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Dof Transcription Factors Are Involved in High CO_2 Induced Persimmon Fruit Deastringency

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Abstract: High CO_2 treatment is a widely used deastringency technology that causes the accumulation of acetaldehyde which precipitates the astringent soluble tannins from persimmon fruit, making them more attractive to consumers. The identification of *DkADH1* and *DkPDC2* (the key genes for acetaldehyde accumulation) and their regulators (e.g., ERFs), has significantly advanced our understanding of the fruit deastringency mechanism, but other TFs are also involved in the high CO_2 response. Here, 32 *DkDofs* genes were identified from 'Gongcheng-shuishi' persimmon, with nine of them shown to differentially respond to high CO_2 treatment. Dual luciferase assay indicated that DkDof3 and DkDof6 could repress the promoters of *DkADH1* and *DkPDC2*, respectively. EMSA assay showed that DkDof3 and DkDof6 physically interacted with probes containing T/AAAAG elements from the DkADH1 promoter, whereas they failed to recognize similar elements from the DkPDC2 promoter. The expression of *DkDof3* and *DkDof6* was also found to be repressed in different persimmon cultivars in response to high CO_2 treatment. It is proposed that DkDof3 and DkDof6 were involved in fruit deastringency by regulating the expression of *DkADH1* and *DkPDC2* in different persimmon cultivars.

Keywords: Dof; persimmon fruit; deastringency; transcription regulation; high CO_2

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1. Introduction

Persimmon (*Diospyros kaki* L.) is a perennial deciduous fruit tree, which is native to China and has a very long cultivation history. Persimmon can be divided into four types according to the astringency of the fruit and the influence of seeds [1]: pollination-constant non-astringent (PCNA), pollination-constant astringent (PCA), pollination-variant non-astringent (PVNA) and pollination-variant astringent (PVA) types. The four types are all astringent when they are immature. The astringency of PCNA and PVNA persimmon is naturally removed on the tree before harvest, but PCA and PVA types are still astringent at harvest and need to be treated after harvest to make them edible [2]. Therefore, it is of great significance to understand the mechanism of postharvest deastringency of persimmon fruit.

The astringency of persimmon fruit is due mainly to the combination of soluble tannins and human oral mucosal proteins, which causes a dry and rough sense in the mouth [3]. Several different artificial deastringency technologies have been discovered, including ethylene treatment [4], vacuum packaging [5], high CO_2 application [6], alcohol and warm water immersion [7]. Of these, high CO_2 treatment is the most widely applied and creates

a low oxygen environment [8]. Under this condition, acetaldehyde is formed and accumulates through anaerobic respiration, and combines with soluble tannins to remove fruit astringency by precipitation [9,10]. Under low oxygen conditions, acetaldehyde is produced in fruit mainly in two ways [11]. First, pyruvate is decomposed into acetaldehyde and CO_2, catalysed by pyruvate decarboxylase (PDC, EC 4.1.1.1); Second, catalytic activity of alcohol dehydrogenase converts ethanol to generate acetaldehyde (ADH, EC 1.1.1.1). Studies have shown that the activities of ADH and PDC both increased during deastringency of persimmon fruit undergoing high CO_2 treatment, with the concomitant accumulation of acetaldehyde and ethanol [11,12]. In a previous study, Min et al. [12] isolated three *DkADH* genes and five *DkPDC* genes from persimmon. Transient overexpression experiments showed that overexpression of *DkADH1* and *DkPDC2* could cause a decrease in soluble tannins, indicating that *DkADH1* and *DkPDC2* are key targets responsible for accumulation of acetaldehyde-mediated postharvest deastringency of persimmon fruit [12,13].

Transcription factors (TFs) involved in the regulation of *DkADH1* and *DkPDC2* expression in persimmon fruit deastringency have been investigated. Min et al. [12] and Zhu et al. [10] found that the expression of *DkADH1* and *DkPDC2* was regulated by DkERF10 and DkERF9/19. Furthermore, *DkERF10*, *DkERF9* and *DkERF19* were themselves regulated by upstream DkERF20/21, DkMYB10, and DkERF18/19, respectively [10,12]. Apart from these TFs, WRKYs [6], NACs [14], ZFs [15] and other family transcription factors have also been reported to be associated with persimmon fruit deastringency caused by high CO_2 treatment. *Dof* genes are plant specific transcription factors [16]. Generally, their N-terminal is a highly conserved single zinc finger Dof domain, which has the dual function of interacting with DNA and protein; The Dof C-terminal is relatively variable, and encodes an important specific transcriptional regulatory domain of Dof proteins [17,18]. Studies have shown that *Dof* genes are involved in many processes of plant growth and development, including tissue differentiation, seed development, and stress responses among others [19]. However, few studies have reported the roles of *Dof* genes in the high CO_2/hypoxia response and acetaldehyde metabolism. Additionally, the potential function of *Dof* genes in persimmon fruit deastringency is still unclear.

