Lycopene Accumulation Affects the Biosynthesis of Some Carotenoid-Related Volatiles Independent of Ethylene in Tomato

Running title: lycopene affects volatiles independent of ethylene

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Abstract

For elucidating the regulatory mechanism of ethylene on carotenoid-related volatiles (open chain) compounds and the relationship between lycopene and carotenoid-related volatiles, transgenic tomato fruits in which ACC synthase was suppressed were used. The transgenic tomato fruit showed a significant reduction of lycopene and aroma volatiles with low ethylene production. 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone, which were suspected to be lycopene degradation products, were lower than those in wild type tomato fruits. In order to identify whether lycopene accumulation affects the biosynthesis of some carotenoid-related volatiles independent of ethylene in tomato or not, the capability of both wild type and transgenic tomato fruits discs to convert lycopene into carotenoid-related volatiles was evaluated. The data showed that external lycopene could convert into 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol in vivo, indicating that the strong inhibition of ethylene production had no effect on enzymes in the biosynthesis pathway of some carotenoid-related volatiles. Therefore, in ACS-suppression transgenic tomato fruits, the low levels of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol was due to decreased lycopene accumulation, not ethylene production. Ethylene only affected the accumulation of lycopene, and then indirectly influenced the level of lycopene-related volatiles.

Key words: aroma volatiles; ethylene; lycopene; tomato

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Nowadays, in addition to nutritional value, volatile profile in tomato fruit is also one of important factors to overall acceptance for consumers. Among all of volatiles in tomato fruit, some volatiles are derived from carotenoid degradation, such as 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and β-ionone (Ishida et al. 1993). It was shown that volatile profiles of tomato varieties differing in flesh color were closely related to the fruit carotenoid composition (Stevens 1970). During the past few years, as the biosynthesis pathway of carotenoids was clear, most studies focused on the regulation of carotenoid formation during tomato fruit ripening (Bramley 2002) and the relationship of carotenoids and volatiles (Ishida et al. 1998; Lewinsohn et al. 2005a,b). In red color tomato fruit, lycopene is the main carotenoid, which accumulation is dependent on ripening and the availability of climacteric levels of ethylene (Theologis et al. 1993). In tomato fruit, phytoene synthase (PSY) is a key enzyme in the synthesis pathway of lycopene (Bramley 2002). Down-regulation of this enzyme in tomato resulted in yellow color fruit with devoiding of lycopene (Bird et al. 1991). The antisense PSY tomato fruit revealed lower level of most volatiles including carotenoid-derived volatiles (6-methyl-5-hepten-2-one, geranylacetone and β-ionone) compared with wild type (Baldwin 2002). When tomato fruit was cultured in vitro and treated with 2-(4-chlorophenylthio)triethylamine, the lycopene of tomato fruit increased as a result of the inhibition of lycopene ε-cyclase, while carotenoids-derived volatiles were changed (Ishida et al. 1998, Phillip and Young 2006,).

Ethylene plays an important role in enhancing biosynthesis of aroma compounds in ripening fruits (Alexander et al. 2002; Zhu et al. 2005). Inhibition of ethylene production by suppression of ACC synthesis can block normal fruit ripening, including degradation of chlorophylls, synthesis of lycopene, solubilization of cell-wall pectins and variation in the levels of volatiles (Oeller et al. 1991, Gao et al. 2007). If these are indeed enzymatical or non-enzymatical derivation from lycopene degradation in vivo, the lack of lycopene might partially account for the lack of some carotenoid-related volatiles in ACS-suppressed transgenic tomato. Generally, ethylene affects the essential enzymes involved in the volatile biosynthetic pathways to
influence the level of volatiles, such as lipoxygenase (LOX), alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT) (Zhu et al. 2005). Ethylene may directly affect the enzymes involved in biosynthesis pathway of carotenoid-related volatiles or affect lycopene accumulation to indirectly influence the level of volatiles. Hence it is necessary to study whether lycopene accumulation affects the biosynthesis of some carotenoid-related volatiles independent of ethylene in tomato or not. In this study, we applied a bioconversion approach to study whether the lack of some carotenoid-related volatiles (6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone) in ACS-suppressed transgenic tomato fruit mainly results from the lack of lycopene in vivo.

Results

Ethylene production of wild type and transgenic tomato fruit
The transgenic tomato fruit, with inhibition of LeACS2 gene expression, showed a marked reduction in ethylene production and an inhibition of the ripening process compared with wild type tomato. The basal level of ethylene evolution in transgenic tomato fruit was below 0.1 nL of C₂H₄ per gram of fruit mass per hour (Figure 1). As the resultant inhibition of ethylene led to delaying maturity and extended the post-harvest life.

