Research article

Ternary complex EjbHLH1-EjMYB2-EjAP2-1 retards low temperature-induced flesh lignification in loquat fruit

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ABSTRACTS

Many transcription factors (TFs), including NACs and MYBs, are involved in regulation of lignin biosynthesis during plant development and in responses to biotic and abiotic stresses. The lignin biosynthesis gene *Ej4CL1* has been identified as a target for cold-induced TFs. We isolated a *bHLH* gene from loquat, *EjbHLH1*, the expression of which was negatively correlated with cold-induced fruit lignification. During low temperature storage (0 °C), *EjbHLH1* transcripts were stable but accumulated during low-temperature conditioning (LTC) treatment, an acclimation process that reduces lignification during subsequent storage at 0 °C. Dual luciferase assays showed EjbHLH1 could repress *Ej4CL1* promoter, but yeast one hybrid assay indicated EjbHLH1 is not able to bind to the *Ej4CL1* promoter. Bimolecular fluorescence complementation (BIFC) indicated that EjbHLH1 could interact with EjAP2-1 and EjMYB2, two previously characterized fruit lignification related transcription factors and firefly luciferase complementation imaging assay indicated EjbHLH1, EjMYB2 and EjAP2-1 could form a ternary complex which enhanced repression of transcription from the *Ej4CL1* promoter, reducing lignification at 0 °C.

1. Introduction

Lignin is an important component of plant secondary cell walls and plays a part in protecting cells and enhancing plant mechanical strength (Rencoret et al., 2011). The biosynthesis of lignin is catalyzed by enzymes in the phenylpropanoid pathway including phenylalanine ammonia lyase (PAL), 4-coumarate:CoA ligase (4CL) and cinnamyl alcohol dehydrogenase (CAD) (Zhao, 2016).

The transcriptional regulation of lignin biosynthesis and regulation of enzymes involved in the phenylpropanoid pathway has been widely investigated. In *Arabidopsis*, *AtMYB58, AtMYB63* (Zhou et al., 2009) and *AtMYB85* (Zhong et al., 2008) are activators of lignification, while *AtMYB4, AtMYB7* and *AtMYB32* repress the expression of lignin biosynthesis genes (Jin et al., 2000; Preston et al., 2004; Fornalé et al., 2014). Two NAC transcription factors (TFs), *AtNST1* and *AtNST2*, regulated secondary wall thickening and overexpression of *AtNST1* in *Arabidopsis* resulted in increased lignin content (Mitsuda et al., 2005). The main TFs reported to be involved in lignin biosynthesis are NACs

and MYBs, but some others have also been identified. *VvWRKY2* could activate the *VvC4H* gene promoter and overexpression of *VvWRKY2* in tobacco modulated the expression of lignin biosynthetic pathway genes (Guillaumie et al., 2010). Overexpression of an *Arabidopsis* AP2/ERF gene (*SHINE*) in rice resulted in ~ 45% reduction in lignin content (Ambavaram et al., 2011). In *Arabidopsis*, overexpression of sorghum transcription factor *SbbHLH1* down-regulated lignin synthesis genes *At4CL1, AtHCT, AtCOMT, AtPAL1* and *AtCCR* (Yan et al., 2013). These results suggested that the other transcription factors, such as those in the bHLH family, are also involved in the regulation of lignin biosynthesis.

bHLH transcription factors have been widely reported to form a bHLH-MYB-WD40 complex (eg. *TT2-TT8-TTG1* in *Arabidopsis*) that regulates synthesis of anthocyanin and proanthocyanidins (Baudry et al., 2004) and tobacco *NtMYC2a* and *NtMYC2b* could form complexes with the *NtJAZ1* repressor to regulate jasmonate-inducible nicotine biosynthesis (Zhang et al., 2012). However, the role of bHLH TFs in regulation of lignin biosynthesis has rarely been reported and their

relationship with other lignin related transcription factors is unknown.

