DkNAC7, a novel high-CO\textsubscript{2}/hypoxia-induced NAC transcription factor, regulates persimmon fruit de-astringency

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Abstract

Artificial high-CO\textsubscript{2} atmosphere (AHCA, 95% CO\textsubscript{2} and 1% O\textsubscript{2}) has been widely applied as a postharvest de-astringency treatment for persimmon fruit. AHCA increases expression of transcription factors, including ethylene response factors (DkERF), that target de-astringency genes. Here, the promoter of DkERF\textsubscript{9}, a previously characterized AHCA-inducible and de-astringency regulator, was utilized to screen a cDNA library by yeast one hybrid assay. A novel NAC transcription factor, named DkNAC7, was identified. Dual-luciferase assay indicated that DkNAC7 could not only trans-activate the promoter of DkERF\textsubscript{9}, but also activated the previously identified deastringency-related gene DkPDC2. Real-time PCR analysis showed that DkNAC7 was up-regulated by AHCA treatment, in concert with the removal of astringency from persimmon fruit and subcellular localization showed DkNAC7 was located in the nucleus. Thus, these results indicate that DkNAC7 is a putative transcriptional activator involved in regulating persimmon fruit deastringency by trans-activation on both DkERF\textsubscript{9} and DkPDC2, which encodes pyruvate decarboxylase.

Introduction

Persimmon (\textit{Diospyros kaki} L.) is a worldwide crop, which originated in Southeast Asia. Persimmon fruit can be divided into astringent and non-astringent types, but most native cultivars in China are of the astringent types [1]. These astringent persimmon fruit have the unique feature of accumulating abundant amounts of condensed tannins (CT) [2]. Astringent persimmon accumulates abundant CTs in fruit even at maturity and soluble CTs (SCT) cause astringency [1,3], which severely affects the persimmon industry and consumer acceptance.

A range of artificial technologies have been developed to remove astringency, including high-CO\textsubscript{2}, ethylene and ethanol [4–8]. Among these, high CO\textsubscript{2} (usually > 90%) is the most...
widely used treatment, in which the O\textsubscript{2} level is reduced to 1%. In plants, hypoxia usually causes the accumulation of products from anaerobic metabolism \cite{9}, and these products (especially acetaldehyde) effectively reduce the SCTs and accelerate deastringency in persimmon fruit \cite{5,7,10}. The activities of alcohol dehydrogenase (ADH, EC 1.1.1.1) and pyruvate decarboxylase (PDC, EC 4.1.1.1) and also their encoding genes (\textit{DkADH1}, \textit{DkPDC1}, \textit{DkPDC2} and \textit{DkPDC3}) have been shown to increase in amount during deastringency \cite{7,11}. Transient over-expression of \textit{DkPDC2} led to a lower level of SCTs in persimmon leaves \cite{7}, suggesting that \textit{DkPDC2} is a key gene for the de-astringency program of persimmon fruit. These results confirmed that CO\textsubscript{2} driven astringency removal involves hypoxia-triggered acetaldehyde metabolism.

In the model plant \textit{Arabidopsis}, a few ethylene response factors (ERFs) have been reported to be involved in the hypoxia response, including \textit{HRE1}, \textit{HRE2}, \textit{RAP2.2} and \textit{RAP2.12}. These ERF genes could transcriptionally regulate \textit{ADH} and \textit{PDC}, and result in hypoxia tolerance \cite{12–15}. As stated above, persimmon fruit deastringency by high CO\textsubscript{2} treatment is considered to operate mainly through the hypoxia fermentation pathway. In persimmon, four \textit{DkERF} were previously reported to be involved in persimmon fruit deastringency, including \textit{DkERF9/10/19/22} \cite{7,8}. Of these, \textit{DkERF9} was characterized as an activator of the promoter of \textit{DkPDC2}, a key gene for deastringency \cite{7}. Due to the low oxygen in high CO\textsubscript{2} treatment, these \textit{DkERFs} were previously termed as hypoxia responsive \cite{8}. But, high CO\textsubscript{2} treatment is an atypical anoxia environment, with effects of both high CO\textsubscript{2} and low O\textsubscript{2}, thus it could be induced either a high-CO\textsubscript{2} or hypoxia response.