Lycopene content in wild type and transgenic tomato fruit
As shown in Figure 2, the lycopene content in transgenic tomato fruit was significant lower than that in wild type tomato fruit. The transgenic tomato fruit did not turn red even at B+40d ripening stage for lacking of lycopene. In contrast, the color of wild type tomato fruit changed to red as fruit ripening, and the lycopene level increased significantly after the B+3 ripening stage in wild type tomato fruit. At the B+10 ripening stage in wild type tomato fruit, lycopene content was over 35 mg/kg FW.

Production of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone in wild type and transgenic tomato fruit treated or not with ethylene
Carotenoid-related volatiles can be grouped as open chain or cyclic according to their molecular structure. 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone are open chain carotenoid-related volatiles. These three volatiles increased during ripening in wild type tomato fruit followed the increased levels of ethylene and lycopene (Figure 3). Compared with wild type tomato fruit, significant reductions in the concentrations of three volatiles were detected in transgenic tomato fruit, along with a major repression of ethylene and lycopene production (Figure 3). When transgenic tomato fruit was treated with ethylene, 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol recovered to the same level like those in wild type tomato fruit treated with exogenous ethylene, however, geranylacetone content increased slightly only and remained lower than that in wild type tomato fruit treated with exogenous ethylene (Figure 4).

Lycopene bioconversion of wild type and transgenic fruit discs in vivo

In order to determine the reason which led to the lack of carotenoid-related volatiles (open chain) compounds in ethylene-inhibited transgenic tomato fruit, the capacity of wild type and transgenic tomato fruit discs to convert lycopene into relative volatiles was assessed. When wild type and transgenic tomato fruit discs were fed with lycopene solution, both of them could generate 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol, but no geranylacetone was generated (Figure 5.). Incubation of wild type tomato tissues with lycopene resulted in a sharp increase in the concentration of 6-methyl-5-hepten-2-one for the first 2h, followed by a decline for the last 4 h. In transgenic tomato tissues, the change of 6-methyl-5-hepten-2-one concentration followed the similar trend as in wild type tomato discs. The concentration of 6-methyl-5-hepten-2-one reached the highest level in transgenic tomato discs after 1h incubation followed with decline. The ability to reduce 6-methyl-5-hepten-2-one into 6-methyl-5-hepten-2-ol was also tested. Both of wild type and transgenic tomato discs fed with lycopene precursor could reduce 6-methyl-5-hepten-2-one into 6-methyl-5-hepten-2-ol. The concentration of 6-methyl-5-hepten-2-ol in both discs was similar and increased along with incubation time. These data, therefore, indicated that suppression of ethylene production had no
significant effect on the activity of enzymes which was involved in the biosynthesis of 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol.

Discussion

At present, consumers are more concerned about the quality of fruit, including nutritive value, aroma, color and shelf life. Colour and aroma volatiles are two major quality attributes of tomato fruits. Generally, long shelf life varieties can be cultured by delaying or reducing the production of plant hormone ethylene (Oeller et al. 1991). However, those varieties usually exhibit a reduction of color and aroma volatiles production. 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone were suspected to be lycopene degradation products (Buttery et al. 1998, Caris-Veyrat et al. 2003). In another previous study (Lewinsohn et al. 2005a), tomato near-isogenic lines differing in fruit carotenogenesis genes accumulated different aroma volatiles. A yellow flesh mutant tomato bearing a nonfunctional psy1 gene was almost devoided of lycopene pigment and appeared to be lacking of 6-methyl-5-hepten-2-one and other norisoprenoid derivatives (Lewinsohn et al. 2005a). Whereas, the tangerine (tg, orange) mutant tomato accumulating the orange pigment prolycopene and lacking in lycopene, showed higher levels of 6-methyl-5-hepten-2-one and geranylacetone compared with wild type red tomato. These results suggested that lycopene, prolycopene, δ-carotene, and neurosporene may give rise to the noncyclic volatiles in vivo, such as 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol. Therefore, if there is a direct and causal relationship between lycopene and carotenoids-derived volatiles, further research in this field is necessary. The events that lead to the regulation of carotenoid formation and degradation into volatile compounds affecting fruit color, nutritional value, and the full flavor of tomato fruit have important economic influence. For better understanding of the correlation between ethylene and tomato fruits quality attributes (aroma volatiles), transgenic tomato fruits in which ACC synthase was suppressed were used. The transgenic tomato fruit showed a significant reduction of lycopene and aroma volatiles (Figure 2, 3).