Low temperatures can induce various types of chilling injuries in stored plant products resulting in internal browning, membrane leakage and lignification (Wang et al., 2006; Ghorbani et al., 2017). An increase in flesh firmness and lignin content is an important adverse effect of low temperature storage in several fruit including loquat (Cai et al., 2006), which significantly reduces fruit quality and storability (Li et al., 2010; Tucker et al., 2017). The first TFs shown to be involved in regulating loquat lignin biosynthesis were MYB genes, *EjMYB1* and *EjMYB2* can activate and repress the promoter of the lignin biosynthesis gene *Ej4CL1* separately (Xu et al., 2014). Further study identified an AP2/ERF gene, EjAP2-1, that trans-represses the *Ej4CL1* promoter by interacting with EjMYB1 and EjMYB2 (Zeng et al., 2015). In grape fruit, transcription factor *VvWRKY2* was also shown to plays a role in regulating lignification by activating the promoter of gene *VvC4H*. Over-expressing *VvWRKY2* in tobacco delayed xylem formation and altered lignin composition in both stems and petioles (Guillaumie et al., 2010). PbrmiR397a regulated lignification of stone cell in pear fruit and overexpressing PbrmiR397 in tobacco resulted in down-regulation of LAC expression and decreased lignin content (Xue et al., 2018). Compared to model plants, however, lignification in fruit is poorly understood.

In the present research, a *bHLH* gene, *EjbHLH1*, was isolated from loquat fruit. Expression of *EjbHLH1* was negatively correlated with fruit lignification and transient expression assay confirmed the repressing role of *EjbHLH1* in the regulation of lignin biosynthesis. Further analysis indicated this negative action of *EjbHLH1* in inhibiting the expression of the lignin biosynthesis gene *Ej4CL1* involved formation of a ternary complex between *EjbHLH1, EjMYB2* and *EjAP2-1*.

2. Materials and methods

2.1. Plant materials and treatments

The fruit of loquat (*Eriobotrya japonica* Lindl. cv. Luoyangqing, LYQ) were collected in 2018 at Luqiao, Zhejiang province, China. After the fruit were transported to the laboratory, uniform fruit were selected and divided into two batches with about 120 fruit in each. For the low-temperature conditioning (LTC) treatment, the fruit were stored at 5 °C for 6 d and then transferred to 0 °C; control fruit were stored at 0 °C. The fruit treatments method was carried out as described previouly (Zeng et al., 2015).

2.2. Determination of fruit firmness and lignin content

Fruit firmness was measured using a TA-XT plus Texture Analyzer (Stable Micro Systems, Surrey, UK) with a 5 mm diameter probe (Xu et al., 2014). Firmness of each fruit was averaged from two measurements 90° apart at the fruit equator, after removal of a small piece of peel. Fruit firmness was expressed as Newtons (N) and 12 individual fruit replicates were used. Lignin content determination was according to the methods described previously (Xu et al., 2014). Absorbance was measured at 280 nm, using a Thermo Scientific Microplate Reader.

2.3. Gene isolation and phylogenetic analysis

The *bHLH* gene, *EjbHLH1,* was identified by RNA-seq and the full sequence determined by PCR sequencing. Alignment was performed using the neighbor-joining method with ClastalX (v1.8.1), the other amino acid sequences of *bHLH* genes were obtained from The Arabidopsis Information Resource (TAIR). The phylogenetic tree was constructed using FigTree (v1.3.1).

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen loquat flesh according to the

protocol described previously (Xu et al., 2014). TURBO DNA-free kit (ambion) was used to remove genomic DNA. The total RNA was quantified using Nanophotometer Pearl (Implen). 1 μg of RNA was used to synthesize cDNA according to the user manual (BioRad).

2.5. Real-time PCR analysis

Gene specific primers for measuring expression of *EjbHLH1* were designed using the online software Primer3 (http://primer3.ut.ee/). The quality and specificity of each pair of primers were tested by melting curves and product sequencing. The *EjACT* gene (GenBank no.JN004223) was chosen as the internal control. Primers for real-time PCR analysis of *EjbHLH1* were as follow: forward primer: 5′-GGAATA AAAGCAAAGGGAACGCT-3′; reverse primer: 5′- ACGGACAGCTCC ATG -3′.

2.6. Dual-luciferase assay

Dual-luciferase assay was used to analyze the role of EjbHLH and previously reported transcription factors in transactivating the promoters of loquat lignin biosynthesis genes. Full-length of *EjbHLH1* was cloned into *EcoR* I and *Sal* I digested pGreen II 0029 62-SK vector (SK).

All recombinant control (SK) and LUC constructs were electroporated into *Agrobacterium tumefaciens* GV3101, which were cultured and then diluted to an OD_{600} of 0.75 and infiltrated into tobacco (*Nicotiana tabacum*) leaves with infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 mM acetosyringone, pH 5.6). Three days after infiltration, the LUC and REN fluorescence intensities were assayed using dual-luciferase assay reagents (Promega), with at least four replicates in each experiment.

