Analysis of PYL Genes and Their Potential Relevance to Stress Tolerance and Berry Ripening in Grape

Yanxia Zhao, Guimei Qi, Fengshan Ren, Yongmei Wang, Pengfei Wang, and Xinying Wu

Shandong Academy of Grape, Shandong Engineering Research Center for Grape Cultivation and Deep-processing, Key Laboratory of East China Urban Agriculture, Ministry of Agriculture and Rural Affairs, Jinan 250100, China

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ABSTRACT. Abscisic acid (ABA) is an essential phytohormone that regulates plant growth and development, particularly in response to abiotic stress. The ABA receptor PYR/PYL/RCAR (PYL) family has been identified from some plant species. However, knowledge about the PYL family (*VvPYLs*) in grape (*Vitis vinifera*) is limited. This study aims to conduct genome-wide analyses of *VvPYLs*. We successfully identified eight *PYL* genes from the newest grape genome database. These *VvPYLs* could be divided into three subfamilies. Exon-intron structures were closely related to the phylogenetic relationship of the genes, and *PYL* genes that clustered in the same subfamily had a similar number of exons. *VvPYL1*, *VvPYL2*, *VvPYL4*, *VvPYL7*, and *VvPYL8* were relatively highly expressed in roots. *VvPYL1*, *VvPYL3*, *VvPYL7*, and *VvPYL8* were expressed in response to cold, salt, or polyethylene glycol stress. *VvPYL6* was up-regulated by cold stress for 4 hours, and the expression of *VvPYL2* was 1.74-fold greater than that of the control under cold stress. *VvPYL8* was up-regulated 1.64-, 1.83-, and 1.90-fold compared with the control when treated with salt, PEG, or cold stress after 4 hours, respectively. Additionally, abiotic stress-inducible elements exist in *VvPYL2*, *VvPYL3*, *VvPYL7*, and *VvPYL8*, indicating that in these four genes, the response to abiotic stress may be regulated by *cis*-regulatory elements. The transcriptional levels of *VvPYL1* and *VvPYL8* significantly increased from fruit set to the ripening stage and decreased in the berry when treated by exogenous ABA. The eight *VvPYL* genes have diverse roles in grape stress responses, berry ripening, or development. This work provides insight into the role of *VvPYL* gene families in response to abiotic stress and berry ripening in grape.

ABA is an important plant hormone that plays a role in the regulation of plant developmental processes, such as seed germination, leaf senescence, root structure, seedling growth, and stomatal closure. ABA also plays an important role in the response of plants to abiotic stress (Fan et al., 2016; Fujita et al., 2011; Zhu, 2002). A previous study has shown that the application of ABA may be a suitable strategy to enable grape (*Vitis vinifera*) to manage stress, thereby increasing the yield and quality of berries at harvest (Murcia et al., 2017).

Recently, many studies have shown that ABA is recognized as an important hormone for ripening initiation, ripening regulation, sugar accumulation, and color development in some

nonclimacteric berries (Ferrara et al., 2015; Fortes et al., 2015; Pilati et al., 2017; Villalobos-González et al., 2016). Color is one of the most important quality parameters of grapes. Anthocyanins primarily determine the color of the berry; one of the best-known roles of ABA is the ability to improve the production of anthocyanins in grape berries, so it can serve as a tool to improve the color of grape berries (Gagné et al., 2011). Numerous studies have found that the exogenous application of ABA improved the coloring and increased the anthocyanin accumulation of grapes berries at veraison (Sun et al., 2011; Wang et al., 2016; Zhu et al., 2016). Previous studies have described the improved color development of red cultivars, including Olympia (*Vitis labrusca* × *V. vinifera*), Kyoho (*V. labrusca* × *V. vinifera*), Crimson Seedless (*V. vinifera*), Rubi (*V. vinifera*), and Fujiminori (*V. labrusca* × *V. vinifera*), with the treatment of ABA (Ban et al., 2003; Cantin et al., 2007; Ferrara et al., 2013; Hiratsuka et al., 2001; Jia et al., 2017; Kretschmar et al., 2016). Phenolic compounds, such as anthocyanins, flavanols, and stilbenes, are the most important secondary metabolites in fruit of grapes; these compounds play a vital role in the sensory properties and quality of grape berries and wines (Gagné et al., 2011). An increase in the ABA content in berries could increase the total anthocyanin content, phenolic content, and antioxidant properties of the grape skins, thus improving the nutritional value of wine (Sun et al., 2011; Wang et al., 2016; Zhu et al., 2016).

In 2009, PYR (pyrabactin resistant)/PYL (PYR-like)/RCAR (regulatory component of ABA receptor), which is referred to as PYL, has been characterized as an ABA receptor (Ma et al., 2009; Park et al., 2009). The PYLs belong to the steroidogenic

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P.W. and X.W. are the corresponding authors. E-mail: fengqiaoyouzi@126.com or echomoon0622@163.com.

