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Genome-Wide Identification of ATP-Binding Cassette (ABC) Transporter Provides Insight to Genes Related to Anthocyanin Transportation in New Teinturier Grape Germplasm 'ZhongShan-HongYu'

Hui Li [1,†], Yaxin Yang [1,†], Qianlan Wang [1], Haoran Li [1], Wu Wang [2], Huan Zheng [1] and Jianmin Tao [1,*]

[1] College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China; 2017104024@njau.edu.cn (H.L.); 2021104024@stu.njau.edu.cn (Y.Y.); 2016104028@njau.edu.cn (Q.W.); 2021204007@stu.njau.edu.cn (H.L.); huanzheng@njau.edu.cn (H.Z.)

[2] Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing 210014, China; 2017204015@njau.edu.cn

* Correspondence: taojianmin@njau.edu.cn

† These authors contributed equally to this work.

Abstract: ATP-binding cassette (ABC) transporters are a large class of superfamily involved in a variety of biological processes with multiple functions, including phytohormone transport, heavy metal ion detoxification, and so on. Anthocyanin pigmentation in grapes is a commercially important feature of this superfamily. To elucidate the mechanisms of the VvABC gene at different stages in grape berries, we analyzed and characterized the ABC family in 'ZhongShan-HongYu' (ZS-HY) berries using RNA-seq analysis. In this study, a total of 146 VvABC genes were identified by comprehensive bioinformatics analysis, which outlined their gene structure, chromosomal location, conserved domains, phylogenetic relationships, and collinearity analysis. The VvABC family could be divided into eight subfamilies based on the phylogenetic analysis. Fifty-eight VvABC genes were identified from the RNA-seq data, of which 31 belong to the ABCG subfamily, 15 belong to the ABCC subfamily, 8 belong to the ABCB subfamily, 2 belong to the ABCF subfamily, and only 1 belongs to each of the ABCA and ABCD subfamilies. We used qRT-PCR to detect the expression of VvABC genes in different organs and found that it changed significantly in different organs. Phylogenetic analysis showed that genes involved in anthocyanin transport in other species were closely related to members of the ABCC subfamily. Subsequently, analysis of the promoter elements and the protein interactions of the VvABCC genes using RNA-seq was performed. This study has improved our understanding of the functions of the ABC gene family and provided a basis for the role of ABC genes in grape anthocyanin transport.

Keywords: genome-wide; ABC; anthocyanin; teinturier grape; gene family

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1. Introduction

The ATP-binding cassette (ABC) transporters are one of the largest superfamilies and are involved in a variety of biological processes and are widespread in the biological community [1,2]. The ABC transporters consists of at least two transmembrane domains (TMDs) embedded in the membrane bilayer and two nucleotide-binding domains (NBDs) located in the cytoplasm [3,4]. Therefore, ABC proteins not only possess a nucleotide-binding domain (NBD) containing highly conserved motifs (such as Walker A and B sequences, ABC characteristic motifs, H ring and Q ring) but also a transmembrane domain (TMD), which is typically composed of six alpha-helices, with 30–40% of its family members possessing the same sequence [2,5]. The specificity of the structure allows ABC transporters to not only utilize ATP hydrolysis to provide energy, but also to specifically recognize substrates for transport [5,6]. The ABC transporters are generally located in the golgi,

plasma, endoplasmic reticulum, mitochondria, endosomes and peroxisomes [7]. The ABC proteins have multiple functions. Most of them are transporters that regulate primary pumps, such as the transport of mineral ions, lipids, and peptides [8–10]. In addition, they are also involved in the transport of pigments, hormones, toxic chemicals, and other secondary metabolites [11–14]. In plants, ABC proteins are classified into eight different subfamilies (ABCA~ABCG and ABCI) [15], of which ABCG is the largest subfamily in plants [5]. Many functions of ABC transporters have not yet been characterized, so it is possible that they are responsible for many other unknown functions. The few studies on ABC genes in grapes have mainly focused on the ABCG subfamily. VviABCG14 was found to be involved in the transport of cytokinins and induced by exogenous hormones CPPU (N-(2-chloro-4-pyridyl)-N′-phenylurea) and tZ (trans-zeatin). Overexpression of VviABCG14 in *Arabidopsis* significantly altered the phenotype of *Arabidopsis* [16]. Another member of the ABCG subfamily, VviABCG20, regulates seed development by participating in the synthesis of suberin in the seed coat [17]. The VvABCG44 was cloned from grape berry skin as a transporter of resveratrol in grape [18]. The ABC transporters are important for plant growth development. The ABC transporters are energized by ATP directly so that complex materials can be transported by overcoming concentration gradients [19]. Many studies on ABC transporters in plants roles mainly focus on detoxification and pathogen defense via exogenous substance and secondary metabolite transport [20], for example, ABC transporters regulate plant development by transporting plant hormones (Auxin, Cytokinin, ABA, Jasmonic acid) [21–26], and translocate monomers (cutin, suberin-sporopollenin and pollen lipid monomers) [27–32]. In addition to the above functions, ABC transporters also participate in the function of anthocyanin transport in plants.

Anthocyanin is a major secondary metabolite that plays an influential role in plants. In addition to providing pigments for plants, anthocyanins also have important protective effects, such as protection against microorganisms, strong light and ultraviolet radiation during stress, and as an antioxidant [33–36]. After endoplasmic reticulum synthesis, anthocyanins are not stable and are further modified by glycosylation, methylation or acylation, followed by transportation to vacuoles to store and accumulate in the form of vacuole inclusions [36–38]. Until now, two major transmembrane transport mechanisms have been proposed: the ligandin transporter and the vesicular transport models [39,40]. However, both of these transmembrane transport models require the participation of transporters. At present, the identified anthocyanin transporters can be divided into three types: glutathione S-transferase (GST); multidrug and toxic compound extrusion (MATE); and ATP-binding cassette (ABC) [41,42]. The GST is localized in the cytoplasm, which can help anthocyanins bind to glutathione (GSH) to form a conjugate and transfer from the cytoplasm to the vacuole with the assistance of ABC transporters [43]. The MATE is a proton or Na^+ electrochemical gradient secondary transporter formed by proton pumps, such as plasma membrane P-type ATPase and vacuolar V-type ATPase. To date, relevant studies have demonstrated that MRP-type ABC primary transporters, especially the ABCC subfamily, are involved in the isolation of vacuolar flavonoids [44,45]. Complete genome sequence analyses of several organisms indicate that there are abundant genes encoding ABCC-type proteins in plants. In maize, anthocyanin is delivered to the vacuole by *ZmMRP3*, which is an ABCC-type transporter [12]. Rye flavonoids are transported to the vacuole by ABC transporters in *Secale cereale* [46]. It is speculated that inhibitors of ABC transporters may significantly reduce the secretion of flavonoids in soybean roots [47].

