



## Article

# Genome-Wide Identification of Laccase Gene Family from *Punica granatum* and Functional Analysis towards Potential Involvement in Lignin Biosynthesis

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**Abstract:** Laccase (LAC) is the key enzyme responsible for lignin biosynthesis. Here, 57 PgLACs from pomegranate were identified and distributed on eight chromosomes and one unplaced scaffold. They were divided into six groups containing three typical Cu-oxidase domains. Totally, 51 *cis*-acting elements in the promoter region of PgLACs are involved in response to ABA, GA, light, stress, etc., indicating diverse functions of PgLACs. The expression profiles of 13 PgLACs during the seed development stage showed that most PgLACs expressed at a higher level earlier than at the later seed development stage in two pomegranate cultivars except PgLAC4. Also, PgLAC1/6/7/16 expressed at a significantly higher level in soft-seed ‘Tunisia’; on the contrary, PgLAC37 and PgLAC50 with a significantly higher expression in hard-seed ‘Taishanhong’. Combined with their distinguishing *cis*-acting elements, it was concluded that PgLAC1/6/7 may respond to GA via TATC-box and GARE-motif, and PgLAC16 repressed the promoter activity of embryo mid-maturation genes via RY-element so as to contribute to softer seed formation, whereas PgLAC37/50 may participate in seed formation and accelerate seed maturity via ABRE and G-box elements. Collectively, the dramatic gene expressions of PgLAC1/6/7/16/37/50 will provide valuable information to explore the formation of soft- and hard-seed in pomegranate.

**Keywords:** pomegranate; lignin biosynthesis; seed hardness; laccase; gene expression



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## 1. Introduction

Pomegranate (*Punica granatum* L.) is a commercial fruit tree with an old cultivation history and belongs to *Lytharceae* family [1]. Nowadays, pomegranate is grown commercially in many counties, such as Iran, China, Spain, and Turkey. Pomegranate fruit is very popular with consumers worldwide due to its delicious taste and benefits to human health. The increasing research demonstrates that pomegranate fruit, as well as its extracts, contain abundant bioactive components, e.g., flavonoids, anthocyanins, organic acids, ellagitannins, phenolic acids, and possess unique antihelminthic, antimicrobial, and antioxidant effects [2–6]. Generally, the edible part of pomegranate fruit is juicy pulp, named arils, by which seeds are surrounded. According to seed hardness, pomegranate cultivars are divided into four groups: soft seed, semi-soft seed, semihard seed, and hard seed [7]. Seeds in soft-seed pomegranate cultivars are easily swallowed and edible. However, the disadvantage is spitting seeds for hard-seed pomegranate cultivars, which seriously affects the taste and consumer appreciation. As we know, lignin is a principal structural component of cell walls in higher plants, and the pomegranate seed formation is closely related to lignin biosynthesis and metabolism [8].

Lignin significantly influences the physical properties and enhances the strength and hardness of cells in plants [9]. Laccase (LAC) is a copper-containing polyphenol oxidase,

which is a key enzyme and is broadly present in plants, bacteria, insects, and fungi [10]. In recent years, LACs have been identified in rice [10], *Arabidopsis thaliana* [11], poplar (*Populus trichocarpa*) [12], and some horticultural plants, such as peach (*Prunus persica*) [13], citrus (*Citrus sinensis*) [14], pear (*Pyrus bretschneideri*) [15], tea plant (*Camellia sinensis*) [16], and eggplant (*Solanum melongena*) [17]. Although the LAC gene family from different species is divided into five to eight groups with quite different amino acid sequences, their catalytic sites are relatively conserved [18]. LAC can catalyze the oxidation of various aromatic and non-aromatic substrates via three catalytic sites with four Cu ions, and thereby LAC family possesses multiple biological functions [18,19]. *AtLAC15* expressed specifically in seed coat cell walls of *Arabidopsis*, and oxidative polymerization of epicatechin and soluble PAs led to seed coat browning of *Arabidopsis* [11,20]. The up-regulation of *DkLAC2* increased proanthocyanidin accumulation in persimmon fruit by short tandem target mimic STTM-miR397 [21]. On the other hand, plant LACs are involved in lignin biosynthesis. Eight *AtLACs* expressed at a high level in the inflorescence stems, leading to deposited lignin. Also, *AtLAC4*, *AtLAC11*, and *AtLAC17* were strongly expressed in stems and promoted the constitutive lignification of *Arabidopsis* stem [20,22,23]. The *LACCASE 5* mutant decreased 10% of Klason lignin content and modified the ratio of the syringyl to guaiacyl units [24]. *AtLAC4* regulated by MiR397b may result in plant biomass production with less lignin in flowering plants [25]. The gene expression of *ChLac8* from *Cleome hassleriana* in the *Arabidopsis caffeic acid o-methyltransferase* mutant led to the C-lignin formation in the stems [26]. In the aspect of fruit trees, *PpLAC20* and *PpLAC30* were identified as candidate genes involved in peach lignin biosynthesis [13]. Six *PbLACs* were likely associated with lignin synthesis and stone cell formation in pear fruit, and *PbLAC1* significantly increased lignin deposition and thickened cell walls in transgenic *Arabidopsis* [15]. These recent progresses further stressed the importance of laccases in lignin biosynthesis; thus, more genetic evidence from other species will contribute to illuminating the LAC function in lignin polymerization.

Seed hardness is not only an important index of fruit quality but also directly decides consumers' preferences. To clarify the function of the laccase family in lignin metabolism and seed formation in pomegranate fruit, *PgLAC* family members were first explored in pomegranate in the present study. The bioinformatic analysis of *PgLAC* family members, including sequence characteristics, exon–intron structures, and conserved motifs, was performed. Moreover, the specific expression patterns of the *PgLAC* members were elucidated during the four seed development stages of soft- and hard-seed pomegranates. The results will provide the key candidate genes for seed formation in pomegranate, contributing to breeding the new germplasm.

## 2. Materials and Methods

### 2.1. Plant Material

*P. granatum* cv. 'Tunisia' and 'Taishanhong' plants were grown in the Fruit Tree Experimental Station, Henan Agricultural University, Zhengzhou, Henan, China. The fruits were collected at 30 d, 45 d, 70 d, and 120 d after full flowering and divided into two groups after removing arils. One group quickly evaluated seeds' seed hardness and lignin content, and the other was stored at  $-80^{\circ}\text{C}$  for qRT-PCR analysis.

### 2.2. Determination of Seed Hardness and Lignin Content

Seed hardness was measured with GY-4 Digital Fruit Hardness Analyzer (Xandpi Instrument Co., Ltd., Leqing, China). The average value of 20 seeds was calculated from three technique replicates and expressed as  $\text{Kg}/\text{cm}^2$ . The seeds were dried at  $80^{\circ}\text{C}$  till reaching the constant weight, ground to powder, and sieved with 0.425 mm aperture. Then, 2 mg samples were evaluated for lignin content using MZS-1-G Kit (Comin Biotechnology Co., Ltd., Suzhou, China), expressed as  $\text{mg}/\text{g}$ .

### 2.3. Identification and Physicochemical Properties of PgLAC Family Members

Pomegranate genome data (ASM765513v2) was downloaded from the website ([https://www.ncbi.nlm.nih.gov/assembly/GCF\\_007655135.1](https://www.ncbi.nlm.nih.gov/assembly/GCF_007655135.1), accessed on 12 March 2022). The sequences of 17 AtLAC proteins were obtained from Uniprot website (<https://www.uniprot.org/>, accessed on 12 March 2022). PgLACs sequences from ‘Tunisia’ were obtained by Blast Wrapper (E-value  $< 1 \times 10^{-5}$ ) in TBtools software with AtLACs sequences and were matched with three Cu-oxidase domains (PFAM00394, PFAM07731, and PFAM07732) on the NCBI CDD (Conserved Domain Database; <https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>, accessed on 18 March 2022). Subsequently, the Genbank Accession Numbers of PgLACs were obtained on NCBI BLAST alignment (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 18 March 2022). The protein isoelectric point (pI) and molecular weight (MW) were accessed using online ExPASy ProtParam ([https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/), accessed on 25 March 2022). The protein isoelectric point (pI) is calculated using pK values of amino acids, and molecular weight (MW) is calculated by the addition of average isotopic masses of amino acids in the protein and the average isotopic mass of one water molecule.

### 2.4. Bioinformation Analysis of PgLAC Family Members

The subcellular localization was predicted on the WoLF PSORT (<https://wolfpsort.hgc.jp/>, accessed on 16 July 2023). The phylogenetic tree was constructed using Clustal W method of MAGE 7.0 software (Mega Limited, Auckland, New Zealand) and optimized on the online website Interactive Tree of Life (<http://itol.embl.de>, accessed on 19 May 2023). The amino acid sequence alignment was performed with neighbor-joining (NJ), and the parameters were set as maximum composite likelihood, complete deletion, and bootstrap 1000 of MAGE 7.0 software. PgLAC proteins were clustered based on the published LAC proteins from other plant species (details in Table S1). Conserved motifs of PgLAC proteins were analyzed using the MEME online software (<https://meme-suite.org/meme/tools/meme>, accessed on 18 May 2023). Exon-intron structures, chromosomal locations, and gene duplication of PgLAC genes were visualized using Gene Structure Shower, Gene Location Visualize, One-Step MCScanX, and Advanced Circos of TBtools software.