Apart from ERFs, some other transcription factors were reported as high-CO\textsubscript{2}/hypoxia responsive in persimmon fruit, such as \textit{DkMYB6} \cite{16} and \textit{DkTGA1} \cite{17}. NAC genes are the main transcription factors reported to be involved in the plant hypoxia response. In \textit{Arabidopsis}, more than 100 NAC genes have been characterized \cite{18} that share highly conserved consensus in the N-terminal region of a Petunia gene (NAM), \textit{Arabidopsis} ATAF1/2 and CUC2 proteins \cite{19}. Among these genes, hypoxia-responsive NAC genes have rarely been reported, and the results from studies on \textit{ANAC102} also indicate that additional NAC genes might exist for the hypoxia response, as \textit{ANAC102} knockout lines did not show altered \textit{ADH} gene transcription in \textit{Arabidopsis} \cite{9}. In persimmon, six NAC genes have been characterized, among which \textit{DkNAC1/3/5/6} were high-CO\textsubscript{2}/hypoxia responsive, however their regulatory roles in persimmon deastringency remain unclear \cite{20}. Thus, the potential role of NAC genes in regulating persimmon deastringency still lacks experimental evidence.

Here, a novel NAC transcription factor (\textit{DkNAC7}) was obtained as a result of yeast one hybrid screening by using the promoter of \textit{DkERF9} as bait and the regulatory role of \textit{DkNAC7} in persimmon de-astringency was investigated using yeast one-hybrid assay, dual-luciferase, real-time PCR and subcellular localization.

Materials and methods

Plant materials and treatment

‘Mopanshi’ (astringent cultivar) persimmon (\textit{D. kaki}) fruit were obtained from a commercial orchard at Fangshan (Beijing, China) in 2012. Fruit without disease or mechanical wounding were selected and treated with artificial high-CO\textsubscript{2} atmosphere (AHCA, 95% CO\textsubscript{2} and 1% O\textsubscript{2}) or air in air-tight containers for 1 d. The physiological data and sampling information were described in Wang et al. \cite{21}.

RNA extraction and cDNA synthesis

Total RNAs were extracted from frozen fruit flesh (2.0 g) and the cDNA synthesis carried out according to the method used previously \cite{6}.

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Abbreviations: AbA, aureobasidin A; AD, pGADT7; ADH, alcohol dehydrogenase; cv, Cultivar; ERF, ethylene response factor; PDC, pyruvate decarboxylase; SCT, soluble condensed tannins.
**Gene isolation and sequence analysis**

A NAC transcription factor was obtained based on the Matchmaker Gold Yeast One-hybrid Library Screening System (Clontech, USA), using the promoter of deastringency-related *DkERF9* [7,16] as the bait DNA sequence. The full-length NAC gene was isolated with a SMART RACE cDNA Amplification Kit (Clontech). The sequences of primers are described in Table 1. For phylogenetic analysis, the NAC genes in persimmon and methods were as described in Min et al [20].

**Yeast one-hybrid assay (Y1H)**

According to library screening results, the protein-DNA interaction was verified with DkNAC7-AD and DkERF9 promoter, individually. Meanwhile, interaction between DkNAC7 and DkPDC2 promoter was also investigated by Y1H. The promoter of DkERF9 was constructed into pAbAi vector (primers are listed in Table 1). The DkPDC2-pAbAi was constructed by Min et al. [8]. The full-length sequence of transcription factor DkNAC7 was subcloned into pGADT7 AD vector (AD) (primers are listed in Table 1). Auto-activation and the interaction analysis were conducted according to the manufacturer’s protocol.

**Dual luciferase assay**

Dual-luciferase assay was used as a rapid and efficient method to detect in vivo trans-activation or trans-repression effects of transcription factors [22]. Full-length *DkNAC7* was inserted into pGreen II 0029 62-SK vector (SK), using the primers listed in Table 1. The dual luciferase assay was carried out with *Nicotiana benthamiana* leaves, using the protocol described by Min et al. [7]. Three independent experiments (with minimum five replicates) were performed to verify the results.

| Table 1. Sequences of the primers used for RACE, full-length amplification, real time PCR and vector construction. |
| Gene | Methods used | Primers (5′-3′) |
|------|---------------|----------------|
| DkNAC7 | 3’RACE (Primary PCR) | CAAGCCTTCCTCAGATGCTGCTGAAT |
| DkNAC7 | 3’RACE (Secondary PCR) | GGAGACAAGAAGAGAGCACAGG |
| DkNAC7 | 5’RACE (Primary PCR) | CTCGTGATCCTCCATATCTCCAC |
| DkNAC7 | 5’RACE (Secondary PCR) | CTCGTGATCCTCCATATCTCCAC |
| DkNAC7 | Full-length clone (FP) | CATCGGCGGTGAACAAACG |
| DkNAC7 | Full-length clone (RP) | CACAAAATGCTCATCTCAGA |
| DkNAC7 | Y1H constructs (FP) | CGGAATTACATGGCCATGATCATGTC |
| DkNAC7 | Y1H constructs (RP) | CATGGAATCCTACCTGAGATCTTAC |
| DkNAC7 | SK vector construction (FP) | CGGCCGCGCGATGCTGATCACG |
| DkNAC7 | SK vector construction (RP) | GATGGATCATCAGATCAGATG |
| DkNAC7 | Q-PCR (FP) | TGAATTCCAAATTTGGAGT |
| DkNAC7 | Q-PCR (RP) | CCCTAGATCCTGAGATG |
| DkNAC7 | GFP vector construction (FP) | CGGCGATCACGATCGATCATGTC |
| DkNAC7 | GFP vector construction (RP) | CATGGATCATCAGATCAGATG |
| DkERF9 | pAbAi vector construction (FP) | CGCGGTAGCTACAGATGCAAG |
| DkERF9 | pAbAi vector construction (RP) | CGCGGTACATACAGATGCAAG |