For most grape cultivars, anthocyanin accumulation in the berry skin is the main reason for the appearance of color. Previous studies have shown that VvABCC1 shares 63% amino acid sequence homology with the protein encoded by the maize ABC transporter gene *ZmMRP3.* In grape skin, ABCC1 is located in the vacuolar membrane and is strictly dependent on GSH to transport glycosylated anthocyanins to the vacuole. Compared to delphinidin 3-*O*-glucoside, ABCC1 preferentially transports malvidin 3-*O*-glucoside [48]. However, anthocyanin accumulation in teinturier grapes occurs not only in the skin, but also in the flesh. Kolor (*Vitis vinifera* L.), Yan73 (*Vitis vinifera* L. (Muscat Hamburg

x Alicante Bouschet)), Alicante Bouschet (*Vitis vinifera* L.) are recognized as teinturier grapes throughout the world. 'ZhongShan-HongYu' is a new teinturier germplasm with genetic and morphological observations that are different from that of Yan-73 and kolor. Anthocyanin accumulation appears in the flesh of 'ZS-HY' 7–10 days after anthesis (DAA), which is different from Alicante Bouschet (52DAA). However, there is still a lack of research on the transport pathways of anthocyanins in the grape flesh, so it is essential to investigate the function of the ABC gene in anthocyanin accumulation in 'ZS-HY' berries. In this study, we used the berries at 3DAA, 6DAA and 22DAA of 'ZS-HY'. In general, the content of anthocyanins increases with the growth and development of grapes [49]. In this study, grape genome information (assembled and annotated version PN40024.v4) was downloaded from the Ensemble Plant database (http://plants.ensembl.org/, accessed on 1 March 2023), and some bioinformatics analyses were performed using this information to help further understand the function of the ABC gene family in teinturier grapes.

2. Materials and Methods

2.1. Plant Materials

In this study, a 3-year-old cultivar 'ZS-HY' ('H' shape and standard growing conditions, 4 m × 8 m spacing) from Nanjing Agricultural University, Jiangsu Province was used. The 'ZS-HY' were grown under a rain shelter with polyvinyl film; the soil type used for planting was thick and fertile loam; and the river water was irrigated by sprinkler using a water pump and through ditching and fertilizing once a year. To verify the specificity of the VvABC gene expression, the leaves, tendrils, stems and berries with similar growth status were collected from the same parts of three vines. All samples were collected and put into liquid nitrogen to bring back to the laboratory and were stored at −80 °C until used.

2.2. RNA Extraction and qRT-PCR Analysis

Samples of leaves, tendrils, stems, and berries were extracted using an RNA Extraction Kit (Tsingke, Beijing, China), and RNA was reverse transcribed into cDNA using the Takara RT Reagent Kit (Takara, Beijing, China).

We selected 22 genes with high gene expression levels from the RNA-seq analysis (SRA accession no. PRJNA898660) for qRT-PCR using the ABI7300 system. The expression levels of all ABC genes in the transcriptome data are shown in Table S1. Primers used for qRT-PCR were designed using Designer 7 software and are listed in Table S2. There were four technical replicates and three biological replicates, and the data were calculated using the $2^{-\Delta\Delta ct}$ method. The gene *Vvactin* was used as the internal reference gene. The total volume of the reaction mixture was 20 µL, containing 10 µL TB Green Supermix, 0.2 µL of each primer, 1 µL cDNA, and 8.6 µL double-distilled water. The PCR procedure began with pre-denaturing at 94 °C for 30 s, which was followed by the following procedure, which required 40 cycles: denaturation at 94 °C for 5 s; annealing at 60 °C for 15 s; and extension at 72 °C for 10 s. Finally, statistical analysis of qRT-PCR was performed using Graph Pad Prism 7 software (version: v7.0).

2.3. Identification of ABC Gene Family Members

The genome data for grape were obtained from the Ensemble Plant database (http://plants.ensembl.org/, accessed on 3 March 2023), and the assembly and annotation version used was PN40024.v4. The genomic information of *Arabidopsis thaliana* was obtained from the Ensemble Plant database. A total of 129 *Arabidopsis* ABC gene family members were used as queries to search for VvABCs with E-value $\leq 1 \times 10^{-5}$ by Blastp. The hidden Markov model profile (PF00005) was downloaded from the Pfam database (http://pfam.xfam.org/, accessed on 3 March 2023) and the grape genome data were searched using HMMER. The genes shared by the two sets of results were identified as candidate genes for grape ABC transporters. Subsequently, the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/?term=, accessed on 4 March 2023), Pfam (https://pfam.xfam.org/search, accessed on 4 March 2023), and the SMART online website (https://

SMART.embl-heidelberg.de, accessed on 4 March 2023) were used to further structure and screen the initial candidate proteins [50,51]. The basic physicochemical properties of the grape ABC transporters were detected by Protpara (http://web.expasy.org/protparam/, accessed on 5 March 2023) in the Expasy website. Subcellular localization prediction was performed using CELLO (http://cello.life.nctu.edu.tw/, accessed on 5 March 2023) [52].

2.4. Phylogenetic Analysis

Multiple sequence alignments of grape ABC gene family members with 129 *Arabidopsis* ABC gene family members were performed using the Muscle program in MEGA X [53]. Phylogenetic trees were constructed using MEGA X software with the bootstrap value set to 1000 and the other parameters were set as default values. Then, the phylogenetic tree file was exported and embellished using the ITOL website (https://itol.embl.de/, accessed on 7 March 2023).

2.5. ABC Gene Chromosome Location and Collinearity Analysis

The length information of each chromosome was first extracted from the genomic information of grapes using TBtools [54], then the location information of all target genes was obtained using GFF files. Finally, TBtools was used to visualize chromosome localization. Furthermore, collinearity analysis was performed using the MCScanX program, then the advanced circos in TBtools was used for visualization [54,55]. The genomes of *Arabidopsis thaliana*, *Malus domestica* and *Prunus persica* for collinearity analysis were downloaded from the Ensemble Plant database; that of *Actinidia chinensis* was obtained from the Kiwifruit Genome Database (https://kiwifruitgenome.org/, accessed on 8 March 2023), and that of *Fragaria vesca* was obtained from the Phytozome database (https://phytozome-next.jgi.doe.gov/, accessed on 8 March 2023).

2.6. Conserved Motif and Gene Structure Analysis of ABC Genes

The conserved patterns in the ABC gene family of grapes were retrieved by logging into the MEME website (http://meme-suite.org/, accessed on 9 March 2023), setting the Motif to 15 and setting the other parameters to default values [56]. Based on grape genome sequence information, the exon-intron structure of the ABC gene was visualized using TBtools.

2.7. Prediction of Cis-Acting Elements of Grape ABC Genes

A 2000 bp sequence upstream of the initiation codon of grape ABC genes was extracted from the genomic data using TBtools software, and then the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 10 March 2023) was used to obtain a prediction of cis upstream sequence-functional elements [57]. The prediction results are visualized via TBtools.

2.8. Analysis of Protein Interactions of VvABCs in RNA-Seq

The STRING online website was used to predict the interaction network of the VvABCs protein sequences screened from the transcriptome, then, the Cytoscape software was used for beautification [58].

2.9. Expression Analysis of VvABC Genes Using RNA-Seq

In order to study the expression profile of the VvABC gene in 'ZS-HY' berries at different stages, the transcriptome data of 'ZS-HY' berries at three stages (3DAA, 6DAA, 22DAA) (SRA accession no.PRJNA898660) were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/, accessed on 15 March 2023) [49]. Gene expression level was estimated by FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) value. The heat map of the VvABC genes was constructed using TBtools.

3. Results

3.1. Identification of the ABC Gene Family in Grape

To screen for all possible ABC transporters in the grape genome, we first used 129 AtABCs in *Arabidopsis* as queries to obtain potential ABC members through the blastp program, and then hmmsearch was used to search for all possible ABC domain genes. The CDD, Pfam and SMART websites were used to predict that the candidate genes contained TMD and NBD domains.

