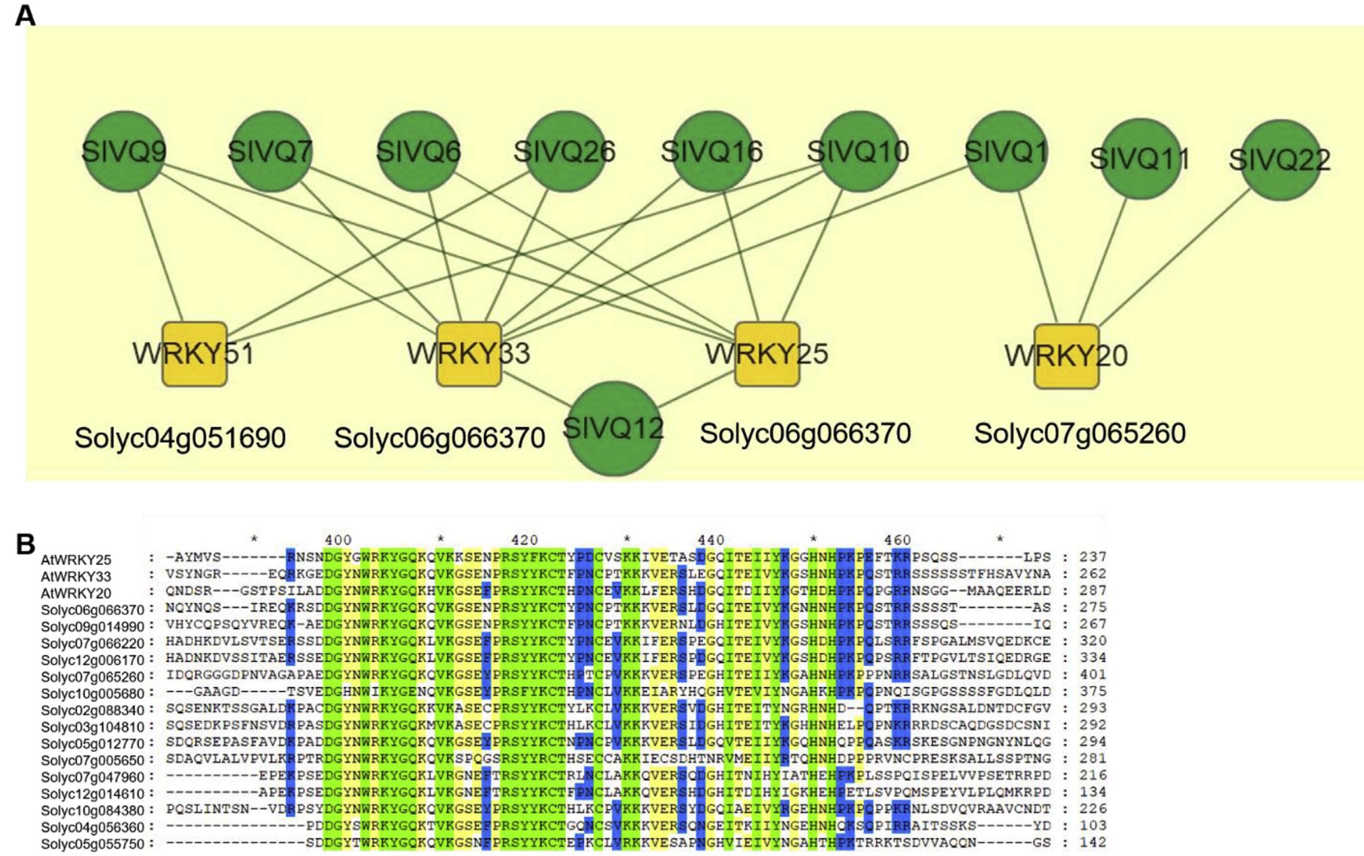

Fig. 5. Interaction of SlVQ proteins with SlWRKY proteins in tomato. A, The prediction of interaction between SlVQ proteins and SlWRKY proteins was drawn by the PAIR and BAR website, and the interaction network was drawn by omicshare tool (http://www.omicshare.com). B, Sequence analysis of the C-terminal WRKY domains of group I SlWRKY proteins (Karkute et al., 2018).

Fig. S3. Four key nodes, including AT4G26640 (AtWRKY20), AT5G64810 (AtWRKY51), AT2G38470 (AtWRKY33), and AT2G30250 (AtWRKY25), were closely interact with different AtVQ proteins. Fig. 4B showed the interactions from BioGrid and the tomato homologous SlVQ proteins were listed. The RGS1, RACK1C and AJH1 are potential interaction proteins of tomato SlVQ proteins. The interaction results showed that WRKY and MAPK proteins were the most involved in VQ protein-mediated network so far.

