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Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates

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Abstract

One of the main characteristics of tomato (Solanum lycopersicum) fruit ripening is a massive accumulation of carotenoids (mainly lycopene), which may contribute to the nutrient quality of tomato fruit and its role in chemoprevention. Previous studies have shown that ethylene (ET) plays a central role in promoting fruit ripening. In this study, the role of jasmonic acid (JA) in controlling lycopene accumulation in tomato fruits was analysed by measuring fruit lycopene content and the expression levels of lycopene biosynthetic genes in JA-deficient mutants (spr2 and def1) and a 35S::prosystemin transgenic line (35S::prosys) with increased JA levels and constitutive JA signalling. The lycopene content was significantly decreased in the fruits of spr2 and def1, but was enhanced in 35S::prosys fruits. Simultaneously, the expression of lycopene biosynthetic genes followed a similar trend. Lycopene synthesis in methyl jasmonate (MeJA) vapour-treated fruits showed an inverted U-shaped dose response, which significantly enhanced the fruit lycopene content and restored lycopene accumulation in spr2 and def1 at a concentration of 0.5 µM. The results indicated that JA plays a positive role in lycopene biosynthesis. In addition, the role of ET in JA-induced lycopene accumulation was also examined. Ethylene production in tomato fruits was depressed in spr2 and def1 while it increased in 35S::prosys. However, the exogenous application of MeJA to Never ripe (Nr), the ET-insensitive mutant, significantly promoted lycopene accumulation, as well as the expression of lycopene biosynthetic genes. Based on these results, it is proposed that JA might function independently of ethylene to promote lycopene biosynthesis in tomato fruits.

Key words: Ethylene (ET), fruit, jasmonic acid (JA), lycopene, mutant, tomato.

Introduction

Carotenoids are a class of 40-carbon terpenoid molecules that are present in most tissues of higher plants. They are one of the most important plant secondary metabolites that play a variety of roles in plant growth and development. For example, they can serve as attractants to pollinators and animals for seed dispersal by providing fruits and flowers with distinct red, orange, and yellow colours (Ronen et al., 2000). They are also important for light harvesting, protecting against excess light energy. In addition, carotenoids are essential components of human diets, providing precursors for vitamin A synthesis and having anti-cancer...
activities (Auldridge et al., 2006; DellaPenna and Pogson, 2006; Tanaka et al., 2008). Lycopene, the most abundant carotenoid found in tomatoes, has been regarded as the bioactive component alternative for the remedy of chronic diseases (Ford and Erdman Jr, 2012). The biosynthetic pathway of carotenoids has largely been elucidated. For example, in Arabidopsis and tomato nearly all the key enzymes have been identified (Ronen et al., 2000; Hirschberg, 2001; Fraser and Bramley, 2004; Römer and Fraser, 2005; DellaPenna and Pogson, 2006). Carotenoids are synthesized in plants through a pathway starting with the formation of phytoene from geranylgeranyl diphosphate (GGPP) in the central isoprenoid pathway and four desaturation steps to produce lycopene.

Tomato fruit is an excellent model for the study of carotenoid synthesis. One of the main characteristics of tomato fruit ripening is a massive accumulation of carotenoids (mainly lycopene). The mechanism controlling fruit ripening is systematic and sophisticated. Despite the detailed knowledge of the carotenoid biosynthetic pathway, the regulation of the synthesis, including the impact of biotic and abiotic factors, many of which are mediated by phytohormones (Srivastava and Handa, 2005) is rarely known (Bramley, 2002). Therefore, much attention has been paid to how carotenoid accumulation is regulated because carotenoid biosynthesis is not only of agricultural importance but also of scientific interest in terms of the chemical, biological, and genetic regulation. Phytohormones play a central role in the signalling networks underlying plant growth and development. They act in a modular fashion and the action can be synergistic under some circumstances and antagonistic under others. Different hormones are involved in various physiological processes, each leading to a specific set of downstream responses (O’Donnell et al., 2003).

