Genome-wide analysis and expression characterization of zinc finger homeodomain (ZHD) family genes responsed to different abiotic stresses and hormonal treatments in grape (*Vitis vinifera* L.)

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Abstract

Background

Zinc finger homeodomain (ZHD) proteins transcription factors (TFs) family occupy a crucial role in regulating plant growth and development. The ZHD TFs family had been studied in diverse organisms such as humans, Drosophila, nematode worms and plants. Especially in plants there is an extensive exploration involved in photosynthesis, defense mechanisms and responses to stress. In this further research, we will provide a better understanding of the ZHD gene family in grape.

Results

In this study, a total of 12 VvZHD genes were identified from the Grape Genome. The exploration of detailed bioinformatics found that the full length of VvZHD genes ranges from 150 bp (base pair) to 26706 bp, isoelectric points (pIs) were 6.25–9.68, molecular weights (MWs) ranged from 10.28 kilodaltons (KDa) to 97.93 kDa. Also, analysis of phylogenetic evolution showed there existed a common ancestor between *Arabidopsis* and grape. Ka (Nonsynonymous) / Ks (Synonymous) was lower than 1 and strong collinearity showed there were tandem duplications of genes between *Arabidopsis* and grape. Codon bias levels of ZHD gene family were relatively higher overall. Gene chip data analysis of cabernet sauvignon seedlings showed VvZHD genes had different expression under different time of different treatments, and with the prolongation of treatment time, most of VvZHD genes were slightly downregulated. The qRT-PCR was carried out under different hormones and stresses to verify expression profiles in grape roots and leaves. Results revealed that most VvZHD genes had higher expressions in roots but lower expressions in leaves. Also, most of the VvZHD gene members were responsed with high expressions after the treatment of ABA, IAA and MeJA in roots. However, *VvZHD9*, *VvZHD11* and *VvZHD12* were highly expressed by inducing of PEG in leaves.

Conclusions

Through genome-wide analysis and expression characterization of ZHD family genes, results showed 12 members had the diversity of function, structure and unique expression characterization under various abiotic stress and hormone treatments. This study will provide us with a newer insight on ZHD gene family of grape and promote to explore the biological functions of ZHD genes in other plants.

Background

TFs are closely related to physiological functions and regulation of expression in plants, which controlls growth, development and response stresses [1, 2]. Different TFs family have a unique DNA-binding domain, forming their own binding specificity zone. About 40 genes encoding DNA-binding protein were isolated from higher plants to confirm the physiological functions of TFs in vitro and in vivo [3]. TFs interact with cis-elements or other TFs proteins respond to various stresses such as drought, high salinity and extreme temperatures. As a signal transduction in the path of responsing to stresses, which regulates the process of growth and development in plants[4, 5]. Thus it can be seen that TFs are of great significant for plants.

The homeodomain (HD) found in many transcription factors is equipped with a 60-amino acids DNA-binding domain. Meanwhile, proteins containing-HD are found in diverse organisms such as humans [6], Drosophila [7, 8], nematode worms [9, 10] and plants, in which they play important roles in development. ZHD subfamily proteins have only been identified in plants, and likely play plant specific roles. These ZHD proteins are expressed predominantly or exclusively in floral tissue, indicating that they may act a likely regulatory role during floral development [11]. Zfh/SIP1 of drosophila is the core gene of the cardiac functional conserved genetic pathway in heart development [7]. *Zfh2* controls the expression of several wing genes in the specification of those cellular properties [8]. Research shows that *zfh-1* and *zfh-2* genes encode novel proteins containing both ZFHD motifs in drosophila [12, 13]. Mouse *ZFH-4* and Xenopus *XSIP1* count a great deal expression in growth and development [14, 15].

A ZHD novel homeodomain protein was first characterized in *Flaveria trinervia* to regulate the gene encoding C4 phosphoenolpyruvate carboxylase (PEPCase) by hybrid experiments [16]. The ZHD class of homeodomain proteins may also be involved in the photosynthesis-related mesophyll-specific gene expression of phosphoenolpyruvate carboxylase in C4 species and in pathogen signaling and plant defense mechanisms [16, 17]. According to the difference between the starting position of the HD domain, sequence and homology of two side of sequence among the HB (Homeobox) protein and other structural domains, plant HB gene families are divided into six types. There are HD-Zip (Homeodomain-leucine-zipper), WOX (Wuschel-relatedhomeobox), KNOX (KNOTTED1-like homeobox), Bell, PHD-Finger (Homeodomain-finger) and ZHD [18]. ZHD TFs with a homeodomain (HD) and a C_2H_2-type zinc finger motif are correlated with plant development, growth and stress responses. ZHD formed an unique structure with conserved DNA binding homeodomain and a zinc finger motif. The conserved motifs Ia and Ib constitute a dimerization domain which is sufficient for the formation of homo- and heterodimers. The zinc finger is an important structural motif, which consists of a central zinc ion and several amino acid residues of cysteines or histidines [18, 19].

To date, it's still unclear on the evolutionary history of the ZHD and MIF (Mini zinc finger) genes, the relationship between the two types of genes. The ZHD and MIF genes contained ZF-HD domain [19]. Based on the presence of Cys and His residues, Zinc finger motifs are classified into different types. There are C_3H, C_2H_2, C_2C_2 and so on [20]. Compared to animals, C_2H_2 zinc finger proteins exhibited the more diversity in a plant. Many Zn-finger TFs proteins and the C_2H_2-type protein factors are involved in various stresses signaling transition pathways [21].

ZHD TFs have been studied in many plants. Seven novel Zn-finger TFs were identified that bind to *OsDREB1B* promoter by the screening of a yeast one hybrid system. There were four ZHD and three C_2H_2 types Zn-Finger TFs. Results revealed that ZHD TFs made a difference on transactivation activity and Zn-Finger TFs play a prominent role in the regulation of *OsDREB1B* [22]. TANDEM ZINC-FINGER PLUS3 (TZP) was active in promoting hypocotyl elongation at the transcriptional level. At the same time, it showed the mechanism of the ZF-HD TFs family in promoting the growth of *Arabidopsis* [23]. The expression of *TaZFHD1* is upregulated by different hormones during spike development [24]. Most GhZHD genes were expressed in ovules and fibers, some were expressed in roots, stems, buds and flowers, seldom expressed in leaves and calli [25]. Co-overexpression of the *ATZFHD1* and NAC genes can improve drought tolerance and promote the expression of *ERD1* in *Arabidopsis* [26, 27]. The expression of *OsZHD1* modulated leaf rolling, shaping rice morphogenesis [28]. The expression levels of *HvZHD1* were upregulated under the dehydration, salinity, and heat stress. Cold stress didn't cause an increase in its expression levels. The *HvZHD1* control in flowering and flower development. And *HvZHD1* is involved in responsing to abiotic stresses in plant [29]. *OsZHD1*, *AtHB22* and *AtHB25* were elected as data, homologous gene *GmZHD1* was cloned to analyse expression level of *GmZHD1*, showing that expression is the highest in flower, secondly seeds and leaves. *GmZHD1* was an important gene in flower, seed and floral development of soybean [30].

By previous research on ZHD family members of other species in plant, we can have a fundmental understanging of ZHD. HD-Zip gene family expression were investigated in regulation of embryo abortion in grapes [31]. However, in order to let us have a further understanding of ZHD TFs. This study identified 12 ZHD genes from grape genome. Through the method of bioinformatics, gene structure, function, motifs and other detailed information of ZHD genes were predicted. We selected some ZHD protein sequences from various species to construct phylogenetic trees, analyzing the history of evolution and classifying based on its tree. The envolutionary direction of pairs of genes was under different selection pressures. The expression of VvZHD was demonstrated by qRT-PCR, indicating expression in roots higher than leaves of grape. Comprehensive analysis, it is possible that we can predict the functions of ZHD genes. This will provide a further study which aimed to reveal the important biological functions and stress tolerance of ZHD proteins, laying a foundation for screening candidate genes in plants.

Results

Identification and physiology characteristic analysis of ZHD genes family in grape

A total of 12 non-redundant putative ZHD genes with the same domain (Accession: PF04770) were identified from Grape Genome. On the basis of their locus name, they were named as *VvZHD1–12* respectively. According to an analysis of physicochemical property (Table 1), the full length of VvZHD genes ranged from 150 bp to 26706 bp. The longest of genes was *VvZHD12*, and the shortest was *VvZHD11*. The size encoded proteins of VvZHD genes varied from *VvZHD4* with 88 aa to *VvZHD7* with 345 aa. The distribution of these genes was mainly located in five chromosomes, *VvZHD10* and *VvZHD11* were marked on 1st chromosome, *VvZHD5* and *VvZHD6* were distributed in 14th chromosome, *VvZHD4, VvZHD7* and *VvZHD9* were distributed in 17th, 4th and 12th chromosome, respectively. *VvZHD2*, *VvZHD3* and *VvZHD12* were labeled in the 18th chromosome but only the distribution of *VvZHD1* and *VvZHD8* was random and unknown. Their pIs were between 6.25 and 9.68 and MWs ranged from 10.28 kDa to 97.93 kDa. In addition, much of VvZHD protein of instability index (II) were more than 60, which indicated these proteins were unstable. Most of these genes possessed a relatively high aliphatic index (A.I) and low hydropathicity (< 0), reaching a conclusion that most of the proteins encoding amino acids were hydrophilic.

