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Cloning of 9-*cis*-epoxycarotenoid dioxygenase (NCED) gene encoding a key enzyme during abscisic acid (ABA) biosynthesis and ABA-regulated ethylene production in detached young persimmon calyx

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Unlike the typical climacteric fruits, persimmons (*Diospyros kaki* Thunb.) produce higher levels of ethylene when they are detached from trees at a younger stage. In order to obtain detailed information on the role of abscisic acid (ABA) in ripening, we cloned the *DKNCED1*, *DKACS2*, and *DKACO1* genes from the calyx. Water loss was first noted in the calyx lobe, and *DKNCED1* was highly expressed 1 d after the fruits were detached, coinciding with an increase in the ABA content. Then, the *DKACS2* and *DKACO1* genes were expressed after some delay. In the calyx, the ABA peak was observed 2 d after the fruits were harvested, and this peak preceded the ethylene peak observed on day 3. The fruit firmness rapidly decreased on day 4, and the fruits softened completely 6 d after they were harvested. The increases in the expressions of ABA, ethylene, and the genes in the calyxes occurred earlier than the corresponding increases in the pulp, although the 3 increases occurred on different days. Exogenous ABA treatment increased ABA concentration, induced expression of both *ACS* and *ACO*, and promoted ethylene synthesis and young-fruit softening; by contrast, treatment with NDGA inhibited the gene expressions and ethylene synthesis and delayed young-fruit softening. These results indicate that ethylene biosynthesis in the detached young persimmon fruits is initially triggered by ABA, which is induced by water loss in the calyx, through the induction of *DKACS2* and *DKACO1* expressions. The ethylene produced in the calyx subsequently diffuses into the pulp tissue, where it induces autocatalytic ethylene biosynthesis, resulting in an abrupt increase in ethylene production.

persimmon fruit, *DKNCED1* gene, ABA, ethylene, calyx, water loss

Fruits have been classified as climacteric and non-climacteric based on their patterns of respiration and ethylene production during maturation and ripening[1]. Persimmon (*Diospyros kaki* Thunb.) fruits are classified as climacteric because they produce ethylene during ripening and induce ripening with autocatalytic ethylene production by exogenously applied ethylene[2–4]. However, unlike other climacteric fruit species, ethylene production in persimmons is substantially greater in fruits harvested at younger stages[5].

It was reported recently that ethylene biosynthesis in the detached young persimmon fruit is initially induced in calyx and is modulated by water loss through transcriptional activation of *DK-ACS2*[6]. Ethylene produced in the calyx subsequently diffuses to other fruit tissues and acts as a secondary signal, which stimulates auto catalytic ethylene biosynthesis in these tissues, leading to a burst of ethylene production[6]. However, the intrin

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sic relation between water-loss in calyx of young fruit and ethylene biosynthesis in fruits and its regulation mechanism are still unclear.

Recent research shows that biologic stress (such as fruit maturation, senescence, seed germination) or a biological stress (such as phytohormone, heavy metal, water stress, chilling damage and high carbon dioxide concentration) affects ethylene biosynthesis[7,8]. Fruits on trees can obtain water continuously. Once detached, they will lose the water supply because of water-loss by respiration. Therefore, detached fruits would suffer from water-loss stress and synthesizes ethylene[6]. We guess that ABA takes part in the process of young fruit ethylene biosynthesis as mentioned above, because ABA not only plays a certain role in many physiological processes during plant growth, but also regulates plant responses to stresses, such as drought, salt alkali and low temperature.

So far, biochemical and genetic studies have suggested that 9-*cis*-epoxycarotenoid dioxygenase (NCED) is the key enzyme in the ABA biosynthetic pathway in plants. In higher plants, ABA derives from C_{40}-*cis*-epoxycarotenoids, either 9′-*cis*-neoxanthin or 9′-*cis*-violaxanthin or both, which are cleaved by the 9′-*cis*-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin, the direct C_{15} precursor of ABA[9–11]. Since its first isolation from the maize vp14 mutant, the *NCED* gene has been cloned and characterized in various plant species, including beans[12], cowpeas[13], avocadoes[14], *Arabidopsis*[9] and citrus[15]. However, the previous studies focused on the mechanisms of drought resistance and signal transduction of the NCED gene[11,16]. Its expression in fruits has only been studied in avocadoes[14], citrus, tomato and peach[17–21], but there is no evidence to show that ABA plays a crucial role either in climacteric or non-climacteric fruits. In this study, we cloned the *NCED* gene from calyx of young persimmon fruits and analyzed the expression of *NCED* genes, action of ABA, and their relation with the mechanism of ethylene action during the young fruit softening process.