In this study, the contents of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol and geranylacetone in transgenic tomato fruits were lower than those in wild type tomato
fruits. In addition, it is shown that external ethylene is capable of restoring the production of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol. This result indicated that an inhibition of the biosynthesis of 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol existed in ethylene-inhibited transgenic tomato fruits. For elucidating the regulatory mechanism of ethylene on carotenoid-related volatiles (open chain) compounds and the relationship between lycopene and carotenoid-related volatiles, the capability of both wild type and transgenic tomato fruits discs to convert lycopene into carotenoid-related volatiles was evaluated. The data showed that external lycopene could convert into 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol in vivo, indicating that the strong inhibition of ethylene production had no effect on enzymes in the biosynthesis pathway of some carotenoid-related volatiles. Therefore, in ACS-suppression transgenic tomato fruits, the low levels of 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol is due to decreased lycopene accumulation, not ethylene production. Ethylene only affect the accumulation of lycopene, and then indirectly influence the level of lycopene-related volatiles.

Materials and methods

Plant material and treatments

The transgenic tomato plant with suppression of ethylene biosynthesis was obtained by transforming wild type tomato (Lycopersicon esculentum L. cv. Lichun) with vectors that express the cDNA of ACC synthase (ACS) gene, LeACS2 in antisense pattern (Luo et al, 1995). Both wild type and transgenic tomato plants with antisense LeACS2 gene were grown in standard green house. Flowers were tagged at anthesis stage and fruit development recorded as DPA (day post anthesis). Mature green fruit were classified as about 40 DPA and were characterized as being fully grown size and green with yellow and mature seeds. Breaker fruit were classified as approximately 45d and also wild type tomato were characterized as showing the first signs of ripening-associated color change from green to yellow. Fruit of subsequent ripening stages were defined in days post-breaker. For wild type tomato, fruit were harvested at five stages of development: mature green(G), breaker(B), B+3d(turning stage),
B+7d(pink stage), B+10d(red ripening stage). Because the ripeness of transgenic tomato fruit was inhibited, we also sampled transgenic tomato fruit till the ripening stages of B+40DPA, at which stages wild type tomato fruit were rotten and could not be sampled. All fruit samples for experiment were taken at corresponding ripening stages.

**Ethylene analysis and treatment with ethylene**

Ethylene production was measured at each ripening stages according to Gao et al (2007). Tomato fruits were weighed and placed in a sealed jar at 22°C and incubated for 60 min to accumulate ethylene. Gas samples (1 mL) for ethylene production were removed from the headspace of the jar by syringe, and analyzed on a gas chromatograph (GC-14C, SHIMADZU) equipped with a flame ionization detector. Each tomato samples has three replicates.

For ethylene treatment, a batch of mature green fruit of both wild type and transgenic tomato fruit were harvested and were exposed to 100 μL·L⁻¹ethylene in a sealed jar until reached to red ripening stage (>90% red).

**Lycopene assay**

Fruit lycopene content were analyzed according to the previous studies (Davies 1976; Fish et al. 2002). 5 g pericarps of fresh tomatoes were finely ground to a puree in 20 mL extraction solvent of n-hexane and acetone (5:4, v/v) at 4°C and then centrifuged at 10000 g for 10 min. Supernatant was collected and precipitation was extracted repeatedly until it became white completely. The supernatants were combined, and took 1 mL from the total volume of the extraction for determining the values of OD₅0₃ on spectrophotometer. The amount of lycopene was calculated in 1mL sample with the following equation: Lycopene (µg/mL) =3.12×OD₅0₂.

**Determination of volatiles**

The pericarp tissue of tomato fruit harvested at each stage was blended for 30s in a kitchen blender and the blended tissue was held for 3 min (Gao et al, 2007). To a 40 g homogenate, 10 mL of saturated calcium chloride were added, and this mixture was blender for 10s. After blending, the homogenate used for volatile analysis was poured into vials, fresh frozen in liquid nitrogen and then stored at -80°C until analysis.
Headspace volatiles was analyzed according to the method described by Speirs et al. (1998) with modification. 6 g of the homogenate was added with internal standards and was transferred to a 10 mL headspace vial sealed with a silicon/Teflon septum. In this analysis, 2-octanone and anethole were used as internal standards. For SPME (solid-phase microextraction) conditions, a Carbowax-divinylbenzene (CW/DVB, 65 μm thickness, Supelco) SPME fiber was used. The vial was equilibrated at 40°C for 10 min with an efficient magnetic stir, then the headspace was sampled by insertion of SPME fiber for 25 min while incubation of the vial continued at 40°C. The absorbed volatiles were then desorbed from the fiber for 4 min into a gas chromatograph (series 6890, Hewlett-Packard) fitted with a capillary DB-wax column (J&W Scientific, 30 m, 0.32 mm inside diameter, 0.25 μm film thickness). Conditions for chromatography were as follows: injector at 220°C, initial oven temperature at 40°C held for 10 min, increased to 100°C at 4 °C min⁻¹, increased to 180°C at 3 °C min⁻¹, increased to 220 °C at 6 °C min⁻¹, and held for 3 min. The flow rate of carrier gas was 1 mL·min⁻¹.

