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Molecular Cloning and Expression Analysis of *PgLAC* in Pomegranate

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Abstract

Decreasing the hardness of pomegranate seeds by reducing the content of lignin is an effective way to develop soft-seeded pomegranate. Laccases (LAC) is a key regulatory factor in lignin synthesis. The full-length sequence of *PgLAC* was obtained from "*Punica granatum* cv. Hongyushizi", by using RACE and RT-PCR methods. *PgLAC* had an open reading frame of 1716 bp and encoded a protein of 571 amino acids. Phylogenetic tree analysis showed that *PgLAC* was most closely related to the *LAC*5 ortholog identified in *Eucalyptus grandis* (*EgLAC*5). Expression analysis showed that expression of *PgLAC* was higher in "Hongyushizi", while lower in "Huiliruanzi" and "Tunisiruanzi"; *PgLAC* was predominantly expressed in stems; From 20 to 80 days after full bloom, the expression of *PgLAC* increased and reached a maximum at 80 d, then gradually decreased. These results suggested that *PgLAC* may be a candidate gene for reducing the hardness of pomegranate seeds.

Keywords

Pomegranate, *LAC*, Gene Cloning, Expression Analysis

1. Introduction

Pomegranate is popular due to the high nutritional value and health benefits [1] [2]. However, most of the cultivars are hard-seeded pomegranate, and the edible parts accounted for only 45% - 52% of the total fruit quality [3]. Therefore, developing new cultivars of soft-seeded is an important direction for pomegranate breeding [4]. Lignin, which can increase cell wall hardness, is considered to be a key factor that determines the hardness of pomegranate seeds [5]. Consequently, reducing the hardness of the pomegranate seeds by decreasing the lignin content is an effective way to increase the edible rate of the pomegranate.

Laccase enzyme, belongs to the family of ceruloplasmin oxidase, which can be

combined with a plurality of copper ions [6]. Studies showed that laccase was involved in the synthesis of lignin and enabled lignin monomers to polymerize into lignin [7] [8] [9]. Members of the laccase family have been identified in Arabidopsis, poplar, tomato, sugar cane, maize etc. [10] [11] [12]. However, *LACs* have no orthologs in lower plant species, indicating that the *LACs* diverged after the evolution of seed plants [13]. Laccase genes usually have multiple members, such as 17 members in Arabidopsis, 49 members in poplar [14] [15]. Single mutation of *LAC*4, *LAC*11 or *LAC*17 in Arabidopsis did not cause significant changes in lignin content, suggesting that these members were usually functionally redundant [10] [13]. Transformation of *SofLAC* from sugarcane into Arabidopsis *LAC*17 mutant restored lignin content [11]. Overexpression of AtmiRNA397b reduced the expression of *AtLAC*4, which led to the reduction of lignin content [16]. Studies in *Populus trichocarpa* showed that the 29 members of *LACs* might be the target genes of Ptr-miR397a. Overexpression of Ptr-miR397a caused a decrease of expression of 17 *LAC* members, and eventually caused the reduction of lignin content in *P. trichocarpa* [17]. According to the existing researches on *LAC*, we found that the functions of the *LAC* in plants were less studied, and *LAC* in pomegranate had not been reported.

In this study, *LAC* homologous gene *PgLAC* was cloned from pomegranate, and the expression level in different pomegranate cultivars, tissues and developmental stages was detected by real-time quantitative PCR. This study lays a foundation for further research on the function analysis of *PgLAC*, and also provides gene resources for breeding new cultivars of soft-seeded pomegranate.

2. Materials and Methods

2.1. Plant Materials

Pomegranate cultivars used in this study were all collected from Germplasm Resources Garden of Pomegranate in Anhui Agricultural University. The fruits of "Huiliruanzi", "Hongyushizi" and "Tunisiruanzi" in similar cultivation environment were collected. The arils and kernels were removed from the seeds, then the seed coats were soaked in liquid nitrogen, and eventually were taken back to the laboratory and store in a −80˚C refrigerator. For the expression analysis experiments, flowers, stems, leaves and the seeds of 20 d, 40 d, 60 d, 80 d, 100 d and 120 d of "Hongyushizi" were collected, respectively. After the same treatments to seeds as mentioned above, the tissues and seed coats were taken back to the laboratory and stored in a −80˚C refrigerator.

