Involvement of *DkTGA1* Transcription Factor in Anaerobic Response Leading to Persimmon Fruit Postharvest De-Astringency

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Abstract

Persimmon fruit are unique in accumulating proanthocyanidins (tannins) during development, which cause astringency in mature fruit. In 'Mopanshi' persimmon, astringency can be removed by treatment with 95% CO_2, which increases the concentrations of ethanol and acetaldehyde by glycolysis, and precipitates the soluble tannin. A TGA transcription factor, *DkTGA1*, belonging to the bZIP super family, was isolated from an RNA-seq database and real-time quantitative PCR indicated that *DkTGA1* was up-regulated by CO_2 treatment, in concert with the removal of astringency from persimmon fruit. Dual-luciferase assay revealed that *DkTGA1* had a small (less than 2-fold), but significant effect on the promoters of de-astringency-related genes *DkADH1*, *DkPDC2* and *DkPDC3*, which encode enzymes catalyzing formation of acetaldehyde and ethanol. A combination of *DkTGA1* and a second transcription factor, *DkERF9*, shown previously to be related to de-astringency, showed additive effects on the activation of the *DkPDC2* promoter. Yeast one-hybrid assay showed that DkERF9, but not DkTGA1, could bind to the *DkPDC2* promoter. Thus, although *DkTGA1* expression is positively associated with persimmon fruit de-astringency, trans-activation analyses with *DkPDC2* indicates it is likely to act by binding indirectly *DkPDC2* promoter, might with helps of DkERF9.

Introduction

Persimmon (*Diospyros kaki* L.) is a crop of high economic importance in China. There are two main types, either non-astringent or astringent [1]. However, most persimmon fruit have astringency at the commercial mature stage [2], caused by accumulation of soluble condensed tannins (proanthocyanidins, SCTs). Artificial postharvest treatments have been developed to

Abbreviations: ADH, alcohol dehydrogenase; ERF, ethylene response factor; PDC, pyruvate decarboxylase; SCT, soluble condensed tannins; AD, pGADT7; BD, pGBKT7; AbA, aureobasidin A; cv, Cultivar.

remove astringency, including high CO_2 (usually >90%), ethylene and ethanol [3–7]. CO_2 is the most effective treatment and depends on the production of acetaldehyde [6,8,9].

The biochemical basis of postharvest de-astringency in persimmon has been comprehensively investigated. The activities of pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase (ADH, EC 1.1.1.1) have been shown to increase during CO_2 treatment [6,10,11] leading to the formation of acetaldehyde and ethanol from pyruvate [12–14] and the acetaldehyde produced precipitates the soluble tannins. More recently, the genes involved in this process have been investigated. Five *PDC* genes and three *ADH* genes have been isolated from persimmon and their expression shown to be induced by the application of CO_2 [6]. Transient over-expression of *DkPDC2* rapidly decreased SCT in persimmon leaves [6], thus, *DkPDC2* is an important structural gene for persimmon fruit de-astringency. Five transcription factors were reported to be involved in persimmon de-astringency regulation, including four ethylene responsive factor genes (*DkERF9/10/19/22*) and a MYB transcription factor (*DkMYB6*) [6,7,15]. All five transcription factor could transcriptionally activated de-astringency related target genes, meanwhile *DkMYB6* could also enhance activity of the *DkERF19* promoter, which suggested possible transcriptional regulatory cascades involving different transcription factors. Thus, de-astringency is based on the induction under anaerobic conditions of genes leading to acetaldehyde production, but more information about the transcriptional regulation of these processes is still limited.

In *Arabidopsis*, at least four *ERF* genes, including *HRE1*, *HRE2*, *RAP2.2* and *RAP2.12* have been identified as the main plant oxygen-sensing regulators. HRE1 and RAP2.2 could transcriptionally regulate the anaerobic fermentation-related *ADH* and *PDC* genes [16,17], and lines containing T-DNA knockouts of *RAP2.2* had lower survival rates than the wild type in *Arabidopsis* [16]. Moreover, some other transcription factors have also been reported as hypoxia responsive, such as *AtMYB2*, which is induced by hypoxia, can bind to the promoter of *AtADH1* [18], and increased the expression of *AtADH1* when overexpressed [19]. Also, in wheat, *TaMYB1* has been shown to be responsive to low oxygen [20] and recent omics-based studies have highlighted more hypoxia responsive genes, and RNA-seq has been used to identify more than 180 transcription factors differentially expressed in response to low oxygen [21]. Thus, it is likely that more transcription factors would be expected to be involved in persimmon fruit postharvest de-astringency.

In the present research, a TGA-like transcription factor, *DkTGA1*, was identified, using data from the RNA-seq analysis performed by Min et al. [7]. The expression pattern of *DkTGA1* in response to the application of 95% CO_2 was studied by real-time PCR. The potential role(s) of *DkTGA1* in regulating previously characterized deastringency-related target genes were investigated, using dual-luciferase assay, testing synergistic interactions between *DkTGA1* and *DkERF* genes and yeast one hybrid analysis.

Materials and Methods

Plant materials and treatment

Commercial mature persimmon (*Diospyros kaki* L. cv. Mopanshi) fruit were harvested and bought from a commercial orchard (Yangjingliang's farm) at Fangshan (Beijing, China) in 2010. We confirm that the field studies did not involve endangered or protected species. Fruit were transported to the laboratory at Zhejiang University (Hangzhou, China) and then were selected with uniform size, free from visible mechanical wounds for postharvest treatments. Selected fruit were divided into two batches, one treated with 95% CO_2 for the de-astringency and the other (control) placed in air, with both batches held in air-tight plastic containers for 1 d. After treatments, the fruit were held in air at 20°C for 4 d. All of the treatments were

performed with three biological replicates. At each sampling point, fruit flesh samples were separately taken from three replicates of four fruit each. The samples were frozen in liquid nitrogen and stored at -80°C until further use.