### 2.5. Analysis of Cis-Acting Elements and Protein Interaction Networks

The promotor sequences were obtained from 2000-bp upstream sequences from the start codon of PgLAC genes and predicted *cis*-acting elements on PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 18 May 2023), and illustrated with TBtools software. To explore gene co-expression patterns, the protein interaction networks were drawn on the website String (<http://cn.string-db.org>, accessed on 20 May 2023), and *Arabidopsis thaliana* was chosen as the species parameter.

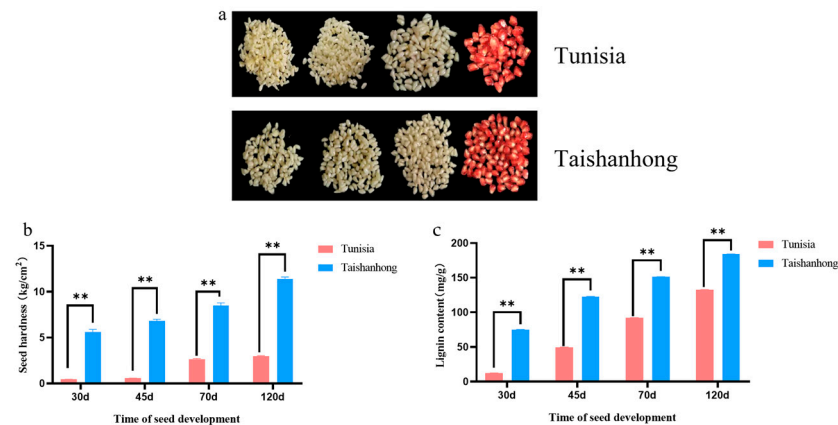
### 2.6. RNA Extraction and Quantitative RT-PCR (qRT-PCR) Analysis of PgLAC Family Members

The total RNA of seeds was extracted using a Quick RNA Isolation Kit (0416-50-GK, Huayueyang, Beijing, China), and the cDNA was synthesized using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). qRT-PCR was run on ABI 7500 PCR instrument (Applied Biosystems, Foster, CA, USA) using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The PCR reaction was performed at 95 °C for 5 min, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. The relative expression level was calculated by  $2^{-\Delta\Delta CT}$  method [27]. The *PgActin* (XM\_031530994.1) was used as an internal reference. All the primers were designed on the website Primer-Blast of NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>, accessed on 6 August 2023) and listed in (Table S2). Statistical analysis was carried out with SPSS Statistics v. 20 (IBM, Chicago, IL, USA) with a significant difference of  $p < 0.05$  and  $p < 0.01$ . The gene expression level was drawn using GraphPad Prism 8 software (San Diego, CA, USA).

### 3. Results

#### 3.1. Comparison of Seed Hardness and Lignin Content during Seed Development Stage

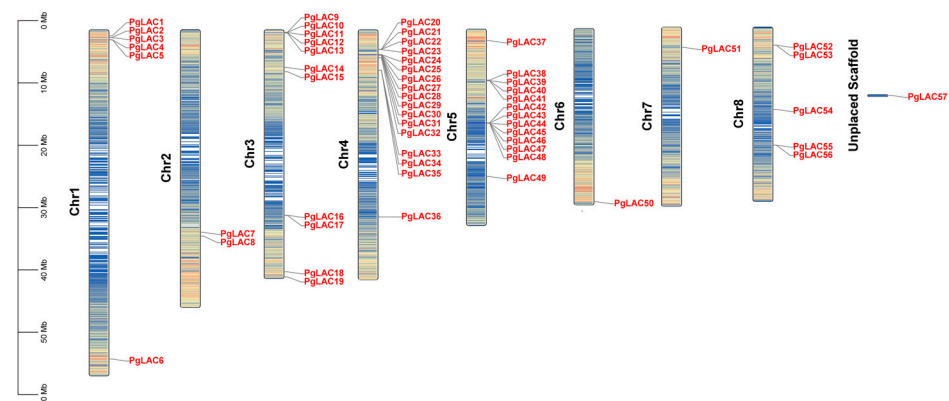
Lignin content was an important index to evaluate the seed hardness in pomegranate. Figure 1a presented seed phenotypic characteristics during four seed development stages of the two pomegranate cultivars. From Figure 1b,c, it was found that seed hardness and lignin content both increased steadily in ‘Taishanhong’ and ‘Tunisia’ seeds as seed development and were highly significantly lower in ‘Tunisia’ seeds than in ‘Taishanhong’ ones ( $p < 0.01$ ).



**Figure 1.** Seed appearance (a), seed hardness (b), and lignin content (c) during four seed development stages from ‘Tunisia’ and ‘Taishanhong’. \*\* indicated significant differences between groups at  $p < 0.01$ , respectively.

#### 3.2. Identification of PgLAC Gene Family Members

In the present study, 57 LAC genes were identified from pomegranate, distributed on eight chromosomes and Unplaced Scaffold, and named PgLAC1–PgLAC57 according to their chromosome positions of the pomegranate genome (Figure 2). It was found that 57 PgLAC genes were mainly distributed Chr 3 (PgLAC9–19), Chr 4 (PgLAC20–36), and Chr5 (PgLAC37–49), containing 41 PgLAC genes, while Chr 6, Chr7, and Unplaced Scaffold only contained one PgLAC gene, respectively.



**Figure 2.** Chromosome distribution of the PgLAC genes. Blue and red represented from less to more of gene density on the chromosome.

Gene duplication was performed in the pomegranate genome to further understand gene evolution. The results displayed that eight PgLACs gene pairs were considered to originate from segmental duplication events across six chromosomes except Chromosome 4 and Chromosome 7, including PgLAC2/39, PgLAC4/7, PgLAC7/38, PgLAC7/50, PgLAC8/19, PgLAC8/53, PgLAC18/37, and PgLAC38/50 (Figure S2 and Table S3). Also, 13 gene pairs were

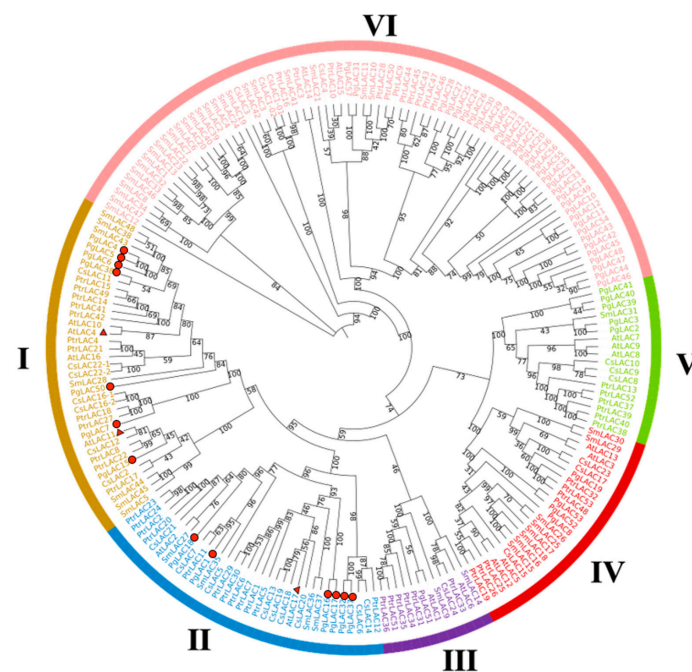


considered as tandem duplication events on Chromosome 1, Chromosome 3, Chromosome 4, and Chromosome 5 (Table S4). The results suggested that both segmental and tandem duplication occurred in the *PgLAC* gene family, which was closely related to the expansion of the *PgLAC* family.

### 3.3. Bioinformatic Characteristics of *PgLAC* Gene Family Members

The amino acids encoded by the *PgLAC* genes ranged from 397 aa (*PgLAC10*) to 614 aa (*PgLAC36*), and only two *PgLAC* genes (*PgLAC10* and *PgLAC52*) were lower than 500 aa (Table 1). Their MWs ranged from 44.53 to 68.98 kDa, and the theoretical isoelectric points (pI) were from 4.51 to 9.90 (Table 1). Moreover, the subcellular localization displayed that 21 *PgLAC* proteins were located on chloroplast; secondly, vacuolar membrane and cytosol each with 11 *PgLAC* proteins, 5 on endoplasmic reticulum, 2 on extracellular, and the least was nucleus and mitochondrion each with one *PgLAC* protein.

To explore the evolutionary relationships of *PgLACs*, the phylogenetic tree of the amino acid sequences of 57 *PgLACs* was constructed with *LAC* proteins from other plant species (including 17 *LACs* from *Arabidopsis thaliana*, 53 from *Populus trichocarpa*, 27 from *Citrus reticulata* Blanco, and 48 from *Solanum melongena*) (Figure 3). The results demonstrated that 202 *LAC* proteins were divided into six groups. Group I had 37 *LAC* proteins, and 33 *LACs* belonged to Group II. Moreover, Group VI contained the maximum *LAC* proteins, up to 77 *LACs*, while only 12 *LAC* proteins were clustered into Group III. Group IV and V had 25 and 18 *LACs*, respectively. Also, the maximum *LAC* proteins from pomegranate were distributed in Group VI, reaching 34, whereas Group III had only one *PgLAC51*. Considering that *AtLAC4*, *AtLAC11*, and *AtLAC17* were responsible for lignin polymerization [23], it was concerned that *PgLACs* (*PgLAC4*, 5, 6, 7, 15, 38, and 50) were clustered into Group I with *AtLAC4* and *AtLAC11*, furthermore, *PgLACs* (*PgLAC1*, 16, 17, 18, 32, and 37) along with *AtLAC17* belonged to Group II (Figure 3), which indicated that they had similar function.