2.2. Isolation of *PgLAC*

The total RNA was extracted from the seed coats of "Hongyushizi" using Trizol reagent (Invitrogen), and digested with DNase I (TaKaRa). Then cDNA was synthesized using reverse transcriptase M-MLV (Promega). According to the *LAC* sequences of other species that have been published, we designed degenerate primers LAC-PF/LAC-PR to clone the intermediate fragment. Based on the obtained

sequence, 5' end specific primers LAC-5'GSP1, LAC-5'GSP2 and 3' end specific primers LAC-3'GSP1, LAC-3'GSP2 were designed. LAC-5'GSP1/UPM Long were used for the first round amplification, and the PCR products were used as template, then LAC-5'GSP2/NUP were used for the second round of amplification to obtain the 5' end sequence of *PgLAC*. Similarly, 3' end sequence of *PgLAC* was obtained. The three parts were spliced, and primers LAC-QC-F/LAC-QC-R were used to amplify the full-length sequence (Table 1). Finally, *PgLAC* was amplified using PrimeSTAR HS DNA polymerase (Takara) to correct the sequence.

2.3. Sequence Analysis and Phylogenetic Tree Construction

PgLAC was compared with the homologous sequences of other species in the NCBI database using DNAMAN 5.0 software; the phylogenetic tree was constructed using MEGA 5.05 and maximum likelihood method with 1000 bootstrap replications [18].

2.4. Quantitative Real-Time PCR

Two grams of RNA from different samples digested by DNase I (TaKaRa) was reverse transcribed into cDNA. Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 Fast Real-time PCR system and SYBR®Premix Ex Taq™ II (TaKaRa). Amplification was carried out by an initial denaturation at 95˚C for 30 s, followed by 40 cycles of amplification (denaturation at 95˚C for 15 s, annealing at 60˚C for 30 s and extension at 72˚C for 1 min). *PgACTIN* was used as the reference gene. The results were analyzed using ABI 7500 software, and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [19].

3. Results and Analysis

3.1. Cloning and Sequence Analysis of *PgLAC*

Based on the conserved regions of *LAC* in Arabidopsis, grape, tobacco, apple,

Table 1. The primers used in this study.

Primers	Sequence 5'-3'	Usage
LAC-PF	GAAGGHACACTTTGGTGGCA	Amplification formiddle fragments of *PgLAC*
LAC-PR	ACATCBGTGGTTTGTCCTGG	
LAC-5'GSP1	TCGTCTCACCAGAATCAATCGG	Amplification for5' race of *PgLAC*
LAC -5'GSP2	CGGTGTCTTGCTTGGTCTCCAT	
LAC-3'GSP1	CTAAGCCAAAGCGTGAAACTCC	Amplification for3' race of *PgLAC*
LAC-3'GSP2	GTGCTCCCAATGTCTCCGATGC	
LAC-QC-F	CACTCTCTGCCACCTTTTGATCAG	Amplification for full-length of *PgLAC*
LAC-QC-R	CTACTTTATCTTATAGAATCAAAT	
LAC-RTPF	CCTGGTAGAAGACGGAGTCGGGGAG	qRT-PCR primers
LAC-RTPR	GGGCGGTGTGACGGTGCTATTATT	
PgACT-PF	AGTCCTCTTCCAGCCATCTC	Amplification for *PgACTIN*
PgACT-PR	CACTGAGCACAATGTTTCCA	

plum and other species, degenerate primers were designed to amplify the intermediate fragment with a length of 449 bp (Figure 1). The 5' end sequence (670 bp) and 3' end sequence (1417 bp) were obtained by nested PCR (Figure 1(b), Figure 1(c)). Three parts were spliced to obtain the full-length of *PgLAC* (2128 bp), which contained 5'-noncoding sequence of 64 bp, 3'-noncoding sequence of 351 bp and an open reading frame of 1716 bp. The gene encoded a protein containing 571 amino acids. The molecular weight was predicted to be 62.8 kD and theoretical isoelectric point was 8.59. The more amino acids in the protein were alanine Ala (8.1%, 46), Thr (8.1%, 46) and Pro (7.7%, 44). Subcellular location prediction showed that the possibility of PgLAC localization in the endoplasmic reticulum was 82%, and the possibility of localization in the cell membrane was 18%.

