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Genome-wide identification and characterisation of phenylalanine ammonia-lyase gene family in grapevine

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) is a rate-limiting enzyme of phenylpropanoid biosynthesis in plants that synthesise many ubiquitous key compounds, including phenolic acids, flavonoids, lignins, and stilbenes. The first draft of the full grape genome assembly was released, but systematic analysis of the PAL family has not been completed in detail. In this study, 15 PALS were identified in the grape genome. Grape PALS are distributed on 6 chromosomes, and 9 PALS are distributed on chromosome 16, in which most of the 2 downstream genes, including chalcone synthase (CHS) and stilbene synthase (STS). Promoter analysis revealed that there were many cis-acting elements in the promoter regions to respond to light, phytohormone, and stress. According to transcriptome-data analysis, VvPALS have extensive expression patterns in various tissues and developmental stages in response to various stresses. Dynamic expression patterns of PAL1/2/3/5 were observed in different developmental stages of 4 grape varieties on the basis of qRT-PCR analysis, and suggested that PALS are involved in a complex mechanism to modulate in anthocyanin biosynthesis. Comprehensive analysis of PALS offers a basis for further understanding the physiological functions of PALS during grape development and their potential role to respond the various stimuli.

INTRODUCTION

Grape (Vitis vinifera L.) is a well-known economic fruit tree due to its high nutritional value. Phenolic compounds produced by the phenylalanine metabolic pathway are key secondary metabolites in grapes. The synthesis of these compounds contributes to organoleptic characteristics, including pigment and mouthfeel (Li & Sun, 2019). Thus, the metabolites of phenylalanine metabolic pathways have attracted widespread attention.

In plants, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) synthesises a variety of secondary metabolites by regulating the nonoxidative deamination reaction of L-phenylalanine, such as lignins, flavonoids, alkaloids, and lignans, especially stilbenes, in grape (Fraser & Chapple, 2011; Vogt, 2010). The major functions of these compounds are involved in structural components, UV light protection, pathogen and predator defence, signalling reproduction and regulation, and chemical modulators for communicating with insects and microbes (Ferrer, Austin, Stewart, & Noe, 2008).

Previous studies showed that PAL is widespread in plants, fungi, and algae, but not animals (Hyun, Yun, Kim, & Kim, 2011; Vance, Bandoni, & Towers, 1975; Young, Towers, & Neish, 1966). The earliest PAL was isolated from Hordeum vulgare (Koukol & Conn, 1961). Many continuous studies were carried out on PAL involvement in light radiation (Gao et al., 2012; Kokalj, Zlatic, Cigic, Kobav, & Vidrih, 2019; Saengnil, Lueangprasert, & Uthaibutra, 2011; Sreelakshmi & Sharma, 2008), tissue-specific expression (de Jong, Hanley, Beale, & Karp, 2015; Lu et al., 2006; Okada, Mikage, & Sekita, 2008), phytohormones (Song, Ma, Tan, & Zhou, 2011; Zhang et al., 2020), and stress (Biliou, Ocampo, & Garcia-Garrido, 2000; Gao et al., 2012; Jiang et al., 2011; Zoufan, Azad, Rahnama Ghahfarokhi, & Kolahi, 2020) among many plant species. Moreover, some studies suggested that PAL possessed therapeutic potential in neoplasms, and markedly inhibited the cell division of human leukaemic and murine leukaemic (L5178Y) lymphoblasts in vitro by depriving these cells of L-phenylalanine (Abell, Stith, & Hodgkins, 1972; Stith, Hodgkins, & Abell, 1973). PAL also worked for phenylketonuria (PKU) patients (Hoskins et al., 1980; van Spronsen & Derks, 2014). Due to the commercial and medical potential of PAL, many studies were conducted to resolve problems associated with the enzyme activity of PALS in yeast (Babich, 2014; D’Cunha, 2005).