Fruit physiology evaluation

In order to reflect fruit astringency, SCTs were measured using Folin-Ciocalteu reagent (Sigma) according to the method described by Yin et al. [5]. The results were expressed as tannin acids equivalents per g^{-1} fresh weight.

Acetaldehyde and ethanol production were measured with a gas chromatograph (Agilent 6890N, USA), fitted with a FID column (HP-INNOWAX, 0.25mm, 30m, 0.25µm, Agilent J&W, CA, USA). Measurements were conducted according to our previous report [6]. In brief, 2 g frozen flesh sample was ground in liquid nitrogen and added to 5 ml saturated NaCl. Three ml of the mixture were transferred to 10 ml air-tight vials with crimp-top caps, and were incubated at 60°C in a water-bath for 1 h. Then, 0.2 ml head-space gas was removed for gas chromatography. The injector, detector and oven temperatures were set at 150, 160 and 100°C, respectively. Sec-butyl alcohol (Sigma) was used as an internal standard. The results were calculated using standard curves for acetaldehyde and ethanol, respectively.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen fruit flesh (2.0 g) by the method developed by Chang et al. [22]. The trace amount of genomic DNA in total RNA was digested with TURBO DNA free kit (Ambion). First strand cDNA was synthesized 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 and subsequent cDNA synthesis.

Gene Isolation and Sequence Analysis

RNA-seq analysis was conducted with CO_2-treated persimmon fruit, which were described in Min et al. [7]. A TGA-like de-astringency responsive unigene was indicated by RNA-seq. The full-length *TGA* gene was isolated with a SMART RACE cDNA Amplification Kit (Clontech). The sequences of primers used for RACE and full-length amplification of *DkTGA1* are described in Table 1. Based on the deduced amino acid sequences, a phylogenetic tree of *TGA* genes was generated using Clustal X (v 1.81) and MEGA (v 6.0). The deduced of amino acid sequences of homologous genes in *Arabidopsis* were obtained from NCBI.

Real-time PCR analysis

Oligonucleotide primers for real-time PCR analysis were designed with primer premier 5.0. The specificity of primers was determined by melting curves and PCR products resequencing. The sequences of oligonucleotide primers are in Table 1. Primers used for *DkADH*, *DkPDC* and *DkERF* genes were the same as in previous reports [7].

Real-time PCR was carried out with LightCycler 480 SYBR Green I Master (Roche) and LightCycler 480 II instrument (Roche) for gene expression studies. The PCR reaction mixture (20 µl total volume) comprised 10 µl 2 × real-time PCR mix (Roche), 1 µl of each primer (10 µM), 2 µl diluted cDNA, and 6 µl Diethy pyrocarbonate (DEPC) H_2O. The PCR program was initiated for 3 min at 95°C, followed by 50 cycles of 95°C for 10 s, 55°C for 30 s, and completed with a melting curve analysis program. The relative expression of each gene was calibrated with values for day 0 fruit set as 1. The housekeeping gene, *Actin*, was used as the internal control [1].

Table 1. Sequences of the primers used for RACE, full-length amplification, real time PCR and vector construction

Genes	Methods used	Primers (5'-3')
DkTGA1	3'RACE (Primary PCR)	CAACAGGGTCTGTATATAGGTGGTGG
DkTGA1	3'RACE (Secondary PCR)	GGTCTGGATAAACTCCAGCAAACT
DkTGA1	5'RACE (Primary PCR)	GTAAGAGCATCTTCCAATTGCTGACA
DkTGA1	5'RACE (Secondary PCR)	TTGGTCAAACTTTTTTGAAGGTAGT
DkTGA1	Full-length clone (FP)	CTCAAAGGTCATATTGGAAACTCA
DkTGA1	Full-length clone (RP)	AGTTCCATCTAAAGGCACTCT
DkTGA1	Q-PCR (FP)	TCATCTTCGCAAGGAAACGC
DkTGA1	Q-PCR (RP)	CGACTAGGCAGGCTCGTGAA
DkTGA1	SK vector construction (FP)	CGCGTCGACATGACCTCTCCAACTGC
DkTGA1	SK vector construction (RP)	GACGGTACCCTAGGCAGGCTCGTGA
DkTGA1	Y1H constructs (FP)	CGCCATATGATGACCTCTCCAACTGC
DkTGA1	Y1H constructs (RP)	GACGGATCCCTAGGCAGGCTCGTGA
DkERF9	Y1H constructs (FP)	CATGAATTCATGGTTGGCTTTGTGAA
DkERF9	Y1H constructs (RP)	GCAGGATCCTCATCCAGCAGCTTCA

Note: underlined sequences show cutting sites for restriction enzymes.

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Dual Luciferase assay

The trans-activation ability of *DkTGA1* on de-astringency related genes was investigated by dual luciferase assay. Full-length *DkTGA1* was inserted into pGreen II 002962-SK vector, using the primers listed in Table 1. Full-length *DkERF* and *DkADH* and *DkPDC* promoter constructs were obtained from our previous studies [6,7]. All constructs were electroporated into *Agrobacterium tumefaciens* GV3101. The dual luciferase assay was performed with *Nicotiana benthamiana* leaves, using the protocol described by Min et al. [6,7]. Dual luciferase assays were carried out in three independent experiments, with five biological replicates for each experiment.

Yeast One-hybrid Assay (Y1H)

The yeast one-hybrid assays were performed, using the Matchmaker Gold Yeast One-hybrid Library Screening System (Clontech, USA). As described in Min et al. [7], *DkADH1* and *DkPDC3* showed auto-activation activities, thus only *DkPDC2* promoter was included for yeast one-hybrid analysis. The full-lengths of *DkERF9* and *DkTGA1* were subcloned into pGADT7 AD vector (AD) (primers are listed in Table 1). Transformed Y1HGold were cultured on SD/-Leu containing 200 ng/ml aureobasidin A at 30°C for 3 d to test interaction. pGADT7-Rec (AD-Rec-P53) was co-transformed with the p53-promoter fragment in Y1HGold as positive control.

Statistical Analysis

Statistical significance of differences was calculated using least significant difference (LSD) with DPS software (v.3.11).

