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# *VvPL11* Is a Key Member of the Pectin Lyase Gene Family Involved in Grape Softening

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**Abstract:** Fruit ripening includes several metabolic changes that lead to sweeter and softer fruit. Pectin depolymerization is one of major factors that softens developing grape berries. Pectin lyases (PLs) play important roles in pectin degradation in the grape berry. However, little is known about the temporal and spatial expression of grapevine (*Vitis* spp.) pectin lyase genes (*VvPLs*) or their function during fruit ripening and softening. In this study, 18 individual *VvPL* genes were identified in the grape genome. All *VvPL* genes were sorted into group I and group II, except *VvPL12* which demonstrated higher and similar expression trends in different tissues and organs. In grape berry, *VvPL1*, *5*, *7*, *11* and *16* were highly expressed, whereas *VvPL18*, *15*, *2*, *13*, *10*, *14*, *17*, *6* and *8* showed lower expression levels at different berry developmental stages. Expression of *VvPL11* firstly increased and then decreased, and the highest expression was shown at 6 weeks after full bloom (WAFB) during berry development. Over-expression of the *VvPL11* gene in tomato caused higher ethylene production and lower firmness compared to wild-type fruit. Moreover, decreased propectin and increased water-soluble pectin (WSP) levels were observed in *VvPL11* transgenic tomato fruit. Consistent with this result, the expression levels of *SlPG2*, *SlEXP*, and *SlPME1*, all of which are genes involved in fruit softening, were up-regulated in *VvPL11*-OE tomato fruit, which supported the idea that *VvPL11* plays an important role in fruit ripening and softening. This study provided a comprehensive analysis of the grapevine PL family and advanced our knowledge of the functions of *VvPLs* during fruit softening.

**Keywords:** pectin lyase; *Vitis labrusca* × *Vitis vinifera*; propectin; ripening; grape berry

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## 1. Introduction

Grapevine is one of the most efficient and economical horticultural crop plants around the world. Recently, grape has become very popular among consumers because of its beautiful color, distinct flavors, and rich nutrients. It is widely cultivated in the Henan Province in China which significantly contributes to local agricultural industry development [1]. However, grapevines are at risk of losing soft berries through fruit drop and decay. Grape softening is an important process before ripening, and the berry softening rate directly affects grape berry firmness [2]. Rapid softening at veraison results in extremely short storage and shelf life after harvest, which causes great losses to the grape industry [3–5]. Previous studies indicate that grape berry softening is a complex process [6] that is associated with changes in the fruit cell wall structure [7,8], cell morphology [9–11], cell spatial arrangement, and cell integrity during berry development [11,12].

The fruit cell wall consists mainly of polysaccharides, which are classified as pectin, hemicellulose, and cellulose, and proteins. Pectin is one of the most complex polysaccharides and is the main component of the primary cell wall and the intermediate layer between the cell wall and the cell membrane. Pectin consists of homogalacturonic acid (HG), rhamnogalacturonic acid (RG), and xylogalacturonic acid [13]. Previous studies suggest

that altering the structure of pectin through depolymerization or breaking of side chains is the major factor determining fruit texture during softening [14]. Pectin degradation is synergistically carried out by various enzymes, including pectin methylesterases (PMEs), polygalacturonases (PGs), galactosidases, and pectin lyases (PLs) [15–17].

PLs are the only known pectin-degrading enzymes and are capable of cleaving glycosidic bonds [18]. PLs belong to a family of polysaccharide lyase enzymes, whose members have been identified in tomato [8], strawberry [19], and peach [20]. Many studies have shown that PL plays a role in a number of physiological and biochemical pathways, including stomatal opening and closing [21], petal abscission [22], pollen development [23], fruit softening [7], leaf senescence [24], and disease resistance [25–27]. The role of PL in regulating fruit softening has been shown in both climacteric and nonclimacteric fruit [28]. During ripening of strawberry and tomato, fruit softening is accompanied by an increase in PL activity [29,30]. Silencing or knocking out *PL* genes leads to a slow rate of fruit softening and increasing fruit firmness, and *SlPL* was verified to be an excellent candidate for improving tomato firmness [13,29,30]. These results suggest that there is an inextricable link between fruit ripening and softening and the *PL* genes.

During grape berry softening, the cell-wall components are important factors, and degradation of pectin polysaccharides has been commonly associated with fruit softening [3,31]. Grapes with higher firmness levels have a lower rate of cell-wall disassembly, and VvPG and VvPL are involved in the difference in firmness between hard and soft berries [31,32]. There are relatively few studies on pectin lyases in grape, and the expression patterns and functions of the VvPLs in grape during fruit softening are not known. Based on these shortcomings in our knowledge, this report first identifies and analyzes the pectin lyase family in the entire grape genome and analyzes the conserved motifs and the evolutionary relationships of the grape pectin lyase proteins. *VvPL11* showed a higher expression in mature berry, and it was over-expressed in tomato. The results of this study provide a preliminary theoretical basis for understanding the function of *VvPL11* in berry softening.

## 2. Material and Methods

### 2.1. Plant Materials

Grape (*Vitis labrusca* × *Vitis vinifera* 'Hanxiangmi') was planted in a commercial vineyard in Zhengzhou of Henan province. 'Hanxiangmi' produces an extremely soft berry. The samples of grape berry clusters were collected at different weeks after full bloom (WAFB), namely 2 W, 4 W, 6 W, 8 W (veraison when almost 50% berries per cluster were colored), and 10 W (ripening). At every sampling time, nine grape clusters were harvested randomly from different vines of the cultivar 'Hanxiangmi' grown in the Yellow River Plain with an average temperature of 14.62 °C and an average precipitation of 744.14 mm [33]. After being subjected to instrumental analyses, the berries from grape clusters were stored at −80 °C for RNA extraction.

### 2.2. Identification and Analysis of VvPL Family Members

To identify grape pectin lyase genes, 26 *Arabidopsis* (AtPL) proteins were downloaded from a search of the TAIR10 database (https://www.arabidopsis.org/index.jsp (accessed on 16 July 2022)) [34], and then AtPL proteins were used as queries to search the JGI database (https://phytozome-next.jgi.doe.gov/ (accessed on 16 July 2022)). Eighteen VvPL proteins were obtained using BLASTP in the JGI database, and each *VvPL* gene was manually checked to confirm whether a conserved PL domain existed. In addition, gene sequences, mRNA, CDS, amino acid sequences and annotation information of the grape PL members were obtained from the JGI database. These data were used in the relevant analyses that follow. Twenty tomato PL genes (*SlPLs*) were also downloaded from the JGI database using the same method according to protein accession numbers [30].

### 2.3. Phylogenetic Analysis and Gene Structure of VvPLs

To investigate the evolutionary relationships between VvPLs and other PLs, the protein sequences of the 18 VvPLs, 26 AtPLs, 22 SlPLs, FaPLc, and FvPLA were aligned to construct a phylogenetic tree by MEGA (7.0) software. Based on the protein sequence alignment results, phylogenetic analysis was performed by using the neighbor-joining (NJ), 1000 bootstrap method. The evolutionary tree file was initially obtained and then plotted using the online website ITOL (https://itol.embl.de/ (accessed on 25 July 2022)). The amino acid sequences of all proteins in the phylogenetic tree are listed in Supplementary Table S1.

The amino acid sequences of the VvPLs were uploaded to SMART (http://smart.embl-heidelberg.de/ (accessed on 11 August 2022)), and information on length of amino acids, relative molecular weight, and theoretical isoelectric point was obtained. These sequences were then uploaded to Cell-Ploc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/ (accessed on 11 August 2022)) for prediction of protein subcellular localizations. The motif analysis was performed using MEME (https://meme-suite.org/meme/tools/meme (accessed on 12 August 2022)). All of these analyses were performed using default parameters. Gene structure and chromosome location of the *VvPLs* were analyzed using Tbtools methods (www.tbtools.com/ (accessed on 12 August 2022)).