2.7. Yeast one-hybrid assay

Yeast one-hybrid assays were conducted using the Matchmaker™ Gold Yeast One-Hybrid Library Screening System, as described in (Xu et al., 2014). The *Ej4CL1* promoter was constructed into pAbAi vector and the full-length coding sequence (CDS) of *EjbHLH1* was inserted into *EcoR* I- and *BamH* I-digested pGADT7 vector. SD medium with aureobasidin lacking leucine (SD-Leu + AbA) was used for yeast one-hybrid assays.

2.8. Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) was used to verify the interaction of different proteins. BIFC assay was conducted according to previous published protocol (Marchler-Bauer et al., 2017). Full-length *EjAP2-1*, *EjMYB2* and *EjbHLH1* sequences were inserted into C- and N-terminal YFP vectors using *Kpn* I and *Sal* I restriction enzyme sites and the primers used listed in Supplementary Table S1. The constructs were transferred into *Agrobacterium tumefaciens* GV3101 and overexpressed in tobacco (*Nicotiana tabacum*) leaves. Two days after infiltration, the results were visualized using a fluorescence microscope (Nikon A1-SHS).

2.9. Firefly luciferase complementation imaging assay

The CDS of *EjbHLH1* and *EjMYB2* without stop codons were inserted into the pCAMBIA1300-nLuc vectors using *Kpn* I and *Sal* I digestion and the CDS of *EjAP2-1* and *EjbHLH1* were cloned into the pCAMBIA1300-cLuc vectors using *Kpn* I and *Sal* I digestion. *Agrobacterium tumefaciens* GV3101 cultures carrying the recombinant vectors were grown on lysogeny broth (LB) agar plates with kanamycin and gentamycin, then suspended in infiltration buffer to OD_{600} 0.5, for *Agrobacterium tumefaciens* carrying pCAMBIA1300 vectors, or OD_{600} 1.5, for *Agrobacterium tumefaciens* carrying pGreenII SK vectors and incubated at room temperature for 3 h before being infiltrated into tobacco (*Nicotiana*

tabacum) leaves. Three days after infiltration, 0.2 mM luciferin was infiltrated into the same position where *Agrobacterium tumefaciens* was infiltrated. The luciferase activity was detected using the NightSHADE LB981 imaging system, according to the previous study (Li et al., 2017).

2.10. Subcellular localization analysis

The CDS of *EjbHLH1* and *EjAP2-1* without stop codons were each inserted as C-terminal fusions in frame with the GFP gene into pCAMBIA1300-35S-eGFP vector using *Kpn* I and *Sal* I digestion. The *Agrobacterium tumefaciens* GV3101 with fusion constructs 35S::*EjbHLH1*-GFP, 35S::*EjAP2-1*-GFP were transfected into tobacco (*Nicotiana tabacum*) according to a previous report (Xu et al., 2014). The visualized results were analyzed using a fluorescence microscope (Nikon A1-SHS).

2.11. Transient expression

In order to explore the function of EjbHLH1, a transient expression system was used in tobacco (*Nicotiana tabacum*) leaves (Tucker et al., 2017). The *Agrobacterium tumefaciens* GV3101 contained pGreen II 0029 62-SK vector (SK) with or without the insertion of EjbHLH1 were infiltrated into the two sides of tobacco leaves. Six replicates were used for lignin analysis.

2.12. Statistical analysis

The statistical significance of differences was calculated using Student's *t*-test. Least significant difference (LSD) at the 5% level was calculated using DPS7.05 (Zhejiang University, Hangzhou, China).

3. Results

3.1. Gene isolation and analysis

A *bHLH* gene was isolated from 'LYQ' loquat fresh based on the RNA-seq data of 0 °C and LTC treatment (Liu et al., 2019), which has negative correlation and regular expression pattern, which was designated as *EjbHLH1* (MK138566). NCBI conserved domain analysis indicated it has a dimerization interface (Supplementary Fig S1) (Marchler-Bauer et al., 2017), suggesting that EjbHLH1 has the potential to physically interact with other proteins. According the phylogenetic analysis, *EjbHLH1* belongs to *bHLH1* subgroup VII and clustered with *AtbHLH39* (Fig. 1), which has been reported to respond to jasmonic acid (Brioudes et al., 2009). A*grobacterium tumefaciens* GV3101 carrying 35S::*EjAP2-1*-GFP and 35S::*EjbHLH1*-GFP vectors were infiltrated into transgenic tobaccos that stably transformed with mCherry and subcellular localization showed red and green protein fluorescence in the nucleus, which is similar to most transcription factors (Fig. 2).