Results

Gene isolation and sequence analysis

One hypoxia responsive *TGA*-like unigene, which showed a 25-fold induction by CO_2 treatment, was identified by RPKM (Reads per kb per million reads) from RNA-seq (data not shown) and

the full-length sequence was obtained using RACE. Sequence analysis indicated that this differentially expressed gene had a `YCQRLRALSMLW` domain ([Fig 1a](#)), which is a characteristic of *TGA* genes [23], and was designated as *DkTGA1* (KU589287).

A phylogenetic tree was generated from the deduced amino acid sequences of the *DkTGA1* and 10 *TGA* genes from *Arabidopsis thaliana*, which have been divided into five clades [24,25]. *DkTGA1* showed the highest homology to clade I that comprises *AtTGA1* (At5g65210) and *AtTGA4* (At5g10030) ([Fig 1b](#)).

Association between *DkTGA1* mRNA accumulation and persimmon fruit postharvest de-astringency

CO_2 treatment (95%) promoted persimmon fruit de-astringency and caused a rapid decrease in the concentration of soluble tannin from 1.214% at 0 d to 0.232% at 1 d while in the control fruit the soluble tannin content was almost constant during storage ([Fig 2](#)). Accumulation of acetaldehyde and ethanol, the products of anaerobic respiration, were observed in CO_2-treated fruit, concomitantly with loss of astringency-related SCT. Acetaldehyde increased from 6.26 µg/g at 0 d to 32.70 µg/g after CO_2 treatment while the ethanol increased from 10.18 µg/g to 72.56 µg/g ([Fig 2](#)). Following removal of CO_2 treatment, acetaldehyde content rapidly declined to the basal level observed at 0 d, while the decrease in ethanol content occurred more slowly than acetaldehyde, and remained higher than that in control fruit ([Fig 2](#)).

Transcripts of *DkTGA1* was transiently induced during CO_2 treatment, peaking after 1 d, with an approximate 50-fold induction at 1 d, and then decreased to similar level of that in control fruit following removal of CO_2 ([Fig 3](#)). All of the previously isolated de-astringency related genes, *DkADH1*, *DkPDC2*, *DkPDC3*, *DkERF9*, *DkERF10*, *DkERF19* and *DkERF22*, were up-regulated by CO_2 treatment ([S1 Fig](#)), which confirmed previous observations [6,7].
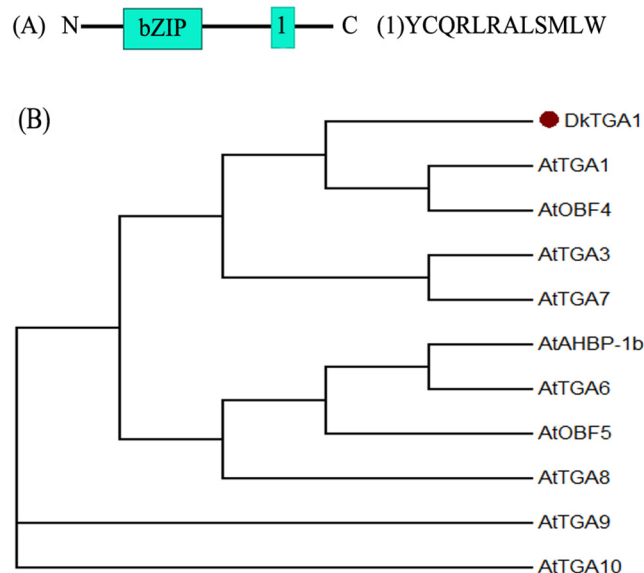

Fig 1. Phylogenetic tree of *TGA*. (A) Schematic analysis of *DkTGA1*. The bZIP domain and TGA related motif are indicated in blue. (B) Persimmon *DkTGA* is indicated with a red dot. The amino acid sequences of AtTGA transcription factors were obtained from The Arabidopsis Information Resource or National Center for Biotechnology Information. The phylogenetic tree was constructed with MEGA (v 6.0)
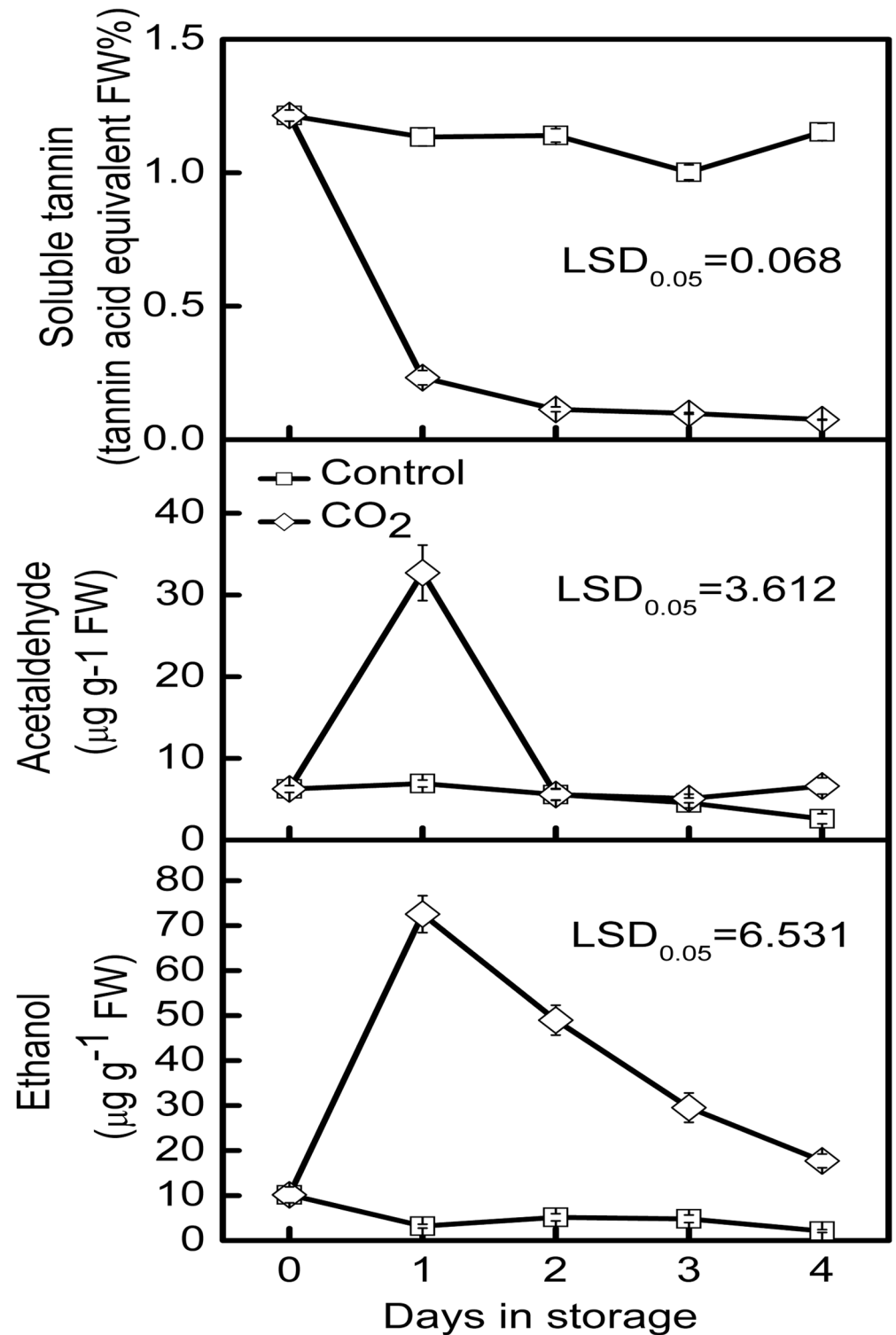
doi:10.1371/journal.pone.0155916.g001