### 2.4. Tissue-Specific Expression Analysis of the VvPL Genes

The expression levels of the *VvPLs* were analyzed in 54 different tissues/organs at different developmental stages of grapes from transcriptome data in the NCBI database (GSE36128) [35]. The identified *VvPLs* were searched in the GEO database (GSE36128), and the expression profile data of these genes were obtained. Heatmaps of the expression levels were created using the $log_2$-RPKM normalized values +1 using OmicStudio (https://www.omicstudio.cn/tool/4/ (accessed on 11 August 2022)).

### 2.5. Expression Analysis of VvPL Genes during Berry Development

The expression levels of 14 *VvPL* genes were obtained by analyzing our previous transcriptome data of the 'Hanxiangmi' berry at four developmental stages (non-published data). The expression of the *VvPLs* was analyzed by clustering, and the heat map was constructed using OmicStudio.

To verify the reliability of the data, reverse transcriptase-quantitative PCR (RT-qPCR) of the *VvPL* genes was performed at different developmental stages of the 'Hanxiangmi' berries. Total RNA was extracted using a plant RNA extraction kit (Huayueyang Co. Ltd., Beijing, China). RT-qPCR was performed as previously described [36]. All PCR primers are listed in Table S2. For each sample, quantifications were made in triplicate. Three biological replicates and three technical replicates were conducted to verify the accuracy of the expression data.

### 2.6. Overexpression of VvPL11 Gene in Tomato

The CDS sequence of *VvPL11* without its stop codon was cloned into pSAK277, and the pSAK277 plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101. Transgenic tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were obtained according to the reference [37]. Transgenic fruits from the T1 generation were collected at breaker, breaker + 3d, breaker + 5d, breaker + 7d, and breaker + 15d, respectively. Fruit firmness was measured using a TA.XT Plus Texture Analyzer (Stable Micro Systems Ltd., Godalming, U.K.) and slightly modified as described by Tan to analyze the ethylene production of fruits [38]. Expression of *VvPL11* and pectin-degradation genes in transgenic tomato fruit were evaluated by the RT-qPCR method.

### 2.7. Analysis of Propectin and WSP Contents

The propectin and water-soluble pectin (WSP) contents of transgenic tomato fruit were determined using the carbazole colorimetric method according to Guan et. al. [39]. The

enzymatic activity of pectin lyase was analyzed using a colorimetric enzyme activity kit (Kemin Co., Ltd., Suzhou, China).

## 3. Results

### 3.1. Identification of Grapevine PL Family Members

A total of 18 genes were identified as candidate *PL* family members in the grapevine genome (Table 1). These 18 *PL* family members were named *VvPL1* to *VvPL18* according to their chromosomal position (from top to bottom). *VvPLs* are unevenly distributed on 12 of the chromosomes of the grapevine genome (Figure 1). Chromosome 1 contains four *VvPL* members, whereas chromosomes 5, 7, 10, 16, and 19 have one member each. Chromosomes 8, 13, 14, and 17 have two *VvPL* members each. The inferred peptide sequence lengths of the VvPL proteins ranged from 78 to 543 amino acid residues, and the predicted molecular weights ranged from 36,103.92 to 57,104.76 (kDa). The predicted isoelectric points of the VvPL proteins were between 4.92 and 9.44 (Table 1).

**Table 1.** The pectin lyase (VvPL) family in grape.

| Protein Name | Number of Amino Acids | Molecular Weight (Da) | Theoretical pI | Subcellular Location |
|---|---|---|---|---|
| VvPL1 | 444 | 49,249.64 | 6.65 | Cell membrane. Chloroplast. Nucleus. |
| VvPL2 | 443 | 49,927.8 | 9.44 | Cell wall. Chloroplast. |
| VvPL3 | 443 | 49,930.33 | 9.66 | Chloroplast. Mitochondrion. |
| VvPL4 | 140 | 15,562.16 | 9.36 | Chloroplast. |
| VvPL5 | 381 | 42,374.55 | 9.06 | Cell membrane. Cell wall. Chloroplast. |
| VvPL6 | 543 | 57,104.76 | 5.65 | Cell membrane. Cell wall. |
| VvPL7 | 496 | 53,897.76 | 5.75 | Cell membrane. Cell wall. Chloroplast. Nucleus. |
| VvPL8 | 403 | 44,441.01 | 6.88 | Cell membrane. Cell wall. Chloroplast. |
| VvPL9 | 370 | 41,484.45 | 9.08 | Cell membrane. Cell wall. Chloroplast. Cytoplasm. Golgi apparatus. Vacuole. |
| VvPL10 | 320 | 36,103.92 | 9 | Cell wall. |
| VvPL11 | 429 | 47,876.52 | 8.48 | Cell wall. Chloroplast. |
| VvPL12 | 403 | 44,091.47 | 6.48 | Cell membrane. Cell wall. Chloroplast. Nucleus. |
| VvPL13 | 400 | 44,196.72 | 4.92 | Cell wall. |
| VvPL14 | 464 | 50,947.37 | 7.64 | Chloroplast. Nucleus. |
| VvPL15 | 445 | 49,642.07 | 7.99 | Chloroplast. |
| VvPL16 | 373 | 41,455.86 | 8.16 | Cell wall. Chloroplast. Cytoplasm. Golgi apparatus. Mitochondrion. Nucleus. |
| VvPL17 | 489 | 53,838.42 | 6 | Cell membrane. Cell wall. Chloroplast. |
| VvPL18 | 331 | 37,066.86 | 6.01 | Cell wall. |

A phylogenetic tree of the 18 VvPL proteins, 26 AtPL proteins, 22 SlPL proteins, FaPLc, and PvPLA clustered into five groups (Figure 2). Group I contained VvPL5 and VvPL6, which were closely clustered with FvPLA and FaPLc from strawberry, respectively. VvPL1, VvPL16, and VvPL12 proteins clustered in group II together with SlPL, which is a candidate gene for tomato firmness. VvPL17, VvPL8, VvPL10, and VvPL11 proteins were clustered in group III, with VvPL11 showing a closer relationship with AtPLL12, which may suggest that VvPL11 is an orthologous proteinof AtPL12. In groups IV and V, there were four (VvPL15/2/3/13) and five VvPL (4/9/14/7/18) proteins, respectively.

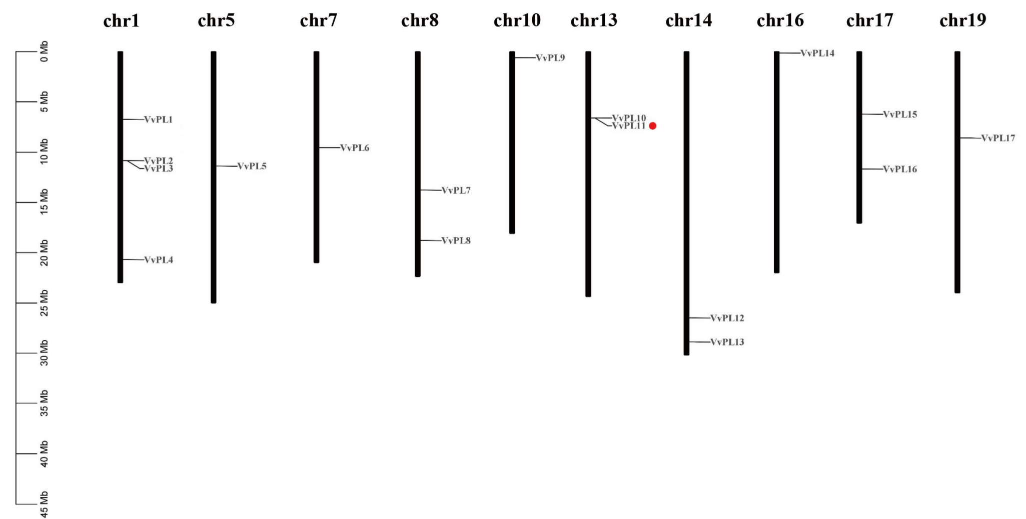


**Figure 1.** Chromosome distribution of pectin lyase (PL) genes in the grapevine genome. Chromosome numbers are shown at the top of each chromosome. Scale is in megabases (Mb).