Compared with other hormones, advances have been made in the regulation mechanism of ethylene on carotenoid biosynthesis in tomato fruits (Lelièvre et al., 1997; Alba et al., 2005). In climacteric fruits, the onset of ripening is characterized by a dramatic increase in ethylene production, which highly correlates with the rapid accumulation of β-carotene and lycopene in tomato fruits. On the other hand, transcription factors in tomato fruit ripening like LeMADS-RIN and LeNOR (Vrebakov et al., 2002) were proven to have a regulatory role prior to ethylene biosynthesis as revealed by physiological studies of their corresponding mutants ripening inhibitor (rin) and non-ripening (nor) (Adams-Phillips et al., 2004), fruits from both mutants failed to synthesize climacteric ethylene or accumulate lycopene (Giovannoni et al., 1995). Six tomato ET receptors, designated LeETR1, LeETR2, LeETR3 (NR), LeETR4, LeETR5, and LeETR6, have been isolated and characterized (Klee and Tieman, 2002; Klee, 2004). Each receptor gene has a distinct pattern of expression throughout plant development and in response to external stimuli. Up to now, LeETR4, LeETR6, and NR genes are the most highly expressed in ripening fruits (Klee and Giovannoni, 2011). In tomato, an ET-insensitive mutation Never ripe (Nr) caused by a single base substitution in the N-terminal coding region of the ethylene receptor gene LE-ETR3 (i.e. NR) has been identified. The receptor is homologous to Arabidopsis ETR1 (Lanahan et al., 1994; Wilkinson et al., 1995). The role of ethylene in carotenoids formation was further demonstrated by the phenotype of the Nr mutant that exhibits reduced ethylene sensitivity and accumulates low amounts of lycopene and β-carotene in ripened fruits (Lanahan et al., 1994). Other plant hormones, like ABA, have also been documented to control carotenoid biosynthesis. The abscisic acid (ABA)-deficient tomato mutants, namely, high-pigment 3 (hp3), flacca (flc), and sitiens (sit) show an increased plastid number and an enhanced level of lycopene content during fruit ripening (Galpaz et al., 2008).

Jasmonates, as a new group of phytohormones, including jasmonic acid (JA), its methyl ester (MeJA), its amino acid conjugates and other metabolites such as 12-OH-JA (Stenzel et al., 2008). JA and its volatile methyl ester MeJA are well-studied plant growth regulators (Fan et al., 1998), and are known to participate in the control of fruit ripening, pollen viability, and plant resistance. In particular, they have also been demonstrated to play a central role in regulating the biosynthesis of many secondary metabolites (Chen et al., 2006). Early studies using 0.5% (w/w) MeJA in lanolin paste to treat tomato fruits at the Mature Green (MG) stage found that lycopene accumulation was almost totally inhibited during treatment (Saniewski and Czapski, 1983). By contrast, lycopene content was not found to change in tomato fruits during MeJA exposure, but it was increased after the treatment (Tzortzakis and Economakis, 2007). Due to the contradictory results observed in these experiments, the limitation of exogenous application tests and the characterization of multiple hormone mutants in tomato, it is interesting to investigate the role of endogenous hormone levels on fruit development and ripening further using phytohormone deficient mutants. In the present study, the JA-deficient mutants def1 (with a defective octadecanoid synthesis pathway) and spr2 [a suppressor of (pro) systemin-mediated responses] mutation with a reduced level of trienoic fatty acids (Howe et al., 1996; Li et al., 2003) were used to investigate the role of JA on carotenoids accumulation during tomato fruit ripening. The lycopene content was significantly decreased in fruits of spr2 and def1, but enhanced in 35S:prosys fruits. The levels of gene expression of the biosynthetic enzymes were examined in the JA mutants since carotenoid synthesis was significantly associated with expression of these genes (Römer and Fraser, 2005; DellaPenna and Pogson, 2006; Sandmann et al., 2006). In addition, the fact that exogenous JA treatments stimulate the production of ethylene in tomato fruits (Imanishi and Nagata, 2003) led us to explore the role of ethylene on JA-induced lycopene accumulation by treatment of Nr mutant fruits with MeJA.