3.2. Association of EjbHLH1 expression and loquat fruit lignification

As shown in Fig. 3, 'LYQ' fruit fresh firmness increased rapidly from about 3.6 N to about 4.3 N on day one and then increased more slowly for the next 5 d during 0 °C storage. LTC treatment significantly retarded this increase, which was less about 7.4% that at 0 °C storage. Lignin content increased gradually over the first 2 d after harvest and remained stable for the next 4 d at 0 °C, whereas the increase in lignin content was delayed by LTC treatment. *EjbHLH1* transcripts were relatively stable during 0 °C storage but were induced in LTC treatment, eventually becoming approximately 4-fold higher after 6 d storage at 0 °C (Fig. 3).

To test the effect of EjbHLH1 on lignin content, an ectopic transient tobacco leaf overexpression system was used. As shown in Fig. 4, the lignin content in leaves was decreased (from 1.97×10^3 to 1.43×10^3 $A_{280}kg^{-1}$ of fresh weight) by EjbHLH1 over-expression.
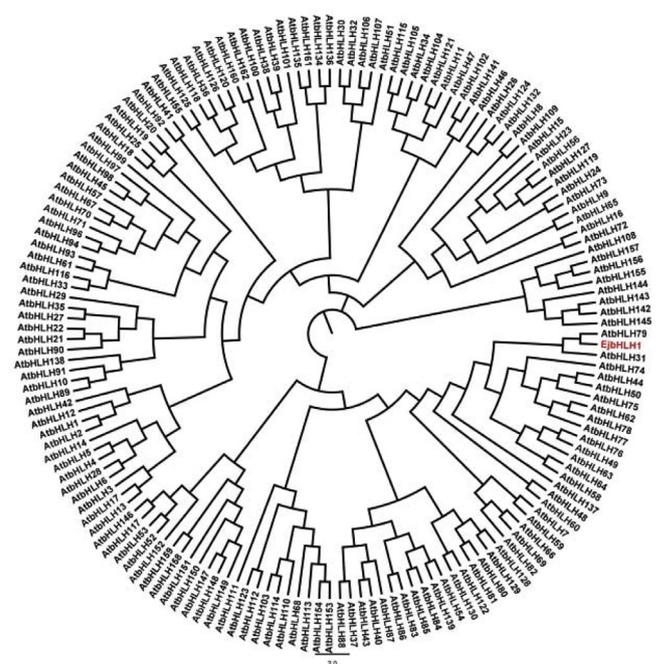

Fig. 1. Phylogenetic analysis of loquat and Arabidopsis bHLH deduced protein sequences. Amino acid sequences of Arabidopsis bHLH genes were downloaded from TAIR (https://www.arabidopsis.org/). The loquat bHLH gene is shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Regulatory effects of EjbHLH1 on expression of lignin biosynthetic genes

To investigate the regulatory effects of *EjbHLH1* on transcription of lignin pathway genes, its ability to transactivate the promoters of loquat lignin biosynthesis genes *Ej4CL1-5* and *EjCAD3* was tested using dual luciferase assay in tobacco leaves. The results indicated that EjbHLH1 could inhibit the activity of the *Ej4CL1* promoter while EjbHLH1 had little effect on promoters of *Ej4CL2* and *Ej4CL4* and had no effect on *Ej4CL3*, *Ej4CL5* and *EjCAD3* promoters (Fig. 4). However, the yeast one-hybrid assay showed EjbHLH1 could not bind the *Ej4CL1* promoter (Supplementary Fig S2).

Two activators, EjMYB1 and EjMYB8, as well as two repressors, EjMYB2 and EjAP2-1, were previously identified as acting on the *Ej4CL1* promoter (Xu et al., 2014; Zeng et al., 2015; Wang et al., 2016). The effects of EjbHLH1 on the promoters of *EjMYB1*, *EjMYB2*, *EjMYB8* and *EjAP2-1* were also investigated using dual luciferase assay. However, the results indicated EjbHLH1 could not regulate these transcription factors (Supplementary Fig S3).