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acute regulatory related lipid transfer (START) protein superfamily and have a conserved ABA binding domain (Park et al., 2009). The interacting domain is composed of four highly conserved loops (CL), CL1–CL4. These loops are essential for ABA binding and the PYL-type 2C protein phosphatase (PP2C) interactions (Lescot et al., 2002). Among them, the conserved domain CL2 is comprised of serine-glycine-leucine-proline-alanine (SGLPA), which is called the gate or proline-cap. The conserved domain CL3 is histidine-arginine-leucine (HRL), known as the latch or leucine-lucker. CL2 and CL3 comprises the Gate-Latch structure that acts as a direct receptor for ABA and is involved in the ABA signaling pathway (Zhang et al., 2015). PYLs inhibit PP2Cs to activate the sucrose nonfermenting 1 related protein kinases 2 (SnRK2s), resulting in phosphorylation of ABA-responsive element binding factors (ABFs) and other effectors of the ABA response pathways (Kobayashi et al., 2005; Umezawa et al., 2009). ABA signaling starts with the recognition of the ABA molecules by the ABA receptor PYLs protein family. Therefore, the study of PYLs as an ABA receptor has become particularly important.

In *Arabidopsis thaliana*, 14 PYL members have been identified (Ma et al., 2009; Park et al., 2009), and these can be divided into two categories: ABA-independent PP2C-inhibitory receptors and ABA-dependent PP2C-dependent receptors (Hao et al., 2011). In *A. thaliana*, PYL4–PYL10 (except PYL7) are monomers in solution that exhibit constitutive inhibitory activity on PP2Cs without the involvement of ABA (Hao et al., 2011; Santiago et al., 2009; Sun et al., 2012). PYR1 and PYL1–PYL3 are homodimers in solution bound by hydrophobic bonds, and ABA must be involved when these receptors bind to the PP2Cs (Hao et al., 2011; Mosquna et al., 2011; Yin et al., 2009). PYL members may be differentially expressed in diverse tissues, exhibit distinct biochemical properties, and possess diverse biological functions. In *A. thaliana*, *PYL2*, *PYL3*, *PYL7*, and *PYL9* are up-regulated in whole seedling tissues under ABA treatment. In contrast, *PYL5*, *PYL6*, and *PYL8* are down-regulated under ABA treatment and are temporally and spatially expressed (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009). *AtPYL8* and *AtPYL9* play an important role in regulating lateral root growth. In the presence of ABA, the overexpression of *PYL9* induced lateral root elongation, and the quiescent phase of the *pyl8-pyl9* double mutant was prolonged (Xing et al., 2016). Recent studies have shown that many PYLs have been characterized at genome-wide levels in strawberry [*Fragaria ananassa* (Chai et al., 2013)], tomato [*Solanum lycopersicum* (González-Guzmán et al., 2014)], rice [*Oryza sativa* (Kim et al., 2012)], soybean [*Glycine max* (Bai et al., 2013)], maize [*Zea mays* (Fan et al., 2016)], and wheat [*Triticum aestivum* (Gordon et al., 2016)]. In a previous study, three grape PYL genes—*VvPYL1*, *VvPYL2*, and *VvPYL3*—were identified (Li et al., 2011). The constitutive level of expression of *VvPYL1* was higher than that of *VvPYL2* and *VvPYL3* in the leaves, stems, and roots. *VvPYL1* was highly expressed in the tissues examined after treatment with ABA, and the level of expression of *VvPYL2* increased in stems, and that of *VvPYL3* was up-regulated in the stems and strongly in roots. *VvPYL1* inhibited the phosphatase activity of *ABII*, a negative regulator of ABA signaling (Li et al., 2011). However, the roles of the grape PYL family genes in stress tolerance and berry ripening are unknown.

With the completion of the newest grape genome database, we were able to identify the PYL family genes in a genome-

wide study. Therefore, we conducted a comprehensive analysis of grape PYLs and investigated their relevance to stress tolerance and berry ripening, which would provide new insight into the roles of grape PYLs in stress tolerance and berry ripening.

Materials and Methods

IDENTIFICATION AND ANALYSIS OF THE PYL FAMILY FROM GRAPE. The reference grape genome database at V2 (Vitulo et al., 2014) was used to search for putative PYLs of grape using the 14 *A. thaliana* PYL protein sequences as queries. The BLAST program was used with default settings [E-value < e^{-10} (Kumar et al., 2008)]. All PYL protein sequences, nucleotide sequences, and promoter sequences that were identified were downloaded and used in the subsequent analyses. All hits that were considered candidate sequences were analyzed using the National Center for Biotechnology Information (NCBI, Bethesda, MD) conserved domain search database to determine whether each protein was a member of the PYL family (Marchlerbauer et al., 2017). Previous research suggests that candidate genes should include PYR_PYL_RCAR-like domains (cd07821), polyketide cyclase domains (pfam03364), CL2 domains (SGLPA), and CL3 domains (HRL) (Guo et al., 2017; Park et al., 2009; Zhang et al., 2015). Any genes that did not contain all four domains at the same time would be removed.