Table 1
Detailed information about physical and chemical properties analysis of 12 VvZHD genes

Gene name	Locus name	Chromosome Site	Amino acid number	pI	Mol. wt (kDa)	Length (bp)	I.I	A.I	Hydropathicity	Domain(start-end)
VvZHD1	GSVIVT00003642001		250	9.37	28.12	750	64.65	48.80	-0.988	48–102
VvZHD2	GSVIVT00014603001	18	251	8.44	26.89	841	60.86	57.57	-0.604	97–151
VvZHD3	GSVIVT00015141001	18	230	7.19	25.12	690	52.77	45.39	-0.879	48–102
VvZHD4	GSVIVT00016336001	17	88	8.88	97.93	264	59.69	39.89	-0.792	26–80
VvZHD5	GSVIVT00020963001	14	323	6.25	34.82	1023	43.95	48.70	-0.903	70–126
VvZHD6	GSVIVT00020964001	14	92	7.67	10.28	258	75.57	65.65	-0.616	26–80
VvZHD7	GSVIVT00024918001	4	345	8.37	37.70	1035	71.93	53.22	-0.764	126–180
VvZHD8	GSVIVT00035356001		281	8.12	30.91	843	68.00	61.42	-0.739	72–126
VvZHD9	GSVIVT00035426001	12	270	9.68	30.80	4126	61.31	68.63	-0.784	32–86
VvZHD10	GSVIVT00036960001	1	326	8.14	35.60	978	65.13	51.47	-0.824	71–127
VvZHD11	GSVIVT00036961001	1	153	8.69	16.29	150	41.59	65.10	-0.409	95–149
VvZHD12	GSVIVT00038656001	18	282	9.22	31.01	26706	68.67	55.07	-0.693	43–99
Note: Bp: base pair										
pI: isoelectric point										
Mol. Wt: Molecular weight										
kDa: kilodaltons										
I.I: Instability index										
A.I: Aliphatic index										

Analysis of phylogenetic tree about ZHD gene family in grape

To explore the relationship of evolution among ZHD gene family, genes amino acid sequence of *VvZHD* (*Vitis vinifera* L.), *OsZHD* (*Oryza sativa* L.), *AtZHD* (*ArabIdopsis thaliana* L.), *NaZHD* (*Nicotiana tabacum* L.), *ZmZHD* (*Zea mays* L.) and *MpZHD (Malus pumila* Mill.*)* were obtained from database websites. These sequences were used to commonly construct a complete phylogenetic tree (Fig. 1). According to classified evidence, ZHD genes were divided into seven classes, namely classI, classII, classIII, classIV, classV, classVI, classVII, respectively. The different colourful triangle was used to represent the main location class of VvZHD genes. VvZHD genes were mainly classified in classIII and classIV, especially in a clade of classIII, only including AtZHD, *VvZHD4*, *VvZHD6* and *VvZHD11.* Other classes also clustered VvZHD gene apart from classII. Results found that a majority of genes were in a close branch with AtZHD. So it can conclude that there is a common ancestor and similar functions between *Arabidopsis* and grape. Multiple sequence alignment shows that all ZHD genes included typical domain with ZHD (Fig. 2). There is a large common character in systematic composition. Result reveals that these genes may have similarity in function and evolutionary process.

The analysis of chromosomal synteny and evolutionary selection pressure among VvZHD genes family

Via the performance of chromosomal synteny and evolutionary selection pressure, illustrated that VvZHD and AtZHD genes had great advantages in the selection and evolutionary course. We perceived several pairs of genes posses higher homology relationships such as *AtZHD3* (AT2G02540) and *VvZHD1*, *VvZHD3*; *AtZHD5* (AT1G75240) and *VvZHD2*, *VvZHD7* gene pairs and so on. These strong collinearity genes always maintained certain genetic chain action meaning a good evolutionary trend under purifying pression (Fig. 3). Using these gene pairs of grapes and *Arabidopsis*, Ka/Ks calculation was carried out. The Result revealed that most of genetic pairs was $0.23 < Ka < 0.70$, and only *VvZHD7* up to its maximum. Only Ks of *VvZHD2* genes were not calculated, and all the others had Ka/Ks data ($Ka/Ks < 1$) (Table 2). Therefore, we can conclude that a purified selective pressure relationship between grape and *Arabidopsis*, realizing an optimizing mutation in the evolutionary course.

Table 2
Evolutionary selection pressure of ZHD gene family between *Arabidopsis* and grape

A pair of genes	Ka	Ks	Ka/Ks
VvZHD1—AT2G02540	0.4074	2.1541	0.1891
VvZHD2—AT1G75240	NaN	NaN	NaN
VvZHD3—AT2G02540	0.4905	53.8560	0.0091
VvZHD3—AT4G24660	0.3245	3.9525	0.0821
VvZHD4—AT1G74660	0.2317	15.3329	0.0151
VvZHD5—AT3G28920	0.4638	50.1379	0.0092
VvZHD5—AT5G15210	0.3587	8.1324	0.0441
VvZHD6—AT1G74660	0.3459	20.8302	0.0166
VvZHD7—AT1G75240	0.5309	7.9834	0.0665
VvZHD7—AT2G18350	0.4190	3.8019	0.1102
VvZHD7—AT2G02540	0.5988	6.9057	0.0867
VvZHD8—AT2G02540	0.4019	1.7590	0.2285
VvZHD9—AT2G18350	0.7009	29.9915	0.0234
VvZHD10—AT5G15210	0.4123	5.2103	0.0791
VvZHD11—AT1G74660	0.3374	40.3660	0.0084
VvZHD12—AT4G24660	0.4949	54.5299	0.0091
Note: Ka: Synonymous			
Ks: Nonsynonymous			

The analysis of codon usage bias

Indexes related to codon usage bias was listed in Table 3. Consequence analysis suggested that CAI and CBI vary from 0 to 1. The CAI of VvZHD is in the value between 0.142 and 0.297, CBI of VvZHD ranges from − 0.141 to 0.243. Both indexes of *VvZHD5* were maximum, indicating its higher expression regulation in ZHD family members. Generally, NC value is from 20 to 61, which indicates that the synonymous codon bias is very larger. Also, NC influenced amino acid composition and genes length. *VvZHD5* had a relative low value with 46.65, showing a larger the synonym codon bias preference. The content of GC encoding amino acid of half of the genes was more than 0.5, the rate of GC3s was the same finding. These findings indicated that most of the grape ZHD prefer to using GC codons and ending in G/C base.

Table 3
Codon usage bias analysis of ZHD gene family in grape

Gene name	T3s	C3s	A3s	G3s	CAI	CBI	Fop	Nc	GC3s	GC
ZHD1	0.2346	0.3687	0.3958	0.3022	0.185	-0.141	0.364	49.73	0.506	0.487
ZHD2	0.2547	0.4104	0.3405	0.2229	0.205	0.011	0.432	53.82	0.519	0.541
ZHD3	0.24	0.4229	0.2059	0.4167	0.23	0.11	0.507	56.11	0.652	0.57
ZHD4	0.3836	0.274	0.3387	0.2623	0.203	-0.075	0.412	54.26	0.424	0.489
ZHD5	0.191	0.5581	0.1223	0.3857	0.297	0.243	0.576	46.65	0.748	0.595
ZHD6	0.2368	0.3158	0.3	0.3971	0.142	-0.061	0.389	57.64	0.567	0.54
ZHD7	0.3208	0.2755	0.4225	0.253	0.198	-0.098	0.376	56.04	0.412	0.492
ZHD8	0.3041	0.424	0.3382	0.2188	0.204	0.045	0.456	48.93	0.496	0.505
ZHD9	0.4381	0.2333	0.3483	0.2674	0.204	-0.058	0.391	55.7	0.379	0.462
ZHD10	0.3032	0.4477	0.188	0.2915	0.236	0.085	0.479	53.64	0.596	0.541
ZHD11	0.359	0.2821	0.3636	0.2435	0.215	-0.045	0.422	55.61	0.415	0.462
ZHD12	0.2589	0.3973	0.2038	0.3689	0.193	-0.018	0.406	56.36	0.62	0.585
Note: CAI: codon adaptation index										
CBI: codon bias index										
Fop: optimal codon usage frequency										
Nc: effective number of codon										

A correlation analysis was performed to further master the preference using of codon bias (Table 4). The correlation analysis showed that the three values FOP, CBI and CAI were extremely significant correlation, and there was a significant correlation between the content of GC and GC3s. Through analysis, we can infer ZHD gene is mainly from the stress of mutation. In addition, The GC (G + C content) was significantly positively correlated with G3s (the frequency of occurrence of the corresponding base in the third position of the synonymous codon) in the grape and GC3s (the G + C content in the third position of the codon) ($p < 0.01$) exists a significantly negatively correlated with T3s and A3s ($p < 0.01$). FOP, CBI and CAI were all significantly positively correlated ($p < 0.01$). GC and GC3s values were significantly negatively related to T3s and A3s ($p < 0.01$), and GC, GC3s and NC (codon effective numbers) were also negative relations. The correlation ($p < 0.05$) among parameters indicated that ZHD proteins focused on the composition of codon preference and gene expression level. The third position base of the synonym codon will affect the level of gene expression and codon usage preference. RSCU and codon number were provided in Table S1. RSCU can directly reflect the level of preference of codon usage without considering gene length and amino acid abundance. All ZHD genes have higher preference for using Met with value 1, and RSCU was not distinctly regular. All in all, codon bias levels of ZHD gene family were relatively higher overall.

