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ORIGINAL ARTICLE

Enhanced flux of substrates into polyamine biosynthesis but not ethylene in tomato fruit engineered with yeast *S*-adenosylmethionine decarboxylase gene

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Abstract *S*-adenosylmethionine (SAM), a major substrate in 1-C metabolism is a common precursor in the biosynthetic pathways of polyamines and ethylene, two important plant growth regulators, which exhibit opposing developmental effects, especially during fruit ripening. However, the flux of various substrates including SAM into the two competing pathways in plants has not yet been characterized. We used radiolabeled ^{14}C-Arg, ^{14}C-Orn, L-[U-^{14}C]Met, ^{14}C-SAM and ^{14}C-Put to quantify flux through these pathways in tomato fruit and evaluate the effects of perturbing these pathways via transgenic expression of a yeast SAM decarboxylase (y*SAMDC*) gene using the fruit ripening-specific promoter E8. We show that polyamines in tomato fruit are synthesized both from Arg and Orn; however, the relative contribution of Orn pathway declines in the later stages of ripening. Expression of y*SAMDC* reversed the ripening associated decline in spermidine (Spd) and spermine (Spm) levels observed in the azygous control fruit. About 2- to 3-fold higher levels of labeled-Spd in transgenic fruit (556HO and 579HO lines) expressing y*SAMDC* confirmed the enzymatic function of the introduced gene. The incorporation of L-[U-^{14}C]Met into Spd, Spm, ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) was used to determine Met-flux into these metabolites. The incorporation of ^{14}C-Met into Spd/Spm declined during ripening of the control azygous fruit but this was reversed in fruits expressing y*SAMDC*. However, incorporation of ^{14}C-Met into ethylene or ACC during ripening was not altered by the expression of y*SAMDC* in the fruit. Taken together these results show that: (1) There is an inverse relationship between the production of higher polyamines and ethylene during fruit ripening, (2) the inverse relationship between higher polyamines and ethylene is modulated by *ySAMDC* expression in that the decline in Spd/Spm during fruit ripening can be reversed without significantly altering ethylene biosynthesis, and (3) cellular flux of SAM in plants is homeostatically regulated based on its demand for competing pathways.

Keywords Fruit metabolism · In vivo flux of metabolism · Plant growth regulators · SAM decarboxylase · Tomato · Transgenics

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Abbreviations

SAM	*S*-adenosylmethionine
ACS	1-aminocyclopropane-1-carboxylate (ACC) synthase
ACO	ACC oxidase
ADC	Arginine decarboxylase
dcSAM	Decarboxylated SAM
MTA	Methylthioadenosine
ODC	Ornithine decarboxylase
SAMDC	SAM decarboxylase
Put	Putrescine
Spd	Spermidine
SPDS	Spd synthase
Spm	Spermine
SPMS	Spm synthase

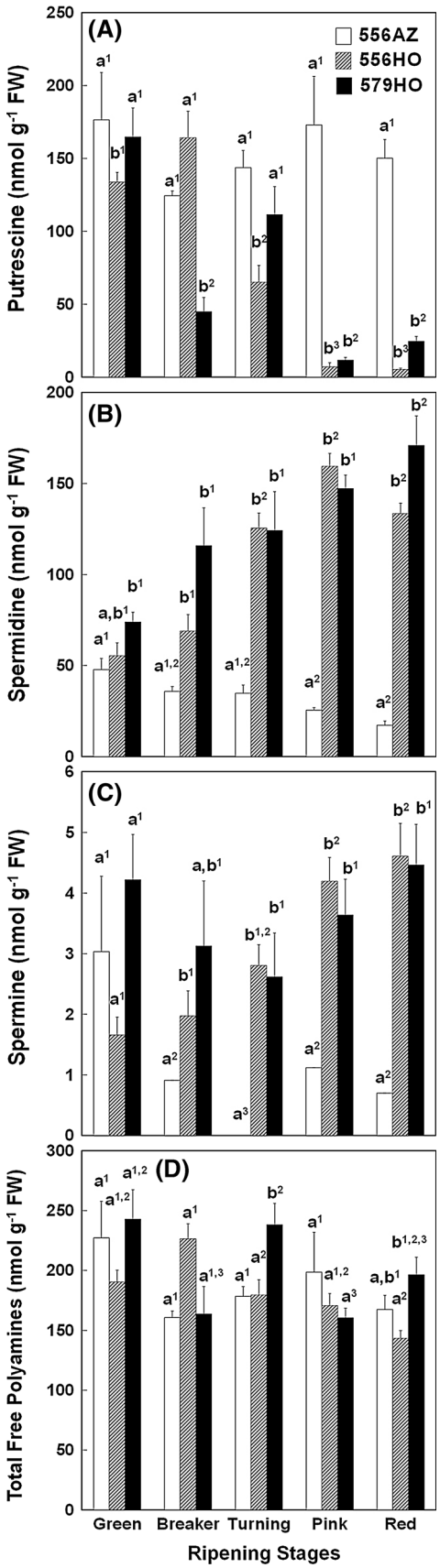
Introduction

Polyamines (PAs), especially putrescine (Put), spermidine (Spd) and spermine (Spm), play critical roles in numerous plant physiological and developmental processes extending from cell division, embryogenesis, root and shoot development, floral initiation and development, and pollen tube growth, to N:C signaling, fruit development and fruit ripening (Mattoo and Handa 2008; Nambeesan et al. 2008; Pegg 2009; Handa et al. 2010). They are often considered as growth promoters and anti-senescence regulators. In contrast, the phytohormone ethylene has been shown to play significant roles in plant senescence and fruit ripening process (Fluhr and Mattoo 1996; Nambeesan et al. 2008; Harpaz-Saad et al. 2012). Since *S*-adenosylmethionine (SAM) is the common precursor for both ethylene and Spd/Spm pathways, a cross talk regulating their biosynthesis and competition for SAM in the tissues has been hypothesized (Fluhr and Mattoo 1996; Quan et al. 2002; Cassol and Mattoo 2003; Harpaz-Saad et al. 2012). The availability of genetic manipulation techniques has also catalyzed studies on influencing fruit ripening in plants via up- or down-regulation of the PA/ethylene pathway genes via transgenic approach (Handa et al. 2010; Gupta et al. 2013; Rudus et al. 2013). However, the flux of met or SAM and other precursors of the PA biosynthesis through the two competing pathways has not been characterized.

Diamine Put, synthesized from either Arg (by Arg decarboxylase—ADC, EC 4.1.1.19) or Orn (by Orn decarboxylase—ODC, EC 4.1.1.17), is the immediate precursor of tri- and tetra-amines, Spd and Spm. Decarboxylated SAM (dcSAM), synthesized from SAM by SAM decarboxylase (SAMDC; *a.k.a.* AdoMetDC, EC 4.1.1.50), donates an aminopropyl group to Put to synthesize Spd and methylthioadenosine (MTA), catalyzed by Spd synthase (EC SPDS, 2.5.1.16). In turn, another aminopropyl moiety from dcSAM reacts with Spd to produce Spm and MTA; the reaction is catalyzed by Spm synthase (SPMS, 2.5.1.22—reviewed in Shao et al. 2012). SAM is also a substrate for 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS, EC 4.4.1.14), which catalyzes the production of ACC and MTA from SAM; ACC is then oxidized by ACC oxidase (ACO, EC 1.14.17.4) to ethylene (Harpaz-Saad et al. 2012; Rudus et al. 2013). MTA in turn is utilized in the methionine salvage cycle. These interrelationships and their possible implications in plant growth and developmental processes have been reviewed recently (Harpaz-Saad et al. 2012).

