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Involvement of PAL, C4H, and 4CL in Chilling Injury-induced Flesh Lignification of Loquat Fruit

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Abstract. **Loquat (*Eriobotrya japonica*) is a model fruit for investigating flesh lignification during storage and response to chilling injury. However, the investigations of enzymes and coding genes and loquat fruit lignification under low-temperature storage are still limited. Here, the activity and transcript levels of up-stream enzymes of the phenylpropanoid pathway, including L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:coenzyme A ligase (4CL), were investigated. The results indicated that activity of these enzymes was positively correlated with loquat fruit lignification and suppression of these increases by heat treatment (HT) and low-temperature conditioning (LTC) significantly alleviated loquat fruit lignification. Coding genes for these enzymes were subsequently isolated based on information from an RNA-seq database and expression of *Ej4CL1* was found to be the most responsive to low temperature and inhibition by HT and LTC treatment, whereas the other genes were less responsive to these treatments. Furthermore, function of *Ej4CL1* was analyzed by transient overexpression in tobacco leaves, where it stimulated lignin accumulation. *Ej4CL1* may be a key candidate that involved in CI-related loquat fruit lignification.**

Texture is an important quality trait for fruit (Li et al., 2010). Postharvest softening usually occurs in most fruit, but lignification is an uncommon phenomenon occurring in ripening fruit, which is usually accompanied by increased fruit firmness, leathery pulp, internal browning, and decreased juiciness (Lin et al., 1999). Loquat (*Eriobotrya japonica* Lindl.) fruit show substantial flesh lignification during postharvest storage, and this is more severe when fruit are subjected to cold storage (Cai et al., 2006a, 2006b). Loquat fruit lignification can be significantly alleviated by various treatments, such as LTC (Cai et al., 2006b), HT (Xu et al., 2014; Zeng et al., 2015), 1-methylcyclopropene (1-MCP) (Cai et al., 2006c), Methyl jasmonate (MeJA) (Cai et al., 2011; Cao et al., 2012), and Nitric oxide (NO) (Xu et al., 2012). Thus, loquat fruit is an ideal material for investigating fleshy fruit lignification.

Generally, lignification is caused by the accumulation of lignin in plant cell walls and the biosynthesis of lignin has been widely studied in the model plant *Arabidopsis*, woody trees and some other crops (Vanholme et al., 2010; Zhao and Dixon, 2011). Monolignol formation is catalyzed by a series of enzymes in the phenylpropanoid pathway, such as PAL, C4H, 4CL, hydroxycinnamoyl-CoA:NADPH moxidoreductase, cinnamyl alcohol dehydrogenase (CAD), etc (Barros et al., 2015; Bonawitz and Chapple, 2012). Many of the coding genes for these enzymes have been functionally verified to be involved in lignin biosynthesis, for instance, 4CL antisense lines in *Arabidopsis* showed lower lignin content, and altered lignin composition compared with wide-type plants (Lee et al., 1997). Similarly, both PAL and C4H suppression lines of tobacco produced lower lignin content and altered lignin composition (Sewalt et al., 1997).

In loquat fruit, the relationship between biosynthetic enzymes and lignification showed CAD activity was closely related to lignin contents of different cultivars (Shan et al., 2008). Moreover, CAD activities, as well as *EjCAD1* expression, was also positively correlated with low temperature-induced loquat fruit lignification (Shan et al., 2008). Recently, *EjCCoAOMT* was newly isolated from loquat fruit and expression also shown to be induced by low temperature (Liu et al., 2015). As mentioned above, various enzymes and their coding genes within phenylpropanoid pathway contributed to lignin biosynthesis. However, the roles of other components within phenylpropanoid pathway for loquat fruit lignification remain unclear.