Note: underlined sequences show cutting sites for restriction enzymes

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Real-time PCR analysis

For real-time PCR, gene specific oligonucleotide primers were designed and are shown in Table 1. The quality and specificity of primers were checked by melting curve and PCR products resequencing. The DkACT was chosen as a housekeeping gene to monitor the abundance of mRNA [7].

Real-time PCR reactions were carried out on a CFX96 instrument (Bio-Rad). The PCR protocols were according to our previous reports, using Ssofast EvaGreen Supermix Kit (Bio-Rad) [6]. The relative expression of this NAC gene was calibrated with values for day 0 fruit set as 1.

Subcellular localization analysis

35S-DkNAC7-GFP was transiently expressed in tobacco leaves by Agrobacterium-mediated infiltration (GV3101) according to previous reports [23,24]. The green fluorescent protein (GFP) fluorescence in tobacco leaves was imaged 2 d after infiltration using a Zeiss LSM710NLO confocal laser scanning microscope. Primers used for GFP construction are described in Table 1.

Statistical analysis

Least Significant Difference (LSD) test was used to compare the statistical significance differences among treatments by using DPS 7.05 or Student’s t-test. The figures were drawn with Origin 8.0.

Results and discussion

Y1H based library screening discovered a novel NAC gene, which targeted the DkERF9 promoter

In our previous reports, DkERF9 transcription factor was shown to be involved in persimmon de-astringency via regulation of the DkPDC2 promoter [7]. In order to obtain further information about the transcriptional regulatory mechanism controlling persimmon fruit deastringency, Y1H based screening was employed to screening the potential interacting transcription factors, using the DkERF9 promoter as bait. A total of 150 PCR products were obtained, among which only one NAC transcription factor gene was characterized. Individual verifications with Y1H indicated that the NAC transcription factor could bind to DkERF9 promoter (Fig 1). As

Fig 1. Protein-DNA interaction between DkNAC7 and the promoter of DkERF9 using yeast one hybrid analysis. Interaction was confirmed on SD medium lacking Leu in the presence of aureobasidin A (-Leu+AbA100). AD-Rec-p53 and p53-AbAi were used as a positive control; AD-empty and pDkERF9-AbAi were used as a negative control.

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six DkNAC genes were reported previously in persimmon fruit [20], this NAC transcription factor was named as DkNAC7 (GenBank no.MG792350) (Fig 1).

Phylogenetic tree analysis indicated that DkNAC7 was close to DkNAC4, but not the other five previously reported DkNAC genes (Fig 2) [20]. Compared to Arabidopsis NAC transcription factors.
factors, DkNAC7 was close to AtNAC078, which was reported to regulate flavonoid biosynthesis under high light in Arabidopsis [25], while it was not clustered with ANAC102, which was shown to be induced by low oxygen (0.1%) in Arabidopsis [9].

In vivo regulatory roles of DkNAC7 on deastringency related genes (DkERF, DkADH1 and DkPDC2)

Further investigations on the possible transcriptional regulatory linkage between DkNAC7 and deastringency related genes were carried out. Three previously studied DkERF genes (DkERF9/10/19) and two structural genes (DkADH1 and DkPDC2) were selected for test. Dual luciferase assay indicated that DkNAC7 could significantly trans-activate the promoters of DkERF9 and DkPDC2 with 1.55 and 1.92-fold enhancement, respectively (Fig 3). The effect of DkNAC7 on the DkERF10 promoter also reached statistical significance, but the response was very limited (about 1.32-fold) and the DkNAC7 gene had no significant effects on the promoters of DkADH1 and DkERF19 (Fig 3).