The basic physicochemical properties of each member of the *VvPYL* gene family were analyzed using the online protein analysis system ProtParam (Wilkins et al., 1999). The subcellular localizations predictor of grape *PYLs* were analyzed online at CELLO (Yu et al., 2006). The grape *PYL* genes were located to chromosomes based on the position of genes using Map Gene2 Chromosome v2 (Chao et al., 2015).

ANALYSIS OF PHYLOGENETIC RELATIONSHIPS, GENE STRUCTURE, AND CONSERVED MOTIFS OF PYL. The *A. thaliana* and grape protein sequence families were multiply aligned using ClustalW (Kyoto University, 1991). Further processing of the alignment files was conducted using ESPrpt 3.0 (Robert and Gouet, 2014). Phylogenetic trees of the *A. thaliana* and grape gene families were constructed using the neighbor joining method with 1000 replicate bootstrap trials, using MEGA version 6.0 software (Tamura et al., 2013). The exon-intron structure of each *VvPYL* was identified by GSDS 2.0 (Hu et al., 2015). The motifs in grape *PYLs* were analyzed by MEME version 5.0.2 (Bailey et al., 2009), with the following parameter settings: output motifs, 20; minimum motif width, 6; maximum motif width, 300.

GENE ONTOLOGY AND KYOTO ENCYCLOPEDIA OF GENES AND GENOMES PATHWAY ANNOTATION. Gene Ontology (GO) function annotation analysis was downloaded from the GO database, and *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway annotation analysis was downloaded from the KEGG database (Kanehisa et al., 2016; Vitulo et al., 2014).

EXPRESSION ANALYSIS OF PYL GENES IN TISSUES AND IN RESPONSE TO ABA OR STRESS. Publicly available grape microarray data were retrieved for tissue-specific expression of *PYL* genes in ‘Corvina’ [*V. vinifera* (Fasoli et al., 2012)]. The microarray expression profiles of short-term abiotic stress samples (GSE31594), high-temperature treated samples (GSE31675), and ABA-treated samples (GSE31664) were retrieved from the publicly available data set Plexdb and GEO databases (U.S. National Library of Medicine, 2016). A

Table 1. Basic information of the *VvPYL* gene family and their putative proteins.

Accession no.	Gene	Coding sequences (bp)	Transcripts (no.)	Protein length (amino acids)	Molecular wt (kDa)	Exons (no.)	Isoelectric point (pH)	Hydrophobicity	Predicted subcellular localization
VIT_202s0025g01340	<i>VvPYL1</i>	570	2	189	21.51	3	5.9	-0.301	Nucleus, cytoplasm
VIT_202s0012g01270	<i>VvPYL2</i>	645	1	214	24.01	1	5.14	-0.458	Nucleus
VIT_204s0008g00890	<i>VvPYL3</i>	558	1	185	20.88	1	5.32	-0.405	Cytoplasm
VIT_208s0058g00470	<i>VvPYL4</i>	684	1	227	24.31	1	8.26	-0.236	Nucleus
VIT_210s0003g01335	<i>VvPYL5</i>	576	1	191	21.10	1	7.79	-0.216	Nucleus, mitochondrion, chloroplast
VIT_213s0067g01940	<i>VvPYL6</i>	639	1	212	22.97	1	6.58	-0.125	Extracellular
VIT_215s0046g01050	<i>VvPYL7</i>	579	2	192	21.32	3	5.97	-0.144	Cytoplasm, nucleus
VIT_216s0050g02620	<i>VvPYL8</i>	558	2	185	20.97	3	5.81	-0.41	Nucleus

Fig. 1. Distribution of grape *PYL* genes on chromosomes (Chrom).

Fig. 2. Phylogenetic analysis of *PYL* protein families from grape and *Arabidopsis thaliana*.

heat map was constructed using the microarray data of grape with Hemi 1.0 software (Deng et al., 2014). The treatment methods of the samples described above were as follows: ‘Cabernet Sauvignon’ (*V. vinifera*) plants were treated with

120-mM salt [NaCl:CaCl, v/v (10:1)], polyethylene glycol (PEG), cold (5 °C), or unstressed, respectively; and then the shoots with leaves were collected at 0, 1, 4, and 8 h for all treatments, and at 24 h for all except cold. ‘Cabernet Sauvignon’ plants were treated with high temperature (35 °C) and a control (25 °C) at 1 week before veraison, and then the berries were collected at 2, 4, and 6 weeks after treatment. ‘Cabernet Sauvignon’ berries were treated with 400 mg·L⁻¹ ABA solution and a control at veraison, and then the berries were collected at 14 and 28 d after treatment [GSE31664 (U.S. National Library of Medicine, 2016)].

