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RESEARCH PAPER

A transcription factor network responsive to high CO_2/hypoxia is involved in deastringency in persimmon fruit

Qing-gang Zhu[1], Zi-yuan Gong[1], Miao-miao Wang[1], Xian Li[1,2], Donald Grierson[1,3], Xue-ren Yin[1,2,*] and Kun-song Chen[1,2]

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

* Correspondence: xuerenyin@zju.edu.cn

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Abstract

Plant responses to anaerobic environments are regulated by ethylene-response factors (ERFs) in both vegetative and productive organs, but the roles of other transcription factors (TFs) in hypoxia responses are poorly understood. In this study, eight TFs (*DkbHLH1*, *DkMYB9/10/11*, *DkRH2-1*, *DkGT3-1*, *DkAN1-1*, *DkHSF1*) were shown to be strongly up-regulated by an artificial high-CO_2 atmosphere (1% O_2 and 95% CO_2). Dual-luciferase assays indicated that some TFs were activators of previously characterized *DkERF*s, including DkMYB10 for the *DkERF9* promoter, DkERF18/19 and DkMYB6 for the *DkERF19* promoter, and DkERF21/22 for the *DkERF10* promoter. Yeast one-hybrid and *cis*-element mutagenesis confirmed these physical interactions with one exception. The potential roles of these TFs in persimmon fruit deastringency were analysed by investigating their transient over-expression (TOX) in persimmon fruit discs, which indicated that $DkMYB6_{TOX}$, $DkMYB10_{TOX}$, $DkERF18_{TOX}$, and $DkERF19_{TOX}$ were all effective in causing insolubilization of tannins, concomitantly with the up-regulation of the corresponding genes. These results indicated that multiple TFs of different classes are responsive to high-CO_2/hypoxia in fruit tissues, and that a TF–TF regulatory cascade is involved in the hypoxia responses involving the Group VII *DkERF10*, and *DkERF*s and *DkMYB*s.

Keywords: Astringency removal, *ERF*, high CO_2, hypoxia, *MYB*, persimmon fruit, transcriptional regulation.

Introduction

Anoxia is a common abiotic stress for plants, usually caused by flooding (Yang *et al.*, 2011). The response to anoxia involves a range of metabolic and morphological responses over different timescales, including a rapid induction of anaerobic metabolism (Kennedy *et al.*, 1992; Voesenek and Bailey-Serres, 2015). Controlled-atmosphere storage in artificially reduced oxygen, usually supplemented with CO_2, has been used for a long time to actively extend post-harvest storage and alleviate physiological disorders for various fruits (Ali *et al.*, 2016; Bekele *et al.*, 2016; Matityahu *et al.*, 2016) and can induce anaerobic responses. A specific benefit for fruit quality conferred by a low-O_2 environment has been reported for astringent-type

Abbreviations ADH, alcohol dehydrogenase; AHCA, artificial high-CO_2 atmosphere (1% O_2 and 95% CO_2); AHNA, artificial high-N_2 atmosphere (99% N_2 and 1% O_2); PDC, pyruvate decarboxylase; ERF, ethylene-response factor; HRE, hypoxia-response ERF; TOX, transient overexpression.

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persimmon (*Diospyros kaki*) incubated in an atmosphere of 1% O_2 and 95% CO_2 (Pesis and Ben-Arie, 1984; Taira *et al.*, 1992, 2001; Min *et al.*, 2012). The low-oxygen environment leads to acetaldehyde accumulation, which removes astringency in persimmon fruit by precipitation of soluble tannins (Taira *et al.*, 2001; Salvador *et al.*, 2007). Controlled atmospheres containing ethylene also promote deastringency, suggesting that ethylene signaling is involved (Ikegami *et al.*, 2007; Min *et al.*, 2012, 2014; Yin *et al.*, 2012). Despite the fact that the commercial application of reduced oxygen for transportation and storage of fruit and some other plant products underpins a major industry and is important for food security, the underlying molecular mechanisms of fruit response to hypoxia are poorly understood.

In recent years, our knowledge of transcriptional regulatory mechanisms controlling hypoxia responses has been advanced significantly by the characterization of subfamily VII of the ethylene-response factors (ERFVII) (Sasidharan and Mustroph, 2011; Xie *et al.*, 2016). In Arabidopsis, five *ERF* genes, namely hypoxia-responsive *ERF1* (*HRE1*), *HRE2*, *RAP2.2*, *RAP2.3*, and *RAP2.12*, have been reported as the main plant oxygen-sensing regulators, and have been shown to control fermentation-related *ADH* and *PDC* genes (Hinz *et al.*, 2010; Licausi *et al.*, 2010; Yang *et al.*, 2011; Bui *et al.*, 2015; Papdi *et al.*, 2015). This sensing system operates via the N-end rule pathway, which controls plant *ERF* hypoxia responses, via post-translational regulation (Gibbs *et al.*, 2011; Licausi *et al.*, 2011*a*). Involvement of *ERF*s in the regulation of hypoxia responses has also been reported in other plants, such as rice submergence tolerance-related *Submergence 1* (*Sub1*; Xu *et al.*, 2006), *ERF VII* in *Rumex* and *Rorippa* (van Veen *et al.*, 2014), and *ERF VII* in apple fruit (Cukrov *et al.*, 2016). Potential roles for *ERF*s in persimmon fruit responses to hypoxia have also been investigated. Eighteen *DkERF* genes were shown to be responsive to treatment with 95% CO_2 (1% O_2), but only *DkERF9*, *10*, *19*, and *22* were capable of trans-activation of the promoters of *DkADH* and *DkPDC* genes (Min *et al.*, 2012, 2014). Moreover, of these four *DkERF* genes, only *DkERF10*, which has similarity to Arabidopsis *HRE2*, belongs to subfamily VII, indicating either that the hypoxia response is more complicated than revealed by investigations in Arabidopsis or that the ERF-VIIs may be regulated mainly at the post-translational level.

*ERF*s are one of the most comprehensively investigated transcription factor (TF) families with regards to involvement in plant hypoxia responses, although a few other hypoxia-related TFs have been reported, such as Arabidopsis *AtMYB2* (Hoeren *et al.*, 1998) and *Heat shock factor A2* (*HsfA2*; Banti *et al.*, 2010), wheat *TaMYB1* (Lee *et al.*, 2007), persimmon *DkMYB6* (Fang *et al.*, 2016) and *DkTGA1* (Zhu *et al.*, 2016). Omics-based analyses, however, have indicated many more TFs are responsive to hypoxia; for instance, at least 22 *ERF*s are regulated by anoxia in coleoptiles of rice (Lasanthi-Kudahettige *et al.*, 2007), and additional differentially expressed TFs have been characterized from Arabidopsis roots, leaves, and seedlings (Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Mustroph *et al.*, 2009; Lee *et al.*, 2011; Licausi *et al.*, 2011*b*). These data indicated the involvement of a variety of TFs in hypoxia responses in plants, although whether and how they interact is unclear.