In vitro studies showing that (1) Put, Spd and Spm inhibited ethylene biosynthesis in a variety of fruit and vegetative tissues (Cassol and Mattoo 2003), and (2) ethylene inhibited the enzymes needed for the biosynthesis of PAs (Apelbaum et al. 1985) brought to light a possible temporal relationship between PAs and ethylene during plant development. Also, these observations fueled the thought that a living plant cell has the potential to commit the flux of SAM either into PA biosynthesis or ethylene biosynthesis, or both. Like ethylene, PA metabolic pathway is highly regulated by a multitude of developmental and environmental signals (see Cohen 1998). Based on these studies, it was hypothesized that a cross talk may exist between the two apparently antagonistic biosynthetic pathways, viz., ethylene and PAs, and thereby influence specific physiological processes in plants (Even-Chen et al. 1982; Roberts et al. 1986; Hyodo and Tanaka 1986; Mehta et al. 1997), although it was later argued that each pathway could prevail only at a specific developmental phase of the plant tissue/organ (see Cassol and Mattoo 2003). For instance, contents of PAs decrease upon the onset of fruit ripening in tomato (Saftner and Baldi 1990; Morilla et al. 1996) and avocado (Kushad et al. 1988) while ethylene production increases. It is unknown if under such developmental situations the two pathways compete for SAM. On the other hand, in a rapidly growing cell culture system of poplar, no competition for ethylene biosynthesis with PA biosynthesis was apparent (Quan et al. 2002).

More impetus to unambiguously test the latter hypothesis came after the technological advances made it feasible to genetically engineer PA pathway in plants (DeScenzo and Minocha 1993; Bastola and Minocha 1995; Bhatnagar et al. 2001, 2002; Mehta et al. 2002; Mayer and Michael 2003; Rea et al. 2004; Alcázar et al. 2005; Wen et al. 2008; Nölke et al. 2008). Transgenic tomato plants were developed in which either yeast *SAM decarboxylase* or *Spd synthase* gene was expressed under the control of a fruit ripening-specific promoter E8 (Mehta et al. 2002; Mattoo et al. 2010; Nambeesan et al. 2010). In these latter studies,



◀ **Fig. 1** Free polyamine content in tomato fruit pericarp of two transgenic (556HO and 579HO) and the control azygous (556AZ) lines during ripening. Putrescine (**A**), spermidine (**B**), spermine (**C**), and total polyamines (**D**). Data are mean ± SE of four replicates. Statistically significant differences ($P \leq 0.05$) among different genotypes at a given stage of ripening are indicated by *different letters*, and differences within the same genotype at different stages of ripening are indicated by *different numbers*

the fruits from independent transgenic plants accumulated high levels of Spd and Spm during ripening while the azygous control plants had relatively low amounts of these two PAs, thus enabling a gain-of-function model system to evaluate the role of PAs in fruit development and ripening. Tomato fruit represents a plant organ at the terminal developmental stage whose cells have stopped dividing and started ripening/senescence programs. Here we present results of a study, which was intended to test the potential competition between the cellular metabolism of ethylene and PAs during the terminal development (i.e., ripening) of control and transgenic tomato fruits using substrate flux experiments.

Materials and methods

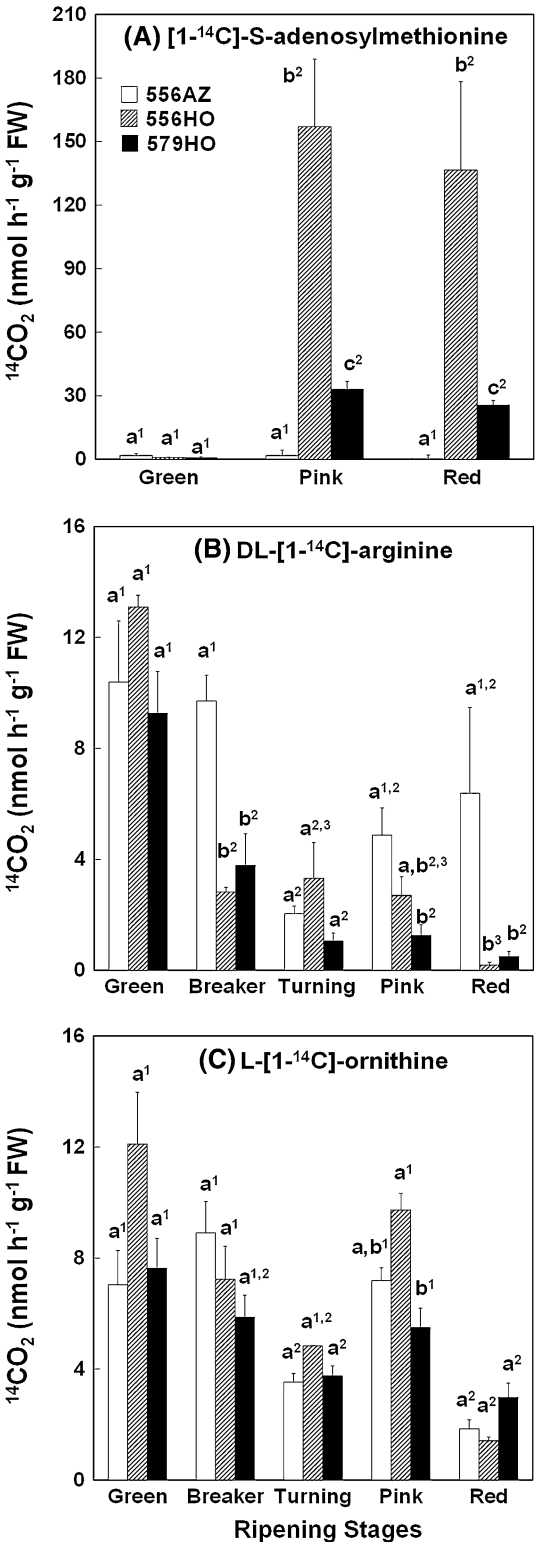
Tomato fruits

Transgenic tomato (*Lycopersicon esculentum* Mill. cv. Ohio 8245) fruits harboring a yeast *SAMDC* gene (*Spe*2) fused to a 2 kb-long fruit-specific E8 promoter, 556HO and 579HO lines, together with the azygous 556AZ control line have been described previously (Mehta et al. 2002). The ripening stages of tomato were established according to color classification of USDA (Mehta et al. 2002). Tomato fruits at different stages of development were collected from the greenhouse-grown plants in Beltsville, MD and transported overnight to the University of New Hampshire, Durham, NH where they were processed immediately for various analyses.

Ethylene and ACC measurements

Ethylene was measured by gas chromatography according to Lizada and Yang (1979) as modified by Quan et al. (2002). Tomato pericarp tissue (2 g FW) was cut into about 20 slices and placed in a 25 ml glass vial, which was capped with a serum stopper. After appropriate periods of incubation, 400 µl of the headspace gas was removed by a syringe and injected into the gas chromatography apparatus for ethylene quantification (Quan et al. 2002).

The cellular ACC content was also determined by modification of the method of Lizada and Yang (1979) as detailed



◀ **Fig. 2** The decarboxylation rates of [1-^{14}C]-*S*-adenosylmethionine (**A**), DL-[1-^{14}C]-arginine (**B**) and L-[1-^{14}C]-ornithine (**C**) by tomato fruit pericarp of 556AZ, 556HO and 579HO lines at different stages of ripening. Data are mean ± SE of four replicates for all stages except for SAM at green stage which had two replicates. Statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by *different letters*, and differences within the same genotype at different stages of ripening are indicated by *different numbers*

in Quan et al. (2002). Half-a-gram FW of tomato pericarp tissue was cut into 5 slices and mixed with 1.0 ml cold 5 % sulfosalicylic acid. The samples were frozen and thawed three times, and centrifuged. Supernatant was saved. Aliquots of 1 ml were sampled, combined with 0.2 ml of 10 mM mercuric chloride, and tubes were stoppered with air-tight serum caps. After leaving them on ice for 10 min, 10 drops of a 2:1 (v/v) solution of commercial Clorox (5.25 % NaClO) and saturated NaOH were slowly added using a 1-ml syringe to release ethylene, which was quantified by gas chromatography. The ACC content (nmol g^{-1} FW) was determined using a standard curve of known quantities of ACC vs. ethylene produced in a similar set-up.