ANALYSIS OF CODON USAGE BIAS AND *CIS*-ACTING REGULATORY ELEMENTS IN THE PROMOTERS OF *VvPYL*. The *cis*-regulatory elements in grape promoter (–2000 bp) *PYLs* were analyzed using PlantCare (Melcher et al., 2009). Codon usage bias of *VvPYL* was analyzed with CodonW 1.4.2 (Peden, 1999). Codon usage indices investigated include codon adaptation index (CAI), codon bias index (CBI), frequency of optimal codons (Fop), guanine (G) cytosine (C) of silent third codon position (GC3s), and GC content (GC) of the gene. SPSS software (version 20.0; IBM, Armonk, NY) was used to determine indices for a correlation analysis.

Results

GENOME-WIDE IDENTIFICATION AND ANALYSIS OF *PYLs* IN GRAPE. Fourteen AtPYLs amino acid sequences were employed as queries to search against the grape genome databases version 2.0 (Vitulo et al., 2014). Eight VvPYLs were identified in grape,

which were designated *VvPYL1*–*VvPYL8* based on their position on the chromosome. The PYL proteins in grape were 185–227 amino acids long; the molecular weights (MWs) of the PYLs varied from 20.88 to 24.31 kDa, and the isoelectric points

(pH) of PYLs ranged from 5.14 to 8.26 with an average of 6.34. Subcellular localization prediction found that *VvPYL2*, *VvPYL4*, and *VvPYL8* proteins were predicted to locate only in the nucleus; *VvPYL3* proteins only locate in the cytoplasm;

Fig. 3. Multiple sequence alignment of the core components of abscisic acid (ABA) signaling of *Arabidopsis thaliana* and *Vitis vinifera*. The four conserved loops (CL1–CL4) are highlighted with red lines.

VvPYL1 and VvPYL7 proteins locate in the nucleus and cytoplasm, and VvPYL5 proteins simultaneously locate in the nucleus, mitochondria, and chloroplasts (Table 1).

VvPYL1 and VvPYL2 were distributed on chromosome 2, and VvPYL3, VvPYL4, VvPYL5, VvPYL6, VvPYL7, and VvPYL8 were located on chromosomes 4, 8, 13, 10, 15, and 16, respectively. The grape *PYL* genes on individual chromosomes were irregularly distributed. Specifically, VvPYL1, VvPYL3, VvPYL5, and VvPYL6 were located on the upper end of the chromosome arms, VvPYL2 and VvPYL4 were in the middle of the arms, and VvPYL7 and VvPYL8 were located on the lower end of the arms (Fig. 1).

PHYLOGENETIC AND STRUCTURAL ANALYSIS OF THE VvPYL FAMILIES. The phylogenetic relationship between the grape *PYL* family and *A. thaliana* *PYL* family is shown in Fig. 2. VvPYLs and AtVvPYLs were divided into three subfamilies in the neighbor-joining tree, which is consistent with the subfamily classification for the *A. thaliana* *PYL* protein family (Park et al., 2009). VvPYL2, VvPYL3, VvPYL4, and VvPYL6 were members of subfamily III; VvPYL5 was a member of subfamily II, and VvPYL1, VvPYL7, and VvPYL8 were members of subfamily I.

Multiple sequence alignment of grape *PYL*s and *A. thaliana* *PYL*s by ClustalW showed that the VvPYLs all contained four identical conserved loops and had CL2 and CL3 conserved domains, which could form the Gate-Latch structure (Fig. 3).

*PYL*s contained one to three exons. There was no intron in VvPYL3, VvPYL4, VvPYL5, or VvPYL6, and three exons and three introns were detected in VvPYL1, VvPYL7, and VvPYL8 (Table 1, Fig. 4B). In addition, most grape *PYL* genes that clustered in the same subfamily had a similar number of exons. For example, most genes in subfamilies II and III had only one exon; three exons and a relatively long intron sequence was detected in subfamily I (Fig. 4B).

The putative motifs in the eight *PYL* proteins were analyzed using MEME software (Bailey et al., 2009). A total of 20 motifs designated as motif 1 to motif 20 were detected (Fig. 4C). The eight *PYL* proteins contained 6–10 motifs, among which VvPYL8 had the fewest motifs and VvPYL2 had the greatest number of motifs. Of the 20 motifs, motif 1, motif 2, and motif 3 emerged in all *PYL*s. Some motifs specifically existed in a certain subfamily. For example, motif 17 was only present in most *PYL*s in subfamily III, and motif 4 only belonged to members of subfamily I.