In the present research, using astringency loss as a reporter of the anaerobic response, we utilized RNA-seq data previously used for *DkERF* isolation (Min *et al.*, 2012, 2014) and identified unigenes for TFs that were up-regulated by an artificial high-CO_2 atmosphere (AHCA; 1% O_2 and 95% CO_2). Another treatment, AHNA (artificial high-N_2 atmosphere; 99% N_2 and 1% O_2) was introduced to distinguish between responses to high CO_2 and hypoxia. Both high-CO_2-responsive and hypoxia-responsive TFs were selected for further analyses. Regulatory interactions of these TFs during hypoxia-triggered deastringency in persimmon fruit were investigated by dual-luciferase assays, yeast one-hybrid interactions, and promoter motif mutations. In the absence of a transformation system for persimmon, the functions of some potential regulators were analysed by transient over-expression in persimmon fruit discs.

Materials and methods

Plant material and treatments

Three astringent-type persimmon (*Diospyros kaki*) fruit were selected for this study, namely two Chinese cultivars, 'Mopanshi' and 'Jingmianshi', and one Japanese cultivar, 'Tonewase', all of which were collected from an orchard at Qingdao (Shandong, China) in 2014. Fruit without disease or signs of mechanical wounding were selected and divided into two batches: (1) the first batch was treated with AHCA (artificial high-CO_2 atmosphere, 1% O_2 and 95% CO_2) in sealed in air-tight containers for 1 d to remove astringency, and (2) the second batch was sealed in similar containers containing air for 1 d, as a control.

In order to distinguish between the effects of high CO_2 and low oxygen, AHNA (artificial high-N_2 atmosphere, 99% N_2 and 1% O_2) treatments were performed using the cultivar 'Gong cheng-shui shi', which was obtained from a commercial orchard at Gongcheng (Guilin, China) in 2017. The fruit were divided into three batches: (1) the first batch was treated with AHCA in sealed air-tight containers for 1 d, (2) the second batch was treated with AHNA in similar containers, and (3) the third batch was sealed in containers containing air, as a control.

The treated fruit were transferred to storage in air at 20 °C. Fruit flesh from three replicate samples each of which consisted of four fruit were sampled for each treatment at all sampling points. The samples were frozen in liquid nitrogen and stored at –80 °C until further use.

Soluble condensed tannins

Soluble condensed tannins are the main source of astringency for persimmon fruit. Here, two different methods were selected to determine the content of soluble condensed tannins. The printing method was used for fruit flesh, according to Min *et al.*, (2015). The whole fruit (1 d after picking, immediately after treatments) was cut into two parts and the cut surface was printed onto processed filter paper that had been soaked with 5% $FeCl_2$ and then oven-dried at 60 °C. The content of soluble tannins was indicated by the intensity of the black color on the filter paper.

A more accurate measure of the content of soluble tannins from frozen samples was obtained with Folin–Ciocalteu reagent, with three biological replicates, according to the method described by Yin *et al.*, (2012). The results were calculated using a standard curve of tannin acid equivalents.

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), using the same parameters described previously by Min *et al.* (2012). In brief, 2 g frozen fruit flesh was ground in liquid nitrogen and added to 5 ml saturated NaCl solution. Then 3 ml of the mixture was transferred to 10-ml air-tight vials with crimp-top caps. The vials were placed in a

water-bath at 60 °C for 1 h, after which 0.2 ml of head-space gas was removed for analysis. 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. All measurements were conducted with three biological replicates.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen persimmon fruit flesh samples (2.0 g for each), using the method described by Chang *et al.* (1993). The total RNA was treated with a TURBO DNA-free kit (Ambion) to remove the genomic DNA. First-strand cDNA synthesis was initiated from 1.0 μg DNA-free RNA, using an iScript™ cDNA Synthesis Kit (Bio-Rad). For each sampling point, three biological replicates were used for RNA extraction and the subsequent cDNA synthesis.

Gene isolation and sequence analysis

Using the same RNA-seq results described by Min *et al.* (2014), predicted TF-related hypoxia-responsive unigenes were isolated. The UTR regions of the transcripts were obtained using a SMART RACE cDNA amplification Kit (Clontech) and the primers are described in Supplementary Tables S1 and S2 at *JXB* online. The sequences of full-length TFs were confirmed and amplified with primers spanning the start and stop codons (Supplementary Table S3) and translated with the ExPASy software (http://web.expasy.org/translate). The newly isolated TFs were named after a BLAST analysis in Genbank and comparison with the reported TFs in persimmon.

Real-time PCR analysis

For real-time PCR, gene-specific oligonucleotide primers were designed (see Supplementary Table S4). The quality and specificity of each pair of primers were checked by melting curves and product resequencing. The housekeeping gene *DkACT* (Min *et al.*, 2012) was chosen as the internal control and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of genes (Livak and Schmittgen, 2001). The expression at the time-point of fruit harvest (0 d) was set as 1 for each gene.

PCR reactions were performed on a CFX96™ Real-Time System (Bio-Rad). PCR reaction mixtures (20 μl) comprised 10 μl of SsoFast™ EvaGreen Supermix (Bio-Rad), 1 μl of each primer (10 μM), 2 μl diluted cDNA, and 6 μl DEPC-treated water. The PCR program was initiated with a preliminary step of 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-curve analysis program. For real-time PCR, three biological replicates were conducted for each gene at each sampling point of each treatment.

Dual-luciferase assay

The trans-activation by the TFs of genes related to deastringency was investigated by dual luciferase assays (Hellens *et al.*, 2005). All constructs were electroporated into *Agrobacterium tumefaciens* GV3101. Full-length TFs were cloned into pGreen II 002962-SK vector (SK), using the primers described in Supplementary Table S5. The promoters of alcoholic fermentation-related genes (*DkADH1* and *DkPDC2*) and deastringency-related *ERF*s (*DkERF9*, *DkERF10*, and *DkERF19*) were originally constructed by Min *et al.* (2012) and Fang *et al.* (2016), and were inserted into the pGreen II 0800-LUC vector.

The dual-luciferase assays were performed with *Nicotiana benthamiana* leaves, using the protocol described by Min *et al.* (2012, 2014). *Agrobacterium* carrying constructs were suspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$. 150 mM acetosyringone, pH5.6) to an OD_{600} of approximately 0.75. TFs and promoters were combined at a ratio of 10:1 (v/v) and infiltrated into tobacco leaves by needleless syringes. Three days after infiltration, leaf discs were punched and assayed with dual-luciferase assay reagents (Promega). Dual-luciferase assays were performed with at least three independent experiments, with five biological replicates in each experiment.