Table 4
The correlation analysis between codon composition and preference parameters (A3s, T3s, G3s, C3s, CAI, CBI, FOP, NC, GC3s, GC)

	T3s	C3s	A3s	G3s	CAI	CBI	Fop	Nc	GC3s	GC
T3s										
C3s	-0.765**									
A3s	0.492	-0.759**								
G3s	− .606*	0.380	− .683*							
CAI	-0.181	0.649*	-0.617*	0.119						
CBI	-0.545	0.839**	-0.845**	0.394	0.823**					
Fop	-0.424	0.811**	-0.805**	0.371	0.864**	0.982**				
Nc	0.362	-0.650*	0.210	0.115	-0.573	-0.463	-0.501			
GC3s	-0.832**	0.885**	-0.890**	0.759**	0.499	0.783**	0.745**	-0.344		
GC	-0.766**	0.796**	-0.851**	0.711**	0.383	0.710**	0.641**	-0.151	0.940**	
Note: CAI: codon adaptation index										
CBI: codon bias index										
Fop: optimal codon usage frequency										
Nc: effective number of codon										

Subcellular locations of the ZHD proteins and the prediction of protein structure

Based on subcellular locations of the ZHD proteins (Table S2), the result showed that all VvZHD genes were highly expressed in the nucleus. Five of them (*VvZHD3*, *VvZHD4*, *VvZHD6*, *VvZHD11* and *VvZHD12*) were also expressed in the chloroplast, which controls path of photosynthesis in the plant. The rest of them were expressed in cytoplasm apart from *VvZHD2*, *VvZHD6*, *VvZHD7* and *VvZHD10*. At the same time, *VvZHD4*, *VvZHD6*, *VvZHD9* and *VvZHD12* were largely expressed in mitochondria. These genes seldom express in peroxisome, only *VvZHD8* and no any genes location existed in nucleus plasma. So it can be found that ZHD genes family mainly focused on expressing in nucleus, cytoplasm, mitochondria, chloroplast, which revealed that VvZHD gene family existed in plant issues involved in stronger respiration and photosynthesis.

The prediction of secondary structure of VvZHD were carried out by predict protein software (Table S3). The secondary structure of VvZHD gene family had alpha helix, beta turn, random coil. Alpha helix was 9.78% − *2*0.00%, beta turn was 1.59% − *2*0.45% and random coil counted a great deal part with 69.32% − *8*6.45% among secondary structure of encoding 12 VvZHD proteins. The result showed that the main secondary structure of ZHD gene family was random coil.

In order to further perceive the structure of *VvZHD*, three-Dimensional Structure (3D) of 12 *VvZHD* genes was predicted and modeled using protein sequence (Table S4 and Fig. S1). Almost all genes were modeled from *Arabidopsis* through the method of NMR and X-ray, most of which were NMR. *VvZHD11* failed to match model protein. Sequence similarity between target proteins and model proteins were over 30% and *VvZHD1*, *VvZHD3*, *VvZHD5*, *VvZHD7*, *VvZHD8*, *VvZHD10 and VvZHD12* up to 50%. *VvZHD2*, *VvZHD4* and *VvZHD6* genes with low similarity and ligands carrying Zinc ion and Sulfate ion were modeled by using X-ray, which equipped similar 3D structure. Entire model GMQE (0 –1) scores has been explored among genes family, they are 0.16, 0.04, 0.18, 0.18, 0.11, 0.18, 0.11, 0.15, 0.12, 0.11 and 0.10, respectively. Above these illustrations, we can have a better master of 3D model of VvZHD.

Motif and gene structure analysis of VvZHD in grape

In order to verify the sequence characterization of VvZHD proteins, the conserved motifs were performed by the MEME tool, using amino acid sequences from the grape, rice and *Arabidopsis* ZHD proteins. The result indicated that 15 motifs were identified in each comparison and named as motif1 to motif15 (Fig. 4 and Table S5). From these motifs, We can find that most ZHD proteins had similar motifs in the same group. All genes contained motif2, which predicted that the gene family with motif1 had a higher conserved domain. Motif3 and motif1 existed in almost all genes, also showing a conservative domain structure and specific functions of proteins.

Through gene structure analysis (Fig. 5) suggested that *VvZHD1*, *VvZHD3* and *VvZHD4* genes had only exons and *VvZHD10*, *VvZHD7* and *VvZHD8* was equipped with exons and upstream or downstream. The rest of VvZHD genes contained exons and introns and *VvZHD12* marked two introns. Most of the genes structures and length are greatly similar in structure composition (Fig. 6).

Cis-elements in the promoter regions of VvZHD genes

PlantCARE was used to predict the functional element of VvZHD genes (Fig. 7). The significant acting elements were filtered out among a variety of elements. Result found that cis-elements include different types of functional elements involved in hormones, light, abiotic stress, defense and tolerance. These elements were unevenly distributed on ZHD family genes in grape. Some acting elements were related to abscisic acid, Methyl jasmonate, gibberellin, salicylic acid and auxin. Elements related to MeJA responsiveness have only emerged in *VvZHD3* and *VvZHD4*. TCA-element, cis-acting element involved in SA responsiveness exists in *VvZHD2*, *VvZHD4*, *VvZHD11* and *VvZHD12*. TC-rich repeats, cis-acting element involved in defense and stress responsiveness were showed in *VvZHD2*, *VvZHD3*, *VvZHD9* and *VvZHD10*. LTR, cis-acting elements involved in low-temperature responsiveness were mainly marked in *VvZHD4*, *VvZHD5*, *VvZHD6*, *VvZHD7* and *VvZHD11*. Elements associated with anaerobic induction almost appeared in all ZHD genes. In addition, light control and auxin action elements counted a great deal in all elements. Comprehensive analysis deduced ZHD gene family might be equipped with growth and development, resistance to stress, improvement in defence in grape function.

Chip - Expression profile analysis of ZHD genes in grape

Through an exploration of expression Profiles, ZHD genes expression regulation from ‘cabernet sauvignon’seedlings was showed under the treatment and different times of ABA, PEG, Salt, Cold (Fig. 8). With stress time prolongation of low temperature, on the whole ZHD genes were significantly downregulated compared to controls. *VvZHD4*, *VvZHD8* and *VvZHD10* were upregulated at 1 h and 4 h then down at 8 h. Only that of *VvZHD6* always upregulate with cold time increasing, and *VvZHD6* possed cold related cis-element LTR. Under the treatment of PEG, all VvZHD genes had extreme significant upregulation expression when PEG treating time length was at 1 h. The *VvZHD8* and *VvZHD10* genes were up to its expression maximum at 1 h stress time. As the stress time prolongation of PEG, the expression *VvZHD1*, *VvZHD3* and *VvZHD7* genes showed a down-up-downregulation trend and had the same rangeability of expression, indicated that the three gene played similar role in plant growth and development. Through the treatment of ABA at 3 d and 10 d, overall expression of VvZHD genes were dramatically decreased, *VvZHD2* and *VvZHD5* of them had a higher expression level than others at ABA treatment or untreament. The level of genes expression under different period of salt treatment, expression showed a unevenly change regulation, and *VvZHD4*, *VvZHD6*, *VvZHD11* and *VvZHD12* was in a relative low expression, *VvZHD6* of which was up to its expression minimum at 24 h. In response to various abiotic stresses, the expression of zinc finger genes was completely different apart from *VvZHD1*, *VvZHD3* and *VvZHD7*.

qRT-PCR analysis of VvZHD genes in response to abiotic stresses and hormones treatment

Under the treatment of various stresses and hormones after 24 h, VvZHD expression level was probed through qRT-PCR in grape leaves and roots (Fig. 9). No matter what kind of treatment, the expression level of ZHD gene family in grape roots was universally higher than in leaves except *VvZHD9*, *VvZHD11* and *VvZHD12*. Also, we find that the expression level of *VvZHD3*, *VvZHD4, VvZHD7, VvZHD8* and *VvZHD10* were significantly downregulated. In the course of responsing different stresses and hormones, the expression of *VvZHD1* was significantly upregulated in roots apart from NaCl, while the expression of *VvZHD1* was significantly upregulated in IAA in grape leaves, other treatments were downregulated than that of control. Under the treatment of IAA, there were several typical genes which possesses higher expression in roots such as *VvZHD2, VvZHD3, VvZHD4* and *VvZHD7*, especially *VvZHD3* and *VvZHD4* had the same pattern with similar expression level in root and leaves. After low temperature treatment, *VvZHD1, VvZHD5, VvZHD6* and *VvZHD7* were significantly upregulated in grape roots, the expression of *VvZHD7* among genes was the largest and was 16.97 folds than controls. The expression of *VvZHD9, VvZHD11* and *VvZHD12* in leaves were relative higher than in roots under the treatment of PEG and ABA. Compared to the control, its expression with PEG was 2.45, 0.3 and 6.61 folds, respectively, its expression with ABA was 1.24, 0.52 and 0.55 folds. Comprehensive above analysis, results shows that *VvZHD3, VvZHD4* and *VvZHD7* played a significant role plant growth in roots, *VvZHD1, VvZHD2* and *VvZHD5* in roots were linked to resistance drought and cold stress. *VvZHD9, VvZHD11* and *VvZHD12* with higher expression might be related to drought tolerance, growth and development in leaves.