Analysis of soluble polyamines

Slices of fruit pericarp were mixed with cold 5 % perchloric acid (PCA) at a ratio of 1:4 (w/v; 0.2 g tissue in 0.8 ml PCA) on ice. The samples were subjected to three cycles of freezing (−20 °C) and thawing (room temperature), spun at 13,000 g for 10 min, and the supernatant was dansylated as described (Minocha et al. 1994). Dansylated PAs were extracted in toluene, vacuum-dried, and the residue dissolved in 1 ml of 100 % methanol. Polyamines were separated and quantified by HPLC (Minocha et al. 1994).

Activities of arginine decarboxylase, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase

The rates of decarboxylation of DL-[1-^{14}C]-Arg (for ADC activity—Moravek Biochemicals Inc., Berea, CA; sp. act. 56 mCi mmol^{-1}), L-[1-^{14}C]-Orn (for ODC activity—New England Nuclear, Boston, MA; sp. act. 58 mCi mmol^{-1}), and L-[1-^{14}C]-SAM (for SAMDC activity—New England Nuclear, Boston, MA; sp. act. 59 mCi mmol^{-1}) were determined as previously described (Minocha et al. 2004). The set-up included thin slices of tomato fruit pericarp tissue of the three genotypes in test tubes fitted with suspended wells containing traps for CO_2. The optimal reaction times were employed, 30 min for ADC and ODC, and 25 min for SAMDC. Enzyme activities are expressed as nmol $^{14}CO_2$ released h^{-1} g^{-1} FW of tissue.

Incorporation of radiolabeled precursors into polyamines

Half-a-gram FW of pericarp tissue from the three genotypes of tomato fruits was cut into 5–8 thin (2–5 mm)

slices and incubated with 1.5 ml of 0.1 M potassium phosphate buffer (pH 6.8) containing either 0.2 or 0.5 µCi of ^{14}C–labeled substrate {L-[U-^{14}C]-Arg (sp. Act. 258 mCi mmol^{-1}—Amersham Life Sciences, Elk Grove, IL), L-[U-^{14}C]-Orn-HCl (sp. Act. 261 mCi mmol^{-1}—Amersham Life Sciences), or [1,4-^{14}C]-Put-diHCl (107 mCi mmol^{-1}—Amersham Life Sciences). After 8 h of incubation, 150 µl of 100 mM solution of the respective unlabeled substrate was added. Quickly, the tissue slices were washed (twice) with distilled water, soaked on paper towels to remove the surface liquid, placed in cold 7.5 % PCA at a ratio of 1 g tissue in 2 ml PCA, and stored at −20 °C until analysis of dansyl-PAs by Thin Layer Chromatography (TLC) as described (Bhatnagar et al. 2002). The PCA extract (50 µl) was counted for radioactivity to determine the uptake of the precursors. Following dansylation and partitioning of dansyl PAs into toluene (Bhatnagar et al. 2002; Majumdar et al. 2013), an aliquot of 20 µl from the toluene fraction was counted for radioactivity. The remaining toluene fraction was dried under vacuum (Speed-Vac) and the dansyl PAs were dissolved in 90 µl of 100 % methanol. A 40 or 60 µL methanol sample was spotted on TLC plates (Whatman LK6D silica gel 60; Whatman Inc., Clifton, NJ, USA), which were developed in a solvent mixture of chloroform: triethylamine (5:1, v/v). After air-drying, the TLC plates were visualized under UV light using NucleoVision 760 Gel Documentation System, and the silica spots corresponding to Put, Spd and Spm were scraped and counted for radioactivity.

Incorporation of ^{14}C-Met into polyamines and ethylene

Tissue slices (500 mg FW) were incubated with 1 ml of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 µCi of L-[U-^{14}C]-Met (285 mCi mmol^{-1}—Amersham Life Sciences) in 16 × 100 mm glass tubes. A 2-ml microfuge tube without cap, containing 1 ml of 0.1 M mercuric acetate (dissolved in 100 % methanol) was suspended in the reaction tube to adsorb ^{14}C-ethylene that was released. In addition, a polypropylene Kontes well (Kontes Scientific Instruments, Vineland, NJ, USA) containing 2 cm^2 filter paper soaked with 50 µl Scintigest was suspended in the tube from a silicon cap to absorb $^{14}CO_2$. After 6 h of incubation, 100 µl of cold L-Met (100 mM in ddH_2O) was injected into the reaction mixture. An aliquot of mercuric acetate solution as well as the filter paper with Scintigest was counted separately for radioactivity in ethylene and CO_2, respectively. The tomato tissue was washed twice with distilled water, soaked on a paper towel, and transferred into cold 5 % SSA at a ratio of 1 g FW to 2 ml SSA. The mixture was kept at −20 °C until further analysis of ^{14}C-ACC and ^{14}C-PAs.

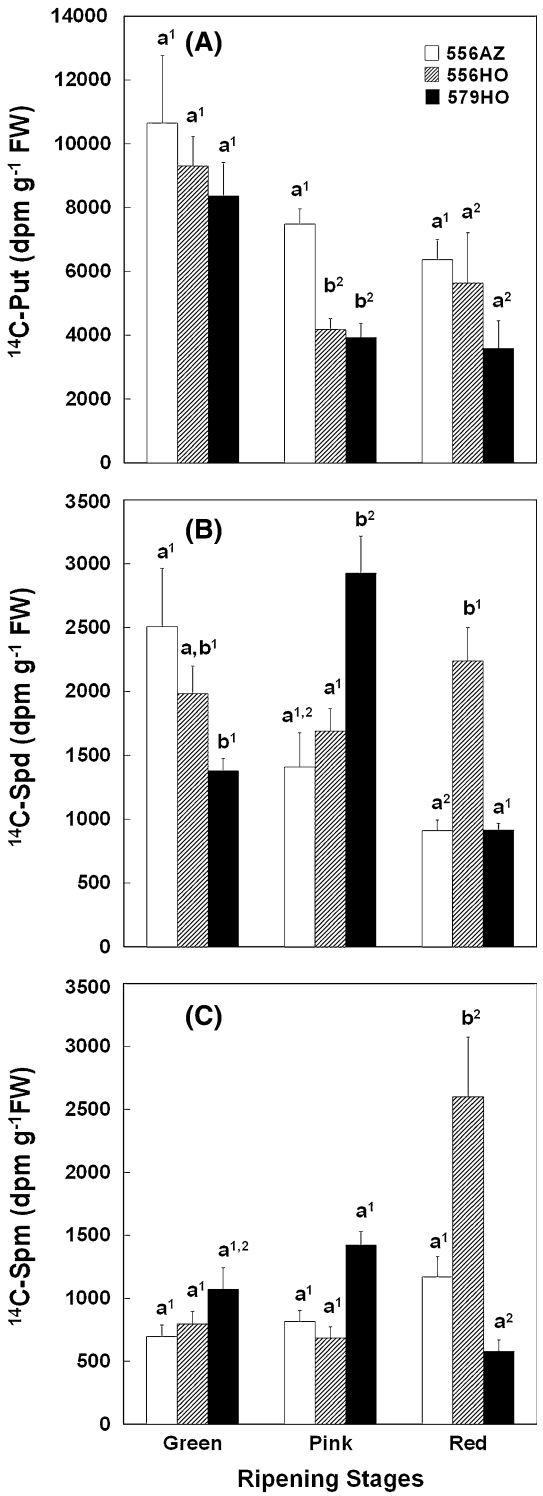


Fig. 3 The amount of radioactivity incorporated from L-[U-^{14}C]-arginine (8 h incubation) into free putrescine (**A**), spermidine (**B**), and spermine (**C**) in tomato fruit pericarp of 556AZ, 556HO and 579HO lines at different stages of ripening. Data are mean ± SE of four replicates. Statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by *different letters*, and the differences within the same genotype at different stages of ripening are indicated by *different numbers*