Materials and methods

Plant materials and growth conditions

Tomato cultivar Castlemart (CA) is the parental line for JA mutants spr2 and def1 as well as the transgenic line 35S:prosys. 35S:prosys seeds were collected from a 35S:prosys homozygote that had been backcrossed five times to its wild-type line cv. CA. Seeds were sown in seedling trays filled with a rich soil mixture after germination on filter paper. Tomato seedlings were grown in a greenhouse, with temperatures ranging from 22–28 °C (night to day air temperature) and a 16 h photoperiod. Three weeks after germination, seedlings were transplanted to plastic pots (34 cm in diameter, 37 cm in depth) filled with perlite and turfy soil (3:1 v/v), followed by daily watering and weekly fertilizing with a half-strength Enshi nutrient solution (Yu and Komada, 1999). Flowers of wild-type tomato plants CA were tagged at anthesis and the number of fruits was limited to fewer than four per cluster. The fruits displaying the first sign of colour change were identified as at
the B (Breaker) stage. The average number of days from anthesis to B for 50 fruits of CA plants was determined to be 50. Fruits at 3 d before B were marked as MG (Mature Green). Fruits at 3 d and 10 d after B were staged as P (Pink) and R (Mature Red), respectively. For jasmonate-deficient mutants (spr2 and def1) and the ET-insensitive Nr mutant, the same sampling standards were used. After harvest, fruits were quickly cut into small cubes of 0.5–0.8 cm³, and then immediately frozen in liquid nitrogen and stored at −80 °C.

Chemical treatment of different plant materials

To test the effect of exogenous JA on lycopene biosynthesis, fruits of CA at the MG stage were harvested and five replicate samples (three fruits each) were placed into 10 l glass jars along with a cotton ball wetted with different concentrations of MeJA (Sigma, MO, USA) (0.05, 0.1, 0.5, 1, 5, and 10 µM). The detached tomato fruits of CA, spr2, def1, and Nr were then treated with solutions containing 0.5 µM MeJA with ethanol as the control. The cotton ball was used to facilitate evaporation. Jars were placed in a growth chamber (Safe Experimental Instrument Company, Ningbo, China) with a 16 h light period at 24 °C, 90% relative humidity, and tightly sealed for 24 h. Fresh air was allowed to replace MeJA afterwards. Samples were collected at different time points (1, 4, 7, and 15 d after treatment), which correspond to the four typical stages (MG, B, P, and R) during tomato fruit ripening. For each day and treatment, after ethylene detection, fruits were rapidly cut into small cubes of 0.5–0.8 cm³ and immediately frozen in liquid nitrogen and kept at −80 °C for further experiments.

RNA extraction and real-time quantitative PCR (qPCR) analysis

Total RNA extraction was carried out using TRIzol reagent (Invitrogen, CA, USA) from 100 mg tomato fruit tissue. Genomic DNA was removed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined by spectrophotometry. RNA integrity was evaluated on a 1.5% (wt/vol) agarose gel. cDNA was synthesized using 5 µg of RNA with the RevertAid first-strand cDNA synthesis kit (Fermentas, Canada). cDNA was diluted in 100 µl of water and used as the template for real-time quantitative PCR.

Quantitative RT-PCR was performed in a total volume of 25 µl, including 1 µl of diluted cDNA, 200 µM for each primer, and 12.5 µl of 2× SYBR Green PCR Master Mix (Takara, Japan) on an iCycler (Bio-Rad Inc., CA, USA). The qPCR programme included a preliminary step of 30 s at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A 2–∆∆CT,actin(TimeX – CT,actin)Time0 (Livak and Schmittgen, 2001). Time X is any time point of Time 0 was set to 1. Primer sequences were used as described by Zhang et al. (2007) for PSY1, GGPS et al. (2007) for GGPS, 5'-CTTCTTCGTCCCTGCAAA-3' (forward) and 5'-GGPSreverse (reverse); for PDS, 5'-AGCTTCCGCCTGGA AACAA-3' (forward) and 5'-CTGACGACATGACGACACC-3' (reverse); for DXS, 5'-GGTTGGTTTGTGCTACGAGACAA-3' (forward) and 5'-ATCCGATCCCTCCGAGATGA-3' (reverse). and a GDX-502 activated alumina glass column held at 200 °C with a detection temperature of 160 °C. The ethylene level was calculated by comparison with commercial standards.

HPLC analysis of carotenoids

Carotenoids were extracted and analysed as described previously (Hart and Scott, 1995; Scott et al., 1996; Barba et al., 2006) with modifications. Tomato fruits (0.6 g) were homogenized with 30 ml of hexane/acetone/ ethanol (1:1:1 by vol.) solution and magnetically stirred for 30 min. The extracts were centrifuged at 3000 g and 4 °C for 10 min and then 15 ml of water was added. The upper layer was placed in a round-bottomed flask, and an aliquot of 6 ml was evaporated to dryness in a rotary evaporator at 30 °C. The residue was dissolved by THF/acetonitrile/methanol (15:30:55 by vol.) solution to a final volume of 3 ml for fruits at the MG and B stages and 6 ml in the case of P and R fruits. The final solution was filtered through 0.45 µm membrane filters and 20 µl were injected onto HPLC. All the procedures were performed under dim light.