1 Materials and methods

1.1 Plant materials

Eight-year-old persimmon (*Diospyros kaki* Thunb. cv. Outanenashi) trees were grown in a persimmon yard at the campus of China Agricultural University (Beijing, China). Flowers on 5 trees were tagged at the date of anthesis. Persimmon fruits at different developmental stages were randomly collected during 60—80 days after anthesis. Ten fruits of uniform size were selected at each sampling time (one replication). The fruits used for determination of ABA content, ethylene production and fruit firmness were cut into small cubes of 0.5—0.8 cm^3 and were quickly frozen in liquid N_2 and stored at −80℃ before RNA extraction.

1.2 RNA extraction, RT-PCR and sequencing

Total RNA was extracted from 10 g flesh or 5 g calyx using the hot borate method[22]. Poly(A)$^+$ RNA was purified using Oligo $(dT)_{30}$-latex (TaKaRa, Kyoto, Japan) following the manufacturer's protocol. Synthesis of the first strand cDNA from the purified poly(A)$^+$ RNA was conducted using Marathon^{TM} cDNA Amplification Kit (ClonTech Co.). The cDNAs were used as a template for amplifying NCED with degenerate primers (forward: 5′-TTYGAYGGIGAY GGIATGGTICA-3′; reverse: 5′-TCCCAIGCRTTCCAI AR RTGRAA-3′) designed from conserved sequences of plant NCEDs (GenBank accession Nos. AF224671, Z97215, DQ028471, DQ028472, AY337613). PCR was performed under the following conditions: 94℃ for 3 min, followed by 30 cycles of 94℃ for 1 min, 50℃ for 2 min, and 72℃ for 3 min, and with the final reaction being terminated at 72℃ for an additional 10 min. The PCR products were ligated into a pGEM-T easy vector (Tiangen, Beijing, China) and subsequently transformed into *E. coli* DH5α. Positive colonies were selected, amplified and then sequenced by Invitrogene^{TM} (Shanghai, China). Sequences encoding plant NCEDs were determined by a homology search of the NCBI databases using the BLAST program. Furthermore, using the same method as mentioned above, we obtained aim genes from persimmon fruits of which the primers are *DK-ACS* (1-aminocyclop ropane-1-carboxylic acid synthase) (Forward, 5′-ATGGGI YYIGCIGARAAYCAGYTI-3′ and reverse, 5′-AAAIA CICKRAACCAICCIGGYTC-3′) and *DK-ACO* (Forward, 5′-TCATGAAGGATTTTGCTGAAAGGTT-3′ and reverse, 5′-TTCAGGGCAGCATACAG-CTTCATG-3′) designed from conserved sequences of persimmon plants.

1.3 Evaluation of the water loss and the water loss rate in detached young fruits at ambient laboratory humidity (40%—50% RH, 20℃)

In order to evaluate the water loss of fruits, 100 fruits harvested (65 days after full bloom) were immediately

marked and weighed with every single fruit. Then the fruits were divided into 10 groups including 10 fruits in each group, and placed in the laboratory of 20℃, at 40%—50% relative humidity (RH). The whole fruits, the pulp and the calyxes (calyx disk and calyx lobe) of group 1 were weighed separately. Then, a group of fruits, the pulps and the calyxes were selected and weighed every day, after the whole fruit was weighed. The pulps and calyxes used for determination of ABA content and ethylene production were cut into small cubes of 0.5—0.8 cm^2, and quickly frozen in liquid nitrogen and stored at –80℃ before RNA extraction. Furthermore, stomata in the fruit surface, the calyx disk and the calyx lobe were observed and the stomata density was calculated. An about 0.5—0.8 cm^2 square area was cut at different parts of the fruits (midsection, top, shoulder and suture of fruit) by hand sectioning, and the tore epidermis was checked in the 400× ordinary optical microscope to observe the stomata number (Stomata density = stomata number/area of epidermis).