Yeast one-hybrid assay

Yeast one-hybrid assays (Y1Hs) were performed in order to verify the gene–gene interactions, using the Matchmaker™ Gold Yeast One-Hybrid Library Screening System (Clontech, USA). The full-length *DkMYB10* was subcloned into the pGADT7 AD vector and the promoter of *DkERF9* was constructed into the pAbAi vector according to the ClonExpress II One-Step Cloning Kit (Vazyme, Nanjing) (primers are listed in Supplementary Table S6). Auto-activation and the interaction analyses were conducted according to the manufacturer's protocol.

Site-directed mutagenesis of gene promoters

Due to auto-activation of the promoters of *DkERF10* and *DkERF19* in yeast (see Supplementary Fig. S7), site-directed mutagenesis was performed for the *DkERF9*, *DkERF10*, and *DkERF19* promoters to eliminate the predicted binding sites for ERF and MYB TF (see Results). Motif mutations were carried out using the Fast Mutagenesis System (Transgene, Beijing) (primers are listed in Supplementary Table S7). Trans-activation effects of TFs on mutated promoters were further analysed by dual-luciferase assays.

Transient overexpression in persimmon fruit discs

In order to further verify the potential roles of TFs in persimmon fruit deastringency, transient overexpression (TOX) was performed with persimmon fruit discs. Discs of 1 cm diameter and 0.5 cm thickness were divided into five batches. The discs were incubated for 1 h with *Agrobacterium* carrying constructs in the same buffer used for the dual-luciferase assay. The discs were then transferred to filter papers (wetted by Murashige and Skoog medium) in tissue-culture dishes, and placed in an incubator at 25 °C for 3 d. All of the experiments (all genes and the empty vector) were performed with three biological replicates. At each sampling point (each day), the discs were dried on filter papers, frozen in liquid nitrogen and stored at −80 °C for further use.

Statistical analysis

The statistical significance of differences was determined using Student's *t*-test by DPS2.05 (Zhejiang University, Hangzhou, China).

Results

Isolation and characterization of deastringency/hypoxia-responsive transcription factors from 'Mopanshi' persimmon fruit

From RNA-seq data from the 'Mopanshi' cultivar (Min *et al.*, 2014), 13 full-length TFs were amplified by RACE and designated according to blast analysis as: *DkbHLH1* and *2* (basic/helix-loop-helix, KY849612-3), *DkMYB9*, *10*, *11*, *12*, and *13* (KY849603-7), *DkRH2-1* and *2* (ring-H2 finger protein, KY849614-5), *DkGT3-1* (trihelix transcription factor GT-3, KY849616), *DkAN1-1* (zinc finger AN1 domain-containing protein, KY849617), *DkHSF1* (heat shock factor, KY849619), and *DkIAA1* (auxin-responsive protein, KY849618). These, together with previously reported TFs from persimmon (*DkERF*s, *DkNAC*s, *DkMYB6*, *DkTGA1*) (Min *et al.*, 2012, 2014, 2015; Fang *et al.*, 2016; Zhu *et al.*, 2016), were used to study transcriptional interactions in anaerobic persimmon fruit. A summary of AHCA-responsive transcription factors from persimmon fruit is given in Supplementary Table S8.

AHCA accelerated deastringency in persimmon fruit and triggered anaerobic fermentation, as indicated by bursts of acetaldehyde and ethanol production (see Supplementary

Fig. S1). Expression of the 13 full-length TFs, which were predicted by RNA-seq, were analysed by real-time PCR. Eight genes were AHCA-responsive in 'Mopanshi', namely *DkbHLH1*, *DkMYB9*,*10*,*11*, *DkRH2-1*, *DkGT3-1*, *DkAN1-1*, and *DkHSF1* (Fig. 1). Of these, *DkbHLH1* showed the most striking response, increasing by about 429-fold after 1 d AHCA treatment, followed by *DkMYB10*, which increased by approximately 55-fold after 1 d. In 'Jingmianshi', *DkMYB11* was the most responsive to high-CO_2 treatment, increasing by about 1422-fold after 1 d, followed by *DkbHLH1* and *DkMYB10*, with 658-fold and 489-fold increases, respectively. In 'Tonewase' only *DkbHLH1* expression was very strongly responsive to AHCA treatment, increasing by about 935-fold after 1d. In contrast, the expression of the other five of the 13 TFs showed limited responses to AHCA treatment at 1 d (Supplementary Fig. S2), at which time the content of soluble tannins had almost reached its lowest level Supplementary Fig. S1). Thus, the subsequent responses (from 2 d onwards) of these genes were probably not related to deastringency.

In addition, a comparison was made between AHCA and AHNA using the cultivar 'Gong cheng-shui shi' (see Supplementary Fig. S3). Among the eight AHCA-responsive TFs, five (*DkbHLH1*, *DkMYB9*, *DkMYB11*, *DkRH2-1*, and *DkHSF1*) were responsive to both AHCA and AHNA, and thus can be termed as hypoxia-responsive; the expression of the other three TFs, *DkGT3-1*, *DkAN1-1*, and *DkMYB10*, remained constant in response to AHNA, and thus these genes were responsive to high CO_2 (Supplementary Fig. S4).

Effect of high-CO_2/hypoxia-responsive TFs on DkADH *and* DkPDC *promoters*

The persimmon genes *DkADH1* and *DkPDC2* were previously shown to be involved in fruit deastringency and to be induced by AHCA treatment (Min *et al*., 2012; Mo *et al*., 2016), and *DkERF9* and *DkERF10* were shown to have direct interactions with the *DkADH1* and *DkPDC2* promoters, respectively. In order to investigate the possible roles of other hypoxia-responsive TFs, the promoters of *DkADH1* and *DkPDC2* were used for dual-luciferase trans-activation assays. The eight AHCA-responsive TFs had either limited or no effects on the *DkADH1* and *DkPDC2* promoters (less than 2-fold increase) (Fig. 2), suggesting that there is no direct regulation by any of these TFs on the promoters of *DkADH1* and *DkPDC2*.