In a previous study, Birsen Çakır identified 135 VvABCs using genomic information from the 12x assembly version [59]. Current grape genome annotation information has been updated to the PN40024.v4 version. In this study, we identified 146 VvABCs using the latest version of the genome information, 11 more genes than in the previous study, including two more members of the VvABCB subfamily, four more members of the C subfamily, one more member of the D subfamily, seven more members of the G subfamily and one member of the I subfamily (Table S3), which may be related to the updated genome version.

The amino acid lengths of the proteins encoded by these 146 ABC genes varied considerably (Table S4). The shortest was VvABCG55, consisting of 204 amino acids, and the longest was VvABCA1, consisting of 1881 amino acids. The molecular weight of the grape ABC transporters ranged from 22355.84 Da (VvABCG55) to 209301.57 Da (VvABCA1), and the isoelectric point values ranged from 5.19 (VvABCG60) to 9.87 (VvABCI3). There were 108 hydrophobic proteins, accounting for 73.97%. The results of subcellular localization prediction showed that 123 ABC transporters were localized to the plasma membrane, accounting for 84.25%. In addition, some other proteins were identified as being localized on the nucleus, golgi vesicles, chloroplast, cytoplasmic and mitochondrion.

3.2. Phylogenetic Analysis

To analyze the evolutionary relationships of the 146 VvABC transporters, we performed phylogenetic tree analysis of the VvABCs and 129 ABC proteins in *Arabidopsis* using MEGA X software (version: v10.0) (Figure S1). The phylogenetic results showed that the grape ABC gene family could be divided into eight subfamilies. Among these subfamilies, the G subfamily had 80 members, followed by the B and C subfamilies with 30 and 27 members, respectively. The remaining subfamilies had fewer members.

3.3. Chromosome Location and Collinearity Analysis of ABC Gene Family

Following chromosome location of the VvABCs based on the annotation information of the grape genome, it was found that the VvABC genes were distributed on 19 chromosomes, but the number distribution was uneven (Figure 1). The largest number of ABC genes was found on chromosomes 6 and 9 with 16 and 20 genes, respectively. These genes are dominated by ABCG members and form large gene clusters. The ABCG genes were most widely distributed on chromosomes with simultaneous distribution on 17 chromosomes. Chromosome 12 had the least number of genes, with only *VvABCG64*.

Gene duplication plays an important role in the expansion of the gene family during evolution. Therefore, we analyzed the location of VvABC duplication in the grape genome and observed four different types of gene duplication. Among the ABC genes in grape, 55 genes were assigned to dispersed duplication, 45 genes were assigned to tandem duplication, 29 were assigned to WGD or segment duplication and 12 were assigned to proximal duplication (Table S5). We identified 45 pairs of duplicated genes in the ABC gene family of grape, and 29 pairs were involved in tandem duplication (Table S6). In addition to tandem duplication, 18 segment duplication events were identified, involving 25 genes belonging to the B or G subfamily (Figure 2A).

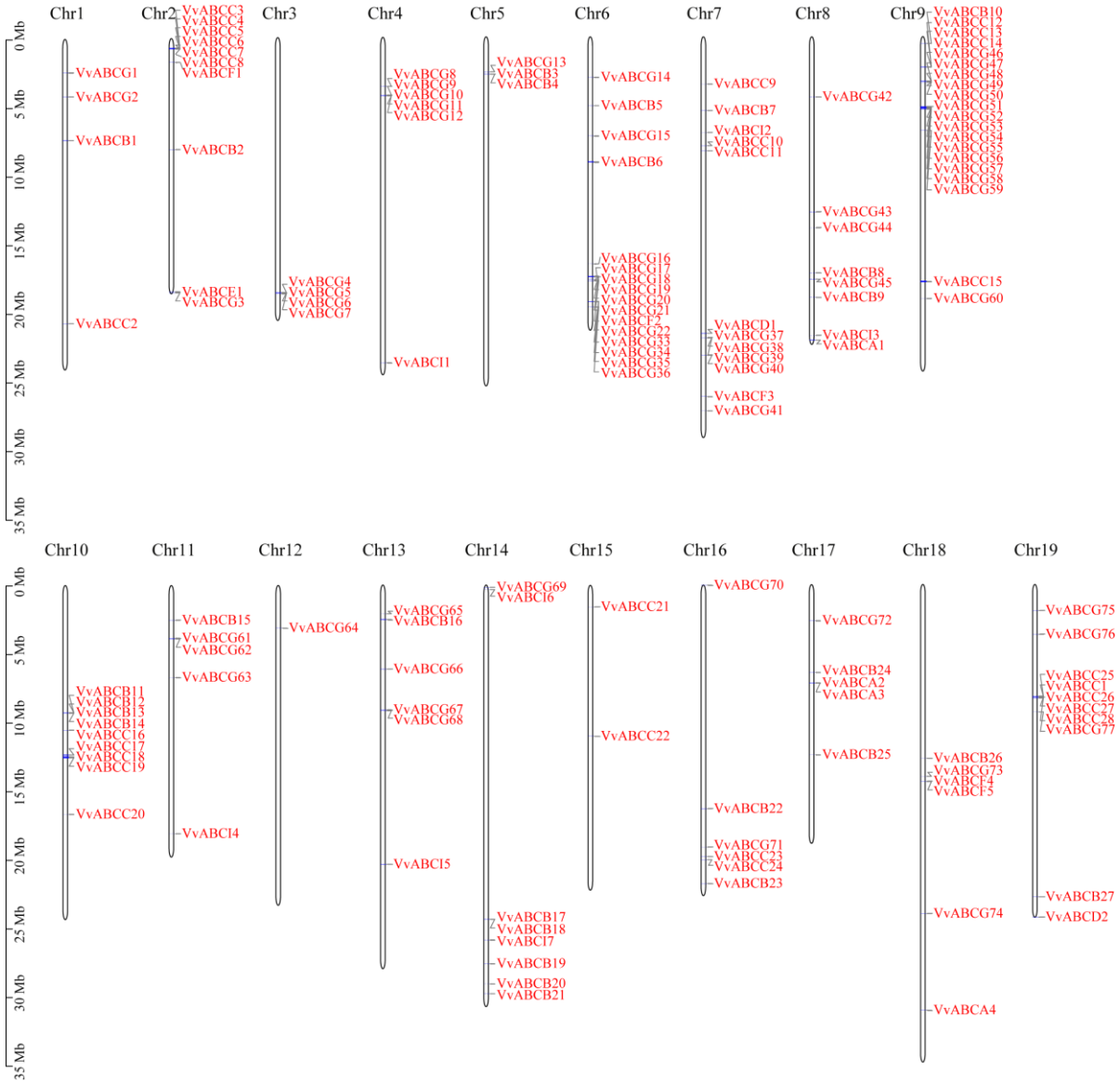

Figure 1. Chromosome localization of grape ABC family members.

In addition to collinearity analysis in the grape genome, we also screened homologous gene pairs between grape and *Arabidopsis thaliana*, *Malus domestica*, *Prunus persica*, *Actinidia chinensis* and *Fragaria vesca*, with the hope of further elucidating the potential evolutionary mechanism of the VvABC family (Figure 2B). There were 123, 92 and 75 pairs of homologous genes between grape and kiwifruit, peach, and strawberry, respectively (Table S7). There were 57 pairs of collinear homologous genes between grape and *Arabidopsis*, with the least number occurring among the five groups of comparisons. There were 133 pairs of homologous genes between grape and apple, including 69 VvABC genes, which could be attributed to the shorter phylogenetic distance between grape and apple.

