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RESEARCH PAPER

Ethylene-responsive transcription factors interact with promoters of *ADH* and *PDC* involved in persimmon (*Diospyros kaki*) fruit de-astringency

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Abstract

The persimmon fruit is a particularly good model for studying fruit response to hypoxia, in particular, the hypoxia-response *ERF* (*HRE*) genes. An anaerobic environment reduces fruit astringency by converting soluble condensed tannins (SCTs) into an insoluble form. Although the physiology of de-astringency has been widely studied, its molecular control is poorly understood. Both CO_2 and ethylene treatments efficiently removed the astringency from 'Mopan' persimmon fruit, as indicated by a decrease in SCTs. Acetaldehyde, the putative agent for causing de-astringency, accumulated during these treatments, as did activities of the key enzymes of acetaldehyde synthesis, alcohol dehydrogenase (ADH), and pyruvate decarboxylase (PDC). Eight *DkADH* and *DkPDC* genes were isolated, and three candidates for a role in de-astringency, *DkADH1*, *DkPDC1*, and *DkPDC2*, were characterized by transcriptional analysis in different tissues. The significance of these specific isoforms was confirmed by principal component analysis. Transient expression in leaf tissue showed that *DkPDC2* decreased SCTs. Interactions of six hypoxia-responsive *ERF* genes and target promoters were tested in transient assays. The results indicated that two hypoxia-responsive *ERF* genes, *DkERF9* and *DkERF10*, were involved in separately regulating the *DkPDC2* and *DkADH1* promoters. It is proposed that a *DkERF–DkADH*/*DkPDC* cascade is involved in regulating persimmon de-astringency.

Key words: ADH, de-astringency, *Diospyros kaki*, ERF, ethylene, hypoxia, PDC, soluble tannins, transcriptional regulation.

Introduction

Ethylene responsive factor (ERF) genes encode plant-specific transcription factors, which are located downstream of the ethylene signalling pathway (Nakano *et al*., 2006). *ERF*s are members of one of the largest transcription factor families,

Abbreviations: ADH, alcohol dehydrogenase; AP2, APETALA2; CBF, C-repeat-binding factor; ERF, ethylene-response factor; HRE, hypoxia-responsive ERF; InSCTs, insoluble condensed tannins; PDC, pyruvate decarboxylase; PG, polygalacturonase; SCTs, soluble condensed tannins; UTR, untranslated region; XET, xyloglucan endotransglycosylase.

considered a super-family, with diverse functions in plants. Recent findings suggest that some *ERF* genes are important regulators of low oxygen tolerance in plants (Gibbs *et al*., 2011; Licausi *et al*., 2011*a*). In fruit, *ERF* genes are key targets for investigating the transcriptional regulatory roles of ethylene in fruit development and ripening. Tomato *LeERF2* was characterized as being a key regulator of ethylene biosynthesis (Zhang *et al*., 2009), and two novel *ERF* members, *SlAP2* and *SlERF6*, have been shown to regulate genes involved in tomato ripening (Chung *et al*., 2010; Lee *et al*., 2012). In perennial fruit, apple *CBF* and kiwifruit *AdERF9* transcriptionally regulate fruit cell wall-related genes (apple *PG1*; Tacken *et al*., 2010; kiwifruit *AdXET5*; Yin *et al*., 2010). However, many aspects of transcriptional regulation of fruit ripening and quality by *ERF* genes remain to be explored.

Recently, hypoxia-responsive *ERF* genes (*HRE*) have been characterized in *Arabidopsis* with *HRE1* and *HRE2* having partially redundant roles in increasing low oxygen tolerance (Licausi *et al*., 2010; Yang *et al*., 2011). Also, the *RAP2.2*, has a similar role in low oxygen signalling in *Arabidopsis* (Hinz *et al*., 2010). At least two of these *ERF* genes, *HRE1* and *RAP2.2*, are involved in regulating the anaerobic fermentation-related genes, *pyruvate decarboxylase* (*PDC*) and *alcohol dehydrogenase* (*ADH*) (Hinz *et al*., 2010; Yang *et al*., 2011).

In various species of fruit, anaerobic environments (low oxygen), generated by controlled atmosphere storage, have been widely applied to prolong their storage life. The persimmon fruit (*Diospyros kaki*) is of particular interest for anoxia-related studies, as an anaerobic environment promotes de-astringency. These fruit are unique for accumulating proanthocyanidins (also known as condensed tannins, CTs), and the fruit astringency is caused by the high content of Soluble CTs (SCTs). Although persimmons can be divided into astringent and non-astringent types (Akagi *et al*., 2009*b*), most native cultivars grown in East Asia are of the astringent type (Yamada *et al*., 1994; Wang *et al*., 1997). Astringent-type fruit are rich in SCTs even at maturity, while the concentration of SCTs is significantly reduced during early development in fruit of the non-astringent type (Akagi *et al*., 2009*a*). Astringent fruit tend to be rejected by consumers and artificial treatments have been developed to remove astringency, including treatments involving CO_2, ethylene, and ethanol (Ikegami *et al*., 2007; Salvador *et al*., 2007), and CO_2 application is usually the most effective (Salvador *et al*., 2007; Del Bubba *et al*., 2009). Furthermore, the physiological and biochemical mechanisms of CO_2-driven de-astringency are much clearer than for other treatments. A high concentration of CO_2 leads to anaerobic fermentation by triggering acetaldehyde metabolism (Matsuo and Ito, 1977; Pesis and Ben-Arie, 2006; Salvador *et al*., 2007). Acetaldehyde plays important roles in the polymerization of SCTs, converting them to insoluble condensed tannins (InSCTs) (Tanaka *et al*., 1994; Taira *et al*., 2001). CO_2-driven de-astringency, therefore, is likely to occur via the modulation of acetaldehyde concentrations. Thus, studying the roles of *ERF* genes in regulating acetaldehyde biosynthetic genes and persimmon de-astringency is particularly important and may suggest new ways to improve the handling of this crop.

In plants, acetaldehyde can be synthesized from pyruvate and ethanol, catalysed by PDC (EC 4.1.1.1) and ADH (EC 1.1.1.1), respectively (Yamada *et al*., 2002; Pesis, 2005; Botondi *et al*., 2012). PDC and ADH have long been associated with persimmon de-astringency. During astringency loss in developing non-astringent persimmon fruit (cv. Nishimurawase), an increase in ADH enzyme activities was observed by Liu *et al*. (2008). The application of CO_2 enhanced ADH and PDC activities during the de-astringency of 'Saijo' persimmons (Tamura *et al*., 1999). ADH and PDC enzymes are both encoded by gene families which have been studied in various plant species (Strommer, 2011), but no *ADH* or *PDC* genes have been characterized from persimmon.

In the present research, eight *DkADH* and *DkPDC* genes were isolated from persimmon fruit, based on the persimmon NCBI EST database. Transcript abundance of each of these genes was analysed in various tissues and in response to de-astringency treatments (CO_2 and ethylene), in the astringent-type 'Mopan' persimmon fruit. *DkADH* and *DkPDC* genes specific to persimmon de-astringency were identified using principal component analysis (PCA) and were characterized by transient overexpression in persimmon leaves. By using RNA-Seq, hypoxia-responsive *ERF* genes were isolated and their activity in regulating target gene promoters was studied *in vitro*.

Materials and methods

Plant materials and treatments

'Mopan' (astringent-type) persimmon (*Diospyros kaki*) fruit were obtained from a commercial orchard at Fangshan (Beijing, China). The fruit were transported to Zhejiang University (Hangzhou, Zhejiang, China) on the second day after harvest. Uniform fruit free of visible defects were chosen for the de-astringency experiments, including CO_2 and ethylene treatments.