Relationship between high-CO_2/hypoxia-responsive TFs

Four *DkERF* genes, *DkERF9*,*10*,*19*, and *22*, were characterized previously as regulators of post-harvest deastringency in persimmon (Min *et al*., 2012, 2014). A further investigation was conducted to test the possible interaction between hypoxia-responsive TFs, including 18 additional *DkERF*s and four *DkMYB*s reported previously (Min *et al*., 2012, 2014; Fang *et al*., 2016), and promoters of *DkERF9*,*10*, and *19*. Dual-luciferase assays indicated various trans-activation reactions, for example between DkMYB10 and the *DkERF9* promoter (approximately 2.1-fold response), DkERF21 and 22 and the *DkERF10* promoter (approximately 2.3- and 2.0-fold, respectively), and DkERF18 and 19 and DkMYB6 and the *DkERF19* promoter (approximately 2.1-, 2.2-, and 3.7-fold, respectively) (Fig. 3). The synergistic effects of DkERF21 and DkERF22 on the promoter of *DkERF10*, and DkERF18, DkERF19, and DkMYB6 on the promoter of *DkERF19* were also investigated, but there were no additive effects of these TFs on their corresponding target promoters (see Supplementary Figs S5 and S6).

Using the yeast one-hybrid assay, it was found that DkMYB10 could physically bind to the *DkERF9* promoter (Fig. 4A). Furthermore, the MBSII (ACCAAC; Grotewold *et al*., 1994) mutation in the *DkERF9* promoter abolished the effects of DkMYB10 (Fig. 4A, B). Since the *DkERF10* and *DkERF19* promoters auto-activated in yeast (see Supplementary Fig. S7), a combination of *cis*-element mutations and dual-luciferase assays was used as an alternative way to test the specificity of this interaction. For the *DkERF10* promoter, two motifs (CAACA, Kagaya *et al*., 1999; ACCGAC, DRE element, Stockinger *et al*., 1997) were mutated to TAATA and TTCGAC, respectively (Fig. 4C). Subsequent dual-luciferase assays indicated that DkERF21 and DkERF22 had similar activation on the *DkERF10* promoter or the mutated *DkERF10* promoter (*DkERF10* m-promoter), suggesting either the absence of a direct interaction or that the interaction occurs with other unknown *cis*-elements (Fig. 4C). Two different mutations were designed to test the interaction between three transcription factors (DkERF18, 19, and DkMYB6) and the promoter of *DkERF19*. Three motifs (TTTGTT/AACAAA, TTTGTT, Dinh *et al*., 2012; GCCGCC, GCC box, Ohme-Takagi and Shinshi, 1995) were mutated to GTTATT/AATAAC and TCCTCC, and designated as *DkERF19m-1* (Fig. 4D). To test the DkMYB6 interaction, the CAGTTG motif (MBSI; Solano *et al*., 1997) in the *DkERF19* promoter was mutated to GAGCTG, designated as *DkERF19m-2* (Fig. 4E). Dual-luciferase assays indicated that these motif mutations abolished trans-activation of the *DkERF19* promoter by DkERF18, 19, and DkMYB6 (Fig. 4D, E).

Transient overexpression analysis in persimmon fruit discs

Due to the difficulty of stable transformation of perennial fruit such as persimmon, transient overexpression (TOX) analyses were performed with fruit discs. *DkMYB6*, *DkMYB10*, *DkERF18*, and *DkERF19* were selected for analysis in view of their direct trans-activation of the *DkERF9* and *DkERF19* promoters (Figs 3 and 4), using tannin removal as a reporter for activity. The content of soluble tannins in the discs treated with transcription factors and the empty vector (SK, control) all declined during the incubation, which may have been due to the experimental manipulation (Fig. 5A). All four transcription factors, however, accelerated insolubilization of tannins from 1 d to 3 d, resulting in significantly lower content of soluble tannins than the controls (Fig. 5A). Interactions between transcription factors were also analysed and the results indicated that TOX of *DkMYB6* and *10* and *DkERF18* and *19* could significantly up-regulate the endogenous *DkERF9* or *DkERF19* transcripts in persimmon fruit discs,

