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 1-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, functionality 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.

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, EjCoAOMT 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. Luoyangqiu (LVQ). 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, ‘LQ’ loquat fruit were collected at commercial maturity from an orchard at Luguiao, Zhejiang, China in 2011. Fruit without disease or mechanical woundings were selected and divided into three groups for treatments. Three treatments are as follows: 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.
(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 (AmBio). 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 a reference gene.

**Gene expression analysis.** Real-time PCR was performed in a total volume of 20 μl containing 10 μl LightCycler 480 SYBR Green I Master Mix (Roche), 6 μl 10 × 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−ΔΔCt method (0 d was set as 1).

**Transient overexpression and lignin measurement.** To verify *Ej4CLI1* function in lignin biosynthesis, transient overexpression was conducted with *N. tabacum* leaves. The full length of *Ej4CLI1* 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 MgCl2, and 0.5 mM aceticosyringone) and OD600 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 (LSD0.05) 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 samples. 

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

| Genes | Method used | Primers | Sequences (5’ to 3’) |
|-------|-------------|---------|---------------------|
| *EjC4H1* | 3’ RACE | Primary PCR | CTTCTCCGATGCGGACGTCCA |
| *EjC4H2* | 3’ RACE | Primary PCR | GACATTCTAAGCGGACCTAGAGACC |
| *Ej4CL8* | 3’ RACE | Primary PCR | ACACCCGAAATTACCTCCCCAGGG |
| *Ej4CL9* | 3’ RACE | Primary PCR | TTACAATTCTCACAGCACGCCG |
| *Ej4CL10* | 3’ RACE | Primary PCR | ACACCCAGAAATTACCCTCCCCAGG |
| *Ej4CL11* | 3’ RACE | Primary PCR | CACGCTGTTTCCACACATCAACAGG |

**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* | TGAGATCACCAGGGGAGGAAAA | GAGCACCATTCTCCTCTCCTCA |
| *EjPAL2* | GGATTGGGAAAGGAGATACC | AGCATGTGAGCCAGCTCGAG |
| *EjC4H1* | ATTTAGGCTGAGGAGGAGGG | CACGGAGGATGTAAGTGGC |
| *EjC4H2* | ACCGTGTGATTAGAAGGAGACG | AGTGAGGAAAGAGGTGTGTG |
| *Ej4CL1* | AGTCACCGTGGGCAA | CATGGCCTCTCATCCGACC |
| *Ej4CL2* | CCTCACCAAAATCCCGAAA | AATTGGCTCTGCTGCTG |
| *Ej4CL3* | TGCAACAAAAAACACACG | GTAGCTGTGCTCTTGGTG |
| *Ej4CL4* | CTTATCCACTCGTCTATCAAA | ATCGATGTAACGCTGGA |
| *Ej4CL5* | TTTTTCCTCTTCTTCTTACA | AATATGCGGAGGATGCG |
| *Ej4CL6* | TGACGAAAAAGGAGGATCCG | AACCATGCACATTTTTGAG |
| *Ej4CL7* | AATACTCACAAATCGTCAGT | TTGGATGCTGAGGAGGATG |
| *Ej4CL8* | ACAACACGACTCGGAGATC | AGTAACTGATGAGGGAGG |
| *Ej4CL9* | AAAAGAATCCTGAGGCAACG | GGCTCGGCCTCCATATT |
| *Ej4CL10* | AAGTACCCTACGGCTCTTCTG | TTGTGATTGCTGAGGAGG |
| *Ej4CL11* | TGTTGACACGCTGCAATTG | AACAACATACCCACACCA |
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 during low-temperature storage, but also decreased in response to LTC and HT. Expression of \textit{EjPAL1} and \textit{EjPAL2} increased at 0 °C and this was significantly inhibited by HT treatment. However, LTC treatment had little effect on either \textit{EjPAL1} or \textit{EjPAL2} (Fig. 4).

Two \textit{EjC4H} genes, \textit{EjC4H1} and \textit{EjC4H2}, exhibited different expression patterns, with the latter showing a much greater increase during LTC than the control. Expression of \textit{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 \textit{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 \textit{Ej4CL} genes could be generally divided into three groups: 1) expression of \textit{Ej4CL1}, \textit{Ej4CL6}, \textit{Ej4CL7}, \textit{Ej4CL8} increased in response to 0 °C; however, only \textit{Ej4CL1} and \textit{Ej4CL6} were significantly repressed by both LTC and HT; 2) \textit{Ej4CL9} had a much smaller response to 0 °C, but was significant inhibited by LTC and HT; 3) other \textit{Ej4CL genes} were measured by real-time polymerase chain reaction. Day 0 fruit values were set as 1. Error bars indicate SR from three biological replicates. Least significant differences (LSDs) represent least significant difference at 0.05.