Here, we identified Dof transcription factors related to astringency removal using high CO_2-treated persimmon fruit. The *Dof* family genes from persimmon fruit were analyzed and the regulatory roles of *DkDofs* in persimmon fruit deastringency were investigated by real-time PCR analysis, dual-luciferase assay and electrophoretic mobility shift assay (EMSA). High CO_2 reduced expression of some Dof genes. DkDof3 and DkDof6 were shown to bind to and inhibit the transcription from the *DkADH1* and *DkPDC2* promoters.

2. Materials and Methods

2.1. Plant Materials and Treatments

Astringent persimmon 'Gongcheng-shuishi' fruit were harvested from a commercial orchard at Guilin (China) in 2018. Fruit of the same size without mechanical injury were selected and treated with air or high CO_2 (95% CO_2 + 1% O_2) for one day in air-tight containers. Then, the control and treated fruit were transferred to 20 °C for storage in air, and nine fruit of each group were sampled every day, containing three biological replicates. The 'Jingmianshi' samples, which were collected and described by Wang et al. [20], were used for the verification of key genes expression in response to high CO_2 treatment.

2.2. Gene Isolation and Sequence Analysis

The predicted coding (CDS) sequences of persimmon *Dof* genes were obtained from 'Youshi' (*Diospyros oleifera*) genome data [21]. The full-length genes were isolated by homologous cloning, and the primers used were listed in Table S1. For phylogenetic analysis, the amino acid sequences of DkDofs were aligned with AtDofs (downloaded from the website: https://www.arabidopsis.org/ [22]) by Clustal X and the results were visualized by FigTree (v1.4.2).

2.3. Soluble Tannins Printing Assay

Soluble tannins contents were visualized by soluble tannins printing assay according to Min et al. [23]. Firstly, filter papers were soaked in $FeCl_2$ solution and then dried. Then, the persimmon fruit was cut lengthwise and immediately printed onto $FeCl_2$-soaked filter paper. The intensity of black indicates the content of soluble tannins.

2.4. RNA Extraction and cDNA Synthesis

Total RNA was extracted by the cetyltrimethylammonium bromide (CTAB) method described in Yin et al. [4]. In short, around 1 g of pulp samples were extracted with CTAB buffer, and then washed twice with trichloromethane/isopentanol (*v*:*v* = 24:1). After precipitation with lithium chloride, it was washed twice with 75% ethanol. Then, the total RNA was obtained after dried and resolved by nuclease-free water.

The genomic DNA was then treated with DNase and synthesis of cDNA were carried out using the PrimeScript ^{TM} RT reagent Kit with gDNA Eraser (Takara, Kyoto, Japan).

2.5. Real-Time PCR Analysis

Gene-specific primers used for real-time PCR were designed using Primer3 (v.0.4.0) and double checked by melting curve and products sequencing. A CFX96 instrument (Bio-Rad) was used to perform the real-time PCR according to Yin et al. [4]. Primers used are listed in Table S2. The reaction system is 20 µL, including 10 µL ssofast evagreen Supermix (Biorad, CA, USA), 1 µL upstream primer, 1 µL downstream primer, 2 µL cDNA and 6 µL water. The PCR reaction procedure was 94 °C for 5 min, and then 94 °C 10 s, 60 °C 10 s for 45 cycles. The persimmon housekeeper gene *DkACT* [12] was selected as internal control to measure the expression level of other genes.

2.6. Dual Luciferase Assay

The dual luciferase assay was used to detect the regulatory effects of TFs on target genes [24]. In brief, the full-length sequences of *DkDofs* were inserted into pGreen II 0029 62-SK vector (SK), and the promoter fragments of *DkADH1* and *DkPDC2* were constructed on pGreen II 0800 Luc vector (Luc) which were completed by Min et al. [12]. Primers used were listed in Table S1. All constructed recombinant SK and Luc plasmids were transferred into Agrobacterium GV3101 and then transiently expressed on tobacco (*Nicotiana benthamiana*) leaves with a volume ratio of 10:1 (transcription factor: promoter). Three days after injection, the luciferase activity in leaves was detected by dual luciferase assay reagents (Promega, Madison, WI, USA).

2.7. EMSA

The full-length sequences of *DkDofs* were connected to the pGEX-4T-1 vector (GE), and then transformed into Rosetta (DE3) pLys bacteria (Novagen) to obtain the recombinant proteins by prokaryotic expression. Primers used are listed in Table S3. The protein induction was by adding 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 37 °C. Before EMSA, the target proteins were purified by the GST-tag Protein Purification Kit (Beyotime Biotechnology). Probes used for this assay were synthesized and 3′-biotin labeled by HuaGene (Shanghai, China) and are listed in Table S4. The upstream and downstream probes were mixed and annealed to form double stranded probes before use. Finally, EMSA was performed using the LightShift Chemiluminescent EMSA kit (ThermoFisher Scientific, Waltham, MA, USA).