For the ethylene treatment, the fruit were harvested in 2009, with mean colour index, firmness, and soluble solids content of 14.70, 52.31 N, and 17.88%, respectively. The fruit were divided into two lots of 180 fruit each. The control fruit were held at 20 °C free of any treatment for the same time periods as used for the ethylene-treated fruit. Continuous ethylene (100 μl l^{-1}) in air (2 d) was applied to the second batch to remove the astringency of the fruit. For the CO_2 treatment, the fruit were harvested in 2010, with mean colour index, firmness, and soluble solids content of 18.11, 43.08 N, and 18.76%, respectively. The fruit were divided into three batches (200 fruit in each) and the first two batches of fruit were treated with 95% CO_2 for 1 d or 2 d, separately, according to Matsuo and Ito (1977). The third batch of fruit was stored in air at 20 °C for 4 d as the control. After treatment, the fruit were held in air at 20 °C.

All of the treatments and controls were performed with three biological replicates. At each sampling point, bulk fruit flesh samples (without skin and core) were taken from three replicates each of four fruit. The samples were frozen in liquid nitrogen and stored at –80 °C for further use.

For tissue-specific gene expression, stems, leaves, and petals were collected at full bloom, frozen in liquid nitrogen, and stored until use.

Fruit firmness and TSS

Fruit firmness and TSS (total soluble solids) were recorded at each sampling time. Fruit firmness measurements were made with a TA-XT2i texture analyser (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 the removal of 1 mm peel. Fruit TSS were measured from samples from both ends of each fruit, using a N20 digital hand-held refractometer (Atago, Japan), according to the methods described in Yin *et al*. (2008). Fruit firmness and TSS were measured on each of 10 fruit.

Soluble condensed tannins content

Soluble condensed tannins (SCTs) were chosen as the main de-astringency index and were also used for comparing the effectiveness of different treatments. SCTs were measured using frozen samples, according to the method described previously (Yin *et al*., 2012), where fruit flesh was extracted with methanol and the SCTs were measured with the Folin–Ciocalteu reagent (Sigma). The results were expressed as tannin acids equivalents g^{-1} fresh weight.

Acetaldehyde and ethanol

Acetaldehyde and ethanol production were measured with a gas chromatograph (Lunan Chemical Engineering Instrument Co. Ltd., model GC-14B, Shandong, China), fitted with a FID column (HP-INNOWAX). A 2 g frozen fruit sample was ground in liquid nitrogen and the ground powder was mixed with 5 ml saturated NaCl solution. Three ml of the mixture were transferred to 10 ml air-tight vials with crimp-top caps. Before measurement, the vials were incubated at 60 °C for 1 h in a water bath. Then, 0.2 ml head-space gas was removed from each vial for acetaldehyde and ethanol measurement. The injector, detector, and oven temperatures were 140, 140, and 100 °C, respectively. Sec-butyl alcohol was added to each vial as an internal control. The results were calculated using standard curves for acetaldehyde and ethanol, respectively.

ADH and PDC activities

ADH and PDC enzymes were extracted and assayed according to the methods described by Botondi *et al*. (2012). One gram of persimmon fruit flesh was homogenized with 3 ml 0.02 M KH_2PO_4 buffer (pH 7.5) (including 2 mM thiamine pyrophosphate, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol), on ice. The homogenate was centrifuged at 17 000 rcf for 20 min at 4 °C and the supernatant was filtered with PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB) and the eluate was used to measure PDC and ADH activity. Three biological replicates were performed at each sampling point.

ADH activity was measured using the increment in NADH by reading the absorbance at 340 nm, in 1 ml of reaction buffer [0.68 ml 100 mM KOH-glycine (pH 9.0), 0.07 ml 11.4 mM NAD^+, 0.15 ml 2 M ethanol, 0.10 ml enzyme extract].

PDC activity was measured using the decrease in NADH. The 1 ml reaction mixture consisted of 0.4 ml 100 mM MES (pH 7.5), 0.1 ml 10 mM thiamine pyrophosphate, 0.1 ml 10 mM $MgCl_2$, 0.1 ml 2.6 mM NADH, 0.1 ml ADH (containing 7 kU ml^{-1}), 0.1 ml of enzyme extract, and 0.1 ml 200 mM pyruvate.

One activity unit (U) was defined as the change in one unit of absorbance at 340 nm min^{-1} and the results were expressed as specific activity (mU g^{-1} flesh). All the reagents were the products from Sigma Aldrich Co. (USA).

RNA extraction and cDNA synthesis

Total RNA was extracted from various frozen tissues (2.0 g for flesh tissues, 0.8 g for leaves, 1.2 g for stems, 1.6 g for petals) with the method developed by Chang *et al*. (1993). The trace contaminating genomic DNA in total RNA was removed swith 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.

Gene isolation and sequence analysis

ADH and *PDC* genes were isolated both using degenerate primers constructed from information for other species and also the persimmon EST database [published by Nakagawa *et al*. (2008) and available from NCBI (National Center for Biotechnology Information)]. The EST databases was blasted using the sequences isolated from using degenerate primers. The redundant sequences were excluded and non-redundant EST sequences were chosen for further experiments. The novel hypoxia (high CO_2)-responsive *ERF* gene was isolated based on RNA-Seq. RNA-Seq was performed and the sequence was assembled and annotated by the Beijing Genome Institute (BGI) (Shenzhen, China). RNA isolation and the preparatory procedures were as previously reported by Feng *et al*. (2012). 26.7 million sequence reads were performed for each library and the sequences were compared by BLASTX to the public protein database.

Rapid amplification of cDNA ends (RACE) was chosen to amplify the 3' end and/or 5' end UTR (untranslated region) of *ADH*, *PDC*, and *ERF*, using a SMART™ RACE cDNA Amplification Kit (Clontech). The sequences of primers used for cloning and RACE experiments are described in Supplementary Table S1 at *JXB* online.

The gene sequences were translated with online software (http://web.expasy.org/translate/) and were confirmed with the BLAST methods in Genbank. The deduced amino acid sequences of homologous genes in other plant species were downloaded from NCBI. Bootstrap Neighbor–Joining trees were generated with ClustalX (v 1.81), using the default parameters.

Oligonucleotide primers and real-time PCR

Oligonucleotide primers for real-time PCR analysis were designed with primer3 (v. 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The specificity of primers was determined by examining the melting curves and re-sequencing the PCR products. The sequences of oligonucleotide primers are described in Supplementary Table S1 at *JXB* online.

Real-time PCR was carried out using a CFX96 instrument (Bio-Rad). The PCR protocols were according to our previous reports, with Ssofast EvaGreen Supermix kit (Bio-Rad) (Yin *et al*., 2012). The relative abundance of each gene was calibrated with samples from mature fruit (for tissue specific studies) or day 0 fruit (for post-harvest experiments). *Actin*, a housekeeping gene, was used as the internal control (Akagi *et al*., 2009*b*).

Transient over-expression in persimmon leaves

In order to determine the roles of *DkADH* and *DkPDC* genes in the regulation of SCTs, an unstable transformation system (transient expression) was adapted. Full-length *DkADH1* and *DkPDC2* coding sequences were constructed into pGreenII 0029 62-SK (Hellens *et al*., 2005). Both of the constructs were electroporated into *Agrobacterium tumefaciens* GV3101. The transient expression system (*Agrobacterium* culture, infiltration buffer) was according to the protocols used for tobacco in our laboratory (Espley *et al*., 2007; Yin *et al*., 2010).

