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The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit

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Abstract

In order to understand more details about the role of abscisic acid (ABA) in fruit ripening and senescence of tomato, two cDNAs (*LeNCED1* and *LeNCED2*) which encode 9-*cis*-epoxycarotenoid dioxygenase (NCED) as a key enzyme in ABA biosynthesis, two cDNAs (*LeACS2* and *LeACS4*) which encode 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and one cDNA (*LeACO1*) which encodes ACC oxidase involved in ethylene biosynthesis were cloned from tomato fruit using a reverse transcription-PCR (RT-PCR) approach. The relationship between ABA and ethylene during ripening was also investigated. Among six sampling times in tomato fruits, the *LeNCED1* gene was highly expressed only at the breaker stage when the ABA content becomes high. After this, the *LeACS2*, *LeACS4*, and *LeACO1* genes were expressed with some delay. The change in pattern of ACO activity was in accordance with ethylene production reaching its peak at the pink stage. The maximum ABA content preceded ethylene production in both the seeds and the flesh. The peak value of ABA, ACC, and ACC oxidase activity, and ethylene production all started to increase earlier in seeds than in flesh tissues, although they occurred at different ripening stages. Exogenous ABA treatment increased the ABA content in both flesh and seed, inducing the expression of both ACS and ACO genes, and promoting ethylene synthesis and fruit ripening, while treatment with fluridone or nordihydroguaiaretic acid (NDGA) inhibited them, delaying fruit ripening and softening. Based on the results obtained in this study, it was concluded that *LeNCED1* initiates ABA biosynthesis at the onset of fruit ripening, and might act as an original inducer, and ABA accumulation might play a key role in the regulation of ripeness and senescence of tomato fruit.

Key words: ABA, ethylene, fruit ripening, *LeACS2*, *LeACO1*, NCED gene, tomato.

Introduction

The plant hormone abscisic acid (ABA) not only plays a regulatory role in plant growth and development, in seed dormancy, and in the adaptation of plants to stress conditions, but also displays a pattern of change similar to ethylene at late stages of fruit development. Some scholars considered that ABA had a crucial role, perhaps even more crucial role than that of ethylene, in fruit maturation and senescence (Giovannoni, 2001, 2004; Rodrigo *et al.*, 2003). To date, the ripening mechanism of climacteric fruit, especially the effect of ethylene, has been well studied (Alexander and Grierson, 2002). However, the mechanism involved in the ripening of non-climacteric fruits remains

unclear, despite similar research efforts being made. The two types of fruits exhibit the same ripening phenomena in terms of colour and texture, with only a difference in ethylene production. Therefore, it is assumed that an upstream gene for ethylene expression should exist in controlling fruit ripening of both fruit types.

On the other hand, tomato has proved to be an excellent model system for the analysis of fruit ripening and development, in part due to the availability of well characterized ripening mutants. The ripening inhibitor (*rin*) and non-ripening (*nor*) mutants are deficient in climacteric respiration and the associated burst peak in ethylene

biosynthesis. Application of exogenous ethylene does not promote fruit ripening, yet it does induce expression of ethylene-regulated ripening genes (Tigchelaar *et al.*, 1978; Yen *et al.*, 1995), which indicated that in addition to general ethylene biosynthesis and signalling, upstream of ethylene there may be other ethylene-independent regulatory factors for climacteric fruit maturation. ABA can be considered as the other ripening control factor, because the ABA content is very low in unripe fruit but increases during the process of fruit ripening in both climacteric (Vendrell and Buesa, 1989; Buesa *et al.*, 1994) and non-climacteric (Inaba *et al.*, 1976; Coombe, 1989; Kojima, 1996; Kondo, 1997, 1998) fruits. At present, ABA deficiency has been thoroughly studied in mutants in various plant species (Taylor *et al.*, 2000; Galpaz *et al.*, 2008). Recently, it was reported (Galpaz *et al.*, 2008) that the ABA content in ABA-deficient mutants is 75% lower than the normal level. Because of this, both the plant and fruit did not show the normal growth observed in the wild type; the total fruit weight and average fruit weight in ABA-deficient mutant fruits were reduced compared with wild-type fruit, and the plant weight was 50% lower in the ABA-deficient plant than in the wild type, indicating that ABA was not only required for plant growth, but was also indispensable for fruit development and ripening. The degreening stage of ABA-deficient orange mutants started later than that of the wild type (Rodrigo *et al.*, 2003), and the sensitivity to ethylene of ABA-deficient orange mutants was also weaker than that of the wild type (Alferez and Zacarias, 1999), which indicated that plants could not grow normally if there was insufficient ABA available.

There is now biochemical and genetic evidence showing that 9-*cis*-epoxycarotenoid dioxygenase (NCED) is the key enzyme of the ABA biosynthesis pathway in higher plants (Tan *et al.*, 1997; Burbidge *et al.*, 1999; Iuchi *et al.*, 2001). Since its was first isolated from the maize *vp14* mutant (Tan *et al.*, 1997), the NCED gene has been cloned and characterized in various plant species including bean (Qin and Zeevaart, 1999), cowpea (Iuchi *et al.*, 2000), *Arabidopsis* (Iuchi *et al.*, 2001), and citrus (Rodrigo *et al.*, 2006). However, previous studies were focused on the mechanisms of drought resistance and signal transduction of the NCED gene (Thompson *et al.*, 2000; Iuchi, 2001; Nambara and Marion-Poll, 2005). Its expression in fruits has only been studied in avocados (Chernys and Zeevaart, 2000) and citrus (Rodrigo *et al.*, 2006). In this study, the NCED gene from tomato fruit was cloned and the expression of the NCED gene, the function of ABA, and their relationship with the mechanism of ethylene action during fruit ripening were analysed.