2.8. Statistical Analysis

The Student's *t*-test was performed by GraphPadPrism7. Figures were drawn with GraphPadPrism7 and Adobe Photoshop CS6.

3. Results

3.1. Analysis of DkDof Genes

Based on the *Diospyros oleifera* genome [21], a total of 32 non-redundant *Dof* genes were found and their deduced amino acid sequences were used to perform phylogenetic analysis with *Dof* genes from Arabidopsis thaliana. These *Dof* family genes, both Arabidopsis and persimmon, could be divided into four major groups, and *Dof* genes from both plants were distributed in all four groups (Figure 1). The distribution of the 32 DkDofs indicated that they have certain specificity in structure and different groups may play different roles and the genes clustered in the same group may have similar functions.

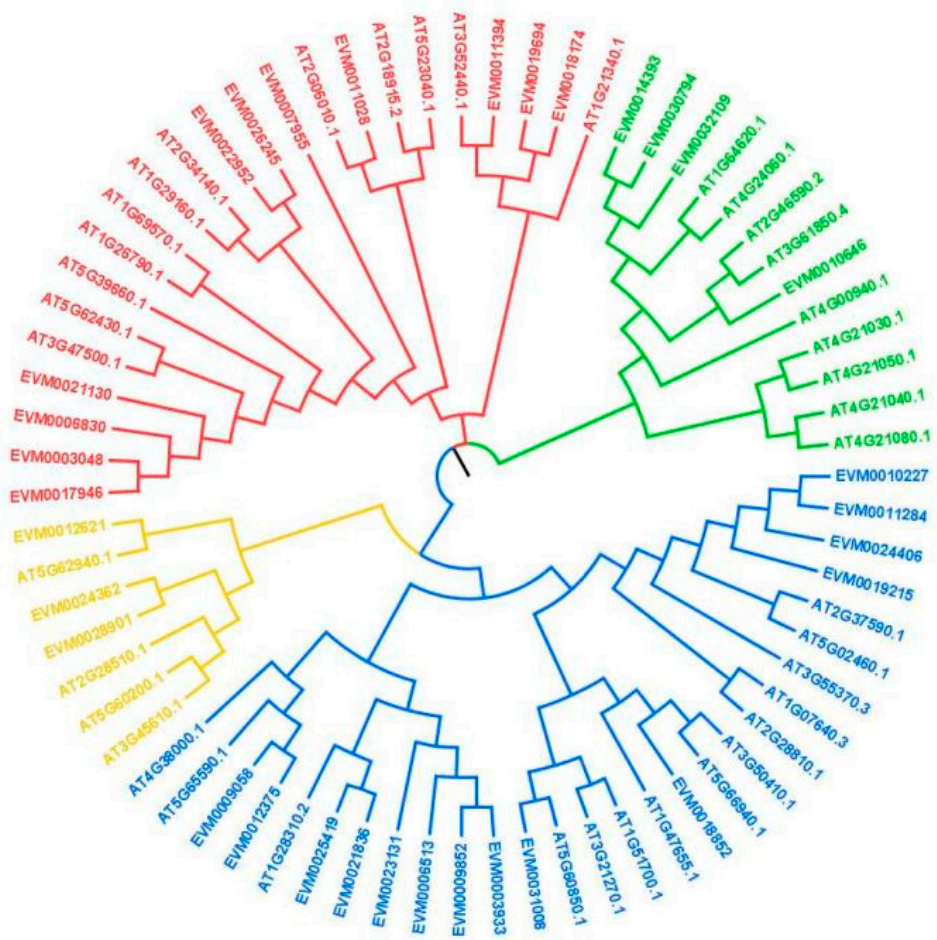

Figure 1. Phylogenetic analysis of *Dof* family genes from Arabidopsis and persimmon. Genes from Arabidopsis start with 'AT' and genes from persimmon start with 'EVM'. All genes were clustered into four groups, highlighted in green, red, yellow and blue, respectively.

3.2. Effects of High CO_2 Treatment on 'Gongcheng-Shuishi' Persimmon Fruit

The high CO_2 deastringency treatment was applied to 'Gongcheng-shuishi' persimmon fruit. The results showed that after 1 d of high CO_2 treatment the soluble tannins content has decreased significantly compared with control and this decreasing trend continued at 2 d (Figure 2). In short, high CO_2 treatment can cause persimmon fruit deastringency, and the effect is more obvious at 2 d.