The VQ protein interaction WRKYs (WRKY20, WRKY25, WRKY33) belongs to the group I WRKY transcription factor and WRKY51 belongs to the group IIc. Their possible tomato SlVQ interaction proteins were shown in Fig. 5A. Multiple sequence alignment of C-terminal WRKY domains of group I SlWRKY proteins (Karkute et al., 2018) and AtWRKY20, AtWRKY25 and AtWRKY33 showed that the core binding domain of these WRKYs was highly conserved (Fig. 5B).

There are ten AtVQ proteins phosphorylated by AtMPK3 and AtMPK6 belonging to Arabidopsis VQ group I, II and III (Pecher et al., 2014). Therefore, to explore the conserved phosphorylation sites, the sequence alignment of VQ proteins of group I in Arabidopsis (AtVQ4, 11, 13, 19, 31,33) and tomato (SlVQ6, 7, 17, 25, 26) were carried out (Fig. 6). The sequence analysis results showed that many phosphorylation sites identified in Arabidopsis VQ proteins could be found in tomato. Therefore, it can be speculated that there is a conserved phosphorylation mechanism of VQ proteins mediated by MAPKs.

3.5. Expression profiles of SlVQ genes in different tissues

To investigate the potential functions of tomato *SlVQ* genes, the gene expression values for each *SlVQ* gene from Tom Express database were studied. As shown in Fig. S4, different *SlVQ* genes were expressed in different tomato tissues. Of the 26 *SlVQ* genes, 14 genes were highly expressed and *SlVQ6* (Solyc02g078030) had the highest expression level, but *SlVQ1* (Solyc01g096510) had the lowest expression level using overall analysis. The *SlVQ* gene expression levels in cultivated tomato Heinz under different development stage (data from bar.utoronto.ca/efp tomato/cgi-bin) also showed that *SlVQ6* expression was highest in different tissues (Fig. 7A). Because of the highest expression level in tomato, SlVQ6 was further studied in the following section.

3.6. SlVQ6 in response to environmental stresses

To explore the possible role of the *SlVQ6* in tomato, the tissue-specific expressions of *SlVQ6* were detected by qRT-PCR, with the total RNA extracted from roots, stems, leaves, flower, green fruit and red fruit. As shown in Fig. 7B, *SlVQ6* is expressed in all tissues with the highest level in red fruit and the lowest level in green fruit. To study the potential role of *SlVQ6* in response to abiotic stress, the expression levels of *SlVQ6* in response to salt, drought and HT stresses were detected by qRT-PCR (Fig. 7C). Under three stresses, the expression levels of *SlVQ6* were significantly decreased, which suggest that *SlVQ6* is involved in the different environmental stress responses like salt, drought and HT and may play diverse roles.

3.7. Overexpression of SlVQ6 decreases HT tolerance

To characterize the biological functions of *SlVQ6*, stable transgenic *Arabidopsis thaliana* lines over-expressing *SlVQ6* were generated. Two independently homozygous lines OE6-3-13 and OE6-7 were verified with the transcription of *SlVQ6* using RT-PCR to the in transgenic plants (Fig. 8B). It is known that Arabidopsis hypocotyl elongation test is a

