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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 promotor 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 °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 Kg/cm^2. The seeds were dried at 80 °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 mg/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, 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 < 1×10^{-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/, 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 ($p < 0.01$).

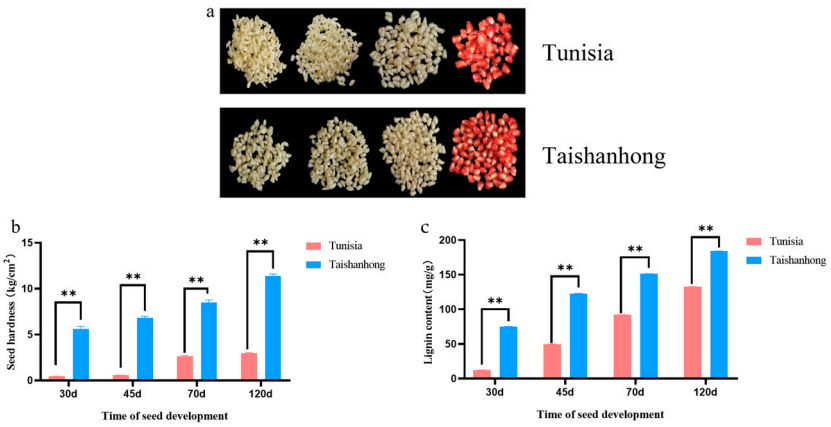

Figure 1. Seed appearance (**a**), seed hardness (**b**), and lignin content (**c**) during four seed development stages from 'Tunisia' and 'Taishanhong'. ** indicated significant differences between groups at $p < 0.01$, respectively.

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.

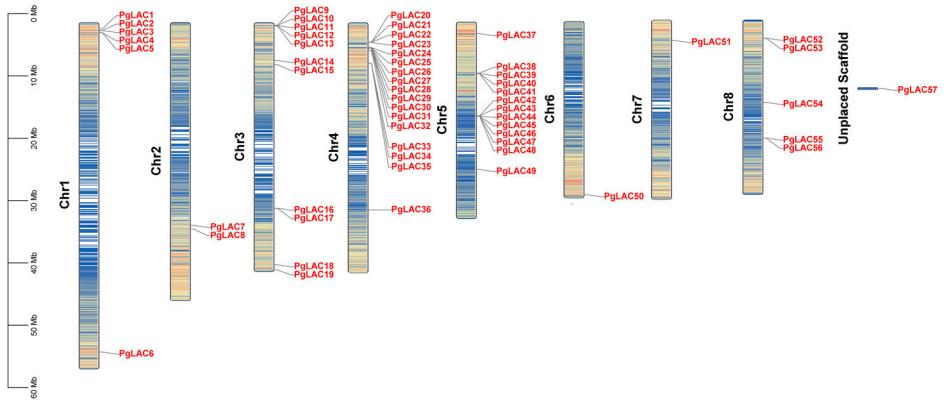

Figure 2. Chromosome distribution of the PgLAC genes. Blue and red represented from less to more of gene density on the chromosome.

Gene duplication was performed in the pomegranate genome to further understand gene evolvement. 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 *PpLAC2/39*, *PgLAC4/7*, *PpLAC7/38*, *PpLAC7/50*, *PpLAC8/19*, *PpLAC8/53*, *PpLAC18/37*, and *PpLAC38/50* (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.