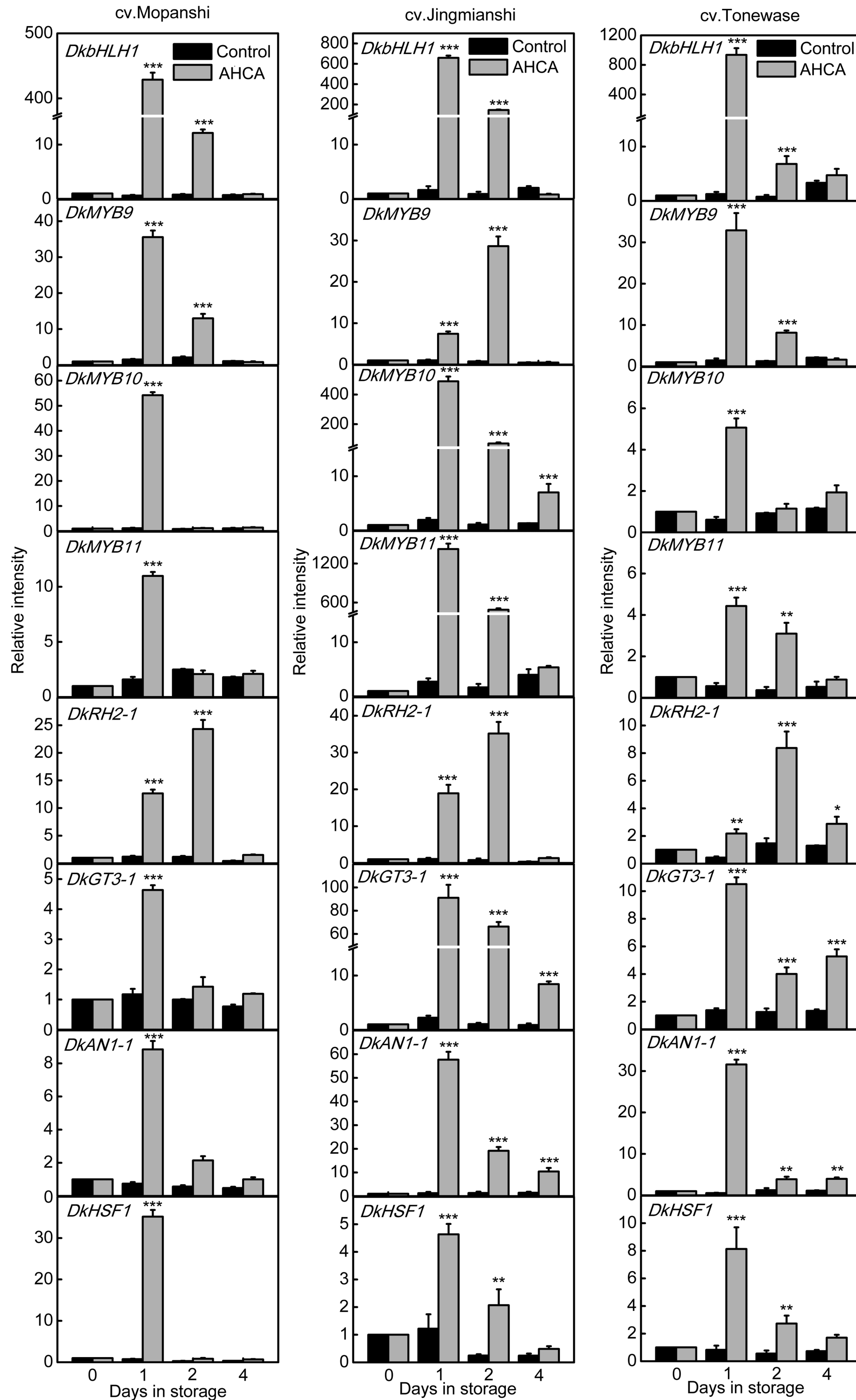

Fig. 1. Expression of TFs in response to treatment with artificial high-CO_2 atmosphere (AHCA, 95% CO_2 and 1% O_2, 1 d) in persimmon fruit cultivars 'Mopanshi', 'Jingmianshi', and 'Tonewase' at 20 °C. For relative mRNA abundance of the genes, the values at day 0 were set as 1. Values are means (+SE) from three biological replicates (*P<0.05, **P<0.01, ***P<0.001).

which further supported the interactions of AHCA-responsive transcription factors with the *ERF* promoters (Fig.5B–E). The expressions of downstream structure genes related deastringency were also analysed, and their expressions were also significantly up-regulated in the fruit discs, indicating that the transcriptional regulatory cascade would ultimately result in the regulation of structural genes (such as *DkADH1* and *DkPDC2*) and hence in regulation of fruit deastringency.

Discussion

Multiple TFs associated with the high-CO_2/hypoxia response that leads to deastringency

AHCA treatment is the most effective commercial method for reducing persimmon fruit astringency. It functions by stimulating the accumulation of anaerobic metabolites, such as acetaldehyde, which precipitate soluble tannins (Supplementary Fig. S1; Pesis and Ben-Arie, 1984; Taira *et al.*, 1992; Arnal and Del Río, 2004), the loss of which acts as a unique reporter of the anaerobic response. Previous work has highlighted the role of *DkERF9*, *10*, *19*, and *22* in this process (Min *et al.*, 2012, 2014). In this present study, eight new TFs belonging to different families, including *MYB*, *bHLH*, *Zinc finger*, *HSF*, and *IAA*, were characterized and their transcripts shown to increase in abundance in response to AHCA treatment in three different cultivars, suggesting that multiple TFs may contribute to the deastringency process. These results are similar to those from omics-based analyses in Arabidopsis, where, in different organs and under different conditions, Liu *et al.* (2005) found 64 differentially expressed TFs in Arabidopsis seeds under hypoxic conditions, and Licausi *et al.* (2011*b*) identified over 180 TF genes, most of which belonged to the *ERF*, *bHLH*, *MYB*, *HSF*, and *Zinc finger* families, that were up- or down-regulated in roots under hypoxic conditions.

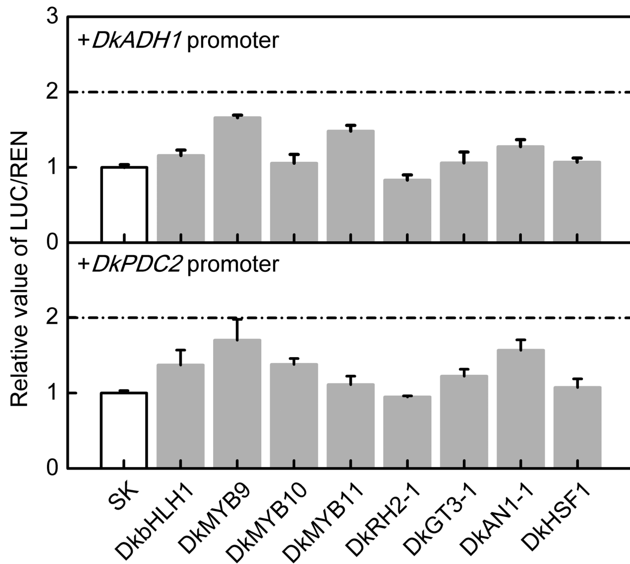

Fig. 2. Regulatory effects of artificial high-CO_2 atmosphere (AHCA)-responsive TFs on the promoters of *DkADH1* and *DkPDC2* determined using the dual-luciferase assay. The ratio of LUC/REN of the empty vector (SK) plus promoter was used as the calibrator (set as 1). Values are means (+SE) from five biological replicates.

The *ERF*s are the best-characterized transcription factor gene family involved in plant hypoxia responses, and members belonging to Group VII play a key role (Hinz *et al.*, 2010; Licausi *et al.*, 2010, 2011*a*; Gibbs *et al.*, 2011; Yang *et al.*, 2011; Min *et al.*, 2012; Gasch *et al.*, 2016). The ERFs detected in this study and earlier research (Min *et al.*, 2014) in persimmon belong to Group VII (*DkERF10*), Group IV (the DREB family) (*DkERF9*), Group IX (*DkERF18*, *19*), and Group X (*DkERF21*, *22*). The DkERF10 protein, the only persimmon Group-VII ERF detected, is assumed to be stabilized due to the MC domain (MCGGAII), which contributes to the stability