1.4 The change of the firmness, ABA, ethylene and the related gene expression in detached young fruits under ambient laboratory humidity (40%—50% RH, 20℃) during spontaneous fruit softening

Seventy young fruits were harvested 70 d fter anthesis and then divided into 10 groups with 10 fruits in each group (5 fruits were used for control). These fruits were placed in the laboratory of 20℃, 40%—50% relative humidity (RH). Fruit firmness, ABA content, ethylene and degree of *DKNCED1* expression in the calyx or fruit were determined 0, 1, 2, 3, 4, 5 and 6 days after harvest. One group of fruits was used every day. Firstly whole fruits, then the flesh and the calyx were tested for analysis of ethylene, ABA and firmness, respectively. Each sample included 10 fruits. Some samples were quickly frozen in liquid nitrogen and stored at –80℃ before RNA extraction. Control fruits were treated with packaging film (with tiny holes in film) also placed in the laboratory of 20℃, 40%—50% RH.

1.5 Effects of exogenous ABA and NDGA treatment on ABA content, ethylene production and gene expression of the *DKNCED1*, *DKACS2, DKACO1* in detached young fruits

In order to evaluate the effect of exogenous ABA or the nordihydroguaiaretic acid (NDGA) (ABA synthetic inhibitor) treatments, 105 young fruits were harvested at the young stage (77 d after full bloom and held at 20℃ at ambient laboratory humidity (40%—50% RH). The fruits were divided into 3 groups and used for the following treatments: ABA (100 μmol/L, group 1), NDGA (100 μmol/L, group 2), and control (CK, distilled water, group 3). Groups 1, 2 and 3 were then vacuum infiltrated into 100 μmol/L each of ABA and NDGA solutions or water for 10 min at room temperature. The all fruits treated above were held at 20℃ and ambient laboratory humidity (40%—50% RH). Three replications were conducted for each treatment. Fruits were sampled every day after treatment for measurement of ABA content, ethylene and fruit firmness. Some samples were quickly frozen in liquid nitrogen and stored at –80℃ before RNA extraction.

1.6 Probe preparation and Northern hybridization

DIG-labeled probes were synthesized using a PCR DIG probe synthesis Kit (Roche Diagnostics). DIG-labeled probes were amplified using pGEM-T Easy plasmids containing the cDNA fragments obtained from the RT-PCR as templates and T7 and SP6 primers corresponding to vector sequences adjoining the multiple cloning sites. For Northern analysis, gene probes of the cDNAs were amplified using plasmids. Aliquots of total RNA (5 μg) were separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 mol/L formaldehyde and blotted onto nylon membranes (Hybond N^+, Amersham biosciences UK). The filters were then hybridized with the DIG-labeled DNA probes in high SDS buffer (7% (w/v) SDS, 5× SSC, 50 mmol/L sodium phosphate, pH 7.0, 2% (w/v) bloking reagent, and 0.1% N-lauroylsarcosine) containing 50% (v/v) formamide overnight at 42℃. After hybridization, filters were washed twice at 37℃ in 2× SSC and 0.1% (w/v) SDS for 15 min and twice at 55℃ in 0.1× SSC and 0.1% (w/v) SDS for 30 min. The membranes were then subjected to immunological detection according to the manufacturer's instructions using CDP-Star™ as a chemiluminescent substrate for alkaline phosphatase (Roche Diagnostics).

1.7 Determination of ABA content

For ABA extraction, 1 g flesh or 0.5 g calyx was ground in a mortar and homogenized in the extraction solution (80% methanol, v/v). Extracts were centrifuged at 10000× *g* for 20 min. The supernatant liquid was eluted

through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) to remove polar compounds, and then stored at –20℃ for enzyme-linked immunosorbent assay (ELISA). The ELISA procedures were conducted according to the instruction provided by the manufacturer (China Agricultural University, Beijing, China). ABA was determined by Thermo Electron (labsystems) Multiskan MK3 (PIONEER Co., China).

1.8 Determination of ethylene production

The rate of ethylene produced by whole fruits was measured by enclosing 5 fruits or 3 g seeds in 1.5 L airtight containers for 1 h at 20℃ by withdrawing (sampling) 1 mL of the headspace gas and injecting it into a gas chromatograph (model Agilent, 6890N, USA) fitted with a flame ionization detector and an activated alumina column. The unit of ethylene production is $nL \cdot g^{-1}FW \cdot h^{-1}$. The measurement condition is as follows: Chromatograph column: HP-5 5% Phenyl Methyl Siloxane, 30 m capillary alumina column (Agilent 19091J-413). The temperature of the column and the detector was 80℃ and 150℃, respectively. The carrier gas flow rate was 40 mL/min N^2 and hydrogen pressure was 0.6 kg/cm^2.