Analysis was performed using an HPLC system consisting of a Waters 2695 separations module and a Waters 2996 photodiode array detector (Waters Corp., Milford, MA, USA). A Hypersil C18 column (5 µm particle size, 4.6 × 250 mm; Elite Analytical Instruments Co., Ltd., Dalian, China) was used with a mobile phase of methanol/acetonitrile (90/10 v/v)+9 µm TEA at a flow rate of 1.2 ml min⁻¹. The total retention time was 30 min. A 20 µl sample was injected into the column by an auto-sampler. Absorbance was detected at 475 nm. Standards (lutein, lycopene, and β-carotene; Sigma, St Louis, MO, USA) were prepared and used to identify and quantify the corresponding carotenoids.

Statistical analyses

Statistical analysis was performed using the SPSS package program version 11.5 (SPSS Inc. Chicago, IL, USA). Data were analysed by one-way ANOVA, followed by the least significant difference (LSD) test at a 95% confidence level (P < 0.05). The values were reported as means with standard error for all the results.

Results

Lycopene synthesis correlated with endogenous JA levels and showed inverted U-shaped dose-response to exogenous JA

HPLC was used to analyse carotenoid contents extracted from tomato fruits. Three principal carotenoids, lutein, lycopene, and β-carotene, were identified by comparing with the corresponding standard solutions (Fig. 1A). To examine the relationship between endogenous JA and the carotenoid level in tomato fruit, carotenoid contents in JA-deficient mutants (spr2 and def1) and their parental isogenic line CA were measured. As shown in Fig. 1B, the lycopene content of the R fruits of the mutants were reduced by at least 40% compared with the wild type. In fact, the differences between these mutants and the wild type were already obvious at the B stage of fruit development. No significant differences in size or fresh weight of the fruits were observed (data not shown). Transgenic tomato plants over-expressing the tomato prosysystemin (PS) cDNA from the Cauliflower mosaic virus (CaMV) 35S promoter exhibited a dramatic increase in the endogenous JA level (Chen et al., 2006). To determine whether the enhanced endogenous JA level could promote lycopene accumulation in tomato fruits, lycopene concentration was measured in 35S::prosys fruits at different development stages. As shown in Fig. 1C, lycopene levels of transgenic fruits were significantly higher than those of the wild type from the B stage to the R stage.
Fig. 1. Effects of JA levels on lycopene contents in tomato fruits. (A) Chromatographic separation of carotenoids extracted from tomato fruits. i is relative to standard solution (mixture of lutein, lycopene, and β-carotene), and ii is about extractives from mature red tomato fruits. Peaks correspond to the following compounds lutein, lycopene, and β-carotene in chronological order. (B) Lycopene contents in the JA-deficient mutants spr2 and def1 and their parental wild-type Castlemart (CA) fruits of four developmental stages. (C) Lycopene contents in fruits of the 35S::prosys transgenic plants and the corresponding wild-type cultivar (Castlemart). Only fruits at the same developmental stages were used for statistical analysis. MG, Mature Green; B, Breaker; P, Pink; R, Mature Red. For each figure, data shown were means ±SE (n=5). Asterisks indicate significant differences at the P <0.05.
To test the JA effect further, different concentrations (0, 0.05, 0.1, 0.5, 1, 5, and 10 µM) of MeJA were used to treat mature green (MG) fruits of CA and carotenoid contents were measured at 1, 4, 7, and 15 d after treatment. The results showed that MeJA promoted lycopene accumulation in tomato fruits in a dose-dependent manner, but inhibited lycopene formation at higher concentrations (Fig. 2A). Increased lycopene accumulation was first detected at 4 d after the 10 µM MeJA treatment, and the maximum level was observed at the 15 d after 0.5 µM MeJA treatment, which was about 46% higher than in the control sample. MeJA treatment had little effect on the levels of lutein and β-carotene in tomato fruits (data not shown). Thus, MeJA application at 0.5 µM appeared to be the most effective as it produced the highest level of lycopene. Upon treatment with 0.5 µM MeJA, spr2 and def1 fruits showed wild-type lycopene levels (Fig. 2B).