3.4. Synergistic effect of EjbHLH1, EjAP2-1 and EjMYB2 on transactivation of the lignin biosynthesis-related Ej4CL1 gene promoter

Using the dual luciferase assay, the synergistic effect of *EjAP2-1*, *EjbHLH1* and *EjMYB2* was investigated. All repressive effects were compared to the control value, set as 1 (Fig. 5). EjbHLH1 and EjMYB2 could repress the activity of the *Ej4CL1* promoter. The paired combinations *EjMYB2* and *EjbHLH1*, *EjAP2-1* and *EjbHLH1*, *EjAP2-1* and *EjMYB2*, generated a stronger suppression effects on the *Ej4CL1* promoter than that obtained with the individual transcription factor. The combination of *EjbHLH1* with *EjAP2-1* and *EjMYB2* generated a 0.46- and 0.42 repressive effect compared to the value of 0.7 for the single transcription factor *EjbHLH1* and *EjMYB2*. The greatest suppression was observed when *EjMYB2*, *EjbHLH1* and *EjAP2-1* were tested in combination, where the activity of *Ej4CL1* promoter was reduced to a value of
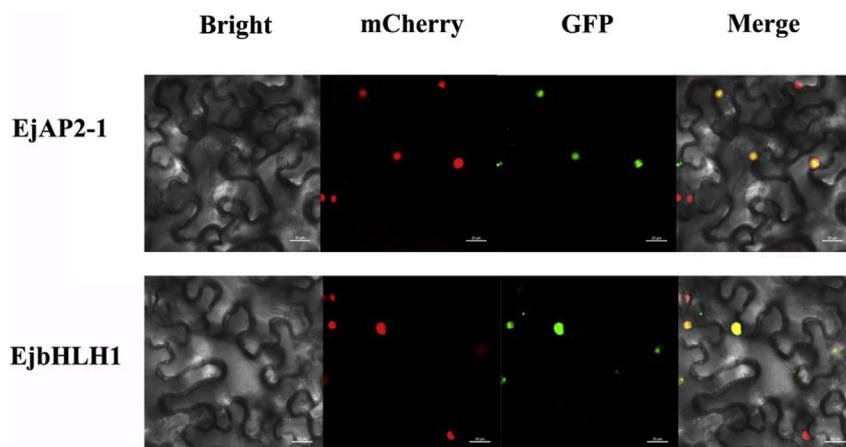
Fig. 2. Subcellular localization of EjAP2-1 and EjbHLH1. *35S::EjbHLH1-GFP* or *35S::EjAP2-1-GFP* vectors was transfected into transgenic tobacco leaves that expressed the nuclear location marker of mCherry stably.
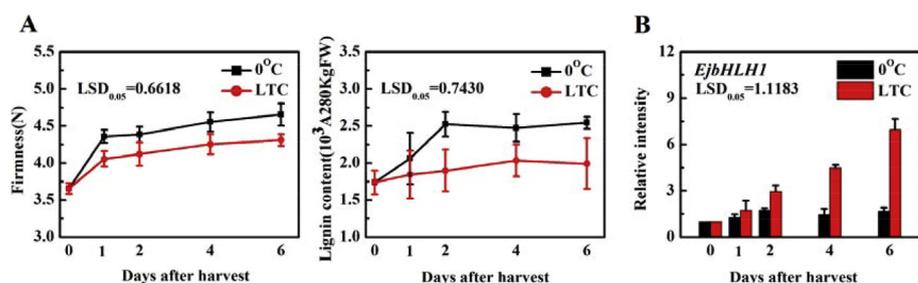

Fig. 3. (A) Comparative analysis of fruit firmness, lignification at 0 °C with and without prior LTC treatment (5 °C storage for 6 d before transfer to 0 °C). The firmness was measured by probe penetration. (B) The expression of *EjbHLH1* during 0 °C storage, with and without prior LTC treatment. Gene expression was expressed as a ratio relative to the harvest time point (0 d), which was set as 1. The error bars indicated stand error (SE) from twelve (firmness) or three (lignin and gene expression) biological replicates.
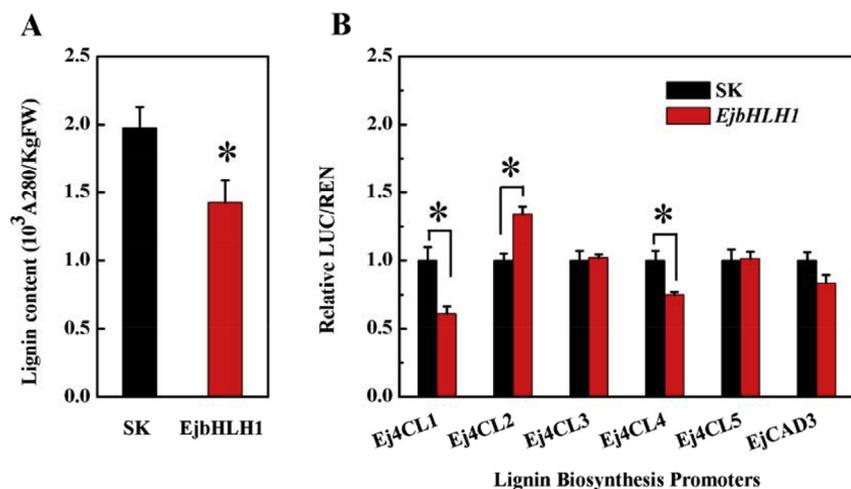