Fig 2. Changes of soluble tannins, acetaldehyde and ethanol in 'Mopanshi' persimmon fruit in response to treatment with 95% CO_2 for 1 day. Error bars represent ±SE from three replicates. LSDs represent least significant difference at $p = 0.05$.

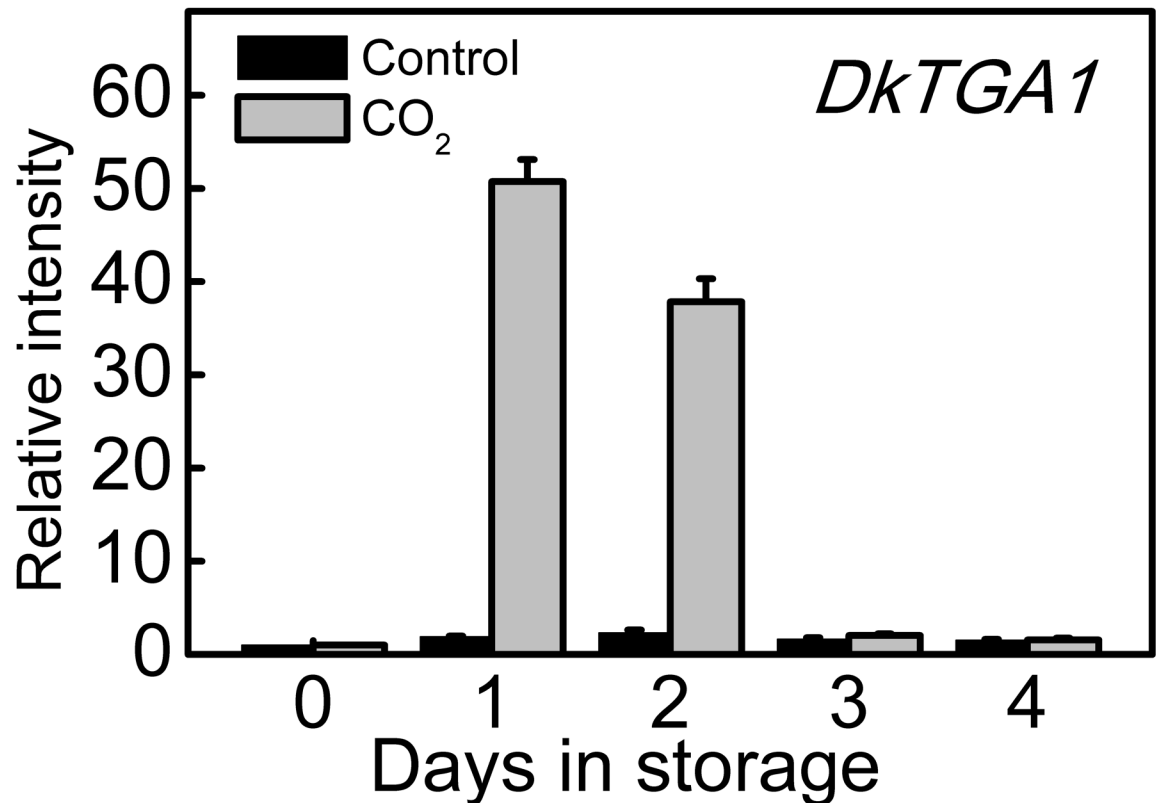doi:10.1371/journal.pone.0155916.g002

Fig 3. Expression of *DkTGA1* in response to CO₂ treatment (95%, 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. LSDs represent least significant differences at $p = 0.05$.

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Trans-activation of persimmon *DkADH1*, *DkPDC2* and *DkPDC3* promoters by DkTGA1

A dual luciferase assay was carried out to investigate the possible transcriptional regulatory linkage between DkTGA1 and target genes known to be involved in astringency removal. The results indicated that *DkTGA1* had a small (but statistically significant) effect on genes induced by anaerobic conditions and involved in the de-astringency process, including *DkADH1*, *DkPDC2* and *DkPDC3*, with an approximately 1.5-fold activation of the promoters of these genes (Fig 4).