Recently, several transcription factors have been reported to be involved in lignin regulation in loquat fruit, including *EjMYB1*, *EjMYB2* (Xu et al., 2014), *EjAP2-1* (Zeng et al., 2015), *EjNAC1* (Xu et al., 2015), and *EjHSF3* (Zeng et al., 2016). All of these transcription factors significantly influenced promoters of genes-encoded PAL and 4CL of the phenylpropanoid pathway, supporting the potential roles of multiple enzymes in loquat lignification regulation. Thus, in the present research, activities of PAL, C4H, and 4CL were analyzed in response to low temperature, LTC and HT in loquat cv. Luoyangqing (LYQ). Based on our previous results (Shan et al., 2008) and using an RNA-seq database, some new coding genes for these enzymes were isolated, their expression studied, and transient overexpression analysis was performed on *N. tabacum* to verify the role of *Ej4CL1* in lignin biosynthesis.

Materials and Methods

Fruit materials and treatments. The fruit materials used in the present research were derived from our previous publication (Xu et al., 2014). In brief, 'LYQ' loquat fruit were collected at commercial maturity from an orchard at Luqiao, Zhejiang, China in 2011. Fruit without disease or mechanical wounding were selected and divided into three groups for treatments. Three treatments are as follow: 1) Control, stored at 0 °C; 2) LTC, storage at 5 °C for 6 d and then transferred to 0 °C; 3) heat treatment (HT), treated at 40 °C for 4 h and then transferred to 0 °C. At each sampling point, three replicates, each of five fruits, were sampled without skins and kernels. The flesh tissues were cut and frozen in liquid nitrogen immediately and stored at –80 °C.

Enzyme activity assays. Activities of three enzymes within the phenylpropanoid pathway were measured according to the previously published methods (Knobloch and Hahlbrock, 1975; Koukol and Conn, 1961; Lamb and Rubery, 1975). One unit of enzyme activity was defined as the amount of enzyme that caused a change of 0.01 in absorbance per min. Protein levels were measured using the method of Bradford

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(1976). Three biological replicates were performed for enzyme activity measurement.

Gene isolation and analysis. Two *EjPAL* genes and five *Ej4CL* genes were previously isolated (Shan et al., 2008; Xu et al., 2014). Using RNA-seq database, two *EjC4H* genes and six *Ej4CL* genes were newly isolated from loquat. Full-length sequences were obtained from partial sequences using SMART RACE cDNA Amplification kit (Clontech), with primers listed in Table 1. Alignment of the deduced amino acid sequences was performed in ClustalX (v 1.81) and the phylogenetic tree was constructed using Figtree (v1.4.2).

RNA extraction and cDNA synthesis. Total RNA was extracted following the previously published methods (Shan et al., 2008). The contaminating DNA was digested using TURBO DNA-free kit (Ambion). The first strand of cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. Three batches of RNA were isolated as three biological replicates for each time point. Diluted cDNA was used as template of real-time polymerase chain reaction (PCR).

Real-time PCR. Oligonucleotide primers for real-time PCR were designed from the untranslated region of individual genes and are listed in Table 2. Loquat actin was used as an internal standard (Shan et al., 2008). Real-time PCR was performed in a total volume of 20 µl with 10 µl 2 × LightCycler 480 SYBR Green I Master Mix (Roche), 6 µl PCR-grade water, 2 µl cDNA, and 2 µl primer mixture on a LightCycler 480 instrument (Roche). The PCR program included initiation at 95 °C for 5 min, followed by 50 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s. A negative control for each primer pair was also performed and at least three replicates of RNA isolation and cDNA synthesis were conducted. Melting curves and Ct value were analyzed to quantify gene abundance, $2^{-\Delta\Delta}C_t$ method (0 d was set as 1).

Transient overexpression and lignin measurement. To verify *Ej4CL1* function in lignin biosynthesis, transient overexpression was conducted with *N. tabacum* leaves. The full length of *Ej4CL1* was inserted into pGreen II 0029 62-SK vector [empty vector (EV)] (Hellens et al., 2005). The constructs were electroporated into *Agrobacterium tumefaciens* GV3101 (MP90) and stored in glycerol at –80 °C for further use. Before formal injection, *Agrobacterium* was activated by streaking on a Luria-Bertani plate for 2 d and re-streaking on a new Luria-Bertani plate for another 1 d. Agrobacterium cultures were then suspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 0.5 mM acetosyringone) and OD_{600} adjusted to 0.75. Target genes and negative controls (EV SK) were injected on each side of the midrib of the same leaf. The infiltrated leaves were sampled 5 d after infiltration for further lignin analysis. Lignin content of flesh and *N. tabacum* were measured as described in our previous publications (Shan et al., 2008; Wang et al., 2010). Data were expressed on a fresh weight basis and all measurements were made in triplicate.