Fig. 4. (A) Transient overexpression of *EjbHLH1* in tobacco leaves. EjbHLH1 was driven by the CaMV 35S promoter. Error bars indicate standard error (SE) from five (for dual luciferase assay) and six (for lignin content) biological replicates (*P < 0.05). (B) *In vivo* interaction of *EjbHLH1* with promoters of lignin biosynthesis genes from LYQ loquat fruit. The ratio of LUC/REN of the empty vector (SK) plus promoter was used as calibrator, set as 1. Error bars indicate SE from at least four replicates.

0.24 compared to the control (Fig. 5).

3.5. Protein-Protein interactions between EjbHLH1 and loquat fruit lignification-related TFs EjMYB2 and EjAP2-1

BiFC assays were performed in order to study the potential physical interactions between EjMYB2, EjAP2-1 and EjbHLH1. The results indicated that EjMYB2 and EjAP2-1 could separately interact with EjbHLH1 (Fig. 6). These interactions between EjbHLH1, EjAP2-1 and EjMYB2 were confirmed by firefly luciferase complementation imaging assays (Fig. 7), which also showed that the strongest fluorescence was observed when the three transcription factors were combined (Fig. 7).

4. Discussion

4.1. EjbHLH1 expression is negatively correlated with loquat fruit flesh lignification

Most fruit undergo softening during postharvest storage (Li et al., 2010). However, the firmness and lignin content of fruit such as red-fresh loquat ('LYQ') increased continuously during low temperature storage. This could be alleviated by LTC treatment (Fig. 3), which confirms the results of previous study (Xu et al., 2014). A bHLH gene, named as *EjbHLH1*, was isolated based on unigene from a loquat RNA-seq database (Liu et al., 2019). The expression of *EjbHLH1* in loquat flesh was relatively low during low-temperature (0 °C) storage but was induced by LTC treatment. Evolutionary analysis showed EjbHLH1 was clustered with AtbHLH31, which regulated late development of petal
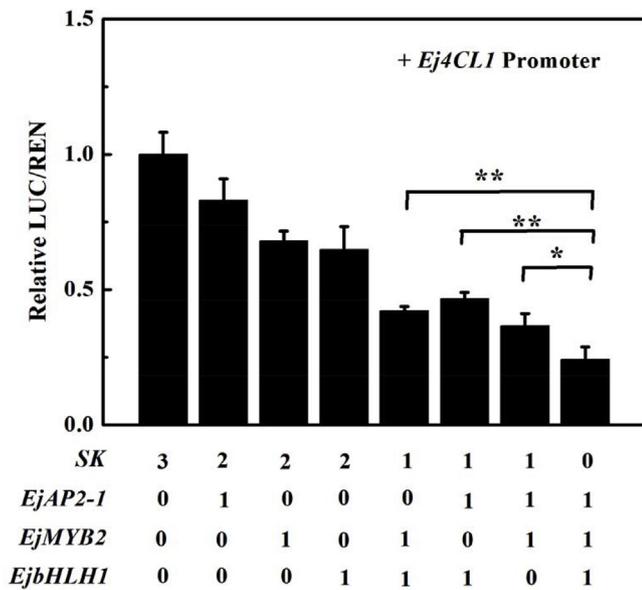
Fig. 5. Effects of *EjbHLH1*, *EjAP2-1* and *EjMYB2* alone and in combination on gene expression from the *Ej4CL1* promoter. The ratio of LUC/REN obtained with the empty vector (SK) plus promoter was used as calibrator, set as 1. Error bars indicate S.E.s from four replicates (*P < 0.05; **P < 0.01). The numbers (0,1) in each column represent the presence or absence of the relevant *Agrobacterium tumefaciens* construct or empty vector (SK).