Measurement of ^{14}C-ACC production

The tissue/SSA suspensions collected in the feeding experiments of L-[U-^{14}C]-Met were frozen (−20 °C) and thawed (room temperature) three times. After centrifugation at 13,000 g for 10 min, 300 µl of the tissue extract (the supernatant) was used for the analysis of ^{14}C-ACC produced in the tomato tissue, while another 400 µl of the tissue extract was used for the analysis of free ^{14}C-PAs. The ^{14}C-ACC was quantified by measuring the production of ^{14}C-ethylene from the reaction, using the procedure similar to that described above for determination of cellular ACC. The ^{14}C-PAs were analyzed as described above.

Statistical analysis

For all experiments involving quantitative analysis, two-to-eight replicates (as indicated in Figure legends) were used for each treatment. Most experiments were repeated two to four times, except for the L-[U-^{14}C]-Met incorporation experiment which was done only once. The results from repeat experiments showed similar pattern of changes even though the exact numbers may have varied. Data from a single representative experiment are shown here for each treatment. The data were subjected to one-way Analysis of Variance (ANOVA) using SYSTAT version 9. Tukey's-test was used to determine significance. The labels of statistical significance are described in Figure legends. In most Figures, statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by different letters, and differences within the same genotype at different stages of ripening are indicated by different numbers.

Results

Profiles of polyamines in the control and the transgenic fruit follow opposite trends during ripening and correlate with SAMDC activity

Putrescine content in the fruit pericarp tissue of the control azygous line (556AZ) varied slightly during ripening, but the differences were not significant (Fig. 1A). In both transgenic lines (556HO and 579HO), however, Put content decreased several-fold during ripening, starting at the breaker or the turning stage. Concomitant with the decreasing trend in Put content in the transgenic fruits, Spd content of the pericarp tissue increased with onset of the ripening process (coincident with the induction of E8 promoter) and continued to rise up to the red stage of ripeness (Fig. 1B). In the control azygous fruit (556AZ), Spd content was generally lower and decreased further as

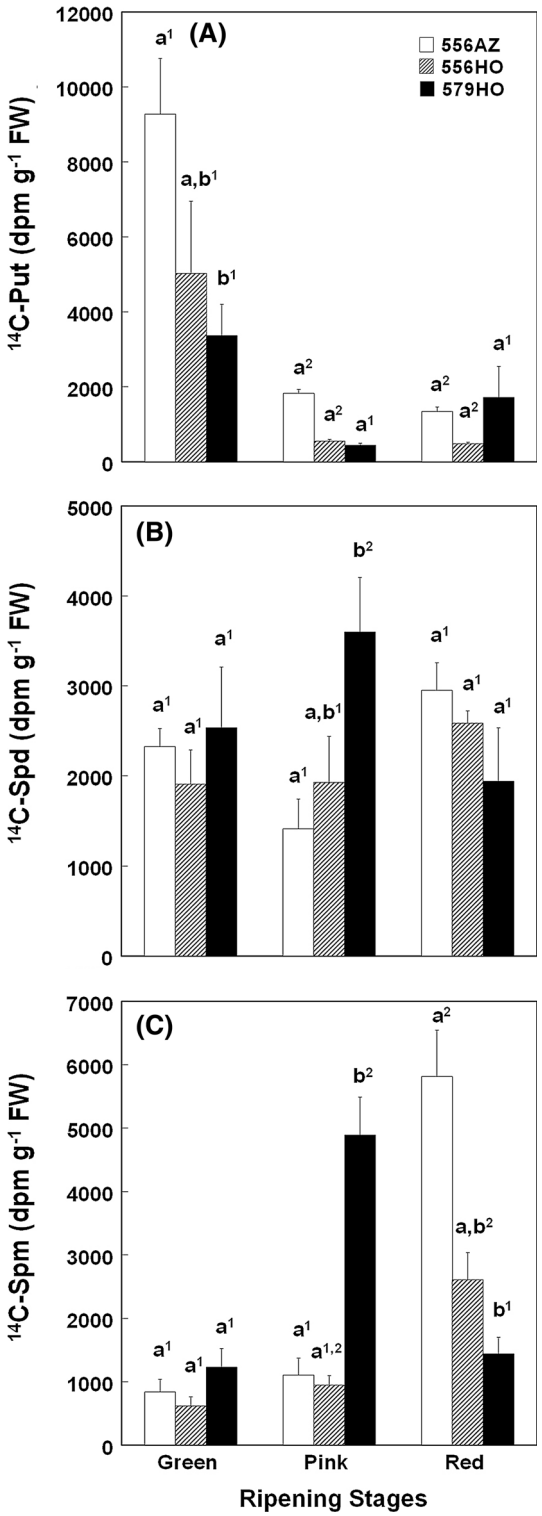


Fig. 4 The amount of radioactivity incorporated from L-[U-^{14}C]-ornithine (8 h incubation) into free putrescine (**A**), spermidine (**B**), and spermine (**C**) in tomato fruit pericarp of 556AZ, 556HO and 579HO lines at different stages of ripening. Data are mean ± SE of four replicates. Statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by *different letters*, and the differences within the same genotype at different stages of ripening are indicated by *different numbers*

Table 1 Radioactivity present in the aqueous and the toluene fractions (combined representing uptake) after dansylation of the PCA ± SE of four replicates

Substrates	Ripening stage	Tomato line	Aqueous (dpm g^{-1} FW) Mean ± SE	Toluene (dpm g^{-1} FW) Mean ± SE
L-[U-^{14}C]-arginine	Green	556AZ	186,988 ± 1,385	21,791 ± 931
		556HO	177,846 ± 6,094	19,866 ± 1,028
		579HO	176,788 ± 6,368	21,387 ± 1,653
	Red	556AZ	251,488 ± 17,359*	15,409 ± 1,601
		556HO	289,842 ± 14,067*	19,193 ± 1,902
		579HO	260,743 ± 13,526*	15,446 ± 1,462
L-[U-^{14}C]-ornithine	Green	556AZ	180,756 ± 8,330	53,644 ± 15,007
		556HO	178,825 ± 7,352	29,414 ± 1,544
		579HO	177,418 ± 13,390	29,535 ± 2,069
	Red	556AZ	186,733 ± 10,178	32,400 ± 5,049
		556HO	198,470 ± 13,402	67,643 ± 27,306
		579HO	211,144 ± 23,890	35,119 ± 4,816
[1,4]-^{14}C-putrescine	Green	556AZ	62,577 ± 3,832	272,952 ± 21,835
		556HO	66,434 ± 9,989	244,507 ± 42,484
		579HO	62,935 ± 2,289	272,810 ± 11,697
	Red	556AZ	104,409 ± 30,661	152,503 ± 46,918
		556HO	62,811 ± 15,897	193,823 ± 75,978
		579HO	100,590 ± 29,300	177,499 ± 48,875