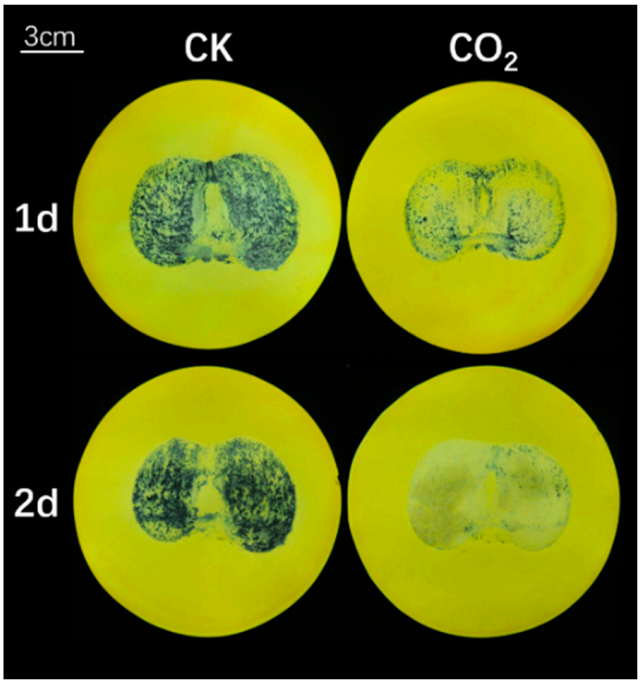

Figure 2. Changes in fruit-soluble tannins in response to high CO_2 treatment. Fruit was treated with high CO_2 (CO_2) or air (CK) for 1 d at 20 °C and then cut and printed onto $FeCl_2$-soaked filter paper to reveal soluble tannins, revealed by the intensity of the black color.

3.3. Expression of DkDofs in Response to High CO_2 Treatment

In order to explore the effects of *DkDofs* on persimmon fruit deastringency, their expression patterns in control and high CO_2-treated samples were analyzed. Real-time PCR experiments were performed to measure the expression of most *DkDofs*. Although we were unable to identify specific primers for genes *EVM0007955*, *EVM0009058*, *EVM0011394*, *EVM0021836* and *EVM0022952*. *EVM0025419* and *EVM0024362* were up-regulated and *EVM0003933*, *EVM0019694*, *EVM0017946*, *EVM0024406*, *EVM0011284*, *EVM0012621* and *EVM0031008* were down-regulated after 1 d in response to high CO_2 (Figure 3). There were also some genes, such as *EVM0018852* and *EVM0023131*, differentially expressed at 2 d (Figure 3). Since the soluble tannins of fruit have decreased at 1 d (Figure 2), the nine *DkDofs* differentially expressed at 1 d were selected as candidate genes for further investigations into the mechanism of deastringency.