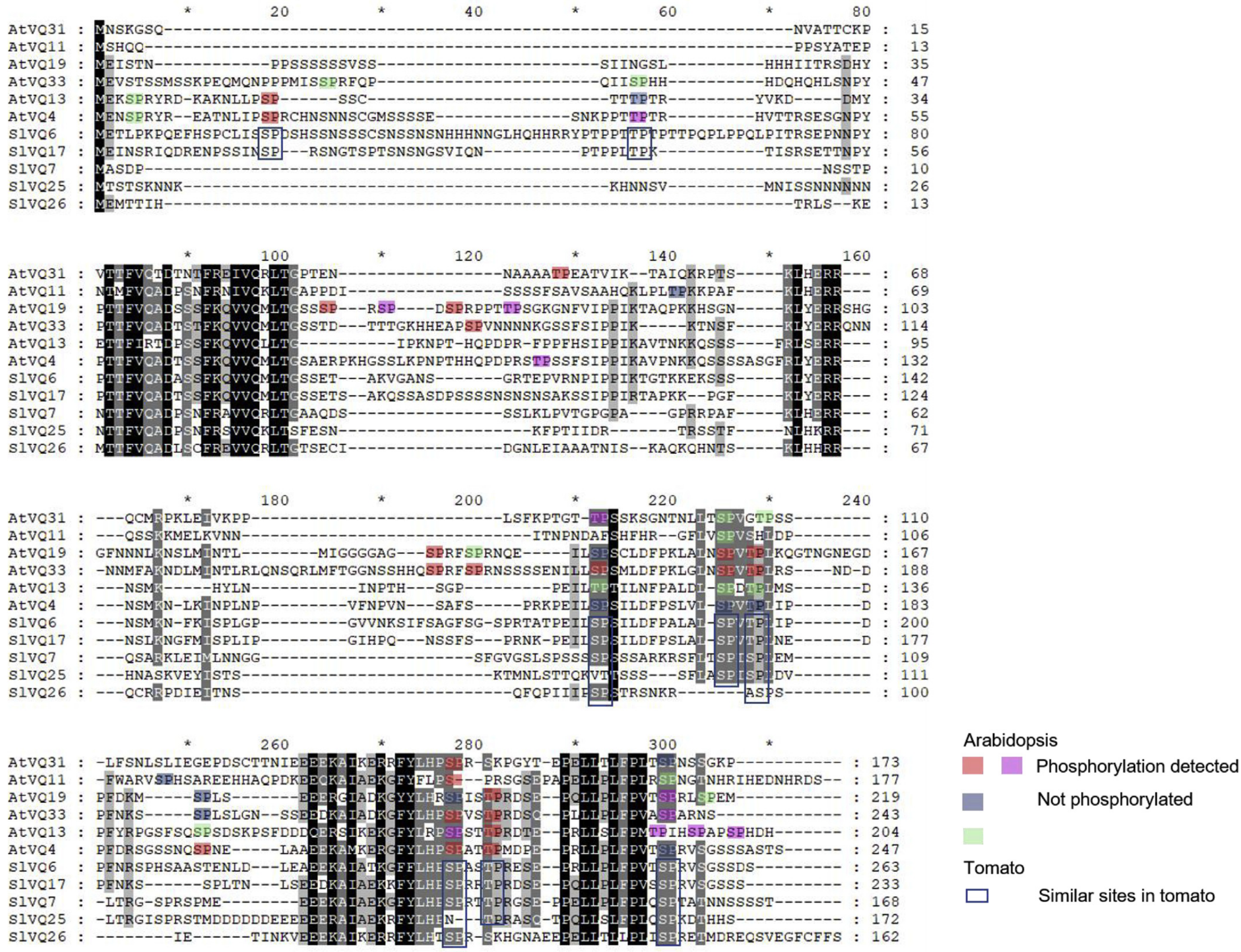

Fig. 6. Phosphorylation of mitogen-activated protein-targeted VQ-motif-containing proteins. Multiple sequence alignment of SlVQ proteins of group I in Arabidopsis and tomato were performed. Sequences were aligned using GeneDoc software. The conserved regions were shown as black or grey shading. In Arabidopsis, the phosphorylated sites have been checked by Pecher et al. (2014). The S/T-P detected as being phosphorylated are marked in red or pink. The non-phosphorylated sites are shown in blue and green. In tomato, the box represents identical or well-conserved sites which is the potential MAPK target sites (S/T-P). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

good method for identifying the heat resistance of Arabidopsis. Here, the hypocotyl length of HT-stressed *SlVQ6*-overexpressing plants were shorter than those of WT (Fig. 8C and D). To further confirm the thermotolerance, the Arabidopsis seedlings were used. As shown in Fig. 8E, most SlVQ6-overexpressing seedlings treated with 45 °C for 120 min died after recovery for 10 days. These results indicate that *SlVQ6* negatively regulates thermotolerance. However, there was no difference between OE lines and WT under drought and salt stresses (data not shown).

3.8. *SlVQ6*-regulated genes

In order to fully understand the gene network regulated by SlVQ6, we performed RNA-seq analysis on overexpressing plants and wild type. Through independent three biological experiments and strict parameter settings, 65 DEG were obtained. Among the 65 DEGs identified, 53 were down-regulated and 12 were up-regulated in overexpression line OE6-7 (Fig. S5A; Table S3). On the basis of gene ontology (GO) annotations, the DEGs were classified into 32 groups including 20 biological processes, 8 cellular components, and 4 molecular function (Fig. S5B; Table S4). For biological process, the DEGs were mainly involved in response to stimulus, metabolic process, and cellular process. The 20 top GO enrichment revealed that a large number of stress responsive DEGs were regulated by *SlVQ6*, including “response to stimulus (36)” and “response to stress (26)” (Fig. S5C). At the molecular function category, except two major categories catalytic activities and binding, “transporter activity” and “nucleic acid binding transcription factor activity” were observed. Six DEGs of transcription factor were down-regulated, including *ERF11*, *ERF109, NAC019*, *MYB75*, *MYB95*, and *ATRL3* (Table S3). To analyze the pathways mediated by *SlVQ6*, the KEGG pathway analysis was performed. KEGG categories were group into “Global and overview maps”, “Lipid metabolism”, “amino acid metabolism”, and so on (Fig. S5D). As shown in Tables S3 and 65 DEGs were classified into different categories including transcription factor, lipid metabolic process, oxidation-reduction process, stress response, defense response, transport, other metabolic process, and unclassified protein/RNA.