Materials and methods

Plant material

Tomato [*Solanum lycopersicum* (formerly *Lycopersicon esculentum*) cv. Alisa Craig] plants were grown in a greenhouse under standard culture conditions [25/20 °C, relative humidity (RH) 70%]. About 1000 flowers on 30 tomato plants were tagged at the date of anthesis. During the development and ripening stage, tomato fruits were collected on the following days (stages) after anthesis: 30 d (immature green), 40 d (mature green), 44 d (breaker 1), 45 d (breaker 2), 47 d (turning), 50 d (pink), 53 d (mature red), and 57 d (over-ripe). Twenty fruits of uniform size were selected at each sampling time (one replication). There were six replications of sampling times in total. The seeds were separated from the fruits. These seeds and fruits, used for determination of ABA and ethylene content, were cut into small cubes of 0.5–0.8 cm^3, quickly frozen in liquid nitrogen, and stored at –80 °C before RNA extraction.

RNA extraction, reverse transcription- PCR (RT-PCR), and sequencing

Total RNA was extracted from 10 g of flesh or 5 g of seed using the hot borate method (Wan and Wilkins, 1994). 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 a Marathon™ cDNA Amplification Kit (Clontech). The cDNA was used as a template for amplifying NCEDs with degenerate primers (forward, 5′-TTYGAYGGIGAYGGIATGGTICA-3′; reverse, 5′-TC-CCAIGCRTTCCAIARRTGRAA-3′) designed from the conserved sequences of plant NCEDs (AF224671, Z97215, DQ028471, DQ028472, and AY337613). PCR was performed under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min, with the final reaction being terminated at 72 °C for an additional 10 min. The PCR products were ligated into a pGEM-T easy vector and subsequently transformed into *Escherichia coli* DH5α. Positive colonies were selected, amplified, and then sequenced by Invitrogen China (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 above mentioned, the desired genes from tomato fruit were obtained using the primers *LeACS2* (5′-CGAGGATTCGGAG-GTTCGTAGGTGT-3′ and 5′-GGTGAGGGAGGAATA-GGTGACGAAAG-3′), *LeACS4* (5′-GCAAGGATTCGGA-TGTTTATGGATGC-3′ and 5′-TGCTCGCACTACGAGC-GAGGAATTG-3′), and *LeACO1* (5′-GCTTGTGAR-AAYTGGGGYTTYTT-3′ and 5′-CATCGCCTCRAAYC-KGGYTTCC-3′) designed from the conserved sequences of tomato plant.

Probe preparation and northern hybridization

Digoxigenin (DIG)-labelled probes were synthesized using a PCR DIG probe synthesis Kit (Roche Diagnostics), and 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 hybridization 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 M formaldehyde, and blotted onto nylon membranes (Hybond N$^+$, Amersham Biosciences, UK). The filters were then hybridized with the DIG-labelled DNA probes in high SDS buffer [7% (w/v)) SDS, 5× SSC, 50 mM sodium phosphate, pH 7.0, 2% (w/v) blocking reagent, and 0.1% N-lauroylsarcosine] containing 50% (v/v) formamide overnight at 42 °C. After hybridization, filters were washed twice at 37 °C in 2× SSC and 0.1% (w/v) SDS for 15 min and twice at 55 °C 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).

ABA content and LeNCED1 expression in dehydrated fruit

In order to evaluate the effect of dehydration stress, fruits were harvested 4 d before the green mature stage and then divided into two groups, with fruits and seeds included in each group. The first group (as control) was stored at 20 °C under high RH (95%) and the second group (dehydration stress) was stored at the same temperature but under low RH (45%, dehydrated fruits). ABA content and the degree of *LeNCED1* expression in the fruits or seeds were determined at 0, 2, 4, 6, 8, 10 and 12 d after treatment. Each treatment included three replications. Each sample included 20 fruits or 5 g of seeds. Some samples were quickly frozen in liquid nitrogen and stored at –80 °C before RNA extraction.

Effect of exogenous ABA, fluridone, and ACC treatments on fruit ripening

In order to evaluate the effect of exogenous ABA, fluridone (an ABA synthetic inhibitor), or 1-aminocyclopropane-1-carboxylic acid (ACC) treatment, tomato fruits were harvested at the green mature stage. They were then divided into four groups and used for the following treatments: ABA (100 μM, group 1), fluridone (100 μM, group 2), ACC (100 μM, group 3), and control (distilled water, group 4). The fruits were treated with 0.5 ml each of the solutions or distilled water, respectively. Three replications were conducted for each solution, with each treatment comprising 20 tomato fruits and 5 g of seeds. Each solution was injected into the fruit from the pedicle with a medical syringe. Then the treated fruits were incubated (stored) at 20 °C and 95% RH for 0, 2, 4, 6, 8 10, and 12 h. Twenty fruits or 5 g of seeds were sampled every 2 d after treatment for measurement of the ABA content and ethylene.