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PAL gene function, enzyme activity, post-translational modifications, and metabolite feedback regulation were extensively studied (Huang et al., 2010; Rohde et al., 2004; Yuan et al., 2019; Zhang, Gou, et al., 2015; Zhang & Liu, 2015). There are between 3 and 10 PALs identified in many plants that form a small gene family (Hou, Shao, Ma, & Lu, 2013; Huang et al., 2010; Lepelley et al., 2012; Raes, Rohde, Christen, Van de Peer, & Boerjan, 2003; Shang, Li, & Dong, 2012; Shi et al., 2010; Xu et al., 2010; Yuan et al., 2019). In addition, the high copy number of PALs was discovered in tomatoes (Chang, Lim, Lee, Robb, & Nazar, 2008). At present, many studies summarised the basic functions of PALs in some plants, but there are differences between each PAL gene, which needs further research (Rohde et al., 2004).

Here, 15 PAL genes were identified on the basis of a genomewide search of the grapevine genome (version 2.1). The intron/exon structures, motifs, and cis-regulatory elements of PAL genes were constructed and analysed. Transcriptome data under different organs and stresses were also investigated. Lastly, the expression profiles of 4 PALs at 6 developmental periods were presented using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Therefore, the purpose of the present study is to lay a foundation for the further investigation of the physiological functions and regulation mechanisms of grape PAL genes to respond to various stimuli.

Materials and methods

Plant materials

In this study, 2 wild Chinese Vitis quinquangularis (Shangnan-24 and Danfeng-2) and 2 Vitis vinifera (Cabernet Sauvignon and Chardonnay) (Table 1) were sampled from the grape germplasm nursery located in Northwest A&F University. According to the modified E–L system (Coombe, 1995), berries from 6 developmental stages (E–L 31, E–L 33, E–L 34, E–L 35, E–L 36, and E–L 38) were selected for quantitative analysis.

Identification of grape PAL genes

The grape genome sequences were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/?term=vitis+vinifera) and EnsemblPlants (http://plants.ensembl.org/Vitis_vinifera/Info/Index) databases, and PALs in grape were identified according to the 12X V1 version (http://genomes.cribi.unipd.it/DATA/). Functional annotations were filtered for the Hidden Markov model identifiers (HMM, Lyase_aromatic, PF00221; http://pfam.janelia.org/) on the basis of HMMER Search (http://www.ebi.ac.uk/Tools/hmmer/); further identification was performed with the BLASTP programme using an E value cut-off of 0.001. Furthermore, we rebuilt the HMM using all possible candidate grape PALs to screen PAL members. Lastly, 15 nonredundant, confirmed grape proteins containing the phenylalanine ammonia-lyase domain were identified as PAL members according to the query of the NCBI Conserved Domains Search website tool (E < 0.01) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Grape Genome Browser 12X V1, and the Pfam database. The nomenclature of the identified PALs was based on their order in grape chromosomes.

| Table 1. Four genotypes of 2 wild Chinese Vitis species and 2 cultivars of V. vinifera. |
|---------------------------------|---------------------------------|-----------------|
| Species                        | Cultivar (Accession No.)        | Grape berry skin colour |
| Vitis vinifera Linn. (Vv)      | Cabernet Sauvignon              | Red              |
|                                | Chardonnay                      | White            |
| Vitis quinquangularis Rehd. (Vq)| Shangnan-24                     | Red              |
|                                | Danfeng-2                       | White            |

Chromosomal localisation, gene duplication and syntenic analysis

The chromosomal-distribution data of putative grape PALs were obtained from genome annotation files (http://plants.ensembl.org/Vitis_vinifera/Info/Index). Chromosomal localisation was constructed by MapGene2Chromosome V2 (http://mg2c.iask.in/mg2c_v2.0/). The identification of gene duplication was obtained using MCScanX software (Wang et al., 2012). The types of gene duplication (singleton, dispersed, proximal, tandem, and segmental) were assigned using the MCScanX command (/duplicate_gene_classifier data) on the basis of the chromosomal positions and blast alignments of all 29,971 grape genes. Syntonic analysis within grape genes, and between grape and Arabidopsis genes were carried out using MCScanX, and the map was constructed using the Circos program (http://circos.ca/) (Krzywinski et al., 2009).

Exon-intron structure and motif analysis

To determine the gene structures of VvPALs, we downloaded the gene sequences from the EnsemblPlants database and identified the exon—intron structure using the GDS version 2.0 tool (http://gds.cbi.pku.edu.cn/) (Hu et al., 2015). Grape PAL protein motifs were constructed with the MEME
version 5.1.0 online program (http://meme-suite.org/tools/meme) (Bailey et al., 2009).