To explore DEGs associated with HT stress, 8 HT-related DEGs were identified using Hana-DB-AT database, including *HSP70-4*, *ERF109*, *ATCPT7*, *FER1*, *GOLS1*, *DIP2*, AT4G36010, and AT2G28400 (Table S3). HSP70 is the star gene for HT response. Furthermore, by integrating the

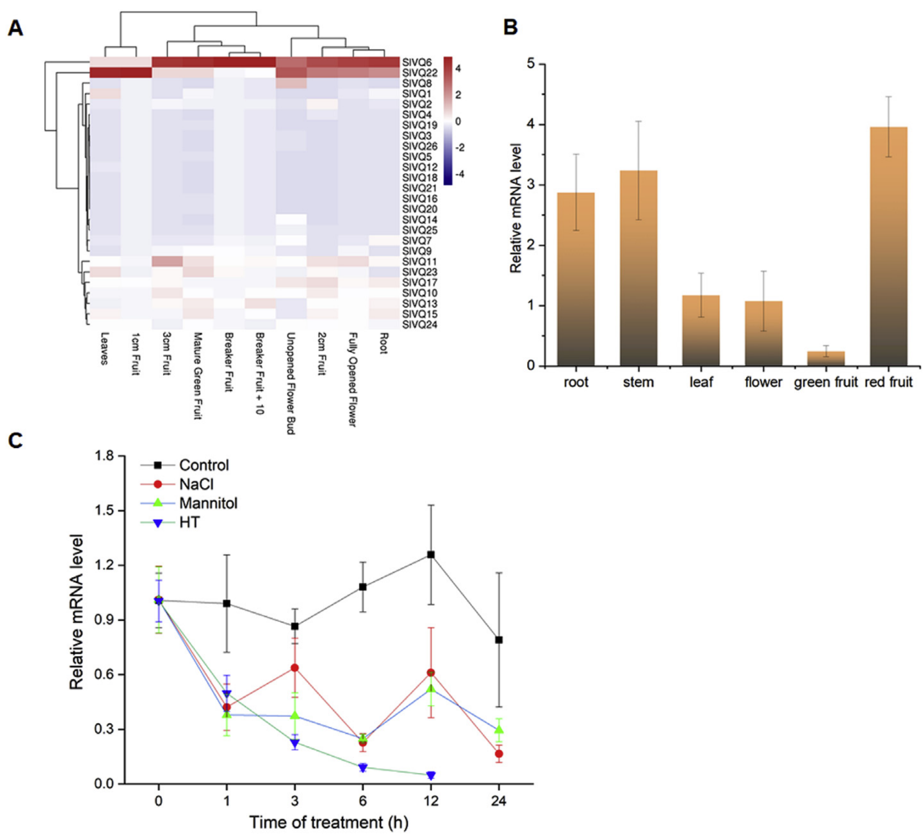

Fig. 7. Gene expression profiles. A, Heatmap of 26 *SlVQ* gene expression levels in cultivated tomato Heinz under different development stage (data from Rosli et al., 2012). The normalized gene expression level is represented by a color scale histogram. B, Expression patterns of *SlVQ6* in different tissues. Tissue-specific expression of *SlVQ6* was detected with qRT-PCR, with the total RNA extracted from roots, stems, leaves, flower, green fruit, and red fruit. C, Expression patterns of *SlVQ6* under different stresses. The tomato seedlings were treated for different time with 125 mM NaCl, 200 mM mannitol and high temperature (HT, 42 °C) stresses. The transcript level of *SlVQ6* was normalized to *Ubq* expression. Error bars are ± SD values of three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

HT-responsive transcriptomic data of Arabidopsis in recent years (Table S3), 25 HT-responsive DEGs were obtained (Table 1), which were all down-regulated in overexpression line OE6-7. qRT-PCR analysis was also performed on four DEGs to validate the data obtained by RNA-seq. As shown in Figs. S6 and 4 tested genes showed similar expression trends at the transcript level. These results suggest that changes in HT-associated DEGs caused by *SlVQ6* may be responsible for the decreased thermotolerance in *SlVQ6* overexpression plants.