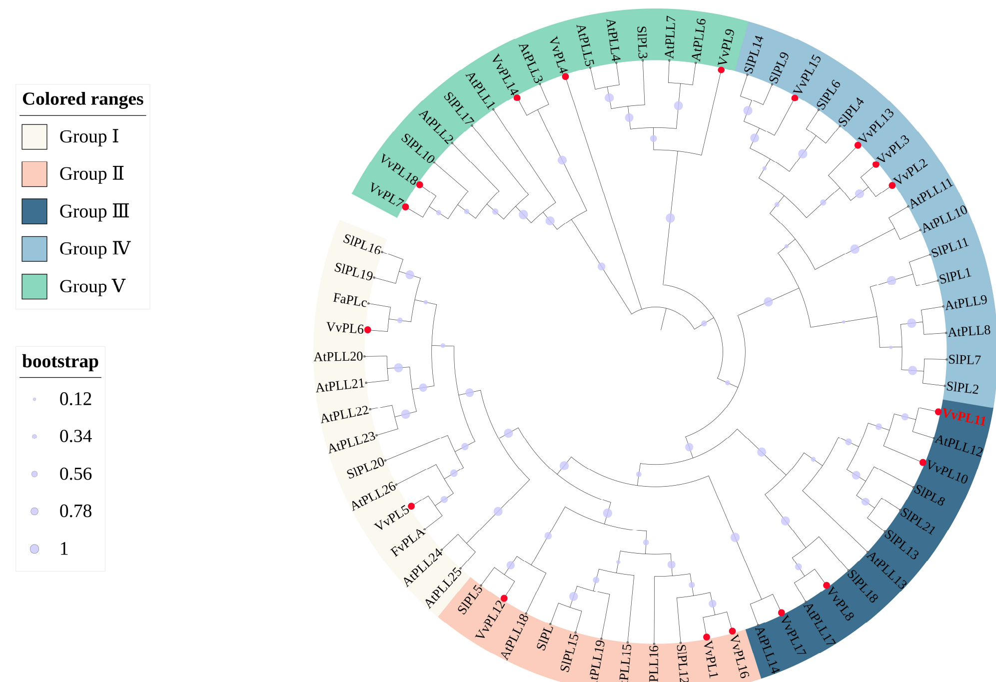


**Figure 2.** Phylogenetic tree of pectin lyase (PL) proteins from grapevine and other species. The proteins clustered into five groups, which were labeled with different colors at the periphery. Grapevine proteins were labeled with red dots. The species shown are *Arabidopsis thaliana* (At), *Vitis vinifera* (Vv), *Solanum lycopersicun* (Sl), *Fragaria ananassa* (Fa), and *Fragaria vesca* (Fa).

The conserved motifs of the VvPL protein family members were identified using Tbtools (Figure 3). Most VvPL members contained 10 motifs, whereas VvPL14 was missing motif 7 and 9, and VvPL4 was missing motifs 1, 2, 4, 5, 7, 8, 9, and 10. Results of intron/exon structural analysis showed that eleven *VvPL* members contained three exons, two *VvPL* members had four exons, two *VvPL* members had two exons, 2 *VvPL* members had six exons, and *VvPL4* contained one exon (Figure 3).

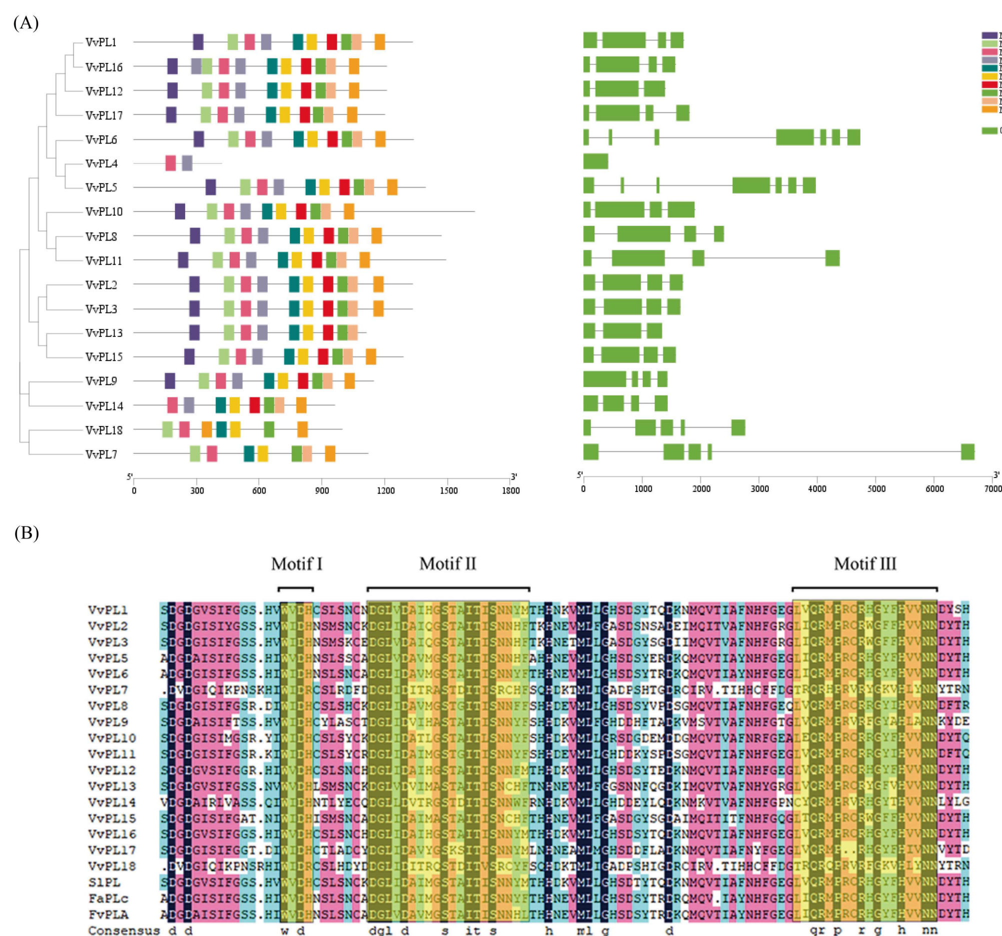


**Figure 3.** Characterization of PL family members in *V. vinifera* and other species. (**A**) Phylogenetic relationships, motifs, and exon/intron structures of *VvPLs*. The left part represents the phylogenetic tree of VvPL proteins. The middle part displays the conserved motifs of VvPLs. Different motifs are represented by blocks of different colors. The right part indicates the exon–intron distribution of *VvPL* genes. (**B**) Amino acid sequence alignment of *V. vinifera* VvPLs with other PLs. Orange shading indicates three typical conserved motifs of PLs, which were referred to as motif I, II and III respectively.

Multiple sequence alignment of the grape, tomato and strawberry pectin lyase genes showed that most of the protein sequences contained conserved motifs I (WIDH), II (DGLIDAIMASTAITISNNYF) and III (LIQRMPRC RHGYFHVVNNDY), with the exception of *VvPL4*, for which the protein sequence lacked three conserved motifs.

### 3.2. Tissue-Specific Expression Analysis of the VvPL Genes

For *V. vinifera*, the available transcriptomic data (GSE36128) were used for the expression profiles of *VvPL* genes. The expression levels of the 18 *VvPLs* in 54 different tissues/organs at different fruit developmental stages indicated that most of the *VvPLs* were expressed in all tissues (Figure 4). Higher expression of *VvPL7* was shown in all tissues and organs, whereas *VvPL5/1/6/11/16*, from different groups in the phylogenetic tree, which are showed similar expression patterns of higher expression levels in inflorescence, flower, and tendril. After berry veraison, expression of *VvPL11* and *VvPL1* were generally down-regulated, which may imply important roles before berry ripening. *VvPL2/3/4/5/8/9/10/12/14/15/17/18* showed scattered expression in different tissues. *VvPL2/3/15/9* showed lower expression in different tissues, except that *VvPL2/3/15* showed higher expression levels in the flowers.