Total amount of radioactivity used in each case was 0.5 μCi

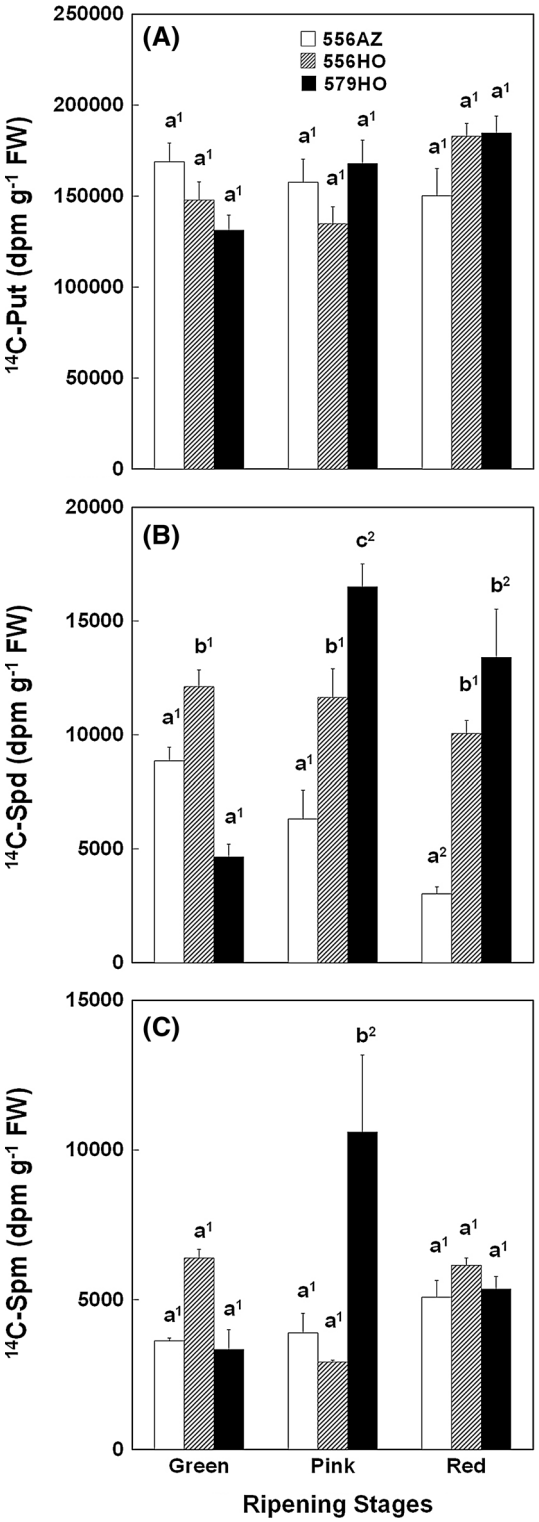
* Statistically significant difference between the green and the red stage for a given genotype

the fruit ripened. Therefore, while the Spd contents in the pericarp tissue of the fruits were quite similar in all three lines at the green stage, in the fruit of both the transgenic lines Spd was several-fold higher than the control fruit at the pink and red stages. Spermine, which constituted only a small proportion (typically <3 %) of total PAs in the fruit, also showed a decline in the control fruit with the onset of ripening but its content considerably increased in the transgenic 556HO fruit; in the fruit of transgenic 579HO line, it remained at a steady but higher level throughout ripening (Fig. 1C). Like Spd, Spm content was several-fold higher in the transgenic fruits at the later stages of ripening as compared to the control fruits. The total PA content of the fruits did not change much in any genotype as the loss in Put was compensated by the increase in Spd/Spm during fruit ripening (Fig. 1D).

Results presented in Fig. 1 indicate that the higher steady state levels of Spd/Spm in the transgenic fruits could involve higher conversion of decarboxylated SAM (dcSAM), likely due to higher activity of the enzyme SAMDC. The decarboxylation of SAM by SAMDC is known to be a rate-limiting step for the biosynthesis of Spd and Spm (Mehta et al. 2002). Our strategy to introduce *ySAMDC* coding sequence in tomato under the control of a fruit ripening-specific promoter was to augment the accumulation of Spd and Spm in the transgenic fruits. In order to confirm if the observed changes in Spd and Spm were consistent with higher SAMDC activity in the transgenic fruit, we directly measured decarboxylation of radioactive SAM (SAMDC activity) in the fruit pericarp tissue. The azygous control fruit had consistently low SAMDC activity at all stages of fruit ripening (Fig. 2A). In contrast to the azygous control fruit, pericarp of both transgenic fruits had higher rates of SAM decarboxylation as the fruit ripened. The most dramatic increase in SAMDC occurred in 556HO fruit at the pink stage, the activity remained high at the red stage as well. The activity of SAMDC in the 556HO fruit was almost tenfold higher than the 579HO fruit, and a hundred-fold higher than the azygous fruit at the pink stage. These data confirm the fruit ripening-specific induction of *ySAMDC* transcripts and their metabolic effects on Spd/Spm production consistent with the properties of the E8 promoter in tomato (Mehta et al. 2002).

The activities of ADC and ODC are not affected by tissue contents of Spd and Spm

The rate of decarboxylation of ^{14}C-Arg (ADC activity) in the three fruit genotypes at the green stage was quite comparable and showed a decline from the green to the turning stage (Fig. 2B); the decline being faster in the transgenic fruits than in the azygous control fruit as ripening progressed (Fig. 2B). While a further decline in ADC with advance in ripening was observed in the two



◀ **Fig. 5** The amount of radioactivity from L-[U-^{14}C]-putrescine present as free putrescine (**A**), spermidine (**B**), and spermine (**C**) in tomato fruit pericarp (at different stages of ripening) of 556AZ, 556HO and 579HO lines. The incubation time was 8 h. Data are mean ± SE of four replicates. Statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by *different letters*, and the differences within the same genotype at different stages of ripening are indicated by *different numbers*

transgenic fruits, it was not the case for the control fruit. The rate of decarboxylation of Orn (i.e. ODC activity) in the three genotypes typically showed smaller changes with ripening, and differences among the three genotypes at any stage of ripening were also small (Fig. 2C).

Increased accumulation of Spd and Spm is due to increased biosynthesis

Although it was apparent from PA analysis and enzyme activity measurements that the increase in Spd/Spm in the transgenic fruit during ripening came from increased use of Put and induced SAMDC activity, we also wanted to determine the source of Put (ADC or ODC) being used in Spd and Spm biosynthesis in the three genotypes. For this we directly measured the incorporation of radioactivity from ^{14}C-labeled substrates of Put (i.e., Arg and Orn), ^{14}C-labeled Put itself and ^{14}C-labeled Met into PAs in the pericarp tissue of the fruit at three stages of ripeness.

Radioactive Put recovered from fruit pericarp tissue fed with ^{14}C-Arg was significantly lower at the pink stage in both transgenic lines and also at the red stage in the 579HO transgenic line as compared to the azygous control fruit during the 8-h incubation period (Fig. 3A). Overall, the ^{14}C-Arg incorporated into Put fraction was lower with the progress in ripening in all three genotypes, the difference being greater in the transgenic fruits than the azygous control. During the same period, the amount of ^{14}C incorporated into Spd was smaller than that in Put, and the trend of changes with progression of ripening was different in the three genotypes (Fig. 3B). While the content of ^{14}C-Spd in the control fruit was lower at pink (by 50 %) and red (by 30 %) stages (vs. the green stage), in the 579HO fruit, radioactivity in Spd was almost twice at the pink stage than the green stage, but it was lower at the red stage (Fig. 3B). The transgenic 556HO fruit showed only a small difference in ^{14}C-Spd with the stage of ripening. Thus, the transgenic fruits retained the ability for sustained Spd biosynthesis at the pink and red stage. Whereas, a low but sustained flow of radioactivity from ^{14}C-Arg into Spm (via Put and Spd) was seen in the control fruit throughout ripening, the amount of radioactivity recovered in this fraction in the 556HO transgenic line was considerably higher at the red fruit stage (Fig. 3C).