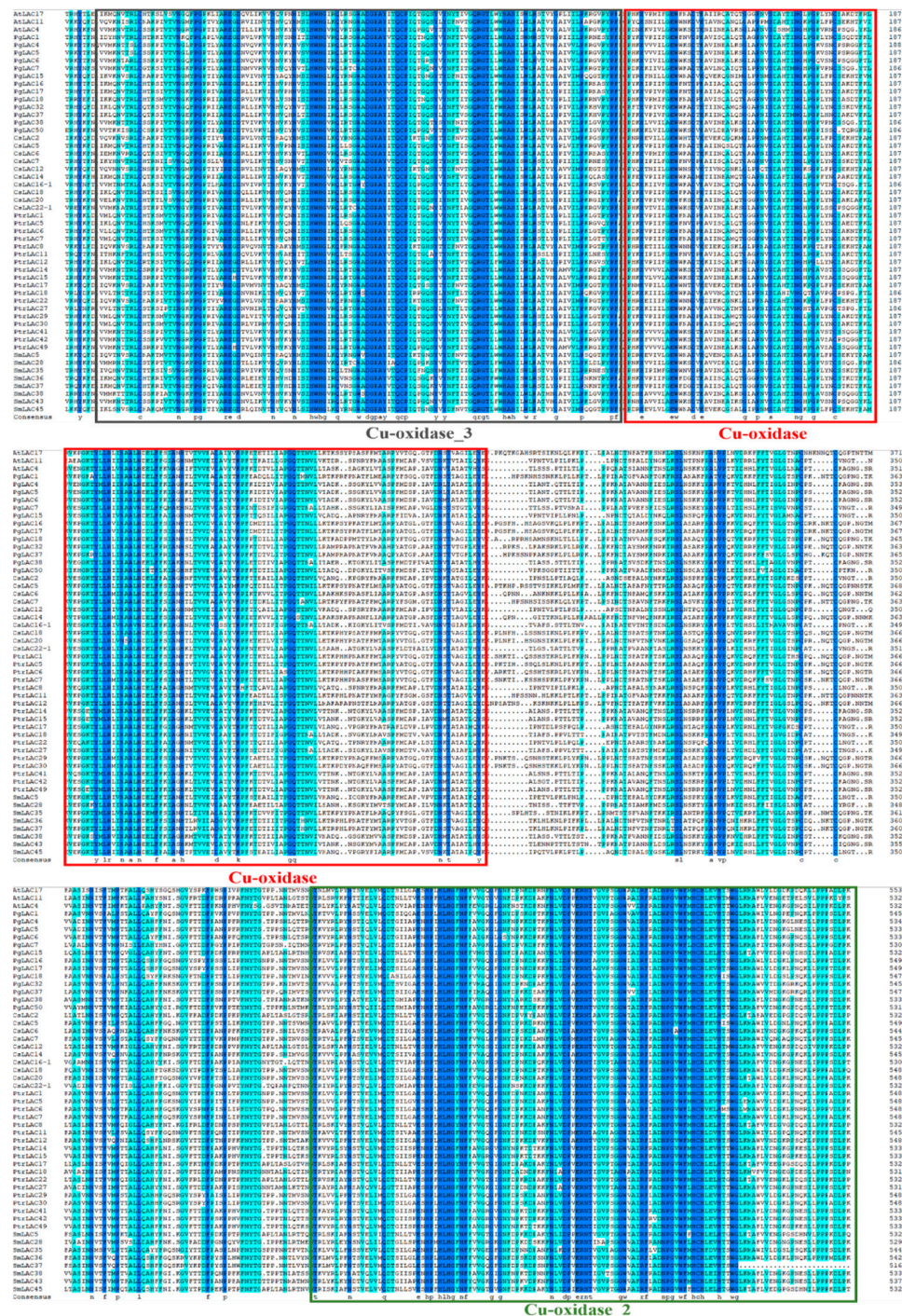
**Figure 3.** The phylogenetic tree of pomegranate, *Arabidopsis thaliana*, *Populus trichocarpa*, *Citrus reticulata* Blanco, and *Solanum melongena*. *PgLACs* from *P. granatum*, *AtLACs* from *A. thaliana*, *PtrLACs* from *P. trichocarpa*, *CsLACs* from *C. reticulata*, and *SmLACs* from *S. melongena*.