**JA regulation of lycopene biosynthesis genes**

Nearly all the genes encoding carotenoid biosynthetic enzymes have been identified. In plants, the pathway starts from isopentenyl pyrophosphate (IPP), the common precursor of all isoprenoids. IPP is condensed to form geranylgeranyl pyrophosphate (GGPP). The first committed step in plant carotenoid synthesis is the head-to-head coupling of two GGPP molecules to produce phytoene, catalysed by phytoene synthase (PSY). Phytoene desaturase (PDS) and β-carotene desaturase (ZDS) then catalyse the desaturation of phytoene into the formation of the red acyclic compound lycopene (Fig. 3A).

To test whether the observed alterations in lycopene content were attributed to changes in gene expression in the carotenoid biosynthetic pathway, quantitative RT-PCR was used to measure the mRNA levels of **DXS**, **GGPS**, **PSY1**, and **PDS** during...

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**Fig. 2.** Effects of exogenous MeJA on lycopene accumulation in tomato fruits. (A) Effects of exogenous MeJA at different concentrations on CA lycopene accumulation. Different concentrations (0, 0.05, 0.1, 0.5, 1, 5, and 10 µM) of MeJA vapour were used to treat mature green (MG) fruits of CA and lycopene contents were measured at 1, 4, 7, and 15 d after treatment. (B) Lycopene contents in 0.5 µM MeJA-treated CA, spr2, and def1. Only fruits at the same day after treatments were used for statistical analysis. Data shown were means ±SE (n=5). Asterisks indicate significant differences at the P <0.05. Open diamond, squares, and triangle represent values statistically significantly higher than MeJA-untreated CA, spr2, and def1, respectively.
Fig. 3. Transcription patterns of lycopene biosynthesis genes DXS, GGPS, PSY1, and PDS in the fruits of CA, spr2, def1, and 35S::prosys and MeJA-treated CA. (A) Biosynthesis pathway of lycopene in tomato. Expression of the genes marked with asterisks have been analysed in this study. DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; IPP, isopentenyl pyrophosphate; IPI, isopentenyl pyrophosphate isomerase; GGPS, geranylgeranyl pyrophosphate synthase; GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase. (B) Expression of lycopene biosynthesis genes in CA, spr2, def1, and 35S::prosys at four developmental stages. The steady-state levels of mRNA were determined by quantitative RT-PCR. MG, mature green; B, breaker; P, pink; R, mature red. (C) Expression of lycopene biosynthesis genes in 0.5 µM MeJA treated CA at 1, 4, 7, and 15 d after treatment. Only fruits at the same developmental stages and the same day after treatments were used for statistical analysis. Data shown were means ±SE (n=5). Asterisks indicate significant differences at the P <0.05. (This figure is available in colour at JXB online.)
fruit development in the JA-deficient mutants spr2 and def1, 35S::prosys transgenic plants and their corresponding wild-type CA. At the P stage, all four gene transcripts were significantly reduced in the mutants and increased in 35S::prosys. By contrast, at the MG and B stages the transcript abundance of these genes did not show any differences among CA, spr2, def1, and 35S::prosys, except for PSY1, which was also repressed in the mutants but markedly enhanced in 35S::prosys at the B stage (Fig. 3B). Since lycopene accumulates mainly at the R stage and the expression levels of lycopene biosynthesis genes reach a maximum at the P stage, the biosynthesis gene transcripts at the P stage may contribute most to lycopene formation in tomato fruits (Télef et al., 2006; Galpaz et al., 2008). In addition, the expression of DXS, GGPS, PSY1, and PDS genes in CA at 1, 4, 7, and 15 d after MeJA application was evaluated. The peak value of GGPS and PDS appeared 4 d after MeJA fumigation, which was 3 days ahead of DXS and PSY1. In agreement with 35S::prosys, the expression levels of all the four genes were up-regulated by MeJA usage in spite of the pattern of non-synchronization (Fig. 3C).