The pattern of changes in the incorporation of ^{14}C-Orn into Put, Spd and Spm was quite similar to that seen for ^{14}C-Arg (Fig. 4); however, the actual amounts incorporated into Put in the transgenic fruit tissue were relatively low (cf. Fig. 3A). Also, differences with the stage of maturity for ^{14}C-Put were more pronounced in that the pink

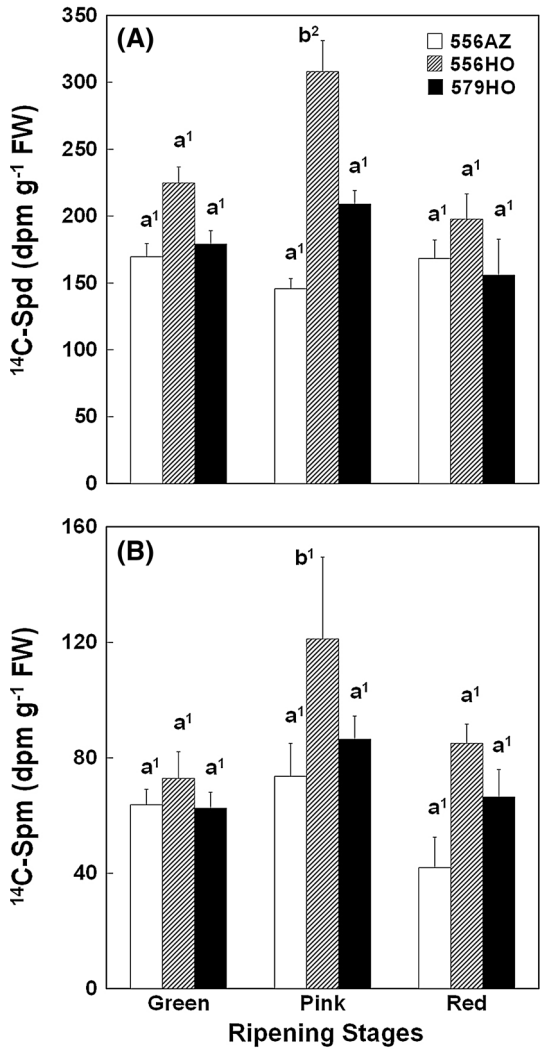


Fig. 6 The amount of radioactivity incorporated from L-[U-^{14}C]-methionine (6 h incubation) into free spermidine (**A**), and spermine (**B**) in tomato fruit pericarp of 556AZ, 556HO and 579HO lines at different stages of ripening. Data presented are mean ± SE of four replicates. Statistically significant differences ($P \leq 0.05$) among the genotypes at a given stage of ripening are indicated by *different letters*, and the differences within the same genotype at different stages of ripening are indicated by *different numbers*

and red stage showed only small amounts of ^{14}C-Orn to be present as Put (Fig. 4A). At the green stage, the amount of ^{14}C-Put derived from ^{14}C-Orn was comparable with that of ^{14}C-Arg in the control fruit, but it was lower in the transgenic fruit; thereafter, it decreased dramatically at the outset of ripening in all three genotypes (Fig. 4A). Despite lesser incorporation of ^{14}C-Orn into Put in the transgenic fruits, its incorporation into Spd was either similar (at the green and red stage) or higher in the transgenic fruit than the control fruit (Fig. 4B). The further transfer of ^{14}C from Spd into Spm in the three genotypes followed a trend similar to that in Spd, the transgenic fruit 579HO having the highest amount of ^{14}C-Spm at the pink stage of ripening (Fig. 4C).

Similar amounts of ^{14}C-Orn and ^{14}C-Arg were taken up by the pericarp tissue of all three genotypes during the 8-h incubation period at the green and the red stages of fruit ripening; the uptake of ^{14}C-Arg was, however, greater in the red than the green stage fruit (Table 1). The distribution of label between the PAs (i.e., the toluene fraction, which may also contain a small amount of non-polar catabolic products of Put) and the unincorporated ^{14}C-Arg/^{14}C-Orn (i.e., the aqueous fraction, which also contains the polar catabolic products of Put) was also similar among the three genotypes for both precursors at the green and the red stage.

While the three genotypes did not differ from one another in the uptake of ^{14}C-Put (Fig. 5A), they differed significantly in the conversion of ^{14}C-Put into Spd (Fig. 5B, C). Radioactive Spd produced from ^{14}C-Put was significantly higher in the two transgenic fruits (compared to the control) at all stages with the exception of 579HO, which was lower at the green stage (E8 promoter is not active at this stage). The conversion of Put into Spd in the azygous control fruit decreased with the progression of ripening, the ^{14}C-Spd at the red stage being less than half of that at the green stage. In the 556HO and 579HO fruits, ^{14}C-Spd accumulation was sustained at levels higher than that in the control fruit at both the pink and the red stage (Fig. 5B). Thus, fruits from both the transgenic lines converted more of ^{14}C-Put into Spd than the control fruit as the ripening commenced. This was coincident with activation of the promoter controlling the *ySAMDC* transgene expression in these fruits. The amount of ^{14}C-Spm was for most part similar among the three genotypes except for 579HO, which had significantly higher incorporation of ^{14}C-Put into Spm at the pink stage (Fig. 5C).

Since the biosynthesis of higher PAs involves the lower PAs as well as aminopropyl groups from dcSAM (Mehta et al. 2002; Shao et al. 2012), we also studied the incorporation of label from ^{14}C-Met into Spd and Spm at three stages of ripening in the three tomato genotypes. The data presented in Fig. 6 show that the results were generally consistent with those for the incorporation of ^{14}C-Put into higher PAs in that greater amounts of ^{14}C-Met were incorporated into these PAs in the transgenic fruit pericarp tissue compared to the control fruit during ripening. Because of the indirect way of Met incorporation into PAs (i.e., via SAM and then dcSAM), the amount of radioactivity incorporated from ^{14}C-Met into Spd and Spm was relatively small in all the three genotypes. The ^{14}C in Spd from ^{14}C-Met in the three genotypes was typically more than twice as much as ^{14}C-Spm within the respective line at any given ripening stage (Fig. 6). The incorporation of ^{14}C-Met into Spd and Spm in the azygous control fruit did not