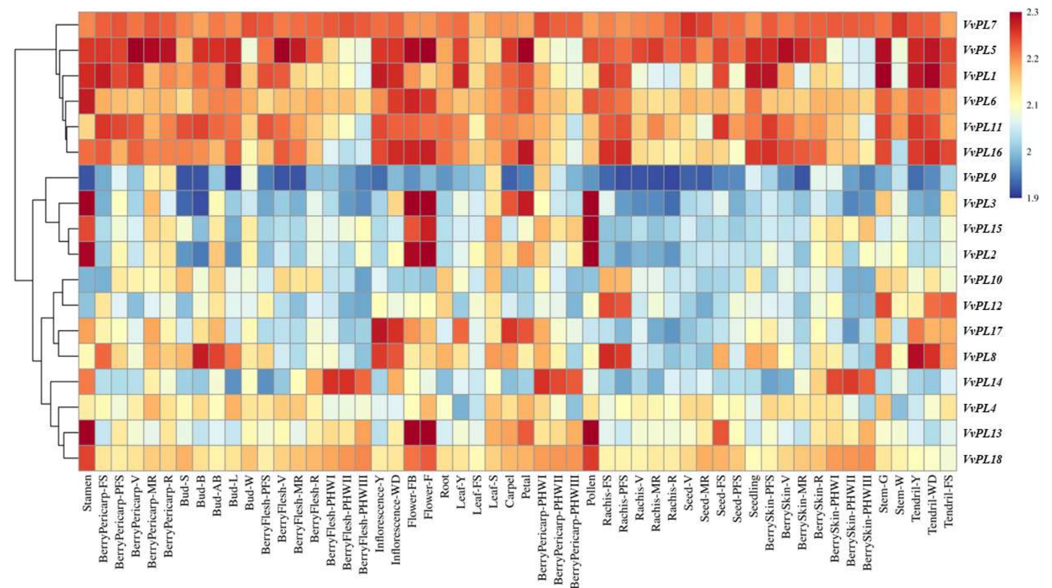


**Figure 4.** Tissue-specific expression patterns of *VvPLs*. The color scale on the right represents the RPKM normalized $\log_2$+1. Blue indicates low expression levels, yellow indicates moderate expression levels, and red indicates high expression levels. Transcriptome data were from the NCBI database (GSE36128) and represent multiple studies. The tissue samples were as follows: Stamen-pool of stamens from 10% and 50% open flowers; BerryPericarp-FS-berry pericarp at fruit set; BerryPericarp-PFS-berry pericarp post-fruit set; BerryPericarp-V-berry pericarp véraison; BerryPericarp-MR-berry pericarp mid-ripening; BerryPericarp-R-berry pericarp ripening; Bud-S-bud swell; Bud-B-bud burst; Bud-AB-bud after-burst; Bud-L-latent bud; Bud-W-winter bud; BerryFlesh-PFS-berry flesh post fruit set; BerryFlesh-V-berry flesh veraison; BerryFlesh-MR, berry flesh mid-ripening; BerryFlesh-R, berry flesh ripening; BerryFlesh-PHWI-berry flesh post-harvest withering I (1st month); BerryFlesh-PHWII-berry flesh post-harvest withering II (2nd month); Berry Flesh PHWIII-berry flesh post-harvest withering III (3rd month); Inflorescence-Y-young inflorescence; Inflorescence-WD-well-developed inflorescence; Flower-FB-flowering begins; Flower-F-flower at flowering; Root-root in vitro cultivation; Leaf-Y-young leaf; Leaf-FS-mature leaf; Leaf-S-senescing leaf; Carpel-pool of carpels from 10% and 50% open flowers; Petal-pool of petals from 10% and 50% open flowers; BerryPericarp-PHWI-berry pericarp post-harvest withering I (1st month); BerryPericarp-PHWII-berry pericarp post-harvest withering II (2nd month); BerryPericarp-PHWIII-berry pericarp post-harvest withering III (3rd month); Pollen-pollen from disclosed flowers at more than 50% open flowers; Rachis-FS-rachis fruit set; Rachis-PFS-rachis post-fruit set; Rachis-V-rachis at véraison; Rachis-MR-rachis mid-ripening; Rachis-R-rachis ripening; Seed-V-seed veraison; Seed-MR-seed mid-ripening; Seed-FS-seed fruit set; Seed-PFS-seed post-fruit set; Seedling-seedling pool of 3 developmental stages; BerrySkin-PFS-berry skin post-fruit set; BerrySkin-V-berry skin at veraison; BerrySkin-MR-berry skin mid-ripening; BerrySkin-R-berry skin ripening; BerrySkin-PHWI-berry skin post-harvest withering I (1st month); BerrySkin-PHWII-berry skin post-harvest withering II (2nd month); BerrySkin-PHWIII-berry skin post-harvest withering III (3rd month); Stem-G-green stem; Stem-W-woody stem; Tendril-Y-young tendril (pool of tendrils from shoot of 7 leaves); Tendril-WD-well-developed tendril (pool of tendrils from shoot of 12 leaves); Tendril-FS-mature tendril (pool of tendrils at fruit set).

### *3.3. Expression Analysis of VvPL Genes during Berry Development*

Our transcriptome data in cultivar 'Hanxiangmi' (Table S3) indicated that the *VvPL* genes in groups I and II were highly expressed during berry growth and development (Figure 5). The expression levels of *VvPL1/5/7/11/16* were higher, and the levels of *VvPL18/15/* 2/13/10/14/17/6/8 were lower across the berry developmental stages (Figure 5A). The highest expression levels for *VvPL5* and *16* were observed at 4 WAFB, whereas *VvPL11* and *VvPL1* were highly expressed at 6 WAFB. RT-qPCR also supported the higher expression levels of *VvPL11* and *1* before berry ripening (Figure 5B). In addition, the expression levels

of *VvPL11* and *1* showed a positive correlation with the content of WSP during berry development (data not shown). Together, these results prompted the choice of *VvPL11* for further experimentation.