**Phylogenetic analysis of PALs**

MEGA X 10 software was used to align sequences and construct phylogenetic trees (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). All 15 grape PAL protein sequences were aligned along 32 PAL from Arabidopsis (Arabidopsis thaliana), tobacco (Nicotiana tabacum), poplar (Populus trichocarpa), rice (Oryza sativa), and maize (Zea mays) using ClustalW software. The rootless phylogenetic trees of PAL sequences were constructed with the maximum-likelihood method and 1000 bootstrap value.

**Promoter cis-acting elements analysis**

To identify the cis-acting regulatory elements of promoter regions, we analysed the 1500 bp upstream sequences of grape PAL genes using the Plantcare online program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002).

**Grape transcriptomic analysis**

Published transcriptome data (GSE36128, GSE37743, and GSE76256) were retrieved from the GEO databases (https://www.ncbi.nlm.nih.gov/gds/). Among these, the GSE36128 dataset contained 54 tissues and organs of V. vinifera Corvina that can survey tissue-specific expression. In GSE37743, expression profile VvPALS under different biotic or abiotic stresses were surveyed, namely, wound, UV-C, and downy. To survey the expression of VvPALS in different varieties during berry development, V. vinifera cv. Cabernet Sauvignon and V. quinquangu laris Danfeng-2 during four growth periods (E–L 33, E–L 34, E–L 35, E–L 38) were analysed in GSE76256. Raw datasets were standardised by using base 2 for the logarithm, and the heatmap was constructed with TBtools software (Chen, Xia, Chen, & He, 2018).

**qRT-PCR and statistical analysis**

All samples were quick-frozen with liquid nitrogen and stored at –80°C. For each developmental-stage sample, total RNA was isolated from grape skin by a modified SDS/phenol method as in a previous study (Reid, Olsson, Schlosser, Peng, & Lund, 2006). The concentration and quality of total RNA were determined with a NanoDrop 2000 UV spectrophotometer (Thermo SCIENTIFIC, USA). For the synthesis of the first-strand cDNA, PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology, Dalian, China) was used according to the kit manual. The primers of VvPALS were designed and are listed in Supplemental S1. The qRT-PCR reaction system (25 μL) was constructed with SYBR Green RT-PCR Master Mix (Takara Biotechnology, Dalian, China), and triplicate quantitative analysis was performed with a IQ5 RT-PCR system (Bio-Rad, Hercules, CA, USA) according to the kit manual. The VvGAPDH gene (GenBank, accession number EF192466) was chosen for internal reference to analyse relative VvPAL expression. Statistical analyses of all quantitative data were performed using SPSS 20.0 (IBM, NY, USA), and plots were generated with SigmaPlot 12.5 (Ashburn, VA, USA).

**Results**

**Identification and chromosomal localisation of VvPALS**

The genomewide identification of PAL family members in grape was carried out with the HMMER search and BLASTP software, and a total of 15 PAL protein sequences were retrieved after comparing the conserved domains of candidate grape genes. Details of those genes are presented in Table 2, including gene name, 12Xv1 genome accessions, 12X genome annotation, ORF length, deduced polypeptide, sublocation, and duplication type. For VvPAL nomenclature, we followed the order of the grape PAL sequences on the chromosome. These 15 VvPALS were mapped on 5 out of 19 grape chromosomes except VvPAL15. VvPAL1 was present on chromosome 6, VvPAL2 on chromosome 1, VvPAL3 and VvPAL4 on chromosome 11, VvPAL5 on chromosome 13, VvPAL6/7/8/9/10/11/12/13/14 on chromosome 16, and VvPAL5 on ChrUn, namely, mapping on unassembled sequences (Figure 1). The full-length coding sequences of VvPALS ranged from 1473 bp (VvPAL15) to 2688 bp (VvPAL3) with proteins of 469 aa to 895 aa, respectively. The isoelectric point (PI) of PALS ranged from 5.93 (VvPAL3) to 6.74 (VvPAL15).