Identification of volatiles was confirmed by GC/MS analysis using reference spectra in NIST98 mass spectra library and by comparison with available authentic chemical standards. MS (series 5973I, Hewlett-Packard) condition was as follows: Mass spectra were collected at a rate of 40 s⁻¹ over the mass range (m/z) 40-450. The electron ionization energy was 70 eV. The temperature of the ion source was 230 °C. The GC/MS transfer line temperature was 180 °C.

The volatiles were quantified by calculating the relative concentrations using regression equations determined by injecting five concentrations of each standard to obtain a peak areas calibration curve. And the areas of volatile components peaks were normalized by the area of internal standard peak.

**In Vivo bioconversion of lycopene**

A modified version of the method described by Francisco was used (Francisco et al. 2002). Tomato fruits at the B ripening stage were surface-sterilized and fruit discs (diameter 7 mm, thickness 2 mm) were cut from the pericarp of tomato fruit at an equatorial band using a cork borer. The discs were rinsed with sterile water to remove the intercellular material. And 50 g discs of each tomato sample were suspended in
100 mL of phosphate buffer (0.1 M, pH 5.8) in 250 mL sterilized Erlenmeyer flasks, and then an emulsion of 2 mg lycopene (Sigma Co., USA, purity > 95%) in a solution of 2 mg of Tween 80 in 500 μL of phosphate buffer were added. Flasks were sealed immediately, shaken (120 rpm) at 25 °C. Aroma volatiles were collected with SPME fiber at the desired periods of time and were analyzed directly. Contrast test without adding emulsion of lycopene were also done. The whole experiment was performed under sterile conditions.

Statistics
All determinations were conducted three times. Analysis of variance (ANOVA) of the data was evaluated by the SPSS 11.0 for windows (SPSS Inc.). Duncan’s multiple-range test was employed to determine the statistical significance of the differences between the means (p<0.05).

References


Figure legend

Figure 1. Ethylene production of wild type and transgenic tomato fruit during fruit ripening. Each point represents the mean of 3 replicates (5 fruits of each) and bars represents SD. WT means wild type tomato fruit (△), and ACS means ACS-suppressed transgenic tomato fruit (□).

Figure 2. Changes in lycopene content of wild type and transgenic tomato fruit during fruit ripening. Each point represents the mean of 3 replicates and bars represents SD. WT means wild type tomato fruit (△), and ACS means ACS-suppressed transgenic tomato fruit (□).

Figure 3. Carotenoid-related (open chain) volatiles contents (ng/g) in wild type and transgenic tomato fruit at each ripening stage. Each point represents the mean of 3 replicates and bars represents SD. WT means wild type tomato fruit (△), and ACS means ACS-suppressed transgenic tomato fruit (□).

Figure 4. Carotenoid-related (open chain) volatiles contents (ng/g) in wild type and transgenic tomato fruit treated with ethylene to red ripening stage. All mature green fruits were treated with ethylene until red ripening stage. Each point represents the mean of 3 replicates and bars represents SD. Means followed by an asterisk are significantly different at p<0.05. WT means wild type tomato fruit (△), and ACS means ACS-suppressed transgenic tomato fruit (□).
Figure 5. Bioconversion of lycopene into 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol in wild type and transgenic tomato fruit discs incubated for 6 h. The fruit discs of wild type and transgenic tomato fruits were cut from the pericarp of tomato fruits at the B ripening stage. The fruit discs were incubated in lycopene solution, then the volatiles were measured by headspace analysis after 0, 1, 2, 4, 6 hours’ incubation. Each point represents the mean of 3 replicates and bars represents SD. WT means wild type tomato fruit (△), and ACS means ACS-suppressed transgenic tomato fruit (□).
Figure 1. Hongyan Gao

![Graph showing the change in nC3H8 gFW⁻¹ h⁻¹ with stage of ripening. The graph compares WT and ACS treatments.](image)

**x-axis:** Stage of ripening

**y-axis:** nl C₃H₈ gFW⁻¹ h⁻¹

Legend:
- WT
- ACS
Figure 2. Hongyan Gao

![Graph showing the stages of ripening of lycopene mg/kg FW for WT and ACS with markers for B, B+3, B+7, B+10, and B+40 stages.](image)
Figure 4. Hongyan Gao

![Bar graph showing concentration (ng/g) for various compounds: 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, and geranylacetone. The graph compares WT and ACS conditions.](image)
Figure 5. Hongyan Gao

[Graph showing the peak area of 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol over incubation time for WT and ACS strains.]