Effect of repeat treatment with exogenous ABA, fluridone, and ACC on postharvest fruit firmness

As the fluridone, exogenous ABA, and ACC treatments have only a short-term effect of several days, for better determination of their effects the fruits were repeatedly treated. A total of 240 tomato fruits on the plants were selected and tagged at 1 week before the mature green stage. These fruits on the tomato plants were treated by injecting them with 0.5 ml of 100 μM ABA, fluridone, and ACC solutions, or water, following the methods described above. At the green mature stage, these fruits were harvested, held in the laboratory under ambient conditions, and repeatedly subjected to the same treatment as described above. Then the fruits were stored at 4 °C, 95% RH for 1 month. During the whole storage period, the fruits were subjected to injections at weekly intervals. Fruits were determined for firmness every week after treatment.

Effect of exogenous ABA, NDGA, and ABA+1-MCP treatments on fruit ripening

Because fluridone cannot completely block ABA accumulation, nordihydroguaiaretic acid (NDGA) (Creelman *et al.*, 1992) was used to block ABA in this test. Preliminary tests showed that NDGA was an ideal inhibitor of the NCED enzyme with regard to its permeating speed and its ability to block ABA biosynthesis. The lowest NDGA concentration which blocks ABA accumulation completely is 100 μM as determined by preliminary tests in tomato fruit. In addition, 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997) was used to block ethylene action during the ripening stage of tomato fruit.

Tomato fruits were harvested at the mature green stage. Immediately after harvest, fruits were divided into four groups and were treated with 0.5 ml per fruit each of 100 μM ABA (group 1), 100 μM NDGA (group 2), 100 μM ABA (group 3), or distilled water (group 4, control) using the methods described above, and held at 20 °C and 95% RH. The fruits in group 3 were treated 4 d after ABA treatment with 5 μl l^{-1} of 1-MCP for 1 h according to Nakatsuka *et al.* (1997). 1-MCP (5 μl l^{-1}) was applied by injecting a measured volume of stock gas into sealed glass jars with the fruits inside. Three replications were conducted for each treatment, using 20 fruits. Fruits were sampled every 2 d after treatment for measurement of the content of ABA and ethylene production. Some pulp tissues were frozen in liquid nitrogen and stored at –80 °C before RNA extraction.

Determination of ABA content

For ABA extraction, 1 g of flesh or 0.5 g of seed were ground in a mortar and homogenized in extraction solution (80% methanol, v/v). Extracts were centrifuged at 10 000 *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 °C for enzyme-linked immunosorbent assay (ELISA). The ELISA procedures were conducted according to the instructions provided by the manufacturer (China Agricultural University, Beijing, China). ABA was determined by Thermo Electron (labsystems) Multiskan MK3 (PIONEER Co., China).

Determination of ACC content

Samples of powdered plant material (1 g of flesh or 0.5 g of seed; three replications) were extracted with 80% aqueous ethanol (v/v). Following oxidative conversion, the ACC content was determined as ethylene by gas chromatography (Agilent, 6890N, USA) according to Lizada and Yang (1979).

Determination of ethylene production

The rate of ethylene produced by whole fruit was measured by enclosing five fruits or 3 g of seeds in 1.5 l air-tight containers for 1 h at 20 °C, withdrawing 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 g^{-1} FW h^{-1}. The measurement conditions were as follows: chromatograph column, HP-5 5% phenyl methyl siloxane, 30 m capillary alumina column (Agilent 19091J-413); the temperature of the column and detector was 80 °C and 150 °C, respectively; the carrier gas flow rate was N^2, 40 ml min^{-1}; and the hydrogen pressure was 0.6 kg cm^{-2}.

Assay of ACO activity

A 1 g aliquot of flesh tissue or 0.5 g of seed powder was added into 1 ml of a solution containing 100 mM TRIS-HCl pH 7.5, 10% glycerine, 30 mM sodium ascorbic acid, 5% polyvinylpyrrolidone (PVP), 0.1 mM FeSO$_4$, and 5 mM dithiothreitol (DTT), and centrifuged at 12 000 *g* (4 °C) for 15 min. A 0.2 ml aliquot of the above solution was added to 1.8 ml of enzyme solution containing 100 mM TRIS-HCl buffer pH 7.5, 10% glycerine, 30 mM sodium ascorbic acid, 30 mM NaHCO$_3$, 1 mM ACC, and 0.1 mM FeSO$_4$, kept at 30 °C for 20 min, and then 1 ml of gas was injected into a gas chromatograph for measurement of ethylene. The ACO activity unit was nl (g h^{-1})$^{-1}$, and measurement was repeated three times.

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 as kg cm^{-2}.

Results

Expression of LeNCED1, LeACS2, LeACS4, *and* LeACO1 *at different ripening stages*

Two different NCED genes were obtained from the cDNA of tomato fruits (mix of samples collected at different stages). These cDNAs differed in the sequence of a 740 bp fragment. A Blast homology search revealed an amino acid similarity of 79.67% between the *LeNCED* genes. One DNA sequence showed 100% homology to *LeNCED1* (located at