We further investigated the duplication types of PAL genes on the basis of MSCscanX software analysis. Four duplication types appeared in this gene family, and tandem was the most prominent duplication type. The main tandem genes were VvPAL6/7/8/9 (blue) and VvPAL11/12/13 (orange) (Figure 1), and VvPAL1, VvPAL2, and VvPAL5 evolved from segment duplication.

**Phylogenetic analysis, gene structure, and motif analysis**

An unrooted phylogenetic tree of 15 PAL amino acid sequences was obtained with the maximum-likelihood method (Figure 2a). The intron/exon structures of VvPAL genes were predicted by an alignment of cDNA to genomic sequences (Figure 2b). This sequence analysis revealed that all coding sequences of the VvPAL genes except VvPAL3 and VvPAL13 have 1 intron and 2 exons. According to the result of protein-motif analysis (Figure 2c), most VvPALS were conserved in sequences and contained all 20 motifs except for VvPAL1/3/4/5/15.
Table 2. Compendium of annotations for the grape PAL gene family.

<table>
<thead>
<tr>
<th>Name</th>
<th>12Xv1 genome accessions</th>
<th>Gene 12Xv1 genome accessions</th>
<th>12X genome annotation</th>
<th>Location (bp)</th>
<th>ORF length&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Deduced polypeptide&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Duplication type</th>
</tr>
</thead>
<tbody>
<tr>
<td>VvPAL1</td>
<td>VIT_06s0004g02620 JGVv4.543</td>
<td>GSVVT01025214001</td>
<td>6: 3,233,462–3,236,945</td>
<td>2421</td>
<td>717</td>
<td>77.97</td>
<td>6.12</td>
</tr>
<tr>
<td>VvPAL2</td>
<td>VIT_08s0004g01710 JGVv4.543</td>
<td>GSVVT01025703001</td>
<td>8: 12,827,707–12,830,497</td>
<td>2347</td>
<td>710</td>
<td>77.48</td>
<td>6.03</td>
</tr>
<tr>
<td>VvPAL3</td>
<td>VIT_11s0016g01520 JGVv16.129</td>
<td>GSVVT01015124001</td>
<td>11: 1,210,039–1,213,113</td>
<td>2688</td>
<td>895</td>
<td>77.97</td>
<td>5.93</td>
</tr>
<tr>
<td>VvPAL4</td>
<td>VIT_11s0016g01640 JGVv16.136</td>
<td>GSVVT01015138001</td>
<td>11: 1,318,994–1,321,292</td>
<td>1998</td>
<td>665</td>
<td>73.18</td>
<td>6.21</td>
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<tr>
<td>VvPAL5</td>
<td>VIT_13s0019g04460 JGVv19.80</td>
<td>GSVVT01016257001</td>
<td>13: 5,881,334–5,885,161</td>
<td>2467</td>
<td>723</td>
<td>78.83</td>
<td>6.27</td>
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<td>VvPAL6</td>
<td>VIT_16s0039g01100 JGVv39.63</td>
<td>GSVVT01024292001</td>
<td>16: 598,409–601,085</td>
<td>2246</td>
<td>710</td>
<td>77.08</td>
<td>6.59</td>
</tr>
<tr>
<td>VvPAL7</td>
<td>VIT_16s0039g01110 JGVv39.64</td>
<td>GSVVT01024293001</td>
<td>16: 603,763–610,090</td>
<td>2649</td>
<td>710</td>
<td>77.05</td>
<td>6.47</td>
</tr>
<tr>
<td>VvPAL8</td>
<td>VIT_16s0039g01120 JGVv39.65</td>
<td>GSVVT01024294001</td>
<td>16: 613,506–616,051</td>
<td>2219</td>
<td>710</td>
<td>77.11</td>
<td>6.7</td>
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<tr>
<td>VvPAL9</td>
<td>VIT_16s0039g01130 JGVv39.66</td>
<td>GSVVT01024295001</td>
<td>16: 619,822–622,337</td>
<td>2142</td>
<td>710</td>
<td>77.13</td>
<td>6.51</td>
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<tr>
<td>VvPAL10</td>
<td>VIT_16s0039g01170 JGVv39.68</td>
<td>GSVVT01024299001</td>
<td>16: 649,907–652,554</td>
<td>2318</td>
<td>710</td>
<td>77.07</td>
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<td>VvPAL11</td>
<td>VIT_16s0039g01240 JGVv39.72</td>
<td>GSVVT01024303001</td>
<td>16: 688,542–691,191</td>
<td>2308</td>
<td>710</td>
<td>77.15</td>
<td>6.43</td>
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<tr>
<td>VvPAL12</td>
<td>VIT_16s0039g01280 JGVv39.73</td>
<td>GSVVT01024305001</td>
<td>16: 700,063–702,582</td>
<td>2178</td>
<td>710</td>
<td>77.08</td>
<td>6.51</td>
</tr>
<tr>
<td>VvPAL13</td>
<td>VIT_16s0039g01300 JGVv39.74</td>
<td>GSVVT01024306001</td>
<td>16: 706,775–712,536</td>
<td>2286</td>
<td>734</td>
<td>80.14</td>
<td>6.5</td>
</tr>
<tr>
<td>VvPAL14</td>
<td>VIT_16s0039g01360 JGVv39.77</td>
<td>GSVVT01024315001</td>
<td>16: 777,110–779,568</td>
<td>2187</td>
<td>710</td>
<td>77.15</td>
<td>6.62</td>
</tr>
<tr>
<td>VvPAL15</td>
<td>VIT_06s2508g00010 JGVv2508.1</td>
<td>GSVVT01006148001</td>
<td>Uni: 42,205,073–42,207,438</td>
<td>1473</td>
<td>469</td>
<td>50.72</td>
<td>6.74</td>
</tr>
</tbody>
</table>

