Genome-Wide Identification of Grapevine's C3H2C3 Type RING E3 Ubiquitin Ligases Family Reveals VyRCHC114, which Confers Tolerance to Drought Stresses

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Abstract

RING is one of the largest E3 ubiquitin ligase families, playing an important role in plants' development and growth and their biotic and abiotic stress responses. The C3H2C3 type is the largest subfamily of RING. Here, 143 C3H2C3-type RING genes (RCHCs) were discovered from the grapevine genome and separated into groups (I˜XI) according to their phylogenetic analysis, with these genes named according to their positions on chromosomes. Gene replication analysis showed that 26 group segmental duplications and 14 tandem duplications of VyRCHCs were identified. All VyRCHCs have introns, VyRCHC29 has a maximum of 19 introns and 96 VyRCHCs have introns of 1 to 3. ARE, ABRE and O2 site Cis-acting elements very conservative in the promoter of VyRCHCs, with a large number. The expression profiles of eight DEGs in RNA-Seq after drought stress were similar to those in qRT-PCR analysis. The in vitro ubiquitin experiment showed that VyRCHC114 had E3 ubiquitin ligase activity, while Arabidopsis thaliana overexpressing VyRCHC114 had stronger tolerance of drought stress than the control. Valuable new information on the evolution of grapevine RCHC family genes and its relevance for studying the functional characteristics of grapevine VyRCHC114 genes under drought stress emerged from this research.

Introduction

To survive in a changing environment, post-translational modification of proteins often occurs when plants perceive and transmit internal or external signals. The acetylation, methylation, phosphorylation, and ubiquitination of proteins are the main types of post-translational modification, which play a key role in different plant development stages and plant-environment interactions. The process of classifying intracellular proteins under the action of a variety of special enzymes, and specifically modifying the screened target proteins, is called ubiquitination(Sadanandom et al., 2012). In eukaryotic cells, the ubiquitin system is complex and mainly involves ubiquitin (a small molecule protein), an intact 26S proteasome, and ubiquitin-activating enzyme (E1), ubiquitin-binding enzyme (E2), and ubiquitin-ligase (E3) (Sharma et al., 2016). The inactivated ubiquitin-dependent ATP is first activated by E1 through the thioester bond formed between the C-terminal of ubiquitin and the cysteine residue of E1; then the ubiquitin signal connected to E1 is transferred to the acetylcysteine of E2. In the next step, the ubiquitin linked to E2 is transferred directly or indirectly to the lysine residue of the target protein via E3. It is noteworthy that E3 ubiquitin ligase is the main factor which determines the specific protein binding during ubiquitination (Kelley, 2018), in that it can repeatedly add ubiquitin to the substrate protein, so that eventually the target protein is degraded by the 26S protease(Stone, 2014).

Recent studies have shown that the RING E3 ubiquitin ligase plays a key role in biological stress responses of plants. For example, in tobacco,*NtRFP1* encodes the C3-H-C4 type RING E3 ligase, which can diminish the

effect of βC1 by regulating the ubiquitination of TYLCCNB-βC1, such that *NtRFP1* -overexpressing transgenic plants consistently developed resistance to toxicity after infection from tomato yellow leaf curl China virus (Shen et al., 2016). Mediated by jasmonic acid, Arabidopsis *JAV1* (JASMONATE-ASSOCIATED VQ-MOTIF GENE1) was a repressor of defense responses against *Botrytis cinerea* and *Spodoptera exigua* larvae; conversely, overexpressing*JUL1* (JAV1-ASSOCIATED UBIQUITIN LIGASE1), which encodes the RING E3 ubiquitin ligase that causes JAV1's degradation by the proteasome, enhanced plant resistance to both *S. litura* and *B. cinerea*(Ali et al., 2019). Arabidopsis *MIEL1* (MYB30-Interacting E3 Ligase1) encodes the RING-type E3 ubiquitin ligase, which interacts with and ubiquitinates MYB30, such that MYB30 proteasome degradation and downregulation of its transcriptional activity were mediated by MIEL1. Accordingly, Arabidopsis *miel1* plants showed increased resistance in response to inoculations *Pst AvrRpm1* and *Pst AvrPphB*with fungus (Marino et al., 2013). Furthermore, the RING-type E3 gene*EIRP1*(*Erysiphe necator* -induced RING finger protein 1) also has a positive role in grapevine defense responses. Speficically, the*EIRP1* induced by *E. necator* targets negative transcription factor VpWRKY11; when *EIRP1* is overexpressed in Arabidopsis it evoked resistance to *G. cichoracearum* and Pst DC300 (Y. Yu et al., 2013). These above findings suggest the defensive response mediated by the E3 ligase may be conserved across plants.

In recent years, mounting research has shown that the RING E3 ligase gene also figure prominently in abiotic stress responses of plants(Shu & Yang, 2017). *SpRing* is a RING-type E3 ubiquitin ligase located in endoplasmic reticulum and participates in salt stress signal transmission in wild tomato variety *Solanum pimpinellifolium*'PI365967'. In addition, *SpRing* is silenced by virus-induced gene silencing, resulting in increased sensitivity of wild tomato to salt stress. Overexpression of *Arabidopsis thaliana* in spring can improve its salt tolerance(Qi et al., 2016). *SDIR1* (SALT- AND DROUGHT-INDUCED REALLY INTERESTING NEW GENE FINGER1) is a RING-type E3 ubiquitin ligase that regulates the salt stress response and ABA signaling in Arabidopsis by degrading the target protein SDIRIP1 (SDIR1-INTERACTING PROTEIN1). The downstream transcription factor ABI5 (ABA-INSENSITIVE5) is regulated by SDIRIP1, and overexpression of ABI5 increases salt tolerance (Zhang et al., 2015). The E3 ubiquitin ligase*OsHTAS* (*Oryza sativa* HEAT TOLERANCE AT SEEDLING STAGE), which regulates the stomatal opening state in the leaves by regulating ROS homeostasis, thus improving the basal heat resistance of the leaves. It involves two pathways, aba dependent and dst-mediated. (Jianping Liu et al., 2016). In Arabidopsis, *CHYR1*(CHYZINC-FINGERANDRINGPROTEIN1) encodes the RING-type E3 ubiquitin ligase which interacts with a related protein kinase KINASE2 (SnRK2) and can be phosphorylated by SnRK2.6 on its Thr-178 residues. When mediated by ABA, CHYR1 promotes the production of reactive oxygen species (ROS), stomatal closure, and drought tolerance in plants (Ding et al., 2015). The capsicum annular E3 ubiquitin ligase, *CaAIRF1*(*Capsicum annuum* ADIP1 INTERACTING RING FINGER PROTEIN1), can interact with protein phosphatase*CaADIP1* and positively regulate ABA signaling pathway to improve drought tolerance (Lim et al.,2017). In *Zea mays* ,*ZmXerico1* encodes a RING-type E3 ligase, which can regulate the stability of ABA8'-hydroxylase protein and thereby enable control of the dynamic balance of ABA; hence, expression of *ZmXerico1* endows maize plants with ABA sensitivity and improves their water use efficiency under drought stress (Brugière et al., 2017). Furthermore, Arabidopsis *AtAIRP1* , *AtAIRP2* , *AtAIRP3* and*Capsicum annuum* CaAIR1 jointly encode a E3 ubiquitin ligase, by regulating ABA signaling transduction to regulate drought responses, the expression of these genes increases ABA-mediated stomatal closure (Cho et al.,2011; Kim & Kim, 2013; C. Park et al., 2015; Ryu et al., 2010). Collectively, the above studies suggest E3 ligase is crucial for responding to abiotic stress.