Discussion

Grape, as one of the four fruit all over the world, which has a higher nutrition value and promotes the development of area economy. Grapes are mainly used for fresh food and processing. Fresh food counts a great deal in grape using. With an increase of people’s demand, the cultivated area of grape is further expanding in the future. However, there still exist some problems in the process of cultivating, such as adverse environment (conditions drought, salty, low temperature), cultivation technique and so on. It will become a big challenge for us that how to tackle these problems to improve grape quality and yield, especially its environment conditions of cultivation. Therefore, It is urgent that let us explore the mechanism of adverse stress resistance and screen and clone several genes related to resist stress. ZHD family are closely related to abiotic stress under different environment.

ZHD genes family play an important regulatory role in various plant, animal, and fungal developmental and physiological processes [32]. Plant homeobox genes (HB) were classified into six type: HD-Zip, PHD finger, BELL, ZF-HD, WOX and KNOX [18]. ZHD family members mainly were involved in pathogen signaling, plant defense mechanisms and abiotic stresses [16, 18]. Previously, researchers had characterized the ZHD subfamily of homeobox genes, consisting of 14 members in *Arabidopsis* [12]. 31 ZF-HD genes were identified in Chinese cabbage and divided them

into ZHD and MIF subfamilies [33], and identified 109 full-length C_2H_2-ZF genes in *Populus trichocarpa* [34]. Different species had various members in number. In the current study, the effects of these ZHD family has been explored and the role of the gene family has been examined in grape.

12 ZHD genes were identified and screened from grape via removing genes without ZHD specific domain. The full lengths of VvZHD genes range from 150 bp to 26706 bp. The size encoded proteins of VvZHD genes varies from *VvZHD4* with 88 aa to *VvZHD7* with 345 aa. The distribution of these genes were mainly located in 1th, 14th and 18th chromosomes. Subcellular location find that all ZHD family were mainly located in the nucleus, *GmZHD1* has the same location of nucleus in Glycine max [30]. Specific motifs of proteins in encoding amino acid sequences are very important regions related to functions. The secondary structure has alpha helix, beta turn, random coil. Alpha helix varies from 9.78–20.00%, beta turn was 1.59% – 20.45% and random coil counted a great deal part with 69.32% – 86.45% in grape. VvZHD gene family were highly similarity in structure.

Through analysis using the MEME server, we identified various conserved motifs in VvZHD proteins, with similar motifs found in the most closely related members in the phylogenetic tree, revealing the functional similarity among the same proteins. Gene structure analysis confirmed that 6 of the VvZHD genes lack introns, A host of plant ZHD genes were previously proved to be intronless whereas the six other genes contain one to two introns [18, 33]. Synteny analysis results showed that almost whole genes had high similarity in evolutionary relationship. A similar study could be harvested, which was Ka/Ks < 1 of whole genes. Therefore, these genes might be copied from the same ancestral fragments. This study divided ZHD genes into seven classes and had 12 members while there are two large subfamalies ZHD and MIF, the ZHD group has 24 members in another study[33]. The consequence of the promoter cis-acting element analysis showed that VvZHD contained some major cis-acting elements related to hormones, light-responsive [35], stress[36], defense and so on. In total, 10 genes had hormone, stresses, light-responsive elements on the upstream promoter. These elements was unevenly distributed on ZHD family genes in grape, LTR related to low temperature [37]. Different genes pairs had the same location in genetic map and gene duplication phenomenon [38], ZHD gene prefer to use G/C bases to encode amino acids, and Ka/KS < 1 [39], showing a closer envolutionary relationship and deriving from common ancestor.

Gene chip results showed that these genes could respond to different duration of various abiotic stress and hormone conditions. Different treatments time had significant expression level, with the prolongation of times, expression level were slightly downregulated or unchanged. The expression of VvZHD was significantly downregulated with controls under the treatment of ABA. In addition, the qRT-PCR analysis of different tissues in different treatments of grape showed that VvZHD was mainly expressed in roots. Most VvZHD genes had a relatively higher expression in roots. However, a previous study showed that the SlZHD genes are widely expressed in various tissues, most genes were preferentially expressed in flower buds and response to different stresses and hormones [40]. BraZF-HD genes are also preferentially expressed in flower. Besides, most of these genes are significantly induced under photoperiod or vernalization conditions, as well as abiotic stresses [33]. Transcription factor *GmZHD1* are mainly expressed in flower, leaves and seeds [30]. especially in the treatment of ABA, IAA and MeJA. The expression of leaves was downregulated and relative low than the controls. In this study, all VvZHD were significantly induced by IAA, ABA, MeJA with highest expression of *VvZHD2*, *VvZHD3*, *VvZHD4* and *VvZHD7*. The expression of *VvZHD9, VvZHD11* and *VvZHD12* in leaves were relative higher than in roots under the treatment of PEG and ABA. *VvZHD3, VvZHD4* and *VvZHD7* had a higher expression in roots than that in leaves. Relative expression of VvZHD genes are relative low in grape leaves than roots. Therefore, Zinc finger family members play a significant role in plant growth and development.

Conclusions

12 ZHD genes were identified in *Vitis vinifera* L. in this study. Through the analysis of some structural and tissue-specific expression diversity among grape ZHD proteins, it indicated they play diverse roles adapting to environmental stress during particular stages of development in plant. We analyzed expression levels of VvZHD genes under different abiotic stress and hormone treatmentsin in roots and leaves of grape. The knowledge from this experiment lay solid foundation for further analysis of the biological functions of ZHD proteins in grape and provide a candidate gene for plant. At the same time, people can better gain these theoretical knowledges into practice to conduct fruit product.

Methods

Plant materials preparation

'Pinot Noir' (*Vitis vinifera* L.) vitro cultivation seedlings were derived from laboratory of fruit physiology and biotechnology, college of horticulture, Gansu Agricultural University. Before experimental treatment, the 'Pinot Noir' vitro cultivation seedlings were propagated, and cultivated in incubator under the growth condition of the light at 16 h, 28 °C and dark at 8 h, 25 °C. Through the cultivation of successive transfer about 35 d, some consistent, strong growth and sterile vitro cultivation seedings were selected as testing materials. Then these seedlings were transformed into medium which contains 200 mmol · L -1 NaCl, 100 mmol · L -1 MeJA, 10% PEG, 100 mmol · L -1 ABA, 10 μmol · L -1 IAA, 5 °C cold treatment. Sterile water was used as a control (CK) treatment, and the treatment time is 24 h. Finally, we adopt three biology duplicates and collect the roots and leaves tissues of grape, and stored in -80 °C for materials preparation.

Identification of VvZHD in grape

To identify members of the VvZHD genes, we download a total of data of AtZHD protein sequence as query in *Arabidopsis thaliana* genome website (https://www.arabidopsis.org/). Gene sequences of VvZHD were obtained by blat-search in the Grape Genome (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), including full length, coding domain sequences (CDS), protein sequences. The accession domain of ZHD gene protein is PF04770 through the Pfam 27.0 database (http://Pfam.sanger.ac.uk/) [41]. 12 VvZHD genes with ZF-HD (accession No. PF04770) were screened as our data.

Identification of physicochemical properties, conserved motifs and gene structure

The whole amino acid sequences were screened from the Pfam database to identify ZF-HD family genes. On the basis of obtained 12 VvZHD genes, we analyzed their physicochemical properties by ProtParam (http://web.expasy.org/protparam/) [42], including the length, Mw, and pI and so on. The CDS and DNA sequences of VvZHD genes were used to predict the gene structure via the tool GSDS (http://gsds.cbi.pkuedu.cn/) [43]. The number of exon/intron can be counted in grape ZHD. To identify the conserved motifs in grape, ZHD proteins employing MEME software (http://memesuite.org/) [44]. The setting of parameters is width of optimum motif ≥ 6 and ≤ 500 nt, maximum number of motifs 10. The subcellular locations of the ZHD proteins in grape were predicted using the Wolf Psort web (https://wolfpsort.hgc.jp/) [45]. The prediction of protein structure was performed by using SWISS-Model (http://swissmodel.expasy.org/) [46, 47].

The phylogenetic relationship analysis of VvZHD proteins

Loading homology genes from various species to construct an evolutional family tree. There are *VvZHD* (*Vitis vinifera* L.), *OsZHD* (*Oryza sativa* L.), *AtZHD* (*ArabIdopsis thaliana* L.), *NaZHD* (*Nicotiana tabacum* L.), *ZmZHD* (*Zea mays* L.) and *MpZHD* (*Malus pumila* Mill.). The amino acid sequences of these ZHD proteins with ZF-HD domain were obtained from NCBI (https://www.ncbi.nlm.nih.gov/pubmed), TAIR (https://www.arabidopsis.org/), and grape Genome Database (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). They were aligned using the multiple alignment tool ClustalX (http://www.clustal.org/clustal2/) [48]. A phylogenetic tree was constructed using MEGA7.0 software following the Neighbor-joining (NJ) algorithm [49] with 1000 bootstrap replicates. Collinearity analysis of genes was performed by Circoletto. Using Ka/Ks_caculator to calculate the selective pressure of a pair of homologous genes with grape and *Arabidopsis* [50].