<sup>a</sup>Systematic designation given to grape PALs in this work.

<sup>b</sup>Length of open reading frame in base pairs.

<sup>c</sup>The number of amino acids, molecular weight (kilo Daltons), and isoelectric point (PI) of the deduced polypeptides.
VvPAL1 and VvPAL5 had 19 motifs without motif 17 and VvPAL3 contained 17 motifs, and 2 of them (motif 4 and motif 9) repeated at the C terminal. VvPAL4 had 15 motifs. VvPAL15 only had 13 motifs, and sequence analysis showed that VvPAL15 had a C-terminal deletion.

To further investigate the evolutionary relationship of PAL members, we constructed the phylogenetic relationship using protein PAL sequences from grape, Arabidopsis, tobacco, poplar, rice, and maize (Figure 3, Supplemental S2). All of these 47 PAL proteins were divided into 2 clades, dicot and monocot. Dicot PAL proteins categorised into 2 subclades, and the 2 grape PALs (VvPAL3 and VvPAL4) clustered into a subclade.

Synteny analysis of grape and Arabidopsis PAL genes

Comparative genome analysis is an effective way to obtain the associated information between a model plant and other plants (Guo et al., 2014). To determine whether grape PAL genes arise from duplication events, synteny analysis was conducted in the present
study (Figure 4). Results suggested that a significant difference existed in the genomic structures of grape and Arabidopsis. The only synteny event of AtPAL4 and VvPAL5 between species indicated that these 2 PAL genes may be the ancestor of other PAL genes. Furthermore, combining the phylogenetic result, the evolution direction of grape PAL genes could be speculated; VvPAL1 and VvPAL2 evolved from VvPAL5, and the cluster genes on chromosome 16 derived from gene-duplication events of VvPAL2.