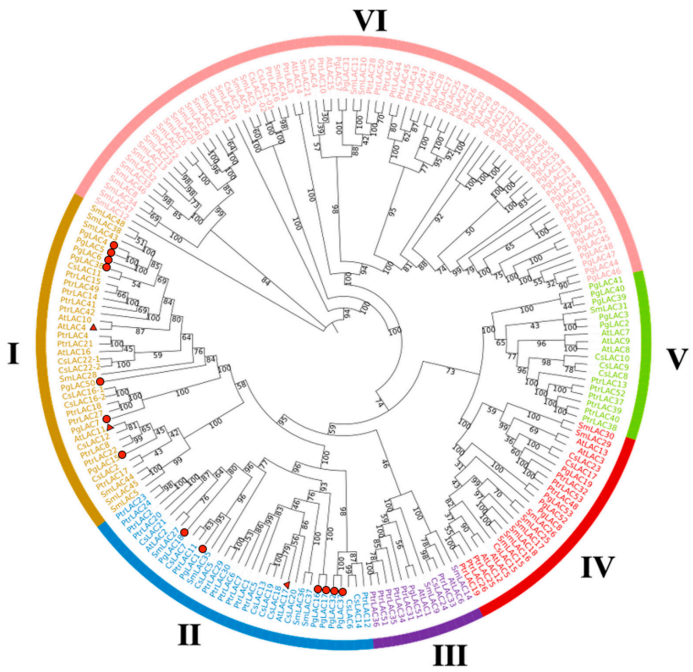

Figure 3. The phylogenetic tree of pomegranate, *Arabidopsis thaliana*, *Populus trichocarpa*, *Citrus reticulata* Blanco, and *Solanum melongena*. PgLACs from *P. granatum*, AtLACs from *A. thaliana*, PtrLACs from *P. trichocarpa*, CsLACs from *C. reticulata*, and SmLACs from S. *melongena*.