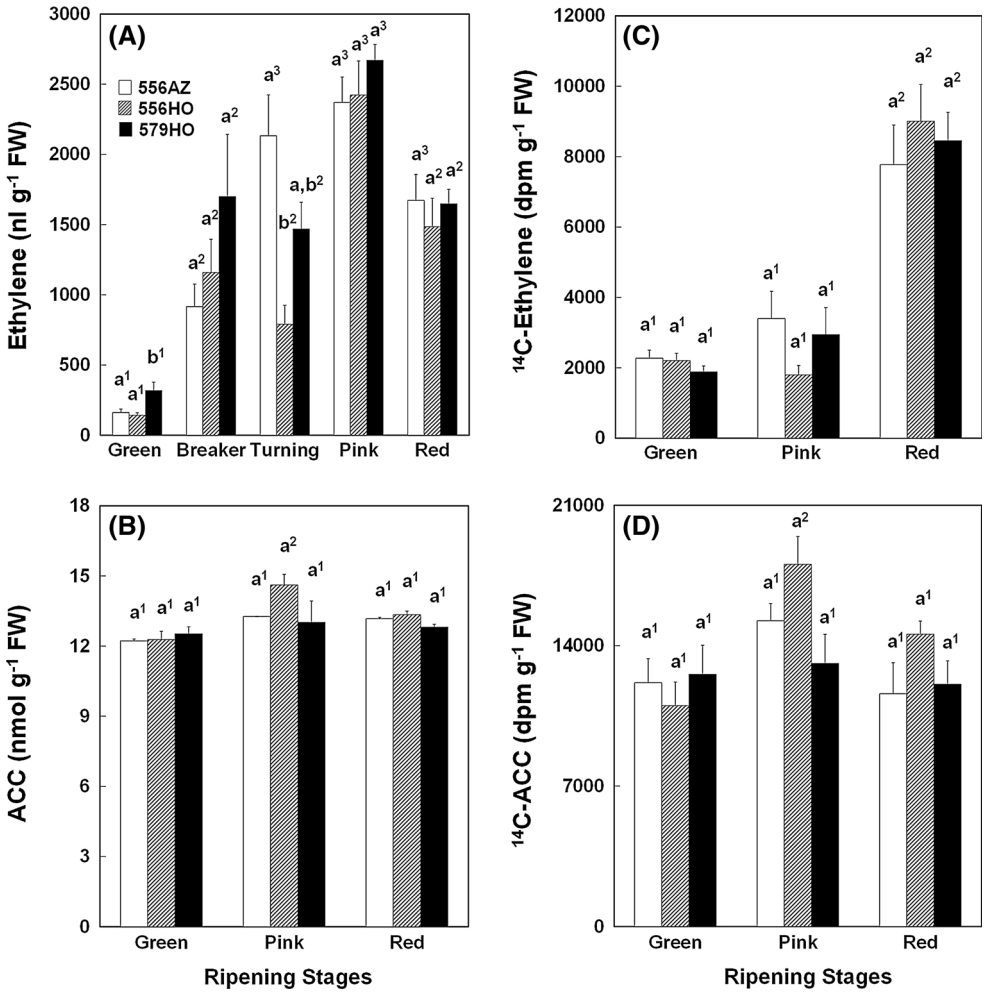


Fig. 7 Cellular ACC content (**A**) and ethylene production (**B**) and the amount of radioactivity incorporated from L-[U-^{14}C]-methionine (6 h incubation) into ethylene (**C**) and ACC (**D**) in tomato fruit pericarp of 556AZ, 556HO and 579HO lines during ripening. Data presented are mean ± SE of four replicates. Statistically significant difference ($P \leq 0.05$) among the genotypes at a given stage of ripening is indicated by *different letters*, and differences within the same genotype at different stages of ripening are indicated by *different numbers*

change much during fruit ripening. The incorporation of ^{14}C-Met into both Spd and Spm by the transgenic fruits was somewhat higher and maximum at the pink stage, the difference being more significant in 556HO.

Ethylene content and its biosynthesis are not affected by changes in polyamines

Following the premise of the study about the question "whether increased use of SAM for Spd/Spm biosynthesis during ripening, particularly in the transgenic tomato would diminish its availability for ethylene production", we analyzed both the steady state levels of ethylene and ACC, and the short-term incorporation of ^{14}C-Met into ACC and ethylene. Figure 7A shows changes in ethylene production in fruit pericarp tissue of the three genotypes over an accumulation period of 25 h. A similar trend in ethylene production during the ripening process was seen for all genotypes, i.e., it generally increased with the progression of ripening, reaching a peak at the pink stage, and then decreasing significantly at the red stage. The pericarp tissue from transgenic tomato fruits produced similar amounts of ethylene as the control fruit at most of the ripening stages, except at the turning stage, where the 556HO transgenic fruit produced less than half the amount of ethylene as the control fruit. The cellular contents of ACC in the three fruit genotypes were also similar and remained unchanged during ripening (Fig. 7B).

The incorporation of ^{14}C-Met into ethylene by the three tomato lines was comparable at a given stage of ripening, except that, at the red stage, ^{14}C incorporation into ethylene was >twofold higher than that at the earlier stages in all cases (Fig. 7C). The flux of Met into ethylene in the three genotypes was therefore not altered. The immediate precursor of ethylene is ACC (derived from Met). We therefore quantified ^{14}C-ACC in the pericarp tissue as well. Except for a somewhat higher amount of ^{14}C-ACC found in the 566HO fruit at the pink stage, there was no significant difference in the amounts of radioactive ACC produced from ^{14}C-Met among the three genotypes or at any stage of fruit ripening. The amount of radioactive ACC produced from ^{14}C-Met was not different during ripening nor among the three genotypes at a given stage of ripening (Fig. 7D). It is also apparent from the data in Figs. 6 and 7

that the amount of Met used for ACC and ethylene production far-exceeded (>tenfold higher) that used for Spd/Spm biosynthesis during the same period of incubation. However, it is important to point out here that the data and conclusions drawn about ethylene/ACC content are related to flux studies in tomato upon wounding (slicing of the pericarp—Li et al. 1992) and may differ from in vivo studies (Mehta et al. 1997, 2002) with whole fruit, which are difficult to design in an unambiguous manner.

Discussion

SAM decarboxylase as a rate-limiting enzyme directs the flux of precursors into the PA with little effect on the ethylene pathway in tomato fruit

Animals and plants use SAM for methylation/transmethylation reactions involving C1 metabolism as well as PA biosynthesis, plants additionally use SAM for the biosynthesis of the plant hormone ethylene. In ethylene biosynthesis pathway, SAM is a substrate for the enzyme ACC synthase that catalyzes the synthesis of ACC, an immediate precursor, which is then terminally converted to ethylene by ACC oxidase (Yang and Hoffman 1984; Fluhr and Mattoo 1996; Harpaz-Saad et al. 2012). In lieu of the generally antagonistic functions of the two growth regulators, ethylene as a ripening and senescence promoter and PAs as promoters of growth and anti-senescence regulators, questions have been raised about possible intracellular competition for SAM between the two pathways, particularly at a stage when developmental switch from the growth phase to a ripening/senescence phase occurs (Mattoo and Suttle, 1991; Cassol and Mattoo 2003; Harpaz-Saad et al. 2012; Gupta et al. 2013). An inter-connection between ethylene and PAs in plant processes was also suggested during rice submergence and potato suberization in studies on acireductone dioxygenase (ARD), a regulatory enzyme which catalyzes the reaction of acireductone with dioxygen and produces 2-keto-4-methylthiobutyrate, the immediate precursor of methionine (Sauter et al. 2005; Bürstenbinder et al. 2007, 2010; Rzewuski et al. 2007; Kim et al. 2008).

We addressed the potential competition between the cellular biosynthesis of ethylene and PAs during the terminal development (i.e., ripening) of tomato fruits by performing substrate flux experiments using azygous control and two independent transgenic tomato lines previously developed to be homozygous with yeast SAM decarboxylase (*ySAMDC*) gene and driven by a ripening-specific E8 promoter (Mehta et al. 2002). Results presented here on flux distributions between substrates and products of the two pathways in the stated tomato lines demonstrate that SAM decarboxylase is a rate-limiting enzyme during ripening and controls the flux of precursors Met/SAM, and indirectly Put, into the higher PA pathway. In the tomato fruit pericarp of ySAMdc lines (556HO and 579HO), Spd and Spm contents were high and/or increased with the progression of ripening. The results on steady state levels of PAs were quite consistent with the changes in the activities of the PA biosynthetic enzymes and the flux of ^{14}C-labeled PA substrates into the various PAs.

