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ORIGINAL ARTICLE

Fruit-specific over-expression of human S-adenosylmethionine decarboxylase gene results in polyamine accumulation and affects diverse aspects of tomato fruit development and quality

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Abstract Polyamines (PAs) have been implicated in fruit ripening where they antagonize the action of ethylene: a ripening inducing phytohormone. S-adenosylmethionine decarboxylase (SAMDC) is a key enzyme involved biosynthesis of higher PAs- spermidine and spermine. Here, we report the genetic modification of tomato fruit ripening and quality by over-expressing human-*SAMDC* driven by fruit-specific promoter (2A11). The PA analysis of ripening fruits from these transgenics showed elevated PA levels in comparison to wild-type (WT). The increased levels of higher PAs are correlated with the accumulation of heterologous *SAMDC* transcripts in such fruits. Transgenic fruits exhibited reduced levels of ethylene (~50 %) production, ~10 days delay in on-vine ripening and extended post-harvest storage of ~11 days as compared to the WT fruits. As a result, these fruits showed improvement in various ripening traits like enhanced lycopene, vitamin C and total soluble solid. In Lesam fruits, an up-regulated expression of Sly*SAMDC*, Sly*EXP*1, Sly*TBG*4, Sly*DXS* 1 and Sly*PSY* 1 was observed, while ethylene biosynthesis genes were down-regulated. Here, we have demonstrated the important role of PAs in altering the molecular and biochemical processes underlying fruit ripening by interfering with the ethylene biosynthesis.

Keywords Tomato · Polyamines · S-adenosylmethionine decarboxylase · Ethylene · Fruit ripening · Transgenic plants

Abbreviations

Put	Putrescine
SAMDC	S-adenosylmethionine decarboxylase
Spd	Spermidine
Spm	Spermine

Introduction

Tomato fruit ripening being climacteric in nature is majorly governed by ethylene. This crop is prone to various post-harvest damages which cost up to 40 % yield loss. Excessive softening and over-ripening are the major causal factors for such losses. Efforts have been made to develop crop plants with reduced post-harvest losses by either delaying the ripening process by down-regulating ethylene metabolism or reducing fruit softening by manipulating the cell wall/membrane metabolism. In order to improve the processing attributes of tomatoes, scientists have attempted to reduce the activity of cell-wall degrading enzymes such as cellulase, polygalacturonase (PG), and pectinesterase (PE) through genetic modification (Sheehy et al. 1988; Phan et al. 2007; Meli et al. 2010). Inhibition of membrane lipid

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catabolism is another alternative for delaying senescence, improving shelf life and quality in ripening fruits (Pinhero et al. 2003). But, all these technologies did not achieve pace, as multigene families govern these enzymes and the isoform of the suppressed enzyme can take up its function. Similarly, by manipulation of ethylene pathway, ripening was delayed but the overall fruit quality was affected (Xiong et al. 2005). These fruits failed to ripen and were inferior in quality with respect to the color, aroma and taste as these characteristics are developed by gradual changes in the metabolism in ripening fruits (Theologis 1992; Hackett et al. 2000). Thus one of the alternate strategies seems to be utilizing the metabolic interactions of pathways related to post-harvest characteristics such as those of polyamines (PAs) and ethylene. The choice of PAs in improving the post-harvest quality of fruits is because of the functional and metabolic intersection of the two biosynthetic pathways. Indeed, the engineering of PA metabolism was considered as an alternate strategy for post-harvest biotechnology (Mehta et al. 2002; Nambeesan et al. 2010).