**Table 1.** Bioinformation analysis and physic–chemical properties of *PgLACs*.

| Gene Name      | Gene ID      | Protein ID     | Gene Length (bp) | Chromosome Position | Amino Acid (aa) | pI   | MW (kDa) | Subcellular Localization |
|----------------|--------------|----------------|------------------|---------------------|-----------------|------|----------|--------------------------|
| <i>PgLAC1</i>  | LOC116200081 | XP_031386621.1 | 1811             | Chr1                | 573             | 9.59 | 63.46    | Chloroplast              |
| <i>PgLAC2</i>  | LOC116192628 | XP_031377084.1 | 1822             | Chr1                | 568             | 8.22 | 62.61    | Vacuolar membrane        |
| <i>PgLAC3</i>  | LOC116193231 | XP_031377897.1 | 1811             | Chr1                | 572             | 8.84 | 62.79    | Vacuolar membrane        |
| <i>PgLAC4</i>  | LOC116193760 | XP_031378369.1 | 1796             | Chr1                | 560             | 9.48 | 61.62    | Chloroplast              |
| <i>PgLAC5</i>  | LOC116193760 | XP_031378376.1 | 1793             | Chr1                | 559             | 9.48 | 61.49    | Chloroplast              |
| <i>PgLAC6</i>  | LOC116192848 | XP_031377387.1 | 1763             | Chr1                | 559             | 9.47 | 61.53    | Chloroplast              |
| <i>PgLAC7</i>  | LOC116195123 | XP_031379957.1 | 1885             | Chr2                | 556             | 7.06 | 61.22    | Vacuolar membrane        |
| <i>PgLAC8</i>  | LOC116197663 | XP_031383717.1 | 1805             | Chr2                | 571             | 8.59 | 62.92    | Chloroplast              |
| <i>PgLAC9</i>  | LOC116199437 | XP_031385642.1 | 1890             | Chr3                | 595             | 5.17 | 66.76    | Endoplasmic reticulum    |
| <i>PgLAC10</i> | LOC116199438 | XP_031385643.1 | 1706             | Chr3                | 397             | 5.44 | 44.53    | Cytoskeleton             |
| <i>PgLAC11</i> | LOC116199436 | XP_031385641.1 | 1896             | Chr3                | 601             | 5.05 | 67.30    | Cytosol                  |
| <i>PgLAC12</i> | LOC116199364 | XP_031385547.1 | 1806             | Chr3                | 601             | 5.04 | 67.60    | Chloroplast              |
| <i>PgLAC13</i> | LOC116199435 | XP_031385640.1 | 1909             | Chr3                | 602             | 5.67 | 67.59    | Vacuolar membrane        |
| <i>PgLAC14</i> | LOC116202020 | XP_031389389.1 | 1877             | Chr3                | 590             | 5.08 | 66.49    | Chloroplast              |
| <i>PgLAC15</i> | LOC116199704 | XP_031386025.1 | 1784             | Chr3                | 564             | 7.70 | 62.49    | Vacuolar membrane        |
| <i>PgLAC16</i> | LOC116198560 | XP_031384589.1 | 1870             | Chr3                | 591             | 9.38 | 65.15    | Endoplasmic reticulum    |
| <i>PgLAC17</i> | LOC116198765 | XP_031384857.1 | 1876             | Chr3                | 591             | 9.38 | 65.12    | Endoplasmic reticulum    |
| <i>PgLAC18</i> | LOC116199682 | XP_031386005.1 | 1817             | Chr3                | 581             | 9.48 | 64.04    | Chloroplast              |
| <i>PgLAC19</i> | LOC116201632 | XP_031388782.1 | 1837             | Chr3                | 584             | 9.10 | 64.85    | Mitochondrion            |
| <i>PgLAC20</i> | LOC116205486 | XP_031393972.1 | 1668             | Chr4                | 550             | 4.94 | 61.26    | Cytosol                  |
| <i>PgLAC21</i> | LOC116205486 | XP_031393974.1 | 1838             | Chr4                | 547             | 4.98 | 60.95    | Cytosol                  |
| <i>PgLAC22</i> | LOC116205486 | XP_031393971.1 | 1846             | Chr4                | 595             | 5.10 | 66.46    | Cytosol                  |
| <i>PgLAC23</i> | LOC116205995 | XP_031394575.1 | 1887             | Chr4                | 595             | 5.26 | 67.21    | Cytosol                  |
| <i>PgLAC24</i> | LOC116202230 | XP_031389549.1 | 1837             | Chr4                | 575             | 5.01 | 63.50    | Vacuolar membrane        |
| <i>PgLAC25</i> | LOC116202230 | XP_031389550.1 | 1747             | Chr4                | 541             | 4.83 | 59.61    | Cytosol                  |
| <i>PgLAC26</i> | LOC116202996 | XP_031390491.1 | 1707             | Chr4                | 568             | 6.16 | 62.63    | Vacuolar membrane        |
| <i>PgLAC27</i> | LOC116203824 | XP_031391629.1 | 1790             | Chr4                | 569             | 6.72 | 63.34    | Extracellular            |
| <i>PgLAC28</i> | LOC116204024 | XP_031391913.1 | 1807             | Chr4                | 569             | 5.92 | 62.36    | Cytosol                  |
| <i>PgLAC29</i> | LOC116206410 | XP_031395142.1 | 1620             | Chr4                | 519             | 4.51 | 56.86    | Cytosol                  |
| <i>PgLAC30</i> | LOC116206410 | XP_031395141.1 | 1765             | Chr4                | 570             | 4.66 | 62.78    | Vacuolar membrane        |
| <i>PgLAC31</i> | LOC116206444 | XP_031395180.1 | 1789             | Chr4                | 564             | 8.89 | 62.93    | Peroxisome               |
| <i>PgLAC32</i> | LOC116205510 | XP_031393999.1 | 1881             | Chr4                | 591             | 9.78 | 65.91    | Chloroplast              |
| <i>PgLAC33</i> | LOC116206011 | XP_031394594.1 | 1809             | Chr4                | 565             | 5.21 | 63.18    | Chloroplast              |
| <i>PgLAC34</i> | LOC116206011 | XP_031394592.1 | 2074             | Chr4                | 599             | 5.21 | 66.79    | Endoplasmic reticulum    |
| <i>PgLAC35</i> | LOC116206011 | XP_031394593.1 | 2074             | Chr4                | 599             | 5.21 | 66.79    | Endoplasmic reticulum    |
| <i>PgLAC36</i> | LOC116206216 | XP_031394887.1 | 1885             | Chr4                | 614             | 5.04 | 68.98    | Chloroplast              |
| <i>PgLAC37</i> | LOC116206933 | XP_031395630.1 | 1852             | Chr5                | 587             | 9.90 | 65.32    | Chloroplast              |
| <i>PgLAC38</i> | LOC116209331 | XP_031398793.1 | 1873             | Chr5                | 563             | 9.31 | 61.69    | Vacuolar membrane        |
| <i>PgLAC39</i> | LOC116207137 | XP_031395863.1 | 1816             | Chr5                | 571             | 8.76 | 63.02    | Chloroplast              |
| <i>PgLAC40</i> | LOC116209617 | XP_031399176.1 | 1813             | Chr5                | 568             | 9.12 | 62.73    | Chloroplast              |
| <i>PgLAC41</i> | LOC116209616 | XP_031399174.1 | 1853             | Chr5                | 568             | 9.4  | 62.76    | Chloroplast              |
| <i>PgLAC42</i> | LOC116209508 | XP_031399023.1 | 1874             | Chr5                | 595             | 5.08 | 67.17    | Chloroplast              |
| <i>PgLAC43</i> | LOC116209509 | XP_031399024.1 | 1876             | Chr5                | 595             | 4.94 | 67.03    | Vacuolar membrane        |
| <i>PgLAC44</i> | LOC116209332 | XP_031398797.1 | 1784             | Chr5                | 512             | 4.77 | 57.58    | Cytosol                  |
| <i>PgLAC45</i> | LOC116209332 | XP_031398799.1 | 1812             | Chr5                | 506             | 5.24 | 57.14    | Chloroplast              |
| <i>PgLAC46</i> | LOC116209332 | XP_031398796.1 | 1739             | Chr5                | 531             | 4.77 | 60.24    | Cytoskeleton             |
| <i>PgLAC47</i> | LOC116209332 | XP_031398795.1 | 1910             | Chr5                | 588             | 5.1  | 66.57    | Cytosol                  |
| <i>PgLAC48</i> | LOC116209332 | XP_031398794.1 | 1925             | Chr5                | 593             | 4.86 | 67.00    | Peroxisome               |
| <i>PgLAC49</i> | LOC116209566 | XP_031399095.1 | 1812             | Chr5                | 585             | 4.87 | 65.04    | Vacuolar membrane        |
| <i>PgLAC50</i> | LOC116210060 | XP_031399722.1 | 1857             | Chr6                | 578             | 8.6  | 63.75    | Chloroplast              |
| <i>PgLAC51</i> | LOC116213035 | XP_031403701.1 | 1851             | Chr7                | 593             | 7.29 | 65.69    | Chloroplast              |
| <i>PgLAC52</i> | LOC116189420 | XP_031374936.1 | 1708             | Chr8                | 481             | 6.41 | 52.59    | Nucleus                  |
| <i>PgLAC53</i> | LOC116189420 | XP_031374934.1 | 1808             | Chr8                | 576             | 6.78 | 63.14    | Chloroplast              |
| <i>PgLAC54</i> | LOC116187280 | XP_031371794.1 | 1855             | Chr8                | 607             | 5.5  | 68.71    | Chloroplast              |
| <i>PgLAC55</i> | LOC116189029 | XP_031374373.1 | 1596             | Chr8                | 603             | 5.05 | 67.58    | Extracellular            |
| <i>PgLAC56</i> | LOC116189029 | XP_031374374.1 | 1910             | Chr8                | 543             | 4.91 | 61.46    | Cytosol                  |
| <i>PgLAC57</i> | LOC116189768 | XP_031375356.1 | 1762             | Unplaced Scaffold   | 564             | 8.54 | 63.07    | Peroxisome               |

Subsequently, the amino acid sequences of the above-mentioned 13 *PgLACs* were aligned with the LACs of other species. The results showed that the *PgLAC* proteins had higher similarity with other LAC proteins and contained three Cu oxidase domains, namely, Cu-oxidase, Cu-oxidase\_2, and Cu-oxidase\_3 (Figure 4).





**Figure 4.** Alignment analysis of PgLAC proteins with other LAC proteins. PgLACs from *P. granatum*, AtLACs from *A. thaliana*, PtLACs from *P. trichocarpa*, CsLACs from *C. reticulata*, and SmLACs from *S. melongena*.

In addition, the LAC family in other species was investigated and listed (Table 2). The LACs were clustered into five or six subfamilies, except eight subfamilies from *S. miltiorrhiza* and *Solanum melongena*. Group I and V from most species contained more LACs. However, *P. granatum* and *S. melongena* had the most members in Group VI and VIII, respectively. A total of 93 LACs were identified from *Glycine max*, ranking first. Secondly, 65 LACs were obtained from *S. miltiorrhiza*. Among four fruit tree plants, *P. granatum* had 57 LACs, *P. persica* for 48, *P. bretschneideri* for 41, and *C. sinensis* for 27. At present, the number of LACs from *A. thaliana* is less, only 17. Based on the phylogenetic relationships,

the 57 PgLACs can be divided into six subfamilies (I–VI), and Group I contained 7 members, 6 members in Group II each contained, 1 member in Group III, 4 members in Group IV, 5 members in Group V, and 34 members in Group VI.

**Table 2.** LACs characteristics from different plant species.

| Species                     | I  | II | III | IV | V  | VI | VII | VIII | Total | Reference  |
|-----------------------------|----|----|-----|----|----|----|-----|------|-------|------------|
| <i>Arabidopsis thaliana</i> | 2  | 4  | 4   | 3  | 3  | 1  | 0   | 0    | 17    | [11]       |
| <i>Camellia sinensis</i>    | 7  | 6  | 4   | 12 | 12 | 2  | 0   | 0    | 43    | [16]       |
| <i>Citrus sinensis</i>      | 9  | 7  | 3   | 1  | 6  | 0  | 1   | 0    | 27    | [14]       |
| <i>Glycine max</i>          | 15 | 13 | 8   | 8  | 49 | 0  | 0   | 0    | 93    | [28]       |
| <i>Oryza sativa</i>         | 7  | 2  | 5   | 5  | 11 | 0  | 0   | 0    | 30    | [10]       |
| <i>Panicum virgatum</i>     | 9  | 4  | 8   | 9  | 19 | 0  | 0   | 0    | 49    | [29]       |
| <i>Populus trichocarpa</i>  | 12 | 12 | 6   | 12 | 6  | 5  | 0   | 0    | 53    | [12]       |
| <i>Prunus persica</i>       | 14 | 4  | 1   | 12 | 17 | 0  | 0   | 0    | 48    | [13]       |
| <i>Punica granatum</i>      | 7  | 6  | 1   | 4  | 5  | 34 | 0   | 0    | 57    | This study |
| <i>Pyrus bretschneideri</i> | 10 | 10 | 2   | 11 | 1  | 7  | 0   | 0    | 41    | [15]       |
| <i>Salvia miltiorrhiza</i>  | 9  | 3  | 2   | 5  | 10 | 32 | 1   | 3    | 65    | [30]       |
| <i>Solanum melongena</i>    | 4  | 7  | 8   | 4  | 1  | 1  | 3   | 20   | 48    | [17]       |
| <i>Sorghum bicolor</i>      | 4  | 3  | 4   | 8  | 8  | 0  | 0   | 0    | 27    | [31]       |