Transient expression in persimmon leaves was performed with the 'Mopan' cultivar, the same cultivar used for astringency removal experiments. Suspensions of *Agrobacterium*, harbouring either *DkADH1* or *DkPDC2*, were prepared with infiltration buffer and infiltrated into persimmon leaves, on the tree. Negative controls were included in the same leaves. Three days after infiltration, the infiltrated leaves were sampled and used for SCT analysis. 0.1 g of tissue from each of the infiltrated leaves was taken for SCTs analysis, using ten single leaf replicates in total.

Genome walking and transient assay

Genome walking and a transient assay were performed according to our previous report (Yin *et al*., 2010). Promoters of *DkADH1* and *DkPDC2* were isolated with a GenomeWalker kit (Clontech), using genomic DNA from 'Mopan' persimmon leaves. The sequences of DkADH1 and DkPDC2 are described in Supplementary Fig. S5 at *JXB* online. Full-length *DkERF9* was inserted into pGreen II 0029 62-SK vector (SK), while the promoters of *DkADH1* (1021 bp) and *DkPDC2* (919 bp) were inserted into pGreen II 0800-LUC vector. As the *DkADH1* promoter has an endogenous *Nco*I site, a 12 bp pGem-T easy vector sequence was incorporated between the *DkADH1* promoter and luciferase reporter. All of the constructs were electroporated into *Agrobacterium tumefaciens* GV3101. The transient assay was performed with *Nicotiana benthamiana* leaves. *Agrobacterium* cultures were prepared with infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 mM acetosyringone, pH 5.6) to an OD_{600} from 0.7 to 1.0. *Agrobacterium* culture mixtures of TFs

(1 ml) and promoters (100 µl) were infiltrated into tobacco leaves by needle-less syringes. Tobacco plants were grown in a greenhouse, under natural light with daylight extension to 16 h. Three days after infiltration, firefly luciferase and renilla luciferase were assayed using the dual luciferase assay reagents (Promega). For each TF-promoter interaction, three independent experiments were performed (at least six replicates in each experiments).

Statistical analysis

Principal component analysis (PCA) was applied to analyse the correlations between de-astringency (soluble tannin concentrations) and gene expression with AlphaSoft version 11.0 (Alpha MOS, Toulouse, France). Software and data manipulations were according to our previous report (Zhang *et al*., 2011). Statistical significance of differences was calculated using Student's *t* test.

Results

Isolation of novel hypoxia (high CO_2)-responsive ERF genes

Four hypoxia responsive *DkERF* genes (*DkERF1*, *DkERF4*, *DkERF5*, and *DkERF6*) were characterized in our previous studies (Yin *et al*., 2012). Two novel hypoxia-responsive *DkERF* genes, designed as *DkERF9* (JX117848) and *DkERF10* (JX145122) were newly isolated after comparing RNA from 2 d CO_2-treated fruit and control fruit using RNA-Seq technology. The RPKM (*R*eads *p*er *k*b per *m*illion reads) values of *DkERF9* and *DkERF10* were 0 in 0 d fruit and 1.5692 and 450.2169 in 2 d CO_2-treated fruit, respectively (data not shown), indicating that both *DkERF9* and *DkERF10* were possibly high-CO_2/hypoxia-inducible. Phylogenetic analysis indicated that hypoxia responsive *DkERF* genes belong to different subfamilies. DkERF1 belongs to subfamily I, while DkERF4-6 belongs to subfamily VIII (according to Nakano *et al*., 2006). The two newly isolated hypoxia (high CO_2)-responsive *DkERF* genes were clustered in subfamily IV (*DkERF9*) and VII (*DkERF10*) (see Supplementary Fig. S1 at *JXB* online). Within the six *DkERF* genes, only *DkERF10* had a conserved MCGGAII motif at the N-end of the deduced protein.

Fruit de-astringency and ERF genes expression

The relationship between hypoxia/high-CO_2-responsive *ERF* genes and fruit de-astringency was evaluated in 'Mopan' persimmon fruit using both CO_2 (95%) and ethylene (100 µl l^{-1}) treatments. Both treatments promoted de-astringency, indicated by the decreasing concentration of SCTs (Fig. 1).

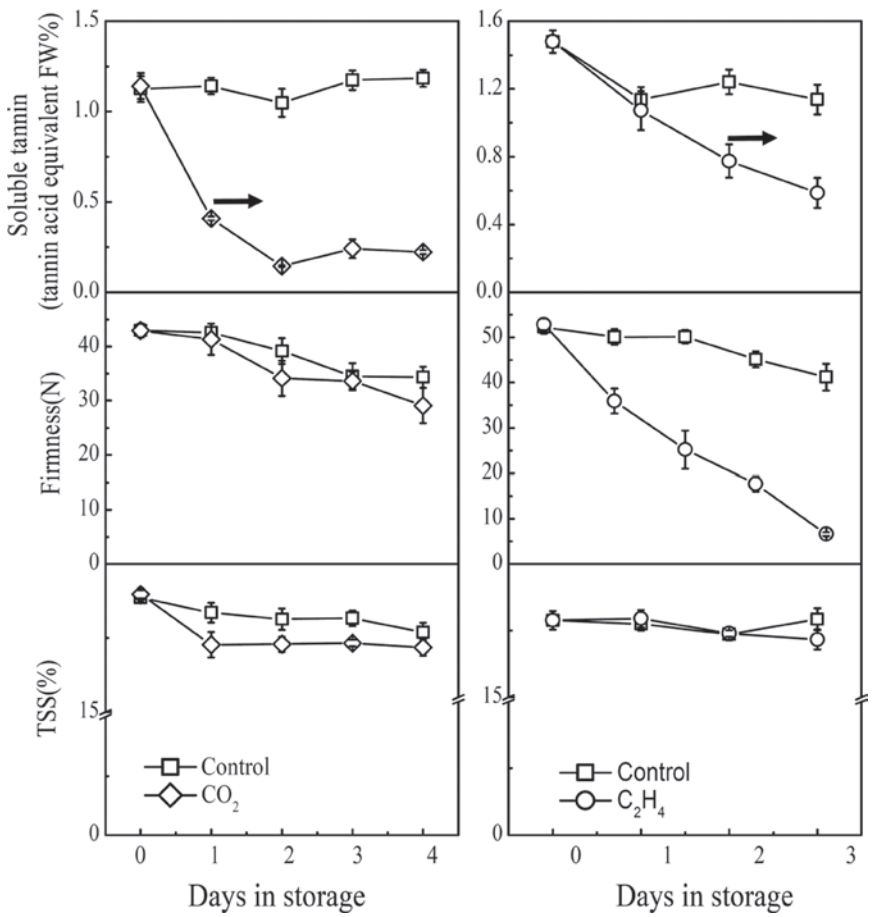

Fig. 1. Effects of CO_2 and ethylene treatments on SCTs, firmness, and TSS of 'Mopan' persimmon fruit at 20 °C. Mature fruit were treated with CO_2 (~95%, v/v, open diamonds, 1 d), ethylene (100 µl l^{-1}, open circles, 2 d), and air (control, open squares), separately. The horizontal arrow represents the end day of the treatment, when the fruit were transferred to 20 °C, free of ethylene. Error bars represent SEs from three (SCTs) or 10 (firmness and TSS) biological replicates.

Table 1. Effect of different astringency removal treatments on acetaldehyde concentration in 'Mopan' persimmon fruit flesh ND indicates that acetaldehyde was undetectable. Errors represent SEs from three biological replicates.

Acetaldehyde (μg g^{-1} flesh weight)					
Days in storage	**Control-1[a]**	**CO_2 1d**	**CO_2 2d**	**Control-2[a]**	**C_2H_4 2d**
0	ND	ND	ND	ND	ND
1	ND	2.51 ± 0.21	2.09 ± 0.18	ND	ND
2	ND	ND	5.94 ± 0.60	ND	0.79 ± 0.09
3	ND	ND	ND	ND	ND
4	ND	ND	ND	—[b]	—[b]

[a] Control-1 and Control-2 correspond to CO_2 treatment and C_2H_4 treatments, respectively.
[b] —: not measured.