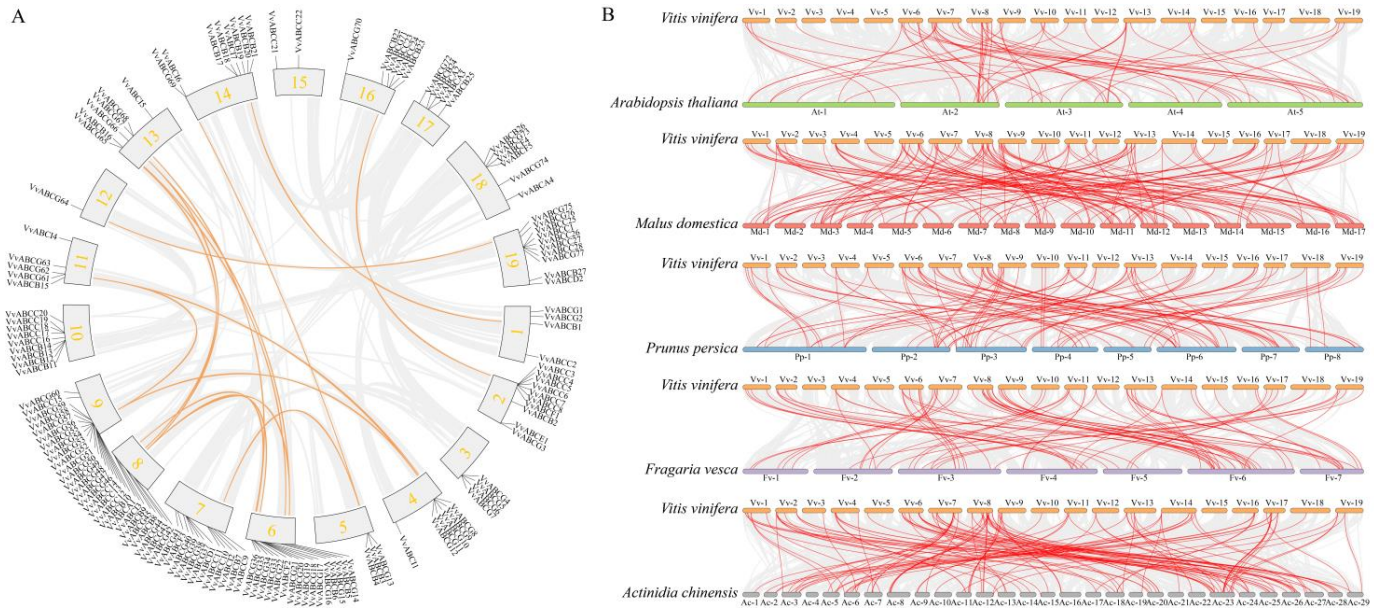

Figure 2. Evolutionary analysis of VvABCs. (**A**) Segmental duplication of ABC gene family in grape. The ABC genes were mapped to different chromosomes. The segmental-duplicated gene pairs of grape ABC genes were connected by red lines, while the gray line delineates synteny blocks in the grape genome; (**B**) Synteny analysis of the ABC genes between grape and five other species (*Arabidopsis thaliana*, *Malus domestica*, *Fragaria vesca*, *Actindia chienesis* and *Prunus persica*). The red line indicates segmental-duplicated gene pairs of ABC genes, while the gray line represents synteny blocks in grape genome and five other species.

3.4. Structure and Motif Analysis of VvABC Gene Family

To explore the sequence structure of the grape ABC family, the structure and motif of each member were analyzed (Figure 3). Motif analysis of the VvABC genes on the MEME website showed that conserved motifs of ABC proteins are different in subfamilies, which may lead to different functions of ABC genes. We identified 15 conserved motifs with a minimum number of amino acids of 15 and a maximum of 50. All protein sequences contain Motif-2, except VvABCG58. Motif-1 and Motif-3 are commonly present in most members. The order of some conserved motifs is similar, such as Motif-2 and-7. The members of the same subfamily show similar conserved motifs and structural composition. Motif-7 exists in all VvABCB subfamilies. Some conserved motifs exist in most members, while some only exist in specific subfamilies. For example, Motif-6, Motif-11, and Motif-14 only exist in the ABCG subfamily.

The exon and intron structures of the VvABC genes were analyzed by using TBtools. The results showed the diversification of intron and exon structures among subfamilies (Figure 4). The number of VvABCs exons ranged from 1 to 41. Gene *VvABCA1* had the largest number of exons, and two ABCF genes (*VvABCF4* and *VvABCF5*) and four ABCG genes (*VvABCG41*, *VvABCG65*, *VvABCG71* and *VvABCG75*) had only one exon. Some gene structures in the same subfamily are similar, but the number of exons varies greatly between different subfamilies.