Statistical analysis. The statistical significance of differences was calculated using Student's *t* test. Least significant differences ($LSD_{0.05}$) were calculated using DPS7.05 (Zhejiang University, Hangzhou, China).

Results

Enzyme activity changes in response to different treatments. In our previous report, HT and LTC treatments significantly alleviated 'LYQ' loquat lignification (Xu et al., 2014). Using the same materials, PAL, C4H, and 4CL activities were measured. As shown in Fig. 1, activities of the three enzymes were all positively correlated with fruit lignification. Activities of PAL and 4CL were induced by low temperature (0 °C) and peaked at 1 d and 4 d, respectively, and these increases were significantly inhibited by LTC and HT. For example, PAL activity increased from 159 to 251 U/mg protein between days 0 and 1, and decreased to 112 and 168 U/mg protein in LTC and HT treated

Table 1. Sequences of the primers used for gene isolation.

Genes	Method used	Primers	Sequences (5′ to 3′)
EjC4H1	3′RACE	Primary PCR	CTCCTCCGCATGGGGCAGTGCAA
		Secondary PCR	GCACATTCTAAGCCGACCTAGAGACC
EjC4H2	3′RACE	Primary PCR	CCACGTCGTTCACAACATCAACGCCG
		Secondary PCR	CCTCGGCCGCGGAGTACAAATCACA
Ej4CL8	3′RACE	Primary PCR	ACACCCAGAAATTACCCTCCCCACGG
		Secondary PCR	TCATCTTCTCACACCAGCACACCGG
Ej4CL9	3′RACE	Primary PCR	ACCTCATTGCTCAGAAGACCGGCGC
		Secondary PCR	GTGCCGAGTTCGTCTTCGCCTTC
	5′RACE	Primary PCR	CGGCAGTGTAAAAGGGGTTGGCGGT
		Secondary PCR	GAAGGCGAAGACGAACTCGGCAC
Ej4CL10	3′RACE	Primary PCR	CCCCGCCAGCTCCAATCCCGAAATC
		Secondary PCR	ACTGGGAGGGTCAAAGGCGTGGAGT
	5′RACE	Primary PCR	GTGCTTCACCAAGGCCACGACCGC
		Secondary PCR	ATCGAACCTTCCCATGAACGCCAGC

Table 2. Sequences of the primers used for real-time polymerase chain reaction.

Genes	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
EjPAL1	TGAGATCACCAGGGGAGGAA	GAGCACCATTCCACTCCTTCA
EjPAL2	GGAGTTGGGAGGAGAGTACC	ACGATGATGCAGGACTCGAG
EjC4H1	AATTTAGGTCGAGGGAGGCG	CACCGAGGGATGTAAAGGCC
EjC4H2	ACCGTTGTTATGAAGCAGACG	AGCTGGAGAAGGATTGTGCT
Ej4CL1	AGTCACCGTCGGGCAAAA	CATGCCCTTCCTTCAAAATG
Ej4CL2	CCTCATCCAAATCCCAGAAA	AATGCCATCTCTGCCGAAC
Ej4CL3	TCGACCAACAACAACAACGC	GTGACTGTTGCTTCTTTGTGT
Ej4CL4	CCTTATCCACTGGTTCATCAAAA	ATCGAATGCAACGCTGAGA
Ej4CL5	TTTTCCCTCACTTTCTCACCA	AAATATCGGGGAGCTTGGAC
Ej4CL6	TGCAGGGAAAAGTGTTGAGG	AAACATCGCACATACTTTGGAG
Ej4CL7	ACAATCTCATCCAATCGTCAGT	TGTGATGTCGAGGGAGTAGT
Ej4CL8	AAACAGAGCACTCCGGATCA	AGGAATCAATGAGGGCAGGT
Ej4CL9	GAAAAGACCTCAGAGCCAAGC	GGCTCGCCCTCCAAATTG
Ej4CL10	AAGTACCCATGGCCTTCGTC	TTGATGAATGCTACGCGTCG
Ej4CL11	TGTGACACAGCTGCAATTGT	AACCAACCTACACCCACACA