bases 669–1408 of the *LeNCED1* sequence) (Burbidge *et al.*, 1997; GenBank accession no. Z97215) and was designated as *LeNCED1* in this study. The full length of *LeNCED1* is 2171 bases, with an open reading frame coding for 605 amino acids. The other DNA sequence (*LeNCED2*, accession no, EU912387) showed 95% homology to *StNCED2* (located at bases 514–1254 of the *StNCED2* sequence, AY662343), and was omitted from the study. In addition, an *ACS* cDNA fragment (*LeACS2-like*) (M34289), an *ACS* cDNA fragment (*LeACS4-like*) (AF548375), and an *ACO* cDNA fragment (*LeACO1-like*) (A35021), were cloned (Zarembinski and Theologis, 1994; Oetiker *et al.*, 1997; Shiu *et al.*, 1998). The expression patterns of these genes during ripening are shown in the Fig. 1. At the six time points at which tomato fruits were sampled, the *LeNCED1* gene was expressed only at the breaker stage, 44–45 d after anthesis when ABA accumulation became high, and no expression was detected during the other ripening stages. The expression of *LeNCED1* was earlier in seed than in fruit, and both were earlier than the expression of *LeACS2*, *LeACS4*, and *LeACO1* (Fig. 1). However, in contrast to *LeNCED1*, the *LeACS2*, *LeACS4* and *LeACO1* genes were expressed from the turning to the over-ripe stage.

Changes in endogenous ABA, ACC content, ethylene production, and ACO oxidase activities at different ripening stages

After the *LeNCED1* gene was expressed, endogenous ABA or ACC in both seeds and fruits increased rapidly to a peak
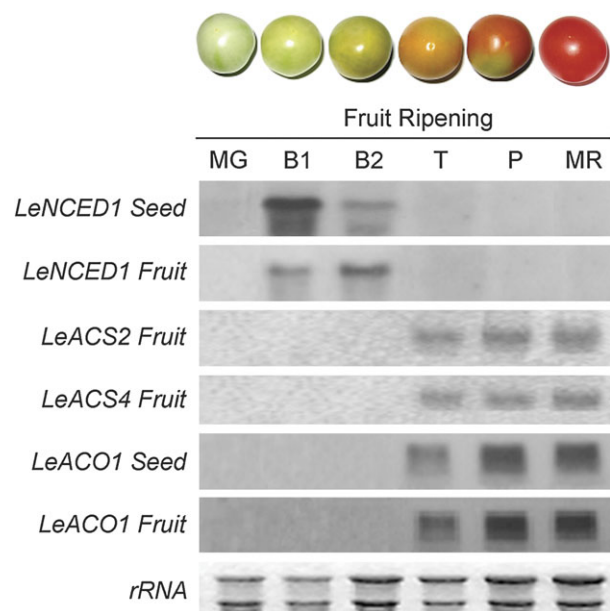
Fig. 1. Expression of *LeNCED1*, *LeACS2*, *LeACS4*, and *LeACO1* during ripening in tomato fruits revealed by northern analysis. Each lane contained 5 µg of total RNA, and the transcript levels of rRNA are shown as an internal loading control. MG, mature green (40 d after anthesis); B1, breaker (44 d after anthesis); B2, breaker (45 d after anthesis); T, turning (47 d after anthesis); P, pink (50 d after anthesis); MR, mature red (53 d after anthesis).

at the breaker or turning stage; this high level was retained until the pink stage, and then declined rapidly at the harvest red stage (Fig. 2). The maximum ABA content preceded ethylene production in both the seeds and the flesh. However, ethylene began to increase in the turning stage and reached its peak value at the pink stage, consistent with ACC oxidase activities during fruit ripening. The ethylene production was lower in the seed than in the fruit. Although the peak value of ABA, ACC, ACC oxidase activity, and ethylene occurred at different ripening stages, they all increased first in seeds and then in flesh tissues.

Effect of dehydration on endogenous ABA and expression of the LeNCED1 gene

When dehydration treatment was performed on both fruit and seed 4 d before the mature green stage, the endogenous ABA rapidly increased in both flesh and seed of tomato fruits (Fig. 3A).

The peak level of ABA was observed at the early mature stage, occurring 2 d earlier than that in the control. The expression of *LeNCED1* was also earlier in dehydration-treated fruits than in control fruits (Fig. 3B). Seed was hypersensitive to dehydration treatment compared with the flesh, but the increased amount of endogenous ABA in the flesh was higher than that in the seed. These results suggested that water stress-induced NCED gene expression and ABA accumulation may regulate the onset of ripening.

Effect of exogenous ABA, fluridone, and ACC treatments on fruit maturation

When the fruits were treated with exogenous ABA, ABA in fruits increased rapidly from 4 d to 6 d (at the maximum); the peak ABA level was found 2 d earlier than in the control fruits. In contrast, in fruits treated with fluridone, the peak ABA level was delayed by 2 d compared with that in the control (Fig. 4). The ABA peak in seed treated with fluridone appeared 2 d later than that in the flesh. In contrast, the ABA peak in seed treated with exogenous ABA occurred 2 d earlier than in the flesh. However, there was no difference in ABA content between ACC treatment and the control.

On the other hand, ethylene increased remarkably in the fruits treated with exogenous ABA or ACC, from 6 d, and reached a peak at 8 d after treatments. The treatment of fruits with fluridone significantly decreased ABA levels and inhibited fruit softening. The experimental results indicated that ABA treatment induced ethylene synthesis, but ethylene (ACC) treatment had no effect on ABA.

