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

Meng Xu,1,2, Shao-jia Li,3,1, Xiao-fen Liu,4, Xue-ren Yin,1,2,* Donald Grierson,1,2,3, Kun-song Chen1,2

1 Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zhejiang University, Zijingang Campus, Hangzhou, PR China
2 The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou, PR China
3 Plant & Crop Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, United Kingdom

ARTICLE INFO

Keywords:
bHLH-MYB-AP2/ERF
Chilling injury
EjbHLH1
Lignin
Loquat fruit
Ternary complex

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 aclimation 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 SbhlH1 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 NMYC2a and NMYC2b 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. They can activate and repress the promoter of the lignin biosynthesis gene Ej4C1L separately (Xu et al., 2014). Further study identified an AP2/ERF gene, EjAP2-1, that trans-represses the Ej4C1L promoter by interacting with EjMYB1 and EjMYB2 (Zeng et al., 2015). In grape fruit, transcription factor VvWRKY2 was also shown to play a role in regulating lignification by activating the promoter of gene VvC4H. Over-expressing VvWRKY2 in tobacco delayed xylem formation and decreased lignin content (Xue et al., 2018). Co-localization of the expression of VvC4H and lignin in xylem vessels indicates this negative action of VvWRKY2 in the regulation of lignin biosynthesis. Further analysis showed the co-localization of expression of the lignin biosynthesis gene Ej4C1L involved formation of a ternary complex between EjWHH1, 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 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. They can activate and repress the promoter of the lignin biosynthesis gene Ej4C1L separately (Xu et al., 2014). Further study identified an AP2/ERF gene, EjAP2-1, that trans-represses the Ej4C1L promoter by interacting with EjMYB1 and EjMYB2 (Zeng et al., 2015). In grape fruit, transcription factor VvWRKY2 was also shown to play a role in regulating lignification by activating the promoter of gene VvC4H. Over-expressing VvWRKY2 in tobacco delayed xylem formation and decreased lignin content (Xue et al., 2018). Co-localization of the expression of VvC4H and lignin in xylem vessels indicates this negative action of VvWRKY2 in the regulation of lignin biosynthesis. Further analysis showed the co-localization of expression of the lignin biosynthesis gene Ej4C1L involved formation of a ternary complex between EjWHH1, EjMYB2 and EjAP2-1.

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 based on 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'-GGATA AAGGCAAAGGAACGCT-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 electro-porated into Agrobacterium tumefaciens GV3101, which were cultured and then diluted to an OD600 of 0.75 and infiltrated into tobacco (Nicotiana tabacum) leaves with infiltration buffer (10 mM MES, 10 mM MgCl2, 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 Ej4C1L 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 the 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.

2.9. 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 OD600 0.5, for 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 OD600 0.5, for Agrobacterium tumefaciens carrying pCAMBIA1300 vectors, or OD600 1.5, for Agrobacterium tumefaciens carrying pCAMBIA1300 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 pCAMBIA1300-3SS-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 AdhHLH39 (Fig. 1), which has been reported to respond to jasmonic acid (Brouids et al., 2009). Agrobacterium tumefaciens GV3101 carrying 35S::EjAP2-1-GFP and 35S::EjbHLH1-GFP vectors were infiltrated into transgenic tobacco 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 was maintained 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
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).

0.24 compared to the control (Fig. 5).

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
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 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 EjMYR2.

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).

Fig. 6. Protein-protein interaction of EjbHLH1 and EjAP2-1 or EjHLH1 and EjMYB2 using biomolecular fluorescence complementation (BiFC) in tobacco leaves. The CDS of EjAP2-1, EjHLH1 and EjMYB2 were fused to the C- and N-terminus of YFP (YFPc and YFPn), respectively. The YFPc or YFPn vectors without genes inserted were used as negative controls. The bars indicated 25 μm.

Fig. 7. Luciferase complementation imaging assay was used to analyze the physical combination of EjblHLH1, EjAP2-1 and EjMYB2. The Agrobacterium tumefaciens contains constructs were infiltrated into tobacco leaves. Luciferase activities were recorded after three days using the NightSHADE LB981 imaging system.
EJMYB8.

Plant bHLH transcription factors typically function as homo- or hetero-dimers (Claria et al., 2003). OsJAZ1 regulates drought tolerance by physically interacting with OsbHLH148 in rice (Seo et al., 2011). AbhHLH38 and AbhHLH39 could form hetero-dimers by interacting with AtFIT1 and co-overexpression of AtFIT1 with AbhHLH38 or AbhHLH39 in Arabidopsis increased iron accumulation, indicating that AtFIT1 co-regulates iron homeostasis in Arabidopsis with AbhHLH38 or AbhHLH39 (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[Lxrx][R]XXlXXXXXXlXXXr) (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-PybHLH33-PyERF3 and PyMYB114-PybHLH33-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 E4CL1 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 E4CL1 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

This research was supported by the National Natural Science Foundation of China (31630067, 31722042), the 111 Project (B17039), and the Natural Science Foundation of Zhejiang Province, China (LR16C150001).

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.