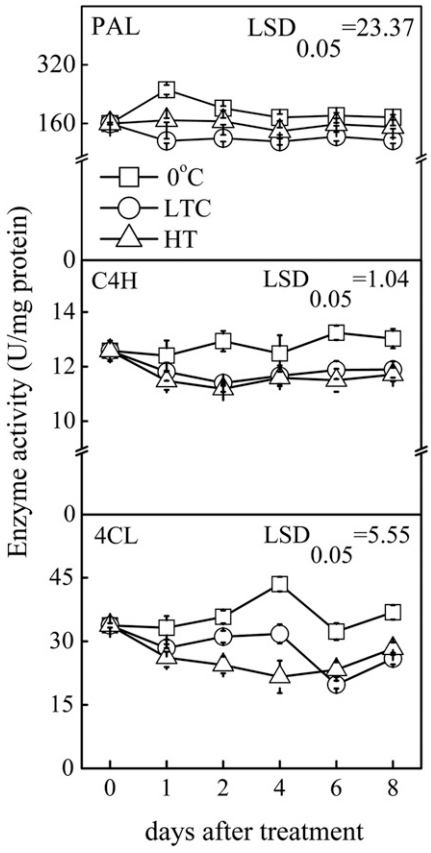

Fig. 1. Effects of HT and LTC treatments on L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate: coenzyme ligase (4CL) activities in LYQ fruit. HT fruit was treated at 40 °C (in air) for 4 h and then was transferred to 0 °C; LTC fruit were stored at 5 °C for 6 d and then was transferred to 0 °C. Error bars indicate SE for three replicates. Least significant differences (LSDs) represent least significant difference at 0.05.

fruit, respectively, at 1 d (Fig. 1). Unlike PAL and 4CL, C4H activities remained constant during low-temperature storage, but also decreased in response to LTC and HT, thus also to some extent correlated with fruit lignification (Fig. 1).

Gene isolation and analysis. As PAL, C4H, and 4CL all appeared to be associated with loquat fruit lignification at the biochemical level, their encoding genes were isolated, including *EjPAL1-2* (EF685343-4) and *Ej4CL1-5* (EF685345, KF767455-8) (Shan et al., 2008; Xu et al., 2014), on the basis of previous reports, and two novel *EjC4H* (*EjC4H1*, KU176088; *EjC4H2*, KU176089) and six new *Ej4CL* genes (*Ej4CL6-11*, KU176090-KU176095) were isolated on the basis of RNA-seq and RACE.

Phylogenetic analysis revealed that *EjC4H1* and *EjC4H2* were clustered with *Genlisea aurea GaC4H* (EPS65721) and *Zostera marina ZmC4H* (KMZ73596) (Fig. 2). Eleven Ej4CL genes, including five previously reported and six newly isolated, were divided into two major classes, class I and class II. *Ej4CL1* and *Ej4CL5* belong to class I and were clustered with *Pro4CL1*, *Pto4CL1*, *Nt4CL1*, and *Nt4CL2*; whereas the other *Ej4CL* genes belong to class II (Fig. 3).

Expression of EjPAL, EjC4H, and Ej4CL genes in response to LTC and HT treatments. The relationship between previously isolated *EjPAL* and *Ej4CL* genes and CI-related loquat lignification had not been investigated (Shan et al., 2008), thus the expression of all *EjPAL*, *EjC4H*, and *Ej4CL* genes was analyzed in response to low temperature, LTC and HT. Expression of *EjPAL1* and *EjPAL2* increased at 0 °C and this was significantly inhibited by HT treatment. However, LTC treatment had little effect on either *EjPAL1* or *EjPAL2* (Fig. 4).