4. Discussion

The VQ proteins are plant-specific proteins, regulating plant growth and development, including responses to environmental stresses (Jing and Lin, 2015). Though VQ protein families have been found in many plants such as Arabidopsis, rice, bamboo and soybean (Guo et al., 2018), most researches so far have centered on the functional studies of VQ proteins in Arabidopsis. There are some differences in the number and types of VQ in plants. The 74 members were found in soybean (Wang et al., 2014), while 18 VQ proteins were found in grapevine (Wang et al., 2015). In this study, 26 SlVQ proteins have been identified based on tomato genome databases and the structure and characteristics of *SlVQ* genes and proteins have been established, which can provide a strong foundation for further in-depth studies of tomato SlVQ proteins.

Most *VQ* genes do not have an intron in higher plants (Jing and Lin, 2015). However, 72% (18/25) of moss *VQ* genes contain introns. Here, only two *SlVQ* genes (*SlVQ3*, *SlVQ14*) have introns (7.7%) (Fig. S2). The results are similar to the findings in Arabidopsis (Cheng et al., 2012), Chinese cabbage (Zhang et al., 2015) and in rice (Li et al., 2014), where there are 30 VQ genes (88.2%), 45 VQ genes (90%) and 37 genes (92.5%) without introns, respectively. It is speculated that a large number of introns might be lost in *VQ* genes during evolutionary time (Wang et al., 2017). Furthermore, most moss encode relatively large proteins, of which most are longer than 300 amino acids. Tomato SlVQ proteins have the average of 236.7 amino acids which is similar to many known plants such as Arabidopsis and tea. The plant VQ gene family tends to lose introns and the overall evolution of the family is relatively conservative (Wang et al., 2017). The 26 SlVQ proteins contained the conserved FxxhVQxhTG motif with seven variations (Fig. 1), including FxxxVQxLTG (13/26), FxxxVQxFTG (5/26), FxxxVQxLTS (4/26), FxxxVQxLTA (1/26), FxxxVQxYTG(1/26), FxxxVQxHTG (1/26), and FxxxVHxVTG (1/26). In previous studies, it is known that there are six motif types in Arabidopsis (Cheng et al., 2012), five in bamboo (Wang et al., 2017), and four in rice (Kim et al., 2013). It is interesting that the FxxxVQxHTG motif is only found in tomato. Meanwhile, 10 conserved motifs were identified in all SlVQ proteins, and there were significant differences in the number of conserved sequences possessed by different groups of SlVQ proteins (Fig. 3). Motif 1 (VQ-containing motif) is distributed in all SlVQ proteins, implying the specific function of SlVQ proteins. The motif 2 and motif 6 correspond to the group IV, while motif 3 and 5 belong to the group I. The same motif in the same group suggests the similar biological function. These results show the more distant evolutionary relationship of these SlVQ proteins and greater divergence between them.

Accumulated evidence suggests that many plant VQ proteins perform biological functions by interacting with WRKY transcription factors, MAPK and other proteins (Pecher et al., 2014; Jing and Lin, 2015; Zhou et al., 2016; Dong et al., 2018). The WRKY transcription factors is one of the largest families of transcriptional regulators regulating plant growth and development. Plant VQ proteins can physically interact with several WRKY proteins to regulate various physiological processes. Their regulatory network has been clearly presented by STRING and Arabidopsis Interactions Viewer (Fig. 4; Fig. S3). In *Arabidopsis*, AtVQ23 (SIB1) and AtVQ16 (SIB2) counteract necrotrophic pathogens through specifically recognizing the C-terminal WRKY domain acting as co-activators of AtWRKY33 in plant defense (Lai et al., 2011). VQ14 (IKU1) interacts with WRKY10 (MINI3) to reduce *IKU2* expression, thereby affecting seed size (Wang et al., 2010). VQ22 negatively