GO AND KEGG PATHWAY ANNOTATION. KEGG annotation indicated that all VvPYL genes were related to environmental information processing and mapped to mitogen-activated protein kinase (MAPK) signaling pathway-plant (ko04016) and plant hormone signal transduction (ko04075). GO annotation results showed that the VvPYL genes were divided into three categories: biological process, molecular function, and cellular component. In the cellular component category, all VvPYLs were annotated as “nucleus.” In biological process, VvPYL2, VvPYL3, VvPYL4, and VvPYL6 were annotated as “abscisic acid mediated signaling pathway” (GO:0009738). VvPYL1, VvPYL5, and VvPYL8 were annotated as “protein response to stress” (GO:00069500), and VvPYL2 and VvPYL5 were annotated as “regulation of seed germination” (GO:0010029). In molecular function, VvPYL1, VvPYL2, VvPYL5, and VvPYL7 were annotated as “abscisic acid binding” (GO:0010427), and VvPYL2, VvPYL3, VvPYL4, and VvPYL6 were annotated as “receptor activity” (GO:0004872) (Table 2).

Fig. 4. Phylogenetic relationships, gene structures, and conserved motifs of *PYL* genes in grape. (A) The phylogenetic tree was constructed using the neighbor-joining method. (B) Exon, intron, and upstream/downstream architecture of grape *PYL* genes. The sizes of exons and introns can be calculated following the scale at the bottom. (C) Distributions of conserved motifs. The motifs are indicated by 20 different color boxes.

ANALYSIS OF VvPYL FAMILY CODON USAGE BIAS. The codon usage bias of VvPYL genes was analyzed (Table 3), and CAI of *PYL* genes in grape were 0.174–0.263. The CAI of VvPYL7, VvPYL1, and VvPYL8 was less than 0.2 in subfamily I. GC3s was greater than 0.5, suggesting that VvPYLs preferentially used codons ending in G/C. The GC of *PYL*s ranged from 0.476 to 0.604, with an average of 0.53, indicating that the GC content of most of *PYL* gene coding regions was greater than the adenine (A) thymine (T) content. The correlation analysis showed that CAI, CBI, and Fop positively correlated with the GC3s/GC content (Table 4).

EXPRESSION OF VvPYL GENES IN TISSUES. Tissue-specific expression patterns of genes could contribute to a better understanding of their biological characteristics. The patterns of expression of seven VvPYL genes were monitored using publicly available microarray data (Fig. 5). The heat map showed that the levels of expression of VvPYL1, VvPYL7, and VvPYL8 genes were relatively higher than those of other

Table 2. Gene ontology (GO) pathway annotation and classification.

Gene	Accession no.	Biological process	Molecular function		
<i>VvPYL1</i>	VIT_202s0025g01340	Protein response to stress Positive regulation of abscisic acid mediated signaling pathway	GO:0006950 GO:0009789	Absciscic acid binding	GO:0010427
<i>VvPYL2</i>	VIT_202s0012g01270	Regulation of seed germination Regulation of protein serine/threonine phosphatase activity Absciscic acid mediated signaling pathway	GO:0010029 GO:0080163 GO:0009738	Receptor activity Absciscic acid binding	GO:0004872 GO:0010427
<i>VvPYL3</i>	VIT_204s0008g00890	Absciscic acid receptor pyl2-like fatty acid catabolic process Absciscic acid mediated signaling pathway	GO:0009062 GO:0009738	Protein homodimerization activity Receptor activity	GO:0042803 GO:0004872
<i>VvPYL4</i>	VIT_208s0058g00470	Regulation of seed germination Absciscic acid mediated signaling pathway	GO:0005515 GO:0009738	Receptor activity Protein binding	GO:0004872 GO:0005515
<i>VvPYL5</i>	VIT_210s0003g01335	Absciscic acid receptor pyl6 positive regulation of abscisic acid mediated signaling pathway Regulation of seed germination Protein response to stress	GO:0009789 GO:0010029 GO:0006950	Absciscic acid binding	GO:0010427
<i>VvPYL6</i>	VIT_213s0067g01940	Absciscic acid mediated signaling pathway	GO:0009738	Protein binding Receptor activity Protein binding Absciscic acid binding	GO:0005515 GO:0004872 GO:0005515 GO:0010427
<i>VvPYL7</i>	VIT_215s0046g01050				
<i>VvPYL8</i>	VIT_216s0050g02620	Protein response to stress Positive regulation of abscisic acid mediated signaling pathway	GO:0006950 GO:0009789		

VvPYLs at different developmental stages and different organelles, particularly in the dormant bud. The levels of expression of *VvPYL2* and *VvPYL4* were different. *VvPYL2* was highly expressed in roots and skin fruit in the seed set stage, and *VvPYL4* was highly expressed in roots and seeds in the ripening stage. In addition, the *VvPYL3* and *VvPYL6* genes were expressed at low levels in most tissues. The highest value in the *VvPYL3* microarray data were only 32.43 in leaves, and the highest value of *VvPYL6* was only 15.64 in the skin of grapes in the ripening stage.