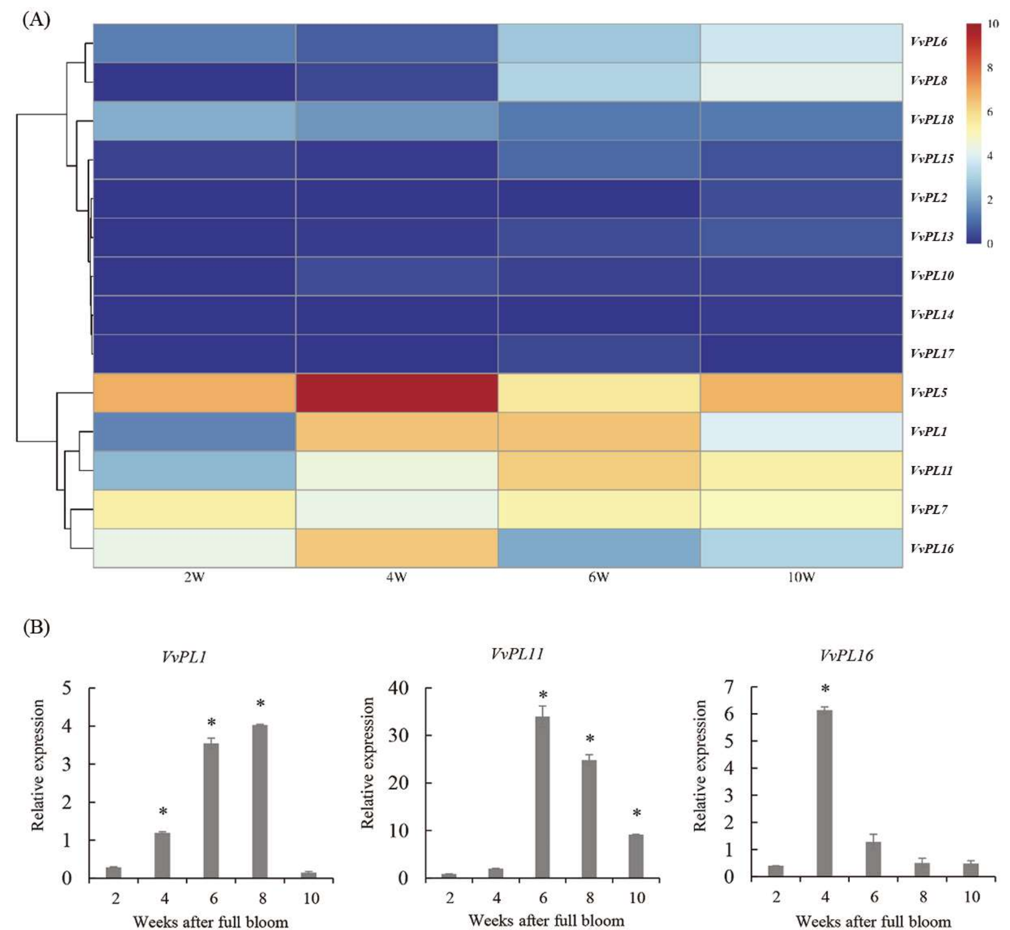


**Figure 5.** Expression patterns of *VvPL* genes at different fruit developmental stages of 'Hanxiangmi'. (**A**) Expression heatmap of *VvPL* genes from the transcriptomes of grape berries at different developmental stages. The color scale on the right represents the RPKM normalized $\log_2$. Blue indicates low expression levels, yellow indicates moderate expression levels, and red indicates high levels. (**B**) Relative expression levels of *VvPL* genes verified by RT-qPCR in developing fruit at 2, 4, 6, 8, and 10 weeks after full bloom (2 W, 4 W, 6 W, 8 W, and 10 W). Data were shown as the means ± standard deviation. * represent significant differences at $p < 0.05$.

### *3.4. Overexpression of VvPL11 in Tomato Decreased Fruit Firmness*

To elucidate any roles of *VvPL11* during fruit ripening, *VvPL11* was overexpressed in tomato plants (Figure 6). A total of 11 *VvPL11*-overexpressing (OE) transgenic lines were obtained and verified by RT-qPCR (Figure 6B). The ectopic *VvPL11* was highly expressed in the leaves of transgenic lines 1, 2, and 3 (Figure 6A,B). There were no phenotypic differences in plant height, flowering time, or fruit set between the OE and WT tomato plants (data not shown). *VvPL11*-OE2 and -OE3 seemed to turn red earlier-specifically, at the Br + 3d and Br + 5d stages-compared to the controls (Figure 6A). After the breaker stage, firmness of the T1 transgenic tomato fruits was lower than that of the control at the 3, 5, 7, and 15 days after breaker, and ethylene production of the fruit was significantly increased compared to control fruits at the same stage (Figure 6C,D). These results suggested that over-expression of the *VvPL11* gene in tomato accelerated tomato fruit ripening and softening.

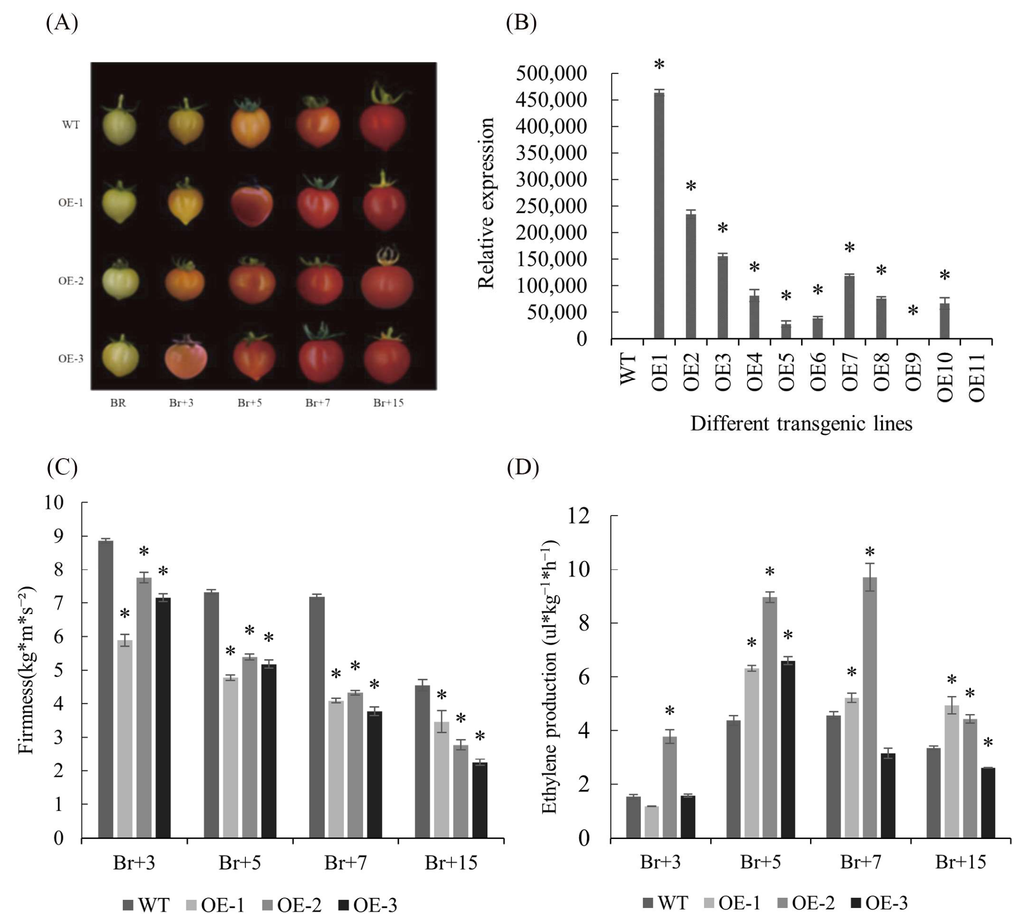


**Figure 6.** Influence of overexpression of *VvPL11* on tomato fruit firmness and ethylene production. (**A**) Phenotypic changes of WT and *VvPL11*-OE (lines OE-1, OE-2, and OE-3) during fruit developing. (**B**) RT-qPCR analysis of *VvPL11* in transgenic plants and wild type 'Micro-Tom' tomato. (**C**) Fruit firmness. (**D**) Ethylene production. Data were shown as the means ± standard deviation. * represent significant differences at $p < 0.05$.

### 3.5. Overexpression of VvPL11 Accelerated Cell Wall Degradation in Transgenic Tomato

To determine how overexpression of *VvPL11* alters the pectin content, the propectin and WSP content in transgenic tomato fruits were analyzed (Figure 7). The propectin content in the *VvPL11*-OE2 and -OE3 lines was greatly decreased at the Br + 3 and Br + 5 stages (Figure 7A), whereas the WSP content consistently increased after the breaker stage compared to controls (Figure 7B). Moreover, the transcript levels of key pectin degradation-related genes, namely *SlPG2*, *SlPME1*, and *SlEXP*, were up-regulated in transgenic tomato fruit at all stages after the breaker stage (Figure 7C–E).