In persimmon, twenty-two ethylene response factors (DkERFs) were differently expressed in response to high CO₂ treatment. Of these 22 genes, only four ERFs (DkERF9/10/19/22) were fund to be involved in persimmon de-astringency [7,8], via the interaction with promoters of de-astringent related target genes (e.g. DkADH1, DkPDC2 and DkPDC3). Furthermore, a MYB transcription factor (DkMYB6) and a bZIP transcription factor (DkTGA1)
were also characterized as regulators of persimmon fruit astringency removal, respectively [16,17]. Thus, these finding with DkNAC7 unveiled a new transcription factor that participates in regulation of persimmon fruit deastringency. Furthermore, DkNAC7 is not closely related to the low oxygen-induced ANAC102 gene [9] from phylogenetic result (Fig 2) which suggests that more than one various type NAC transcription factor may contribute to the hypoxia response.

**Cascade regulations of DkNAC7-DkERF9-DkPDC2**

Comparing the effects DkNAC7 and the previously characterized TFs on the DkPDC2 promoter, DkNAC7 was shown to have only a relatively limited action, which was only slightly stronger than DkTGA1 [17]. Y1H analyses indicated that DkNAC7 cannot bind to and is therefore an indirect regulator for hypoxia responsive DkPDC2 (S1 Fig). As the present results indicated that interaction between DkNAC7 and DkERF9 promoter (Fig 1) and our previous study indicated that DkERF9 could physically bind to DkPDC2 promoter [17]. Thus, it could be proposed that a regulatory cascade involving DkNAC7-DkERF9-DkPDC2 contributes to persimmon fruit deastringency. The regulatory roles of NAC transcription factors in hierarchical interactions with ERFs have also been reported in other fruits, for instance MdNAC029/MdNAP, an apple NAC gene, was reported to directly repressed the expression of two ERF genes (MdCBF1 and MdCBF4) by binding to their promoters, thus negatively regulating cold tolerance via the CBF-dependent pathway [26]. These finding from persimmon not only partial explain the transcriptional regulations during deastringency, but also provided a new example of NAC-ERF regulation. Moreover, since the NAC-ERF cascade contributes to persimmon deastringency (high-CO\textsubscript{2}/hypoxia response) and apple cold tolerance, this raises the question whether other NAC-ERF may be involved in abiotic stress responses.

**Expression and subcellular localization analyses for DkNAC7**

The above-mentioned regulatory effects of DkNAC7 on deastringency related genes encouraged us to study the response of DkNAC7 to deastringency treatment. From previous results, AHCA treatment (also called CO\textsubscript{2} treatment or high CO\textsubscript{2} treatment: 95% CO\textsubscript{2} and 1% O\textsubscript{2}) was very effective in removing astringency in various persimmon [5,7,21]. Therefore, using previously described materials [21], the expression of DkNAC7 was analyzed. The DkNAC7 gene exhibited a sharp increase in expression in response to AHCA treatment, with the highest level at 1 d (Fig 4). After removal of CO\textsubscript{2} treatment, transcripts of DkNAC7 decreased concomitantly, but remained statistically significantly higher than in control fruits. Such expression was similar to most of the previously identified deastringency related transcription factors. Furthermore, subcellular localization analysis of DkNAC7 in tobacco leaves using GFP tagging, showed strong signals in the nucleus (Fig 5).

Taken all the results of Y1H, dual-luciferase assay, expression and subcellular localization together, we propose DkNAC7 as a novel regulator of persimmon fruit deastringency, acting via direct regulation of the DkERF9. Again, DkNAC7 was not closest homolog to the low oxygen-induced ANAC102 gene [9], indicating either potential differences between species or organs, or the complexity of NAC-regulatory roles. Another possible explanation would be the differences between experimental treatments, as in model plant or crops, anoxia treatments were generally low O\textsubscript{2}, but AHCA treatment in persimmon involves high CO\textsubscript{2} and low O\textsubscript{2}. Thus, the deastringency related DkNAC7, as well as the previously characterized transcription factors, could be termed as high-CO\textsubscript{2}/hypoxia responsive.
Fig 4. Expression of DkNAC7 in response to AHCA treatment (95% CO₂, 1% O₂, 1 day). Relative mRNA abundance was evaluated by real-time PCR. Day 0 fruit values were set as 1. Error bars represent ± SE from three replicates (\( p < 0.05; ** p < 0.001 \)).

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Fig 5. Subcellular localization of DkNAC7-GFP in tobacco leaves transformed by agroinfiltration. GFP fluorescence of DkNAC7 is indicated. Bars = 25 μm.

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Supporting information

S1 Fig. Yeast one-hybrid analysis of DkNAC7 binding to promoter of DkPDC2. (TIF)

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