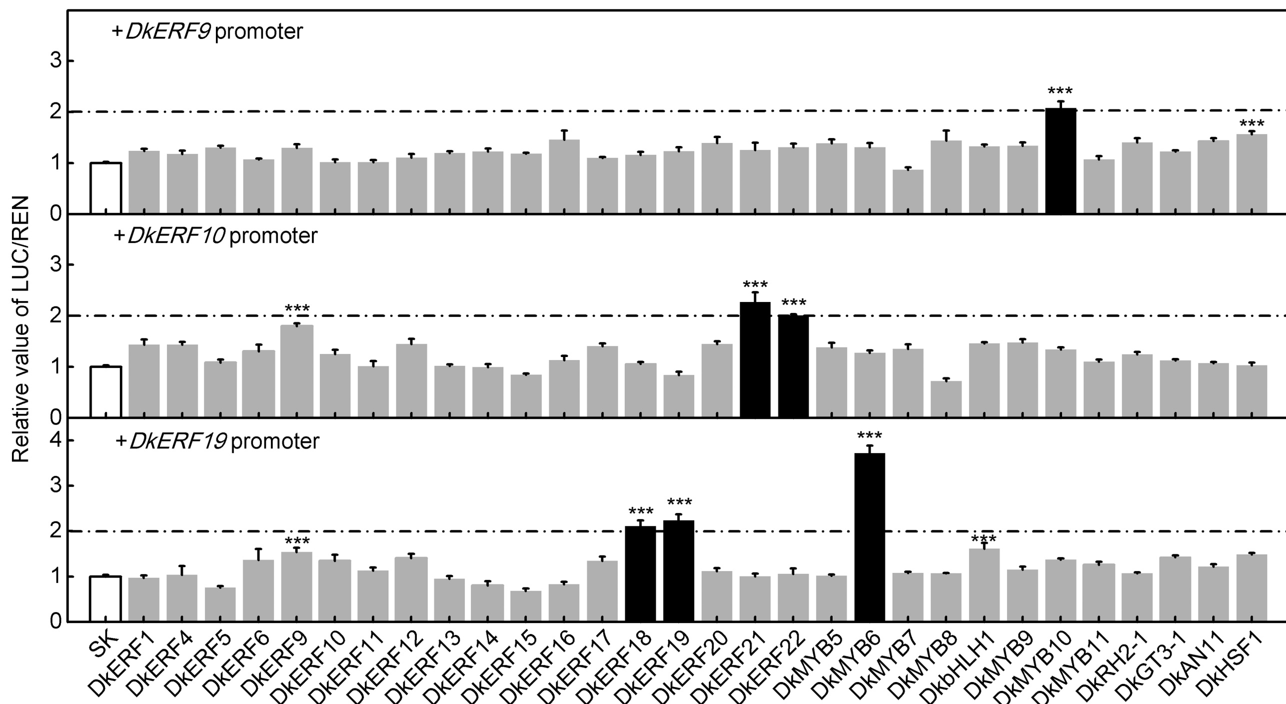

Fig. 3. Regulatory effects of artificial high-CO_2 atmosphere (AHCA)-responsive TFs on the promoters of deastringency-related *DkERF9*, *DkERF10*, and *DkERF19* determined using the dual-luciferase assay. AHCA-responsive *DkERF* and *DkMYB5-8* were isolated by Min *et al.* (2012) and Fang *et al.* (2016). The ratio of LUC/REN of the empty vector (SK) plus promoter was used as the calibrator (set as 1). Black columns highlight inductions of at least 2-fold. Values are means (+SE) from five biological replicates (***P<0.001).

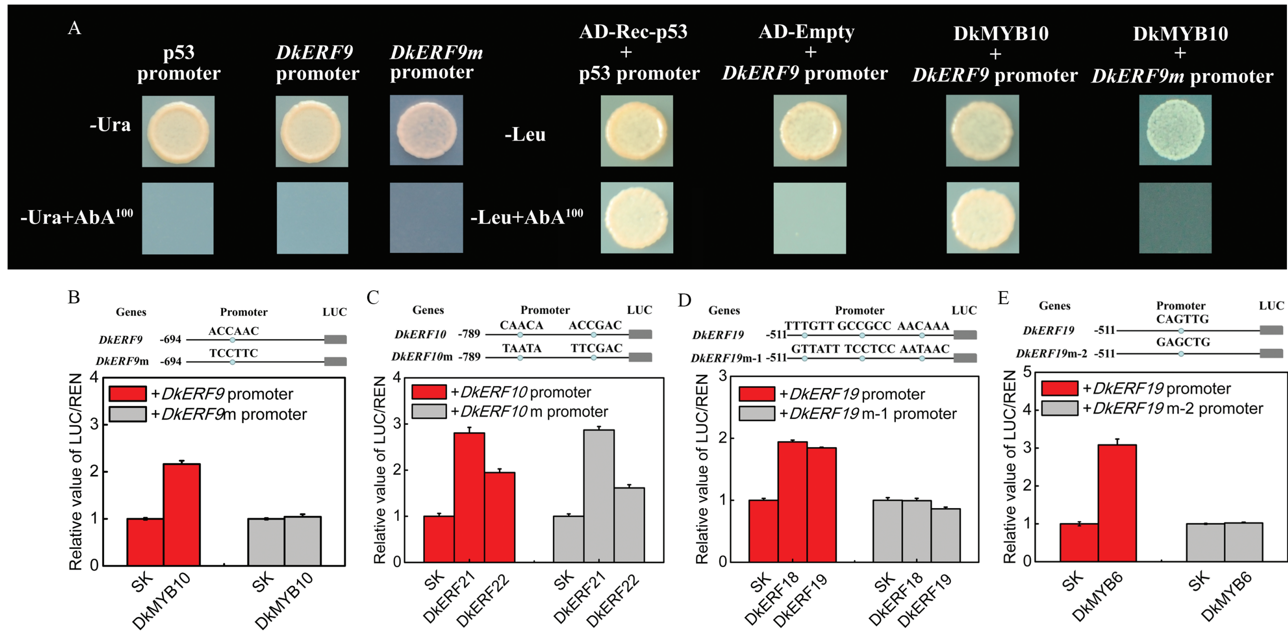

Fig. 4. Analysis of interactions between TFs and promoters determined by (A) yeast one-hybrid and (B–E) motif mutation assays. (A) Yeast one-hybrid analysis. Auto-activation of promoters was tested on synthetically defined (SD) medium lacking Ura in the presence of aureobasidin A (–Ura+AbA^{100}). Interactions were determined on SD medium lacking Leu in the presence of aureobasidin A (–Leu+AbA^{100}). Positive control: AD-Rec-p53+p53 promoter, provided with the kit; negative control: AD-Empty+*DkERF9* promoter. (B–E) Schematic diagrams of motif mutations for the *DkERF9/10/19* promoters and results of dual-luciferase assays performed with original and mutated promoters. The ratio of LUC/REN of the empty vector (SK) plus promoter was used as the calibrator (set as 1). Values are means (+SE) from five replicates.

of hypoxia-responsive ERFs (Gibbs *et al.*, 2011; Licausi *et al.*, 2011*a*), but there is also a major increase in *DkERF10* mRNA on day 1 of anoxia (Min *et al.*, 2012). The other *DkERF* genes involved in the response lack this MC domain, but our results indicate that they nevertheless participate directly in the regulatory cascade. In Arabidopsis and other plants, multiple groups of *ERF*s have also been shown to be associated with the responses to hypoxic treatments (Licausi *et al.*, 2011*b*; Cukrov *et al.*, 2016). Moreover, all these eight AHCA-responsive TFs were not homologous to the known 'core 49' hypoxia-responsive genes as identified by Mustroph *et al.* (2009). Although the expression of MYBs and many other transcription factors has been correlated with hypoxia tolerance (Hoeren *et al.*, 1998; Abe *et al.*, 2003; Mattana *et al.*, 2007; Mustroph *et al.*, 2009; Fang *et al.*, 2016), it is worth emphasizing that among the eight AHCA-responsive TFs, only five genes were characterized as hypoxia-responsive with AHNA treatment, and the other three (*DkGT3-1*, *DkMYB10*, and *DkAN1-1*) were only responsive to high-CO_2, and are thus not hypoxia-responsive (Supplementary Fig. S4). These findings indicated the similarities and also the differences between AHCA treatment in persimmon and hypoxia responses in model plants.