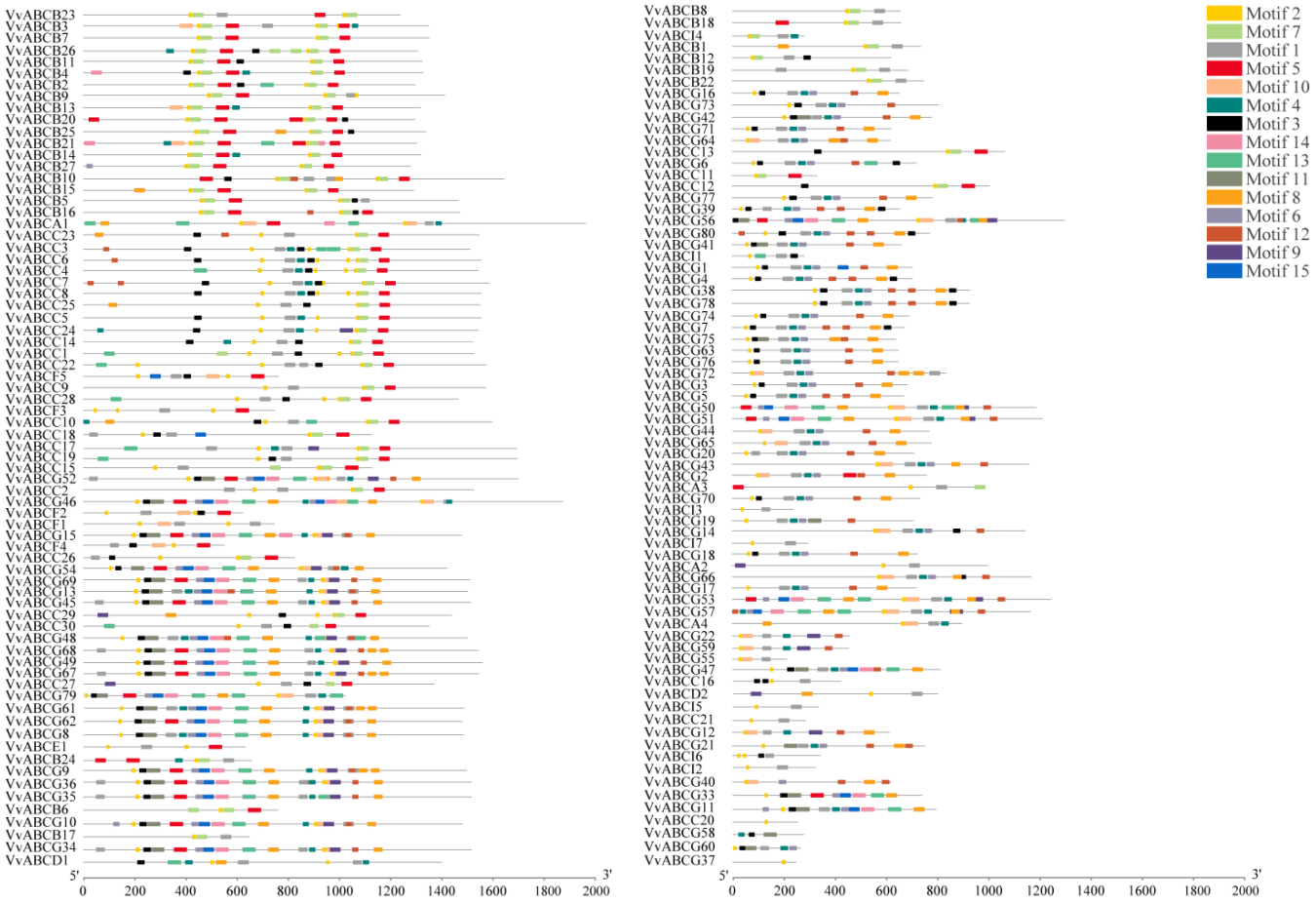

Figure 3. Conserved motif in ABC proteins. Different color boxes represent different motifs.

3.5. Prediction of Cis-Acting Elements in the Promoter Region of VvABC Genes

After downloading the grape genome file from the Ensemble Plant database, the promoter sequence of ABC gene about 2000 bp upstream of the CDS start site was extracted using TBtools. Then, PlantCARE was used to analyze the cis-acting elements in the promoter region, and it was found that the VvABC gene contained 48 elements, including G-BOX, ABRE, MRE, ARE and so on (Figure 5). These elements can respond to a variety of environments and stresses, including light-responsive and regulatory elements, methyl jasmonate response elements, and anaerobic sensing elements as the more common ones. Each ABC gene promoter sequence contains light-response and regulatory elements. In addition, some elements related to low temperature, salicylic acid and wound response were also identified in the ABC gene promoter region (Table S8).

3.6. Analysis of Protein Interactions of the VvABCs in RNA-Seq

Fifty-eight VvABCs were screened by using RNA-seq analysis of 'ZS-HY' in three stages (Table S1), including ABCA (two numbers), ABCB (eight numbers), ABCC (sixteen numbers), ABCD (only one), ABCG (thirty-one numbers) and ABCF (two numbers). The STRING database was used to predict potential interactions among the VvABC proteins (Figure 6). There were 81 nodes predicted from the VvABC protein interaction network, and each node interacted with other nodes. Some of these proteins interacted with each other in a simple point-to-point mode, while some proteins exhibited complex polygenic interaction patterns. Gene *VvABCB6* was predicted to be the central node that radiates fifty-five connections to other genes; *VvABCE1* radiates to 20 other genes; *VvABCF1* radiates to 18 other genes; and *VvABCD2* radiates to 12 other genes. We screened ABC genes with high gene expression from the RNA-seq analysis and marked them in red. It was found

that, except for *VvABCA1*, *VvABCC3*, *VvABCB22*, *VvABCG41* and *VvABCG62*, other genes showed polygenic interaction.

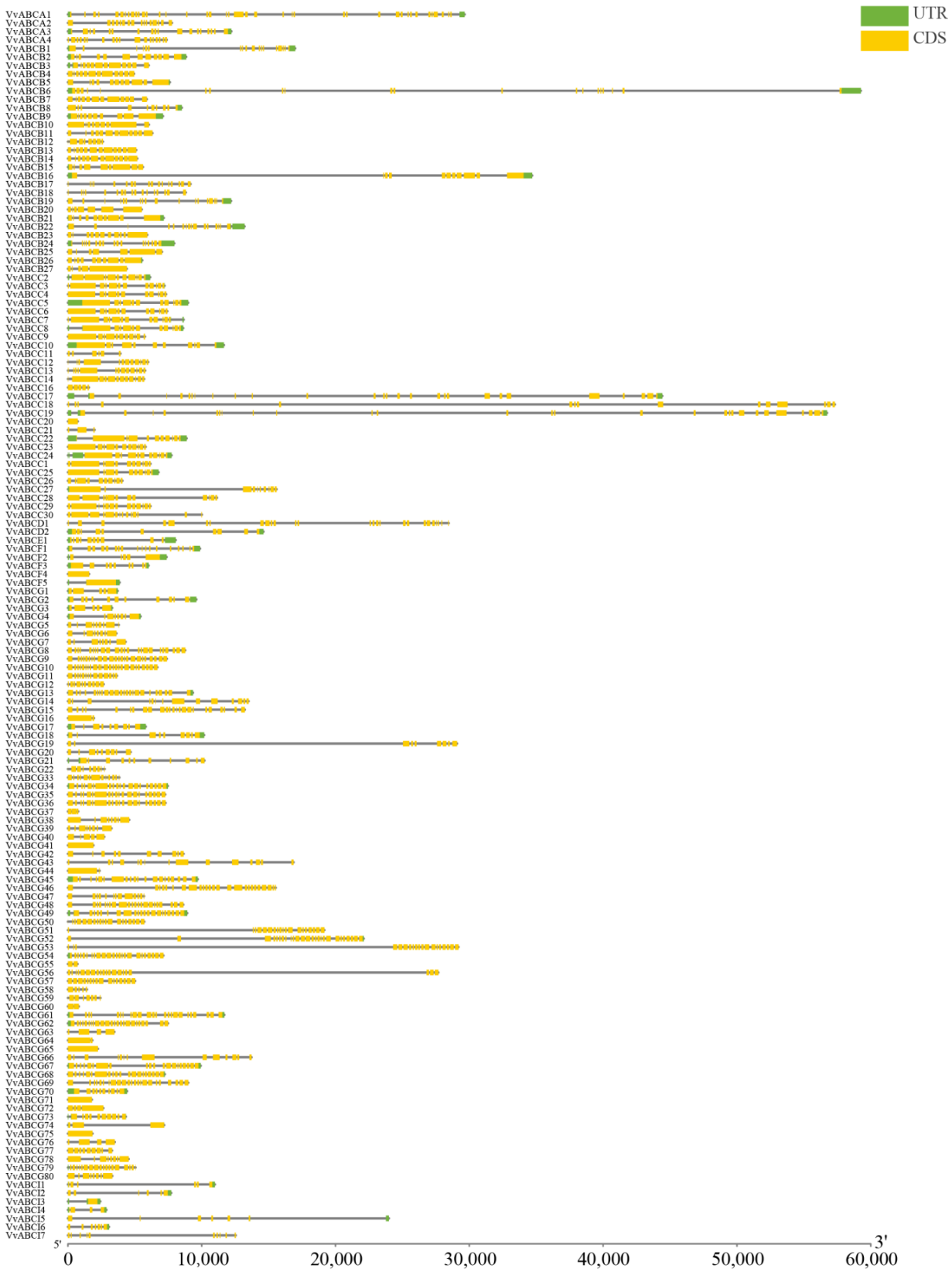

Figure 4. VvABC gene structures in the grape genome. The yellow boxes, green boxes and black lines in the gene structure diagram represent CDS, UTR and introns, respectively.