**JA effect on ethylene production rate is relevant to endogenous JA levels and exogenous MeJA application**

Exogenous MeJA treatment stimulates ethylene production in tomato fruits (Imanishi and Nagata, 2003), and ethylene plays a key role in the processes of tomato fruit ripening including carotenoids accumulation (Lelièvre et al., 1997; Alba et al., 2005). To determine if the effect of JA on lycopene accumulation was associated with ethylene, the ethylene production rate was measured in spr2, def1, and 35S::prosys. As can be seen in Fig. 4A, it was found that ethylene production was notably repressed in the spr2 and def1 mutants, but enhanced in the 35S::prosys plants, indicating that ethylene production was closely correlated with endogenous JA levels in tomato fruits at the B, P, and R stages. Ethylene production was also measured in MeJA-treated CA, spr2, and def1. It was observed that ethylene production was significantly recovered after MeJA treatment (Fig. 4B). In this context, JA-promoted lycopene appeared to correlate with JA-stimulated ethylene production.

**Enhanced lycopene accumulation in Nr after MeJA application**

In order to obtain further insights into the cross-talk of JA and ET, the ET-insensitive mutant Nr, which carries a semi-dominant mutation in NR was used. MeJA-treated Nr accumulated many more pigments (Fig. 5A) and released markedly more ethylene (Fig. 5B). This is consistent with existing findings that Nr is not impaired in any step of ET biosynthesis (Lanahan et al., 1994). Furthermore, the lycopene accumulation in Nr fruits altered dramatically upon exogenous MeJA application, which could be visually recognized from longitudinal sections of Nr fruits taken on the 7th day after 0.5 µM MeJA application (Fig. 5C). In addition, the expression of DXS, GGPS, PSY1, and PDS genes was evaluated. The GGPS transcript peaked at 15 d and the transcript abundance of DXS and PSY1 reached a maximum at 7 d, while the PDS transcript exhibited a peak at 4 d after treatment (Fig. 5D). Here, the MeJA-induced expression levels of all the four genes in Nr were all markedly higher than those in untreated fruits (Fig. 5D). The results demonstrated that the perception of ET is not required in the induction of lycopene biosynthesis by MeJA.

**Discussion**

In plants, jasmonates regulate the synthesis of various secondary metabolites including anthocyanins (Devoto et al., 2005; Chen et al., 2007; Shan et al., 2009; Qi et al., 2011), glucosinolates (Mewis et al., 2005; Sasaki-Sekimoto et al., 2005), caffeoylputrescine (Chen et al., 2006) as well as other antioxidants.
Mutants in biosynthesis and signalling pathways contribute most to the understanding of JA functions in plants, especially in Arabidopsis and tomato. In the current study, JA-deficient mutants, spr2 and def1, were used to investigate the role of JA in carotenoid biosynthesis in tomato fruits. Lycopene synthesis was reduced by about 40% in these mutants during fruit ripening (Fig. 1B), indicating a positive role of JA in lycopene formation. Several earlier studies suggested that exogenous application of MeJA inhibits lycopene accumulation (Saniewski and Czapski, 1983). To address this inconsistency, different concentrations of MeJA were applied to wild-type fruits and the result showed that MeJA functions in a dose-dependent fashion. Treatments at lower concentrations (0.05–0.5 µM) promoted lycopene synthesis, while higher concentrations (5 µM and 10 µM) repressed the synthesis of lycopene (Fig. 2A). Despite the concentration parameters, the contrary results could also be due to the different application processes. In previous studies, lanolin was used as a MeJA carrier to paste the fruit surface, most likely prolonging the treatment, whereas in the present study, MeJA vapour was used and the treatments lasted for 24 h. A similar result was also observed in a previous survey (Tzortzakis and Economakis, 2007), in which the lycopene concentration did not change significantly during MeJA exposure, but increased after the treatment. Several exogenous application experiments also observed a stimulation of β-carotene by MeJA, while no remarkable increase was found in this study (data not shown). It seems that the stimulatory effect of JA on carotenoids production is restricted to lycopene in tomato fruit.