Effects of repeatedly applied exogenous ABA, fluridone, and ACC on postharvest fruit firmness
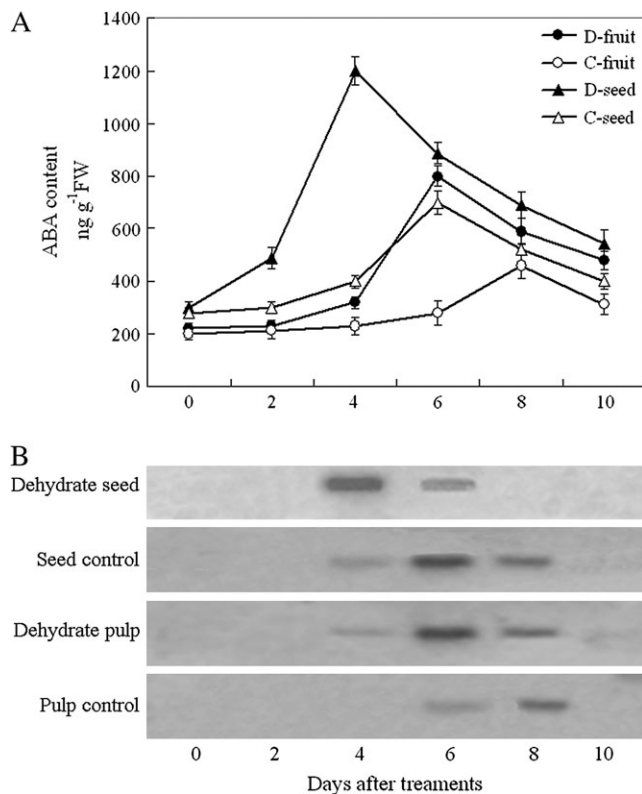
The application of ABA or ACC accelerated the rise in ethylene production and respiration associated with changes in skin colour and fruit softening compared with control fruits treated only with water (Fig. 6). Flesh firmness was higher in fruits treated repeatedly with fluridone than in
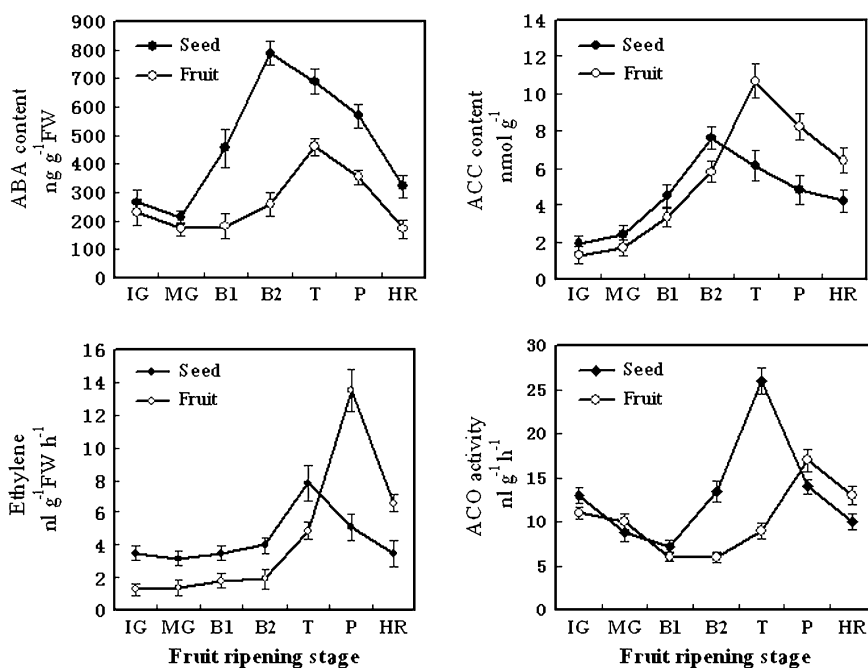
Fig. 2. Changes in the endogenous ABA content, ethylene production, ACC content, and ACO activity during fruit ripening in the seed and flesh of tomato. Fruit samples were collected from the immature green to the red ripe stage. Twenty fruits were collected for each treatment with three replications. Each point represents the mean of three replications, and vertical bars represent ±SE. IG, immature green (20 d after anthesis); MG, mature green (40 d after anthesis); B1, breaker (44 d after anthesis); B2, breaker (45 d after anthesis); T, turning (47 days after anthesis); P, pink (50 d after anthesis); MR, mature red (53 d after anthesis).

fruits treated with ABA or ACC, and in control. The flesh firmness of the ABA- and ACC-treated fruits at the sixth week after treatment were ~36.3% and 50.0% lower than that of control fruits, respectively (Fig. 5). The reduction of the ABA level in fruit resulted in delayed ripening and softening, indicating that the fruit ripening and softening were closely related to ABA accumulation; that is, ABA may play an important role in regulating the later stage of fruit ripening.

Expression of the LeACS2 and LeACO1 genes in the fruit after treatment with exogenous ABA or NDGA

Northern analysis showed that in the pulp of control fruit, lower expression of the *LeNCED1* gene occurred 4 d after harvest, and peaked at 6–8 d, whereas *LeACS2* and *LeACO1* were expressed at 8–12 d after harvest (Fig. 7). NDGA treatment suppressed and deferred the expression of *LeACS2* and *LeACO1* to lower levels, and ABA treatment, in contrast, promoted them. All treatments with ABA or NDGA had no effect on expression of the *LeNCED1* gene. These observations indicate that ABA may mediate the ACC synthase gene (*LeACS2*) and ACC oxidase gene (*LeACO1*) in tomato fruit during ripening.