Analysis of putative cis-element in the promoter regions of VvZHD genes

Approximately 5 to 10 bp putative cis-elements in VvZHD genes were detected using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [51, 52]. We gained cis-elements related information at 2000 bp prior to gene initiation. Then using TBtools (https://github.com/CJ-Chen/TBtools) to draw a map of ZHD gene family .

Codon usage bias analysis

Codon bias features intending to use unequally synonymous codons for an amino acid. The index can reflect the protein expression level [53, 54]. CDS sequences of VvZHD genes were employed to calculate the frequency of optimal codons (FOP), GC (effective number of codon content), GC content at the third site of the synonymous codon (GC3s content), relative synonymous codon usage (RSCU), codon adaptation index (CAI), and codon bias index (CBI) through the online software CodonW v.1.4 (http://codonw.sourceforge.net) [55]. A correlation analysis between codon composition and preference parameters of VvZHD genes, including A3s, T3s, G3s, C3s, CAI, CBI, FOP, NC, GC3s and GC, was worked out using SPSS v.22.0 statistical software.

Gene chip data analysis of VvZHD

Searching for abiotic statistics of grape from NCBI and GEO, we will acquire some stress datas of NaCl, PEG, ABA and cold in 'Cabernet Sauvignon' grape. They were showed login number (Affymetrix Gene Chip 16 K, GSE31662 and GSE31594) with cDNA of VvZHD genes and downloaded. Therefore, all of the similar genes ID were gained [56]. We select abiotic stresses datas in excel, Heatmap was drawed by using TBtools (https://github.com/CJ-Chen/TBtools).

qRT-PCR analysis under NaCl, PEG, ABA, MeJA, IAA and cold stress

RNA from leaves and roots of grape was extracted using a Spectrum Plant Total RNA kit (Sigma St. Louis, MO, USA) under different treatments. 12 VvZHD genes primers were designed and synthesized in the online software (the Sangon Biotech (Shanghai) Company). A total RNA as template to synthesize cDNA using the Prime Reverse Transcriptase Kit (Takara Bio, Shiga, Japan). Reverse transcription process was following the Reverse transcription system. Real-time fluorescent quantitative PCR (qRT-PCR) was performed by the application of Light Cycler® 96 real-time PCR system (Roche, Switzerland). The *VvGAPDH* gene as reference gene, A sample of cDNA (3 μL) was subjected to qRT-PCR in an eventual of 20 μL, that contained 1.6 μL primers (upstream primer was 0.6 μL, downstream primer was 0.6 μL), 10 μL SYBR Green Master Mix Reagent (Takara Bio, Shiga, Japan) and 5.4 μL ddH_2O. PCR amplification procedure was 40 cycles of 95 °C for 30 s, 95 °C for 10 s, 58 °C for 30 s, 72 °C for 20 s. The relative expression of the gene was calculated and analyzed using $2^{-\Delta\Delta CT}$ method and SPSS v.22.0, and Duncan's multiple range tests were employed to test significant differences ($P < 0.05$).

Abbreviations

ZHD
Zinc finger homeodomain
TFs
transcription factors
Bp
base pair
PIs
Isoelectric Points
MWs
molecular weights
kDa
kilodaltons
Ka
Nonsynonymous
Ks
Synonymous
I.I
Instability index
A.I
Aliphatic index
3D
three-Dimensional Structure
TIGR
Rice Genome Annotation Project
MeJA
Methyl jasmonate
ABA
Abscisic Acid
SA
Salicylic Acid
CAI
Codon Adaptation Index
CBI
Codon Bias Index
Fop
Optimal codon usage frequency
Nc
effective number of codon
RSCU
Relative Synonymous Codon Usage
A.I
Aliphatic index
CDS
Coding domain sequence
qRT-PCR
Quantitative Real-time PCR
SO4
Sulfate ion;;
ZN
Zinc ion
NRM
Nuclear magnetic resonance
GMQE
Global model quality estimation

Declarations

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Contributions

GJ N and SX L conceived and designed the experiments. YM L, GJ N and ZH M performed the experiments and prepared the plant materials and treated the samples. J M, XM S, BH C and AK revised the paper. GJ N analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Availability of data and materials

All sequences analyzed in this study and the raw data are included in this manuscript and its additional information files.

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Supplementary Information

Supplementary materials

Additional file1 Figure S1 Three-Dimensional Structures prediction of ZHD gene family in grape.

Additional file2 Table S1 The codon number and RSCU of VvZHD genes

Additional file3 Table S2 Subcellular location prediction of ZHD genes in grape

Additional file4 Table S3 The secondary structure prediction of ZHD genes in grape

Additional file5Table S4 Some related parameters of three-Dimensional Structure (3D) prediction of VvZHD genes

Additional file6 Table S5 Different motifs protein sequence of VvZHD

Figures

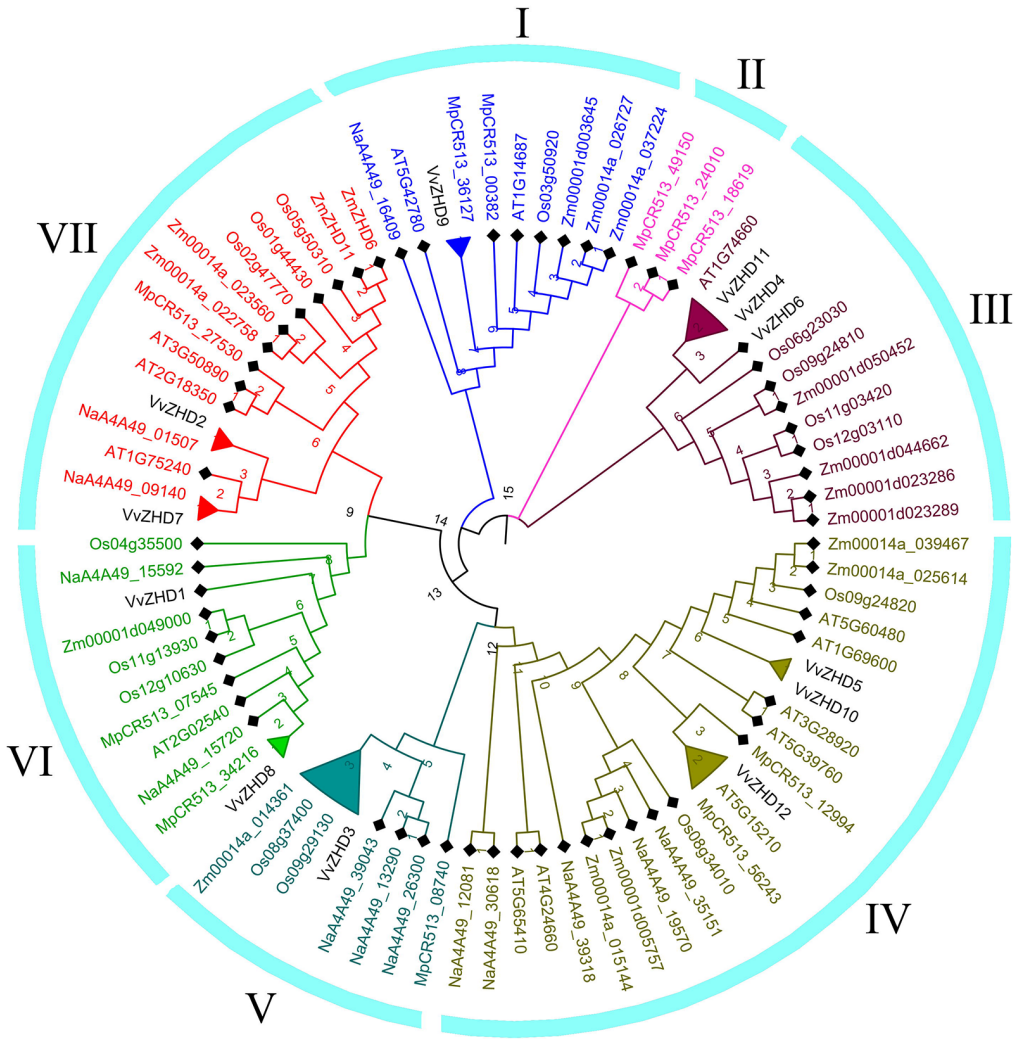

Figure 1

The phylogenetic analysis of exploring ZHD family members relationship in grape. A whole phylogenetic tree was constructed and divided seven groups, it is called ClassI, ClassII, ClassIII, ClassIV, ClassV, ClassVI and ClassVII, respectively. Each class was marked by various colourful branches. Different colourful triangle was used to represent the main location class of VvZHD genes. Seven species included Vitis vinifera L., Oryza sativa L., Arabidopsis thaliana L., Nicotiana tabacum L., Zea mays L. and Malus pumila Mill., respectively.