EXPRESSION PROFILES OF *VvPYLs* IN RESPONSE TO STRESS AND ABA. With the increase in stress time of salt or PEG, the level of expression of *VvPYL1* increased gradually compared with the control, but it was down-regulated by the expression of cold stress (Fig. 6). After 8 h of cold stress treatment, the levels of expression of *VvPYL2* were 1.74-fold greater than those of the control. After 4 h of PEG or salt stress, *VvPYL3* was significantly down-regulated. From 1–4 h of cold stress, *VvPYL3* increased gradually compared with the control. *VvPYL6* was up-regulated by cold stress for 4 h. The level of expression *VvPYL7* was higher than that of the control in the salt or PEG stress treatment after 24 h. When subjected to salt stress, the level of expression of the *VvPYL7* gene was up-regulated 2.27-fold than that of the control. *VvPYL8* was up-regulated 1.64-, 1.83-, and 1.90-fold compared with the control when treated with salt, PEG, or cold stress after 4 h, respectively. The profiles of expression of *VvPYLs* in response to high temperature were analyzed; and we found that compared with the control, the level of expression of *VvPYLs* was not obvious under the high-temperature treatment (Supplemental Fig. 1).

We detected the expression patterns of the *VvPYL* genes in ‘Cabernet Sauvignon’ berries treated with ABA solution at

veraison. In general, the transcriptional levels of most of *VvPYLs* in berries decreased in response to exogenous application of ABA for 14 and 28 d. Compared with the control, the expression of *VvPYL1*, *VvPYL2*, *VvPYL3*, and *VvPYL8* decreased after both 14 and 28 d when the plants were treated with ABA. *VvPYL3*, which was reduced to more than half of the control, was particularly down-regulated after 14 d of ABA treatment. The expression of *VvPYL5* and *VvPYL6* increased first for 14 d and then decreased for 28 d with the berry development. *VvPYL7* only decreased after 14 d of ABA treatment but did not change after 28 d of treatment (Fig. 7).

ANALYSIS OF CIS-REGULATORY ELEMENTS IN THE PROMOTERS OF *VvPYLs*. Cis-regulatory elements (CREs) of the upstream 2000 bp were analyzed for each member of the *VvPYL* gene family. In addition, putative CREs were identified—including ABA-responsive element (ABRE), auxin response element (Aux RR), gibberellin-responsive element (TATC, GARE motif, P-box), ethylene-responsive element (ERE), jasmonic acid-responsive element (CGTCA motif, TGACG motif), salicylic acid-responsive element (TCA element), seed-specific regulation [purin and pyrimidin nucleotides (RY) element], meristem-specific regulation (CCGTCC motif), zein metabolism regulation (O₂ site), and abiotic stress-inducible elements, such as thymine cytosine (TC)-rich repeat, myelocytomatosis protein binding sites (MBS), dehydration responsive element (DRE) core, and low temperature responsive (LTR). All *PYL* promoter sequences had an ethylene-responsive element. ABA-responsive element existed in *VvPYL2*, *VvPYL5*, *VvPYL7*, and *VvPYL8* promoters. An element of the TC-rich repeat involved in abiotic stress induction was present in *VvPYL2* and *VvPYL5*. The drought-inducibility

element (MBS) was detected in *VvPYL2*, *VvPYL3*, *VvPYL4*, and *VvPYL8*. In addition, the cold stress-inducible element (DRE core, LTR) presented in *VvPYL2*, *VvPYL3*, *VvPYL4*, *VvPYL7*, and *VvPYL8* (Fig. 8).

Table 3. Index of codon usage bias in *VvPYL* genes in grape.

Gene	CAI	CBI	Fop	GC3s	GC
<i>VvPYL1</i>	0.187	−0.036	0.39	0.538	0.476
<i>VvPYL2</i>	0.263	0.137	0.502	0.686	0.564
<i>VvPYL3</i>	0.216	−0.029	0.403	0.53	0.476
<i>VvPYL4</i>	0.248	0.183	0.522	0.701	0.59
<i>VvPYL5</i>	0.209	0.069	0.451	0.565	0.532
<i>VvPYL6</i>	0.257	0.221	0.543	0.76	0.604
<i>VvPYL7</i>	0.182	−0.032	0.387	0.554	0.519
<i>VvPYL8</i>	0.174	−0.026	0.391	0.536	0.488

CAI = codon adaptation index, CBI = codon bias index, Fop = frequency of optimal codons, G = guanine, C = cytosine, GC3s = GC of silent third codon position, GC = GC content.

Table 4. Correlation analysis of the index of codon usage bias.

	CAI	CBI	Fop
GC3s	0.886318**	0.967332**	0.964969**
GC	0.818273**	0.964036**	0.948819**

CAI = codon adaptation index, CBI = codon bias index, Fop = frequency of optimal codons, G = guanine, C = cytosine, GC3s = GC of silent third codon position, GC = GC content.