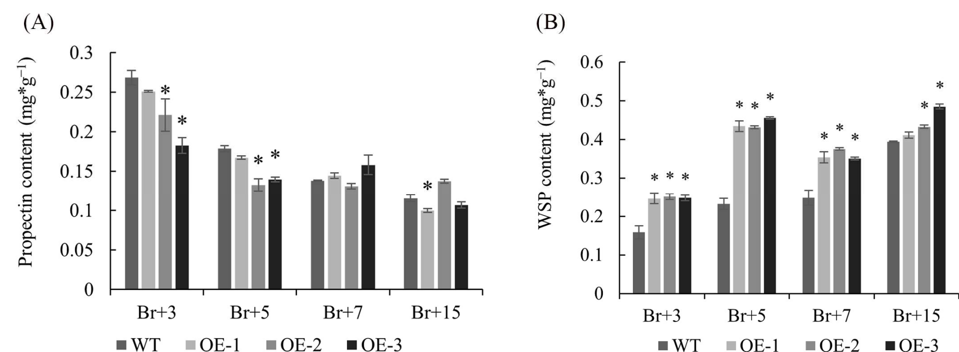


**Figure 7.** *Cont.*

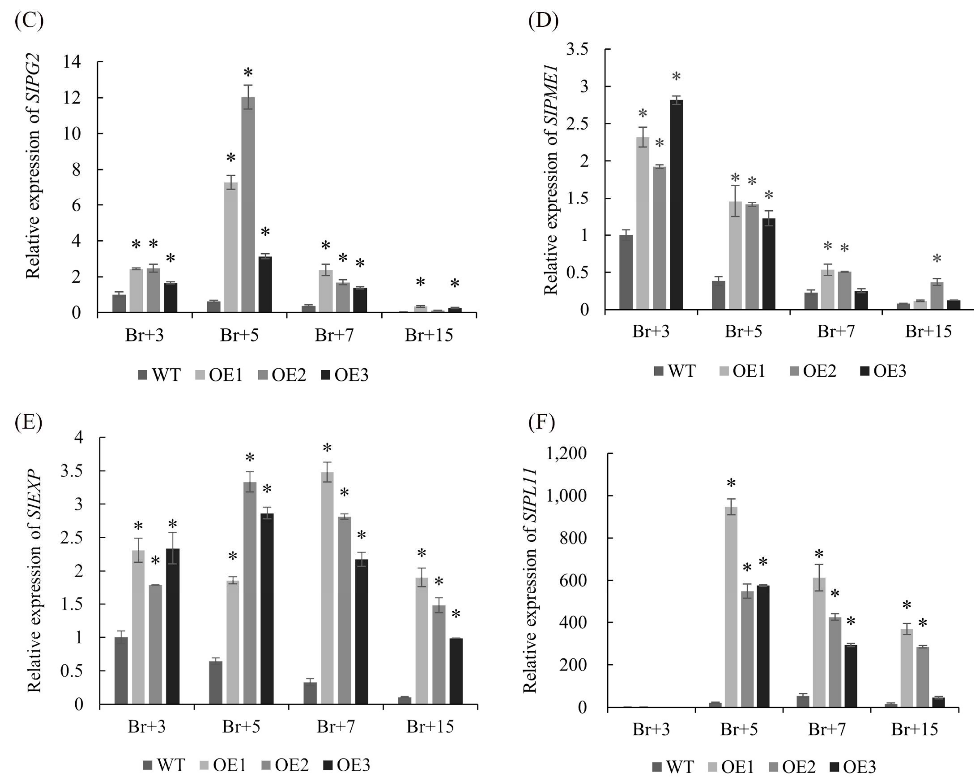


**Figure 7.** Overexpression of *VvPL11* affected pectin degradation and the expression levels of pectin-related genes in tomato. (**A**) Propectin; (**B**) WSP; (**C**) *SlPG2*; (**D**) *SlPME1*; (**E**) *SlEXP*; and (**F**) *SlPL11*. (* $p < 0.05$, Student's *t*-test). Data were shown as the means ± standard deviation. * represent significant differences at $p < 0.05$.

## 4. Discussion

During grape berry ripening, degradation of cell-wall components leads to cell-wall structural disruption and fruit softening, and depolymerization of pectin in the primary wall is an important factor [3,32]. Until now, the identification and characterization of the PL family have been conducted in different plants, such as *A. thaliana*, *S. lycopersicun*, *Fragaria ananassa*, and *Fragaria vesca*, in order to identify the crucial PL genes associated with fruit development processes [34]. However, the expression pattern and function of *VvPL* genes, which code for the only known pectin-degrading enzymes cleaving glycosidic bonds [18], have remained poorly understood with regard to berry softening. In this study, we identified 18 *VvPL* genes, characterized them in term of their phylogenetic relationships, gene conservation patterns, and gene structure, and analyzed the possible function of the *VvPL11* gene in fruit softening.

### *4.1. Most of VvPL Genes in Groups I and II Were Highly Expressed in Different Tissues and Organs*

In a search of the grape genome database, 18 *VvPL* genes were identified and were classified into five major groups. Most of VvPL members exhibit the conserved motifs of PL proteins in *S. lycopersicun*, *F. ananassa*, and *F. vesca* [34], whereas VvPL4 has the shortest predicted amino acid sequence and seems to lack most motifs and conserved domains, suggesting that *VvPL4* is an incomplete gene. However, the *VvPL4* gene showed differential expression among the tissue and organ samples, indicating that this conflicting result needs to be further investigated. The proteins in the same group have similar exon–intron structures, except for group 5. The difference in exon numbers indicated that the *VvPL* members possibly have their own diversified function [40].

The expression analysis performed by GEO Datasets (GSE36128) and our previous transcriptome profiles showed that all *VvPL* genes in group I and group II except *VvPL12*

demonstrated higher and similar expression trends in different tissues and organs. In group I, expression levels of *VvPL5* and *VvPL6* were higher, and the amino acid sequences of *VvPL6* and *VvPL5* were highly similar to *FaPLc* and *FvPLA*, which may imply *VvPL6* and *VvPL5* were orthologs of *FaPLc* and *FvPLA*, respectively. *FaPLc* and *FvPLA* have proven roles in ripening and softening of strawberry [41], suggesting that *VvPL6* and *VvPL5* may be associated with softening in grape berries. In group II, *VvPL1* and *VvPL16* clustered with *SlPL12*, *SlPL15*, and *SlPL*, which show dominant expression during fruit maturation; *SlPL* is a candidate gene for tomato firmness [30]. In the phylogenetic tree, *VvPL12* was close to *SlPL5*, which is an important gene that contributes to fruit softening [42]. The clustering and expression results suggested that *VvPL* genes in groups I and II may be involved in fruit ripening and softening. Moreover, transcriptome analysis of berries at different developmental stages from the grape cultivar 'Hanxiangmi' showed that *VvPL6/5* in group I and *VvPL1/16* in group II had higher expression levels, which further implies that these *VvPLs* play vital roles in berry ripening and softening.

### *4.2. VvPL11 Plays an Important Role in Fruit Softening*

As enzymes that take part in pectin degradation, PL genes have been proven to be highly expressed in mature fruit but not in unripe fruit, which indicates PLs are associated with fruit ripening and softening of banana and peach [43,44]. *VvPL11* belonged to group III and was the only gene with higher expression during berry development. Because a positive correlation between *VvPL11* gene expression and WSP content in berry was observed, the *VvPL11* gene was overexpressed in tomato plants to elucidate its role in fruit softening. Overexpression of *VvPL11* resulted in lower fruit firmness and higher ethylene production in transgenic tomato compared to WT, which suggested that the protein VvPL11 was an accelerator on fruit softening. Cell wall component analysis indicated that there was a lower propectin and higher WSP content in transgenic tomato fruit, which suggests that fruit softening and texture changes were advanced by accelerating degradation of propectin. This result is consistent with the findings in banana [45,46], strawberry [47,48], tomato [49], and peach [44], for which inhibition of fruit ripening by external treatments was often found to be accompanied by a decrease in pectin lyase expression. Furthermore, antisense expression of a pectin lyase gene delayed postharvest softening of strawberry [50].

The present study also showed that an overexpression of the *VvPL11* gene resulted in increased transcript levels for the *SlPG*, *SlPL*, and *SlPE* genes, similar to results observed in tomato fruit [51]. In transgenic tomato plants overexpressing apricot *PaPL9*, genes related to ethylene biosynthesis, fruit softening, and chlorophyll degradation showed induced expression, which suggested that PL genes play a core role in fruit ripening and that their overexpression may modulate the expression of ripening-related genes. In this study, our research was mainly concentrated on the crucial function of the *VvPL11* gene, which is associated with cell wall degradation, fruit softening, and ripening. Nevertheless, further work in molecular and genetic identification is necessary to explore the regulatory network of berry ripening and softening mechanisms in the future.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/xxx/s1. Figure S1: Characterisation of VvPL5/6, FvPLA and FaPLc. Table S1: The sequence of protein used in phylogenetic trees. Table S2: The information of the primers used for RT-qPCR and cloning. Table S3: Transcriptome data of *VvPLs* in the cultivar 'Hangxiangmi'.