Effect of NDGA and 1-MCP on ABA accumulation and ethylene synthesis

The changes in content of ABA and ethylene production after treatment with exogenous ABA, NDGA, or 1-MCP are shown in Fig. 8. When the fruits were treated with exogenous ABA, both alone and together with 1-MCP, the ABA content in fruits increased rapidly from 4 d and reached the peak level at 6 d after treatment, which was 2 d earlier than that in the control fruits. In contrast, ABA accumulation was completely blocked when fruit was treated with NDGA, but NDGA could not completely block ethylene biosynthesis in the later ripening stage. On the other hand, ethylene increased remarkably in the fruits treated with exogenous ABA, from 6 d, and reached the peak at 8 d after treatment. The ethylene production was completely blocked by 1-MCP treatment (Fig. 8). Treatment with both NDGA and 1-MCP inhibited fruit ripening and softening. The roles of both ABA and ethylene in the later ripening of fruit are complex.
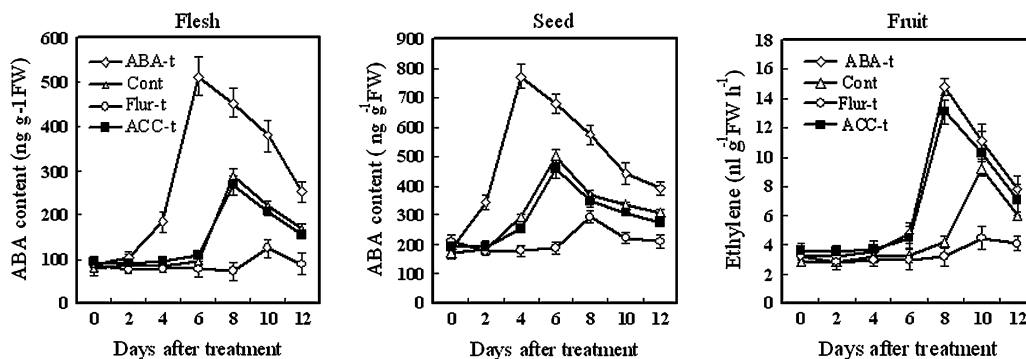

Fig. 3. Changes in the endogenous ABA contents (A) and *LeNCED1* expression (B) in the dehydrated flesh and seed of tomato fruit held in low (45%) or high (95%) RH conditions (temperature 20 °C). Each lane in B contained 5 μg of total RNA, and the transcript levels of rRNA are shown as an internal loading control. Each point in A represents the mean of three replications and vertical bars represent ±SE. Filled triangles, dehydrated seed; open squares, control (seed); filled circles, dehydrated flesh; open circles, control (flesh)

Fig. 4. Effects of exogenous ABA, fluridone, and ACC treatments on ABA levels and ethylene production in the flesh and seed of tomato fruits during the mature green stage. Each point in A represents the mean of three replications and vertical bars represent ±SE. Open diamond, 100 μM ABA; open square, control (distilled water); open circle, 100 μM fluridone; filled square, 100 μM ACC.

Discussion

The initiation of ABA biosynthesis by the LeNCED1 *gene at the stage of onset of fruit ripening*

Cloning and characterization of NCEDs in fruits facilitate understanding of the role of ABA in fruit ripening. Two NCED genes have been isolated from avocado (*Persea Americana* Mill.), a climacteric fruit. *PaNCED1* and *PaNCED3* were expressed consistently with ethylene production during fruit ripening but earlier than ABA accumulation. Thus it has been proposed that ethylene induces NCED gene expression and ABA accumulation, which results in post-ripeness (Chernys and Zeevaart, 2000). Recently, two NCED genes, *CsNCED1* and *CsNCED2*, have been isolated from *Citrus sinensis* L. Osbeck, a non-climacteric fruit (Rodrigo *et al.*, 2006). Only *CsNCED1* was expressed in fruit in accordance with ABA accumulation, suggesting that *CsNCED1* may play a key role in ABA biosynthesis.

In this study, two cDNAs of NCED gene were cloned from tomato fruit, which shows the existence of at least two NCED genes. The expression of the *LeNCED1* gene and ethylene-associated genes (*LeACS2, LeACS4,* and *LeACO1*) was also analysed in tomato fruit during ripening. The comparison of the expression of these genes led to the important discovery that the *LeNCED1* gene was expressed only at the stage of onset of ripening in both the flesh and seed when the ABA content becomes high, in response to which the expression of *LeNCED1* occurred earlier than the expression of *LeACS2, LeACS4,* and *LeACO1.* Therefore, it was proposed that the expression of the *LeNCED1* gene initiated ABA biosynthesis at the beginning of fruit ripening, acting as an original inducer for maturation. On the other hand, ethylene biosynthesis during the climacteric stage has been demonstrated to be due to the accumulation of transcripts of two ACC synthase genes, *LeACS2* and *LeACS4* (Rottmann *et al.*, 1991; Lincoln *et al.*, 1993), and two ACC oxidase genes, *LeACO1* and *LeACO4* (Barry *et al.*, 1996; Nakatsuka *et al.*, 1998). Moreover, fruit ripening in both *rin* and *nor*, ripening-impaired tomato mutants, was not induced by exogenous ethylene treatment, although the expression of some ripening-associated genes is induced by ethylene treatment in both mutant fruits (Lanahan *et al.*, 1994; Yen *et al.*, 1995; Giovannoni, 2001). These results suggest that an ethylene-independent regulator may exist upstream of ethylene for controlling the onset of ripening in both fruit types. Exogenous ABA treatment might induce gene expression of *LeACS2* and *LeACO1* (Fig. 7) and initiate the ripening process in the tomato fruits (Figs 6–8), suggesting that ABA might act as an upstream regulator before ethylene in fruit ripening.