Expression of \textit{EjPAL}, \textit{EjC4H}, and \textit{Ej4CL} genes in response to LTC and HT treatments. The relationship between previously isolated \textit{EjPAL} and \textit{Ej4CL} genes and CI-related 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 \textit{EjPAL1-2} (EF685343-4) and \textit{Ej4CL1-5} (EF685345, KF767455-8) (Shan et al., 2008; Xu et al., 2014), on the basis of previous reports, and two novel \textit{EjC4H} (\textit{EjC4H1}, KU176088; \textit{EjC4H2}, KU176089) and six new \textit{Ej4CL} genes (\textit{Ej4CL1-6}, KU176090-KU176095) were isolated on the basis of RNA-seq and RACE.

Phylogenetic analysis revealed that \textit{EjC4H1} and \textit{EjC4H2} were clustered with \textit{Genlisea aurea GaC4H} (EP56721) and \textit{Zostera marina ZmC4H} (KMZ73596) (Fig. 2). Eleven \textit{Ej4CL} genes, including five previously reported and six newly isolated, were divided into two major classes, class I and class II. \textit{Ej4CL1} and \textit{Ej4CL5} belong to class I and were clustered with \textit{Pro4CL1}, \textit{Pro4CL6}, \textit{Nov4CL1}, and \textit{Nov4CL2}; whereas the other \textit{Ej4CL} genes belong to class II (Fig. 3).

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

Two \textit{EjC4H} genes, \textit{EjC4H1} and \textit{EjC4H2}, exhibited different expression patterns, with the latter showing a much greater increase during LTC than the control. Expression of \textit{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 \textit{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).
leaves of N. tabacum and injected into Agrobacterium GV3101, and transferred to the SK vector. 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).

Transgenic overexpression of Ej4CL1 in N. tabacum. The results of enzyme activity 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 $1.78 \times 10^3$ A280 kg$^{-1}$ of fresh weight, which was significant higher than that of leaves treated with the EV, with lignin content of $1.45 \times 10^3$ 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 EjCCoIOMT (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 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.

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, Ej2H1, and Ej2H4 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 Pn4CL1 (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-resistant replication, which further supported that loquat Ej4CL1 is an important candidate for lignin biosynthesis, whereas several other members of this gene family are not involved in this response.

**Literature Cited**

Barros, J., H. Serk, I. Granlund, and E. Pesquet. 2015. The cell biology of lignification in higher plants. Ann. Bot. (Lond.) 115:1053–1074.

Baucher, M., B. Chabbert, G. Pilate, J. Dooreselaere, M.T. Tollier, M. Petit-Conil, D. Cornu, B. Monties, M. Montagu, D. Inzé, L. Jouanin, and W. Boerjan. 1996. Red xylem and higher lignin extractability by down-regulating a cinamyl alcohol dehydrogenase in poplar (*Populus tremula* x *P. alba*). Plant Physiol. 112:143–150.

Bonawitz, B.D. and C. Chapple. 2012. The genetics of lignin, an important material component that provides structural support and protection to plants. Plant Physiol. 159:236:2692–2698.

Cai, C., W.P. Xu, W.S. Zhang, X. Li, I.B. Ferguson, and K.S. Chen. 2006b. Accumulation of lignin in relation to change in activities of lignification enzymes in loquat fruit flesh after harvest. Postharvest Biol. Technol. 40:163–169.

Cai, C., J.J. Xu, L.L. Shan, X. Li, C.H. Zhou, W.S. Zhang, I.B. Ferguson, and K.S. Chen. 2006d. Low temperature conditioning reduces postharvest chilling injury in loquat fruit. Postharvest Biol. Technol. 41:252–259.

Cao, S.F., Y.T. Cai, Z.F. Yang, and Y.H. Zheng. 2012. MEJA induces chilling tolerance in loquat fruit by regulating proline and Y-amino butyric acid contents. Food Chem. 133:1466–1470.