Detection of cis-acting elements in PAL gene promoter regions

Multiple cis-acting elements in VvPAL promoters were found, as detailed in Figure 5 and Supplemental S3. We mainly investigated 5 types of cis-acting elements, namely, development-related, light responses, phytohormone responses, stress responses, and site-binding related elements. The elements of light-related response were abundant (Figure 5); each VvPAL promoter had 2–10 light responsiveness elements, and VvPAL3 and VvPAL4 contained fewer light-responsiveness elements, which suggested that they differentially responded under light compared with the other VvPALs. Ten types of phytohormone response elements were found that are involved in the response of abscisic acid (ABA), methyl jasmonate (MeJA), auxin, gibberellin (GA), and salicylic acid (SA) (Supplemental S3). Most VvPALs contained ABRE, a cis-acting element involved in abscisic acid (ABA) responsiveness. The TATC-box and TGA-box only existed in VvPAL13 and VvPAL2, respectively, which are involved in gibberellin (GA) and auxin. In addition, VvPAL15 did not contain a phytohormone response element. Moreover, 7 development-related elements, 5 stress-response elements, and 3 site-binding-related elements were detected. CAT-box was present in 8 VvPALs and is related to meristem expression. Stress-response elements were important cis-acting elements of grape PAL promoters. ARE was ubiquitous in most VvPAL gene promoters except VvPAL3/4 and responded to anaerobic induction. VvPAL2/5/10/11/14 contained the LTR element, which was able to respond to low temperature. The MYB binding site (MBS element) related to drought response was present in VvPAL6 and VvPAL8. The stress- and defence-responsiveness (TC-rich repeats) and wound-response (WUN-motif) elements were detected in VvPAL3 and VvPAL2, respectively. The remaining type of cis-acting elements was site-binding-related elements, including AT-rich sequence, CCAAT-box, and MBSI.

Expression profiles of grape VvPAL genes revealed by transcriptome analysis

To investigate the expression patterns of VvPALs to different stresses and developmental stages, an online
A gene-expression dataset was retrieved, and the detailed expression of these VvPALs is provided in Supplemental 4. To analyse the expression profiles of VvPALs in different tissue types, a total of 54 grape samples were collected from GSE36128 (Figure 6). VvPAL1 and VvPAL2 were expressed at a relatively higher level in

Figure 4. Synteny analysis of grape and Arabidopsis PAL genes. The chromosomes of grape and Arabidopsis are drawn as a circle. The distribution of AtPAL genes and VvPAL genes are marked beside the circle. Coloured curves indicate the details of syntenic regions between grape and Arabidopsis PAL genes.

Figure 5. Cis-acting elements of grape PAL gene in promoter regions. The black blocks at the top show five different types of cis-acting elements, and the horizontal axis represent the different cis-acting elements. Details of cis-acting elements are listed in Supplemental S3.
most grape tissue types, including stamen, berry pericarp, buds, inflorescence, flowers, leaves, seeds, and berry skin. VvPAL3 and VvPAL4 were expressed at a lower level in all tissue types. The expression of other VvPALS was similar in different tissue types.

The expression levels of GSE37743 data were analysed to reveal the response of VvPALS to different abiotic or biotic stresses (Figure 7a). Three VvPALS (VvPAL1/2/5) represented upregulated expression under wound treatments compared with the control. Except for VvPAL1/3/4, showing downregulation expression, the expression patterns of VvPALS under UV-C treatments were significantly upregulated expression. VvPAL1/2/5 exhibited a high expression level under downy treatment, whereas the difference in the expression of the other VvPALS was similar to those in the control.

We analysed the expression levels in V. vinifera Cabernet Sauvignon and V. quinquangularis Danfeng-2 during berry development to determine PAL expression in different grape genotypes and development stages (GSE76256, Figure 7b). Most VvPALS in Cabernet Sauvignon exhibited high expression levels at the veraison and ripening stages (E–L 35 and E–L 38). By contrast, PALs in Danfeng-2 showed no significant expression level during berry development.

**Expression patterns of PALs from four grapes during different developmental stages**

For further understanding the change of VvPAL genes in berry skin during grape-berry development, the expression patterns of 4 PALs (PAL1/2/3/5) in 2 wild Chinese V. quinquangularis (Shannan-24 and Danfeng-2) and 2 V. vinifera (Cabernet Sauvignon and Chardonnay) were analysed by qRT-PCR (Figure 8). Because sequences of VvPAL2/6/7/8/9/10/11/12/13/14/15 are highly conserved, the expression patterns of those genes were detected by 1 primer. In the study, a total of 4 pairs of