In traditional assays for determination of the activities of ADC, ODC and SAMDC, $^{14}CO_2$ released from decarboxylation of the respective [1-^{14}C]-labeled substrate by tissue homogenates is trapped in an alkaline solution and counted. In this study, in vivo assays using intact tissue (pericarp slices) of developing fruit allowed us to avoid the laborious steps of homogenization and, at the same time, minimizing effects of the pH of external buffer. Also, such assays would likely mimic the cellular environment for measurement of enzyme activities; the experimental procedure was previously used to quantify ADC, ODC and SAMDC activities in a manner comparable to traditional assays with relatively small amounts of live tissue (Minocha et al. 1999). Furthermore, we complemented the enzyme activity assays with the incorporation of [U-^{14}C]-labeled substrates into PAs, ACC and ethylene to substantiate the increase in flux of the PA substrates toward higher PA accumulation at different stages of fruit ripening, and found a positive correlation between the two approaches. The observation that the activities of ADC and ODC declined with the ripening process and that of SAMDC was higher in the transgenic fruit during ripening are consistent with the reduced amounts of ^{14}C-Put and higher amounts of ^{14}C-Spd and ^{14}C-Spm being produced from the substrates in later stages of fruit ripening.

Fruits over-expressing the *ySAMDC* coding sequence have delayed ripening and other useful agronomic traits (Mattoo and Handa 2008; Nambeesan et al. 2008; Handa et al. 2010; Rudus et al. 2013). The increased production of higher PAs (in the *ySAMDC* transgenics) during ripening seems to delay the ripening process without affecting ethylene production. While several-fold increase in ethylene production was apparent during the progression of ripening, we did not observe a significant change either in the cellular ACC content or in the incorporation of ^{14}C-Met into ACC in this study. From these observations it is apparent that the biosynthesis of ACC keeps pace with its conversion to ethylene by ACC oxidase, even though its substrate SAM is being diverted toward Spd/Spm biosynthesis in larger quantities, maintaining a homeostatic steady state level of this metabolite in the fruit tissue. It can thus be argued that the increase in SAM utilization in the transgenic fruits does not come at the expense of its reduced use/demand for ethylene production, supporting

the view that there is little competition for SAM utilization between the biosynthesis of higher PAs and ethylene in the tomato fruit during ripening. This conclusion is consistent with previous studies showing that ethylene and higher PAs can be synthesized concurrently without a drain on cellular SAM (Mehta et al. 2002; Quan et al. 2002; Harpaz-Saad et al. 2012). Thus, we provide direct experimental evidence for the increased flux of SAM toward the PA pathway in the *ySAMDC*-transgenic fruit to have no effect on the production of ACC or ethylene during the later stages of fruit ripening. We validate this through two complementary approaches; i.e. enzyme assays and the incorporation of the labeled substrates for Spd and Spm biosynthesis. Moreover, the 'competition concept' need not hold under particular developmental phase of a plant tissue when only one or the other pathway is predominant. Notably, in tomato [(Saftner and Baldi 1990; Morilla et al. 1996; experiments in this and prior study (Mehta et al. 2002) with control azygous tomato fruit)] and avocado (Kushad et al. 1988) fruits, the content of Spd and Spm decreases as fruits ripen with a concurrent increase in ethylene synthesis.

Catabolism of Put decreases during ripening and tomato fruit have insignificant back conversion of Spd and Spm into Put

Both the analysis of steady state levels of ACC as well as the incorporation of ^{14}C-Met into ACC and C_2H_4 in the control and the transgenic wounded tomato pericarp tissues show the results to be similar to those with poplar cells (Quan et al. 2002). It appears that under the experimental conditions employed cellular pools of Met and SAM in the pink stage tomato pericarp tissue are large, so that ^{14}C-Met from exogenous supply is only present as a low percentage of the total Met or SAM. As the fruit ripening proceeds, intracellular Met in wounded tomato tissue may become more saturated with exogenously supplied ^{14}C-Met, thus leading to the production of more ^{14}C-ethylene. This is evident from the increased production of the radioactive ethylene by the red stage tomato. The radioactivity in ACC fraction in the wounded tissue remained roughly the same throughout the ripening process, which was similar to the trend of steady state cellular ACC content in the tissue. Similar amounts of ^{14}C-labeled ACC present in the tomato tissue throughout tomato fruit ripening suggest that ACC biosynthesis is regulated by a homeostatic mechanism throughout the ripening process. Apparently, therefore, concentrations of SAM channeled into ethylene and PA biosyntheses may be relatively small compared to SAM utilization for other metabolic reactions as proposed earlier (Quan et al. 2002), and possibly therefore plant cells maintain large pool(s) of SAM. This also seems to be true for wound ethylene. The high rate of ethylene production (>100 nl g^{-1} FW h^{-1}) from fruit slices is indicative of wound-associated instead of ripening-specific ethylene (10–20 nl g^{-1} FW h^{-1}). Expression of transgene *SAMDC* did not influence production of wound ethylene in tomato fruit slices suggesting that SAM was not limiting even when plant tissue was producing higher amounts of ethylene due to wounding and further confirming that the two pathways are not limited by the amount of SAM present in fruit tissue.

While the incorporation of ^{14}C-Met into PAs represented a small fraction of Met taken up by the tissue, the amounts of ^{14}C-Spd and ^{14}C-Spm in the transgenic fruits were higher than the control fruit especially at later stages of ripening. This is consistent with the effects of *SAMDC* transgene that results in higher SAMDC activity during ripening of the transgenic fruits. There was hardly any radioactivity found in the Put fraction from the incorporation of ^{14}C-Met, showing that there was little if any back conversion of Spd and Spm into Put in the tomato tissues. The conversion of Spd and Spm back into Put has been reported in animal cells (Cohen 1998) and also in some plants (Caffaro et al. 1993; De Agazio et al. 1996; Moschou et al. 2008a, b; Shao et al. 2012). When the tomato pericarp tissue was incubated with ^{14}C–Put, $^{14}CO_2$ mainly came from Put catabolism since its conversion to Spd does not release CO_2. The production of $^{14}CO_2$ from ^{14}C–Put by all three lines of tomato decreased with fruit ripening; the transgenic fruits produce larger amounts of $^{14}CO_2$ than the control fruit at any given stage of ripening. These data indicate that Put catabolism in the tomato pericarp tissue slows down with the ripening process, but its catabolism is faster in the transgenic fruit than the control fruit as ripening progresses mainly because SAM decarboxylase activity is greatly activated by the introduced yeast SAM decarboxylase gene (Mehta et al. 2002).

Are SAM levels under a homeostatic regulation?

A major difference between the in vitro and in vivo experiments is that, by design, in vitro studies reveal a short-term response while the in vivo studies provide a much longer duration of a response. We had previously demonstrated that ACS transcript levels in tomato slices (an in vitro system) are inhibited when incubated with Spd and Spm within the first 10–12 h of incubation (Li et al. 1992). Therefore, we were surprised when we discovered higher rates of ethylene biosynthesis in transgenic tomato fruits that accumulate high levels of Spd and Spm during a long term (over a week and more) on the shelf or on the vine (Mehta et al. 2002). If there is a competition for SAM in plant cells, it may be a transient one, which could signal a homeostatic mechanism to maintain intracellular SAM levels. The nature of this homeostasis is not known. In mammalian cells, studies with glycine *N*-methyltransferase (GNMT)-deficient humans and GNMT knocked-out mouse revealed this enzyme to be a driving

regulator for cellular SAM levels (Luka et al. 2009). The authors proposed that this regulation is likely critical for SAM homeostasis, occurring post-translationally (via inhibition of the enzyme by folate) or transcriptionally. It remains to be shown if plant cells also invoke a similar mechanism to regulate cellular SAM levels.

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Conflict of interest The authors declare no conflicts of interest.

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