1.9 Fruit firmness measurement

Flesh firmness was measured after the removal of fruit skin on three sides of each fruit using a KM model fruit hardness tester (FUJIHARA Co., Japan). The strength of flesh firmness was recorded in kilogram per square centimeters (kg/cm^2).

2 Results

2.1 The cloning and sequencing of the *NCED* gene in the calyx of persimmon fruit

We obtained one 740-bp-fragment of NCED cDNA (*DKNCED1*) from the calyxes of young persimmon fruits (Figure 1). A BLAST homology search revealed that the DNA sequence of *DKNCED1* (GenBank accession number: EU925812) showed 74.19% identity with the *PpNCED1* sequence (EF625684) from peaches, 75.07% identity with the *VVNCED1* sequence (GenBank accession number: EF625685) from grapes, and 72.57% identity with the *LeNCED1* sequence (GenBank accession number: Z97215) from tomatoes (located at 540—1280 bp of the *LeNCED1* sequence). We also cloned an ACS cDNA fragment (*DK-ACS2-like,* 983 bp) and an

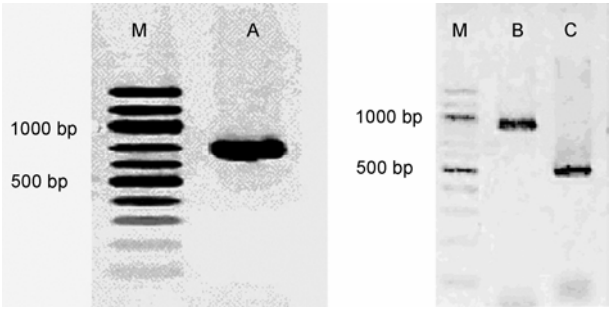

Figure 1 RT-PCR amplification of *DKNCED1* (GenBank accession number, EU925812), *DKACS2* and *DKACO1* genes from persimmon (*Diospyros kaki* Thunb. cv. Outanenashi) calyx. M, DNA marker; A, *DKNCED1*; B, *DKACS2*; C, *DKACO1*.

ACO oxidase gene (*DK-ACO1-like,* 520 bp); the former showed 99% homology with the *DK-ACS2* sequence (GenBank accession number: AB073006), and the latter showed 98% homology with the *DK-ACO1* sequence (GenBank accession number: AB073008), which is located at 400—920 bp of *DK-ACO1*. For the Northern blot analysis, we prepared a DNA probe using these 3 fragments.

2.2 The water-loss route and the dehydration rate in the detached young fruits incubated at room temperature and ambient laboratory humidity (40%—50% RH)

We observed that the pericarp surface of the persimmon fruits showed a thick stratum corneum and wax, but it did not show any stoma. The stomas on the calyx lobe were similar to those on the leaves. The stoma densities on the face and the reverse of the calyx lobe were significantly different (1.70/mm^2 and 5.62/mm^2, respectively), and there were no stomas on the calyx disk, except for some floss. These results suggested that water evaporation from the persimmon fruit occurs mainly through the stoma on the calyx lobe. As shown in Figure 2, the calyxes of the detached young persimmon fruits showed rapid water loss at room temperature and ambient laboratory humidity (40%—50% RH). Considering the water content in a freshly detached calyx as 100%, the dehydration rate was 16.7% 1 day after harvest, 35.7% 2 days after harvest, 36.9% 3 days after harvest, 45.1% 4 days after harvest, and 48.5% 5 days after harvest. The leaves had dried up 5 days after harvest. By contrast, the fruit lost little water until 5 days after harvest. This result proved that after the harvest, water loss in the calyx occurs earlier than that in the fruit, and the calyx is the part responsible for evaporative water loss in detached young fruits.