Figure 5. Predicted cis-acting elements in promoter region of grape ABC genes. Different colors represent different elements.

The ABCB transporters are thought to be responsible for auxin transport according to research [14,22,60–63]. Auxin is essential to plant interactions with the environment, and it is the core of many plants' developmental processes, from embryogenesis to organ senescence [64]. However, the regulatory network in plants is complex, and secondary metabolites and plant hormones in plants may be co-regulated by different subfamilies. The VvABCB6 may be responsible for the growth and development of berries, so this may be the reason for the central node of VvABCB6.

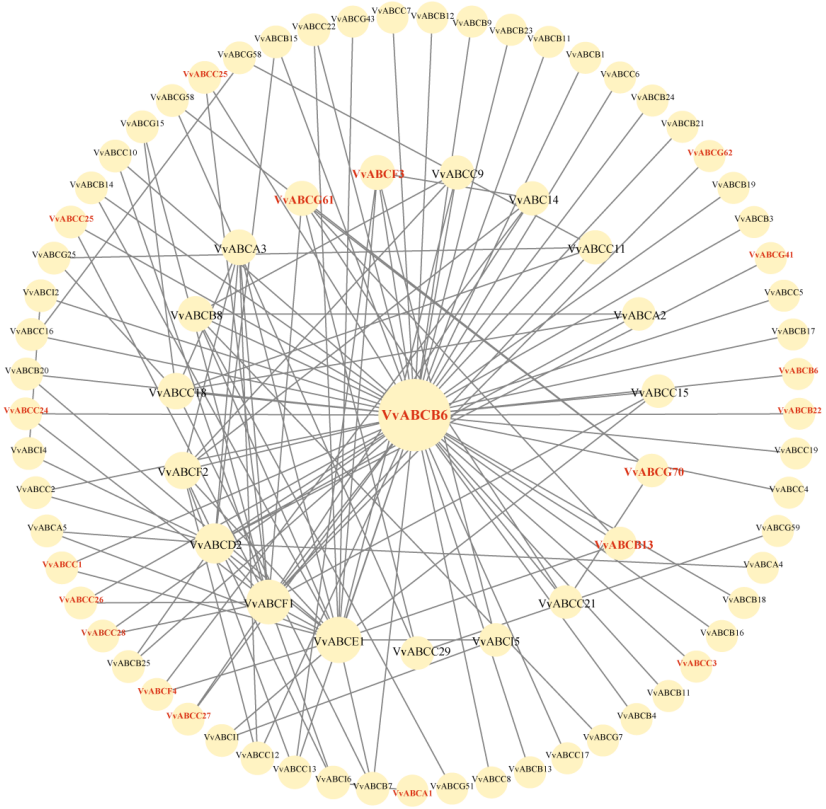

Figure 6. The interaction network diagram of ABC proteins in grape. The VvABCs in red font represent high FPKM values in the transcriptome data.

3.7. Expression Analysis of the VvABC Genes in Three Stages of Berries Growth and Development

According to the expression pattern of genes in berries, clustering analysis was performed among ABC genes, and these genes were divided into three categories. According to the values of genes' FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced), it was found that the expression of genes of the A subfamily (*VvABCA1*), B subfamily (*VvABCB6*, *VvABCB7*, *VvABCB13* and *VvABCB26*), C subfamily (*VvABCC1*, *VvABCC3*, *VvABCC22*, *VvABCC29*, *VvABCC25*, *VvABCC26*, *VvABCC27* and *VvABCC28*), G subfamily (*VvABCG4*, *VvABCG17*, *VvABCG41*, *VvABCG61*, *VvABCG62*, *VvABCG67*, *VvABCG70* and *VvABCG71*), and F subfamily (*VvABCF3* and *VvABCF4*) were higher in berries during three stages of 'ZS-HY'. These results indicated that the VvABCs show different expression levels even if these genes belong to the same subfamily. This may be due to the fact that ABC transporters, as the largest gene family, are involved in various physiological activities with a wide range of reaction substrates, so they play an important role in the growth and development of grape fruits, not just in anthocyanin transport.

We investigated the genetic relationships between ABC genes and ABC family members involved in anthocyanin transport in *Arabidopsis thaliana* ((*AtABCC1*, *AtABCC2*) AF008124, AF020288), *Zea mays* ((*ZmMRP3*) AY609318) and *VvABCC1* (JX245004). In the phylogenetic tree, *VvABCC1* was homologous to *ZmMRP3*, followed by *VvABCC3* and *VvABCC13*; *VvABCC19* was homologous to *AtABCC1* and *AtABCC2* (Figure 7B). Therefore, the ABCC family may play an important role in the regulation of anthocyanin in 'ZS-HY'.