3.2. Homology Analysis of PgLAC

PgLAC displayed high similarities with other LAC protein sequences. For example, the similarity between PgLAC and EgLAC5, VvLAC12, RcLAC12 reached to 86.51%, 83.54% and 81.11%, respectively. Sequence analysis indicated that the conserved PgLAC binding domains contained three copper ions of laccase gene family: T1 (84 - 131) with four H (histidine) residues, T2 (232 - 270) containing N (aspartic acid), L (leucine), V (valine), K (Lai Ansuan), P (proline), Q (glutamine) and T (threonine) residues, T3 (472 - 545) with two H (histidine) residues, C (cysteine), and L (leucine) residues (Figure 2).

3.3. Phylogenetic Analysis of PgLAC Homeodomain Proteins

In order to determine the phylogenetic relationship between PgLAC and other LACs, MEGA 5.05 software was used for multiple sequence alignments and phylogenetic analysis. The results showed that PgLAC was identical with the origin of LACs in other species, and was indeed a homologue of the LAC family. The phyologenetic tree revealed that PgLAC was placed in one clade with EgLAC5,

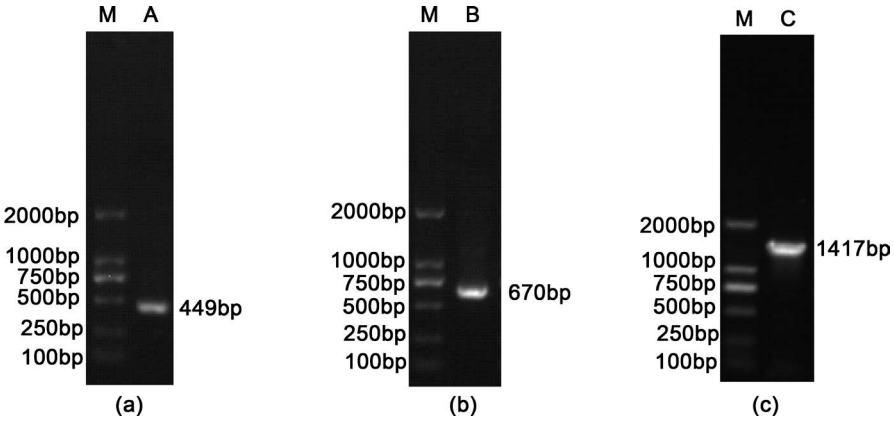


Figure 1. The amplification of pomegranate *PgLAC*. M: DL 2000 DNA marker; (a) The detection of middle fragments cDNA amplification of pomegranate *PgLAC*; (b) The detection of 5'-RACE cDNA amplification of pomegranate *PgLAC*; (c) The detection of 3'-RACE cDNA amplification of pomegranate *PgLAC*.