### 3.4. Motif Distribution and Exon/Intron Analysis of PgLAC Family Members

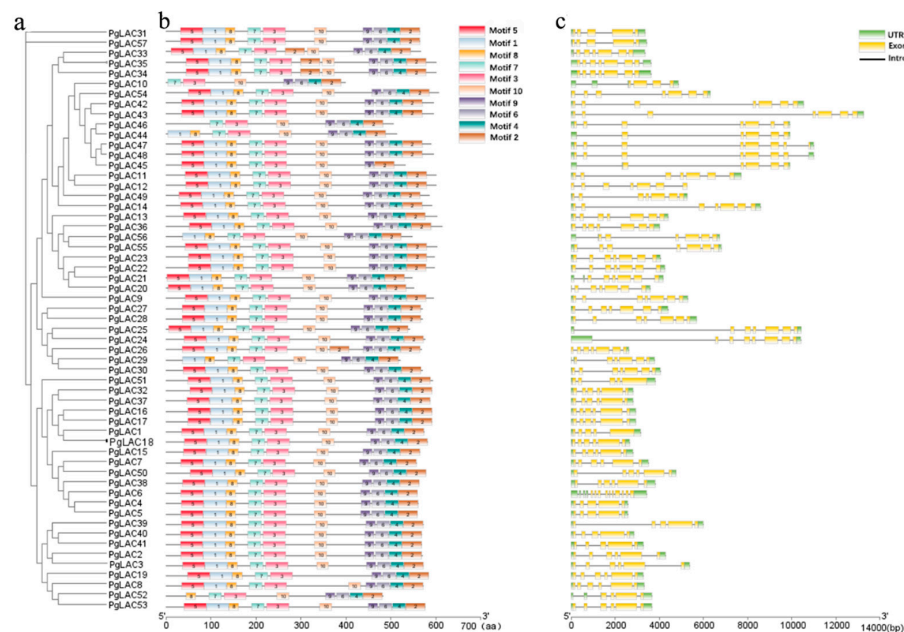
The MEME result showed that 10 conserved motifs were presented in PgLACs (Figure 5). The length of the 10 motifs was 21–50 aa, and the motif sequences were provided in Figure S1, which encoded multicopper oxidase and belonged to typical plant laccases. Among them, motif1, motif5, motif8 encoded multicopper Cu-oxidase\_3; motif3 and motif7 encoded multicopper Cu-oxidase; motif2, motif4, motif6, and motif9 encoded multicopper Cu-oxidase\_2. As shown in Figure 5, 50 PgLACs all contained the 10 motifs, and most PgLACs ended with the order of motif9, motif6, motif4, and motif2 except PgLAC45, suggesting that PgLAC gene members possessed relatively conserved sequences. Additionally, among 57 members of PgLAC family, PgLAC26, PgLAC33, PgLAC34, and PgLAC35 all contained one more motif 2; motif 5 did not occur in PgLAC10, PgLAC29, PgLAC44, PgLAC46, PgLAC52, and PgLAC56; PgLAC10 and PgLAC46 also missed motif 1 and motif 8. Therefore, the motif distribution displayed the specificity of the gene structure of PgLACs, perhaps resulting in different functions. To better understand the structural characteristics of PgLAC genes, their exon–intron structures were explored. As shown in Figure 5, 57 PgLACs exhibited diverse intron/exon patterns, and the number of exons ranged from 4 to 10. Among PgLACs, 23 and 20 PgLACs contained 7 and 6 exons, respectively.

### 3.5. Analysis of Cis-Acting Elements in PgLACs Promoters

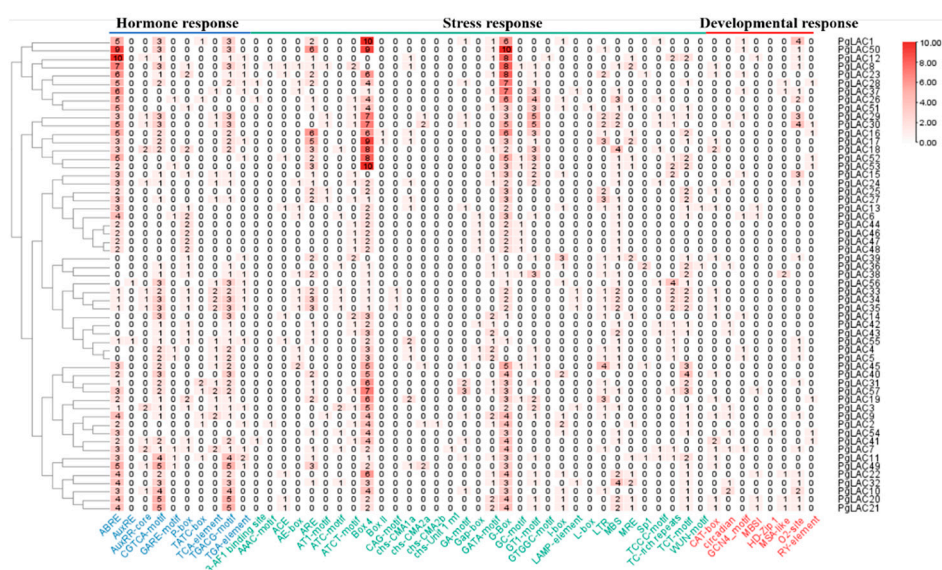
The cis-acting elements were obtained from the 2000-bp upstream sequence of PgLACs, so as to investigate the possible function of PgLACs. As shown in Figure 6, in PgLACs promoters were observed 51 cis-acting elements involving hormone response, stress response, and development response. Among hormone-responsive elements, the abscisic acid responsiveness element (ABRE) was the most common and appeared on the upstream sequences of 48 PgLACs, reaching a maximum of 10 on the upstream sequences of PgLAC12. The second one was MeJA responsiveness elements (CGTCA-motif and TGACG-motif), which existed on the upstream sequences of 42 PgLACs with 1–3, furtherly, 5 PgLACs (PgLAC10, PgLAC11, PgLAC20, PgLAC21, and PgLAC49) contained 4 or 5 (Figure 6). Also, other important hormone-responsive elements were discovered, such as salicylic acid responsiveness element (TCA-element), auxin responsiveness elements (TGA-element, AuxRR-core, and AuxRE), and gibberellin responsiveness elements (GARE-motif, P-box, and TATC-box). Thereby, abscisic acid and methyl jasmonate may greatly participate in modulating the expression of PgLACs. Among the cis-acting elements involved in stress response, the light-responsive elements were the most abundant in 53 PgLACs promoters



and G-box in 51 *PgLACs* promoters. In particular, the upstream sequences of *PgLAC1* and *PgLAC53* contained 10 Box 4, respectively, and the upstream sequences of *PgLAC50* had 10 G-box. Meanwhile, the low-temperature responsiveness element (LTR), MBSI (involved in flavonoid biosynthesis), drought stress responsiveness element (MBS), defense and stress responsiveness element (TC-rich repeat), etc., were discovered. Regarding plant development, eight *cis*-acting elements were detected on the upstream sequences of *PgLACs*. O<sub>2</sub>-site involved in zein metabolism regulation was the most common, existing in 22 *PgLACs* promoters, while the least for HD-Zip 1, only 1 in *PgLAC50* promoter (Figure 6).



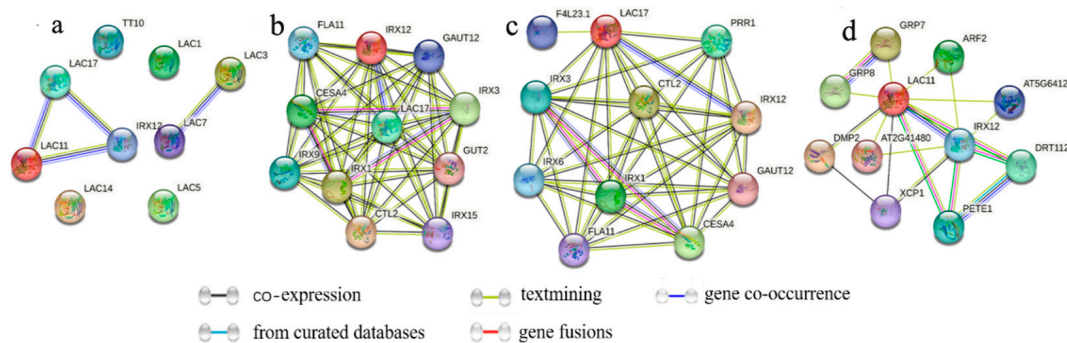
**Figure 5.** Motif structure and exon/intron analysis of *LAC* family members from pomegranate. (a) The phylogenetic tree of 57 pomegranate laccase proteins. (b) motif distribution. (c) exon/intron analysis. The scales at the bottom of the image indicated motif length (amino acid numbers) and the estimated exon/intron length (bp). Yellow box indicated exons, while black lines indicated introns.



**Figure 6.** *Cis*-acting elements analysis on the promoter of *PgLACs*. The numbers represented the number of *cis*-acting elements in each *PgLAC* promoter. The blue represents the hormone response, the green the stress response, and the red for the developmental response.

### 3.6. Analysis of Protein Interaction Networks

The co-expression of 57 PgLAC proteins was predicted using the String protein interaction database; *A. thaliana* was the model species. The stronger reaction between the two proteins, the thicker the linkage line. As Figure 7a shown, PgLAC proteins were identical to AtLAC1, AtLAC3, AtLAC5, IRX12 (AtLAC4), AtLAC7, AtLAC11, AtLAC14, TT10 (AtLAC15), and AtLAC17, respectively. Also, IRX12, AtLAC11, and AtLAC17 had high homology and co-expressed. PgLAC4, PgLAC5, PgLAC6, PgLAC7, PgLAC38, and PgLAC50 were identical to IRX12 and co-expressed with fasciclin-like arabinogalactan-protein FLA11 which was related to secondary cell-wall cellulose synthesis, and galacturonosyltransferase GAUT12 which involved in pectin assembly (Figure 7b). PgLAC1, PgLAC16, PgLAC17, PgLAC18, PgLAC32, and PgLAC37 were identical to AtLAC17, and co-expressed with IRX12, chitinase-like protein CTL2, cellulose synthase A catalytic subunit 4 (CESA4), and cellulose synthase A catalytic subunit 8 (IRX1) (Figure 7c). PgLAC15 was identical to AtLAC11 and co-expressed with DMP2, which is involved in membrane remodeling and xylem cysteine peptidase XCP1 (Figure 7d).