Synergistic effect of *DkTGA1* and *DkERF* on trans-activation of promoters of de-astringency-related target genes

It was shown previously that DkERF9 and DkERF19 could activate the *DkPDC2* promoter while DkERF10 activated the *DkADH1* promoter [6,7]. As shown in Fig 4, DkTGA1 could trans-activate the *DkADH1*, *DkPDC2* and *DkPDC3* promoters. Thus, further investigations were conducted using *DkERF9*, *DkERF10* and *DkERF19* promoters, however, the results indicated that DkTGA1 also had very limited effects on promoters of these *DkERF* genes (S2 Fig).

Subsequently, the effect of combining DkTGA1 and DkERF transcription factors was analyzed. DkTGA1 and DkERF9 together significantly enhanced promoter activation, compared
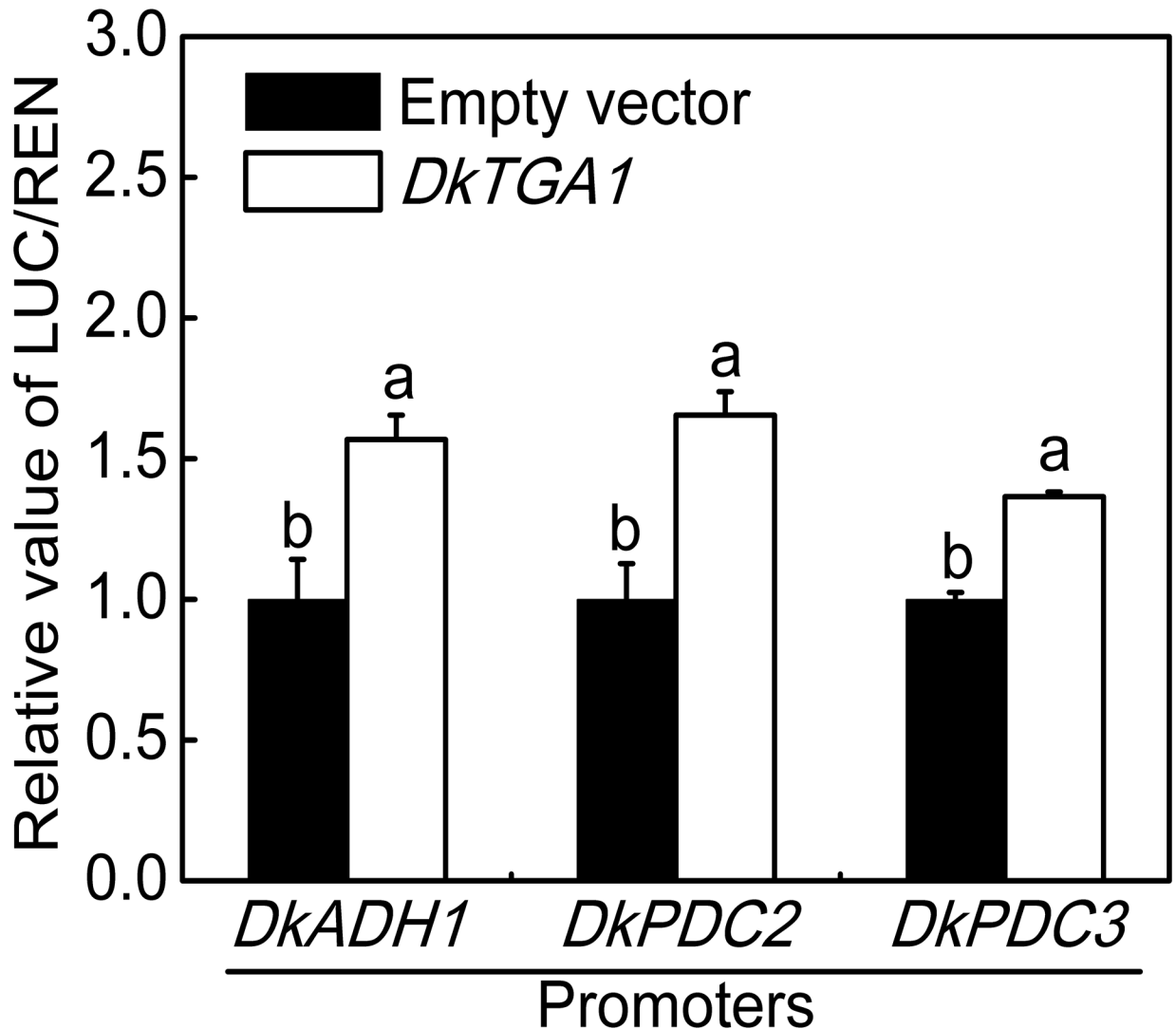
Fig 4. Trans-activation effects of *DkTGA1* on the promoters of deastringency-related genes (*DkADH1*, *DkPDC2*, *DkPDC3*) using the dual-luciferase assay. The value of LUC/REN for the empty vector (SK) was set as 1. Error bars indicate S.E.s from five replicates. Different letters above the columns indicate significant differences ($P < 0.05$)

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with the effects of DkTGA1 or DkERF9 singly (Fig 5). However, no such enhancement was found when DkTGA1 was tested in combination with DkERF10, DkERF19 or DkERF22 (Fig 5).