Transcriptional regulatory cascade of AHCA-responsive TFs

Although the mRNAs for *DkbHLH1*, *DkMYB9,10,11*, *DkRH2-1*, *DkGT3-1*, *DkAN1-1*, and *DkHSF1* increased in abundance in response to AHCA, the corresponding proteins did not have a significant direct trans-activation effect on the *DkADH1* and *DkPDC2* promoters (all responses being significantly below 2-fold) (Fig. 2). This suggested that the newly identified factors might function indirectly in stimulating deastringency, and a further investigation was conducted to test possible interactions between the AHCA-responsive TFs and *DkERF9,10*, and *19*, which recognize and trans-activate the *DkADH1* or *DkPDC2* promoters. The results indicated that there are at least two main types of transcriptional interactions between TFs: MYB–ERF and ERF–ERF interactions (Fig. 6). At least two MYBs, DkMYB6 and DkMYB10, physically bound to, and were putative activators of, the *DkERF19* and *DkERF9* promoters, respectively. ERF–ERF interactions included an indirect effect of DkERF21 and DkERF22 on the *DkERF10* promoter and a direct regulation by DkERF18 and DkERF19 of the *DkERF19* promoter (Fig. 6). *DkERF19* showed auto-activation in dual-luciferase assays, indicating that its own protein can bind and trans-activate its promoter, and as our knowledge of the TF cascade expands it will be important to test for similar interactions and auto-regulations between specific TFs (e.g. MYB and ERF) that may contribute to regulation of the high-CO_2/hypoxia response (Fig. 6). It also worth highlighting *DkbHLH1*, which was significantly up-regulated by deastringency treatments in all three examined cultivars and had a limited (less than 2-fold, but nonetheless significant) effect on the *DkERF19* promoter. Compared to the TFs considered above, the regulatory mechanisms of *DkbHLH1* (as well as the other responsive TFs) in the response of fruit to hypoxia require further investigation.

Regulatory cascades between TFs have been widely reported in various plants; for example, *AtSND1*, a NAC transcription factor, is involved in the regulation of secondary wall biosynthesis in Arabidopsis through trans-activation of *AtMYB46* (Zhong *et al.*, 2007), and the MdMYB10 protein can bind and transactivate *MdMYB10*, which is involved in anthocyanin production

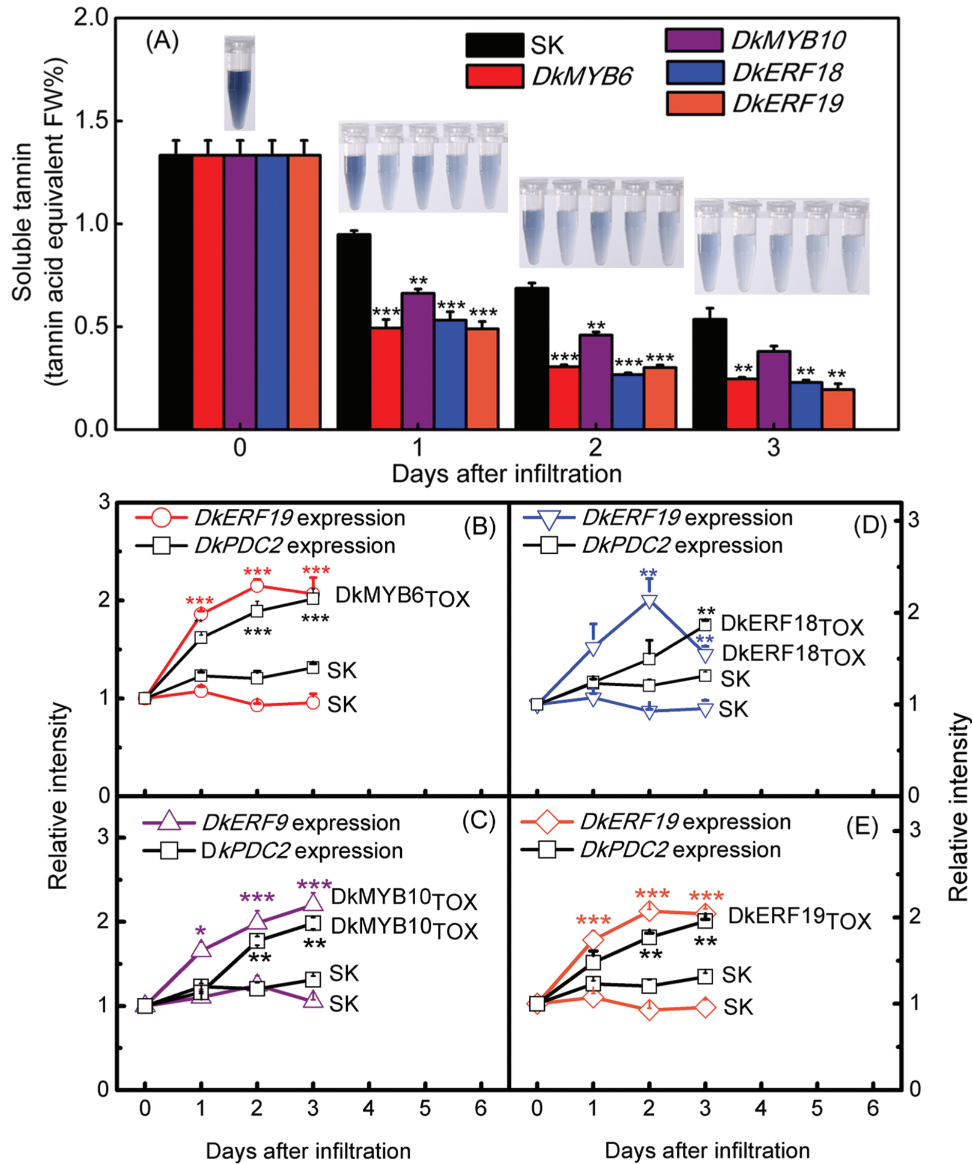

Fig. 5. Transient over-expression of TFs in persimmon fruit discs. The transient over-expression experiments were conducted with the empty vector pGreen II 002962-SK (SK) and DkMYB6/10 and DkERF18/19. Tissues from each of the infiltrated discs were taken to measure the content of soluble tannins (A) and the relative gene expression levels of related downstream genes compared with the SK control (B–E) during the 3 d of infiltration. Soluble tannin contents were measured using Folin–Ciocalteu reagent and were quantitated as tannin acid equivalents. The images above the bars in (A) show the reaction liquids used for measuring the soluble tannin content: the darker the colour of the test solution the higher the content of soluble tannin. Values are means (±SE) from three biological replicates. (**P*<0.05, ***P*<0.01, ****P*<0.001).