Table 2. Effect of different de-astringency treatments on ethanol concentration in 'Mopan' persimmon fruit flesh Errors represent SEs from three biological replicates.

Ethanol (μg g^{-1} flesh weight)					
Days in storage	**Control-1[a]**	**CO_2 1d**	**CO_2 2d**	**Control-2[a]**	**C_2H_4 2d**
0	24.51 ± 4.25	24.51 ± 4.25	24.51 ± 4.25	18.08 ± 1.71	18.08 ± 1.71
1	6.83 ± 1.02	48.69 ± 4.46	58.50 ± 9.63	5.48 ± 1.94	4.49 ± 0.51
2	6.32 ± 1.16	42.74 ± 6.19	117.12 ± 2.96	3.78 ± 0.75	34.82 ± 2.08
3	6.56 ± 1.31	23.74 ± 0.92	126.07 ± 11.32	2.93 ± 0.57	23.13 ± 1.24
4	4.62 ± 0.26	8.08 ± 0.45	5.45 ± 4.26	—[b]	—[b]

[a] Control-1 and Control-2 correspond to CO_2 treatment and C_2H_4 treatments, respectively.
[b] —: not measured.

However, the effects of CO_2 and ethylene treatment on fruit ripening were different. Ethylene accelerated fruit texture change, and fruit softened from an initial value of 52.31 N to 14.19 N after ethylene treatment compared with 45.15 N in control fruit after 3 d in storage. By contrast, the firmness of CO_2-treated fruit was similar to that of control fruit (Fig. 1). The TSS content decreased generally during both treatments, however, a larger reduction was found in CO_2-treated fruit (Fig. 1).

Acetaldehyde and ethanol, the main products of anaerobic fermentation, were also analysed in CO_2- and ethylene-treated fruit. Acetaldehyde, the main compound that interacts with the SCTs to render them insoluble, was only detectable during treatment, and was undetectable once the treatments were removed (Table 1). The acetaldehyde content was much higher in CO_2-treated fruit (5.94 μg g^{-1} at the end of 2 d continuous CO_2 exposure) than ethylene-treated fruit (0.79 μg g^{-1} at the end of 2 d continuous ethylene exposure). Ethanol was generally detectable, although the de-astringent treatments also enhanced its accumulation several fold, and it was at its highest concentration in CO_2-treated fruit (Table 2).

Real-time PCR analysis indicated the two newly isolated *DkERF* genes, *DkERF9* and *DkERF10*, were both induced by the different de-astringent treatments (Fig. 2). Expression of *DkERF10* was more responsive to these treatments than *DkERF9* and the other ERFs.

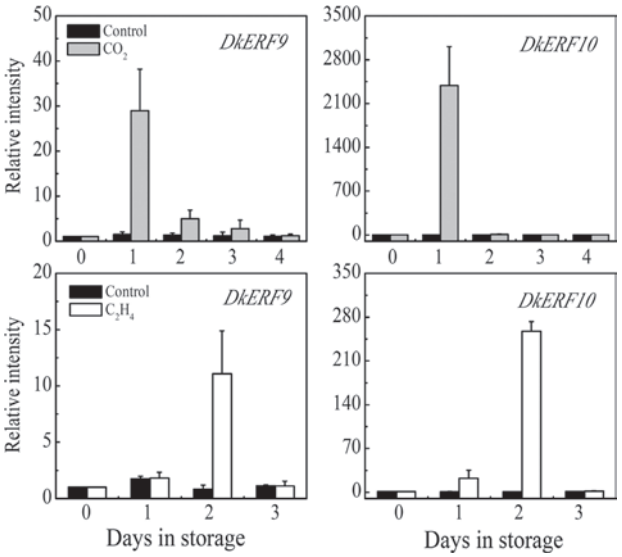

Fig. 2. Transcriptional analysis of hypoxia responsive *DkERF9* and *DkERF10*. Transcripts of *DkERF9* and *DkERF10* were measured by real-time PCR in response to CO_2 and ethylene treatment. Mature fruit were treated with ethylene (100 μl l^{-1}, open circles, 2 d) and air (control, open squares), separately. A horizontal arrow represents the end day of the treatment, when the fruit were transferred to air at 20 °C. Day 0 fruit values were set as 1. Error bars indicate SEs from three biological replicates.

Isolation of anaerobic fermentation-related structural genes

Three *ADH* (*DkADH1–3*, JX117841–JX117843) and five *PDC* (*DkPDC1–3*, JX117844–JX117846; *DkPDC4*, DC592775; *DkPDC5*, JX117847) cDNAs were isolated from 'Mopan' persimmon fruit (astringent type). *DkADH1*, *DkADH2*, and *DkPDC2* were full-length coding sequences (CDS), while others were partial CDS.

Phylogenetic analysis of the deduced amino acid sequences showed that *DkADH1* clustered close to *LeADH1*, *LeADH5*, and *StADH1*; *DkADH2* was close to *AtADH2* and *LeADH4*; while *DkADH3* was similar to *PaADH1* and *CmADH2* (Fig. 3). Unlike *DkADH* sequences, *DkPDC* coding sequences could be divided into two groups, with *DkPDC1*, *DkPDC3*, and *DkPDC4* in one group with homology to *LePDC1* and *CmPDC1*; while *DkPDC2* and *DkPDC5* were similar to each other and closely related to *VvPDC1* (Fig. 3). Tissue-specific expression analysis revealed that *DkADH* and *DkPDC* genes were differentially expressed in various persimmon tissues (see Supplementary Fig. S2 at *JXB* online).

Enzymatic and transcriptional response of ADH and PDC during fruit de-astringency

ADH activities were induced by both the 1 d CO_2 and 2 d ethylene treatments. The levels in the other tissues were very high but the fruit levels were lower, but still at effective levels of activity. With the CO_2 treatment, activity was induced from 1 d and reached a peak at 3 d (Fig. 4), and increased more gradually in ethylene-treated fruit (Fig. 5). Expression of *DkADH1* mRNA was particularly responsive to these treatments, and paralleled the changes in enzyme activity. *DkADH2* and *DkADH3* expression was relatively more constant and at much lower levels. There was a stronger differential response to ethylene compared with CO_2 (Figs 4, 5).

The PDC activities exhibited similar patterns, which increased in CO_2-treated fruit, the increase being observed from 1 d (Fig. 6),