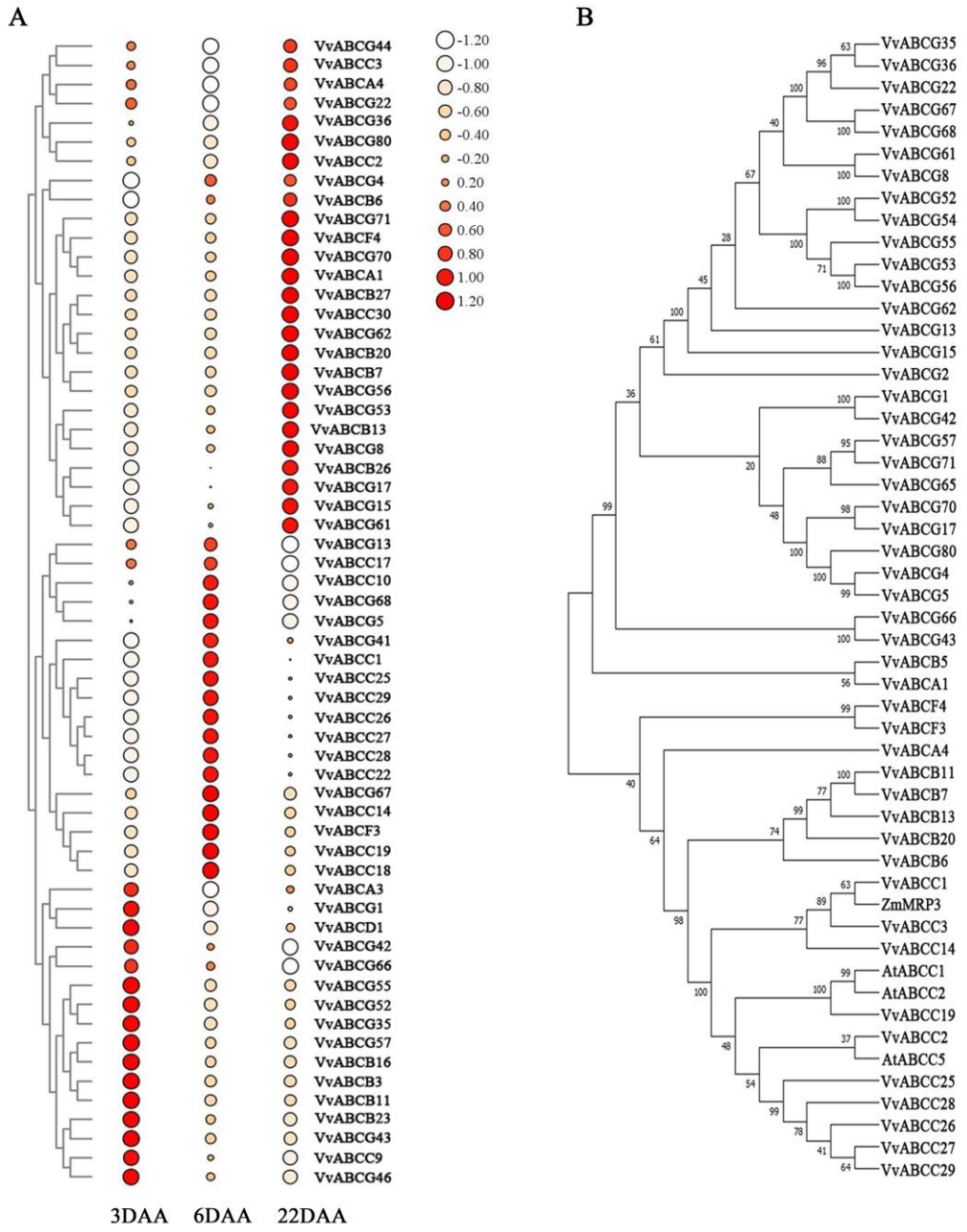

Figure 7. Heatmap of expression patterns of the VvABCs in three stages and evolutionary analysis of VvABC proteins. (**A**) The expression levels of ABC genes were represented in different colors, with increasing levels from white (−1.20) to red (1.20); (**B**) Phylogenetic tree of the VvABCs with AtABCC1, AtABCC2 and ZmMRP3.

In order to further study the VvABCC genes, we selected 10 VvABCC transporters according to the RNA-seq analysis and evolutionary tree analysis to identify cis-elements in the promoter region (Figure S2). The MBS elements (MYB binging site involved in flavonoid biosynthesis genes regulation) were found in all genes except *VvABCC3* and *VvABCC14*, MBS elements (MYB binging site involved in flavonoid biosynthesis genes regulation) were only found in *VvABCC26*, *VvABCC27*, *VvABCC29*. It is suggested that the ABCC genes are also regulated by upstream TF MYB, and there are more binding sites with MYB in *VvABCC26*, *VvABCC27*, *VvABCC29* promoters than other genes.

3.8. Expression Analysis of ABC Genes in Different Organs

To explore the expression patterns of the ABC gene family in different organs, we screened genes with high FPKM value in each subfamily from RNA-seq data to analyze the VvABC genes in the leaves, tendril, stem, and berries (22DAA) of 'ZS-HY'. As shown in Figure 8, the expression of gene *VvABCB20* was the highest in the stem, and the same pattern also appeared in the *VvABCC1*, *VvABCC3* and *VvABCG13* genes. In contrast, those of the ABCC subfamily (*VvABCC25*, *VvABCC26*, *VvABCC29* and *VvABCC28*) and ABCG subfamily (*VvABCG22, VvABCG55* and *VvABCG62*) had higher expression levels in berries.

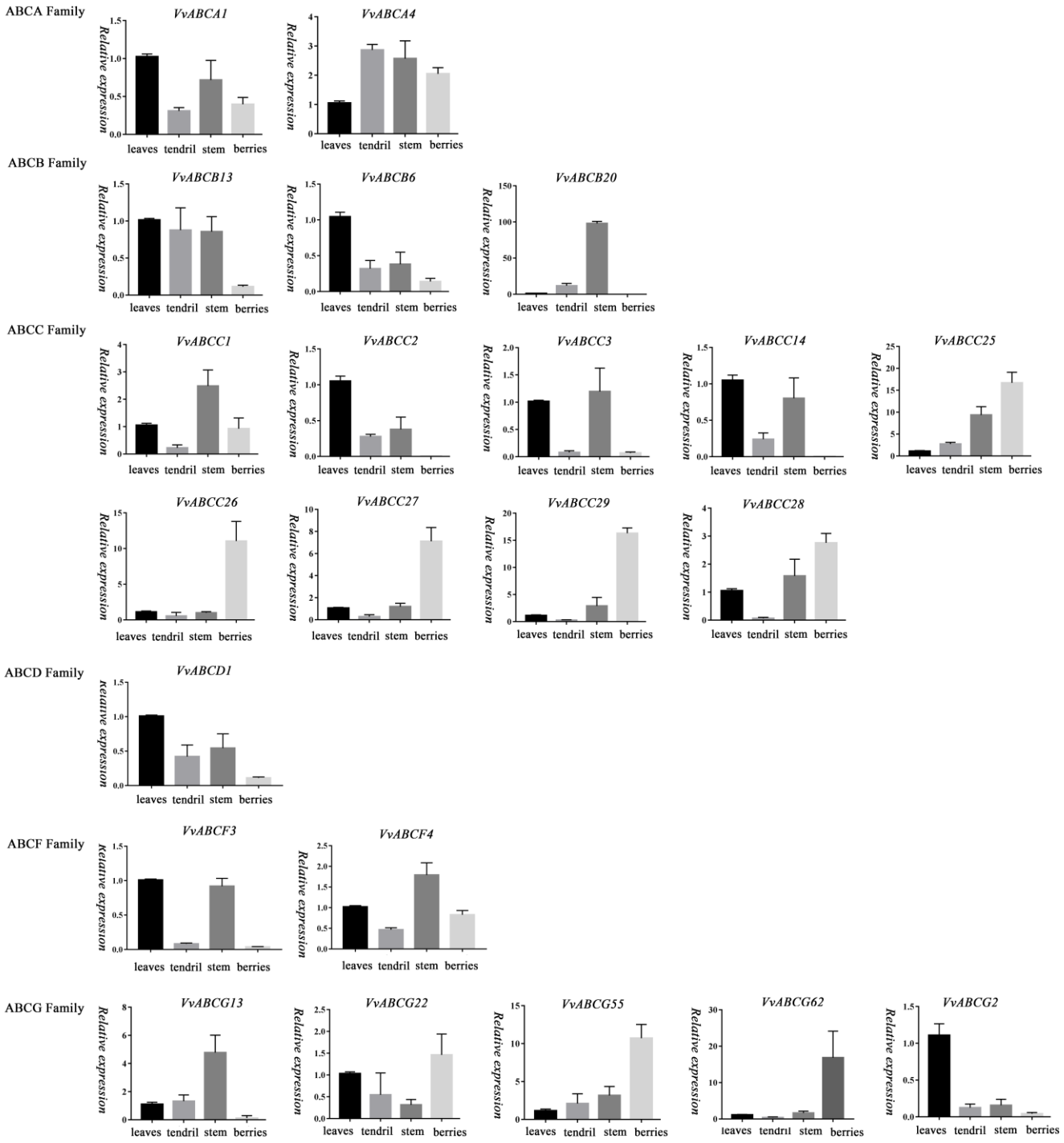

Figure 8. Relative expression levels of 22 VvABC genes in leaves, tendrils, stems, and berries in 'ZS-HY'.

