Postharvest Biology and Technology 111 (2016) 161–167

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

DkMYB6 is involved in persimmon fruit deastringency, via transcriptional activation on both *DkPDC* and *DkERF*

Fang Fang[a,b,c], Miao-miao Wang[a,b,c], Qing-gang Zhu[a,b,c], Ting Min[a], Donald Grierson[a,d], Xue-ren Yin[a,b,c,*], Kun-song Chen[a,b,c]

[a] College of Agriculture & Biotechnology, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
[b] Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
[c] The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China
[d] Plant & Crop Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

ARTICLE INFO

Article history:
Received 28 June 2015
Received in revised form 30 July 2015
Accepted 20 August 2015
Available online 30 August 2015

Keywords:
MYB
Persimmon
Postharvest deastringency
Transcriptional regulation

ABSTRACT

Postharvest deastringency is important for persimmon fruit, since most cultivars are of the astringent type. In the present research, astringency as indicated by the decrease in soluble tannin content, was removed from 'Mopan' persimmon by placing them in an atmosphere of 95% CO_2. In contrast to the decrease in soluble tannin, increases in concentrations of acetaldehyde and ethanol were transiently triggered by CO_2 treatment. Four *DkMYB* genes, *DkMYB5–8*, belonging to the R2R3 MYB family, were isolated. mRNA accumulation studies indicated that *DkMYB5–8* were up-regulated by CO_2 treatment, and that expression was positively correlated with persimmon fruit astringency removal. However, using a dual-luciferase assay, only one of these four *DkMYB* genes, *DkMYB6*, showed the ability to trans-activate the promoters of the previously identified persimmon fruit deastringency-related genes *DkPDC2* and *DkPDC3*. Furthermore, *DkMYB6* were also observed to be an activator of the previously characterized deastringency regulators, *DkERF9* and *DkERF19*. Thus, *DkMYB6* is a putative transcriptional activator, induced by high CO_2, which is involved in persimmon fruit deastringency, by operating on both *DkPDC* structural genes and *DkERF* transcription factors.

1. Introduction

Hypoxia is a common abiotic stress for many plants, often caused by reduced oxygen level due to flooding or submergence. Plant roots and seedling have often been chosen for hypoxia response analysis, and a series of genes involved in the anaerobic response have been identified, including well-characterized fermentation-related alcohol dehydrogenase (*ADH*) and pyruvate decarboxylase genes (*PDC*), as well as many transcription factors (ethylene response factor, *ERF*; *MYB*, *WRKY*, etc.) (Hwang et al., 2011; Licausi et al., 2011b). Among the transcription factors, the *ERF* family was recently comprehensively investigated and considered as novel and predominant regulators of the plant hypoxia response (Licausi et al., 2010; Sasidharan and Mustroph, 2011; Yang et al., 2011). Analysis of the effects of transgenic expression of subfamily VII *ERF*s, such as *HRE1*, *HRE2* and *RAP2.2*, indicated that these *ERF* genes were positive regulators of low oxygen tolerance in plants (Hinz et al., 2010; Licausi et al., 2010). Mechanisms of action of *ERF*s in hypoxia tolerance involve both of transcriptional regulation of *ADH*/*PDC* genes (Yang et al., 2011) and protein destabilization and turnover by the N-end rule pathway (Gibbs et al., 2011; Licausi et al., 2011a).

Apart from the role of *ERF*s, the regulatory mechanisms of other transcription factors involved in the plant hypoxia response remain largely unknown. However, the other transcription factors, such as MYB, appear to be involved earlier in the hypoxia response than *ERF*. *Arabidopsis AtMYB2* has been shown to physically interact with the *AtADH1* promoter (Hoeren et al., 1998), and over-expression of *AtMYB2* enhanced *AtADH1* expression (Abe et al., 2003). Similarly, wheat *TaMYB1* was responsive to low oxygen (Lee et al., 2007) and rice *Mybleu* overexpression increased hypoxia tolerance in *Arabidopsis* (Mattana et al., 2007). Despite the potential function of *MYB* in response to plant hypoxia, the underlying mechanisms have rarely been investigated.

Abbreviations: ADH, alcohol dehydrogenase; ERF, ethylene response factor; PDC, pyruvate decarboxylase; SCT, soluble condensed tannins.

* Corresponding author at: College of Agriculture & Biotechnology, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China. Fax: + 86 571 88982224.
E-mail address: xuerenyin@zju.edu.cn (X.-r. Yin).

http://dx.doi.org/10.1016/j.postharvbio.2015.08.012
0925-5214/

Unlike the situation during plant development, the production and storage of horticultural fruit crops can benefit from reduced oxygen levels, particularly when accompanied by increased CO_2 concentration, to extend storage life (Latocha et al., 2014). However, reduced oxygen level should also trigger anaerobic metabolism (e.g., ethanol and acetaldehyde), as occurs in all hypoxia response in other plants (Botondi et al., 2012). Besides improving fruit storability, there is an additional unique benefit of low oxygen/high CO_2 in persimmon fruit. A high concentration of CO_2 accelerates a decrease of soluble condensed tannins (SCT) content, removing astringency (Salvador et al., 2007; Del Bubba et al., 2009; Yin et al., 2012; Min et al., 2015). This of great importance since most cultivated persimmon fruit are of the astringent type, and even at maturity the fruit are rich in SCT (Yamada et al., 1994; Luo et al., 2014). Although low oxygen/high CO_2 are beneficial for fruit production, especially for persimmon fruit, our understanding of the fundamental underlying mechanisms affecting low oxygen/high CO_2 responses in fruit is still very limited and has not been addressed in model plants. High CO_2 treatment effectively removed astringency in 'Mopan' persimmon fruit, by triggering increases in the activities of ADH and PDC, leading to the accumulation of acetaldehyde (Min et al., 2012). A concomitant increase in expression of *DkADH1*, *DkPDC1* and *DkPDC2* genes was closely correlated with persimmon fruit deastringency (Min et al., 2012). Furthermore, four *DkERF* genes, *DkERF9*, *DkERF10*, *DkERF19* and *DkERF22*, were characterized and shown to be involved in persimmon fruit deastringency by virtue of their ability to trans-activate promoters of *DkADH* and *DkPDC* genes (Min et al., 2012, 2014). However, the potential roles of the other transcription factors in persimmon fruit deastringency remain unknown.