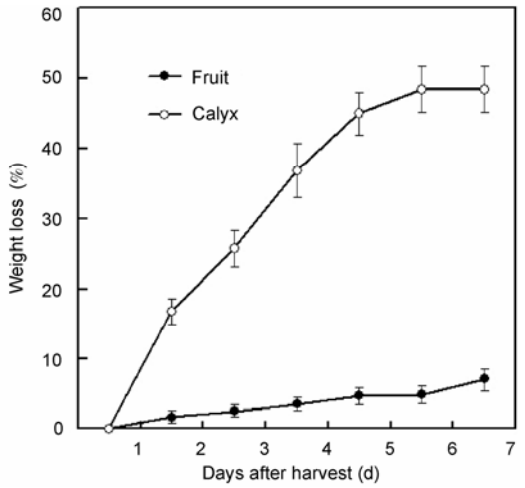

Figure 2 The changes in the water-loss rate in the calyx and the pulp of detached young persimmon fruits (65 d after full bloom) at room temperature (20℃) and ambient humidity (40%—50% RH). A total of 100 fruits were marked and weighed immediately after harvesting. Then, the fruits were divided into 10 groups with 10 fruits in each group, and they were placed in the laboratory at 20℃ and 40%—50% relative humidity. The whole fruits, the pulps, and the calyxes (calyx disk and calyx lobe) were separately weighed. Then, a group of fruits, the pulps, and the calyxes were selected and weighed every day after the whole fruit was weighed.

2.3 Changes in the levels of ABA, ethylene and gene expression in the detached young fruits incubated at room temperature and ambient laboratory humidity (40%—50% RH)

The peak level of ABA in the calyx was observed on the second day, and the peak level of ABA in the pulp was observed on the third day; the increases in the ABA contents of both calyx and pulp occurred earlier than the increases in ethylene production (Figure 3). Fruit firmness decreased rapidly from day 4 after harvest, the calyx lobe exfoliated on day 5 after harvest, and the control fruits showed softening.

2.4 Effects of exogenous ABA and NDGA treatments on the ABA and ethylene levels in detached young fruits

When the fruits were treated with exogenous ABA, the ABA content increased rapidly. The peak ABA level in the calyx was observed 1 d earlier than that in the pulp, and the ABA contents in both calyx and pulp were detected 1 day before ethylene production was detected. However, NDGA treatment inhibited ABA accumulation