4. Discussion

It has been reported that there are many types of ABC proteins in plants. The ABC genes are found in many species. In *Arabidopsis thaliana*, 129 ABC genes have been identified [65], 130 members in maize [66], 128 members in rice [67], 154 members in tomato [68], 162 members in flax (*Linum usitatissimum* L.) [69], and 115 members in strawberry [70]. Most ABC transporters require ATP hydrolysis to provide energy for intracellular and extracellular transmembrane transport [2,71]. At first, ABC transporters in plants were discovered during detoxification. However, to date, the function of ABC transporters has been extended beyond the scope of detoxification mechanisms [72]. Studies have shown that ABC transporters play an essential role in the regulation of heavy metals and in the transport of secondary metabolites, as well as in the response to plant pathogenic microorganisms [11,14,73]. Anthocyanins are a class of flavonoids, which are major families of secondary metabolites [74]. Whilst studying the transporter-mediated model, a series of evidence has indicated that multidrug and toxic extrusion (MATE) and ATP-binding cassette (ABC) proteins are involved in anthocyanin transport [12,41,75,76]. However, the involvement of ABC transporters in anthocyanin transport in teinturier 'ZS-HY' grapes has not been confirmed. In this study, we identified the whole genome of the ABC gene based on grape genome to screen the key genes involved in anthocyanin transport.

The diversity and specificity of the ABC gene family functions are determined by the number of family members. In grape, 146 ABC genes were identified and grouped into eight subfamilies according to their homology and domains of conserved sequences (Figure S1). All VvABC genes were distributed unevenly among all chromosomes (Figure 1). The prediction of subcellular localization showed that most ABC genes were located in the plasma membrane (Table S4), which may be consistent with their function as transporters. We also analyzed the collinearity of gene pairs between grape and *Arabidopsis thaliana*, *Malus domestica*, *Prunus persica*, *Actinidia chinensis* and *Fragaria vesca*. The closer the evolutionary relationship, the more collinear gene pairs were found, such as kiwifruit and apple (Figure 2B). Gene duplication events (tandem replication, fragment replication, whole genome replication, etc.) are important forces for the rapid expansion and evolution of gene families. In our study, we found that 55 of 146 VvABC genes were associated with discrete duplication and 45 genes were associated with tandem duplication (Figure 2A). Therefore, the main driving factor of the expansion of the ABC gene family may be related to repetitive events. The structural differences between genes are caused by the differences between introns and exons [77]. The analysis of motif and gene structure shows that members of the same subgroup may share similar gene structures (Figures 3 and 4).

A series of studies have shown that ABCC transporters are involved in anthocyanin transport in plants. The ABCC is a multidrug-resistance-associated protein (MRP) [44]. The OsMRP15, an ABCC transporter from rice (*Oryza sativa*), also plays a role in the vacuolar uptake of glycosylated anthocyanins [78]. Previous studies have shown that reducing MRP3 expression can alter the color of maize leaves [12]. Maize lacks a glutathione S-transferase encoded by the *bz2* gene, which leads to inappropriate accumulation of anthocyanins. Because the activity of the MRP transporter binding substrate is strongly influenced by glutathione S-transferase, it is possible that ABC-type MRP transporters are involved in the anthocyanin transport process in maize [79]. The first discovered transporters of the ABCC subfamily were AtABCC1 and AtABCC2 [45,80,81]. They were originally thought to transport the GSH conjugate of malvidin 3-*O*-glucoside in vitro [45,81]. However, because most of the accumulation in *Arabidopsis* vacuoles is of acylated anthocyanins, the transporters of acylated anthocyanins have been continuously investigated. So far, it has been shown that AtABCC1, AtABCC2 and AtABCC14 can rely on the MgATP to transport acylated anthocyanins in *Arabidopsis* vegetative tissues, which is different from the previously reported GSH-dependent transport of malvidin 3-*O*-glucoside [82].

In grape, *VvABCC1* is involved in the accumulation of malvidin 3-*O*-glucoside in the skin [48]. Gene *VvABCC17* has been shown to be involved in the transport of glycosylated anthocyanins. In addition, *VvABCC1*, *VvABCC2*, *VvABCC8* and *VvABCG11*

were found to be more highly expressed in black grapes than in white grapes [83]. Phylogenetic analysis showed that genes involved in anthocyanin transport in other species were closely related to members of the ABCC subfamily, especially *VvABCC1*, *VvABCC2*, *VvABCC3*, *VvABCC14* and *VvABCC19* (Figure 7B). Meanwhile, the expression level of *VvABCC1*, *VvABCC3* and *VvABCC14* was significantly upregulated in our RNA-seq analysis (Figure 7A). The gene expression of other C subfamilies (*VvABCC22*, *VvABCC29*, *VvABCC25*, *VvABCC26*, *VvABCC27* and *VvABCC28*) also showed an upward trend. Analysis of gene expression level in different organs by qRT-PCR showed that the *VvABCC1*, *VvABCC3* and *VvABCC14* transcription level in berries is low. However, *VvABCC25*, *VvABCC26*, *VvABCC27*, *VvABCC29* and *VvABCC28* are highly expressed in berries (Figure 8). Combined with the analysis of promoter elements in the gene (Figure S2), we hypothesize that *VvABCC25*, *VvABCC26*, *VvABCC27*, *VvABCC29* and *VvABCC28* may play an important role in the anthocyanin accumulation in 'ZS-HY' (Table S9).

The analysis of protein interaction in RNA-seq showed that there was also interaction between different subfamilies, indicating that there might be cross-complementation between the functions of genes among the subfamilies of ABC (Figure 6). Through this relationship, ABC genes jointly regulate the regulation and transport of different substances in plants.

5. Conclusions

We identified and analyzed 146 VvABC genes in terms of evolutionary relationship, chromosome distribution, collinearity, gene structure and motif, promoter element analysis and other factors. We also used RNA-seq analysis to identify and analyze the expression patterns of VvABC genes. These results provide a functional prediction analysis of ABC proteins involved in anthocyanin transport in berries of novel germplasm teinturier grape 'ZS-HY', as well as a foundation for further functional verification. However, how ABC genes regulate anthocyanin transport may involve complex network regulation, which requires further investigation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9050532/s1. Figure S1: Phylogenetic tree constructed with 135 ABC protein sequences with the ABC from *Arabidopsis thaliana*. Different colors represent different subfamilies of ABC genes. The MEGA X software was used to construct the tree by Neighbor-Joining method; Figure S2: Putative cis-elements exist in the upstream 2 kb region of ABCC protein genes (1 to 10 represent *VvABCC1*, *VvABCC2*, *VvABCC3*, *VvABCC14*, *VvABCC19*, *VvABCC25*, *VvABCC26*, *VvABCC27*, *VvABCC29* and *VvABCC28*, respectively). The orange box indicates that a particular element exists, and the white box indicates that it does not exist; Table S1: FPKM value of VvABC gene in RNA-seq; Table S2: The primer sequences of genes used in qRT-PCR analysis; Table S3: The extra genes compared to the 135 previously identified VvABC genes; Table S4: Members of the ABC gene family in grape genome; Table S5: Duplication type of VvABC genes; Table S6: The segmentally and tandemly duplicated VvABC gene pairs; Table S7: ABC gene pairs with collinearity relationships identified in grape, *Arabidopsis thaliana*, *Malus domestica*, *Fragaria vesca*, *Actinidia chinensis* and *Prunus persica*; Table S8: Type and number of cis-acting elements in promoter region of ABC gene in grape; Table S9: Anthocyanin composition and content of grape berry at 3DAA, 6DAA and 22DAA.

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