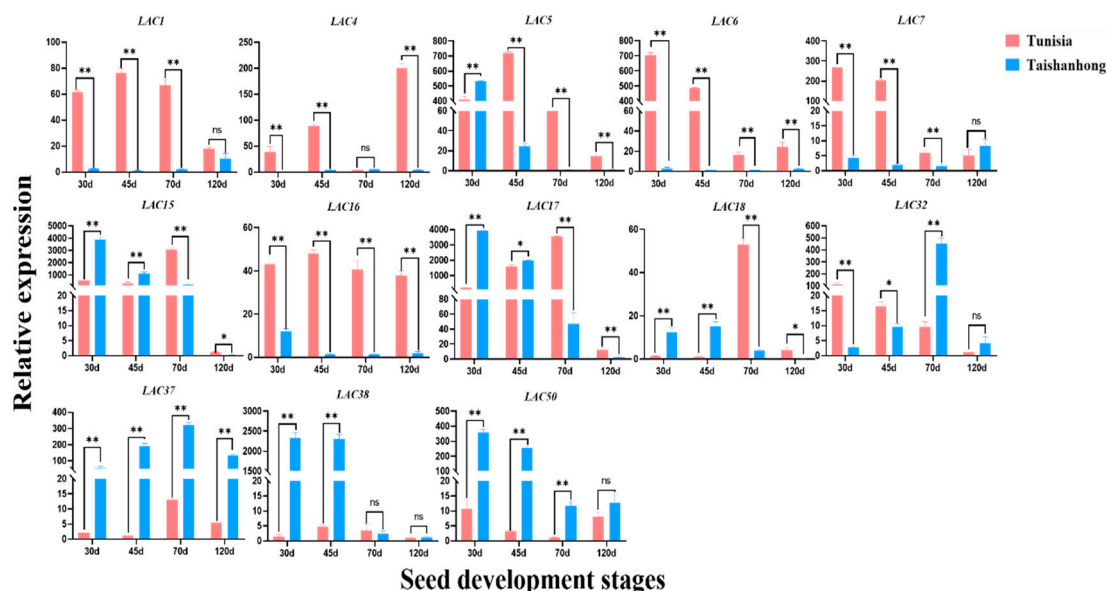


**Figure 7.** Analysis of interaction networks of PgLAC proteins (a); PgLAC4, PgLAC5, PgLAC6, PgLAC7, PgLAC38 and PgLAC50 (b); PgLAC1, PgLAC16, PgLAC17, PgLAC18, PgLAC32 and PgLAC37 proteins (c); PgLAC15 protein (d). CESA4: cellulose synthase A catalytic subunit 4; CTL2: chitinase-like protein 2; GAUT12: galacturonosyltransferase 12; IRX1: cellulose synthase A catalytic subunit 8; IRX15: irregular xylem protein; IRX3: cellulose synthase A catalytic subunit 7; XCP1: xylem cysteine peptidase 1; DMP2: transmembrane protein.

### 3.7. Expression Profiles of PgLACs during the Seed Development

To clarify the potential function of PgLACs, the expression level of 13 PgLACs (PgLAC1, PgLAC4, PgLAC5, PgLAC6, PgLAC7, PgLAC15, PgLAC16, PgLAC17, PgLAC18, PgLAC32, PgLAC37, PgLAC38, and PgLAC50) was assessed during four seed development stages of soft/hard-seed pomegranate using RT-PCR method. The PgLACs exhibited different expression levels during seed development; importantly, a significant difference in expression levels was found between soft- and hard-seed pomegranates (Figure 8). And, the expression of most PgLACs was higher during the earlier stage of seed development (30–70 d after full flowering) than at the later stage of seed development (120 d after full flowering) between the two pomegranate cultivars except PgLAC4. Moreover, 5 PgLACs (PgLAC1/7/32/38/50) had not a significant difference in the gene expression level at 120 d after full flowering, which demonstrated that the formation of seed hardness depended on the earlier stage of seed development. Meanwhile, during the earlier seed development stage, PgLAC1 and PgLAC7 expressed at a significantly higher level in soft-seed ‘Tunisia’ than in hard-seed ‘Taishanhong’; on the contrary, PgLAC50 displayed a significantly higher expression in ‘Taishanhong’ than in ‘Tunisia’ ( $p < 0.01$ ). Similarly, during the whole seed development, PgLAC6 and PgLAC16 expressed at a significantly higher level in ‘Tunisia’ than in ‘Taishanhong’, while the expression of PgLAC37 always kept a significantly higher level in ‘Taishanhong’ than in ‘Tunisia’ pomegranate ( $p < 0.01$ ) (Figure 8). Therefore, it was inferred that the soft-seed development might be a close relationship with PgLAC1, PgLAC6,

*PgLAC7*, and *PgLAC16*; correspondingly, *PgLAC37* and *PgLAC50* may greatly participate in hard-seed development in pomegranate. In addition, the peak of gene expression at 30 d, 45 d, and 70 d after full flowering each appeared 4 *PgLACs* gene in ‘Tunisia’, while in ‘Taishanhong’, 7 *PgLACs* at 30 d after full flowering, and 3 *PgLACs* at 70 d and 2 *PgLACs* at 120 d after full flowering. Collectively, Combined with Figure 1, the earlier stage of seed development was the key to the formation of seed hardness and lignin accumulation for hard-seed pomegranate.



**Figure 8.** Gene expression profiles of *PgLACs* during seed development stage. \* and \*\* indicated significant differences between the two cultivars at  $p < 0.05$  and  $p < 0.01$ , respectively.

#### 4. Discussion

In general, hard-seed pomegranate cultivars are more resistant to cold stress, whereas soft-seed ones are more popular with consumers due to easily swallowed seeds [32,33]. Seed hardness has become the first preference for more customers. In China, ‘Taishanhong’ (hard-seed) and ‘Tunisia’ (soft-seed) are widely cultivated pomegranate cultivars, which play an important role in promoting the pomegranate industry. In the present study, it was found that seed hardness and lignin content both increased steadily in ‘Taishanhong’ and ‘Tunisia’ seeds as seed development, with a significantly lower level in ‘Tunisia’ seeds than in ‘Taishanhong’ ones. Furthermore, lignin content at 45 d after full flowering increased by 7.7% than 30 d after full flowering, whereas that at 120 d after full flowering increased by 3.3% than 70 d after full flowering; thereby, more lignin deposition may be conducted at the earlier stage of seed formation. Similarly, Niu et al. also proved that the soft-seeded variety contained lower lignin at the early fruit developmental stage [34]. To explore the formation of seed hardness is essential to breed new soft-seed pomegranate germplasm, furtherly, other fruit trees.

Plant laccase enzymes belong to copper-containing polyphenol oxidase and polymerize monolignols into lignin, participating in lignin biosynthesis and various abiotic/biotic stresses [14,35]. Laccases have a multiple-gene family, ranging from 17 (*Arabidopsis*) to 94 members (soybean) [11,28]. In the current study, 57 laccase genes were identified from the *P. granatum* genome, and 41 of 57 *PgLACs* distributed Chr 3, Chr 4, and Chr5. The putative sequences of amino acid ranged from 397 aa (*PgLAC10*) to 614 aa (*PgLAC36*), and the large difference also presented 367–1559 aa in peach, 133–595 aa in citrus, and 485–1136 aa in pear [13–15]. Most *PgLACs* belong to alkaline pI, similar to *PbLACs* [15]. Most *PgLAC* proteins were located on chloroplast with 21 vacuolar membranes and cytosol, each with 11 by the predicted subcellular localization. They also contained the three typical conserved Cu-oxidase domains and had high homology with *A. thaliana*, *P. trichocarpa*,



*C. reticulata*, and *S. melongena*. The exon number of 57 *Pg*LACs varied from 4 to 10, and for other horticultural plants, 3–6 exons for citrus [14], 4–12 exons for pear [15], and 1–13 exons for eggplant [17]. The comparison of exons among species indicated diverse functions in pomegranate laccases.

The proteins interaction networks of 57 *Pg*LAC showed *Pg*LAC1, *Pg*LAC16, *Pg*LAC17, *Pg*LAC18, *Pg*LAC32, and *Pg*LAC37 were co-expressed with CTL2, IRX1, and IRX12 involved in cell wall biosynthesis, further supporting the involvement of the *Pg*LACs in lignin biosynthesis. A previous study reported that the three *Arabidopsis* LACs (*At*LAC4, *At*LAC17, and *At*LAC11) were responsible for lignin polymerization [23]. The phylogenetic tree was constructed and showed that 57 *Pg*LACs were divided into six groups, seven *Pg*LACs (*Pg*LAC4, 5, 6, 7, 15, 38, and 50) in Group I with *At*LAC4 and *At*LAC11, and six *Pg*LACs (*Pg*LAC1, 16, 17, 18, 32, and 37) in Group II along with *At*LAC17. The results implied the 13 *Pg*LACs may possess similar functions to the *Arabidopsis* LACs. Therefore, the expression profiles of the 13 *Pg*LACs were investigated during the four seed development stages. We provided the evidence that most *Pg*LACs expressed at a higher level during the earlier stage of seed development than at the later stage of seed development in the two pomegranate cultivars except *Pg*LAC4, which was in line with higher lignin content at the earlier seed development stage. However, the distinct difference in the gene expression levels of individual *Pg*LAC existed between cultivars, higher *Pg*LAC1, *Pg*LAC6, *Pg*LAC7, and *Pg*LAC16 in the soft-seed cultivar, whereas higher *Pg*LAC37 and *Pg*LAC50 in the hard-seed pomegranate cultivar.