Grapevine (*Vitis vinifera* L.) is a major cash crop, whose cultivated varieties have a total worldwide output of nearly 70 million tons of the fruit berries from more 7 million hectares of harvested land (Y. Yu et al., 2020). This plant is mainly grown to produce table grapes, fruit juices, and wine (M. Wang et al., 2014). Most grapevine producing areas in the world incur seasonal droughts. According to global climate modeling, droughts will intensify in the near future. Drought can adversely affect the growth and development of grapevines, because under drought stress the concentration of cytokinin in their stems decreases , vegetative and reproductive growth is inhibited (Hardie & Considine, 1976). When a grapevine is in full bloom, drought stress will also affect its pollination process, which decreases the fruit setting rate and affects the size of

the individual fruit berries produced (Santos et al., 2003). With worsening water shortages, drought stress may well become a key factor impacting grapevine and wine production worldwide (Chaves et al., 2009). Therefore, it is of great significance to grapevine production and breeding to study the drought resistance of wild grapevine plants as this could uncover the molecular mechanisms enabling them to withstand drought effects. *Vitis yeshanensis* is a wild grapevine plant native to arid areas of China, whose morphological characteristics indicate adaptability to arid environments in many aspects(Wan et al., 2008). Several studies have shown that wild *Vitis yeshanensis* has stronger drought resistance than other cultivars(Jing & Wang, 2013; Yang et al., 2012).

Although the RING-type gene family has been found in more and more plant species, and its importance for plants' stress responses and growth and development increasingly recognized, RING-type genes have yet to be fully identified in grapevine. The RING type E3 ubiquitin ligase is reportedly involved in grapevine's stress and growth, but too few studies have investigated E3 ubiquitin ligase's involvement in the regulation of grapevine response to drought stress. This study aimed to characterize the RING-type E3 ubiquitin ligase in grapevine's genome and its relevance for drought stress. To do this, genome-wide identification of C3H2C3 genes, the largest subfamily of grapevine RING-type, was carried out, coupled to their phylogenetic analysis, gene structure analysis, chromosome mapping, gene replication analysis, and cis-acting element analysis in gene promoter regions. We also quantified the expression levels of these genes under simulated drought treatment, and obtained a group of DEGs. The *VyRCHC114* gene was confirmed by RT-qPCR, and then the ubiquitin ligase activity of this gene verified. The functioning of this gene under drought conditions was elucidated using Arabidopsis transgenic plants. Our study provides an important basis for ubiquitin regulation of drought stress in grapevine.

Materials and Methods

Plants and treatments

Arabidopsis thaliana plants (ecotype Columbia, Col-0) were grown in a soil mix of peat moss, perlite and vermiculite (3:1:1, v/v/v) under a 12/12 h day/night cycle at 25 with 60% humidity. For drought stress, plants were transformed with an empty vector (EV) or to overexpress *VyRCHC114* (OE#2, OE#5, or OE#13), all of which grown on individual MS medium plates for 7 days before transplantation into soil. After 3 weeks, these plants received 15 days of drought stress, after which they were re-watered and their survival recorded after six days. This experiment was repeated three times.**Identification of RING-type C3-H2-C3 genes in the grapevine genome**

To identify the C3H2H3 type of RING, the most recent grapevine genome file in the Ensembl Plants Database (http://plants.ensembl.org/index.html) was downloaded and used. The HMM profiles (PF13639 and PF12678) of related RING C3H2C3 domain sequences were downloaded from the Pfam (http://pfam.xfam.org/), SMART (http://smart.embl-heidelberg.de/), and CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) databases to identify the RING C3H2C3 domain genes, by using HMMER software (3.0) set to its default parameters. Finally, 143 genes were confirmed as RING C3H2C3 genes, after manually checking whether their protein sequences had eight definite metal ligands. The physicochemical properties of each RING-type C3H2C3 protein were predicted using the Protparam online tool (https://web.expasy.org/protparam/). The 143 *VyRCHCs* were named according to their positional information on the chromosomes.

NJ phylogenetic analysis of RING-type C3H2C3 genes

A phylogenetic tree was constructed based on the respective protein sequence of *VyRCHCs* , by comparing their conserved amino acids. These were analyzed with 1000 bootstrap repetitions to determine their adjacency (method of Maximum Likelihood) in MEGA7.0 software.

Chromosome localization and gene duplication of *VyRCHCs*

According to the annotated positions in the grapevine genome data, 143 grapevine *VyRCHCs* were located on 20 chromosomes. By referring to the previous studies, the tandem repeat gene pairs and segment repeat

gene pairs of grapevine RING C3H2C3 were reliably identified (Ni, Ji, & Guo, 2020). Their visualization was implemented using TBtools software (Chen et al., 2018). The differentiation process after replication was detected by Ka and Ks, and the selection pressure of replication gene pairs was measured by Ka/Ks. Generally speaking, Ka/Ks < 1 indicates negative selection or purification selection, Ka/Ks = 1 represents neutral selection, and Ka/Ks > 1 indicates positive selection to accelerate evolution. The non-synonymous (Ka) and synonymous substitution rates (Ks), as well as Ka/Ks ratio values, of *VyRCHCs* repeat gene pairs were calculated in the kaks_calculator2.0 program in the Linux system.

Visual analysis of amino acid sequence and gene structure

The conserved motif of each *VyRCHC* gene was identified by the MEME program (v4.12.0). Parameter settings used were a motif length of 6-35 aa residues and a maximum detection amount of 7. Then, the *VyRCHCs* structure was visualized in TBtools (Chen et al., 2018).

Cis-acting component analysis of *VyRCHCs*

The *VyRCHCs* upstream 2 kb promoter sequences were uploaded to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), the XML file to TBtools results visualization (Lescot et al., 2002).

Expression analysis of *VyRCHCs* in grapevine under drought stress

To analyze the grapevine *RCHC* genes' expression levels under drought stress, we obtained from the NCBI database (registration number: SRA110531) two different drought resistance genes (101.14 and M4) which were compared under two different treatments WS(Water Stress) and WW (Well-Watered) in roots and in different periods (T1–T4:2d、4d、7d、10d) RNA-Seq data set. Based on the expression values of RING C3H2C3 in the roots of the two genotypes, we calculated the log2(WS/WW) values (fold-change) in each time period. The R package 'pheatmap' was used to produce a heatmap for this data.