Two *EjC4H* genes, *EjC4H1* and *EjC4H2,* exhibited different expression patterns, with the latter showing a much greater increase during LTC than the control. Expression of *EjC4H1* slightly decreased during 1–3 d at 0 °C and increased to initial level of 0 d at 6 and 8 d, whereas LTC and HT treatment had little effect on expression (Fig. 5). Expression of *EjC4H2* was increased 3-fold in fruit stored for 2 d at 0 °C and 6-fold in fruit stored 4 d after LTC treatment (Fig. 5).

Transcript accumulation patterns for the eleven *Ej4CL* genes could be generally divided into three groups: 1) expression of *Ej4CL1*, *Ej4CL6*, *Ej4CL7*, *Ej4CL8* increased in response to 0 °C; however, only *Ej4CL1* and *Ej4CL6* were significantly repressed by both LTC and HT; 2) *Ej4CL9* had a much smaller response to 0 °C, but was significant inhibited by LTC and HT; 3) other *Ej4CL*

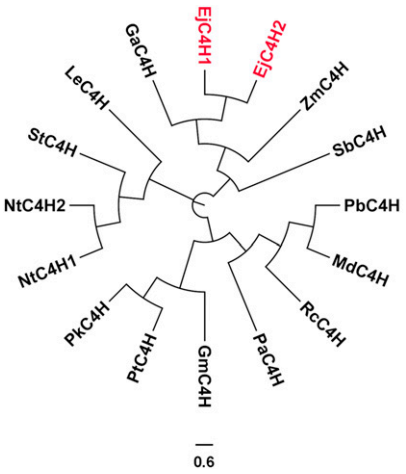

Fig. 2. Phylogenetic tree of cinnamate 4-hydroxylase (C4H) from loquat and other plants. Loquat EjC4Hs are highlighted in red. The NCBI accession numbers of the protein sequences are as follows: *Genlisea aurea* GaC4H (EPS65721); *Lithospermun erythrorhizon* LeC4H (BAB71717); *Malus domestica* MdC4H (AAY87450); *Prunus avium* PaC4H (AEA02458); *Pyrus bretschneideri* PbC4H (AEK94316); *Rubus coreanus* RcC4H (ABX74779); *Sorghum bicolor* SbC4H (XP002461939); *Solanum tuberosum* StC4H (DQ341174); *Zostera marina* ZmC4H (KMZ73596); *Glycine max* GmC4H (FJ770486); *Nicotiana tabacum* NtC4H1 (DQ350352), NtC4H2 (DQ350353).

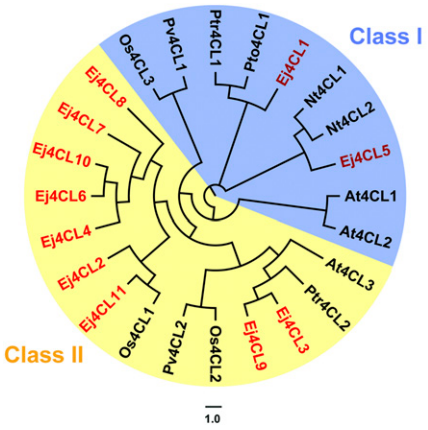

Fig. 3. Phylogenetic tree of 4-coumarate:coenzyme ligase (4CL) from loquat and other plants. Loquat Ej4CLs are highlighted in red. The NCBI accession numbers of the protein sequences are as follows: *Glycine max* Gm4CL1 (AAL98709), Gm4CL2 (P31687), Gm4CL3 (AAC97389); *Nicotiana tabacum* Nt4CL1 (O24145), Nt4CL2 (O24146); *Arabidopsis thaliana* At4CL1 (NP_175579), At4CL2 (NP_188761), At4CL3 (NP_176686); *Populus tremuloides* Ptr4CL1-2 (AAC24503-4); *Populus tomentosa* Pto4CL1 (AAL02145.1); *Lolium perenne* Lp4CL1-3 (AAF37732-4); *Oryza sativa* Os4CL1(BAD05189), Os4CL2 (Q42982), Os4CL3 (AB234050).