![Figure 6. Expression patterns of grape PAL genes in 54 tissues based on the GSE36128. The log2 (RNA-normalised value+1) is used to show the gene expression levels. The red and blue represent the high and low expression levels, respectively. The vertical axis shows the different tissues, and the details of tissue abbreviation name are listed in Supplemental 4.](image-url)
specific primers were designed. Expressions of grape PAL genes were detected while they exhibited dynamic expression patterns. PAL1 showed peak expression in E-L stage 31 in all 4 grape varieties, and regained another peak expression in E-L 36 of the black grape varieties, but did not appear in the white grape varieties. PAL2 showed peak expression in E-L 36 in all 4 grape varieties. The expression pattern of PAL3 was puzzling because of its large difference between the 2 white and 2 black grape varieties. In the white grapes, peak expression was presented in E-L 36. However, it presented in E-L 34 in the black grapes. PAL5 expressed at a lower level in the white grapes, and it still had peak expression in E-L 31 and 36. We observed that the mRNA accumulation of the PAL gene was related to grape berry skin colour. Grapes with the same berry skin colour showed similar expression profiles of all PAL genes.

Discussion

PAL is the key enzyme for secondary metabolites, and PAL activity is correlated with flavonoid and stilbene accumulation (Sparvoli, Martin, Scienza, Gavazzi, & Tonelli, 1994). In the study, 15 grape PAL genes were identified that had high similarity with well-characterised PALs from Arabidopsis (Raes et al., 2003). Chromosomal localisation of grape PAL genes indicated that most of the genes (VvPAL6-14) were located on chromosome 16. Interestingly, Most of the chalcone synthase (8 in 14) and stilbene synthase (42 in 48) gene families, which are well-known as downstream genes of PAL in the phenylalanine metabolism pathway, are also located on chromosome 16 (Parage et al., 2012). The high copy numbers of the PAL, CHS, and STS gene families suggested that a gene-duplication event may occur on chromosome 16, or one related to the hexaploid ancestral genomes of grape (Jaillon et al., 2007; Velasco et al., 2007); the expression relevance between these genes needs further study. The duplication types of PAL genes were analysed, and VvPAL6-9 and VvPAL11-13 were found to be tandem repeat genes; these grape PALs may have a redundant function in the biosynthesis of secondary metabolites. The PAL expanded in grape, but not in tobacco, maize, and poplar, which each has 4 PAL genes. This indicated that the increase in grape PAL genes may be conducive to improving adaptation to the external environment. Phylogenetic analysis of VvPALs, OsPALs, AtPALs, NtPALs, PtPALs, and ZmPALs showed that all PALs were divided into monocots and dicots. Cluster results indicated that PALs and the phenylpropanoid biosynthesis pathway could exist before monocots and dicots differentiation. In addition, grape PALs fell into the same subclades except VvPAL3 and VvPAL4, which were independent of monocots and dicots, and possibly originated from older ancestors.

To investigate the biochemical processes involved in VvPALs, cis-acting elements of promoter regions were analysed. Five types of cis-acting elements,
namely, development-related, light responsiveness, phytohormone responses, stress responses, and site-binding-related elements, were abundant in VvPALs. These elements lead PAL genes to participate in reactions associated with cis-acting elements contained therein. Many studies suggested that PALs are involved in many environmental stimuli, including stress conditions such as UV irradiation, droughts, pathogens, wounds, and extreme temperatures (Huang et al., 2010; Rohde et al., 2004). In Arabidopsis, AtPAL1/2/4 mainly participate in lignin synthesis, and AtPAL1/2 are related to flavonoid synthesis. VvPAL promoters contained 17 types of light-responsiveness elements, which was consistent with their significant difference in expression under UV-C treatments (Figure 7a). The research of Sun et al. (2017) revealed that the expression of four members of PAL (VvPAL6, VvPAL7, VvPAL8, and VvPAL9) was highly significantly correlated with the accumulation of flavonoids in berries from different light treated groups. A similar result was also found in other research; in Arabidopsis, the mutant of PAL1/2 can enhance sensitivity to UV-B and decrease the synthesis of flavonoid compounds (Huang et al., 2010). An ABA response element (ABRE) was also found in most VvPAL genes that had relatively lower expression in the ripe berry flesh, leaves, rachis, and tendrils (Figure 6), which indicated that VvPALs also play an important role during tissue development and maturation. Previous reports showed that increased leaf ABA can partially inhibit PAL activity under biotic stress (Audenaert, De Meyer, & Höfte, 2002). In addition, VvPAL2/5/10/11/14 contained the LTR element, which was able to respond to low temperature. A study showed that low temperature induced the expression of VvPAL11 (Zhang, Jia, et al., 2015).