G-box widely presents on the promoter of many plant genes with a palindromic DNA motif of CACGTG [35]. Previous reports found that G-box participated in the co-expression system for nuclear photosynthetic genes and influenced organ differentiation [36]. In the current study, the gene expression of *Pg*LAC37 and *Pg*LAC50 during the first 70 d after full flowering displayed higher with a significant difference in ‘Taishanhong’ than in ‘Tunisia’; further, the more G-box elements presented in *Pg*LAC37 with 7 G-box and *Pg*LAC50 with 10 ones, suggesting that the two genes were the key candidate gene for the formation of the hard seed in pomegranate. Similarly, more ABRE elements involved in abscisic acid (ABA) responsiveness were also observed in the *Pg*LAC37 (6 ABRE) and *Pg*LAC50 (9 ABRE). ABA is involved in secondary cell-wall formation [37], and the late-wood formation of *Pinus radiata* and *Pinus sylvestris* is correlated with an increase in ABA concentration [38,39]. Taken together, the G-box and ABRE elements in the promoters of *Pg*LAC37 and *Pg*LAC50 may be an essential reason for modulating the higher expression of *Pg*LAC37 and *Pg*LAC50 during the earlier seed development stage of ‘Taishanhong’ pomegranate, thus, the two genes greatly participated in seed formation and accelerated seed maturity.

Gibberellin (GA) is a primarily growth-regulating phytohormone and regulates diverse biological processes. Previous studies revealed that GA induced berry seedless and regulated flower development, berry set, expansion, and ripening in grapes [40–42]. TATC-box and GARE-motif are known to both respond to GA. Interestingly, we found TATC-box in only *Pg*LAC1 promotor and GARE-motif in the promoters of *Pg*LAC6 and *Pg*LAC7, as well as *Pg*LAC4 and *Pg*LAC5 among 13 *Pg*LACs. Collectively, higher expression of *Pg*LAC1/4/6/7 in ‘Tunisia’ may be induced by GA and then produce softer seeds. The five *Pg*LACs played an indispensable role in the formation of softer seeds. RY-element is found predominantly in seed-specific promoters [43] and mediates repression of embryo mid-maturation genes involved in the accumulation of storage compounds [44]. In the current study, RY-element was observed only in the *Pg*LAC16 promotor, which suggests that the dramatically higher expression of *Pg*LAC16 may inhibit the accumulation of storage compound during the seed development stage, thus hindering the formation of seed hardness in soft-seed pomegranate. In conclusion, *Pg*LAC1/4/6/7/16 with higher expression in ‘Tunisia’ will greatly contribute to exploring the soft seed formation, which is potential candidate gene for breeding soft-seed pomegranate with GA application.

## 5. Conclusions

Laccase is the key enzyme on the lignin biosynthesis pathway, closely correlated with seed hardness. The LAC family was first identified from the pomegranate genome. A total of 57 *PgLACs* were divided into six groups containing typical Cu-oxidase domains. Exon-intron structure and motif analysis predicted that the *PgLACs* had diverse functions in lignin biosynthesis. Combined with *cis*-acting elements and the gene expression patterns, *PgLAC37* and *PgLAC50* were the key candidate genes for the formation of hard seed in pomegranate, attributed to more G-box and ABRE elements in their promoters, which regulated the expression of *PgLAC37* and *PgLAC50*, participated in seed formation, accelerated seed maturity, finally, produced harder seed. In soft-seed pomegranate, higher expression of *PgLAC1/4/6/7* may contribute to soft-seed formation responsive to GA via GARE motif and TATC-box. And the *PgLAC16* promoter containing RY-element may regulate soft seed development by reducing the accumulation of storage compounds in seeds. Collectively, the results of our study will provide important gene information and a new perspective for breeding hard- and soft-seed pomegranate cultivars.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae9080918/s1>, Figure S1: The sequence information for each motif (Motif 1–Motif 10); Figure S2: Segmental duplication of the *PgLAC* genes in pomegranate; Table S1: LACs information in the phylogenetic tree; Table S2: The primers used in qRT-PCR; Table S3: Segmental duplication pairs of the *PgLAC* genes within the pomegranate genome; Table S4: The identified tandem duplication pairs of *PgLAC* genes.

**Author Contributions:** Conceptualization, J.S. and J.Y.; formal analysis, R.T. and S.W.; visualization, J.Y., R.T., S.W. and M.L.; investigation, J.Y., R.T. and R.W.; resources, C.S. and J.J.; writing—original draft, J.S. and J.Y.; writing—review and editing, J.S., J.Y. and X.Z. All authors have read and agreed to the published version of the manuscript.

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## References

1. Yuan, Z.; Fang, Y.; Zhang, T.; Fei, Z.; Han, F.; Liu, C.; Min, L.; Xiao, W.; Zhang, W.; Wu, S.; et al. The pomegranate (*Punica granatum* L.) genome provides insights into fruit quality and ovule developmental biology. *Plant Biotechnol. J.* **2017**, *7*, 1363–1374. [CrossRef] [PubMed]
2. Bishayee, A.; Mandal, A.; Bhattacharyya, P.; Bhatia, D. Pomegranate exerts chemoprevention of experimentally induced mammary tumorigenesis by suppression of cell proliferation and induction of apoptosis. *Nutr. Cancer* **2016**, *68*, 120–130. [CrossRef] [PubMed]
3. Karimi, M.; Sadeghi, R.; Kokini, J. Pomegranate as a promising opportunity in medicine and nanotechnology. *Trends Food Sci. Technol.* **2017**, *69*, 59–73. [CrossRef]
4. Kandyli, P.; Kokkinomagoulos, E. Food applications and potential health benefits of pomegranate and its derivatives. *Foods* **2020**, *9*, 122. [CrossRef]
5. Ambigaipalan, P.; de Camargo, A.C.; Shahidi, F. Phenolic compounds of pomegranate byproducts (outer skin, mesocarp, divider membrane) and their antioxidant activities. *J. Agric. Food Chem.* **2016**, *64*, 6584–6604. [CrossRef]
6. Morittu, V.M.; Mastellone, V.; Tundis, R.; Loizzo, M.R.; Tudisco, R.; Figoli, A.; Cassano, A.; Musco, N.; Britti, D.; Infascelli, F.; et al. Antioxidant, biochemical, and in-life effects of *Punica granatum* L. natural juice vs. clarified juice by polyvinylidene fluoride membrane. *Foods* **2020**, *9*, 242. [CrossRef]
7. Zarei, A.; Zamani, Z.; Fatahi, R.; Mousavi, A.S.; Seyed, A. A mechanical method of determining seed-hardness in pomegranate. *J. Crop Improv.* **2013**, *27*, 444–459. [CrossRef]
8. Xue, H.; Cao, S.; Li, H.; Zhang, J.; Niu, J.; Chen, L.; Zhang, F.; Zhao, D. De novo transcriptome assembly and quantification reveal differentially expressed genes between soft-seed and hard-seed pomegranate (*Punica granatum* L.). *PLoS ONE* **2017**, *12*, e0178809. [CrossRef]
9. Zarei, A.; Zamani, Z.; Fatahi, R.; Mousavi, A.; Salami, S.A.; Avila, C.; Canovas, F.M. Differential expression of cell wall related genes in the seeds of soft- and hard-seeded pomegranate genotypes. *Sci. Hortic.* **2016**, *205*, 7–16. [CrossRef]