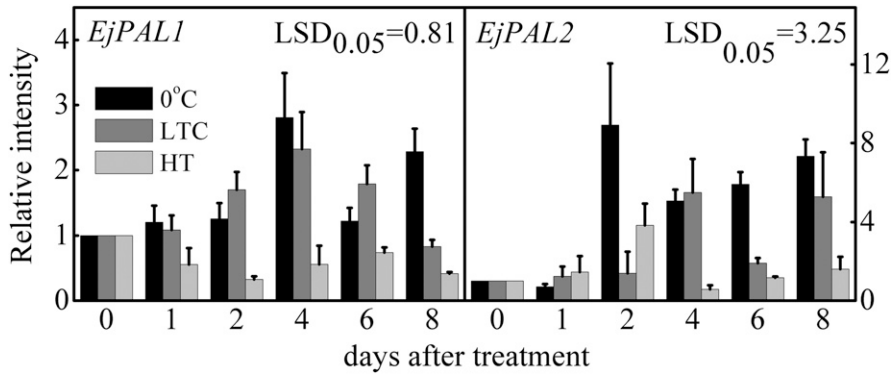

Fig. 4. Effects of low-temperature conditioning (LTC) and heat treatment (HT) on *EjPAL* expression. Transcripts of *EjPAL genes* were measured by real-time polymerase chain reaction. Day 0 fruit values were set as 1. Error bars indicate SE from three biological replicates. Least significant differences (LSDs) represent least significant difference at 0.05.

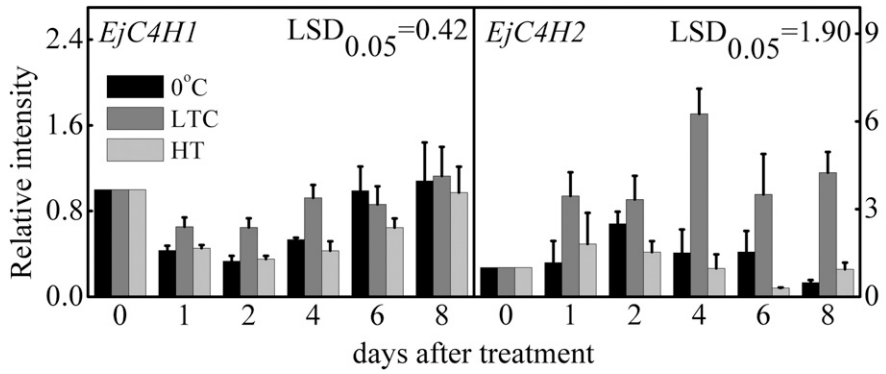

Fig. 5. Effects of low-temperature conditioning (LTC) and heat treatment (HT) on *EjC4H* expression. Transcripts of *EjC4H genes* were measured by real-time polymerase chain reaction. Day 0 fruit values were set as 1. Error bars indicate SE from three biological replicates. Least significant differences (LSDs) represent least significant difference at 0.05.