growth by responding to jasmonate (Brioudes et al., 2009). The responses of *EjbHLH1* during LTC treatment indicated that *EjbHLH1* may act as a repressor in loquat fruit lignification, which was confirmed using an ectopic transient expression system and dual-luciferase assay (Fig. 4). Heterologous overexpression of Sorghum *SbbHLH1* in *Arabidopsis* resulted in lower plant lignin content and down-regulated the lignin synthesis genes *4CL1*, *HCT*, *COMT* and *CCR* indirectly (Yan et al., 2013). Although the protein sequence of EjbHLH1 shared only 12.85% similarity with SbbHLH1, both SbbHLH1 and EjbHLH1 resulted in decreased lignin content and down-regulated lignin biosynthesis gene
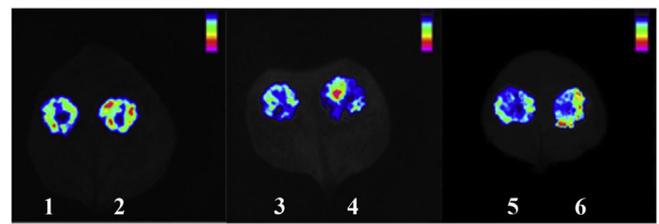
1   EjMYB2-nLuc / EjAP2-1-cLuc / SK

2   EjMYB2-nLuc / EjAP2-1-cLuc / SK-EjbHLH1

3   EjbHLH1-nLuc / EjAP2-1-cLuc / SK

4   EjbHLH1-nLuc / EjAP2-1-cLuc /SK-EjMYB2

5   EjbHLH1-nLuc / EjMYB2-cLuc / SK

6   EjbHLH1-nLuc / EjMYB2-cLuc /SK-EjAP2-1

Fig. 7. Luciferase complementation imaging assay was used to analyze the physical combination of EjbHLH1, EjAP2-1 and EjMYB2. The A*grobacterium tumefaciens* contains constructs were infiltrated into tobacco leaves. Luciferase activities were recorded after three days using the NightSHADE LB981 imaging system.

expression.

4.2. *EjbHLH1 indirectly represses lignification by forming a ternary complex involving EjbHLH1-EjMYB2-EjAP2-1 which acts on the Ej4CL1 promoter*

Although EjbHLH1 could trans-repress activities of the *Ej4CL1* promoter, it could not physically interact with the *Ej4CL1* promoter, which suggested that EjbHLH1 is an indirect regulator (Supplementary Fig S2). Synergetic regulations between transcription factors have been widely reported in fruit. VvMYC1 (a bHLH transcription factor) has been shown to participate in the regulation of anthocyanin and proanthocyanidin synthesis by physically interacting with MYB5a, MYB5b, MYBA1/A2, and MYBPA1, while single VvMYC1 could not activate the promoters of flavonoid pathway genes (Hichri et al., 2010). The results of the present study, however, indicated that EjbHLH1 had no significant effect on the promoters of *EjAP2-1*, *EjMYB1*, *EjMYB2* and
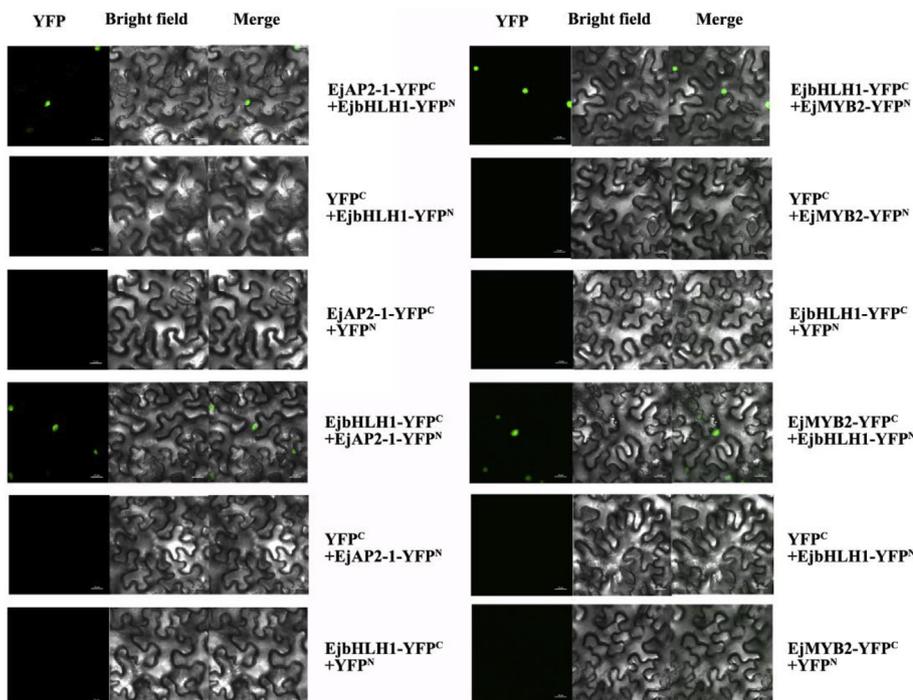