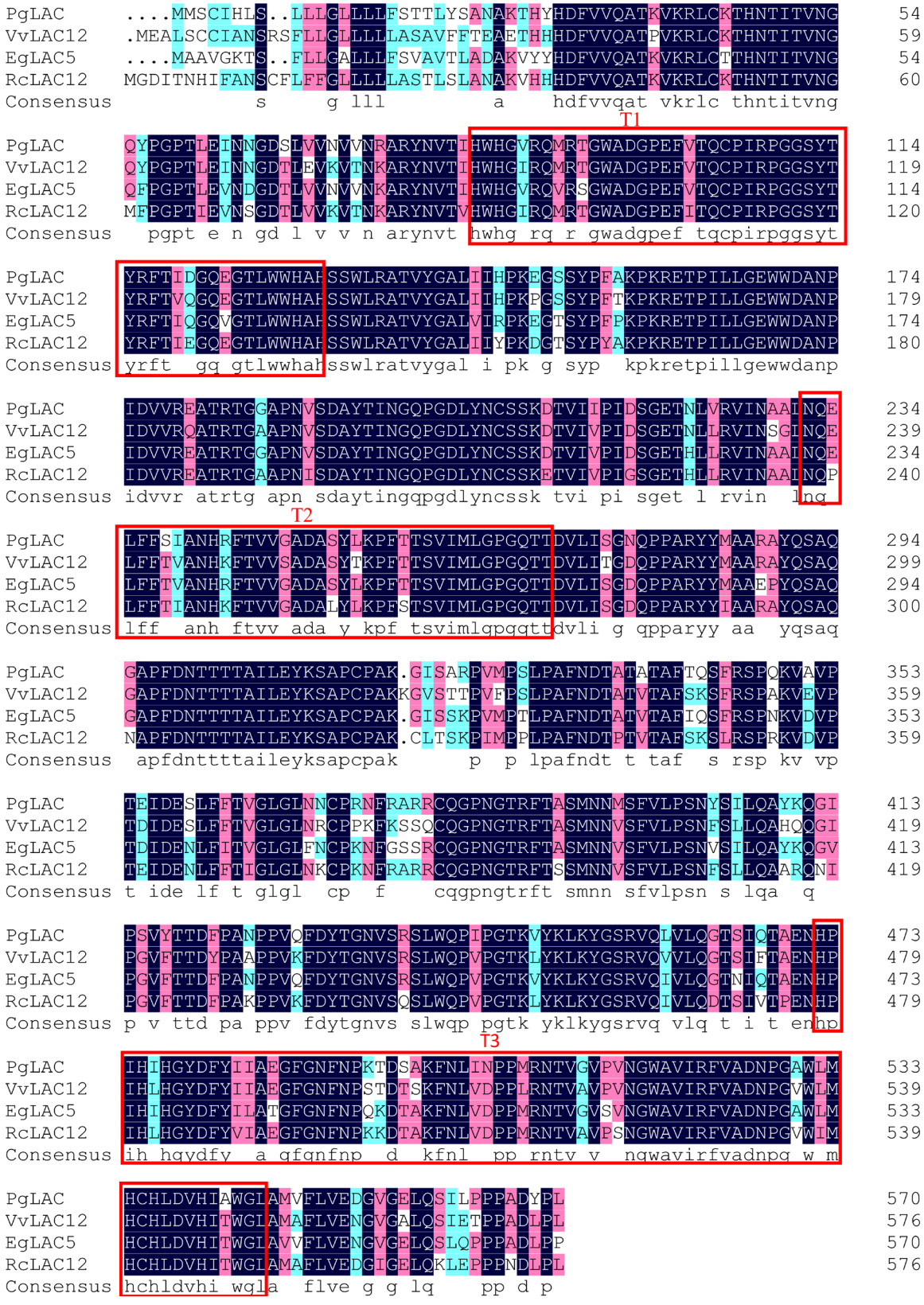
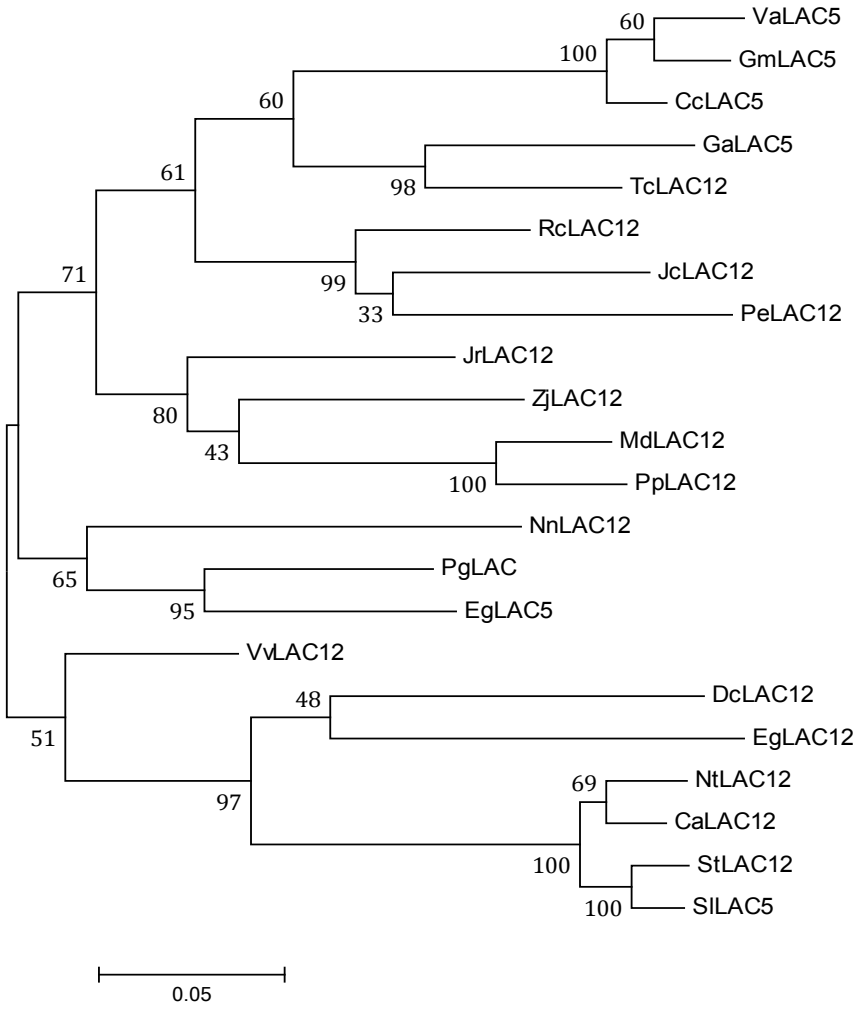