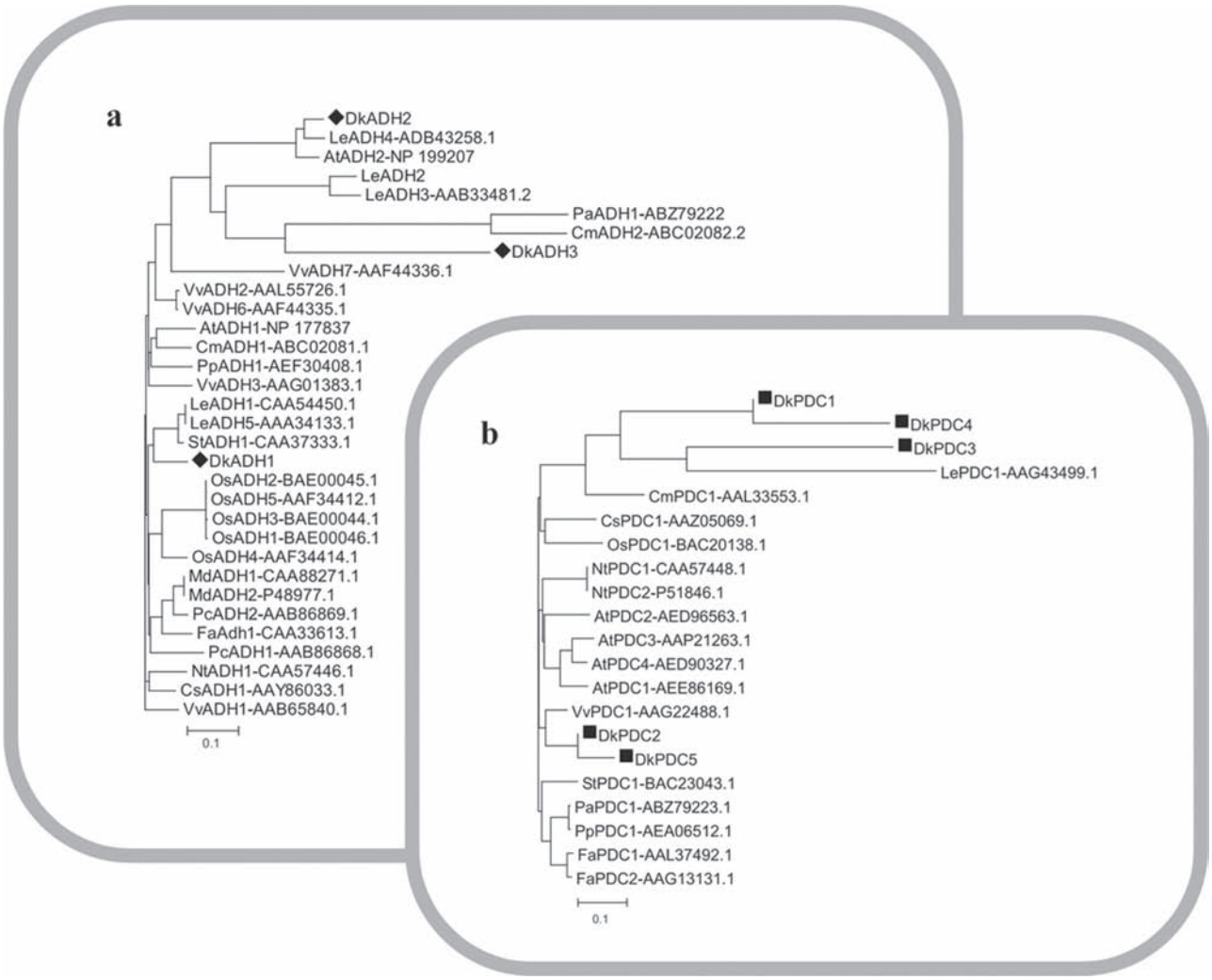

Fig. 3. Phylogenetic analysis of *ADH* (a) and *PDC* (b) genes. The deduced amino acid sequences were obtained from the NBCI and the accession numbers are indicated. At, *Arabidopsis thaliana*; Cm, *Cucumis melo*; Cs, *Citrus sinensis*; Fa, *Fragaria ananassa*; Le, *Lycopersicon esculentum*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Pa, *Prunus armeniaca*; Pc, *Pyrus communis*; Pp, *Prunus persica*; St, *Solanum tuberosum*. The phylogenetic trees were constructed using ClustalX (v 1.81).

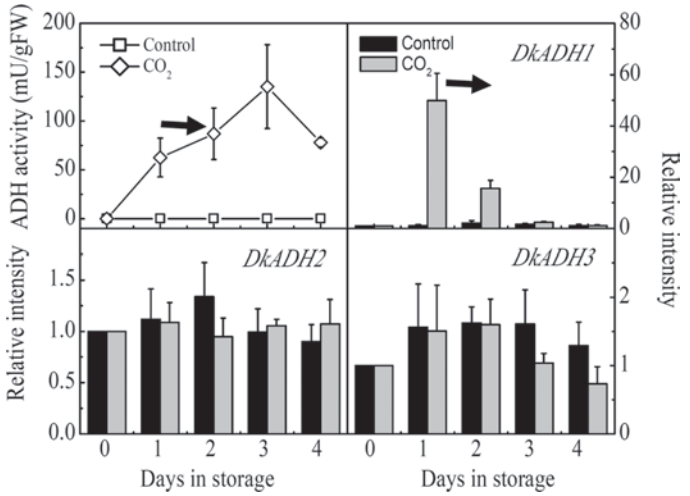

Fig. 4. Changes in ADH enzyme activity and mRNA amounts from specific genes in response to CO_2 treatment. Mature fruit were treated with CO_2 (~95%, v/v, open diamonds, 1 d) and air (control, open squares), separately. The horizontal arrow represents the end day of the treatment, when the fruit were transferred to air at 20 °C. Day 0 fruit values were set as 1. Black columns and white columns represent relative mRNA abundance of the gene transcripts in control and ethylene treated fruit, respectively. Error bars indicate SEs from three biological replicates.

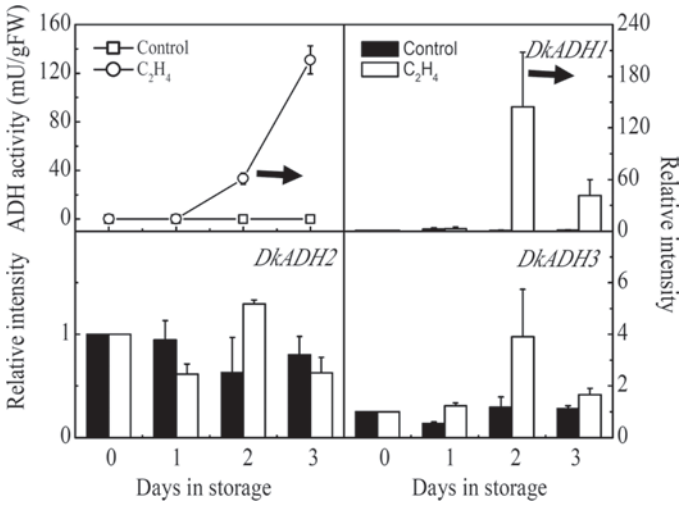

Fig. 5. Changes in ADH enzyme activity and mRNA amounts from specific genes in response to ethylene treatment. Mature fruit were treated with ethylene (100 µl l^{-}, open circles, 2 d) and air (control, open squares), separately. The horizontal arrow represents the end day of the treatment, when the fruit were transferred to air at 20 °C. Day 0 fruit values were set as 1. Black columns and white columns represent relative mRNA abundance of the gene transcripts in control and ethylene-treated fruit, respectively. Error bars indicate SEs from three biological replicates.

while in ethylene-treated fruit there was a 1 d delay before PDC increased (Fig. 7). Among the *PDC* gene family, the transcript abundance of *DkPDC1* and *DkPDC2* was increased by more