Yeast one/two-hybrid assays were performed in view of the results obtained from the dual luciferase assay. Motif analysis indicated the existing of the potential ERF and TGA binding sites (Fig 6A). Yeast one-hybrid results indicated that DkERF9 could bind to *DkPDC2* promoter, whereas no physical interaction was found between DkTGA1 and the *DkPDC2* promoter (Fig 6B). However, yeast two-hybrid studies indicated that there was no direct protein-protein interaction between DkTGA1 and DkERF9 (S3 Fig), despite the additive effects observed between DkTGA1 and DkERF9 on expression from the *DkPDC2* promoter.
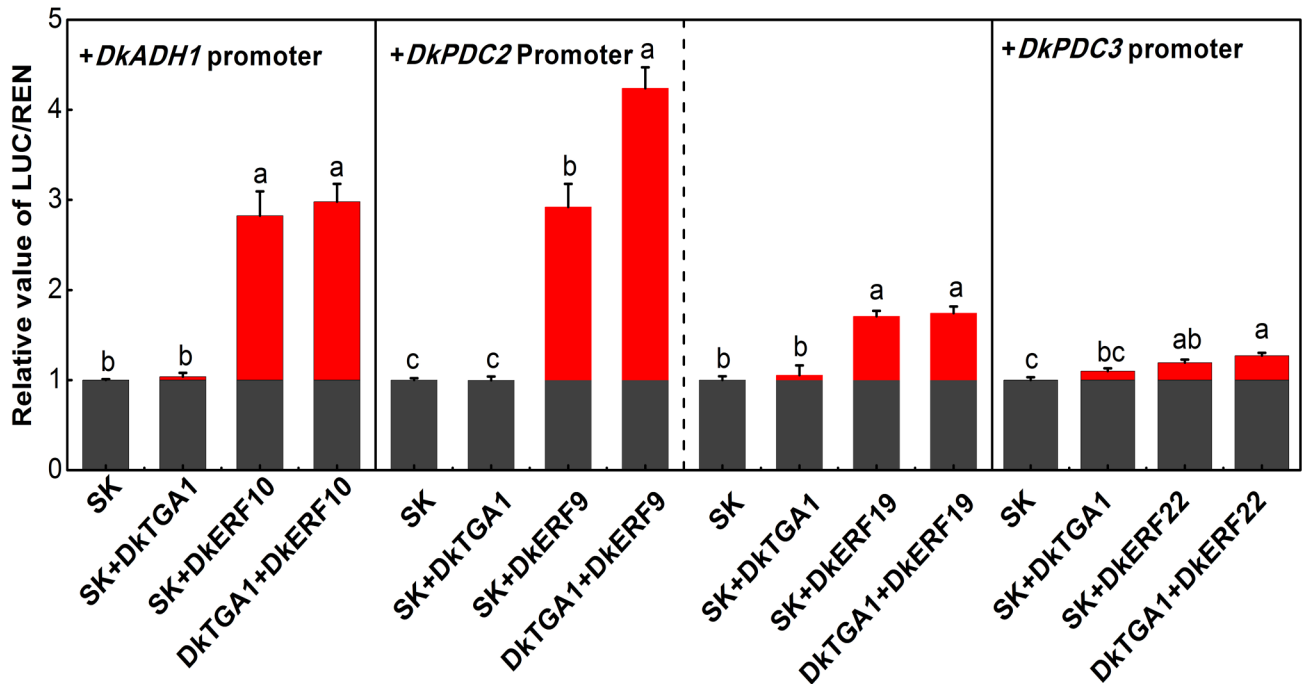
Fig 5. Synergistic effect of DkTGA1 and DkERF on trans-activation of the promoters of deastringency-related genes (*DkADH1*, *DkPDC2*, *DkPDC3*) using the dual-luciferase assay.

Discussion

Persimmon astringency removal had been widely studied and several technologies had been applied to reduce the SCTs in persimmon fruit, including treatment with high concentrations of CO_2 or N_2 [4,6,7,12,26], C_2H_4 treatment [5], dipping in hot water [27], all of which can lead
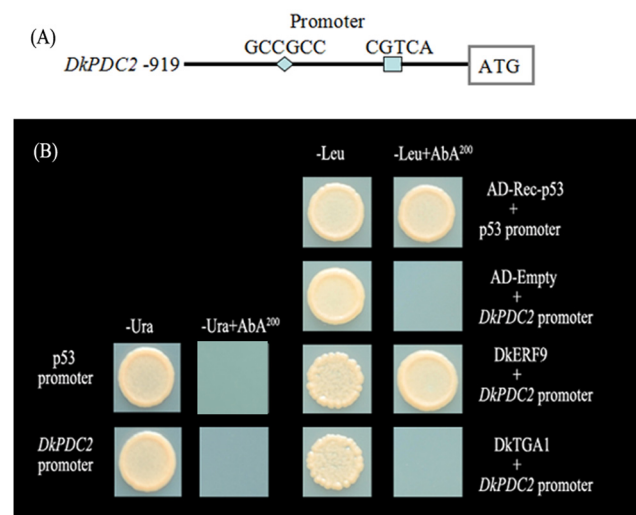

Fig 6. Yeast one-hybrid analysis of DkTGA1 and DkERF9 binding to promoter of *DkPDC2*. (A) Schematic representation of *DkERF* (GCCGCC) and *DkTGA* (CGTCA) possible binding sites in *DkPDC2* promoter. (B) Auto-activation of promoters were tested on SD medium lacking Ura in presence of aureobasidin A (AbA). Interaction was determined on SD medium lacking Leu in presence of AbA.

to the persimmon fruit astringency removal. The effectiveness of CO_2 treatment is widely considered to be due to the hypoxia response, which stimulates acetaldehyde production in persimmon fruit by anaerobic respiration, which precipitates the insoluble tannins, thereby removing astringency [6,15,28]. The results obtained in the present research are entirely consistent with this, as a rapid decrease in SCTs and transient stimulation of acetaldehyde and ethanol were observed in 'Mopanshi' persimmon in response to 95% CO_2 treatment.