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

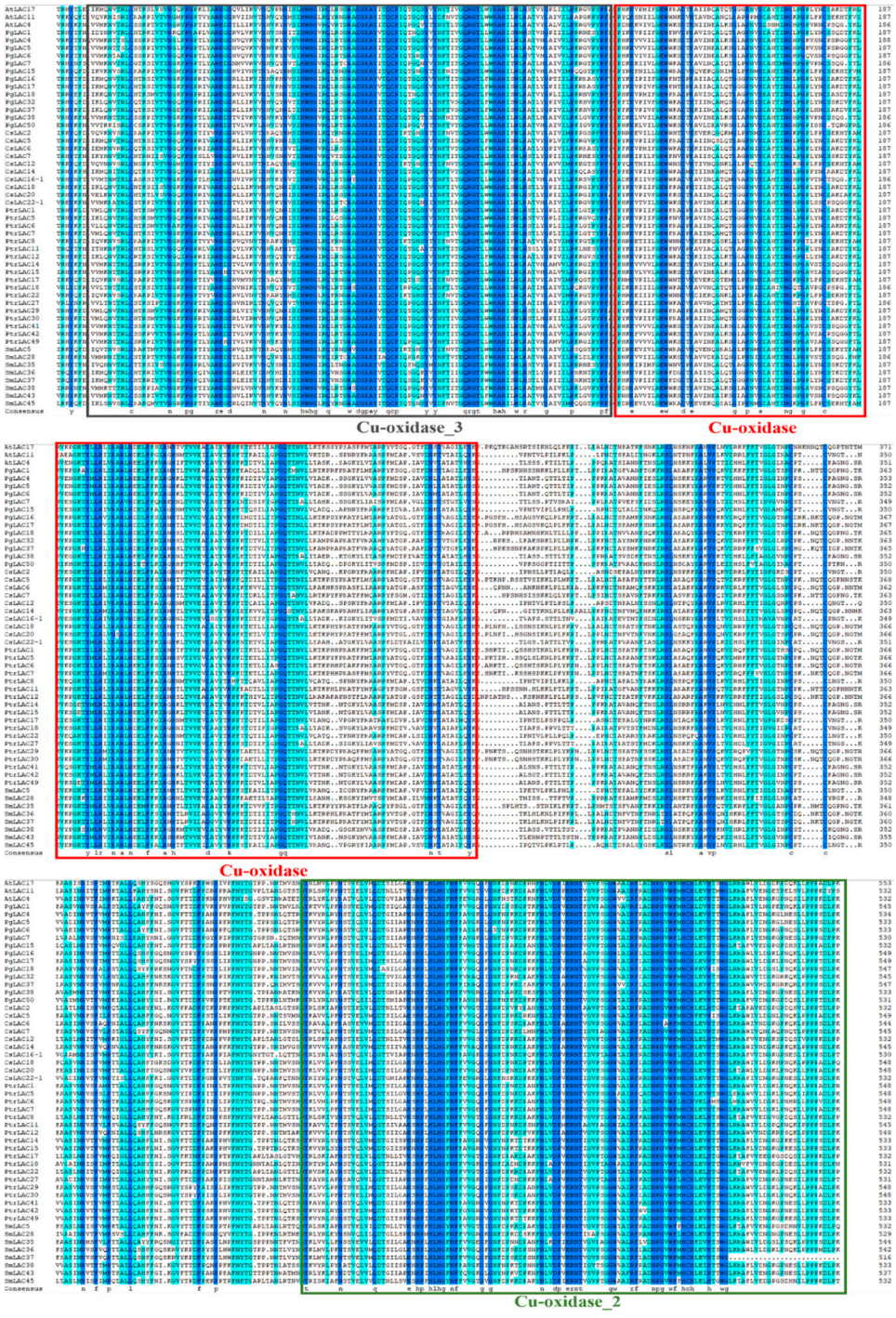

Figure 4. Alignment analysis of PgLAC proteins with other LAC proteins. PgLACs from *P. granatum*, AtLACs from *A. thaliana*, PtrLACs from *P. trichocarpa*, CsLACs from *C. reticulata*, and SmLACs from *S. melongena*.

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
Arabidopsis thaliana	2	4	4	3	3	1	0	0	17	[11]
Camellia sinensis	7	6	4	12	12	2	0	0	43	[16]
Citrus sinensis	9	7	3	1	6	0	1	0	27	[14]
Glycine max	15	13	8	8	49	0	0	0	93	[28]
Oryza sativa	7	2	5	5	11	0	0	0	30	[10]
Panicum virgatum	9	4	8	9	19	0	0	0	49	[29]
Populus trichocarpa	12	12	6	12	6	5	0	0	53	[12]
Prunus persica	14	4	1	12	17	0	0	0	48	[13]
Punica granatum	7	6	1	4	5	34	0	0	57	This study
Pyrus bretschneideri	10	10	2	11	1	7	0	0	41	[15]
Salvia miltiorrhiza	9	3	2	5	10	32	1	3	65	[30]
Solanum melongena	4	7	8	4	1	1	3	20	48	[17]
Sorghum bicolor	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 *PgLAC*s (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 *PgLAC*s all contained the 10 motifs, and most *PgLAC*s 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 Promotors

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* promotors 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* promotors. 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_2-site involved in zein metabolism regulation was the most common, existing in 22 *PgLACs* promotors, while the least for HD-Zip 1, only 1 in *PgLAC50* promotor (Figure 6).

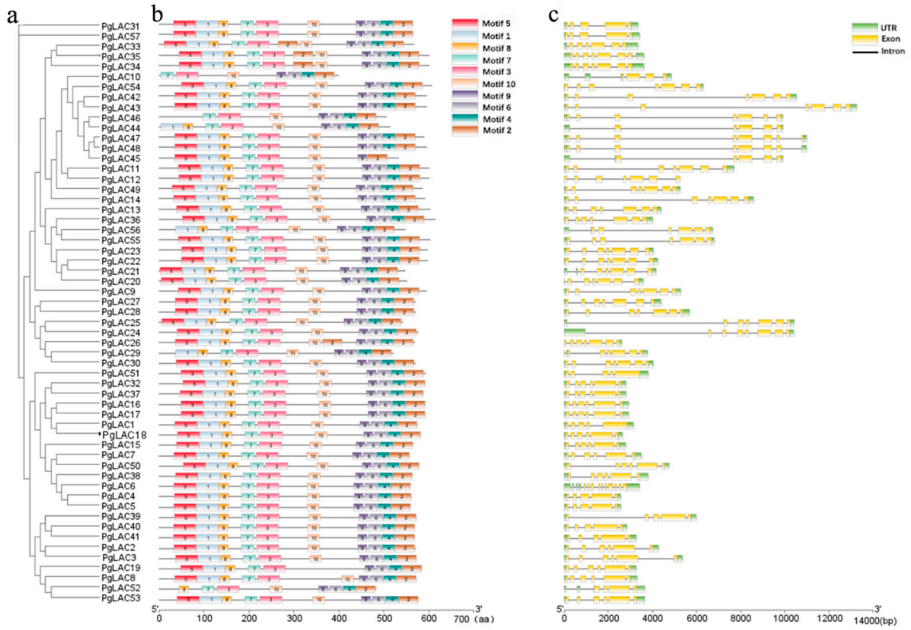

Figure 5. Motif structure and exon/intron analysis of *LAC* family members from pomegranate. (**a**) The phylogenetic tree of 57 pomegranate laccase proteins. (**b**) motif distribution. (**c**) exon/intron analysis. The scales at the bottom of the image indicated motif length (amino acid numbers) and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