10. Liu, Q.; Luo, L.; Wang, X.; Shen, Z.; Zheng, L. Comprehensive analysis of rice laccase gene (*OsLAC*) family and ectopic expression of *OsLAC10* enhances tolerance to copper stress in *Arabidopsis*. *Int. J. Mol. Sci.* **2017**, *18*, 209. [\[CrossRef\]](#)
11. Turlapati, P.V.; Kim, K.W.; Davin, L.B.; Lewis, N.G. The laccase multigene family in *Arabidopsis thaliana*: Towards addressing the mystery of their gene function(s). *Planta* **2011**, *233*, 439–470. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Liao, B.; Wang, C.; Li, X.; Man, Y.; Ruan, H.; Zhao, Y. Genome-wide analysis of the *Populus trichocarpa* laccase gene family and functional identification of *PtLAC23*. *Front. Plant Sci.* **2023**, *13*, 1063813. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Qui, K.; Zhou, H.; Pan, H.; Sheng, Y.; Yu, H.; Xie, Q.; Chen, H.; Cai, Y.; Zhang, J.; He, J. Genome-wide identification and functional analysis of the peach (*P. persica*) laccase gene family reveal members potentially involved in endocarp lignification. *Trees* **2022**, *36*, 1477–1496. [\[CrossRef\]](#)
14. Xu, X.; Zhou, Y.; Wang, B.; Ding, L.; Wang, Y.; Luo, L.; Zhang, Y.; Kong, W. Genome-wide identification and characterization of laccase gene family in *Citrus sinensis*. *Gene* **2018**, *689*, 114–123. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Cheng, X.; Li, G.; Ma, C.; Abdullah, M.; Zhang, J.; Zhao, H.; Qing, J.; Cai, Y.; Lin, Y. Comprehensive genome-wide analysis of the pear (*Pyrus bretschneideri*) laccase gene (*PbLAC*) family and functional identification of *PbLAC1* involved in lignin biosynthesis. *PLoS ONE* **2019**, *14*, e0210892. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Yu, Y.; Xing, Y.; Liu, F.; Zhang, X.; Li, X.; Zhang, J.; Sun, X. The laccase gene family mediate multi-perspective trade-offs during tea plant (*Camellia sinensis*) development and defense processes. *Int. J. Mol. Sci.* **2021**, *22*, 12554. [\[CrossRef\]](#)
17. Wan, F.; Zhang, L.; Tan, M.; Wang, X.; Wang, G.; Qi, M.; Liu, B.; Gao, J.; Pan, Y.; Wang, Y. Genome-wide identification and characterization of laccase family members in eggplant (*Solanum melongena* L.). *PeerJ* **2022**, *10*, e12922. [\[CrossRef\]](#)
18. Janusz, G.; Pawlik, A.; Świdorska-Burek, U.; Polak, J.; Sulej, J.; Jarosz-Wilkolazka, A.; Paszczyński, A. Laccase properties, physiological functions, and evolution. *Int. J. Mol. Sci.* **2020**, *21*, 966. [\[CrossRef\]](#)
19. Liu, M.; Dong, H.; Wang, M.; Liu, Q. Evolutionary divergence of function and expression of laccase genes in plants. *J. Genet.* **2020**, *99*, 23. [\[CrossRef\]](#)
20. Pourcel, L.; Routaboul, J.M.; Kerhoas, L.; Caboche, M.; Lepiniec, L.; Debeaujon, I. TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *Plant Cell* **2005**, *17*, 2966–2980. [\[CrossRef\]](#)
21. Zaman, F.; Zhang, M.; Liu, Y.; Wang, Z.; Xu, L.; Guo, D.; Luo, Z.; Zhang, Q. DkmR397 regulates proanthocyanidin biosynthesis via negative modulating *DkLAC2* in Chinese PCNA Persimmon. *Int. J. Mol. Sci.* **2022**, *23*, 3200. [\[CrossRef\]](#)
22. Berthet, S.; Demont-Caulet, N.; Pollet, B.; Bidzinski, P.; Cézard, L.; Bris, P.L.; Borrega, N.; Hervé, J.; Blondet, E.; Balzergue, S.; et al. Disruption of *LACCASE4* and 17 results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. *Plant Cell* **2011**, *23*, 1124–1137. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Zhao, Q.; Nakashima, J.; Chen, F.; Yin, Y.; Fu, C.; Yun, J.; Shao, H.; Wang, X.; Wang, Z.; Dixon, R.A.; et al. *LACCASE* is necessary and nonredundant with *PEROXIDASE* for lignin polymerization during vascular development in *Arabidopsis*. *Plant Cell* **2013**, *25*, 3976–3987. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Wang, Y.; Bouchabke-Coussa, O.; Lebris, P.; Antelme, S.; Soulhat, C.; Gineau, E.; Dalmais, M.; Bendahmane, A.; Morin, H.; Mouille, G.; et al. *LACCASE5* is required for lignification of the *Brachypodium distachyon* culm. *Plant Physiol.* **2015**, *168*, 192–204. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Wang, C.; Zhang, S.; Yu, Y.; Luo, Y.; Liu, Q.; Ju, C.; Zhang, Y.; Qu, L.; Lucas, W.J.; Wang, X.; et al. MiR397b regulates both lignin content and seed number in *Arabidopsis* via modulating a laccase involved in lignin biosynthesis. *Plant Biotechnol. J.* **2014**, *12*, 1132–1142. [\[CrossRef\]](#)
26. Wang, X.; Zhuo, C.; Xiao, X.; Wang, X.; Docampo-Palacios, M.; Chen, F.; Dixon, R.A. Substrate specificity of *LACCASE8* facilitates polymerization of caffeoyl alcohol for c-lignin biosynthesis in the seed coat of cleome hassleriana. *Plant Cell* **2020**, *32*, 3825–3845. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* **2001**, *25*, 402–408. [\[CrossRef\]](#)
28. Wang, Q.; Li, G.; Zheng, K.; Zhu, X.; Ma, J.; Wang, D.; Tang, K.; Feng, X.; Leng, J.; Yu, H.; et al. The soybean laccase gene family: Evolution and possible roles in plant defense and stem strength selection. *Genes* **2019**, *10*, 701. [\[CrossRef\]](#)
29. Li, R.; Zhao, Y.; Sun, Z.; Wu, Z.; Wang, H.; Fu, C.; Zhao, H.; He, F. Genome-Wide identification of switchgrass laccases involved in lignin biosynthesis and heavy-metal responses. *Int. J. Mol. Sci.* **2022**, *23*, 6530. [\[CrossRef\]](#)
30. Li, C.; Li, D.; Zhou, H.; Li, J.; Lu, S. Analysis of the laccase gene family and miR397-/miR408-mediated posttranscriptional regulation in *Salvia miltiorrhiza*. *PeerJ* **2019**, *7*, e7605. [\[CrossRef\]](#)
31. Wang, J.; Feng, J.; Jia, W.; Fan, P.; Bao, H.; Li, S.; Li, Y. Genome-Wide identification of sorghum bicolor laccases reveals potential targets for lignin modification. *Front. Plant Sci.* **2017**, *8*, 714. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Shi, J.; Gao, H.; Wang, S.; Wu, W.; Tong, R.; Wang, S.; Li, M.; Jian, Z.; Wan, R.; Hu, Q.; et al. Exogenous arginine treatment maintains the appearance and nutraceutical properties of hard-and soft-seed pomegranates in cold storage. *Front. Nutr.* **2022**, *9*, 828946. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Luo, X.; Li, H.; Wu, Z.; Yao, W.; Zhao, P.; Cao, D.; Yu, H.; Li, K.; Poudel, K.; Zhao, D.; et al. The pomegranate (*Punica granatum* L.) draft genome dissects genetic divergence between soft-and hard-seeded cultivars. *Plant Biotechnol. J.* **2019**, *18*, 955–968. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Niu, J.; Cao, D.; Li, H.; Xue, H.; Chen, L.; Liu, B.; Cao, S. Quantitative proteomics of pomegranate varieties with contrasting seed hardness during seed development stages. *Tree Genet. Genomes* **2018**, *14*, 14. [\[CrossRef\]](#)



35. Giuliano, G.; Pichersky, E.; Malik, V.S.; Timko, M.P.; Scolnik, P.A.; Cashmore, A.R. An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7089–7093. [[CrossRef](#)]
36. Kobayashi, K.; Obayashi, T.; Masuda, T. Role of the G-box element in regulation of chlorophyll biosynthesis in *Arabidopsis* roots. *Plant Signal. Behav.* **2012**, *7*, 922–926. [[CrossRef](#)]
37. Liu, C.; Yu, H.; Rao, X.; Li, L.; Dixon, R.A. Absciscic acid regulates secondary cell-wall formation and lignin deposition in *Arabidopsis thaliana* through phosphorylation of NST1. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, 23. [[CrossRef](#)]
38. Jenkins, P.A.; Shepherd, K.R. Seasonal changes in levels of indole-acetic acid and abscisic acid in stem tissues of *Pinus radiata*. *N. Z. J. Bot.* **1974**, *4*, 511–519.
39. Wodzicki, T.J.; Wodzicki, A.B. Seasonal abscisic acid accumulation in stem cambial region of *Pinus silvestris*, and its contribution to the hypothesis of a late-wood control system in conifers. *Physiol. Plant.* **1980**, *48*, 443–447. [[CrossRef](#)]
40. Cui, M.; Wang, W.; Guo, F.; Fan, X.; Guan, L.; Zheng, T.; Zhu, X.; Jia, H.; Fang, J.; Wang, C.; et al. Characterization and temporal-spatial expression analysis of *LEC1* gene in the development of seedless berries in grape induced by gibberellin. *Plant Growth Regul.* **2020**, *90*, 585–596. [[CrossRef](#)]
41. Cheng, C.; Xu, X.; Singer, S.D.; Li, J.; Zhang, H.; Gao, M.; Wang, L.; Song, J.; Wang, X. Effect of GA3 treatment on seed development and seed related gene expression in grape. *PLoS ONE* **2013**, *8*, e80044. [[CrossRef](#)] [[PubMed](#)]
42. Wang, P.; Xuan, X.; Su, Z.; Wang, W.; Abdelrahman, M.; Jiu, S.; Zhang, X.; Liu, Z.; Wang, X.; Wang, C.; et al. Identification of miRNAs-mediated seed and stone-hardening regulatory networks and their signal pathway of GA-induced seedless berries in grapevine (*V. vinifera* L.). *BMC Plant Biol.* **2021**, *21*, 442. [[CrossRef](#)] [[PubMed](#)]
43. Lelievre, J.M.; Oliveira, L.O.; Nielsen, N.C. 5'-CATGCAT-3' elements modulate the expression of glycinin genes. *Plant Physiol.* **1992**, *98*, 387–391. [[CrossRef](#)] [[PubMed](#)]
44. Guerriero, G.; Martin, N.; Golovko, A.; Sundström, J.F.; Rask, L.; Ezcurra, I. The RY/Sph element mediates transcriptional repression of maturation genes from late maturation to early seedling growth. *New Phytol.* **2009**, *184*, 552–565. [[CrossRef](#)]

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