The induction of ethylene synthesis and initiation of fruit ripening by ABA accumulation

Generally, the ripeness and senescence of respiratory climacteric fruits are regulated by ethylene. However, the content of ABA increases during the ripening of respiratory climacteric fruits such as apples (Scriren and Wills, 1984; Kondo *et al.*, 1991), peaches (Looney *et al.*, 1974; Tsuchida, 1990), and pears (Rudnicki *et al.*, 1968). In this study, endogenous ABA accumulated before ethylene production in tomatoes. The results suggested that it is endogenous ABA, but not ethylene, that is critical for the onset of ripening, while the role of ethylene is limited to later ripening stages. The results indicated that exogenous ABA accelerated fruit ripening and, in contrast, fluridone treatment delayed the ripening by inhibiting ABA.

The production of ethylene was triggered by applying ABA to tomato fruits during the ripening stage, which indicated that increasing ABA to a certain level in the fruit
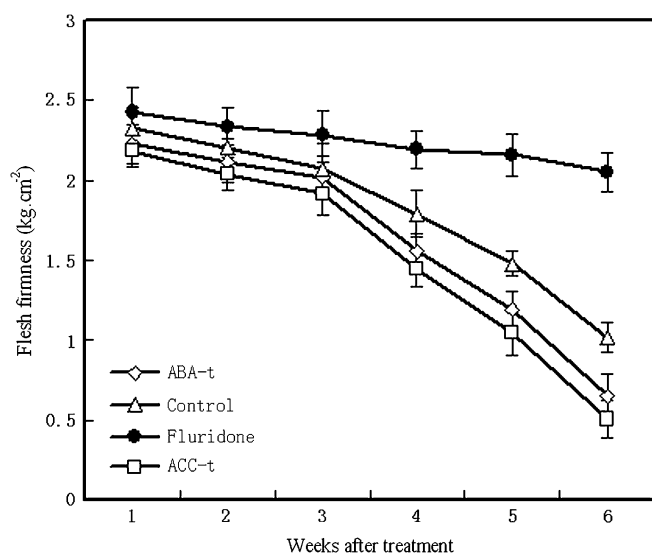
Fig. 5. Changes in flesh firmness in tomato fruits treated with exogenous ABA or fluridone or ACC solution. Non-treated fruits were taken as the control. Each point represents the mean of three replications and vertical bars represent ±SE. Open diamond, 100 μM ABA; open square, control (distilled water); filled circle, 100 μM fluridone; open square, 100 μM ACC.
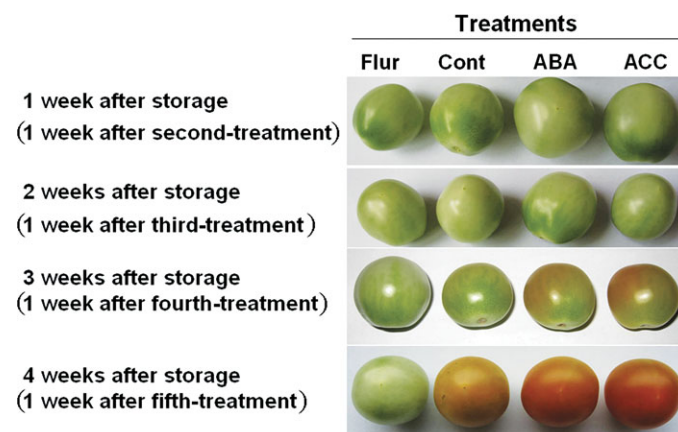
Fig. 6. Changes of skin colour in tomato fruits treated with exogenous ABA or fluridone or ACC solution. Non-treated fruits were taken as the control. In the green mature stage, tomato fruits were harvested and treated with 0.5 ml per fruit each of 100 μM ABA, 100 μM fluridone, or 100 μM ACC, or 0.5 ml of distilled water per fruit (control).
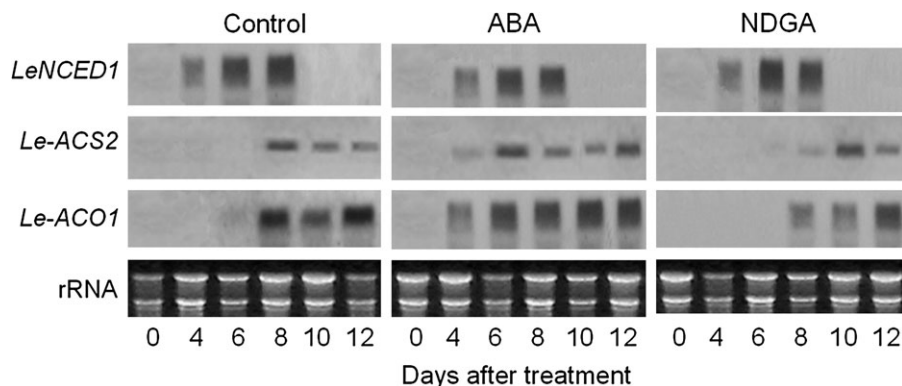
Fig. 7. Changes in the expression of *LeNCED1*, *LeACS2*, and *LeACO1* in tomato fruit treated with exogenous ABA or NDGA. Each lane contained 5 μg of total RNA, and the transcript levels of rRNA are shown as an internal loading control.
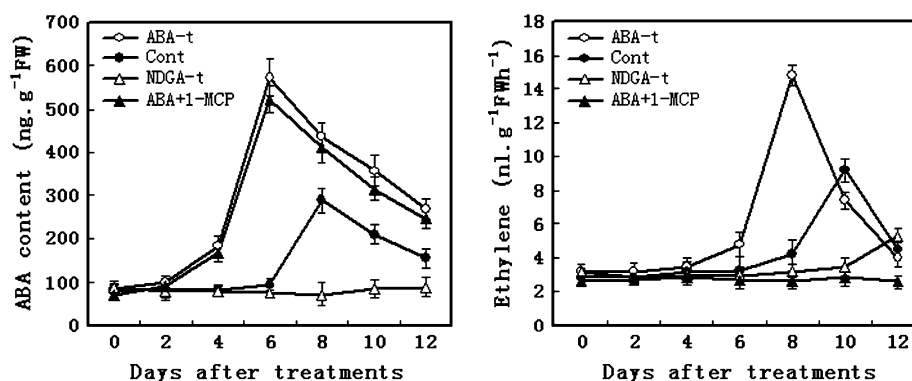
Fig. 8. Effects of exogenous ABA, NDGA, and ABA+1-MCP treatments on ABA levels and ethylene production in tomato fruits. Each point represents the mean of three replications and vertical bars represent ±SE. Open circles, 100 μM ABA; filled circles, control (distilled water); open squares, 100 μM NDGA; filled triangles; 100 μM ABA+5 μl l^{-1} 1-MCP.