Figure 2. Alignment of the predicted amino acid sequences of PgLAC compared with *Eucalyptus grandis* (EgLAC), *Vitis vinifera* (VvLAC) and *Ricinus communis* (RcLAC). Amino acids highlighted in black, red and blue respectively represent residues completely conserved, partially conserved and similar to consensus. Conserved domain (T1, T2, T3) were marked by red frames.

whlie it had a distant relationship with VaLAC5 (Figure 3).

3.4. Expression Analysis of *PgLAC* in Different Pomegranate Cultivars

In order to investigate the expression level of *PgLAC* in different cultivars of pomegranate, qRT-PCR was used to detect the relative expression of *PgLAC* in



The phylogenetic tree was constructed using the maximum likelihood method. The scale bar indicated 0.05 substitutions per site. The accession numbers of selected LACs were listed as follows: *Vigna angularis* VaLAC5 (XP_017429457.1); *Glycine max* GmLAC5 (XP_003519418.1); *Cajanus cajan* CcLAC5 (XP_020208524.1); *Gossypium arboreum* GaLAC5 (XP_017645108.1); *Theobroma cacao* TcLAC12 (XP_007008849.2); *Ricinus communis* RcLAC12 (XP_002520796.1); *Jatropha curcas* JcLAC12 (XP_012086668.1); *Populus euphratica* PeLAC12 (XP_011004316.1); *Juglans regia* JrLAC12 (XP_018838961.1); *Ziziphus jujuba* ZjLAC12 (XP_015877482.1); *Malus domestica* MdLAC12 (XP_008343632.1); *Prunus persica* PpLAC12 (XP_007218932.1); *Nelumbo nucifera* NnLAC12 (XP_010264533.1); *Punica granatum* PgLAC (AJD79906.1); *Eucalyptus grandis* EgLAC5 (XP_010067566.1); EgLAC12 (XP_010031156.1); *Vitis vinifera* VvLAC12 (XP_002273875.2); *Daucus carota* subsp. Sativus DcLAC12 (XP_017238938.1); *Nicotiana tabacum* NtLAC12 (XP_016460452.1); *Capsicum annuum* CaLAC12 (XP_016575563.1); *Solanum tuberosum* StLAC12 (XP_006355637.1); *Solanum lycopersicum* SlLAC5 (XP_004240885.1).

Figure 3. Phylogenetic analysis among different LACs.