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## References

1. Lu, Z.W.; Zhang, K.; Wang, P.; Lou, Y.S.; Fan, H.J.; Wu, W.Y.; Zhang, X.F. Status and Development Trends of Grape Industry in Henan Province. *J. Agric. Sci.* **2019**, *48*, 120–126.
2. Chang, B.; Zhang, Y.; Keller, M. Softening at the onset of grape ripening alters fruit rheological properties and decreases splitting resistance. *Planta* **2019**, *250*, 1293–1305. [CrossRef] [PubMed]
3. Balic, I.; Ejsmentewicz, T.; Sanhueza, D.; Silva, C.; Peredo, T.; Olmedo, P.; Campos-Vargas, R. Biochemical and physiological study of the firmness of table grape berries. *Postharvest Biol. Tec.* **2014**, *93*, 15–23. [CrossRef]
4. Zhang, S.T.; Fu, M.M.; Li, Z.Q.; Li, J.W.; Hai, L.F.; Chen, C.Y.; Feng, J.C. *VvEIL2* and *VvEIL4* regulate ethylene synthesis and carotenoid metabolism during senescence of grape rachis. *Postharvest Biol. Tec.* **2022**, *187*, 111853. [CrossRef]
5. Liu, H.N.; Pei, M.S.; Wei, T.L.; Yu, Y.H.; Guo, D.L. ROS scavenger Hypotaurine delays postharvest softening of 'Kyoho' grape by regulating pectin and cell metabolism pathway. *Postharvest Biol. Tec.* **2022**, *186*, 111833. [CrossRef]
6. Yahuaca, B.; Martinez-Peniche, R.; Reyes, J.L.; Madero, E. Effect of ethephon and girdling on berry firmness during storage of 'Malaga Roja' grape. *Acta Hortic.* **2006**, *727*, 459–465. [CrossRef]
7. Yang, L.; Cong, P.; He, J.; Bu, H.; Qin, S.; Lyu, D. Differential pulp cell wall structures lead to diverse fruit textures in apple (*Malus domestica*). *Protoplasma* **2022**, *259*, 1205–1217. [CrossRef]
8. Zheng, X.; Yuan, Y.; Huang, B.; Hu, X.; Tang, Y.; Xu, X.; Deng, W. Control of fruit softening and Ascorbic acid accumulation by manipulation of *SlIMP3* in tomato. *Plant Biotechnol. J.* **2022**, *20*, 1213–1225. [CrossRef]
9. Wang, Z.; Tang, Y.; Jin, X.; Liu, Y.; Zhang, H.; Niu, H.; Lan, H. Comprehensive evaluation of Korla fragrant pears and optimization of plucking time during the harvest period. *Int. J. Agric. Environ.* **2022**, *15*, 242–250. [CrossRef]
10. Yan, R.; Han, C.; Fu, M.; Jiao, W.; Wang, W. Inhibitory effects of $CaCl_2$ and pectin methylesterase on fruit softening of raspberry during cold storage. *Horticulturae* **2021**, *8*, 1. [CrossRef]
11. Amanullah, S.; Osae, B.A.; Yang, T.; Abbas, F.; Liu, S.; Liu, H.; Luan, F. Mapping of genetic loci controlling fruit linked morphological traits of melon using developed CAPS markers. *Mol. Biol. Rep.* **2022**, *49*, 5459–5472. [CrossRef] [PubMed]
12. Du, J.; Anderson, C.T.; Xiao, C. Dynamics of pectic homogalacturonan in cellular morphogenesis and adhesion, wall integrity sensing and plant development. *Nat. Plants* **2022**, *8*, 332–340. [CrossRef] [PubMed]
13. Wang, D.; Yeats, T.H.; Uluisik, S.; Rose, J.K.; Seymour, G.B. Fruit softening: Revisiting the role of pectin. *Trends Plant Sci.* **2018**, *23*, 302–310. [CrossRef]
14. Brummell, D.A.; Harpster, M.H. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol.* **2001**, *47*, 311–340. [CrossRef] [PubMed]
15. Song, X.; Dai, H.; Wang, S.; Ji, S.; Zhou, X.; Li, J.; Zhou, Q. Putrescine Treatment Delayed the Softening of Postharvest Blueberry Fruit by Inhibiting the Expression of Cell Wall Metabolism Key Gene *VcPG1*. *Plants* **2022**, *11*, 1356. [CrossRef]
16. Brummell, D.A. Cell wall disassembly in ripening fruit. *Funct. Plant Biol.* **2006**, *33*, 103–119. [CrossRef]
17. Posé, S.; Paniagua, C.; Matas, A.J.; Gunning, A.P.; Morris, V.J.; Quesada, M.A.; Mercado, J.A. A nanostructural view of the cell wall disassembly process during fruit ripening and postharvest storage by atomic force microscopy. *Trends Food Sci. Tech.* **2019**, *87*, 47–58. [CrossRef]
18. Seymour, G.B. Pectate lyase action in vivo and fruit softening. a commentary on: 'fruit softening: Evidence for pectate lyase action in vivo in date (*Phoenix dactylifera*) and rosaceous fruit cell walls'. *Ann. Bot.* **2021**, *128*, i–ii. [CrossRef]
19. Santiago-Doménech, N.; Jiménez-Bemúdez, S.; Matas, A.J.; Rose, J.K.C.; Muñoz-Blanco, J.; Mercado, J.A.; Quesada, M.A. Antisense inhibition of a pectate lyase gene supports a role for pectin depolymerization in strawberry fruit softening. *J. Exp. Bot.* **2008**, *59*, 2769–2779. [CrossRef]
20. Hayama, H.; Shimada, T.; Fujii, H.; Ito, A.; Kashimura, Y. Ethylene-regulation of fruit softening and softening-related genes in peach. *J. Exp. Bot.* **2006**, *57*, 4071–4077. [CrossRef]
21. Chen, Y.; Li, W.; Turner, J.A.; Anderson, C.T. PECTATE LYASE LIKE12 patterns the guard cell wall to coordinate turgor pressure and wall mechanics for proper stomatal function in *Arabidopsis*. *Plant Cell* **2021**, *33*, 3134–3150. [CrossRef]
22. Singh, A.P.; Pandey, S.P.; Pandey, S.; Nath, P.; Sane, A.P. Transcriptional activation of a pectate lyase gene, *RbPel1*, during petal abscission in rose. *Postharvest Biol. Tec.* **2011**, *60*, 143–148. [CrossRef]
23. McCormick, S.; Twell, D.; Vancanneyt, G.; Yamaguchi, J. Molecular analysis of gene regulation and function during male gametophyte development. *Symp. Soc. Exp. Biol.* **1991**, *45*, 229–244.