RNA extraction and quantitative real-time PCR (qRT-PCR)

The qRT-PCR primers were designed using Primer Premier 5 software. The RNA from Arabidopsis and grapevine leaves was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Beijing, China), after which reverse transcription of RNA into cDNA was done using the Prime Script RT Reagent Kit (Takara, Dalian, China). The qRT-PCR was performed in an IQ^5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR Premium EX Taq II (Takara, Dalian, China). The reaction volume was 25 μl. The relative expression level corresponding to a given gene was calculated by using the 2^{-Ct} method; each reaction was prepared in triplicate and repeated three times. Primer sequence information in

Supplementary Table 5.

E3 ubiquitin ligase activity assay

The open reading frame (ORF) of VyRCHC114 and the different site mutants C320S, H341A, C328S, and N355A were separately cloned into the SalI/KpnI site of the pMAL-c5X vector (New England Biolabs UK Ltd, Hitchin, UK). According to the manufacturer's instructions, the pMAL protein fusion and purification system (New England Biolabs) was used to purify the fusion protein. Ubiquitination activity was then measured that according to the method described above (Y. Yu et al., 2013), albeit with the following modifications made: 250 ng of purified E3 (MBP-*VyRCHC114* , C320S, H341A, C328S, and N355A) in the ubiquitination buffer (50 mM Tris-HCl (pH 7.5), while the other reagents and steps used were the same. Primer sequence information in**Supplementary Table 5** .

Determination of chlorophyll content

To measure the Fv/Fm and Pn values, liquid nitrogen was used as a substrate. Leaf powder (100 mg) with 80% acetone (to extract pigments) were used to determine chlorophyll content. Chlorophyll was detected

by spectrophotometry at 645 nm and 663 nm. Ten leaves from each plant were analyzed and their values calculated by Handy PEA software (v1.31) and Biolyzer 4HP software (v4.0.30.03.02).

Physiological analysis of drought treatment and drought stress response of transgenic Arabidopsis

For this experiment, 3-week-old wild type (WT) and transgenic Aribidopsis plants were treated with drought. These plants were placed under drought conditions for 15 days, and then kept fully hydrated for 6 days, after which their survivorship was determined. Each sample contained 14 seedlings, and each experiment was done in triplicate.

For their physiological analysis, APX, SOD, POD and CAT activity levels were measured as described in two other recent studies (Chen et al., 2019; Gill et al., 2015). Each sample consisted of a pond of three seedlings, and each experiment was conducted in triplicate.

Results

Genome-wide identification of RING C3H2C3 type finger proteins in grapevine

The results of the Hidden Markov Model (HMM) were analyzed, and the gene sequences were extracted and given to SMART, CDD, and Pfam for domain authentication. From this, 143 *VyRCHC* genes were obtained by comparing and screening genes with eight conservative metal ligands, and the alignment members were not abandoned. The physicochemical properties of each of the 143 *VyRCHCs* were identified (**Supplementary Table 1**). The number of amino acids encoded by the 143 *VyRCHCs*ranged from 70 (*VyRCHC50*) to 763 (*VyRCHC98*). For these genes, the molecular weights of their products varied from 7.83 kDa to 83.58 kDa, while their isoelectric points varied from 3.88 to 9.95.

Analysis of *VyRCHCs* in the C3H2C3 domain

The typical RING domain is considered to be an octahedral group of metal-bound cysteine and its residues, which can chelate two zinc ions in a spherical cross-supported structure, in which the metal ligands 1 and 3, and 2 and 4, each bind to one zinc ion. This structure, however, requires a certain distance between adjacent metal ligands, it being variable between ml2-ml3 and ml6-ml7. We calculated statistics for this distance between adjacent metal ligands (**Supplementary Table 2**). It was found that, except those between ml2-ml3 and ml6-ml7, the distances between other metal ligands were constant, while those from ml2 to ml3 spanned 11 to 24 amino acids, and for ml6-ml7 the distance varied from 8 to 14 amino acids. The 143 *VyRCHCs* C3H2C3 domains have two amino acids between ml1-ml2 and ml5-ml6, while ml3-ml4 contains one amino acid, ml7-ml8 contains two amino acids as does ml4-ml5. To understand whether these RING C3H2C3 structural domains are conserved apart from their eight special metal ligands, their comparative analysis was conducted (**Supplementary Figure 1**). This revealed that some amino acids in the structural domain of RING C3H2C3 have a typical position bias (**Figure 1A**). In the C3H2C3 type RING region, the ml2 located in front of amino acid residues is the most common Ile (I) or Val (V); likewise, the phenylalanine (Phe, F) residue is typically before ml5, the leucine residue (Leu, L) is always next to ml2, and the aspartic acid (Asp, D) residue is usually positioned after ml6, while the tryptophan residue (Trp, W) is usually the fourth following ml6. Notably, a very conservative proline (P) was found situated after ml7. According to the RING-type C3H2H3 domain schematic diagram, two pairs of metal ligands bind to a zinc ion (**Figure 1B**). The total amino acid length of the C3H2C3 domain per *VyRCHC* gene and the corresponding number of different lengths were calculated: the vast majority of these were 41 and 42, accounting for 88.8% of all genes**(Figure 1C)** .

Phylogenetic analysis of *VyRCHCs*

To infer the evolutionary relationships of grapevine's *VyRCHCs* , Phylogenetic analysis of RCHC protein sequences of Arabidopsis, tomato, and grapevine were constructed (using the Maximum Likelihood method). According to the phylogenetic analysis, these 180 genes could be divided into 6 subgroups: I-VI (Figure 2). Group I has the least number of members, only 12, and the group with the largest number of members

is group III, while the *RCHC* gene of *Arabidopsis thaliana* or tomato is found in each group. It is worth noting that more *RCHC*genes of *Arabidopsis thaliana* and tomato are gathered in group VI. Most of the RING-type C3H2C3 genes of grapevine display some homology to*RCHC* genes of Arabidopsis or tomato. In addition, in different groups, some gene pairs showed high similarity, which were confirmed in the distance of evolutionary relationship, the location of RING conserved domain and the length of protein sequence. For instance,*SlATL33* and *VyRCHC62* , *SlATL46* and *VyRCHC108* ,*SlATL51* and *VyRCHC110* , *AtBRH1* and *VyRCHC116* ,*AtRHA1A* and *VyRCHC13* , *AtSDIR1* and *VyRCHC97* ,*AtRHC1A* and *VyRCHC59* etc. Next, a phylogenetic tree containing only 143 VyRCHC protein sequences was constructed (using the NJ method). To facilitate their study and analysis, the 143 members were divided into 11 groups (I-XI) according to the classification and phylogenetic analysis of **Supplementary Figure 2** , from which 27 pairs of genes with high homology were found. Based on their color-coded names, the *VyRCHCs* were then divided into six groups according to the number of conserved amino acids in their protein sequence.