could promote the transformation of ACC to ethylene and result in a series of physiological and biochemical responses that facilitate fruit ripening. Thus, it was proposed that ABA may act at upstream metabolic events of ethylene action/perception and initiate the ripening process by inhibiting or activating general metabolic events. In addition, ABA might induce the expression of non-ethylene-dependent genes and initiate the ripening process in respiratory climacteric fruits.

Since 1-MCP effectively blocks ethylene receptors and represses ethylene-mediated effects in plant tissues (Sisler and Serek, 1997), it was used to study ethylene-regulated processes during later ripening in this study (Fig. 8). The result showed that fruit ripening and softening were effectively inhibited by 1-MCP treatment, although ABA was fully biosynthesized by exogenous ABA stimulation. These results indicate that ABA plays an important role in fruit ripening, and ethylene is more important for later ripening stages.

Effects of exogenous ABA, fluridone, and NDGA on ABA accumulation, ethylene synthesis, and fruit ripening

In higher plants, ABA is formed by oxidative cleavage of epoxicarotenoids, such as neoxanthin and violaxanthin.

Fluridone specifically inhibits the biosynthesis of epoxicarotenoid, and thus is considered as an effective inhibitor of ABA biosynthesis (Bartels and Watson, 1978; Rasmussen *et al.*, 1997). Fluridone inhibits phytoene desaturase, which converts phytoene to phytofluene in the carotenoid biosynthesis pathway. Since carotenoids are the precursors of ABA in plants, carotenoid biosynthesis inhibitors should also prevent the biosynthesis of ABA. The results in this study showed that exogenous applications of ABA to mature green fruit could induce ethylene synthesis, accelerated fruit colouring, and accelerated softening (Figs 5, 6). In contrast, treating the fruits with fluridone led to an obvious reduction of endogenous ABA and impeded fruit maturity and softening (Figs 4–6). Because colour change and fruit softening are dependent on both the presence and perception of ethylene (Blankenship and Sisler, 1993), the results suggest that ABA has a direct effect on ethylene synthesis although fluridone could not completely block ABA accumulation (Fig. 4). In order to block ABA accumulation fully, NDGA was used in the test (Figs 7, 8). NDGA is now emerging as a useful compound to study ABA function in plants (Creelman *et al.*, 1992). Preliminary tests showed that NDGA was an ideal inhibitor of the NCED enzyme with regard to its permeating speed and ability to block ABA biosynthesis. ABA accumulation in detached mature green

tomato is very sensitive to NDGA. Because a concentration of 100 μM could totally block ABA accumulation, it was then used for the assessment of the role of ABA. There are two ways for NDGA to inhibit ABA accumulation: one is to inhibit the expression of ABA synthase genes at the transcriptional level while the other is to inhibit synthase activities at the post-transcriptional level, or both. The results of northern analysis of ABA synthase genes showed that the expression of *LeNCED1* was not influenced by NDGA compared with the control samples. This indicates that the blocked ABA accumulation caused by NDGA was due to its direct inhibition of the synthase activities at the post-transcriptional level (Fig. 7). As shown in Figs 7 and 8, ABA accumulation was totally blocked by 100 μM NDGA, but ethylene production increased ∼12 d after NDGA treatment, suggesting that fluridone or NDGA were acting only during a limited period of time, and the increase of ethylene and fruit ripening at later stages could be explained by a cessation of the effectiveness of fluridone or NDGA with time after treatment.

In conclusion, a relationship between ABA and ethylene during ripening and senescence was indicated in the tomato fruit: (i) the expression of the ABA biosynthetic gene (*LeNCED1*) occurs before that of ethylene biosynthesis genes; (ii) ABA content also preceded the climacteric increase in ethylene production; (iii) ABA may induce ethylene biosynthesis via the regulation of ACS and ACO gene expression; (iv) exogenous ABA accelerates fruit ripening, and fluridone or NDGA treatment delayed fruit ripening by inhibition of ABA; and (v) ethylene plays a key role in the later stages of fruit ripening.

Acknowledgements

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