24. Wu, H.; Wang, B.; Chen, Y.; Liu, Y.; Chen, L. Characterization and fine mapping of the rice premature senescence mutant *ospse1*. *Theor. Appl. Genet.* **2013**, *126*, 1897–1907. [CrossRef]
25. Yang, Y.; Zhang, Y.; Li, B.; Yang, X.; Dong, Y.; Qiu, D. A *Verticillium dahliae* pectate lyase induces plant immune responses and contributes to virulence. *Front. Plant Sci.* **2018**, *9*, 1271. [CrossRef] [PubMed]
26. Fagard, M.; Dellagi, A.; Roux, C.; Périno, C.; Rigault, M.; Boucher, V.; Expert, D. *Arabidopsis thaliana* expresses multiple lines of defense to counterattack *Erwinia chrysanthemi*. *Mol. Plant Microbe Interact.* **2007**, *20*, 794–805. [CrossRef] [PubMed]
27. Marín-Rodríguez, M.C.; Orchard, J.; Seymour, G.B. Pectate lyases, cell wall degradation and fruit softening. *J. Exp. Bot.* **2002**, *53*, 2115–2119. [CrossRef]
28. Yoo, S.D.; Gao, Z.; Cantini, C.; Loescher, W.H.; Van Nocker, S. Fruit ripening in sour cherry: Changes in expression of genes encoding expansins and other cell-wall-modifying enzymes. *J. Am. Soc. Hortic. Sci.* **2003**, *128*, 16–22. [CrossRef]
29. Zhang, W.W.; Zhao, S.Q.; Gu, S.; Cao, X.Y.; Zhang, Y.; Niu, J.F.; Xing, Y. FvWRKY48 binds to the pectate lyase *FvPLA* promoter to control fruit softening in *Fragaria vesca*. *Plant Physiol.* **2022**, *189*, 1037–1049. [CrossRef]
30. Yang, L.; Huang, W.; Xiong, F.; Xian, Z.; Su, D.; Ren, M.; Li, Z. Silencing of *SlPL*, which encodes a pectate lyase in tomato, confers enhanced fruit firmness, prolonged shelf-life and reduced susceptibility to grey mould. *Plant Biotechnol. J.* **2017**, *15*, 1544–1555. [CrossRef]
31. Ejsmentewicz, T.; Balic, I.; Sanhueza, D.; Barria, R.; Meneses, C.; Orellana, A.; Campos-Vargas, R. Comparative study of two table grape varieties with contrasting texture during cold storage. *Molecules* **2015**, *20*, 3667–3680. [CrossRef] [PubMed]
32. Ma, L.; Sun, L.; Guo, Y.; Lin, H.; Liu, Z.; Li, K.; Guo, X. Transcriptome analysis of table grapes (*Vitis vinifera* L.) identified a gene network module associated with berry firmness. *PloS ONE* **2020**, *15*, e0237526. [CrossRef] [PubMed]
33. Zhang, Z.G.; Geng, Y.X.; Cai, M.T.; Zhang, X.L.; Sun, Z.X.; Yin, J.Y. Spatial-Temporal Evoltion and Trend Analysis of Climatic Potential Productivity in Henan Province during 1978–2017. *J. Soil Water Conserv.* **2020**, *27*, 247–253.
34. Benítez-Burraco, A.; Blanco-Portales, R.; Redondo-Nevado, J.; Bellido, M.L.; Moyano, E.; Caballero, J.L.; Muñoz-Blanco, J. Cloning and characterization of two ripening-related strawberry (*Fragaria* × *ananassa* cv. Chandler) pectate lyase genes. *J. Exp. Bot.* **2003**, *54*, 633–645. [CrossRef]
35. Fasoli, M.; Santo, S.D.; Zenoni, S.; Tornielli, G.B.; Farina, L.; Zamboni, A.; Porceddu, A.; Venturini, L.; Bicego, M.; Murino, V.; et al. The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *Plant Cell* **2012**, *24*, 3489–3505. [CrossRef]
36. Cheng, C.; Wang, Y.; Chai, F.; Li, S.; Xin, H.; Liang, Z. Genome-wide identification and characterization of the 14-3-3 family in *Vitis vinifera* L. during berry development and cold- and heat-stress response. *BMC Genom.* **2018**, *19*, 579. [CrossRef]
37. Guo, J.; Cao, K.; Deng, C.; Li, Y.; Wang, L. An integrated peach genome structural variation map uncovers genes associated with fruit traits. *Genome Biol.* **2020**, *21*, 258. [CrossRef] [PubMed]
38. Tan, D.; Li, T.; Wang, A. Apple 1-aminocyclopropane-1-carboxylic acid synthase genes, *MdACS1* and *MdACS3a*, are expressed in different systems of ethylene biosynthesis. *Plant Mol. Biol. Rep.* **2013**, *31*, 204–209. [CrossRef]
39. Guan, X.Q.; Yang, Y.; Wang, H.Z.; Guo, C.S.; Guan, J.F. Effects of spraying calcium on contents of calcium and pectin and fruit quality of Red Globe Grape (*Vitis vinifera* L.). *J. Plant. Nutr. Soil Sci.* **2014**, *20*, 179–185.
40. Palusa, S.G.; Golovkin, M.; Shin, S.B.; Richardson, D.N.; Reddy, A.S. Organ-specific, developmental, hormonal and stress regulation of expression of putative pectate lyase genes in *Arabidopsis*. *New Phytol.* **2007**, *174*, 537–550. [CrossRef]
41. Youssef, S.M.; Jiménez-Bermúdez, S.; Bellido, M.L.; Martín-Pizarro, C.; Barceló, M.; Abdal-Aziz, S.A.; Mercado, J.A. Fruit yield and quality of strawberry plants transformed with a fruit specific strawberry pectate lyase gene. *Sci. Hortic.* **2009**, *119*, 120–125. [CrossRef]
42. Yang, Y.; Lu, L.; Sun, D.; Wang, J.; Wang, N.; Qiao, L.; Wang, C.L. Fungus polygalacturonase-generated oligogalacturonide restrains fruit softening in ripening tomato. *J. Agric. Food Chem.* **2021**, *70*, 759–769. [CrossRef]
43. Marin-Rodriguez, M.C.; Smith, D.L.; Manning, K.; Orchard, J.; Seymour, G.B. Pectate lyase gene expression and enzyme activity in ripening banana fruit. *Plant Mol. Biol.* **2003**, *51*, 851–857. [CrossRef]
44. Xu, Z.; Dai, J.Y.; Kang, T.Y.; Shah, K.; Li, Q.; Liu, K.; Xing, L.B.; Ma, J.J.; Zhang, D.; Zhao, C.P. *PpePL1* and *PpePL15* Are the Core Members of the Pectate Lyase Gene Family Involved in Peach Fruit Ripening and Softening. *Front. Plant Sci.* **2022**, *13*, 844055. [CrossRef] [PubMed]
45. Chen, J.; Li, Y.; Li, F.; Hong, K.; Yuan, D. Effects of procyanidin treatment on the ripening and softening of banana fruit during storage. *Sci. Hortic.* **2022**, *70*, 759–769. [CrossRef]
46. Chopsri, A.; Sekozawa, Y.; Sugaya, S. Effects of hot air treatment on cell wall-degrading enzymes, pulp softening and ripening in bananas. *Food Res. Int.* **2018**, *25*, 2195–2203.
47. Pose, S.; Kirby, A.R.; Paniagua, C.; Waldron, K.W.; Morris, V.J.; Quesada, M.A.; Mercado, J.A. The nanostructural characterization of strawberry pectins in pectate lyase or polygalacturonase silenced fruits elucidates their role in softening. *Carbohydr. Polym.* **2015**, *132*, 134–145. [CrossRef] [PubMed]
48. Youssef, S.M.; Amaya, I.; López-Aranda, J.M.; Sesmero, R.; Valpuesta, V.; Casadoro, G.; Mercado, J.A. Effect of simultaneous down-regulation of pectate lyase and endo-β-1,4-glucanase genes on strawberry fruit softening. *Mol. Breed.* **2013**, *31*, 313–322. [CrossRef]
49. Uluisik, S.; Chapman, N.H.; Smith, R.; Poole, M.; Adams, G.; Gillis, R.B.; Seymour, G.B. Genetic improvement of tomato by targeted control of fruit softening. *Nat. Biotechnol.* **2016**, *34*, 950–952. [CrossRef] [PubMed]

50. Jiménez-Bermudez, S.; Redondo-Nevado, J.; Munoz-Blanco, J.; Caballero, J.L.; López-Aranda, J.M.; Valpuesta, V.; Mercado, J.A. Manipulation of strawberry fruit softening by antisense expression of a pectate lyase gene. *Plant Physiol.* **2022**, *128*, 751–759. [CrossRef] [PubMed]
51. Shi, L.; Liu, Q.; Qiao, Q.; Wang, X.; Ren, Z.; Zhu, Y.; Huang, W. Exploring the effects of pectate and pectate lyase on the fruit softening and transcription profiling of *Solanum lycopersicum*. *Food Control* **2022**, *133*, 108636. [CrossRef]

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