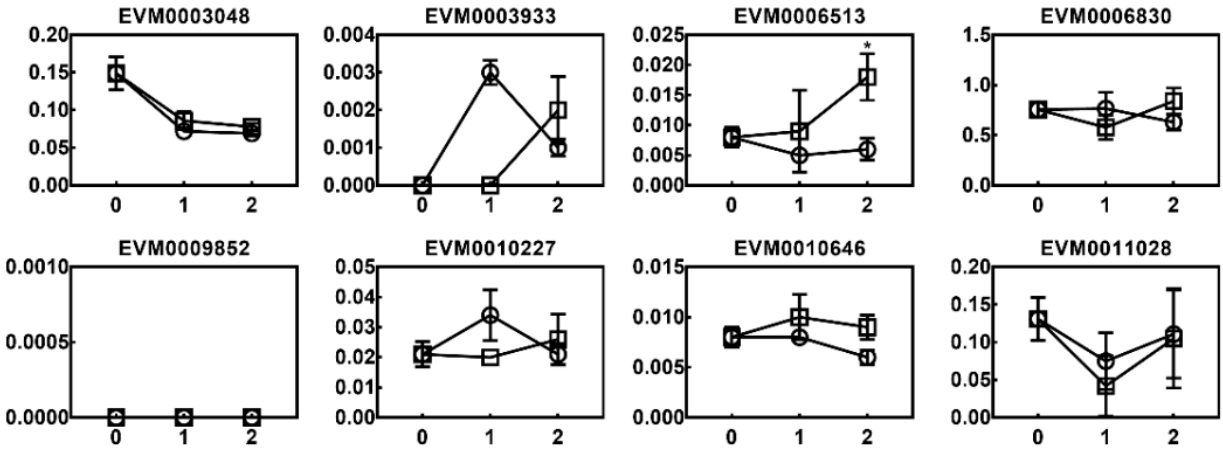

Figure 3. *Cont.*

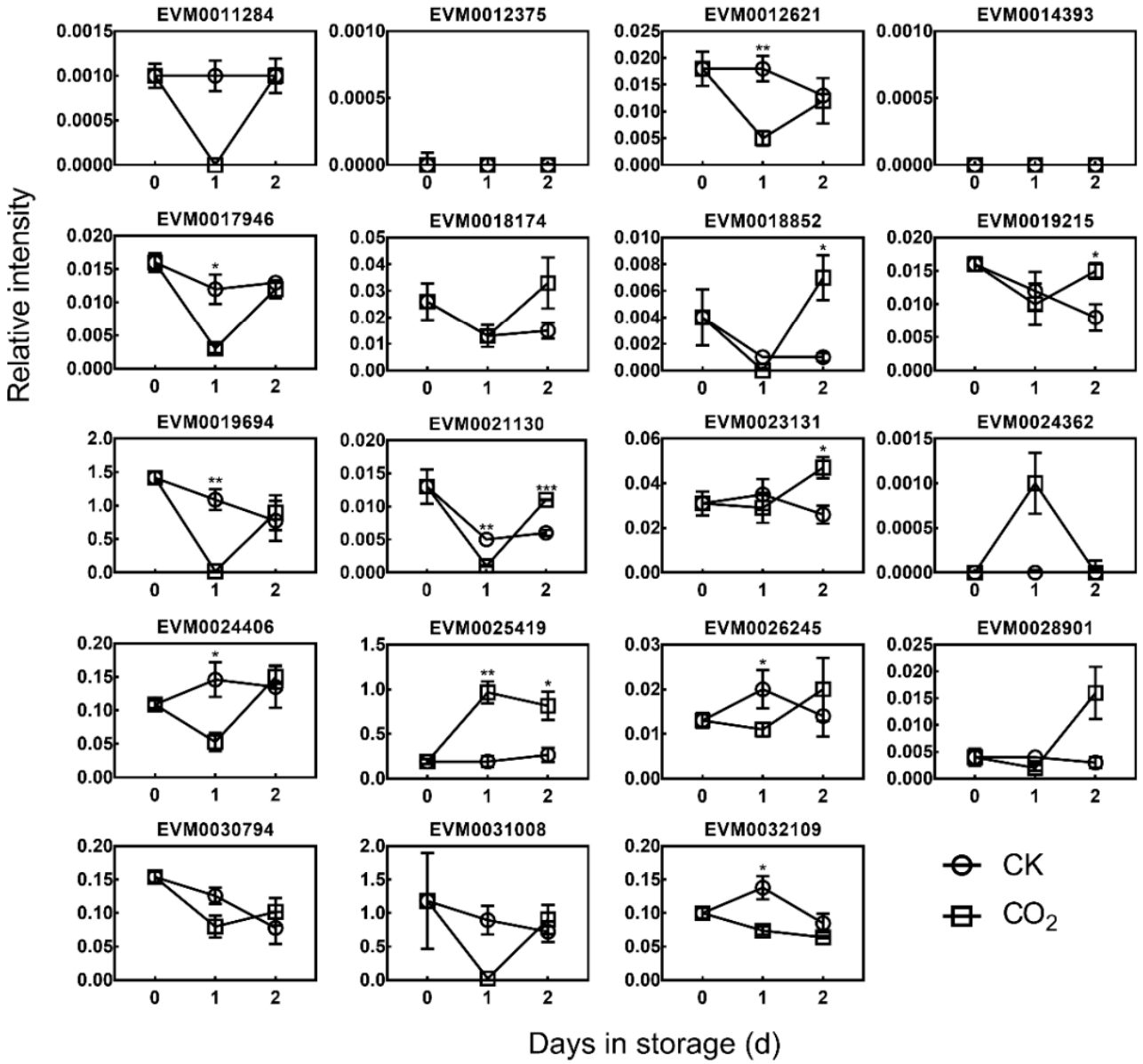

Figure 3. Expression of *DkDofs* in response to high CO_2 treatment in 'Gongcheng-shuishi' fruit. *EVM0031008*, *EVM0019694*, *EVM0011284*, *EVM0025419*, *EVM0003933*, *EVM0024406* and *EVM0012621* were designated as *DkDof1-7*. *DkACT* was used as internal control. Error bars represent standard errors for three replicates (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3.4. Regulatory Effects of DkDof1-7 on DkADH1 and DkPDC2 Promoters

Full-length sequences of seven *DkDofs* were isolated (designated as *DkDof1-7*, corresponding to *EVM0031008*, *EVM0019694*, *EVM0011284*, *EVM0025419*, *EVM0003933*, *EVM0024406* and *EVM0012621*). Since *DkADH1* and *DkPDC2* have been identified as the key targets involved in the high CO_2-induced deastringency process, the regulatory effects of DkDof1-7 on their promoters were analyzed. Dual luciferase assay found no significant trans-activation effect, but DkDof3 and DkDof6 were found to be repressors of the *DkADH1* and *DkPDC2* promoters with a 0.5-fold reduction in expression (Figure 4). The transcriptional effects of DkDof2 and DkDof7 on transcription from the *DkADH1* promoter and DkDof5 on the *DkPDC2* promoter also reached statistical significance, but the differences did not reach the selected thresholds (more than 2-fold or less than 0.5-fold) (Figure 4).