Characterization of the motifs and gene structure of*VyRCHCs*

To further understand the diversity in motif composition between *VyRCHCs* , the MeMe analysis of VyRCHC proteins from groups I to XI was carried out. From this, 12 conserved motifs were identified in the VyRCHC protein, respectively named motif 1 to motif 12 (**Figure 3B**), in which motif 1 and motif 2 is found in almost every VyRCHCs, this motif combines to form the eight most important metal ligand (C-C-C-H-H-C-C-C) structures of every *VyRCHC* gene. The sequence information of motif 1-12 is presented in **Supplementary Table 6**(motif data). We next analyzed the exons, introns, and several key structures of *VyRCHCs* (**Figure 3C**). All the VyRCHC genes have introns, with a maximum of 19 introns in *VyRCHC29* and at least one intron in 57 *VyRCHCs* **(Supplementary Figure 3)** . The longest intron length was found in VyRCHC141. Importantly, each *VyRCHC* gene contained a special structure type we know as RING, and there are 13 such structures in some genes, such as PA, CUE, DUF1117, zinc_ribbon_9, and zf-CHY, among others. These structures could be relevant for the function of *VyRCHCs* (**Figure 3B**).

According to the phylogenetic analysis of *VyRCHCs*(**Figure 3A**), 45 pairs of genes can be found in the evolutionary tree. The results of the MeMe and gene structure analyses of these gene pairs were also similar (**Figure 3B and 3C**). For example, the conserved motifs in the protein sequences of *VyRCHC44/64* are highly similar, and the gene's structure type and length are also similar, such as for *VyRCHC94/95, VyRCHC38/97, VyRCHC18/78, VyRCHC28/67* and *VyRCHC11/107,* to name a few. Unexpectedly, the MeMe analysis of *VyRCHC22/23/24, VyRCHC55/127, VyRCHC105/133,* and *VyRCHC13/116* gene pairs gave near identical results to those from the gene structure analysis, revealing a remarkably similar protein sequence length, gene structure length and the intron number among them. We thus speculate these four gene pairs may perform similar functions in grapevine plants.

Chromosomal localization and gene replication analysis of*VyRCHCs*

According to the location of *VyRCHCs* in the grapevine genome, 143 *VyRCHCs* were placed on 20 chromosomes (**Figure 4**), albeit unevenly distributed among them. Imprinting of the *VyRCHCs* was found in each chromosome of grapevine, but the number of genes on different chromosomes varied. The most found were 12 *VyRCHCs* on chromosome 11, the 11 *VyRCHCs* were identified on chromosome 1,7,13 and 18. Further, we also observed that these most of these *VyRCHCs* are likely distributed at both ends of the chromosome, leaving only a small portion of them in its middle part. Gene replication events include tandem replication and segmental replication, both of which are very vital for expanding the number of members of the gene family. To clarify the amplification mechanism of *VyRCHCs*during their evolution, we studied their potential repetitive events of *VyRCHCs* . According to the intraspecific alignment of 143 *VyRCHCs* , 40 pairs of genes, 14 and 26, were respectively identified as associated with tandem or segmental replication events. Among the 40 pairs of gene events, the tandem repeat frequency between chromosomes 1 and 7 was the highest, and there were eight tandem replication events, with three pairs of genes in chromosome 2 and one pair of genes on chromosomes 12, 13, and 18 identified as tandem replication genes. In addition, the frequency of segmental repetition was greatest in chromosomes 6, 8 and 13, for which six, seven, and nine events were found, respectively. It was worth noting that no repetitive events were found in chromosomes 10 or 17.

Together, these results suggested that the main replication event mode of grapevine *VyRCHCs* family is via segmental replication; hence, it could have played a crucial role in the amplification of *VyRCHCs* during their evolutionary history.

To explore the selection of grapevine *VyRCHCs* in terms of their repetition and differentiation, the non-synonymous (Ka), synonymous (Ks), and Ka/Ks of each duplicated *VyRCHCs* were calculated. Among the 40 pairs of repetitive genes in grapevine, the Ka/Ks values of 31 pairs were all less than 0.5, while the average Ka/Ks value was 0.246. The Ka/Ks value of 8 pairs was between 0.5 and 0.787, with additional pair of genes being the sole having a Ka/Ks value greater than 1, at 1.269. It is worth noting that 60% of the *VyRCHCs* pairs had Ka/Ks values less than 0.3, indicating that most of the repeated grapevine *VyRCHCs* were under negative selection during evolution (**Supplementary Table 3**).

Chromosomal localization and gene replication analysis of *VyRCHCs*

To clarify the evolutionary relationship between grapevine, Arabidopsis, and tomato *RCHC* genes, their interspecific homologous genes were identified by comparative analysis. This uncovered 56 pairs of homologous genes among the three species, including 13 *VyRCHCs* 10 Arabidopsis and 13 tomato *RCHC* genes (**Figure 5**). This shows that grapevine, Arabidopsis, and tomato all retained similar *RCHC* genes in their evolutionary history. It is worth noting the absence of homologous genes with *VyRCHC29* in tomato, but their presence in Arabidopsis, which may have arisen from gene deletions in the process of evolution, given that the same genes are *VyRCHC11* , *VyRCHC38* , *VyRCHC107* , *VyRCHC119* , and *VyRCHC137* . Nonetheless, two or more *RCHC* genes in Arabidopsis and tomato were found homologous to one *VyRCHC* gene; for example, *VyRCHC89* and *Solyc07g053850.3/Solyc12g005470.2* and *AT4G28370/AT2G20650* , as well as those of *VyRCHC1* , *VyRCHC32* , *VyRCHC97* , *VyRCHC104* , *VyRCHC118* , and *VyRCHC142* . Hence, these genes may be parallel gene pairs and the putative source of amplifications of *RCHC* genes during evolution.

Cis-acting element analysis in *VyRCHCs* promoter

To further investigate the transcriptional regulation of *VyRCHCs* , cis-acting elements in the 2000bp region upstream of the *VyRCHCs* ' codon were predicted. The predicted cis-acting elements can be divided into seven categories according to their functions: namely, light response (32), hormone response (11), growth and development response (9), stress response (6), enhanced promoter cis-acting (6), binding site cis-acting (6), and other functional cis-acting (2) elements. Most promoters of grapevine *VyRCHCs* contained the CAAT-box or TATA-box, which are involved in the enhanced promoter cis-acting elements. In addition, 127 *VyRCHCs* promoters harbored the stress response element ARE, more than half of the promoters of the *VyRCHCs* having the hormone response elements ABRE, TGACG-motif, CGTCA-motif, and over half of the *VyRCHCs* also featured the G-box, GT1-motif, and Box 4 in their promoters (**Supplementary Table 4**). In the 2000kb region upstream of *VvRCHCs* , discovered very many different functions of cis element, in addition to the common cis element with light response and enhanced the promoter, also found that the more growth and adversity stress related cis element, this suggests that *VyRCHCs* may widely participating in various life activities of plant.