A few transcription factors have been suggested to be involved in the de-astringency response in persimmon fruit, however, only five transcription factors have been verified as having trans-activation ability on promoters of de-astringent related target genes (eg. *DkADH1*, *DkPDC2* and *DkPDC3*). These include four *DkERF* genes (*DkERF9/10/19/22*) [6,7] and a MYB transcription factor (*DkMYB6*) [15]. Here, a de-astringency responsive bZip transcription factor, detected in an RNA-seq database and designated as *DkTGA1*, based on phylogenetic comparisons with related genes from other plants. TGA is a member of a sub-class of bZIP transcription factors that have been widely reported to be involved in stress responses, for instance, *AtTGA1-7* has been characterized with respect to their interaction with *pathogenesis-related gene1* (*NPR1*) [29]; *AtTGA1* and *AtTGA4* also played important roles in Arabidopsis root developmental responses to nitrate [30]. *DkTGA1* was clustered with *AtTGA1* and *AtTGA4*, suggesting *DkTGA1* may also participate in response to stress in persimmon fruit. Although no TGA genes have been reported as being responsive to low oxygen in other plants, the present results indicate a significant (50-fold) induction of *DkTGA1* expression in response to high CO_2/low O_2 treatment ([Fig 3](#)). This suggests that the involvement of TGA genes in the anoxia response in other plants should be investigated. *DkTGA1* had a small transactivation effect on the promoters of *DkADH1*, *DkPDC2* and *DkPDC3*, which further indicated its possible involvement in persimmon de-astringency and the hypoxia response. However, the effects of *DkTGA1* on de-astringency related target genes were weaker than our previously reported results for other transcription factors such as *DkERF9/10/19/22* and *DkMYB6* [6,7,15]. It is notable that increasing expression of *DkTGA1* only occurred at 1 d and 2 d, which is similar to *DkERF9/10/19/22* [6,7] and differ to *DkMYB6* [15]. As deastringent process for 'Mopanshi' fruit in response to high CO_2 treatment, generally happened on 1 d or 2 d, thus expression patterns of *DkTGA1*, as well as *DkERF9/10/19/22*, suggested that these transcription factors might be more specific for deastringency.

It is worth emphasizing that the synergistic effects of DkTGA1 and DkERF9 were observed on *DkPDC2* promoter. Synergistic effects of different transcription factors have been widely reported, for example MaNAC1 is involved in cold tolerance of banana fruits, and interacts with MaCBF1 [31] and EjAP2-1 interacts with EjMYB1/2 to form a complex involved in loquat fruit lignification [32]. Moreover, other TGAs and ERFs have been shown to interact with each other, for instance, AtTGA4 interacts with AtEBP (ethylene-responsive element binding protein, the previous name for *ERF*), which binds the ethylene response promoter elements and may therefore be important in regulating gene expression during the plant defense response [33]. However, additive effects of DkTGA1 and DkERF9 were not due to direct protein-protein interaction between, as Y2H experiments indicated no interaction between two transcription factors. Thus, synergistic effects of DkTGA1 and DkERF9 not only raised a possible involvement of a new transcription factor(s) in persimmon fruit de-astringency, but also provided a new evidence for relationships, with unknown mechanisms, between members of the TGA and ERF families, different from those previously reported. It is clear that DkERF9 can physically interact with the *DkPDC2* promoter, while *DkTGA1* is not able to bind directly to the promoter. These results suggest that *DkTGA1* is a novel and indirect regulator of persimmon fruit postharvest de-astringency, and may function via indirect interaction with DkERF9, a direct

regulator on *DkPDC2*. As persimmon fruit deastringency related transcription factors are still limited, future researches might bridge the gap between *DkTGA1* and deastringency.

Conclusion

In conclusion, CO_2 (95%) treatment is effective in promoting and accelerating persimmon fruit postharvest de-astringency. The present study isolated a de-astringency inducible *DkTGA1*, which is an indirect activator of anaerobic response target genes. Furthermore, DkTGA1 and DkERF9 were shown to have an additive effect on activation of *DkPDC2*. As CO_2-driven persimmon fruit postharvest de-astringency is mainly based on fermentation under anoxia, the activity of *DkTGA1* suggests the likely involvement of *TGA* transcription factors in hypoxia responses in other plants.

Supporting Information

S1 Fig. Expression of previously characterized deastringency related *DkERF*, *DkADH* and *DkPDC* genes in response to CO_2 treatment.
(TIF)

S2 Fig. Extent of trans-activation of the *DkERF* promoters by DkTGA1, using the dual-luciferase assay.
(TIF)

S3 Fig. Yeast two-hybrid analysis of interaction of DkTGA1 and DkERF9.
(TIF)

Author Contributions

Conceived and designed the experiments: QGZ XRY. Performed the experiments: QGZ MMW ZYG FF. Analyzed the data: QGZ XRY XL DG KSC. Contributed reagents/materials/analysis tools: QGZ MMW ZYG NJS. Wrote the paper: QGZ XRY DG KSC.

References

1. Akagi T, Ikegami A, Tsujimoto T, Kobayashi S, Sato A, Kono A, et al. DkMyb4 is a Myb transcription factor involved in proanthocyanidin biosynthesis in persimmon fruit. Plant Physiol. 2009a; 4, 2028–2045.

2. Akagi T, Ikegami A, Suzuki Y, Yoshida J, Yamada M, Sato A, et al. Expression balances of structural genes in shikimate and flavonoid biosynthesis cause a difference in proanthocyanidin accumulation in persimmon (*Diospyros kaki* Thunb.) fruit. Planta 2009b; 230, 899–915.

3. Ikegami A, Eguchi S, Kitajima A, Inoue K, Yonemori K. Identification of genes involved in proanthocyanidin biosynthesis of persimmon (*Diospyros kaki*) fruit. Plant Sci. 2007; 172, 1037–1047.

4. Salvador A, Arnal L, Besada C, Larrea V, Quiles A, Pérez-Munuera I. Physiological and structural changes during ripening and deastringency treatment of persimmon fruit cv. 'Rojo Brillante'. Postharvest Biol Technol. 2007; 46, 181–188.

5. Yin XR, Shi YN, Min T, Luo ZR, Yao YC, Xu Q, et al. Expression of ethylene response genes during persimmon fruit astringency removal. Planta 2012; 235, 895–906. doi: 10.1007/s00425-011-1553-2 PMID: 22101946

6. Min T, Yin XR, Shi YN, Luo ZR, Yao YC, Grierson D, et al. Ethylene-responsive transcription factors interact with promoters of ADH and PDC involved in persimmon (*Diospyros kaki*) fruit de-astringency. J Exp Bot. 2012; 63, 6393–6405. doi: 10.1093/jxb/ers296 PMID: 23095993

7. Min T, Fang F, Ge H, Shi YN, Luo ZR, Yao YC, et al. Two novel anoxia-induced ethylene response factors that interact with promoters of deastringency-related genes from persimmon. PLOS One 2014; 9, 97043.