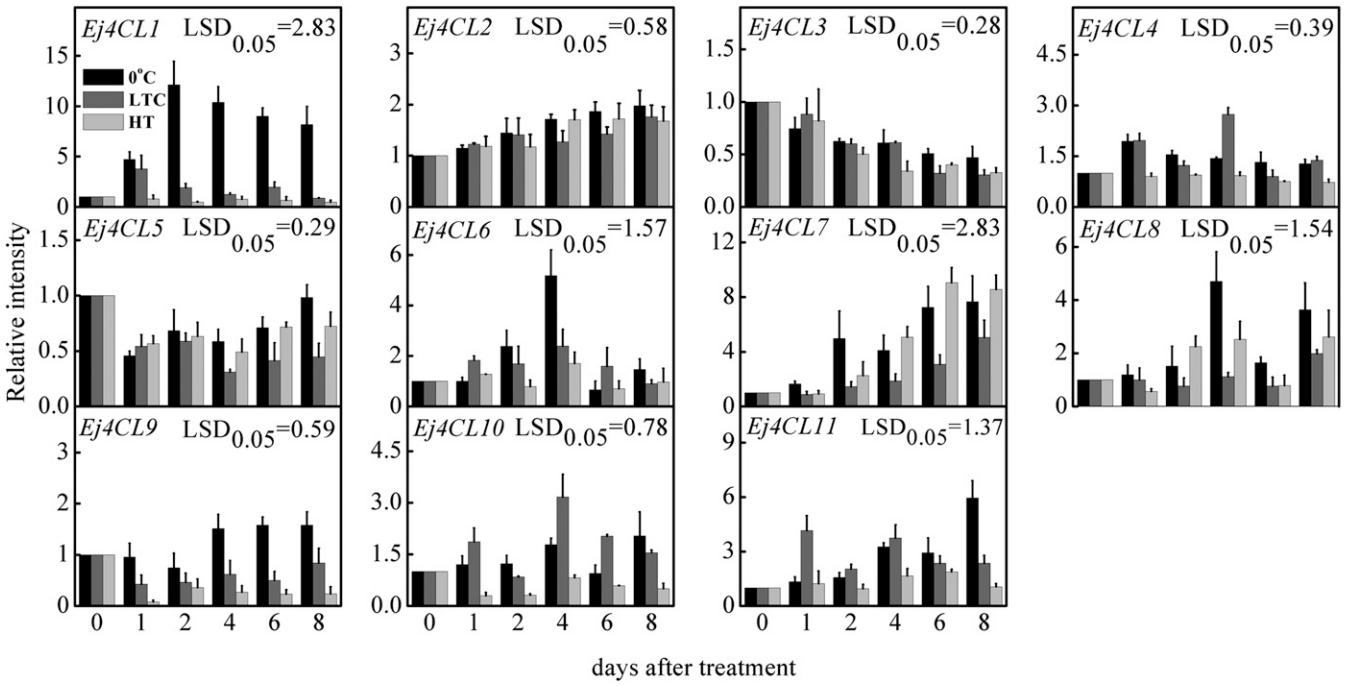

Fig. 6. Effects of low-temperature conditioning (LTC) and heat treatment (HT) on *Ej4CL* expression. Transcripts of *Ej4CL genes* were measured by real-time polymerase chain reaction. Day 0 fruit values were set as 1. Error bars indicate SE from three biological replicates. Least significant differences (LSDs) represent least significant difference at 0.05.

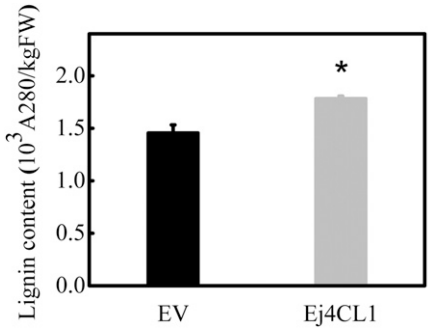

Fig. 7. Transient overexpression of *Ej4CL1* in *N. tabacum* leaves. *Ej4CL1* was driven by the CaMV 35S promoter. EV represents empty vector. Error bars indicate SE from three biological replicates (*$P < 0.05$).

genes showed little response and less difference between treatments. Of all the *Ej4CL* genes, *Ej4CL1* was the most responsive, showing an ≈12-fold increase after 2 d storage at 0 °C (Fig. 6).

Transient overexpression of Ej4CL1 in N. tabacum. The results of enzyme activity changes, phylogenetic tree and gene expression analysis all supported the putative role of *Ej4CL1* in loquat fruit lignification. As loquat is a perennial fruit, making transgenic experiments difficult, the function of *Ej4CL1* was tested in *N. tabacum*. Here, full length *Ej4CL1* was cloned into pGreen II 0029 62-SK vector, transferred to *Agrobacterium tumefaciens* GV3101, and injected into leaves of *N. tabacum*. The results indicated that leaves (half-blade) infiltrated with *Ej4CL1* had a lignin content of 1.78×10^3 A280 kg^{-1} of fresh weight, which was significant higher than that of leaves treated with the EV, with lignin content of 1.45×10^3 A280 kg^{-1} of fresh weight (Fig. 7).