According to the results of transcriptomic analyses, the expression patterns of VvPAL genes were different in different tissue types, stages, and stresses. Huang et al. (2010) reported that the PAL gene is essential in regulating plant growth, development, and stress response in Arabidopsis. Changes in some VvPAL genes (VvPAL2, VvPAL9, VvPAL10, and VvPAL15) expression have been observed during pathogens infection (Ahn, Kim, Cho, & Yun, 2014; Boubakri et al., 2013; Patricia et al., 2015). Moreover, when stimulated by the change of growth conditions, the

Figure 8. Expression patterns of grape PAL genes in berry skins during different developmental stages. The name of corresponding genes was indicated upper left of each histogram. Different grape varieties are shown in colour as the graph legends. E-L 31, E-L 33, E-L 34, E-L 35, E-L 36, and E-L 38 represent different developmental stages of grape.
expression of PAL genes also changed accordingly. Recent studies showed that root restriction resulted in the downregulation of VvPAL1, VvPAL5, VvPAL6, VvPAL7, and VvPAL8 (Leng et al., 2020). Increasing evidence shows that the PAL activity of grape has a significant effect on downstream secondary metabolites, such as flavonoid and resveratrol, which are involved in defence mechanisms. After 15 hours of exposure to UV light, VvPAL activity was found to coordinate induction with stilbene synthase (STS) and cinnamate 4-hydroxylase (C4H), and reached a maximum (Fritzeimeier & Kindl, 1981). Moreover, the mRNAs of PAL and STS1 reached their maximal increment after 24 hours by various levels of light irradiation in Campbell (Ahn et al., 2015). In terms of qRT-PCR analysis, we studied the expression profiles of grape PAL1/2/3/5 during grapefruit development and ripening, and results showed that grape PALs were dynamically regulated in grape berry skin and their change folds associated with grape skin colour. The difference in the expression profiles in red and white grapes suggested that grape PAL genes, especially VvPAL1 and VvPAL5, are involved in anthocyanin biosynthesis.

However, by considering the function difference in PtPAL gene family in poplar (Shi et al., 2013), PtPALS in subgroup A, namely, PtPAL2, PtPAL4, and PtPAL5, clustered with VvPAL1 and VvPAL5 (Figure 3), play an important role in manipulating PAL activity for lignin synthesis. Further extended study into the function of members of the grape PAL family is needed. For other PAL genes, a potential function exists in responding to various biotic or abiotic stresses. Several studies also showed that PALs are regulated by post-translational modifications such as ubiquitination (Yu, Kim, Yun, Suh, & Lee, 2019; Zhang, Gou, & Liu, 2013). Kelch repeat F-box (KFB) proteins (KFB01, KFB20, KFB39, KFB50, and SAGL1) mediated the degradation of PALs by physical interaction. In the future, we will further screen ubiquitin involved in phenylalanine metabolic pathways to precisely regulate the biosynthesis of grape anthocyanins, flavanols, flavanols, and resveratrol.

Conclusions

A total of 15 PAL genes were identified in grape via genome screening, and 9 VvPAL genes were found to be located on chromosome 16. Comprehensive analysis of gene duplication, phylogenetic relationship, gene structure, conserved motifs, synteny, promoter cis-acting elements, and expression profiles was also performed. Gene-duplication analysis indicated that the tandem duplication triggered the expansion of the PAL gene family in grape compared to Arabidopsis. Cis-acting elements and transcriptome data provided evidence for the roles of the PAL gene family in responding to various growth and development processes and stresses. The potential function of VvPAL3 and VvPA4 needs to be considered on the basis of the difference in expression patterns of VvPALS. Further, dynamic expression analysis of PAL1/2/3/5 in different grape varieties revealed that VvPAL1 and VvPAL5 may be involved in the modulation mechanisms of anthocyanin biosynthesis due to their differential expression in white and red grapes. In conclusion, the identification and characterisation of PAL family members in grapes provide a basis for further understanding functional characterisation to respond to various biotic and abiotic stresses.

Disclosure statement

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