***P* < 0.01.

Fig. 5. Tissue specific gene expressions of *VvPYLs*.

Fig. 6. Profiles of the expression of *VvPYLs* under salt, polyethylene glycol (PEG), and cold stress; CK = control.

Discussion

In this work, we identified eight genes (*VvPYLs*) in grape genomes encoding putative ABA receptors based on their amino acid sequence similarity with PYLs in *A. thaliana*. This is less than the number of *VvPYL* genes compared with *A. thaliana* [14 (Park et al., 2009)], strawberry [10 (Chai et al., 2013)], tomato [15 (González-Guzmán et al., 2014)], rice [13 (Kim et al., 2012)], soybean [23 (Bai et al., 2013)], maize [11 (Fan et al., 2016)], and wheat [13 (Gordon et al., 2016)]. However, together with their orthologous PYLs from other plants, phylogenetic analysis showed that the putative *VvPYL* proteins were also classified into three subfamilies (Park et al., 2009). Aside from *VvPYL2*, most grape *PYLs* in subfamilies II and III had no intron, indicating that these genes are highly conserved and may be a more primitive type (Kolkman and Stemmer, 2011). The *PYLs* in subfamily I had three exons, indicating that these genes obtained new introns during evolution, which is consistent with the *PYLs* in rice, maize, tomato, and rubber tree (*Hevea brasiliensis*) (Fan et al., 2016; González-Guzmán et al., 2014; Guo et al., 2017; He et al., 2014).

All grape *PYLs* contained four identical conserved loops that play important roles in ABA binding and PP2C interaction, which is consistent with the structure of AtPYLs in *A. thaliana* (Ma et al., 2009; Park et al., 2009). The protein motif analysis found that *VvPYL* members that clustered in the same subfamily contain similar motifs, indicating a functional similarity between members of the same subfamily. The results of the GO and KEGG annotations showed that all *VvPYL* genes were related to environmental information processing and plant hormone signal transduction (Bai et al., 2013; Ma et al., 2009; Tian et al., 2015).

The CAI of *VvPYLs* was significantly less than 1.0. This indicated that codon usage bias was weak in the grape *PYL* genes (Sharp and Li, 1987). The GC content of most coding regions of the *PYL* genes was higher than the AT content, which might be a result of different genomic organization and mutational pressures (Ikemura, 1985). *VvPYL* genes more commonly had codons ending in G/C; this is consistent with the G/C codon usage reported in *A. thaliana* (Kawabe and Miyashita, 2003). The CAI, CBI, and Fop of the grape were positively correlated with GC content, indicating that the codon usage bias of the grape was affected by the mutation pressure (Chen et al., 2004; Wang and Roossinck, 2006).

GO annotation results showed that the function of *VvPYL2* was involved in regulation of seed germination, which might be consistent with the high level of expression of *VvPYL2* in grape seed. Simultaneously, one RY element (seed

specific regulation element) was found in the upstream non-coding sequence of *VvPYL2*. We suspect that *VvPYL2*, which is highly expressed in grape seed, might be regulated by the RY element. In *A. thaliana*, *AtPYL8* and *AtPYL9* play an important role in regulating lateral root growth in subfamily I (Xing et al., 2016). We found that *VvPYL1*, *VvPYL7*, and *VvPYL8* belonged to subfamily I and were relatively highly expressed in roots. Our results were like those from *A. thaliana*, suggesting that subfamily I may be closely related to root development. *VvPYL1*, *VvPYL7*, and *VvPYL8* were relatively highly expressed in dormant buds, implying that these receptors may be related to ABA signaling during bud dormancy. This is consistent with a previous study that showed that ABA plays a key role in the regulation of endodormancy in grape buds (Vergara et al., 2017). In rice, *OsPYL1* was predominantly expressed in roots, *OsPYL3* and *OsPYL5* in leaves, *OsPYL7* and *OsPYL8* in embryos, and *OsPYL2* and *OsPYL9* in all tissues (Tian et al., 2015). In maize, *ZmPYL11* and *ZmPYL6* were expressed in roots, and *ZmPYL10* was expressed in the leaves (Fan et al., 2016). This indicates that different *PYLs* may play a role in different tissues in plants.