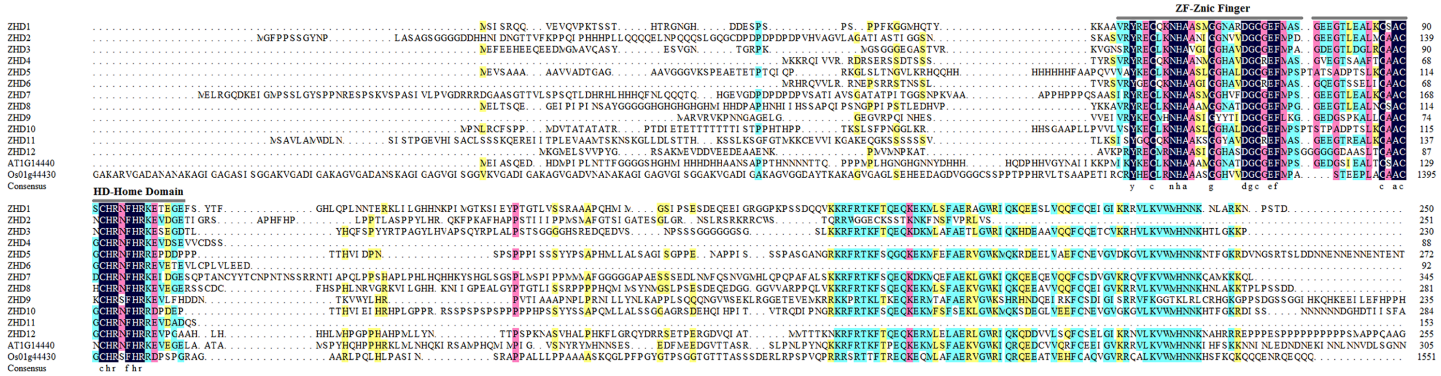

Figure 2

Multiple-sequence alignments of ZHD gene family member proteins. Lineation part is its functional domain (Zinc Finger-Homedomain).

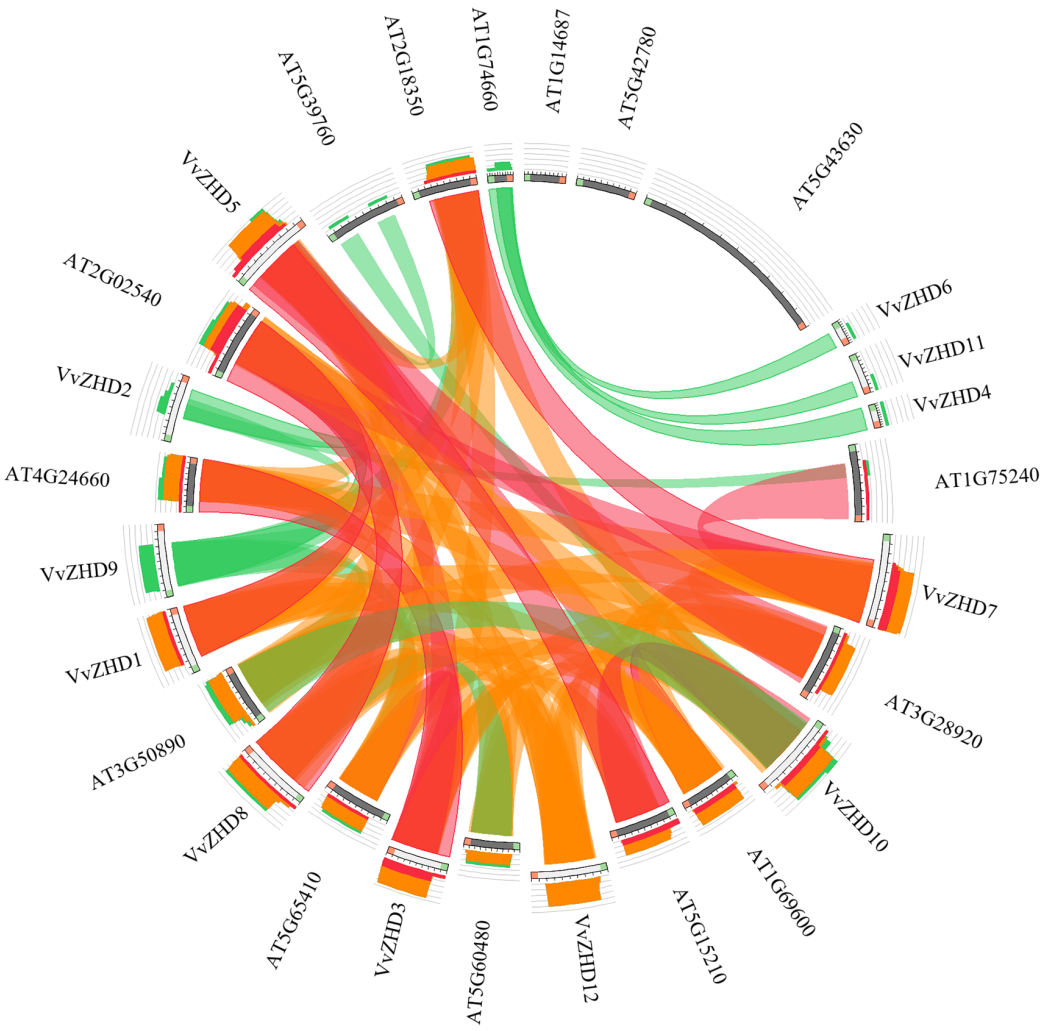

Figure 3

The collinearity analysis of VvZHD and ATZHD genes. The synteny relationship between each pair of VvZHD-ATZHD genes was predicted using Circoletto online website. Using 'score/max' ratio colouring with blue<=0.25, green<=0.50, orange<=0.75, red>0.75. Each synteny relationship is filled with different color.

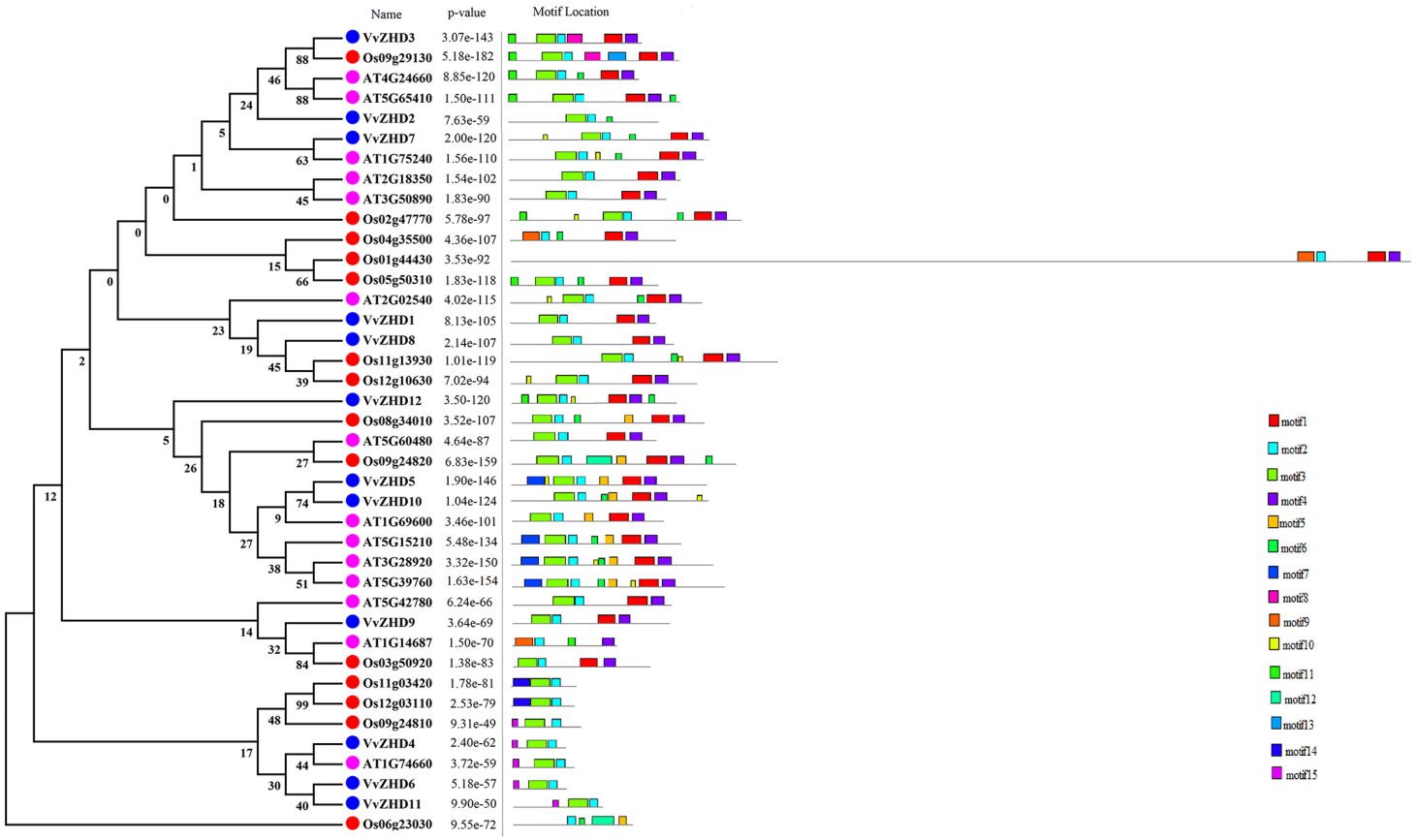

Figure 4

The analysis of motifs about ZHD genes in grape, rice and Arabidopsis thaliana.