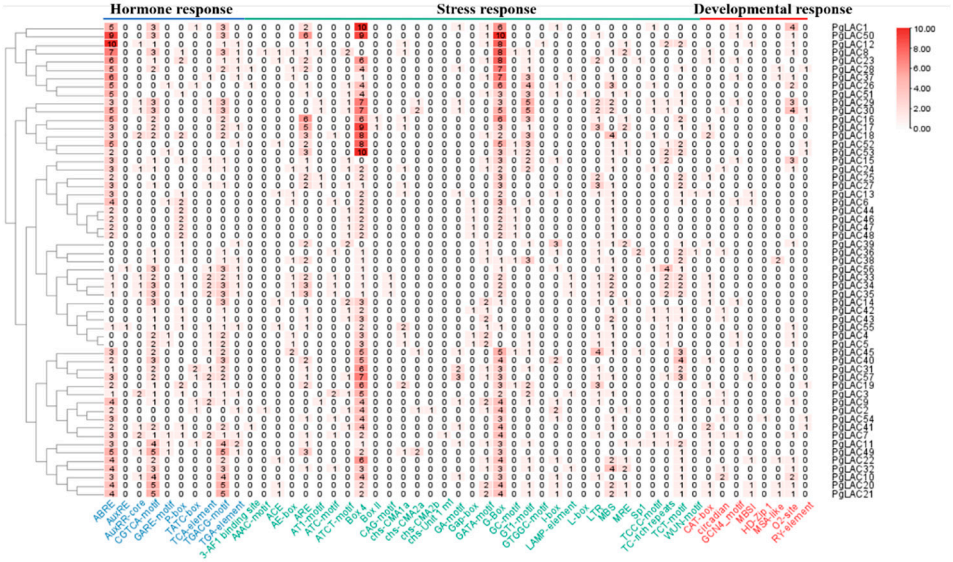

Figure 6. *Cis*-acting elements analysis on the promoter of *PgLACs*. The numbers represented the number of *cis*-acting elements in each *PgLAC* promotor. The blue represents the hormone response, the green the stress response, and the red for the developmental response.

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).

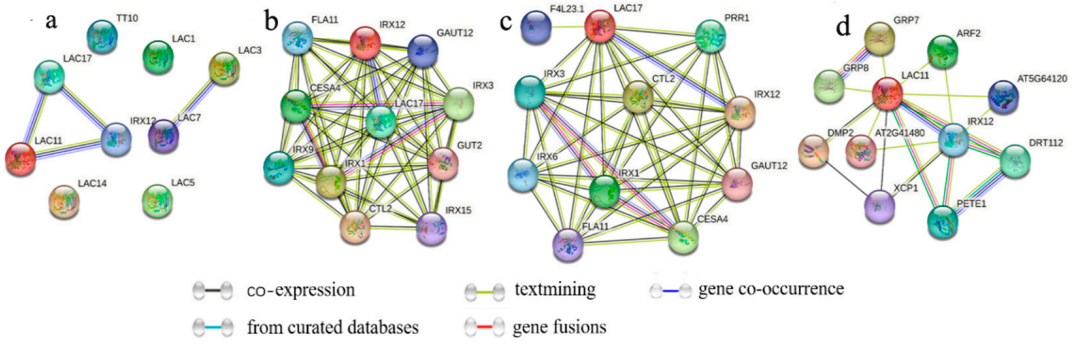

Figure 7. Analysis of interaction networks of PgLAC proteins (**a**); PgLAC4, PgLAC5, PgLAC6, PgLAC7, PgLAC38 and PgLAC50 (**b**); PgLAC1, PgLAC16, PgLAC17, PgLAC18, PgLAC32 and PgLAC37 proteins (**c**); PgLAC15 protein (**d**). CESA4: cellulose synthase A catalytic subunit 4; CTL2: chitinase-like protein 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.