The *PYL* proteins were identified as ABA receptors and located upstream of the ABA signaling network. ABA plays an important role in stress response (Fujita et al., 2011; Zhu, 2002). In cotton, the expression of *GhPYL1*, *GhPYL6*, *GhPYL8*, *GhPYL10*, *GhPYL12*, and *GhPYL26* were down-regulated during drought treatment, and only three genes (*GhPYL22/23/25*) were up-regulated under drought stress (Yun et al., 2017). In maize, except *ZmPYL1*, all of the remaining *ZmPYL* genes

were activated by osmotic stress (Fan et al., 2016). Our results are consistent with these previous studies in that the different *PYLs* of grape expression patterns also respond to diverse stress. In this work, GO function annotations showed that some grape *PYL* members, such as *VvPYL1*, *VvPYL5*, and *VvPYL8*, are involved in stress responses. We found that the expression of *VvPYL1*, *VvPYL3*, *VvPYL7*, and *VvPYL8* was activated in response to cold, salt, or PEG stress. Additionally, the analysis of promoters of *VvPYL2*, *VvPYL3*, *VvPYL7*, and *VvPYL8* showed that these *PYLs* contained abiotic stress-inducible elements. For example, a cold stress-inducible element (LTR) existed in the *VvPYL2* promoter, and *VvPYL2* was up-regulated by cold stress. Therefore, this research showed that some *PYLs* may be regulated by the CREs. This different response pattern indicated that different *PYLs* in the grape participated in different stress responses.

In ‘Kyoho’, the expression level of *VvPYL1* was highest in the tissue of grape berry, and the expression of *VvPYL1* increased during fruit development (Gao et al., 2018). In tomato, *SIPYL1*, *SIPYL2*, *SIPYL3*, and *SIPYL6* were the major genes involved in the regulation of fruit development (Sun et al., 2011). In strawberry, *FaPYR1* was expressed in green and red fruit (Chai et al., 2013). ABA appears to play an important role in accelerating the ripening of grape berries. The beginning of the maturity of grapes relied on the rapid rise of ABA (Jia et al., 2017). In the grape flesh, we found that the levels of expression of *VvPYL1*, *VvPYL4*, *VvPYL7*, and *VvPYL8* significantly increased from fruit set to ripening. Notably, *VvPYL7* increased to nearly five times that of fruit set in the ripening stage. In the berry skin, the level of expression of *VvPYL1* and *VvPYL8* improved from fruit set to ripening, indicating that *VvPYL1* and *VvPYL8* may be involved with fruit coloring. This information provides additional evidence that the ABA receptors may play an important role in fruit development in grape.

The application of ABA may be a suitable strategy for grape to adapt to stress and improve the yield and quality of the grapes at harvest (Murcia et al., 2017). We found that most of the levels of expression of *VvPYLs* were affected by exogenous ABA. The transcriptional levels of *VvPYL1*, *VvPYL2*, *VvPYL3*, and *VvPYL8* decreased under both 14 and 28 d of ABA treatment in ‘Cabernet Sauvignon’. The expression levels of *VvPYL5* and *VvPYL6* were down-regulated by treatment with ABA for 14 d. The ABRE was a *cis*-regulatory element that may be regulated by ABA signaling (Kim et al., 2011). However, among the *PYLs* affected by exogenous ABA, only the promoters of *VvPYL2*, *VvPYL5*, and *VvPYL8* contained ABRE. This suggests that some levels of the expression of *VvPYLs* that responded to

ABA signals could be related to ABRE, while the expression of other responses to ABA signals was not. Tian et al. (2015) reported that *OsPYL1*, *OsPYL2/9*, and *OsPYL3* were down-regulated after ABA treatment. In addition, Fan et al. (2016) reported that *ZmPYL4–11* was found to be down-regulated by ABA treatment. However, in rice and maize, some *PYLs* were up-regulated; after ABA treatment, *OsPYL4* was up-regulated in rice and *ZmPYL1–3* was up-regulated in maize roots (Fan

Fig. 7. Profiles of the expression of *VvPYLs* when *Vitis vinifera* fruit are treated with abscisic acid (ABA); CK = control.

Fig. 8. Analysis of *cis*-regulatory elements in the 2000-bp promoter regions of *VvPYLs*; ABRE = abscisic acid responsive element, TC = thymine cytosine, MBS = myelocytomatosis protein binding sites, DRE = dehydration responsive element, LTR = low temperature responsive, ERE = ethylene-responsive element, RY = purin and pyrimidin nucleotides.

et al., 2016; Tian et al., 2015). These data indicate that different *PYL* members of different plant species have differential expression patterns in response to ABA. This could be because the ABA signals are perceived differently by different tissues, reflecting the diversity of *PYLs* in different plants.

We successfully identified and analyzed eight *VvPYL* genes in grape. The *VvPYL* proteins were classified into three subfamilies, namely subfamily I, II, and III. The exon/intron organizations of grape *PYLs* were closely related to the phylogenetic relationship of the genes. All the *VvPYLs* had CL2 and CL3 domains, which were essential for ABA binding and were involved in the ABA signaling pathway. We provide evidence that different grape *PYLs* exhibit diverse roles in grape stress response, berry ripening, or development. Our results provide insight into the roles of *VvPYLs* in stress tolerance and berry ripening.

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Supplemental Fig. 1. Profiles of the expression of *VvPYLs* under high temperature (HT); CK = control.