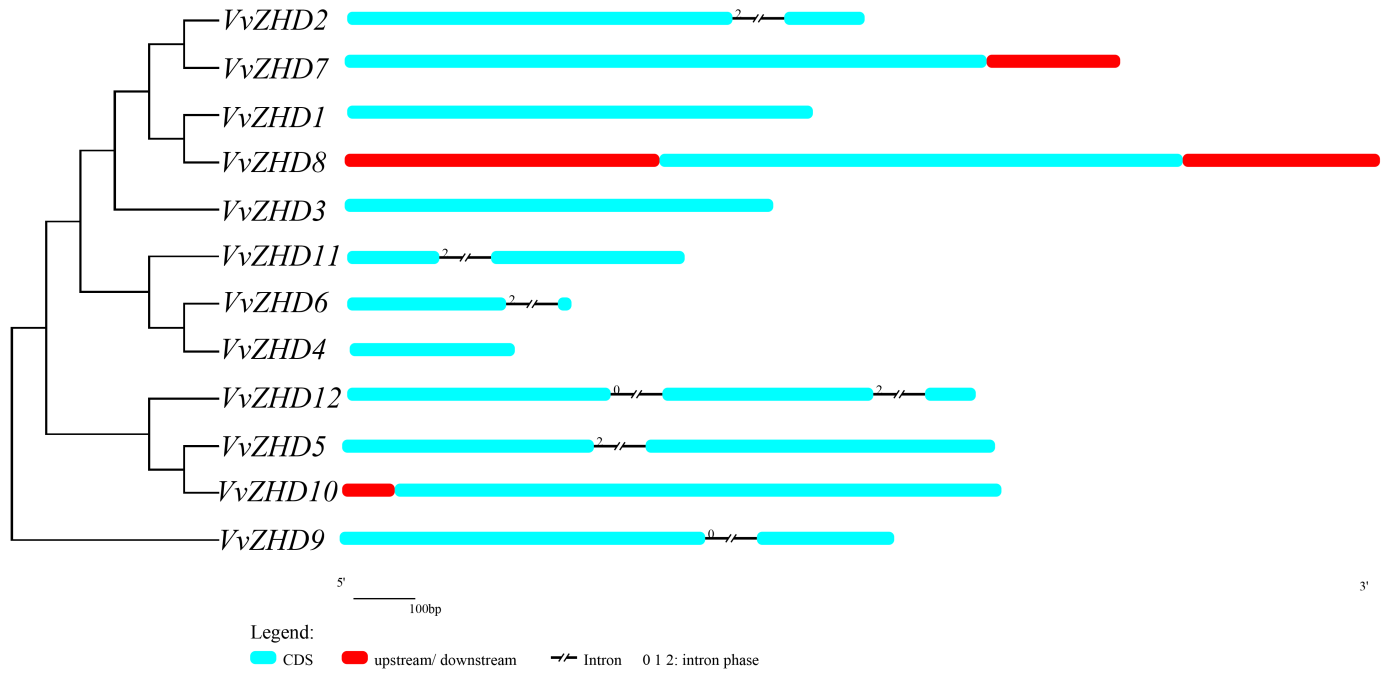

Figure 5

Structures and compositons of ZHD genes in grape. Through GSDS online software, we obtained gene specific structural information. Lines filled with sapphirine was exon and black was intron, and red line represents upstream or downstream. 0, 1 and 2 were intron phase.

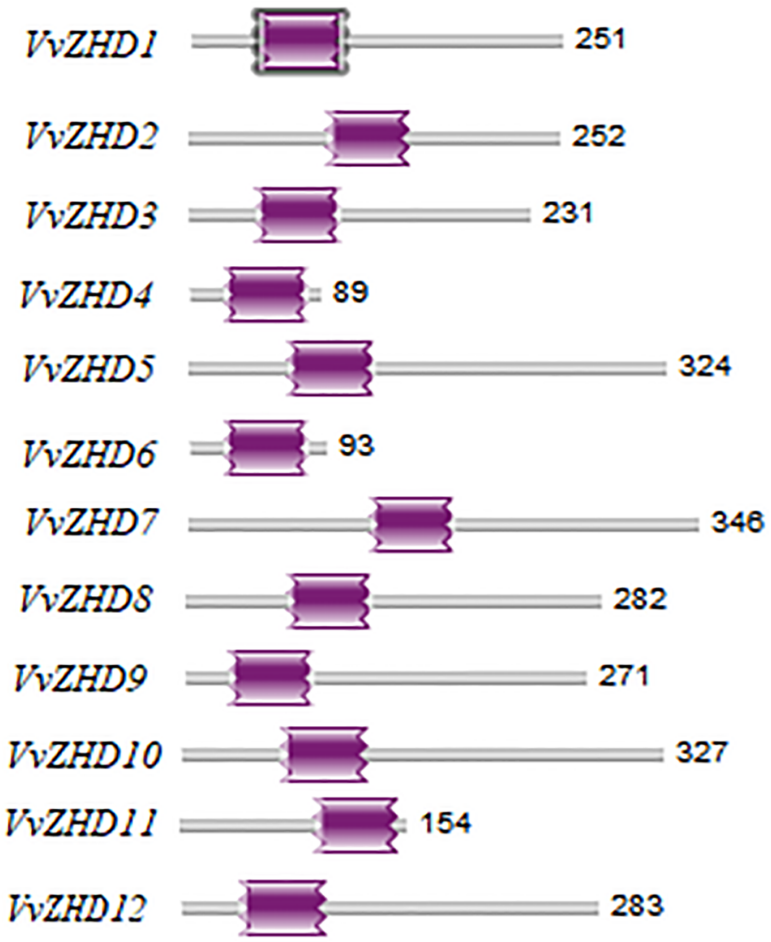

Figure 6

Structures and compositons of ZHD genes in grape. Through GSDS online software, we obtained gene specific structural information. Lines filled with sapphirine was exon and black was intron, and red line represents upstream or downstream. 0, 1 and 2 were intron phase.

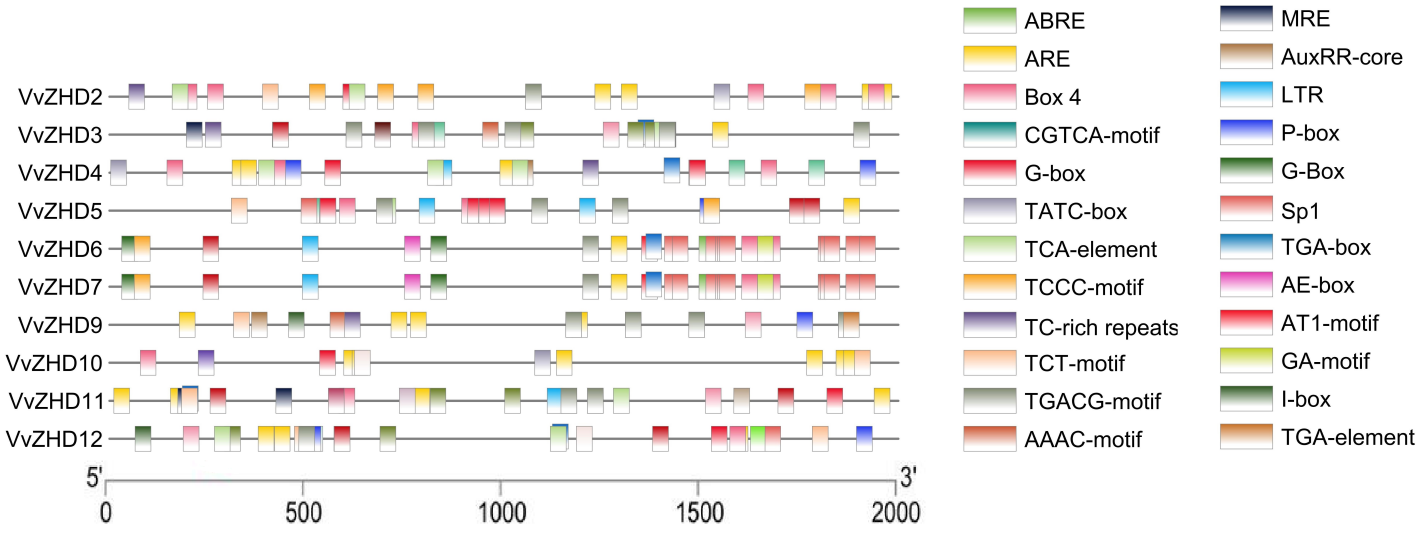

Figure 7

The cis-acting elements function involved in 12 ZHD genes in grape. The first 2000bp of gene sequences of 12 VvZHD genes was used to analysize promoter function. Each element was marked with different colors. ARE, cis-acting regulatory element was essential for the anaerobic induction. ABRE, cis-acting element was involved in the abscisic acid responsiveness. Box 4, AE-box, AT1-motif and part of a conserved DNA module were involved in light responsiveness. CGTCA-motif, cis-acting regulatory element was involved in the MeJA-responsiveness. G-box, TCCC-motif, TGACG-motif, AAAC-motif, TCT-motif, MRE, G-box, Sp1, GA-motif and I-box were involved in light responsiveness. TATC-box and P-box cis-acting element were involved in gibberellin-responsiveness. TCA-element cis-acting element was involved in salicylic acid responsiveness. TC-rich repeats cis-acting element were involved in defense and stress responsiveness. AuxRR-core, TGA-box and TGA-element cis-acting regulatory element were involved in auxin responsiveness. LTR, cis-acting element was involved in low-temperature responsiveness.