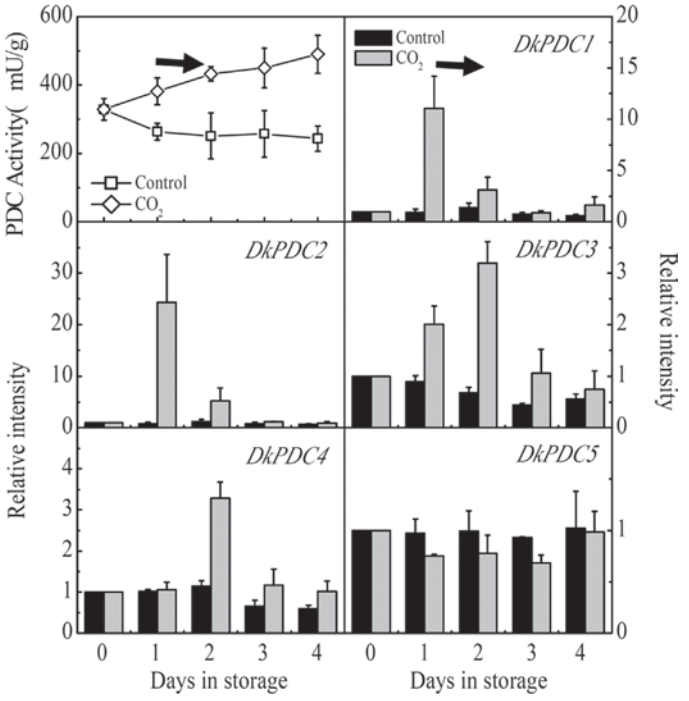

Fig. 6. Changes in PDC enzyme activity and mRNA amounts from specific genes in response to CO_2 treatment. Mature fruit were treated with CO_2 (~95%, v/v, open diamonds, 1 d) and air (control, open squares), separately. A horizontal arrow represents the end day of the treatment, when the fruit were transferred to air at 20 °C. Day 0 fruit values were set as 1. Black columns and white columns represent relative mRNA abundance of the gene transcripts in control and ethylene-treated fruit, respectively. Error bars indicate SEs from three biological replicates.

than 50-fold (Figs 6, 7). *DkPDC3* and *DkPDC4* mRNA accumulation increased only in the CO_2-treated fruit and the levels were relatively lower than for *DkPDC1* and *DkPDC2*. *DkPDC5* was partially repressed by exogenous ethylene treatment (Fig. 7) The above expression patterns from the 1 d CO_2 treatment (Figs 4, 6) were confirmed in the 2 d CO_2-treated fruit (see Supplementary Fig. S3 at *JXB* online).

PCA analysis was performed to evaluate the relations between de-astringency and expression of *ADH/PDC* genes, with the mean data for soluble tannin contents and levels of gene expression. The analysis showed that, for both treatments, PC1 and PC2 accounted for over 99% of the total variation, and PC1 was dominant with 99.757% and 99.754% for ethylene and CO_2 treatments, respectively (Fig. 8). As expected, de-astringency treatments showed negative correlations with soluble tannin contents. In the ethylene treatment, *DkADH1*, *DkADH3*, *DkPDC1*, and *DkPDC2* were distributed along the negative axis of PC1, and were far from SCTs (Fig. 8). Similarly, with the CO_2 treatment, *DkADH1*, *DkPDC1*, and *DkPDC2* were the most distant from SCTs (Fig. 8). As PC1 was the main contributor to the total variation, these results suggest that *DkADH1*, *DkPDC1*, and *DkPDC2* are most likely to be involved in de-astringency (decrease in SCTs).

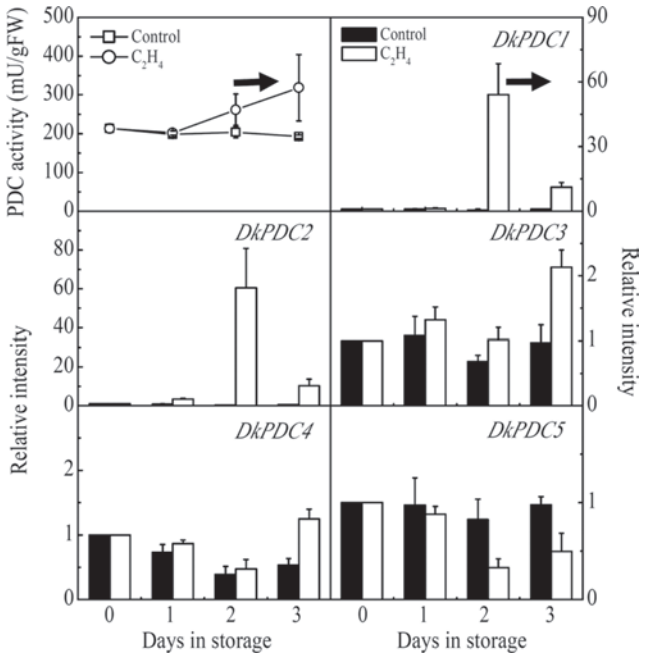

Fig. 7. Changes in PDC enzyme activity and mRNA amounts from specific genes in response to ethylene treatment. Mature fruit were treated with ethylene (100 µl l^{-1}, open circles, 2 d) and air (control, open squares), separately. A horizontal arrow represents the end day of the treatment, when the fruit were transferred to air at 20 °C. Day 0 fruit values were set as 1. Black columns and white columns represent relative mRNA abundance of the gene transcripts in control and ethylene-treated fruit, respectively. Error bars indicate SEs from three biological replicates.

Transient over-expression of DkADH1 *and* DkPDC2 *in persimmon leaves*

Persimmon is a perennial fruit and it takes years to generate transgenic fruit with stable transformation technologies, thus a transient overexpression system was chosen for quick gene functional analysis. *DkADH1* and *DkPDC2* constructs, driven by the CaMV 35S promoter, were introduced into *Agrobacterium*, which was then infiltrated into persimmon leaves. Analysis of SCTs in leaves indicated that the effects of *DkADH1* and *DkPDC2* in decreasing SCTs were different. The levels of SCTs in the leaves infiltrated with *DkADH1* were similar to those in leaves infiltrated with empty vectors (SK, Fig. 9). Transient expression of *DkDPC2*, on the other hand, led to a small but significant (P <0.05) decrease in levels of SCTs in the leaves (Fig. 9).

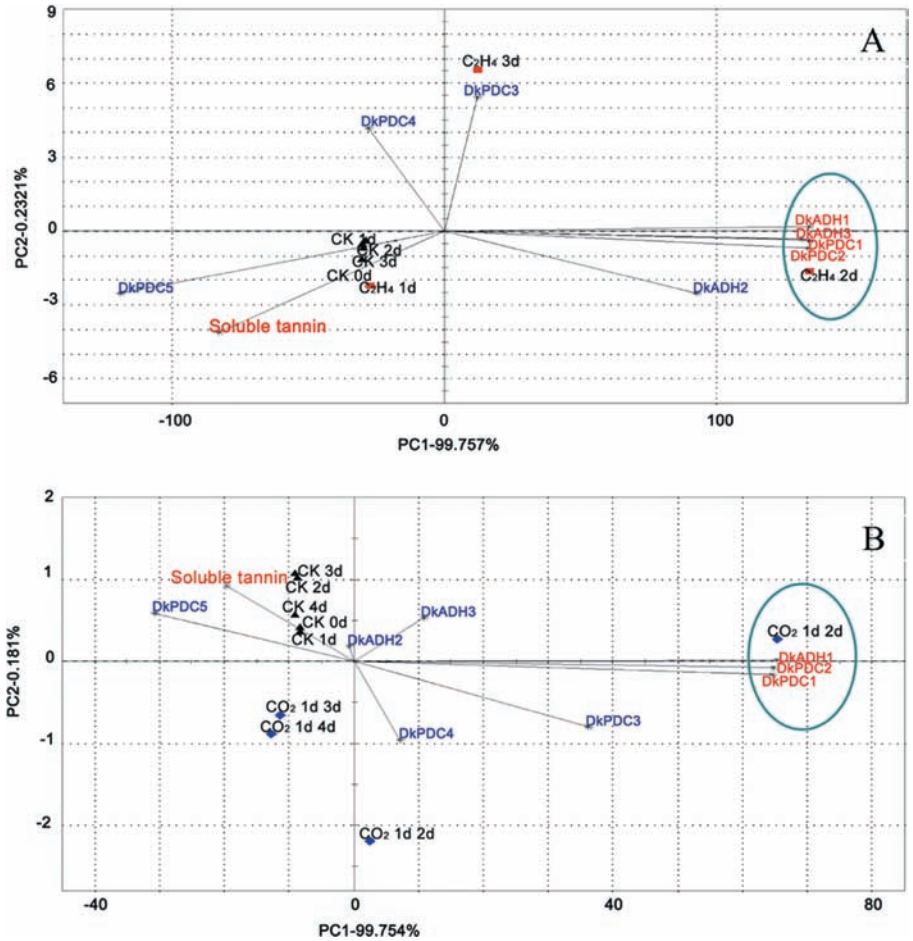

Fig. 8. PCA analysis of the relationship between *DkADH* and *DkPDC* gene expression and persimmon fruit de-astringency. Soluble tannin contents indicate the extent of fruit de-astringency. Ethylene treatment (a) and CO_2 treatment (b). The mean data were used for PCA analysis.