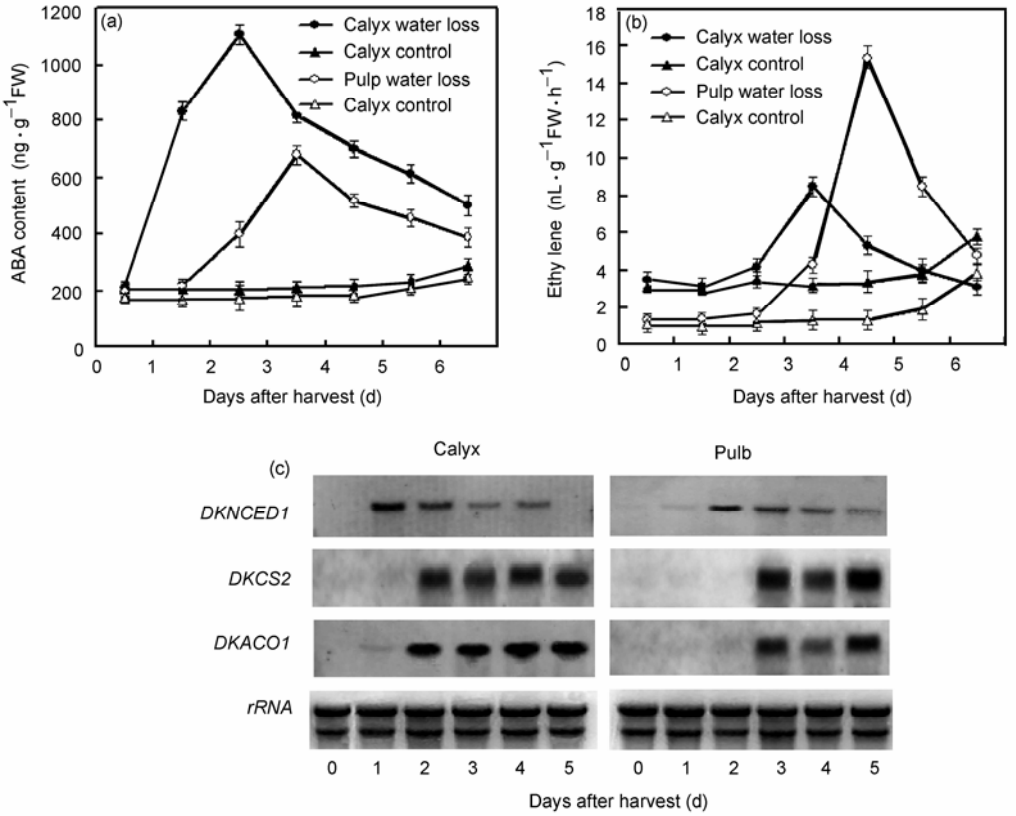

Figure 3 The ABA content (a), ethylene production (b) and the related gene expression (c) in both calyx and pulp of young persimmon fruits. Fruits were harvested at the young stage (65 d AFB) and incubated at 20℃ and ambient humidity (40%—50% RH). The control fruits were treated with packaging film and incubated at 20℃ and 40%—50% RH.

in both calyx and pulp (Figure 4). NDGA treatment significantly blocked ABA accumulation, decreased ethylene production in both calyx and pulp, and inhibited fruit softening. The experimental results indicated that ABA induced ethylene synthesis and initiated fruit softening.

2.5 Expression of the *DKNCED1*, *DK-ACS2* and *DK-ACO1* genes in the calyxes after treatments with exogenous ABA or NDGA at room temperature and ambient laboratory humidity (40%—50% RH)

Northern blotting analysis of the calyxes revealed that the *DKNCED1* gene was expressed at low levels 0 d after harvesting, and the expression peaked on day 1—2, while *DKACS2* and *DKACO1* were expressed 2—4 d after harvesting (Figure 5). NDGA treatment suppressed and reduced the expressions of *DKACS2* and *DKACO1*, while ABA treatment promoted the expressions of these genes. The ABA and NDGA treatments did not have any effect on the expression of the *DKNCED1* gene. These observations indicate that ABA may trigger ethylene production in the detached young persimmon fruits by inducing the expressions of the *ACC synthase* gene (*DKACS2*) and the *ACC oxidase* gene (*DKACO1*).

3 Discussion

In this study, we cloned a cDNA of the *NCED* gene (*DKNCED1*) from the calyxes of persimmon fruits, and we also analyzed the expressions of the *DKNCED1* gene and the ethylene-associated genes (*DKACS2, DKACO1*) in the detached young fruits. The results show that when the ABA content increased, the expression of the *DKNCED1* gene was induced by water loss in both the pulp and the calyx; at high concentrations of ABA, *DKNCED1* gene expression preceded the expression of the *DKACS2* and *LeACO1* genes. Therefore, it was proposed that *DKNCED1* expression initiated ABA biosynthesis in the dehydrating calyx and acted as an original inducer for fruit maturation. By contrast, ethylene biosynthesis during the climacteric stage has been demonstrated to be caused by the accumulation of transcripts of the *ACC synthase* and *ACC oxidase* genes[6].

Moreover, some studies have suggested that the onset of ripening in fruits is controlled by an ethylene-independent regulator that exists upstream of the ethylene biosynthesis region. Exogenous ABA treatment may induce expressions of *DKACS2* and *DKACO1* (Figures 4 and 5) and initiate the softening process in detached