Discussion

Lignin biosynthesis has been extensively studied in the model plant *Arabidopsis* and woody plants (Baucher et al., 1996; Voo et al., 1995), because of its roles in providing mechanical support, as a contaminant in paper manufacture, and as a substance adversely impacting on bioconversion and energy production from plants (Carroll and Somerville, 2009; Xu et al., 2011). In the past decade, the influence of lignin accumulation on fruit quality has been studied in various fruit species, such as loquat (Cai et al., 2006a, 2006b), where flesh lignification can be severe at low-temperature storage. A few biosynthetic enzymes in the phenylpropanoid pathway have been reported to be involved in loquat lignification, for instance PAL and CAD were associated with senescence-related lignification in loquat fruit (Cai et al., 2006a) and the activity of CAD was also related to the lignin content in different cultivars of loquat fruit (Shan et al., 2008). However, the role of other enzymes, such as C4H, have been generally ignored in previous research on CI and only *EjCAD1* (Shan et al., 2008) and *EjCCoAOMT* (Liu et al., 2015) have been reported to be related to lignification associated with loquat fruit CI.

Here, three enzymes of the phenylpropanoid pathway were investigated. Enzyme activities of PAL, C4H, and 4CL were all positively correlated with loquat fruit CI-induced lignification, and their activities were significantly repressed by two treatments (LTC and HT), which cause reduced lignification (Xu et al., 2014). This suggested that additional enzymes within the phenylpropanoid pathway were involved in loquat fruit lignification, although some enzymes, such as PAL and 4CL, have previously been considered as having no correlation with lignin contents of different cultivars (Shan et al., 2008). Accordingly, the expression patterns of 16 genes coding for PAL, C4H, and 4CL were analyzed. Accumulation of mRNAs for *Ej4CL1*, *Ej4CL6* and *Ej4CL9* were positively correlated with loquat fruit lignification in response to both LTC and HT; *EjPAL1* only responded to HT treatment, but not LTC; while the remaining genes showed little association with fruit lignification. The present results indicate that expression of *EjPAL1*, *EjPAL2*, *EjC2H1*, and *EjC2H4* showed limited correlation with activities of PAL and C2H, which suggest the potential of the other coding genes exist but remain unknown for loquat fruit, due to the lack of genome information. It is worth emphasizing that *Ej4CL1* was most responsive to low temperature (>10 folds) and also showed the most significant responses to HT and LTC treatments. *Ej4CL1* was clustered with class I *4CL* genes, although class I and class II *4CL* genes have previously been considered as lignin specific, such as *Ptr4CL1* (Hu et al., 1998). Taken together, the enzyme activity, phylogenetic tree and gene expression data, indicate that *Ej4CL1* is a key candidate gene for understanding the regulation of CI-induced lignification in loquat fruit.

Transient overexpression systems are widely used in various perennial fruit for gene function analysis, such as apple and crabapple anthocyanin-related genes (Espley et al., 2007); persimmon tannin-related genes (Min et al., 2012; Mo et al., 2015), kiwifruit aroma-related genes (Nieuwenhuizen et al., 2015). Using similar technologies, function of *Ej4CL1* on lignin biosynthesis was verified in *N. tabacum*. Transient overexpression of *Ej4CL1* significantly triggered lignin accumulation, which further supported that loquat *Ej4CL1* is an important candidate for lignin biosynthesis.

In conclusion, PAL, C4H and 4CL were positively associated with loquat fruit CI-induced lignification in addition to CCoAOMT and CAD, reported by others. Moreover, *Ej4CL1*, a class I member, was characterized as a candidate gene for control of lignin biosynthesis, whereas several other members of this gene family are not involved in this response.

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