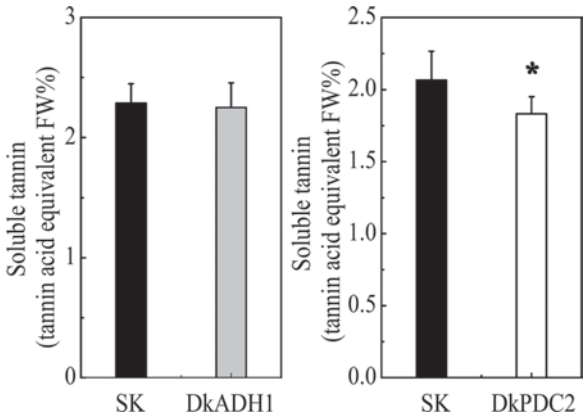

Fig. 9. Transient expression of *DkADH1* and *DkPDC2* in 'Mopan' persimmon leaves. Soluble tannins contents in leaves infiltrated with *DkADH1* or *DkPDC2*, driven by the CaMV 35S promoter. SK represents empty vector. Error bars indicate SEs from ten biological replicates (*P <0.05).

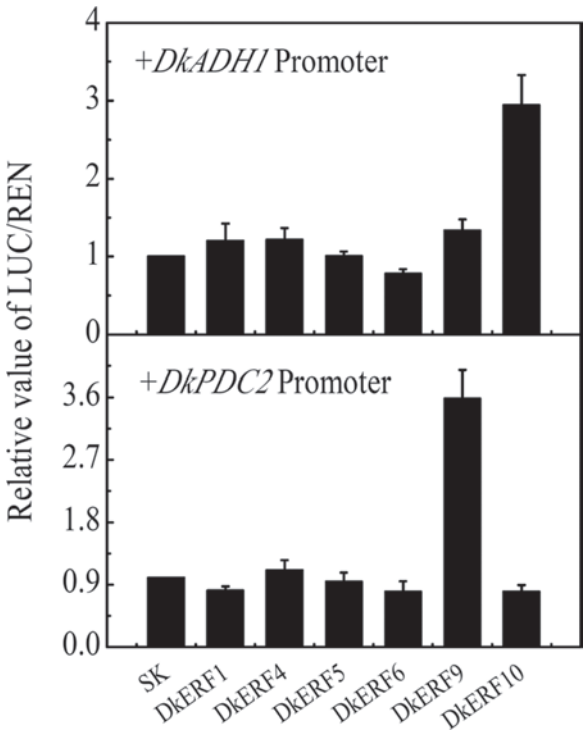

Fig. 10. *In vivo* interaction of hypoxia-responsive *ERF* gene and target promoters. 100 µl samples were infiltrated into *Nicotiana benthamiana* leaves. Firefly luciferase and renilla luciferase were assayed 3 d after infiltration. For each TF-promoter interaction, three independent experiments were performed (at least six replicates in each experiment). The ratio of LUC/REN of the empty vector (SK) plus promoter was used as calibrator (set as 1). Error bars indicate SEs from six biological replicates.

Association and interaction of hypoxia-responsive ERF*s and* DkADH1 *and* DkPDC2

Linear correlation analysis was performed on gene expressions of *DkERF* genes and the target genes. Except for *DkERF6*, expressions of other *DkERF* genes were closely related with *DkADH1* and *DkPDC2* (see Supplementary Fig. S4 at *JXB* online).

A transient assay indicated that *DkERF9* and *DkERF10* acted as activators, while the other five *ERF* genes had less effect on *DkADH1* and *DkPDC2* promoters (Fig. 10). Activity of the *DkPDC2* promoter was induced by interaction with *DkERF9*, while activity of the *DkADH1* promoter was only induced by *DkERF10* (Fig. 10).

Discussion

The majority of persimmon fruit in production are unique in that they remain astringent even at maturity, whereas astringency only occurs at the unripe stages in other fruit. Thus, the process of astringency removal is one of the most important aspects of postharvest handling of persimmon fruit. Persimmon fruit de-astringency has been extensively studied at the physiological level, and several successful technologies for promoting astringency loss have been developed, such as CO_2 and ethylene treatment. The mechanisms of action of astringency removal have mainly been studied in relation to CO_2 treatment, which is thought to occur by the promotion of hypoxia-related fermentation. Despite these physiological and biochemical investigations, however, molecular insights into persimmon de-astringency are still lacking. Nevertheless, persimmon fruit provide a good model for investigating both the hypoxia response and de-astringency.

Both CO_2 and ethylene treatments accelerate persimmon fruit de-astringency

By utilizing the astringent type persimmon 'Mopan', two de-astringency treatments, CO_2 and ethylene, were compared. The results showed that CO_2 was more effective than ethylene in inducing de-astringency, indicated by the decrease in SCTs (Figs 3, 34) and the increase in acetaldehyde formation (Tables 1, 12), which is in agreement with previous reports (Arnal and Del Río, 2003; Pesis and Ben-Arie, 2006; Salvador *et al*., 2007). This is consistent with acetaldehyde being the main compound involved in the insolubilization of SCTs.

Isolation of hypoxia-responsive ERF *genes from persimmon*

In model plants, *HRE* and *RAP* type *ERF* genes were recently shown to be the key regulators in plant responses to hypoxia (Gibbs *et al*., 2011; Hinz *et al*., 2010; Licausi *et al*., 2010, 2011*a*; Sasidharan and Mustroph, 2011; Yang *et al*., 2011). Comparative analysis of the predicted amino acid sequences encoded by the *HRE* and *RAP* genes revealed that all of the five proteins had conserved N-ends, initiated by the MC domain (MCGGAII), which has recently been shown to be involved in controlling the stability of regulators of the plant oxygen-sensing mechanism (Gibbs *et al*., 2011; Licausi *et al*., 2011*a*; Sasidharan and Mustroph, 2011). In persimmon, four *ERF* genes, *DkERF1*, *DkERF4*, *DkERF5*, and *DkERF6* were characterized as inducible by both low oxygen and ethylene, and proposed to be

de-astringency-related in our previous study (Yin *et al*., 2012). By utilizing RNA-Seq methodology, two additional novel hypoxia (high CO_2)-responsive genes, *DkERF9* and *DkERF10*, were isolated. However, only *DkERF10*, encoded the conserved MC domain. Phylogenetic analysis also indicated that *DkERF10* was closely related to *HRE* and *RAP* genes, and, like them, belongs to subfamily VII. Thus, the phylogenetic and domain analyses indicated that *DkERF10* had a high probability of being functionally involved in low oxygen sensing.