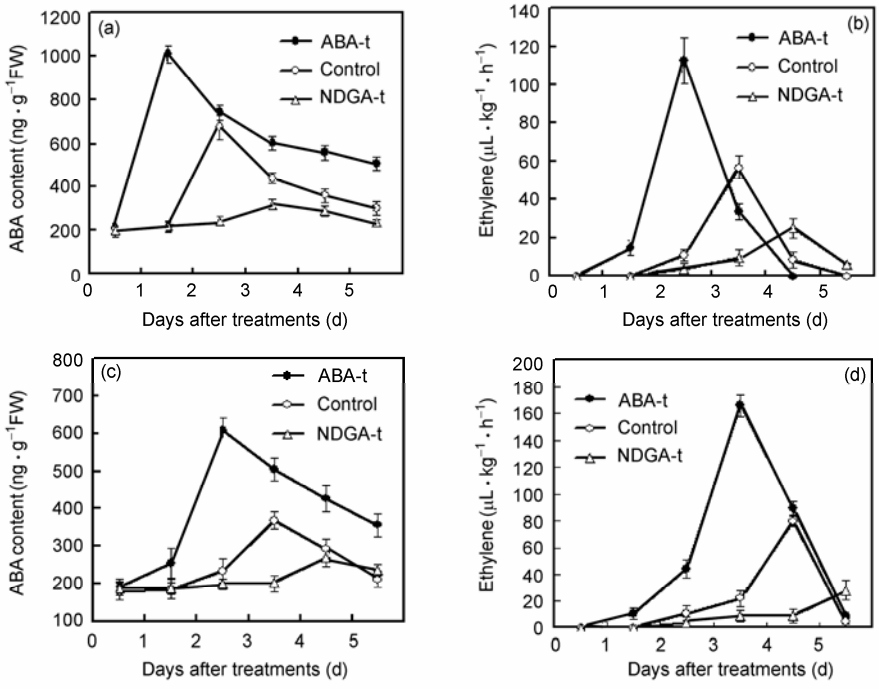

Figure 4 The changes in ABA content and ethylene production in detached young persimmon fruits. (a) and (b) Calyx; (c) and (d) pulp; (a) and (c) ABA content; (b) and (d) ethylene production. The fruits were harvested at a young stage (70 d AFB), and then, they were divided into 3 groups and used for the following treatments: ABA (100 μmol/L, group 1), NDGA (100 μmol/L, group 2), and control (CK, distilled water, group 3). Then, the fruits of groups 1, 2 and 3 were vacuum infiltrated into water or 100 μmol/L solutions of ABA or NDGA for 10 min at room temperature. These treated fruits were incubated at 20℃ and ambient laboratory humidity (40%—50% RH)

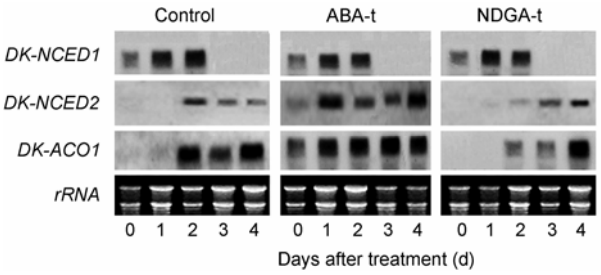

Figure 5 Northern-blot analysis of the expression of *DKNCED1*, *DKACS2* and *DKACO1* genes in the calyxes of young persimmon fruits (70 d AFB) treated with 100 μmol/L of NDGA or 100 μmol/L of ABA during storage at ambient laboratory humidity (RH, 40%−50%; 20℃). Each lane contained 5 μg of total RNA, and the transcript levels of rRNA are shown as an internal loading control.

young fruits (Figures 4 and 5), suggesting that ABA may act as an upstream regulator before ethylene production in fruit ripening and softening processes.

In the present study, we synthesized 3 probes (*DK-NCED1*, *DK-ACS2* and *DK-ACO1*) and used these probes in Northern blotting analyses for analyzing the interaction between endogenous ABA accumulation, ethylene biosynthesis, and fruit softening in detached young persimmon fruits. We found that the persimmon peel has a thick cuticle and wax layer but no stomas; the calyx lobes had massive stomas, and the calyx disk did not have any stomas but showed tomenta on the surface. The dehydration rate of the persimmon fruits reduced after the calyxes were sealed by wax, indicating that the calyx is the principal route for water loss in detached young persimmon fruits, which is similar to the findings of a previous report[6]. The *DKNCED1* expression was induced under water stress and initiated ABA biosynthesis (Figures 4 and 5). In the calyxes of detached young persimmon fruits, ABA accumulation reached a peak value and decreased rapidly without a stable plateau. Theoretically, after ABA accumulation reaches the peak value, constant expression of the *NCED* gene is responsible for maintaining a constantly high level of ABA accumulation. In addition, the other synthase genes and enzymes involved in ABA biosynthesis are also responsible for ABA accumulation. Therefore, the constant accumulation of ABA depends on sufficient supply of the ABA precursors. The undetached and detached fruits showed different patterns of water-stress-induced ABA accumulation, because the synthesis pathways of the ABA precursors were different. While undetached fruits received ABA precursors from the mother plant, detached fruits did not receive any supply of precursors and could not maintain constant ABA accumulation. Therefore, in the detached fruits, water-stress-induced ABA accumulation declined rapidly after reaching the peak value. In grape fruits, the absolute content of endogenous ABA is not the key factor for triggering fruit maturation, since fruit maturation is also related to the content and activity of the signal receptor of the hormone. Low ABA content can induce physiological reactions. Our results indicated that in the calyxes of persimmon fruits, the water-stress-induced ABA was sufficient for initiating physiological reactions related to the softening of young persimmon fruits, and the ABA production was rapidly followed by softening of the fruits. In addition, ABA shows prolonged and multipronged effects on the development and maturation of persimmon fruits.