8. Taira S, Ikeda K, Ohkawa K. Comparison of insolubility of tannins induced by acetaldehyde vapor in fruit of three types of astringent persimmon. J Jpn Soc Hortic Sci. 2001; 48, 684–687.

9.   Pesis E, Ben-Arie R. Involvement of acetaldehyde and ethanol accumulation during induced deastringency of persimmon fruits. J Food Sci. 2006; 49, 896–899.

10. Tamura F, Tanabe K, Itai A, Hasegawa M. Characteristics of acetaldehyde accumulation and removal of astringency with ethanol and carbon dioxide treatments in 'Saijo' persimmon fruit. J Jpn Soc Hortic Sci. 1999; 68, 1178–1183.

11. Liu CP, Zheng ZM, Liang YN, Li B. Relationship between activity of alcohol dehydrogenase and soluble tannin in persimmon. Acta Hortic Sin. 2008; 35, 741–746. (In Chinese with English abstract)

12. Yamada M, Taira S, Ohtsuki M, Sato A, Iwanami H, Yakushiji H, et al. Varietal differences in the ease of astringency removal by carbon dioxide gas and ethanol vapor treatments among Oriental astringent persimmons of Japanese and Chinese origin. Sci Hortic. 2002; 94: 63–72.

13. Pesis E. The role of the anaerobic metabolites, acetaldehyde and ethanol, in fruit ripening, enhancement of fruit quality and fruit deterioration. Postharvest Biol Technol. 2005; 37, 1–19.

14. Botondi R, Russo V, Mencarelli F. Anaerobic metabolism during short and long term storage of kiwifruit. Postharvest Biol Technol. 2012; 64, 83–90.

15. Fang F, Wang MM, Zhu QG, Min T, Grierson D, Yin XR, et al. *DkMYB6* is involved in persimmon fruit deastringency, via transcriptional activation on both *DkPDC* and *DkERF*. Postharvest Biol Technol. 2016; 111, 161–167.

16. Hinz M, Wilson IW, Yang J, Buerstenbinder K, Llewellyn D, Dennis ES, et al. Arabidopsis *RAP2.2*: an ethylene response transcription factor that is important for hypoxia survival. Plant Physiol. 2010; 153, 757–772. doi: 10.1104/pp.110.155077 PMID: 20357136

17. Yang CY, Hsu FC, Li JP, Wang NN, Shih MC. The AP2/ERF transcription factor AtERF73/HRE1 modulates ethylene responses during hypoxia in Arabidopsis. Plant Physiol. 2011; 156, 202–212. doi: 10.1104/pp.111.172486 PMID: 21398256

18. Hoeren FU, Dolferus R, Wu YR, Peacock WJ, Dennis ES. Evidence for a role for AtMYB2 in the induction of the Arabidopsis alcohol dehydrogenase gene (ADH1) by low oxygen. Genetics 1998; 149, 479–490. PMID: 9611167

19. Abe H, Urao T, Ito T, Seki M. Arabidopsis AtMYB2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 2003; 15, 63–78. PMID: 12509522

20. Lee TG, Jang CS, Kim JY, Kim DS, Park JH, Kim DY, et al. A Myb transcription factor (TaMyb1) from wheat roots is expressed during hypoxia: roles in response to the oxygen concentration in root environment and abiotic stresses. Physiol Plantarum 2007; 129, 375–385.

21. Licausi F. Regulation of the molecular response to oxygen limitations in plants. New Phytol. 2011; 190, 550–555. PMID: 21091695

22. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep. 1993; 11, 113–116.

23. Schindler U, Beckmann H, Cashmore AR. TGA1 and G-box binding factors: two distinct classes of arabidopsis leucine zipper proteins compete for the G-box like element TGACGTGG. Plant Cell 1992; 4, 1309–1319. PMID: 1446171

24. Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, et al. bZIP transcription factors in *Arabidopsis*. Trends Plant Sci. 2002; 7, 106–111. PMID: 11906833

25. Gatz C. From pioneers to team players: TGA transcription factors provide a molecular link between different stress pathways. Mol Plant Microbe In. 2013; 26, 151–159.

26. Arnal L, Del Río MA. Removing astringency by carbon dioxide and nitrogen-enriched atmospheres in persimmon fruit cv.'Rojo Brillante'. J Food Sci. 2003; 68, 1516–1518.

27. Kitagawa H. Studies on the removal of the astringency and storage of Kaki (Oriental persimmons). 5. A relation between removal of the astringency and acetaldehyde formation during the warm water treatment for removal of the astringency. J Jpn Soc Hortic Sci. 1969; 37, 379–382. (in Japanese with English abstract).

28. Del Bubba M, Giordani E, Pippucci L, Cincinelli A, Checchini L, Galvan P. Changes in tannins, ascorbic acid and sugar content in astringent persimmons during on-tree growth and ripening and in response to different postharvest treatments. J Food Compos Anal. 2009; 22, 668–677.

29. Kesarwani M, Yoo J, Dong XN. Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol. 2007; 144, 336–346. PMID: 17369431

30. Alvarez JM, Riveras E, Vidal EA, Gras DE, Contreras-Lopez O, Tamayo KP, et al. Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of *Arabidopsis thaliana* roots. Plant J. 2014; 80, 1–13. PMID: 25039575

31. Shan W, Kuang JF, Lu WJ, Chen JY. Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. Plant Cell Environ. 2014; 37, 2116–2127. PMID: 24548087

32. Zeng JK, Li X, Xu Q, Chen JY, Yin XR, Ferguson IB, et al. EjAP2-1, An *AP2/ERF* gene, is a novel regulator of fruit lignification induced by chilling injury, via interaction with *EjMYB* transcription factors. Plant Biotechnol J. 2015; 13, 1325–1334. PMID: 25778106

33. Butiner M, Singh KB. *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. P Natl Acad Sci USA 1997; 94, 5961–5966.