PAs: putrescine (Put), spermidine (Spd), and spermine (Spm) are low molecular weight, polycationic compounds that affect large number of developmental and physiological responses in a number of organisms, including plants (Kusano et al. 2008; Schwartz et al. 2011). Free PAs are known to act as anti-senescence agents and also influence early fruit development and ripening (Valero et al. 2002). In tomato, both ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) pathways for Put biosynthesis are active (Adiga and Prasad 1985). Put is either synthesized directly from ornithine via the action of ornithine decarboxylase (ODC) or indirectly from arginine via arginine decarboxylase (ADC). Higher PAs (Spd and Spm) are synthesized by the addition of aminopropyl group to Put with the help of biosynthetic enzymes Spd synthase (SPDSYN) and Spm synthase (SPMSYN) respectively. The required aminopropyl groups are provided by the decarboxylated S-adenosylmethionine (dcSAM), a product of SAM decarboxylation (Evans and Malmberg 1989; Tiburcio et al. 1990). SAMDC (SAM decarboxylase) is the enzyme involved in this decarboxylation reaction of SAM. SAM has been established as a common precursor for both PA and ethylene biosynthesis pathways. Numerous physiological effects of ethylene in plants seem to be antagonized by PA treatment (Parra-Lobato and Gomez-Jimenez 2011). PAs along with salicylic acid, an inhibitor of wound-responsive genes in tomato, have been suggested to regulate ethylene biosynthesis at the level of ACC synthase transcript accumulation (Li et al. 1992). The exogenous application of PAs has been demonstrated to delay fruit ripening, impede color change, increase fruit firmness, prolong the shelf life, improve quality characteristics of fruits, increase the fruit size and yield, and also reduced ethylene and respiration rate emissions, induced mechanical stress resistance, and reduced chilling symptoms (Martýnez-Romero et al. 2002). Tomato transgenic lines over-expressing yeast *SAMDC* showed increase in Spd and Spm along with enhanced lycopene and ethylene levels and better fruit juice viscosity in tomato fruit (Mehta et al. 2002). Similar results were obtained with over-expression of yeast Spd synthase (y*SPDSYN*) in tomato (Nambeesan et al. 2010). The increased PAs in both these transgenics seem to surpass the senescence effects of higher ethylene levels. Thus, PAs and ethylene while competing for SAM as a common substrate, influence plant growth and senescence antagonistically (Kumar and Rajam 2004).

Apart from their role in improvement of fruit characteristics PA fortification in fruit also make it more nutritious since PAs when supplemented in diets increases feeding efficiency and weight by countering the effect of anti-nutritional factors (Sousadias and Smith 1995; Bardocz et al. 1998).

Thus, it is evident that PA-ethylene nexus plays a crucial role in fruit ripening. Looking at the multifunctional and regulatory aspects of PA and ethylene, it is possible that controlled manipulation of such a key regulator rather than a structural or a regulatory gene operating in a single branch of the biosynthesis pathway would have the advantage of allowing the ripening to occur in a near normal way and may result in better improvement of fruit shelf life and quality traits. By over-expressing PA biosynthesis genes the SAM flux can be diverted towards the PA synthesis, which in turn would retard ethylene biosynthesis. Moreover, this will also provide insight into the metabolic involvement of PAs during fruit development and ripening. Therefore, the present study was undertaken to improve the tomato fruit characteristics by expressing human-*SAMDC* under the control of fruit-specific promoter (2A11) and also an attempt was made to determine the molecular basis for improved fruit characteristics.

Materials and methods

Plasmid constructs and plant material

SAMDC gene from human source was chosen for the present study as it was accessible during the initiation of this work. The 4.0 kb 2A11 promoter was excised from pGEM-T-2A11 (provided by Dr. V. S. Reddy, ICGEB, New Delhi) by digesting with *Sma*I and *Eco*RI and was ligated into the pBinAR, replacing CaMV 35S promoter. Plasmid pGEM3Z-f-human *SAMDC* (provided by Prof. Tony Pegg, Hershey Medical Center, Hershey, USA), was digested with *Sac*I and *Xba*I enzymes to release 1.2 kbh-*SAMDC* gene fragment. This fragment was end filled with klenow fragment (Fermentas, Canada) and was subsequently ligated into the *Sma*I digested pBin2A11 vector in the sense orientation with respect to the promoter. In addition, the pBin-T-DNA contained the neomycin phosphotransferase II (*NPT*-II) gene from the Tn5

transposon of *E. coli* (K12), under the control of the nos promoter from *Agrobacterium tumefaciens* (Fig. 1). The seeds of tomato (*Solanum lycopersicum* Mill. cv. Pusa Ruby) were procured from National Seeds Corporation, New Delhi. Fully expanded cotyledons from 10 to 12 days old tomato seedlings grown in pots containing garden soil and vermiculite (1:1) under controlled growth conditions (26±1 °C, 16 h photoperiod with irradiance of 40 μE $mol m^{-2} s^{-1}$) were utilized for transformation experiments.

Tomato transformation

The *A. tumefaciens* (LBA4404) mediated tomato transformation with pBIN-SAMDC binary vector was performed to develop tomato transformants according to a protocol developed by Madhulatha et al. (2007). The expression of NPTII activity was used as a selectable trait to screen transformed plants. Transformed and wild-type (WT) regenerants, with well-developed roots were then transferred to transgenic green-house for further studies.

Transgene integration and expression analysis of tomato transformants

Genomic DNA was isolated from the leaves of WT and tomato transformants using CTAB method (Doyle and Doyle 1990). Utilizing 100 ng of genomic DNA, the transformants were analyzed by PCR for the presence of the transgene. The primer pairs used for the amplification of 750 bp fragment of *NPT*-II gene at 59 °C annealing, were 5'-TCAGAAGAACTCGTCAAGAA-3' and 5'-ATGGGGATTGAACAAGATGG-3' and for 570 bp amplicon of h-*SAMDC* gene at 53 °C annealing, were 5'-GCTGCACATTTTTTCGAAG-3' and 5'-AGGTTTGATCTGGCTGAC-3'.