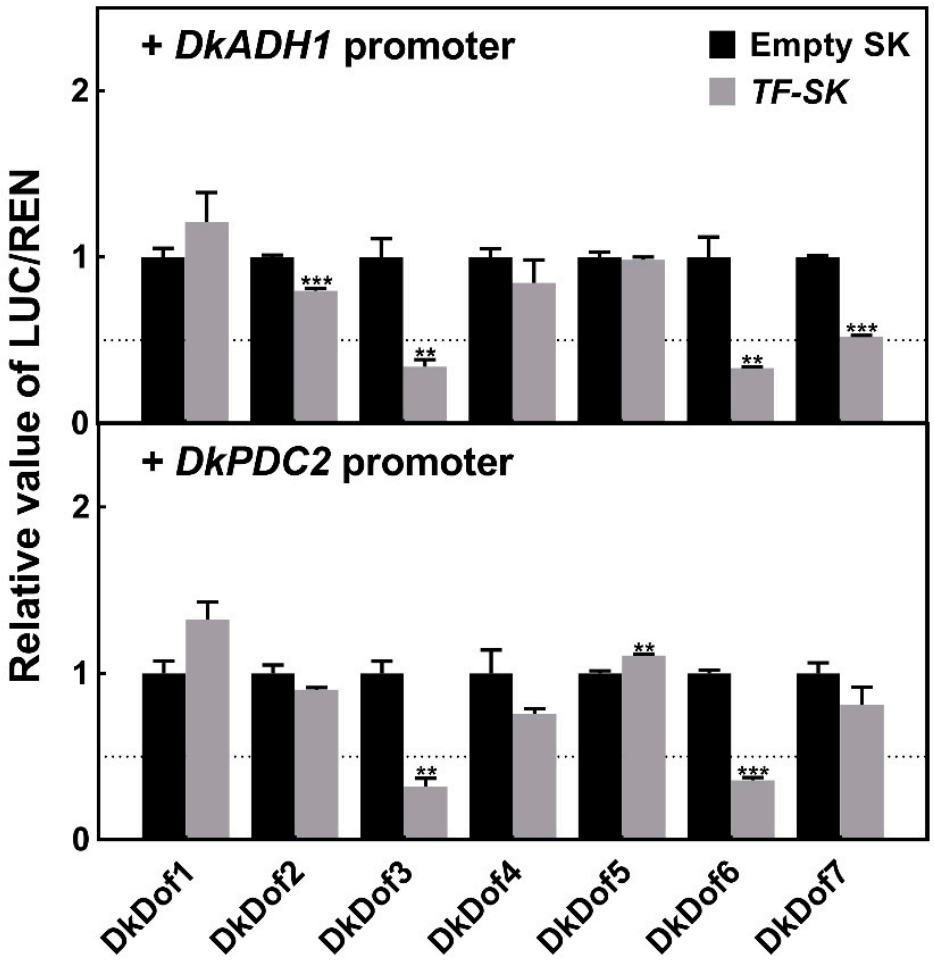

Figure 4. The regulatory effects of DkDof1-7 on the promoters of *DkADH1* and *DkPDC2*. Empty SK and *TF*-SK means empty SK plasmid and recombinant SK construct containing the TF gene, respectively. The LUC/REN value of empty SK plus corresponding promoter was set as 1. Error bars represent standard errors from three replicates (**, $p < 0.01$; ***, $p < 0.001$). DkDof2, 3, 6 and 7 inhibit the activity of the promoter of *DkADH1* whereas *DkDof3* and *6* inhibit activity of the *DkPDC2* promoter.

3.5. Binding Abilities of DkDof3 and DkDof6 on DkADH1 and DkPDC2 Promoters

In order to explore whether the regulatory effects of DkDof3 and DkDof6 on the *DkADH1* and *DkPDC2* promoters were direct or indirect, their abilities to bind these two promoters were tested. The specific DNA binding sequence for Dof family proteins is T/AAAAG [25]. There were five and three T/AAAAG motifs in the *DkADH1* and *DkPDC2* promoters, respectively, which were designed as probes with nearby sequences of about 30 bp (Figure 5A) (Sequences of probes were listed in Table S4). EMSA results indicate that DkDof3 could bind to the P1, P2, P4 and P5 probes from *DkADH1* promoter and DkDof6 could bind to the P2, P3, P4 and P5 probes from *DkADH1* promoter (Figure 5B). The unlabeled *DkADH1*-P4 probe was designed to verify the specificity of the binding and the results show that adding the unlabeled DkADH1-P4 probe could reduce the binding of labeled *DkADH1*-P4 probe to DkDof3 and DkDof6 proteins (Figure 5D). These results indicate that both DkDof3 and DkDof6 directly bind to the *DkADH1* promoter. However, neither of them was found to bind to the probes from the *DkPDC2* promoter, which suggested that their regulatory effects on *DkPDC2* promoter might be indirect (Figure 5C).