Real-time PCR indicated that all six *DkERF* genes were responsive to hypoxia/high CO_2. Of these six, *DkERF10* was the most responsive to the de-astringent treatment, especially to CO_2 treatment (greater than a 2000-fold increase, Fig. 2). Moreover, some of hypoxia/high CO_2-responsive *ERF* genes were also modulated by ethylene, with different patterns. *DkERF4*, *5*, *9*, and *10*, were responsive to both ethylene and CO_2 (present results and Yin *et al*., 2012*b*), while *DkERF1* and *6* only seemed to respond to CO_2. This observation is similar to that for the hypoxia-response *ERF* genes in *Arabidopsis*, where *HRE1* is both responsive to ethylene and hypoxia (Hinz *et al*., 2010; Yang *et al*., 2011), while *Rap2.12* is not regulated by 1-aminocyclopropane-1-carboxylic acid (ACC) (Licausi *et al*., 2011*b*).

Characterization of persimmon structural genes related to anaerobic fermentation

As mentioned previously, acetaldehyde is one of the main compounds that render the SCTs insoluble and cause de-astringency. Synthesis of acetaldehyde is generally catalysed by PDC, which converts pyruvate to acetaldehyde. ADH is then involved in the potentially reversible interconversion of acetaldehyde and ethanol (Strommer, 2011). In persimmon fruit, both PDC and ADH have been proposed as candidates involved in acetaldehyde formation (Yamada *et al*., 2002). In plants, PDC has been associated mostly with acetaldehyde formation (Nguyen *et al*., 2009), whereas ADH is best known for its role in the anaerobic response (Strommer, 2011), although it has also been linked in fruit to the regulation of the formation of volatile six-carbon alcohols (Speirs *et al*., 1998; Zhang *et al*., 2010). Phylogenetic analysis of persimmon *DkADH* and *DkPDC* genes revealed that the isolated genes were clustered with different homologues from *Arabidopsis*, tomato, tobacco, etc (Fig. 1). However, it is not possible to predict gene function simply based on phylogenetic tree analysis, particularly as the genes with high similarity were found to have different expression patterns in tissue-specific analysis (e.g. *DkPDC3* and *DkPDC4* were similar in sequence, but differentially expressed in various tissues).

The expression patterns of *DkADH* and *DkPDC* genes were monitored during de-astringency. The up-regulation by ethylene of *DkADH1*,*3* and *DkPDC1*,*2* is similar to the response of *ADH* and *PDC* genes to ethylene in other fruit, e.g. apricot *PaADH* (EU395433), and melon *CmADH1* (DQ288986) and *CmADH2* (DQ288987) (Manríquez *et al*., 2006; González-Agüero *et al*., 2009). During CO_2 treatment, no matter whether for 1 d or 2 d durations (Fig. 6; see Supplementary Fig. S2 at *JXB* online), *DkADH1*, *DkPDC1*, *DkPDC2*, and *DkPDC3* were transiently induced, similar to tomato *LeADH2* (X77233) and tobacco *TobADH1* (X81853) and *TobPDC1* (X81854) genes (Longhurst *et al*., 1994; Bucher *et al*., 1995). The expression patterns of these genes have also been shown in previous reports, to coincide with the increase in related enzyme activities (Tamura *et al*., 1999). *DkADH1*, *DkPDC1*, and *DkPDC2* were substantially more responsive to both persimmon de-astringency treatments than the other gene family members. Furthermore, the increase in expression of *DkADH1*, *DkPDC1*, and *DkPDC2* occurred in parallel with the changes in ADH and PDC enzyme activities (Figs 4, 45, 46, 47), suggesting that they might be the key genes involved in persimmon de-astringency, and this suggestion is supported by the PCA results (Fig. 8). Transient expression of *DkADH1* and *DkPDC2* in persimmon leaves had different effects on leaf SCTs contents. Leaves infiltrated with *DkPDC2* exhibited a small, but significant reduction in levels of SCTs, while *DkADH1* had no affect (Fig. 9). Although further functional analysis needs to be performed with stable transformation systems, all the present results strongly indicate that *DkADH1*, *DkPDC1*, and *DkPDC2* should be considered as the most important candidates for persimmon fruit de-astringency.

DkERF *genes involved in the transcriptional regulation of persimmon* DkADH1 *and* DkPDC2 *and in relation to de-astringency*

Transcription factors (TFs) act as the switches for regulating target genes (Riechmann *et al*., 2000). In model plants, expression of *ADH* and *PDC* genes is modulated by novel hypoxia-responsive *ERF* genes. Our results indicate that, with the exception of *DkERF6* (see Supplementary Fig. S4 at *JXB* online), changes in transcript abundance of *DkERF* genes and the target genes were closely correlated. Using the transient assay, gene–gene screening indicated that only *DkERF9* and *DkERF10* are involved in regulating the promoters of *DkPDC2* and *DkADH1* target genes (Fig. 10). These results were similar to the finding obtained by RNAi methodology that *Arabidopsis HRE1* is an activator of *ADH1*, *PDC1*, and *PDC2* (Yang *et al*., 2011). Thus, *DkERF9* and *DkERF10* might act as the major regulators of persimmon fruit low oxygen signalling, and thus be the key regulator genes in causing de-astringency.

Interestingly, the present results indicate that two *ERF* genes act as switches for different targets. *DkERF10* interacted with the *DkADH1* promoter, while *DkERF9* only induced activation of the *DkDPC2* promoter. These results suggest that different *ERF* genes may have different targets, which is in contrast to the observations for *HRE1* in *Arabidopsis*. This indicates that these two *DkERF* genes have different regulatory roles in persimmon de-astringency.

DkERF10 is a homologue of *Arabidopsis HRE* and *RAP* genes, and belongs to subfamily VII. Thus, it is not surprising that *DkERF10* was characterized as a regulator of persimmon fruit hypoxia response and de-astringency. On the other hand, *DkERF9* belongs to subfamily IV, which comprises the DREB subfamily. DREB type *ERF* genes are the main regulators of plant responses to abiotic stresses, such as drought and heat (Lata and Prasad, 2011). However, the roles of many DREB-type *ERF* genes have not yet been elucidated. Based on the present results, in addition to members of subfamily VII, subfamily IV *ERF* genes may also be involved in plant hypoxia responses.

In conclusion, both CO_2 and ethylene treatments were effective in reducing 'Mopan' persimmon fruit astringency, by promoting the increase of acetaldehyde, ADH and PDC activities and the decrease of SCTs. Three genes, *DkADH1*, *DkPDC1*, and *DkPDC2*, are the most likely candidates for increasing acetaldehyde concentrations and causing de-astringency, and the role of *DkPDC2* in decreasing SCTs was functionally confirmed. Two hypoxia-responsive *ERF* genes, *DkERF9* and *DkERF10*, are involved in the regulation of the *DkPDC2* and *DkADH1* promoters, respectively. Based on the present findings, we propose that both of *DkERF9* and *DkERF10* are involved in the persimmon fruit anoxia response and de-astringency and increase acetaldehyde accumulation by modulating different target genes. As there are at least three hypoxia responsive *ERF* genes in *Arabidopsis* (Hinz *et al*., 2010; Licausi *et al*., 2010), additional hypoxia-responsive *ERF* genes may exist in persimmon fruit. Furthermore, the effects high CO_2 and ethylene on the *DkERF* transcriptional regulation of *DkADH* and *DkPDC* genes need to be investigated further.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Sequences of the primers used for gene isolation and expression analysis.

Supplementary Fig. S1. Phylogenetic tree of *ERFs*.

Supplementary Fig. S2. Differential expression of *DkADH* and *DkPDC* genes in various 'Mopan' plant tissues.

Supplementary Fig. S3. Differential expression of *DkADH* and *DkPDC* genes in response to 2 d CO_2 treatment at 20 °C.

Supplementary Fig. S4. Correlation of transcripts abundance of *DkERF* genes with *DkADH1* and *DkPDC2* expression.

Supplementary Fig. S5. Sequences of *DkADH1* and *DkPDC2* promoters.

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