in red-fleshed apples (Espley *et al.*, 2009). For hypoxia responses, some TFs have been characterized at the transcript level and correlated with the expression of hypoxia-responsive genes (e.g. *ADH* and *PDC*, Abe *et al.*, 2003; Licausi *et al.*, 2011*b*). Our TF–promoter interaction results showed that, although Group-VII ERFs may play a leading role in sensing anaerobic conditions, there is a transcriptional cascade that leads to the up-regulation of the fermentation genes *DkADH1* and *DkPDC2* that involves the Group-VII DkERF10, and DkERFs from other Groups (DkERF9,18,19,21,22) and DkMYB6 and 10 (Fig. 6). The model presented here is supported by physical-binding and trans-activation studies (Figs 3 and 4; Min *et al.*, 2012, 2014; Zhu *et al.*, 2016) that provide insight into a hierarchy of interactions between the components of a regulatory cascade, leading to anaerobic responses. It enhances our understanding of the mechanism of the fruit hypoxia response, and may also apply to similar responses in other plant organs. One mechanism for the action of DkERF10 is that the protein may be stabilized by the effect of low O_2 on the MC domain. No TFs were found that could directly regulate the *DkERF10* promoter (Figs 3 and 4), although there was an indirect enhancement by DkERF21 and DkERF22. The possibility that there may be another unknown TF that can directly regulate the *DkERF10* promoter and/or that an unknown *cis*-element exists in the *DkERF10* promoter to which ERFs can bind requires further investigation.

In vivo *interactions between high-CO_2/hypoxia-responsive TFs and their roles in insolublization of tannins in persimmon fruit*

TOX analyses showed that $DkMYB6_{TOX}$ and 10_{TOX} and $DkERF18_{TOX}$ and 19_{TOX} could significantly accelerate

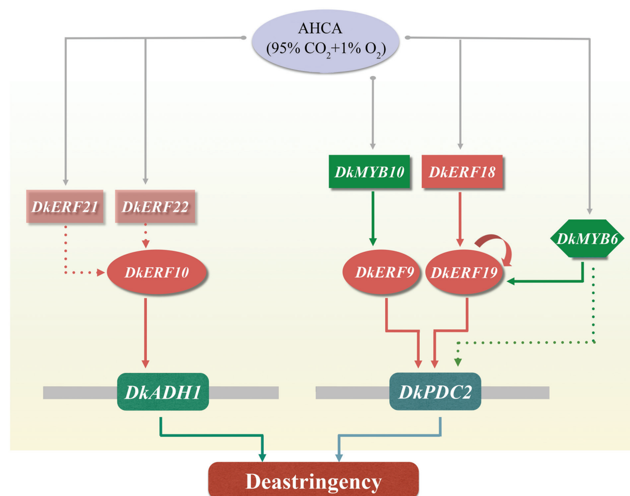

Fig. 6. Model of TF interactions in response to artificial high-CO_2 atmosphere (AHCA). AHCA treatment triggers the expression of various TFs, but only those with confirmed interactions are shown here. DkERF9, 10, and19 bind directly to, and activate promoters of, the anoxia-related genes *DkADH1* and *DkPDC2*, which subsequently accelerate deastringency. DkERF18, 19, and DkMYB6, 10 physically bind and trans-activate *DkERF* promoters; DkERF21 and 22 are indirect regulators of *DkERF10*. DkERF19 exhibits auto-activation and binds to its own promoter. Solid arrows indicate direct interactions, while dashed arrows indicate indirect regulation.

insolubilization of tannins in persimmon fruit discs, indicating that they participate in causing deastringency, which results directly from anaerobiosis (Taira *et al.*, 2001; Salvador *et al.*, 2007). The advantage of the TOX system is that it allows the analysis of the regulation of the endogenous genes and of the role of their transcriptional regulators and tannin content. Examples of the successful use of TOX include overexpression of *DkMYB4* in kiwifruit calluses, which significantly enhanced tannin biosynthesis (Akagi *et al.*, 2009), and expression of *DkPDC2* in persimmon leaves, which decreased soluble tannin content (Min *et al.*, 2012). The expression of *DkERF9*, *DkERF19*, and *DkPDC2* was up-regulated by $DkMYB6_{TOX}$ and 10_{TOX} and $DkERF18_{TOX}$ and 19_{TOX} in discs over a 1–3 d period, indicating the rapid and continuous responses of endogenous genes to these TFs, which occurred concomitantly with the decrease in soluble tannins in fruit discs. These *in vitro* results (Fig. 5) confirm the potential interactions and roles for *ERF* and *MYB* TFs in the response of persimmon fruit to AHCA treatment (Fig. 6).

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Effects of AHCA treatment on post-harvest deastringency in fruit of persimmon 'Mopanshi' at 20 °C.

Fig. S2. Expression of transcription factors that were relatively less responsive to AHCA treatment.

Fig. S3. Comparison of tannin printing assays for control and AHNA- and AHCA-treated 'Gong cheng-shui shi' fruit at 1 d.

Fig. S4. Expression of transcription factors in response to AHCA and AHNA treatment in 'Gong cheng-shui shi' fruit.

Fig. S5. Synergistic trans-activation effects of combinations of DkERF21 and DkERF22 on the *DkERF10* promoter.

Fig. S6. Synergistic trans-activation effects of combinations of DkMYB6 and DkERF18/19 on the *DkERF19* promoter.

Fig. S7. Auto-activation test for the *DkERF10/19* promoters.

Table S1. Primer sequences for 3´-RACE analysis.

Table S2. Primer sequences for 5´-RACE analysis.

Table S3. Primer sequences for full-length TFs.

Table S4. Primer sequences for real-time PCR analysis.

Table S5. Primer sequences for the dual-luciferase assays.

Table S6. Primer sequences for the yeast one-hybrid assay.

Table S7. Primer sequences for site-directed mutagenesis of the *DkERF9/10/19* promoters.

Table S8. Summary on hypoxia-responsive transcription factors from persimmon fruit.

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