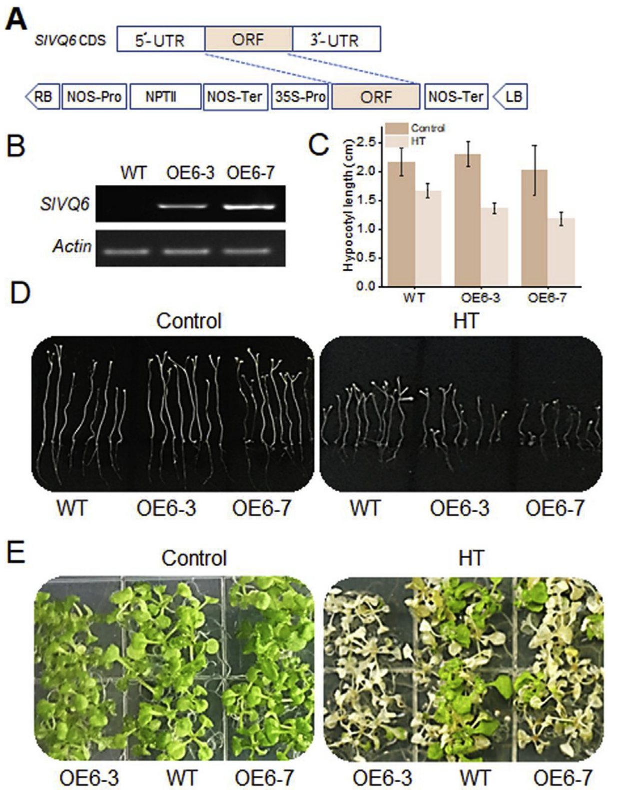

Fig. 8. The phenotype of *SlVQ6* overexpressing Arabidopsis seedlings under high temperature (HT) stresses. A, Schematic diagram of the expression *SlVQ6* construct. The coding region of *SlVQ6* was inserted in the expression vector pBI121 controlled by CaMV 35S. B, The expression of *SlVQ6* in the Arabidopsis overexpression (OE) lines. RT-PCR analyzed the transcript abundance of *SlVQ6* in the two independent OE transgenic plants. *Actin* was used as internal control. C, D, Phenotype of *SlVQ6*-OE transgenic plants under HT stress. Hypocotyl length of wild-type (WT) and *SlVQ6*-OE Arabidopsis lines in ½ MS medium under HT stress. After germination for 3 d, the plates covered with foil were subjected to HT treatment at 45 °C for 120 min, followed by vertical culturing in the dark for 8 d. Then, the length of hypocotyl was measured (C) and the phenotype was photographed (D). E, Phenotype of *SlVQ6*-OE transgenic seedlings after HT test. The 20 d old seedlings of WT and *SlVQ6*-OE lines on 1/2 MS media were subjected to 45 °C for 150 min and then recovered at 22 °C for 10 d. Experiments were repeated three times and the representative images were shown.

controls JA-mediated defense by interacting with WRKY28 and WRKY51 to (Hu et al., 2013a). WRKY2 and WRKY34 interact with VQ20 protein to regulate pollen development and function (Lei et al., 2017). In Arabidopsis, the VQ protein only physically interacts with members of the WRKY transcription factor of Group I or Group IIc (Cheng et al., 2012; Pecher et al., 2014). In apple and soybean, at least 20 MdVQ proteins and GmVQ proteins interact with the C-terminal WRKY domains of Group I and the single WRKY domain Group IIc WRKYs (Zhou et al., 2016; Dong et al., 2018). In this study, the SlVQ interaction networks were integrated using STRING (Fig. 4A) and SlVQ11 interacting WRKYs (SlWRKY28, SlWRKY30, SlWRKY71) belongs to the Group IIc of tomato WRKY (Huang et al., 2012). While SlWRKY18, SlWRKY20, SlWRKY31, and SlWRKY33 were classified in the Group I of tomato WRKY. In Arabidopsis, there are 34 VQ proteins and 32 group I and IIc WRKY proteins (Cheng et al., 2012). Soybean contains 74 VQ proteins and 72 group I and IIc WRKY proteins (Zhou et al., 2016). In tomato, there are 26 VQ proteins (Table S2, Fig. 1) and 15 group I and 16 group IIc WRKY proteins (Huang et al., 2012). As also shown in Fig. 6, the C-terminal WRKY domains in group I SlWRKY proteins are highly conserved using the alignment of the Arabidopsis WRKYs. All these results indicate that the mechanism of VQ-WRKY interactions also exist in tomato.