Fig. 6. Protein-protein interaction of EjbHLH1 and EjAP2-1 or EjbHLH1 and EjMYB2 using biomolecular fluorescence complementation (BiFC) in tobacco leaves. The CDS of *EjAP2-1*, *EjbHLH1* and *EjMYB2* were fused to the C- and N-terminus of YFP (YFP^C and YFP^N), respectively. The YFP^C or YFP^N vectors without genes inserted were used as negative controls. The bars indicated 25 μm.

EjMYB8.

Plant bHLH transcription factors typically function as homo- or hetero-dimers (Ciarapica et al., 2003). OsJAZ1 regulates drought tolerance by physically interacted with OsbHLH148 in rice (Seo et al., 2011). AtbHLH38 and AtbHLH39 could form hetero-dimers by interacting with AtFIT1 and co-overexpression of *AtFIT1* with *AtbHLH38* or *AtbHLH39* in *Arabidopsis* increased iron accumulation, indicating that AtFIT1 co-regulates iron homeostasis in *Arabidopsis* with AtbHLH38 or AtbHLH39 (Yuan et al., 2008). Thus, EjbHLH1 may participate in regulation of lignin biosynthesis by forming a dimer or higher order complex.

MYBs that interact with bHLHs share a conserved domain [DE]Lxx [RK]xxxLxxxxxxLxxxR (Zimmermann et al., 2004; Serna et al., 2006) and protein domain analysis indicated the EjMYB2 possesses this conserved domain (Supplementary Fig S4). Interestingly, the results of BIFC and firefly luciferase complementation imaging assays showed that EjbHLH1 could interact with both EjAP2-1 and EjMYB2. Moreover, our previous study indicated EjAP2-1 interacts with EjMYB2 (Zeng et al., 2015), thus EjbHLH1 may form a ternary complex to regulate lignin biosynthesis. Protein complexes such as dimers or ternary complexes are very important for the regulation of gene expression. For example, the expression of anthocyanin biosynthesis pathway genes is activated by a transcription initiation ternary complex of TT2-TT8-TTG1 in *Arabidopsis* (Baudry et al., 2004). In pear, PyMYB114 and PyMYB10 could form different ternary complexes (PyMYB10-PybHLH3-PyERF3 and PyMYB114-PybHLH3-PyERF3) to regulate anthocyanin biosynthesis (Yao et al., 2017).

Unlike these ternary transcriptional activator complexes, combination of EjbHLH1, EjMYB2 and EjAP2-1 significantly repressed transcriptional activation from the *Ej4CL1* promoter. Moreover, the firefly luciferase complementation imaging assay showed that the combination of three transcription factors generated brighter signals compared with the combination of two transcription factors, which strongly supports the possibility that a ternary complex is formed.

5. Conclusion

In conclusion, we identified a novel transcription factor, *EjbHLH1*, and showed it was an indirect regulator of the *Ej4CL1* promoter and participated in regulating loquat fruit lignification by forming a ternary complex, EjbHLH1-EjMYB2-EjAP2-1. This not only advanced the understanding of fleshy fruit lignification, but also demonstrated the role of a ternary complex in regulating fruit quality.

Conflicts of interest

The authors declare that they have no conflict of interest.

Funding

Author contributions

Xue-ren Yin and Kun-song Chen conceived and designed the experiments, Meng Xu and Shao-jia Li performed the experiments and analyzed the data, and Meng Xu and Xiao-fen Liu wrote the manuscript. Prof. Don Grierson improved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.032.

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