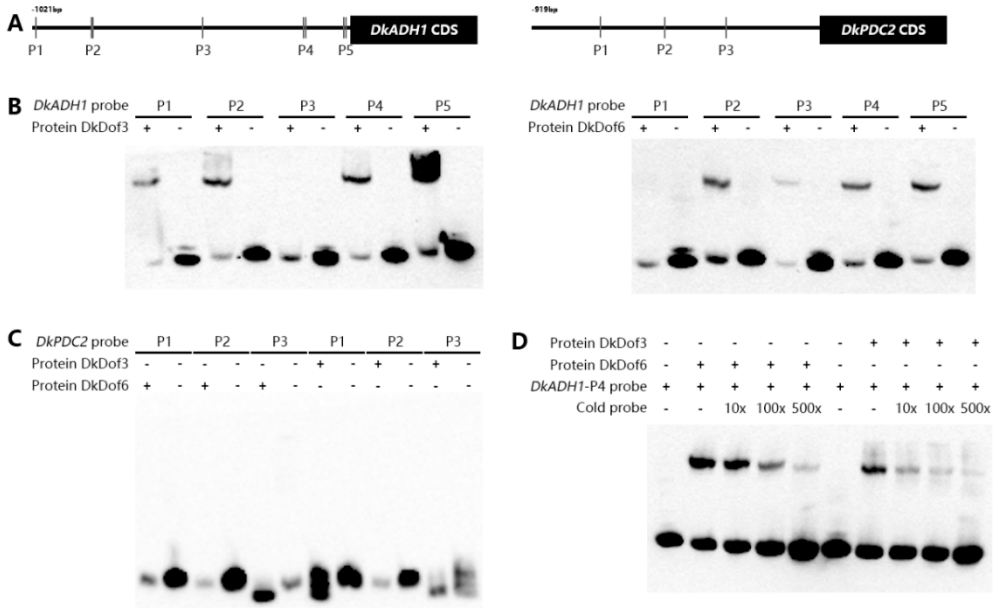

Figure 5. The binding abilities of DkDof3 and DkDof6 to the promoters of *DkADH1* and *DkPDC2*. (**A**) The location of binding motifs (T/AAAAG) for Dof family TFs in the promoters of *DkADH1* and *DkPDC2*. (**B**,**C**) The binding abilities of different probes with the core motif. (**D**) Verification of the binding effects by competition of unlabeled *DkADH1*-P4 probe (cold probe).

3.6. Expression of DkDof3 and DkDof6 in 'Jingmianshi' Fruit

In order to further verify the relations between DkDof3 and 6 and persimmon fruit deastringency, the changes in their expression were analyzed in another persimmon cultivar ('Jingmianshi'). As found for 'Gongcheng-shuishi', high CO_2 treatment also effectively accelerated deastringency in 'Jingmianshi' fruit [20], and inhibited the expression of *DkDof3* and *DkDof6* (Figure 6), which confirmed that these two *Dof* genes were negatively correlated with persimmon fruit deastringency.

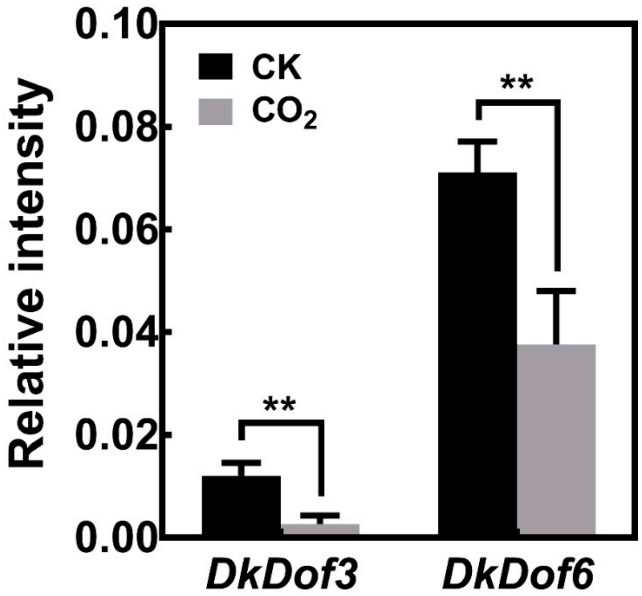

Figure 6. Expression of *DkDof3* and *DkDof6* after one day of high CO_2 treatment of 'Jingmianshi' fruit. *DkACT* was used as internal control. Error bars represent standard errors from three replicates (**, $p < 0.01$).