The 35S::prosys transgenic plants have been used as a positive control in studies on endogenous JA effects (Chen et al., 2006). In addition to a significantly enhanced level of endogenous JA, 35S::prosys plants also exhibited constitutive induction of secondary metabolites such as caffeoylputrescine (Chen et al., 2006). In the current study, lycopene content was significantly increased in 35S::prosys fruits during ripening. Together with the results observed in JA-deficient mutants, the role of JA in lycopene formation is apparent.
The accumulation of carotenoids is mainly controlled at the gene expression level of the biosynthetic enzymes (Römer and Fraser, 2005; DellaPenna and Pogson, 2006; Sandmann et al., 2006) as well as anthocyanin accumulation in Arabidopsis (Shan et al., 2009). Phytoene synthase is a major control point during carotenoid biosynthesis in tomato fruits (Fraser et al., 2002).

There are two genes encoding phytoene synthase in tomato plants, PSY1 and PSY2. The former encodes a fruit- and flower-specific isoform whose expression is strongly induced during fruit ripening. Expression of the latter gene is predominantly in green tissues and it does not contribute to carotenoid synthesis in ripening fruit (Fraser et al., 1999, 2002). In addition to PSY1, other biosynthetic enzymes, including DXS, GGPS, and PDS, are also up-regulated during fruit ripening and contribute to the formation of lycopene (Bramley, 2002). MeJA is previously shown to regulate carotenoid accumulation (Saniewski and Czapski, 1983; Tzortzakis and Economakis, 2007). Nevertheless, the effect of JA on carotenoid biosynthetic gene expression has not been investigated. In the current survey, we determined that the maximum expression levels of related genes are at the P stage, consistent with previous observations (Téléf et al., 2006; Gal paz et al., 2008). Furthermore, the effect of JA on lycopene formation was obvious in fruits of JA-deficient mutants and transgenic plants at this stage (Fig. 3B). Moreover, the expression pattern was strongly correlated with the lycopene contents observed in mutants and the over-expression line. Therefore, it is concluded that endogenous JA regulates the transcription of biosynthetic genes, subsequently leading to altered levels of lycopene in fruits of JA-deficient mutants and 35S::prosys.

Although the interactions between JA and ET on plant defence have been widely studied in Arabidopsis (Lorenzo et al., 2003), few reports were found concerning their co-operation on plant secondary metabolism. Exogenous JA application improved the production of ethylene and the expression of ethylene biosynthetic genes in tomato fruits (I manishi and Nagata, 2003). Considering the essential role of ethylene in lycopene biosynthesis, its involvement in JA-induced lycopene accumulation was explored in this study. It was observed that the production rate of ethylene was lower in JA-deficient mutants and higher in 35S::prosys and MeJA-treated plants in all four stages compared with CA (Fig. 4A, 4B). The result indicates that the endogenous JA level has a positive impact on ethylene production. Similar results were also obtained from exogenous MeJA application (Imanishi and Nagata, 2003). It is acknowledged that climacteric fruits including tomato are characterized by an increment in the synthesis of the phytohormone ET upon the initiation of ripening (Klee and Giovannoni, 2011). The climacteric ET leap may arise from other phytohormone facilitation. Ethylene production rate and lycopene accumulation in Nr fruits after MeJA treatment was measured here with the objective of exploring possible cross-talk between JA and ET. It was found that MeJA still enhanced Nr ethylene production (Fig. 5B). In addition, after MeJA treatment, lycopene content was markedly induced in Nr mutants (Fig. 5C). There are at least two possible explanations for this phenomenon. One is that the JA-enhanced ethylene production triggers other unknown mechanisms that can initiate the accumulation of lycopene and the other is that ethylene signal transduction is not required for the JA-induced lycopene accumulation, i.e. JA may act without ET in the lycopene biosynthesis process to confer a fundamental pigment requirement for Nr normal growth. Just as it is generally believed that JA-mediated secondary metabolite elicitation was evolved early in the higher plant lineage and is conserved (De Geyter et al., 2012). In conclusion, endogenous JA and exogenous MeJA application positively regulate lycopene accumulation as well as its biosynthetic gene expression. Furthermore, ethylene production closely relates to the endogenous JA levels and exogenous MeJA application. Moreover, exogenous application of MeJA could restore the repressed lycopene accumulation and ethylene production in ET-insensitive mutant Nr. The results indicate that the JA-induced lycopene biosynthesis in tomato fruits is independent of ethylene signal transduction (Fig. 6). The findings have extended our understanding of JA and ET interaction during lycopene biosynthesis in ripening tomato fruits, providing strong evidence that JA is essential for plant secondary metabolism, suggesting a potential strategy in increasing the commercial value of tomato fruits.

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