The mitogen-activated protein kinase (MAPK) cascade is an evolutionally conserved signaling pathway. AtVQ21/MKS1 is first discovered as a substrate of AtMPK4 (Andreasson et al., 2005). Multiple Ser residues of AtVQ21 can be phosphorylated by AtMPK4. MKP4 phosphorylates VQ21 to release VQ21 and WRKY33 and then WRKY33 binds to the promoter of PHYTOALEXIN DEFICIENT3 (Andreasson et al., 2005; Petersen et al., 2010). It is speculated that the AtVQ21 facilitates the recruitment of the kinase to WRKY factors to activate the AtMPK4-mediated pathway. AtVQ9 interacts with AtMPK3 (Cheng et al., 2012). AtVQ4 interacting with WRKY inhibits WRKY, which is released by MPK3/6-activated phosphorylation of AtVQ4. The phosphorylation of AtVQ4 promotes its destabilization and degradation (Pecher et al., 2014). Furthermore, MPK3 and/or MPK6 can phosphorylate ten AtVQ proteins, including VQ4 (MVQ1), VQ13 (MVQ2), VQ33 (MVQ3), VQ19 (MVQ4), VQ11 (MVQ5), VQ31 (MVQ6), VQ32 (MVQ7), VQ6 (MVQ8), VQ14 (MVQ9), and VQ9 (MVQ10) (Pecher et al., 2014). Hence, it is speculated that there is a triangular relationship between VQ proteins, WRKYs, and MAPKs was proposed (Weyhe et al., 2014; Jing and Lin, 2015). It is interesting that only group I, II and III AtVQ proteins can be phosphorylated by MAPKs. In tomato, there are five SlVQ proteins of group I (SlVQ6, 17, 7, 25, 26) and two of group II (SlVQ11, 13). The similar phosphorylation sites identified in Arabidopsis VQ proteins were found in tomato (Fig. 6). The SlMPK5, SlMPK6, and SlMPK7 can interact SlVQ24 (Fig. 4A). Therefore, it can be speculated that there is a conserved phosphorylation mechanism of VQ proteins mediated by MAPKs.

SlVQ6 caught our attention because of its highest expression level in different tissues (Fig. 7). SlVQ6 is homologous to AtVQ4 (MVQ1). MVQ1 inhibits PAMP-induced gene expression in a VQ-motif-dependent manner, acting as a negative regulator of PAMP induced responses to modulate resistance to pathogens (Pecher et al., 2014). In tomato, *SlVQ6* is expressed in all tissues with the highest level in red fruit and the lowest level in green fruit (Fig. 7B). *SlVQ6* could response to salt, drought and HT stresses (Fig. 7C). However, the specific function of the SlVQ6 was still unclear. To study the possible function of SlVQ6, the transgenic Arabidopsis over-expressing *SlVQ6* were generated. *SlVQ6* overexpression reduced plant tolerance to HT (Fig. 8). Arabidopsis AtVQ15 and AtVQ9 have been shown to act as a negative regulator of plant tolerance to osmotic and salinity stress, respectively (Perruc et al., 2004; Hu et al., 2013b). All these results suggest that VQ proteins belonging to different subfamilies play different biological functions. To further elucidate the possible molecular mechanisms, the relevant regulatory genes were identified in *SlVQ6* overexpressing lines by RNA-seq (Fig. S5). Among the 65 screened DEGs, 53 DEGs were down-regulated by *SlVQ6*. Specifically, 22 DEGs enriched in the “response to stress” term. Five more genes such as *NAC019* encoding NAC domain-containing protein 19, *RD20* encoding a stress-inducible caleosin, *GolS1* encoding a galactinol synthase, *HSP70-4* encoding a heat shock protein 70 gene, and *AT4g36010* (thaumatin-like protein) encoding a PR-5 like protein, were shown to be HT-inducible. These genes may play an important role in improving plant HS stress tolerance. For example, NAC019 controls abiotic stress responses including dehydration, ABA and high salt. Moreover, overexpression of *NAC019* significantly induces stress response genes (Xie et al., 2010). MYB75 (AtPAP1) increases anthocyanin accumulation in plants, which can confer abiotic stress tolerance components (Lee et al., 2016). RD20 (responsive to dehydration) appears to be one of the most highly expressed genes and is now often dehydrated, salt stress and ABA as stress marker genes. RD20 has putative peroxidase activity and may play a role in lipid modification and/or degradation. RD20 is involved in stomatal control, transpiration and drought resistance in Arabidopsis (Aubert et al., 2010). Lipoxygenase activity is required for the production of oxylipin signaling molecules. Plant ferritin gene expression can be induced by drought, salt, cold, heat and other link factors. Overexpression of the wheat ferritin gene *TaFER-5B* enhances tolerance to HT and other

Table 1
HT-responsive DEGs in 35S:*SlVQ6* compared to wild-type Arabidopsis.