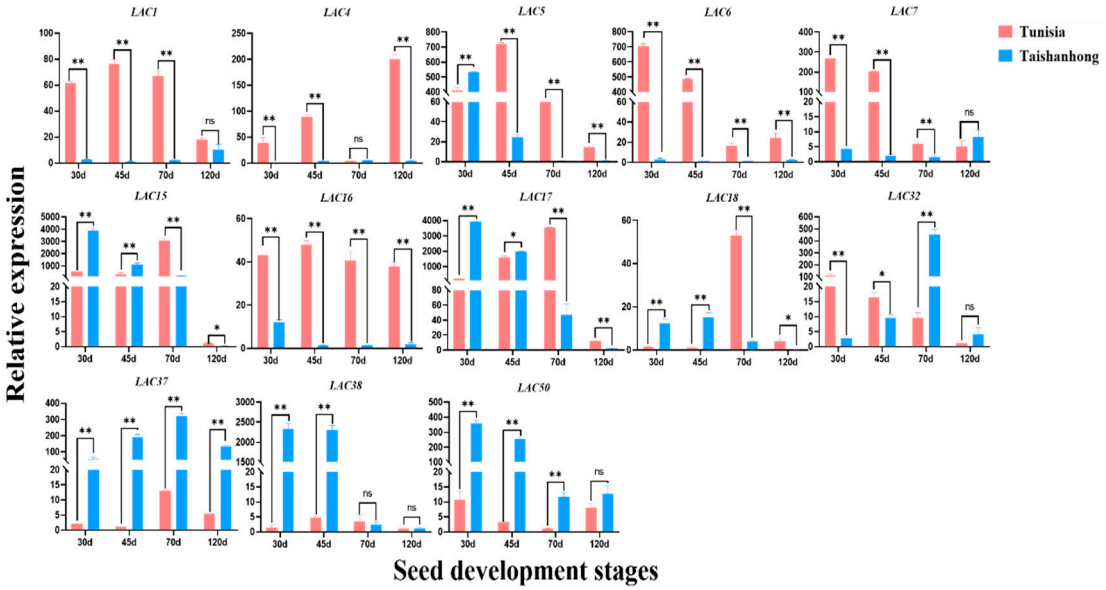

Figure 8. Gene expression profiles of *PgLACs* during seed development stage. * and ** indicated significant differences between the two cultivars at $p < 0.05$ and $p < 0.01$, respectively.

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 *PgLACs* 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 PgLAC showed PgLAC1, PgLAC16, PgLAC17, PgLAC18, PgLAC32, and PgLAC37 were co-expressed with CTL2, IRX1, and IRX12 involved in cell wall biosynthesis, further supporting the involvement of the PgLACs in lignin biosynthesis. A previous study reported that the three *Arabidopsis LACs* (*AtLAC4*, *AtLAC17*, and *AtLAC11*) were responsible for lignin polymerization [23]. The phylogenetic tree was constructed and showed that 57 *PgLACs* were divided into six groups, seven *PgLACs* (*PgLAC4*, *5*, *6*, *7*, *15*, *38*, and *50*) in Group I with *AtLAC4* and *AtLAC11*, and six *PgLACs* (*PgLAC1*, *16*, *17*, *18*, *32*, and *37*) in Group II along with *AtLAC17*. The results implied the 13 *PgLACs* may possess similar functions to the *Arabidopsis LACs*. Therefore, the expression profiles of the 13 *PgLACs* were investigated during the four seed development stages. We provided the evidence that most *PgLACs* 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 *PgLAC4*, 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 *PgLAC* existed between cultivars, higher *PgLAC1*, *PgLAC6*, *PgLAC7,* and *PgLAC16* in the soft-seed cultivar, whereas higher *PgLAC37* and *PgLAC50* 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 *PgLAC37* and *PgLAC50* 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 *PgLAC37* with 7 G-box and *PgLAC50* 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 *PgLAC37* (6 ABRE) and *PgLAC50* (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 promotors of *PgLAC37* and *PgLAC50* may be an essential reason for modulating the higher expression of *PgLAC37* and *PgLAC50* 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 *PgLAC1* promotor and GARE-motif in the promotors of *PgLAC6* and *PgLAC7*, as well as *PgLAC4* and *PgLAC5* among 13 *PgLACs*. Collectively, higher expression of *PgLAC1/4/6/7* in 'Tunisia' may be induced by GA and then produce softer seeds. The five *PgLACs* 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 *PgLAC16* promotor, which suggests that the dramatically higher expression of *PgLAC16* 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, *PgLAC1/4/6/7/16* 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 promotors, 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* promotor 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.

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