"Hongyushizi", "Huiliruanzi" and "Tunisiruanzi". The results showed that the expression of *PgLAC* in "Hongyushizi" was 3.5 times higher than that of "Tunisiruanzi". The expression of *PgLAC* in "Huiliruanzi" and "Tunisiruanzi" was lower, and there was no significant difference between the two cultivars (**Figure 4**).

3.5. Expression Analysis of *PgLAC* in Different Tissues

The expression level of *PgLAC* in different tissues of pomegranate was detected, with "Hongyushizi" as the material. The results showed that *PgLAC* was all detected in leaves, petals and stems. The expression in stems and petals was 19.59 times and 3.74 times higher than that in leaves，respectively (**Figure 5**).

3.6. Expression Analysis of *PgLAC* in Different Developmental Stages

In order to reveal the expression characteristics of *PgLAC* in different developmental stages of seeds, the pomegranate seeds were collected from 20th day to

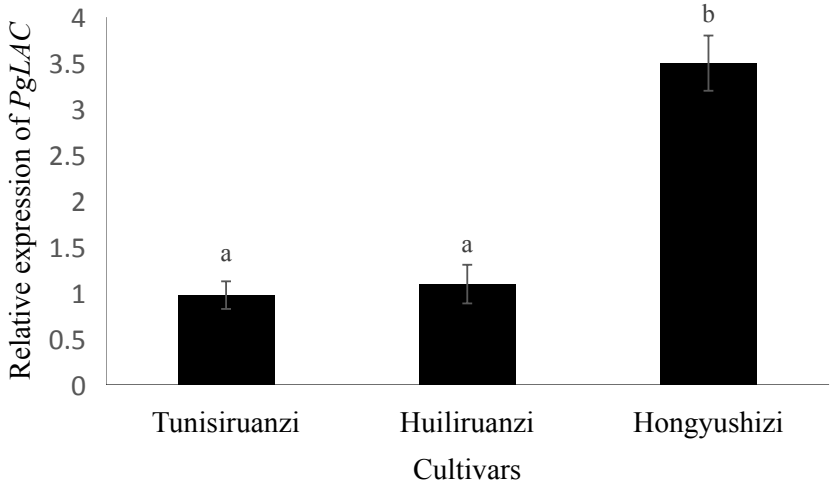


Figure 4. The relative expression of *PgLAC* in three cultivars of pomegranate seed coats.

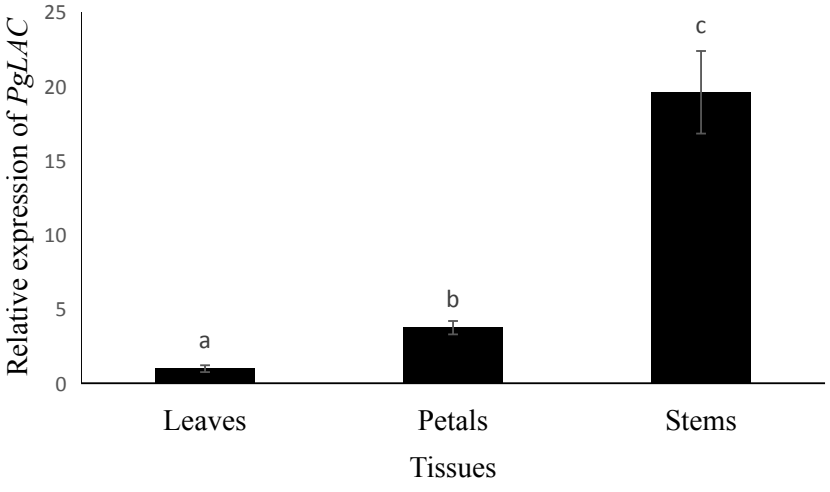


Figure 5. The relative expression of *PgLAC* in different tissues of "Hongyushizi".

120th day, with the time interval of 20 days. The results showed that *PgLAC* was expressed in all stages of seeds, showing the overall downward trend after rising first. The relative expression remained a low level from 20 d to 60 d, and reached a maximum value at 80 d, then decreased gradually (Figure 6).

4. Discussion

Pomegranate is widely cultivated all over the world, and seed hardness is one of the most important economic traits. Compared to hard-seeded pomegranate, soft-seeded pomegranate has a broader market prospect. Lignin content is an important factor in determining the hardness of pomegranate seeds.