In the present research, four *DkMYB* genes were identified using data from the RNA-seq analysis performed by Min et al. (2014), and their expression patterns in response to deastringency treatment (95% CO_2) were analyzed by real-time PCR. Utilizing a dual luciferase assay, transcriptional regulatory roles of *DkMYB* genes on previously characterized deastringency-related target genes (*DkADH1*, *DkPDC2*, *DkPDC3*) and transcription factors (*DkERF9* and *DkERF19*) were examined.

2. Materials and methods

2.1. Plant material and treatments

Astringent-type 'Mopan' persimmon (*Diospyros kaki*) fruit were harvested from a commercial orchard at Fangshan (Beijing, China) in 2013. Fruit with no sign of disease or mechanical wounding and with mean firmness of 47.08 N, were selected and divided into two batches, each comprising 100 fruit. The first batch was treated at 95% CO_2 for 2 days to remove astringency, then were transferred to 20 °C storage in air; the second batch was sealed in similar containers as the first batch fruit, without any treatment, for 2 days, then transferred to 20 °C storage (Control). The treatments and controls were performed with three replicates. At each sampling time, flesh samples were collected and frozen in liquid nitrogen. The frozen samples (three replicates) stored at −80 °C until further use (soluble tannins, acetaldehyde and ethanol measurements; RNA extraction)

2.2. Fruit firmness

Fruit firmness was measured with a TA-XT2i texture analyzer (Stable Micro Systems, UK), and the penetration measurements were carried out according to our previous report (Yin et al., 2012). Firmness was measured at two positions 90° apart at the fruit equator, after removal of 1 mm peel. At each sampling point, fruit firmness was measured with 10 fruit replicates for each treatment.

2.3. Soluble condensed tannins content

Persimmon fruit astringency was mainly caused by soluble condensed tannins (SCT). SCTs were measured using frozen samples with the Folin–Ciocalteu reagent (Sigma), according to the method described previously (Yin et al., 2012). The results were calculated using the standard curve of tannin acids equivalents g^{-1} fresh weight. Soluble condensed tannins content were measured with three biological replicates.

2.4. Acetaldehyde and ethanol

Acetaldehyde and ethanol production were measured with a gas chromatograph (Agilent 6890N, USA), fitted with a FID column (HP-INNOWAX, 0.25 mm, 30 m, 0.25 μm, Agilent J&W, CA, USA). Measurement protocols were according to our previous report (Min et al., 2012). A 2 g frozen fruit sample was ground in liquid nitrogen and added to 5 ml saturated NaCl solution. Three ml of the mixture were transferred to 10 ml air-tight vials with crimp-top caps. The vials were incubated at 60 °C for 1 h and head-space gas (0.2 ml) was removed and injected into a gas chromatograph. The injector, detector and oven temperatures were set at 150, 160 and 100 °C, respectively. Sec-butyl alcohol (Sigma) was used as an internal control. The results were calculated using standard curves for acetaldehyde and ethanol, respectively.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from various frozen persimmon fruit flesh samples (2.0 g for each) by the method described by Yin et al. (2013). The trace contaminating genomic DNA in total RNA was removed with TURBO Dnase (Ambion). cDNA synthesis was initiated from 1.0 μg DNA-free RNA, using iScript™ cDNA Synthesis Kit (Bio-Rad). For each sampling point, three biological replicates were used for RNA extraction.

2.6. Gene isolation and analysis

Using the same transcript resources described by Min et al. (2014), up-regulated transcripts related to MYB transcription factors were selected. The UTR region of the transcripts were obtained using SMART RACE cDNA amplification Kit (Clontech) and the primers described in Table S1.

Alignment was performed using the neighbor-joining (NJ) method in ClustalX (v. 1.81) and a phylogenetic tree was constructed with online software Figtree (http://tree.bio.ed.ac.uk/software/figtree/). The deduced amino acid sequences of *Arabidopsis* MYBs were obtained from TAIR, and the related genes in persimmon (Akagi et al., 2009; Su et al., 2014) or hypoxia-responsive *MYB* in other plants (Abe et al., 2003; Lee et al., 2007), though very limited, were also included in the phylogenetic analysis. -W-(X19)-W-(X19)-W-....-F/I-(X18)-W-(X18)-W- is an N terminal DNA-binding domain, also known as the MYB domain (Dubos et al., 2010), was also analyzed in *DkMYB* genes.

2.7. Real-time PCR analysis

For real-time PCR, gene-specific oligonucleotide primers were designed and are described in Table S2. Gene specificity of each pair of primers was double checked by melting curve and product resequencing. The *DkACT* gene was employed as the internal control for monitoring the abundance of the mRNA (Min et al., 2014).

Real-time PCR reactions were performed on a LightCycler 1.5 instrument (Roche), initiated by 30 s at 95 °C, followed by 45 cycles of 95 °C for 5 s, 60 °C for 5 s, and completed with a melting