Carroll, A. and C. Somerville. 2009. Cellulosic biofuels. Annu. Rev. Plant Biol. 60:165–182.

Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kurty-Ammu, and A.C. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. 49:414–427.

Hellens, R.P., A.C. Allan, E.N. Friel, K. Boltho, K. Grafton, M.D. Templeton, S. Karunairetnam, A.P. Gleave, and W.A. Laing. 2005. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods 1:13.

Hu, W.J., A. Kawaoka, C.J. Tsai, J. Lung, K. Osakabe, H. Ebinuma, and V.L. Chiang. 1998. Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). Proc. Natl. Acad. Sci. USA 95:5407–5412.

Knobloch, K.H. and K. Hahlbrock. 1975. Isoenzymes of p-coumarate: CoA ligase from cell suspension cultures of *Glycine max*. Eur. J. Biochem. 52:311–320.

Koukol, J. and E.E. Conn. 1961. The metabolism of aromatic compounds in higher plants. IV. Purification and properties of the phenylalanine deaminase of *Hordeum vulgare*. J. Biol. Chem. 236:2692–2698.

Min, T., X.R. Yin, Y.N. Shi, Z.R. Luo, Y.C. Yao, J.K. Zeng, J.K. Zeng, X. Li, J.Y. Chen, X.R. Yin, I.B. Ferguson, and K.S. Chen. 2015. 4-Coumarate:CoA ligase from cell suspension cultures of *Glycine max* regulates chilling injury. J. Expt. Bot. 61:179–190.

Xu, B., L.L. Escamilla-Trevino, N. Sathitsuksanoh, Z.X. Shen, H. Shen, Y.H.P. Zhang, R.A. Dixon, and B.Y. Zhao. 2011. Silencing of 4-coumarate:coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. New Phytol. 192:611–625.

Xu, M.J., J.F. Dong, M. Zhang, X.B. Xu, and L.N. Sunday. 2012. Cold-induced endogenous nitric oxide generation plays a role in chilling tolerance of loquat fruit during postharvest storage. Postharvest Biol. Technol. 65:5–12.

Xu, Q., X.R. Yin, J.K. Zeng, H. Ge, M. Song, C.J. Xu, X. Li, I.B. Ferguson, and K.S. Chen. 2014. Activator- and repressor-type MYB transcription factors are involved in chilling injury induced flesh lignification in loquat via their interactions with the phenylpropanoid pathway. J. Expt. Bot. 65:439–459.

Xu, Q., W.W. Wang, J.K. Zeng, J. Zhang, D. Grierson, X. Li, X.R. Yin, and K.S. Chen. 2015. A NAC transcription factor, *EjNAC1*, affects lignification of loquat fruit by regulating lignin biosynthesis. *Plant Cell Environ.* 39:1780–1789.

Zeng, J.K., X. Li, Q. Xu, J.Y. Chen, X.R. Yin, I.B. Ferguson, and K.S. Chen. 2015. *EjAP2-1*, an *AP2/ERF* gene, is a novel regulator of fruit lignification induced by chilling injury, via interaction with *EjMYB* transcription factors. Plant Cell Environ. 38:1253–1268.

Zeng, J.K., X.L. Li, J. Zhang, H. Ge, X.R. Yin, and K.S. Chen. 2016. Regulation of loquat fruit low temperature response and lignification involves interaction of heat shock factors and genes associated with lignin biosynthesis. Plant Cell Environ. 39:1778–1789.

Zeng, Y.K., Q. Xu, Y.K. Zeng, H. Ge, X.R. Yin, and K.S. Chen. 2017. Expression profiles in loquat fruit with different lignin accumulation and genes associated with lignification and their expression profiles in loquat fruit with different lignin accumulation. Planta 227:1243–1254.

Zhou, V.J.H., W. Ni, J.W. Blount, H.G. Jung, S.A. Masoud, P.A. Howles, C. Lamb, A. Sameer, A. Paul, C. Lamb, and R.A. Dixon. 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol. 115:41–50.

Shan, L.L., X. Li, P. Wang, C. Cai, B. Zhang, C.D. Sun, W.S. Zhang, C.J. Xu, I.B. Ferguson, and K.S. Chen. 2008. Characterization of cDNAs associated with lignification and their expression profiles in loquat fruit with different lignin accumulation. Plant Cell Environ. 31:985–995.