More and more studies showed that *LACs* were involved in lignin synthesis. In this study, the homologous gene of *LAC* was cloned from pomegranate. The putative amino acid sequence of this gene contained the conserved binding domains of three copper ions, hence it was named *PgLAC*. Phylogenetic tree analysis suggested that all the LACs originated from the same ancestral origin, which subsequently diverged at different phases of evolution. PgLAC were most closely related to EgLAC5.

The relative expression of *PgLAC* varied among different cultivars. *PgLAC* expression was higher in the seeds of "Hongyushizi" with high lignin content, while lower in "Huiliruanzi" and "Tunisiruanzi" with lower lignin content, which indicated that *PgLAC* expression was correlated with lignin content, and *PgLAC* may be an important factor affecting seed hardness in different cultivars. The roles of *LAC* members in plant growth and development varied, and therefore tissue-specific expression were different. For example, studies in Arabidopsis showed that *LAC*17 was mainly expressed in the interfascicular fibers, whereas *LAC*4 was expressed in vascular bundles and interfascicular fibers [10]. To analyze the expression characteristics of *PgLAC* in different tissues of pomegranate, the expression levels of *PgLAC* in leaves, petals and stems were detected. *PgLAC* was expressed in all three tissues, with the highest expression in

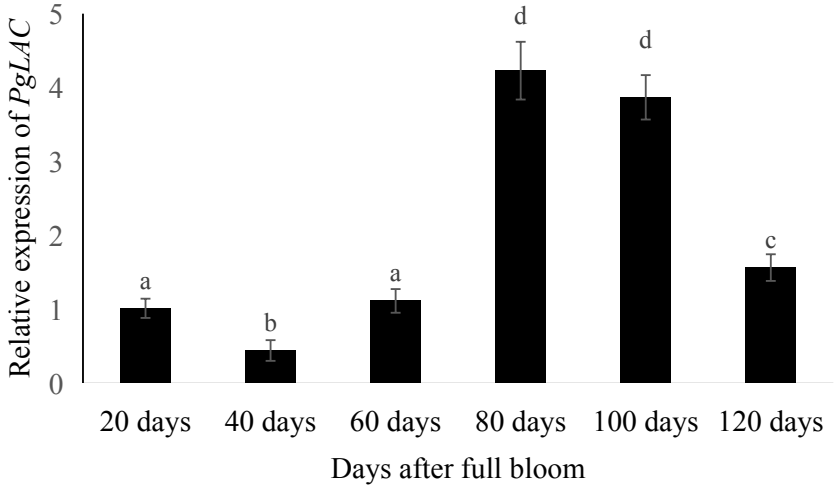


Figure 6. The relative expression of *PgLAC* in different stages of "Hongyushizi" grains.

stems and lowest expression in leaves. The result indicated that *LAC* was highly expressed in tissues with high lignin content, which further supported the involvement of *PgLAC* in the synthesis of lignin in Pomegranate. *PgLAC* expression in seeds at different stages was also detected. The expression of *PgLAC* increased rapidly from 20 d to 80 d, suggesting that lignin was rapidly synthesized and accumulated during this period. When the content of lignin reached a certain range, the expression of *PgLAC* decreased, also the synthesis of lignin reduced. These results implied that *PgLAC* might play an important role in the synthesis of pomegranate lignin.

Since *PgLAC* was expressed in several tissues of pomegranate, reducing the total expression level of *PgLAC* will affect multiple developmental processes. Our study found that *PgLAC* expression gradually increased in the early stages of seed development, and eventually resulted in lignin accumulation. Therefore, reducing lignin content by regulating the expression level of *PgLAC* during seed development, may be an effective way to reduce the hardness of the pomegranate seeds. This study lays a theoretical foundation for developing new cultivars of soft-seeded pomegranate by using genetic engineering methods.

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Abbreviations

C, cysteine;
H, histidine;
K, Lai Ansuan;
LAC, Laccases;
L, leucine;
N, aspartic acid;
P, proline;
Q, glutamine;
qRT-PCR, Quantitative real-time PCR;
T, threonine;
V, valine.