It is known that the RING gene can play a key role in plants' growth and response to abiotic stresses. Accordingly, the cis-acting elements related to abiotic stress, growth and hormone regulation were focused upon here. The respective locations of the five major acting elements associated with hormone response, binding sites, growth and development, and stress of our concern, on the promoter of the *VyRCHCs*(**Figure 6A**) were determined. To accurately identify the stress-related elements, we focused on four kinds (anaerobic induction, injury response, low temperature, drought response (**Figure 6C**), low temperature response, defense and stress response), whose locations are also depicted. In addition, we counted the number of major elements related to stress, growth and development, and hormone responses in the *VyRCHC* gene promoter (**Figure 6B**). Evidently, concerning growth and development, the number of O2-sites is the largest, there are 5 promoters of *VyRCHC6* and 4 promoters of *VyRCHC40* . In terms of stress, the number of ARE is very large, found in 89% of the *VyRCHCs* promoters, moreover, 5 of the most promoters

of *VyRCHC14* and *VyRCHC81* occurred. In terms of hormone response, the number of ABRE is dominant, found in 64% of the *VyRCHCs* promoters, moreover, 9 of the most promoters of *VyRCHC3* and *VyRCHC16* occurred. Surprisingly, 22 of the *VyRCHC74* gene promoters were found and 11 of the *VyRCHC128* gene promoters were found. These results suggested that *VyRCHCs* may be associated with cis-acting elements of different functions; in other words, these genes may be regulated by these elements and thereby influence related plant life activities.

Expression analysis of *VyRCHCs* in roots of two grapevine rootstocks with different drought sensitivity

To investigate differential *VyRCHCs* ' expression between plants having contrasting drought-resistant genes (101.14 vs. M4) under drought stress and their potential functioning, the grapevine RNA-Seq transcriptome database of the published dataset was used(Corso et al., 2015). We checked the expression of 143 *VyRCHCs* ; of them, 7 *VyRCHCs* were not expressed at any time, so we excluded them.

To understand the expression of these *VyRCHCs* under the drought treatment, we used the ratio of WS (Water Stress) to WW (Well-Watered) gene expression of the two genotypes to draw an expression heat map, expression values are reported as log2 of the fold change (WS/WW) fold change. (**Figure 7A**). However, more than 60% of the *VyRCHCs* in the two genotypes were highly expressed under the imposed drought. To screen out the key genes, in each time period of the treatment, the gene that conforms to $|\log2 (WS/WW)| > 1$ is considered a differential gene, and the Venn diagram was made using the differentially screened genes of the drought - tolerant genotype M4 at different times (**Figure 7B**). By looking at the different genes in each period, there are finally 8 genes that are different in three periods. To robustly verify the gene expression levels, we quantified the expression levels of these 8 key genes (**Figure 8**), whose pattern basically conformed to the trend shown in **Figure 7C** . That is, the *VyRCHC114* gene was significantly down-regulated at 2 days, with a strong downward trend through 4, 7, and 10 days of the drought treatment. The *VyRCHC66* , *VyRCHC68* , *VyRCHC69* and *VyRCHC95* genes had a similar expression trend, being slightly up-regulated at 2 days of drought, but strongly down-regulated at 4, 7, and 10 days thereafter. These results suggested eight key genes are probably involved in regulating the plant response to drought.

Identification of E3 ubiquitin ligase activity of *VyRCHC114*

To clarify whether *VyRCHC114* has E3 ubiquitin ligase activity, we conducted an in vitro ubiquitin activity assay, achieved by using purified MBP-*VyRCHC114* fusion protein mixed with ubiquitin, E1, and E2 and by western blotting with the MBP antibody. Ubiquitin molecules were detected on the fusion protein linked by MBP antibody (**Figure 9A**). This same method was used to detect ubiquitin antibody tags. The *VyRCHC114* protein was detected in the fusion protein linked by the ubiquitin antibody, which indicated it had E3 ligase activity.

We knew the RING-C3H2C3 type protein can form a RING structure for ubiquitin regulation, but this process depends on the interaction between the eight conserved metal ligands. To further illustrate whether and how E3 ligase activity of *VyRCHC114* depends on these conserved metal ligands, as shown in **Figure 9C** , we selected four different amino acid sites for mutation (two key conservative and two non-conservative metal ligand sites). Four corresponding proteins (C320S, C328S, H341A, N355A) were obtained, and their ubiquitin activity in vitro was tested by the same method. After the immuno-blotting analysis of MBP antibody and ubiquitin antibody, evidently the two mutant proteins C320S and H341A lost their E3 ubiquitin ligase activity due to mutations at key sites, but the two mutant proteins C328S and N355A maintained theirs (**Figure 9B**). These results indicated these conserved metal ligand sites are crucial factors for demonstrating the *VyRCHC114* 's ligase activity.

Overexpression of *VyRCHC114* enhances Arabidopsis drought tolerance

To clarify the effects of *VyRCHC114* 's role in plant responses to drought, we selected transgenic Arabidopsis (OE #2, #5, #13) with high expression levels of the *VyRCHC114* gene for subsequent experiments (**Figure 10B**). After 15 days of drought imposed upon wild plants and transgenic plants, followed by normal watering

for 6 days, phenotype observations revealed plants overexpressing *VyRCHC114* had significantly improved the drought tolerance (**Figure 10A**). Further, on average, more than 70% of the plants overexpressing *VyRCHC114* survived the drought stress, which was significantly higher than the 30% survival of the EV-transformed group (**Figure 10C**).

To understand the relationship between plant growth and drought resistance, electrolyte leakage rate (**Figure 11A**) and chlorophyll content (**Figure 11B**) were both measured. These were similar between *VyRCHC114* -overexpressed and EV-transformed plants in the non-stress treatment, but after 8 days of drought stress, the electrolyte permeability of the former was significantly lower than the latter's, while the chlorophyll content was significantly higher in overexpressing than EV-transformed plants. Additionally, the changes in photosynthesis under drought stress were further analyzed by measuring potential photosynthetic efficiency (**Figure 11C**) and capacity storage capacity (**Figure 11D**). Each was not significantly different between EV-transformed and *VyRCHC114* overexpression plants when they were non-stressed; however, Fv/Fm was significantly higher in the latter than the former at 4 days, and especially at 7 days, of drought stress. At 4 days, energy storage capacity of *VyRCHC114* -overexpressed plants was not significantly different from that of EV-transformed plants, but at 7 days of drought stress, that of the former exceeded the latter. Hence, these results suggested *VyRCHC114* could enhance the drought resistance of plants by participating in the regulation of photosynthesis.