4. Discussion

It is very important for the development of the persimmon industry to study fruit deastringency technologies. In recent years, research to precisely control fruit astringency without affecting other quality attributes has evolved from simple technology development [11,26] to investigations into the molecular basis of the mechanisms of deastringency [10,12]. Previous studies have identified *DkADH1* and *DkPDC2* as key target genes of high CO_2-induced persimmon fruit deastringency [12]. Moreover, several TFs have been reported to regulate high CO_2-induced persimmon fruit deastringency, such as ERFs [12], MYBs [10] and WRKYs [6]. However, these TFs cannot fully explain the whole process of fruit deastringency. Here, the involvement of *Dof* families in deastringency regulation was studied which was little reported before. A total of 32 *Dof* genes were identified, based on the *Diospyros oleifera* genome [21], and nine *DkDofs* were probably involved in deastringency regulation as they were differentially expressed in response to high CO_2 treatment (Figure 3). Among them, DkDof3 (EVM0011284) and DkDof6 (EVM0024406) could trans-repress the promoters of *DkADH1* and *DkPDC2* (Figure 4), which enriches the regulation targets of *Dof* family. Besides, DkDof3 and DkDof6 were clustered together (blue group) and were homologous to Arabidopsis AT2G37590.1 (Figure 1), which has been shown to be involved in vascular development [27]. As ADH and PDC are also the key enzymes involved in hypoxia response, whether this blue group of *Dof* genes including AT2G37590.1 have flood resistance function is worth exploring.

TFs can be divided into activators and repressors according to their regulatory effects on the target genes [28]. Many TFs have been reported to regulate hypoxia responses or fruit deastringency. Some of these TFs have been found to form complexes, such as DkERF24 and DkWRKY1 [6]. Some are involved in a regulatory cascade, for example, DkMYB10 was identified as the upstream activator of DkERF9 [10]. Some TFs individually regulate the expression of *ADH* or *PDC*, such as DkERF23 [6]. However, these reported that TFs are all activators. It has been reported previously that *Dof* family genes could play roles as repressors. For example, ZmDof1 inhibited pollen formation by binding to and suppressing the promoter of *Zm401*, which was the key gene for pollen formation [29]. AtDAG1 (DOF AFFECTING GERMINATION1) controls seed dormancy and germination by inhibiting the expression of abscisic acid degradation gene *CYP707A2* and gibberellin synthesis gene *AtGA3ox1* [30]. Here, DkDof3 and DkDof6 were verified to be involved in fruit deastringency by suppressing transcription of *DkADH1* and *DkPDC2* (Figure 4), which showed that the deastringency process is jointly regulated by transcriptional activators and repressors. Besides, DkDof3 and DkDof6 could regulate both *DkADH1* and *DkPDC2* promoters and might play a greater role in fruit deastringency, as most previously identified TFs only regulated one of them (Figure 7).

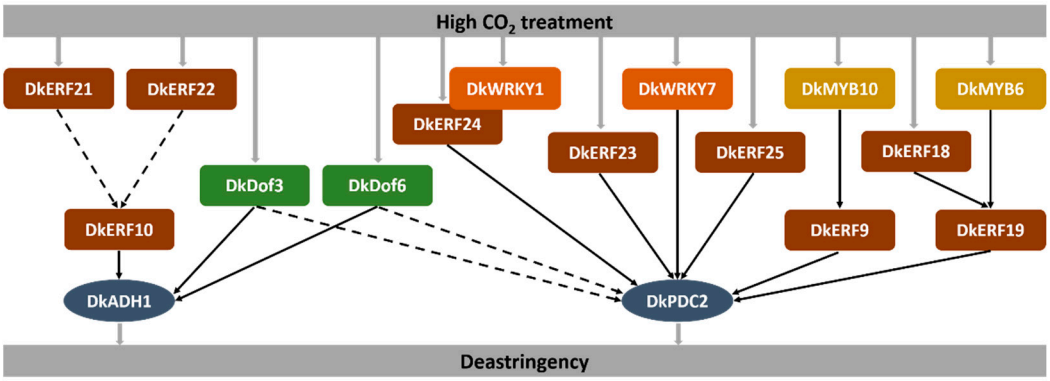

Figure 7. The proposed regulatory roles of TFs to the promoters of *DkADH1* and *DkPDC2*. DkDof3 and DkDof6 were newly discovered in this study. Black solid arrows indicate direct regulation and black dashed arrows mean indirect regulation. DkDof3 and DkDof6 were repressors.

In conclusion, 32 *DkDofs* were identified from persimmon fruit, with nine of them differentially expressed in response to high CO_2 treatment, which were considered to be involved in persimmon fruit deastringency. Of these, DkDof3 and DkDof6 were identified as repressors that could directly and indirectly trans-repress the promoters of *DkADH1* and *DkPDC2*, respectively. Moreover, the expression of *DkDof3* and *DkDof6* was repressed in another persimmon cultivar in response to high CO_2 treatment, which suggested that the regulatory roles of them may be conserved in different cultivars. These findings add to our understanding of persimmon fruit deastringency and the function of Dof family genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8070643/s1, Table S1: Primers used for genes isolation; Table S2: Primers used for real-time PCR analysis; Table S3: Primers used for pGEX-4T-1 vector construction; Table S4: Sequences of the probes used for EMSA.

Author Contributions: Conceptualization, X.Y.; methodology, W.W. and X.L.; validation, W.W. and X.Y.; investigation, R.J.; writing—original draft preparation, R.J. and W.W.; writing—review and editing, K.C. and X.Y. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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