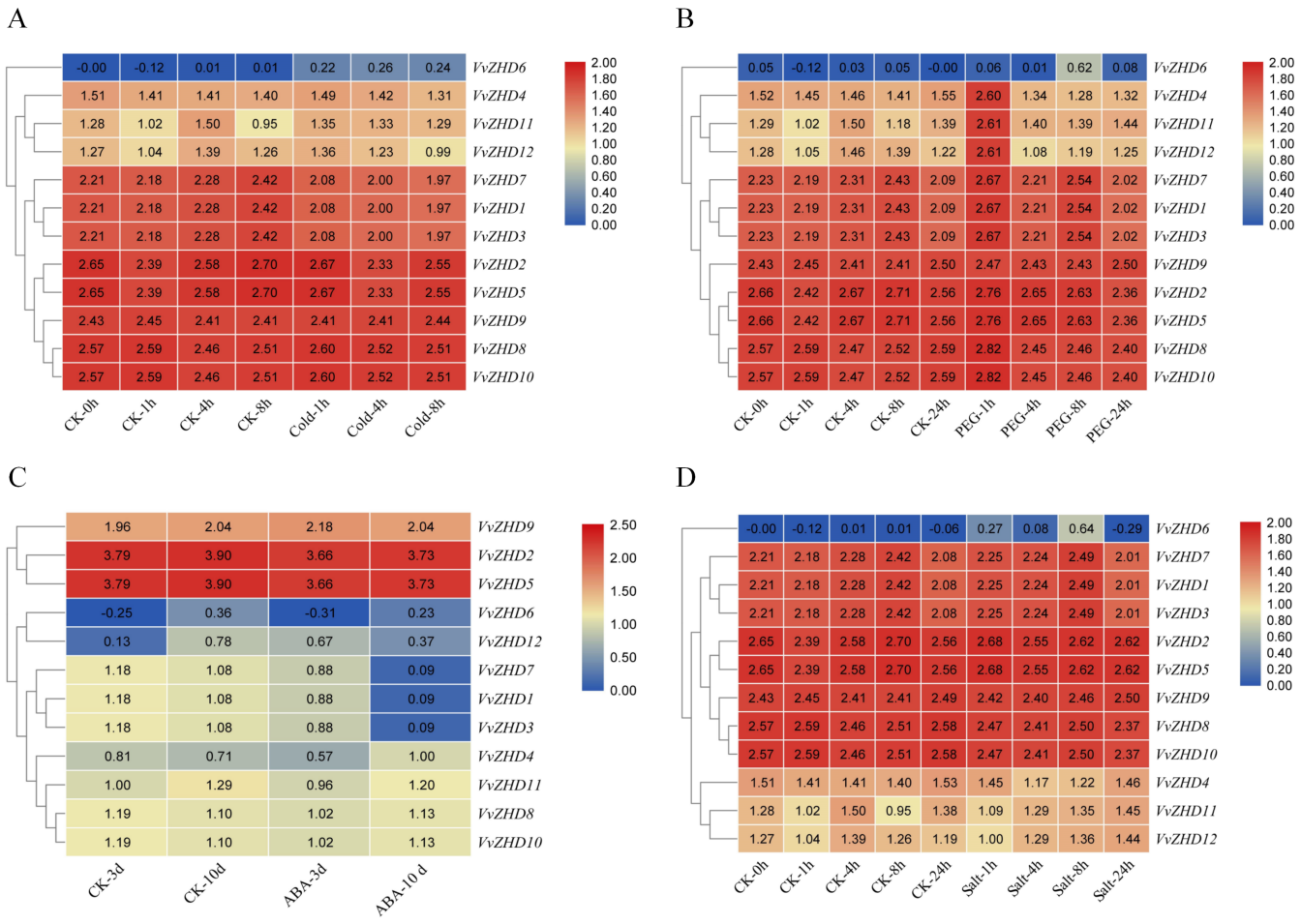

Figure 8

RNA-Seq expression profiles of ZHD genes under the stress of Salt, PEG, Cold and ABA. Color scale of the dendrogram represents the scale value of RPKM in the heatmap. Colourful shadow is bright or dark, which represents the level of gene expression, one of which was high expression with dark red and low expression with dark blue.

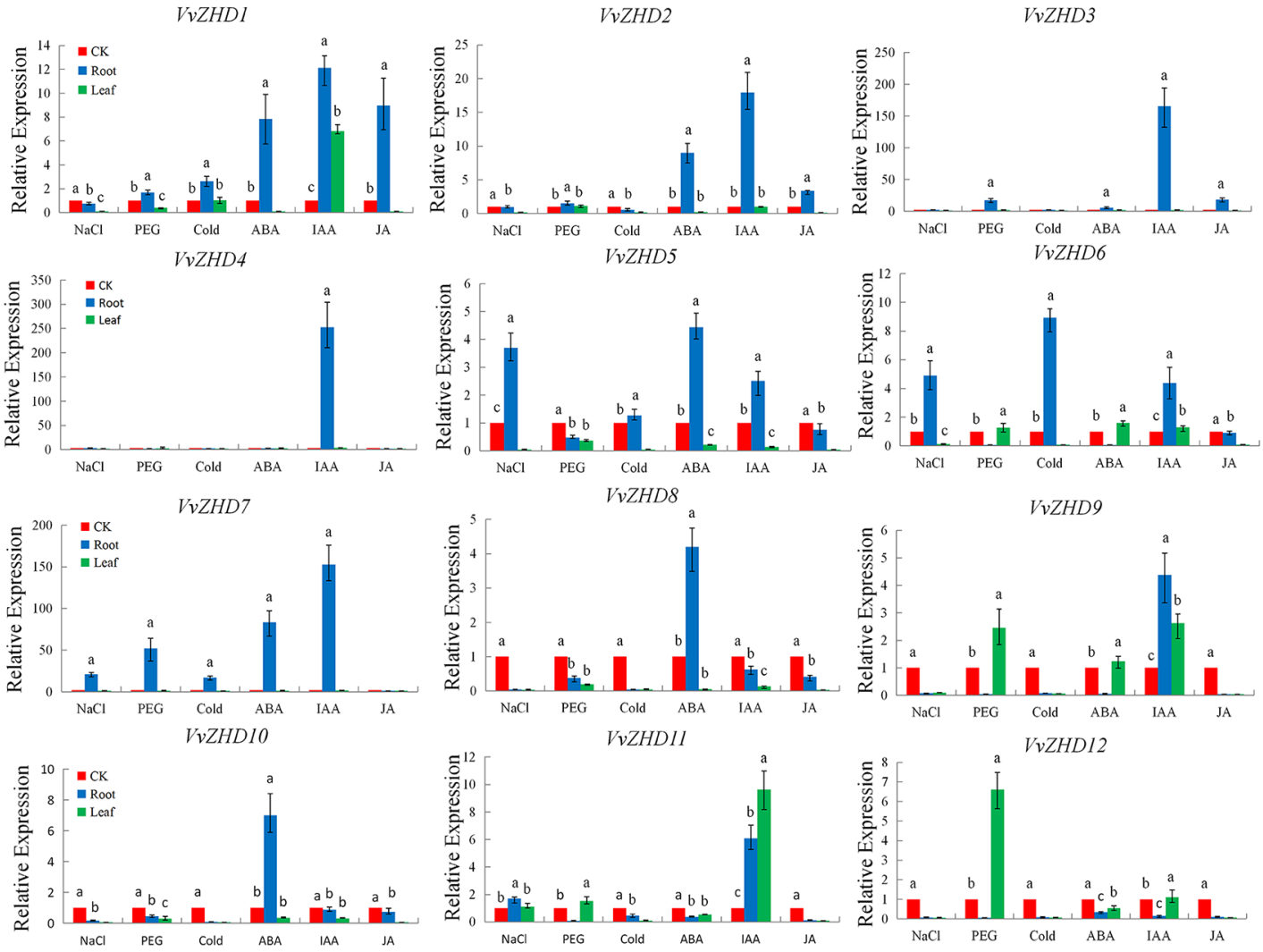

Figure 9

The expression level of VvZHD genes under the treatment of NaCl, PEG, Cold ABA, MeJA and IAA in leaves and roots of grape. The grape tissue culture seedlings were treated at NaCl, PEG, Cold(5°C), ABA, MeJA and IAA. Column diagram with red, blue and green represented control, root and leaves respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- qRTPCRData.xlsx
- GeneChipData.xlsx
- FigS1.tif
- TableS5.docx
- TableS4.docx
- TableS3.docx
- TableS2.docx
- TableS1.docx