Moreover, much research has shown that antioxidant enzymes can influence plants' drought tolerance. Common antioxidant enzymes are ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), so we examined their activity. As **Figure 12**shows, under non-stress conditions, the activity of these antioxidant enzymes was similar between the plants, whereas when drought stressed for 4 and 7 days, the activities of APX (**Figure 12A**), SOD (**Figure 12B**), POD (**Figure 12C**) and CAT (**Figure 12D**) were significantly higher in plants overexpressing *VyRCHC114* than those EV-transformed. Taken together, these data indicate *VyRCHC114* may also improve drought tolerance by elevating antioxidant enzyme activity.

AtCOR15a , *AtERD15* , *AtP5CS1* , and *AtRD29A* are known to be key genes for regulating plant responses to drought stress, so we quantified their expression under imposed drought. As expected, when non-stressed, there was no significant difference between plants overexpressing *VyRCHC114* overexpression and those EV-transformed. By contrast, under drought stress, all four genes were significantly higher in *VyRCHC114* -overexpressed plants than in those EV-transformed (**Figure 13**).

Discussion

The RING C3H2C3 gene family has since been identified in many plant species (Alam et al., 2017; Li et al., 2011; Qanmber et al., 2018; L. Yang et al., 2019). Related studies have shown that RING genes are involved in a variety of biological processes, growth and development and hormonal responses, as well as plant responses to abiotic stresses (Hua & Vierstra, 2011). However, for grapevine, the RING C3H2C3 gene had not yet been identified in its whole genome, with few reports available on its relevance for grapevine's growth and developmental regulation or response to abiotic stress. In our study, we analyzed the whole genome of grapevine for the RING C3H2C3 gene family members. Using the criteria of whether the eight conserved metal ligands are present, a total of 143 non-redundant RING C3H2C3 genes were thus identified. Studies have shown that grapevine's genome size is about 0.5 times that of tomato, containing 0.75 times as many genes as tomato (Consortium, 2012; Jaillon et al., 2007). According to the known RING C3H2C3genes in tomato, the genes account for 58multiples of genome length and the number of genes (Yang et al., 2019).

Many RING C3H2C3 of E3 ubiquitin ligases belong to the ATL gene family et al., 2006). According to Arabidopsis and tomato RING C3H2C3 genes. we divided grapevine's RING C3H2C3 genes into six categories (I-VI) (**Figure 2A**). Each group has Arabidopsis or tomato in the same branch. This shows that grapevine genes have sequence similarity with*Arabidopsis thaliana* and tomato genes. Based on the phylogenetic analysis and taxonomic analysis of 143 *VyRCHCs,* they were divided into 11 groups, named I-XI (**Figure 2B**). Our evolutionary analysis of grapevine, Arabidopsis, and tomato provided evidence that the

replication events of these genes occurred after their retention during the differentiation from common ancestors before species differentiation. Gene replication can arise from fragment replication, tandem replication, transposable events and even whole genome replication, which not only provide the evolutionary potential for species to produce new functional traits but also are a main driving force for species differentiation (Moore & Purugganan, 2003; Ren et al., 2018). In the identification of gene families from many species, gene replication events have proven instrumental in their expansion (Wang et al., 2016). We observed that some grapevine *VyRCHCs*correspond to one or more intraspecific homologous genes, thus indicating *VyRCHC* gene family's amplification in this plant may have been caused by gene duplication. Studies have shown that tandem replication often occurs in widely and fast evolving gene families, a good example being Nucleotide Binding Sites Leucine Rich Repeat (NBS-LRR) resistance families (Cannon, Mitra, Baumgarten, Young, & May, 2004). Segmental replication is more common in slow evolving gene families, like the MYB gene family (Cannon et al., 2004). We found 26 of 40 pairs of RING-type C3H2C3 genes participated in segmental replication (**Figure 4**), about twice the number of tandem copies. Therefore, we speculate that the *VyRCHCs* comprise a relatively slow evolution gene family, for which both segmental and tandem replications have contributed to its amplification. Yet, of the two modes, segmental replication was more important, a finding consistent with other grapevine gene families, like the WRKY family genes in the autopolyploid*Saccharum spontaneum* (Li et al., 2019). We calculated the Ka/Ks values of 40 pairs of repetitive genes in grapevine (**Supplementary Table 3**), finding that repeated *VyRCHCs*were in a strong state of purification and selection in their evolution, with an average Ka/ Ks of 0.379.

VyRCHCs structure studies have shown that most *VyRCHCs* in the same group have similar gene structure, including motif composition and exon/intron distribution. But different groups, which may be related to the functional diversity of *VyRCHC* gene members (**Figure 3A and 3C**). Further, among the 45 pairs of parallel*VyRCHCs* , most of them have a conservative gene structure and motif composition, with few showing a certain degree of differentiation. For example, *VyRCHC104* has six introns while *VyRCHC118* has three introns (**Supplementary Figure3**), but the results of MeMe analysis of their protein sequences are basically similar (**Figure 3B**). This supposed difference also exists among*VyRCHC63/98, VyRCHC50/51, and VyRCHC12/115* pairs, which may be due to the loss or increase of a single intron during the evolution of gene structure. Similarly, this situation commonly occurs in the identification of other gene families (Q. Wang et al., 2015). The same group of *VyRCHCs* are conserved in the process of evolution, and to a certain extent accompanied by gene tissue variation, thus indicating that some *VyRCHCs* members can achieve functional diversification via differential expansion. In general, the analysis of*VyRCHCs* was consistent with their phylogenetic analysis, and this robust correspondence demonstrates the latter's reliability as well as providing insight into the conservative evolutionary relationships among*VyRCHC* proteins of grapevine.

Cis-acting elements in gene promoter regions may be critical for gene regulation (**Figure 6**). For example, the DELLA protein and its interacting RING finger protein inhibit the gibberellin response, by binding to the promoter of a subset of the gibberellin response gene in Arabidopsis (Park et al., 2013). Analysis of cis-acting elements in grapevine's *VyRCHCs* revealed the existence of different types of cis-acting elements upstream of the C3H2C3 genes. Except for a large number of components related to optical responses, plant hormone regulation, growth and development, and stress were also common upstream of different *VyRCHCs* . This situation is in fact rather common in RING genes of all species (Alam et al., 2017; Li et al., 2011; Qanmber et al., 2018; Yang et al., 2019). ARE is a functional element related to antioxidant induction, which is present in 89% of *VyRCHCs* and contains many elements. For example, there are eight promoters upstream of the *VyRCHC29* gene, 6 upstream of the *VyRCHC86, VyRCHC119, VyRCHC27* genes, and five promoters upstream of*VyRCHC81, VyRCHC14, VyRCHC121, VyRCHC103, VyRCHC63, VyRCHC102, VyRCHC51* genes. Similarly, TC-rich repeats are an action element related to plant defense against stress, present in 44% of*VyRCHCs* , while MBS is an action element related to drought induction, found in 41% of *VyRCHCs* . Among them, the prevalence of MBS is four-fold higher in the upstream promoter of the*VyRCHC114* gene. Among the functional elements related to plant hormone regulation, 64% of the upstream promoters for *VyRCHCs*contain ABRE elements, 9 of the most promoters of *VyRCHC3* and*VyRCHC16* occurred. Surprisingly, 22 of the *VyRCHC74* gene promoters were found and 11 of the *VyRCHC128* gene promoters