GeneID	Symbol	Description	log2Fold Change	Padj	Pvalue
AT4G34410	*ERF109*	Ethylene-responsive transcription factor ERF109	−1.7656159	0.0292609	0.0001049
AT1G52890	*NAC019*	NAC domain-containing protein 19	−1.6117165	0.0489443	0.0002068
AT1G56650	*MYB75*	Transcription factor MYB75	−1.295724	3.481E-07	7.429E-11
AT1G74430	*MYB95*	Encodes a putative transcription factor (MYB95)	−1.1568064	0.0461724	0.0001912
AT2G33380	*RD20*	Probable peroxygenase 3	−1.5454267	0.0005784	6.666E-07
AT1G73480	*MAGL4*	Alpha/beta-Hydrolases superfamily protein	−1.1937144	0.0009646	1.318E-06
AT5G58770	*ATCPT7*	Dehydrodolichyl diphosphate synthase 2	−1.1730723	0.0183226	5.631E-05
AT5G24150	*SQE5*	Squalene epoxidase 5	−1.1483694	0.0174543	5.082E-05
AT5G01600	*FER1*	Ferritin-1, chloroplastic	−1.2434777	7.083E-05	3.023E-08
AT2G32510	*MAPKKK17*	Mitogen-activated protein kinase kinase kinase 17	−1.7334613	0.0017974	2.685E-06
AT4G23600	*CORI3*	Cystine lyase CORI3	−1.4160737	0.0004622	5.102E-07
AT2G47180	*GOLS1*	Galactinol synthase 1	−1.3359483	9.109E-05	4.277E-08
AT3G12580	*HSP70-4*	ARABIDOPSIS HEAT SHOCK PROTEIN 70	−1.2895823	0.0013108	1.846E-06
AT1G02820	*LEA2*	LEA3	−1.2186066	0.0132962	3.292E-05
AT5G03210	*DIP2*	A small polypeptide contributing to resistance to potyvirus	−2.3034195	3.54E-08	6.044E-12
AT4G08870	*ARGAH2*	ARGAH2	−1.3172627	0.0002138	1.46E-07
AT1G65490	*STMP5*	Secreted peptide in plant growth and pathogen defense.	−1.1586415	0.0002138	1.369E-07
AT4G36010	*AT4G36010*	Thaumatin-like protein	−1.0487232	0.0007316	9.329E-07
AT3G27170	*CLC-B*	CLC-B	−1.2641473	0.0031434	5.10E-06
AT4G13800	*ENOR3L3*	Magnesium transporter NIPA	−1.1316624	0.000378	3.55E-07
AT4G16590	*ATCSLA1*	Cellulose synthase-like A01	−1.8473936	0.0156537	4.21E-05
AT2G35070	*AT2G35070*	Transmembrane protein	−1.8065839	0.0003639	3.11E-07
AT5G15500	*AT5G15500*	Ankyrin repeat family protein	−1.6085961	0.0032698	5.443E-06
AT2G28400	*AT2G28400*	Senescence regulator	−1.4931369	0.0049203	9.451E-06
AT1G24145	*AT1G24145*		−1.2099571	0.020932	6.783E-05

Note: Fold change = < 0.5, FDR < 0.05.

abiotic stresses associated with ROS clearance (Zang et al., 2017). *AtGOLS1* can be induced by drought, salinity and heat stress. Heterologous expression of *Medicago falcate GolS1* in tobacco increases tolerance to freezing, low temperature, drought, and salt stress in transgenic tobacco plants (Zhuo et al., 2013). Therefore, the down-regulation of these stress-related genes, especially those associated with HT, may partly explain the decreased thermotolerance in *SlVQ6* transgenic Arabidopsis.

5. Conclusion

The present study provides the first systematic analysis of tomato VQ proteins. A total of 25 SlVQ proteins were identified and classified into 8 subgroups in tomato. Genome-wide bioinformatics analysis was carried out to study gene structure, conserved protein motif, phylogenetic relationship, and functional interaction network including WRKY transcription factors and MAPKs. In addition, *SlVQ6* was highly expressed in tissues and could response to different stresses. *SlVQ6* showed negative regulation of high temperature tolerance. Transcriptome analysis revealed that SlVQ6 down-regulates the expression of several stress responsive genes, such as *HSP70-4*, *RD20*, *GolS1*, and *AT4g36010* in overexpression plants compared with those in WT under normal growth conditions. The present study provides critical information about tomato *SlVQ* genes and their encoded proteins, as well as further research on SlVQ proteins, especially on SlVQ6.

Author contributions

Conceived and designed the research: HD, CG. Conducted the experiment: HD, GY, SM. Analyzed the data: HD, GY. Partly participated in the experiment: YQ, YW, QC, XX, XW. Wrote the paper: HD. Writing Guidance: CG.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.019.

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