Persimmon is a special respiration climacteric fruit. In comparison with other fruits, the calyx lobe of persimmon has a bigger size, and higher chlorophyll content, and shows a higher photosynthetic capacity than the leaves. Moreover, there are a number of stomas on the calyx lobe of persimmon fruits, and these stomas serve to regulate gas exchange. The CO_2-exchange rates in persimmon fruits reduced significantly and fruit ripening was inhibited after the calyx lobe was excised and the wounds were sealed by Vaseline. In addition, we found that the calyx lobe was more sensitive to environmental stresses such as high CO_2 content and salt stress (date not shown) than the other parts of the fruits were.

In this work, NDGA was used to specifically inhibit ABA biosynthesis. The results showed that detached young persimmon fruits were sensitive to NDGA, which is a suitable ABA inhibitor for studying the physiological function of ABA during fruit development and maturation. The results of the Northern blot analysis (Figures 4 and 5) show that the expression of *DKNCED1* was not influenced by NDGA or exogenous ABA treatment, in comparison with the expression in the control fruits. These results indicate that NDGA blocks ABA accumulation by directly inhibiting NCED enzyme activity.

NDGA is a useful tool for studying fruit maturation and senescence, because it effectively inhibits ABA biosynthesis and ABA-mediated physiological effects. NDGA significantly blocked ABA activity, and it also blocked ethylene production by inhibiting the expression of the ACC gene (Figures 4 and 5). The results indicated that ethylene biosynthesis in detached young persimmon

fruits was regulated by ABA, and the expression of the *ACC synthase* gene was positively regulated by ABA.

In this study, *DK-ACS2* gene expression and ethylene production in both calyx and pulp were inhibited by NDGA treatment (Figure 5). These results indicated that in calyxes subjected to water stress, ethylene biosynthesis was regulated by ABA and not by ethylene. ABA may regulate the ripening and senescence of young persimmon fruits via 2 pathways: (i) ABA may initiate ripening and senescence of fruits by acting upstream of ethylene biosynthesis and inducing some biosynthetic activities by inhibiting/inducing system-2 ethylene production; (ii) ABA may initiate ripening and senescence of fruits by inducing the expression of ethylene-independent genes. Our results indicate that the ABA and ethylene-regulation mechanics in the detached persimmon fruits shows interactions that accelerate fruit softening by regulating the expression of ethylene-biosynthesis-enzyme genes and ethylene production.

In addition, to investigate the involvement of water loss in the production of ABA and ethylene in young persimmon fruits, we harvested fruits at a young stage (70 d AFB) and packed these fruits individually in polyethylene films. The packaged fruit and the nonpackaged control fruits were incubated at room temperature and ambient laboratory humidity (40%—50% RH), and the weight loss, ABA content, and ethylene production were monitored daily. The packaged fruits did not soften after 3 weeks of incubation at room temperature (the control fruits softened after 5 d), but they showed abnormal firming that might have been caused by oxygen deficit. Further physiological studies are required. Most persimmon varieties, such as "Mopanshi" Persimmon, show some of the above-mentioned characteristics of young persimmon fruits, such as production of fruit-softening-inducing ethylene under water stress, even if the fruits are harvested at the optimum maturity stage. This is one of the principal reasons for the low market share of persimmon fruits in China. Presently, we are studying the packaging of persimmon fruits by using breathable films that can reduce water losses and extend the shelf lives of persimmons.

In conclusion, we proposed a model in which the *DKNCED1* gene initiates ABA biosynthesis in the dehydrating calyxes of detached young persimmon fruits: (i) ABA may induce ethylene biosynthesis via the regulation of *ACS* and *ACO* gene expressions; (ii) The produced ethylene diffuses into the pulp of the fruit, where autocatalytic ethylene biosynthesis is reduced and results in an abrupt increase in ethylene production and fruit softening.

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