were found, respectively. The TCA elements involved in salicylic acid- regulated and jasmonic acid regulated CGTCA element and TGACG element are also ubiquitous in *VyRCHCs* , which exist in 51% and 55% of*VyRCHCs* , respectively. These findings suggest that *VyRCHCs*may be involved in the regulation of different life activities. For example, ubiquitin ligase SDIR1 regulates stress-responsive abscisic acid signal by interacting with ABRE abscisic acid response element (Y. Zhang et al., 2007). ABA, GA, ethylene, trauma, drought, heat stress, and pathogen response elements are present in the promoter region of*OsRING* genes of rice plants, for which pathogen infection, SA, ABA, JA, and ethephon (ET) treatments could induce target genes expression to different degrees (Meng et al., 2006). A similar analysis of RING C3H2C3 gene *mRHCP1* was recently done in maize (Li et al., 2017). Therefore, we speculate that these *VyRCHCs* may be involved in a variety of different regulatory mechanisms through these cis-acting elements.

According to the analysis of RNG-Seq data set (**Figure 7A**), more than 60% of *VyRCHCs* were significantly up-regulated or down-regulated under drought stress, indicating those genes may play a key role in how grapevine responds to drought. Studies have revealed the molecular mechanism of many circular genes involved in the drought stress. For example, in Arabidopsis, *XERICO, SDIR1, AtAIRP1, AtAIRP2, AtAIRP3* and *AtAIRP4* has been found to play a key role in the drought response of plants. In addition, an E3 ubiquitin ligase*atrzf1* mutation increased the proline content of Arabidopsis and improved this plant's drought tolerance (Ju, Min, Chung, & Kim, 2013). Similarly *GpDSR7* encodes an E3 ubiquitin ligase, which when overexpressed in Arabidopsis increased its tolerance to drought stress (Li et al., 2016). In our study, and according to previous screening methods (Ji et al., 2019), we focused on genes which were significantly up-regulated or down-regulated at four time periods during the drought treatment, as they more likely to play a key role in grapevine's drought stress response (**Figure 7B and 7C**). Comparing the RNA-Seq dataset with the RT-qPCR data (**Figure 7D**), it was found that the *VyRCHC114* gene was significantly down-regulated within 2 days of experiencing drought, which continued to decline strongly through 10 days after this treatment. Hence, we postulated the *VyRCHC114*gene may possess E3 ubiquitin ligase activity enabling it to participate in the regulation of grapevine drought stress responses. Verifying this, we detected that *VyRCHC114* has E3 ubiquitin ligase activity and the *VyRCHC114* gene is overexpressed in Arabidopsis. Some related indexes of Arabidopsis plant fitness under drought stress were observed. That experiment demonstrated that the survival rate of overexpressed plants and the activities of many antioxidant enzymes were significantly increased. Drought stress significantly inhibited the greater electrolyte permeability of overexpressed plants; correspondingly, the decreasing trend of chlorophyll content was eliminated, while reductions in photosynthetic efficiency and energy storage capacity were significantly inhibited.

Drought stress greatly impacts the photosynthesis of plants, by affecting their photosynthetic rates and carbon metabolic pathways(Teng et al., 2014). A lowered rate of photosynthesis can lead to excessive accumulation of reactive oxygen species (ROS), leading to cytotoxicity, membrane lipid peroxidation, and even cell death (Apel & Hirt, 2004; Park et al., 2011) which can be countered by antioxidant enzymes as a form of plant defense. The overexpression of maize E3 ubiquitin ligase gene in transgenic tobacco can reportedly improve the drought resistance of tobacco (J. Liu et al., 2013). Not only that, other abiotic stresses may also be regulated by photosynthesis, thus enabling plants to adapt to stress conditions (Le Hir et al., 2017). According to our results,*VyRCHC114* overexpressing plants maintained a strong photosynthetic rate and energy storage capacity while under drought stress. The reason for this may be an increase of their chloroplast content, pointing to *VyRCHC114* 's possible involvement in the regulation of chlorophyll biosynthesis pathway as an E3 ubiquitin ligase. Nonetheless, we also examined the expression of genes known to play a major role in drought stress responses of plants (Hsieh et al., 2010; Ma et al., 2015; Yu et al., 2017). Our results revealed that the expression levels of these genes were significantly higher in*VyRCHC114* -overexpressed than EV-transformed Arabidopsis*plants* . Moreover, antioxidant system may be involved in plant abiotic stress tolerance mediated by the E3 ubiquitin ligase. Here, we provide physiological evidence that *VyRCHC114* heterologous expression enhances drought resistance by increasing the activity of antioxidant enzymes, which can scavenge for and eliminate ROS to indirectly reduce membrane damage.

To sum up, *VyRCHC* may act as an E3 ligase to mediate substrate degradation through the ubiquitin proteasome mechanism. This interaction may cause the target protein to be labeled by ubiquitin signaling,

which leads to proteasome degradation. Since *VyRCHC114* likely represents a new class of negative regulatory factors in the drought signal pathway, we think the degraded protein is a positive regulator of drought signaling, so that more of this substance may activate drought signaling. Therefore, *VyRCHC114* may improve the water retention ability and antioxidant defense of plants by regulating their chlorophyll content and antioxidant system, thus participating in drought stress response. So far, however, the target protein of the plant *VyRCHC114* gene has not been determined, nor is the mechanism of augmented SOD, POD, APX and CAT activities clearly understood. In future work, we will focus on the identification of *VyRCHC114* target proteins under drought stress and activation mechanisms of the antioxidant system in *VyRCHC114* -transgenic plants.

Conflict of interest

The authors have no conflict of interest to report.

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Figure legends

Figure 1 *VyRCHCs*' C3H2H3 domain analysis.

(A) SeqLogo of *VyRCHCs* ' C3H2H3 domain. (B) Schematic diagram of zinc ion binding to the C3H2C3 structure of the *VyRCHCs* . Cysteine and histidine are metal ligands marked numerically in ellipses. The hexagon represents a zinc ion. The number on the line between metal ligands is the number of amino acids between them. (C) The number of different amino acids in the *VyRCHCs* in the metal ligand region.

Figure 2 Phylogenetic analysis of RCHC protein in grapevine and other plants.

Via the Maximum Likelihood method, MEGA7.0 was used to construct phylogenetic trees of grapevine, Arabidopsis, and tomato (*Solanum lycopersicum*) with RING-type C3H2H3 proteins. The number of bootstrap repeats was n = 1000. Displayed are the percentages of bootstrap scores greater than 50%. Conservative domains, grouping, and information on different species are shown in the form of a, b, c.

Figure 3 Phylogenetic tree, gene domain, and structure analysis of *VyRCHCs* in grapevine.

(A) The phylogenetic tree of *VyRCHCs* was constructed using the NJ method. Different background colors represent different grouping branches. (B) Domain analysis of VyRCHCs proteins. At the bottom of the line, different colored squares represent different types of conserved amino acid sequences and based on MeMe analysis. The modules of different colors above the line represent the functional domains that have been identified. (C) Genetic structure of *VyRCHCs* , the CDS sequence is represented by a blue square/rectangle, the introns by black lines, the UTRs by yellow squares/rectangles.

Figure 4 Location information of *VyRCHCs* on the grapevine chromosomes.

The colored boxes contain the tandem duplication pairs, and the colored lines connect segmental replication pairs.

Figure 5 Repeated analysis of *RCHC* genes in grapevine, Arabidopsis and tomato (*Solanum lycopersicum*).

The inner circle is the density distribution of all the genes on the chromosome. In the outer ring are the chromosomes of different species: grapevine in blue, tomato in red, and Arabidopsis in green. The duplicated genes involved were labeled around the chromosome, with the orange lines for Sl and Vy and the green lines for At and Vy.

Figure 6 Analysis of cis-acting elements in the *VyRCHCs*.

(A) A list of VyRCHCs to facilitate correspondence. (B) Color-coded numbers of cis-acting elements of the three major types of *VyRCHCs* promoters. (C) Different types of cis-acting elements are represented by different colored squares and their position on each *VyRCHC* gene promoter.

Figure 7 Differential heat map of *VyRCHCs*' expression in plants under drought stress conditions.

(A) Heat maps of two different genotypes, based on their log2(WS/WW) values from the RNA-Seq data set, under drought stress and normal conditions at times T1–T4. (B) The Venn diagram of DEGs obtained from the analysis of the expression of M4 genotype at different periods. (C) Selected eight candidate genes in genotype M4 at different stages of WS/WW heat map.

Figure 8 Expression of 8 candidate genes were screened in qRT-PCR for plants under drought stress and control conditions.

The x-axis represents the different days during the treatment and the y-axis the relative levels of a gene's expression. Each treatment group had three biological repeats whose averages are plotted with the standard deviation. The asterisks indicate the significant level (* $P < 0.05$, ** $P < 0.01$).

Figure 9 E3 ubiquitin ligase activity of *VyRCHC114*.

(A) Determination of E3 ubiquitin ligase activity of *VyRCHC114* ; an immunoblot analysis was performed with the ubiquitin antibody (right) and MBP antibody (left). (B) Determination of E3 ubiquitin ligase activity of *VyRCHC114* mutants; an immunoblot analysis was performed with ubiquitin antibody (right) and MBP antibody (left). (C) Schematic diagram of *VyRCHC114* C3H2C3 domain and putative mutation sites. C328S and N355A affect a non-conserved site of the *VyRCHC114* C3H2C3 domain. Mutations in C320S and H341A affect the ubiquitin activity of *VyRCHC114* .

Figure 10 *VyRCHC114* overexpression (OE) enhances drought resistance in Arabidopsis.

(A) Phenotypes of three transgenic and an EV-transformed Arabidopsis lines after 15 days of drought stress and a 6-day recovery period. (B, C) Relative expression levels of *VyRCHC114* and survival of transgenic and EV-transformed Arabidopsis plants. Data are the mean ± SD (standard deviation). The asterisk, (*) and (**), indicate that OEs and EV-transformed groups were significantly different at $P < 0.05$ and $P < 0.01$ (Student's t-test).

Figure 11 Physiological indices of the EV-transformed and overexpressing (OE) Arabidopsis plants after drought stress.

(A) Electrolyte leakage, (B) chlorophyll content, (C) PSII maximal photochemical efficiency (Fv/Fm), and (D) net photosynthetic rate of leaves (Pn) were evaluated. Data are the mean ± SD (standard deviation). The asterisk, (*) and (**), indicates that OEs and EV-transformed groups were significant different at $P < 0.05$ and $P < 0.01$ (Student's t-test).

Figure 12 *VyRCHC114*-overexpressed and EV-transformed plants' activity of various antioxidant enzymes in Arabidopsis.

Under drought stress for 0, 4, and 7 days, are ascorbate peroxidase (APX, A), superoxide dismutase (SOD, B), peroxidase (POD, C) and catalase (CAT, D) activities of overexpressed (OE) and EV-transformed plants were determined. Data are mean ± SD (standard deviation). The asterisk, (*) and (**), indicates that OEs and EV-transformed groups were significantly different at $P < 0.05$ and $P < 0.01$ (Student's t-test).

Figure 13 Relative expression levels of drought resistance genes in transgenic and EV-transformed Arabidopsis after drought stress.

(A) AtCOR15a, (B) AtERD15, (C) AtP5CS1, (D) AtRD29A. Data are the mean ± SD (standard deviation). The asterisk, (*) and (**), indicates that overexpressed (OEs) and EV-transformed groups of plants were significantly different at $P < 0.05$ and $P < 0.01$ (Student's t-test).

Supplementary Data

Supplementary Figure1 Schematic diagram of C3H2C3 conserved sequence alignment of VyRCHCs.

Cys(C) and His(H) amino acids were added on a blue and pink background. The C3H2C3 conserved amino acid sequence length of these genes is shown later in the sequence.

Supplementary Figure2 Phylogenetic analysis of RCHC protein in grapevine.

Via the NJ method, MEGA7.0 was used to construct phylogenetic trees of grapevine. The number of bootstrap repeats was n = 1000. Displayed are the percentages of bootstrap scores greater than 50%. According to phylogenetic analysis, 143 *VyRCHCs* were divided into 11 groups, which were divided into 6 groups according to the total number of amino acids in the C3H2C3 conserved domain in VyRCHC protein sequence, and marked with 6 different colors.

Supplementary Figure3 Number of introns in *VyRCHCs*.

Supplementary Table 1 Detailed information of all 143 *VyRCHCs* identified in grapevine genome.

Supplementary Table 2 The distance between conserved metal ligands in the C3H2H3 domain of 143 *VyRCHCs*.

Supplementary Table 3 Ka/Ks analysis and divergence time estimated for grapvine duplicated *VyRCHCs* paralogs.

Supplementary Table 4 Functions of the cis-acting elements that found in the promoter region of each of *VyRCHCs*.

Supplementary Table 5 The sequences of the primers used in these experiments.

Supplementary Table 6 Motif data Information in MeMe Analysis of *VyRCHCs*