Genomic DNA (10 μg), restricted with *Xba*I enzyme was subjected to Southern hybridization using radiolabelled h-*SAMDC* probe. The probe was made using nick translation kit according to manufacturer's guidelines (Bangalore Genei, India). Blots were prepared using the standard protocol (Sambrook et al. 1989), using nylon membrane (Hybond N, Pharmacia). Pre-hybridization and hybridization were carried out according to Sambrook et al. (1989).

Each of the ~100 mg of Leaf, flower bud (FB), Pericarp tissue from mature green (MG), breaker (BR), pink red (PR) and red ripe (RR) fruits was pulverized to homogenate

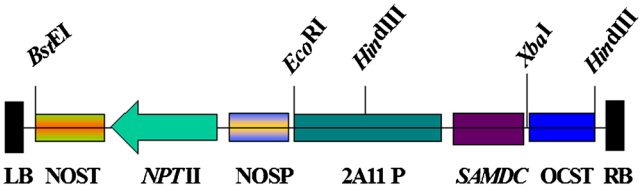

Fig. 1 T-DNA map of SAMDC gene construct

powder. The homogenate was used for isolation of total RNA with the TriZol reagent following manufacturer's guidelines (Invitrogen, USA) with subsequent RNase free DNase (Fermentas, Canada) treatment. Semi-Quantitative RT-PCR was performed to check the transcript level of the transgene and various other genes involved in fruit ripening. DNA-free RNA (200 ng) was used for the one-step RT-PCR reaction as specified in the kit manual (Taurus-Scientific, India). The reaction conditions were as follow: 45 °C for 50 min, followed by 94 °C for 5 min, n cycles of 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 30 s and finally 72 °C for 15 min. The products were analyzed on ethidium bromide stained 1.5 % agarose gel. The reaction conditions and probes are detailed in Table S1. All semi-quantitative-RT-PCR experiments were carried out thrice. In each independent experiment, the pulverized tissue of three fruits from same plant was pooled to get 100 ng RNA.

Transgene segregation analysis

The leaves from T_1, T_2 and T_3 progenies (40 each) of transgenic lines along with few WT plants were utilized for segregation analysis studies. Segregation pattern was confirmed by PCR in all the 40 progenies from T_1, T_2 and T_3 generations using transgene specific primers.

Polyamine analysis

PAs were estimated by TLC method with 100 mg of tissues each from leaf, FB and pericarp of the MG, BR, PR and RR fruits. Tissue was homogenized in 1 mL of 10 % perchloric acid. The resultant extract was fractionated, dansylated, chromatographed and quantified as described by Bajaj and Rajam (1996) using dual wavelength fluorometer (Bio-Rad, VersaFluor, USA) with an excitation wavelength of 350 nm and emission wavelength of 495 nm. The PA content was estimated in three independent experiments.

Ethylene estimation

For the determination of ethylene levels, three RR fruits (pre-weighed) were enclosed in 100 mL of air-tight container for about 1 h at room temperature. 3 mL of the headspace atmosphere of the container was withdrawn with a syringe and injected into a gas chromatograph (Model HP 5890, Hewlett Packard, USA) (Singh and Pal 2008). The ethylene estimation was carried out in ten independent experiments with three experimental replicates.

Estimation of respiration rate

RR fruits (pre-weighed) were sealed in an air-tight container for 1 h and their respiration rate was determined by head

space gas analysis technique using CO_2/O_2 analyzer (Model Checkmate 9900 O_2/CO_2, PBI Dansensor, Denmark). The respiratory activity was measured in ten independent experiments with nine experimental replicates.

On-vine ripening period determination

Flowers were tagged at anthesis and the days were noted for the fruit formation. The MG fruits displaying first sign of colour change were identified as BR stage. Average days for BR to reach RR were noted to determine the on-vine ripening period. The on-vine ripening period was measured for nine fruits as biological replicates each in ten independent sets, in three generations.

Determination of fruit shelf life

The RR fruits were kept at room temperature and the time was noted for the first visual sign of shriveling. The shelf life of fruits in three generations was noted for nine fruits each in ten independent sets.

Determination of physiological loss of water (PLW)

PLW in RR fruits during storage at room temperature was estimated by subtracting the sample weights from their previous recorded weights and was represented as % PLW compared to initial weight. The data was recorded over three generations for nine biological replicates each in ten independent sets.

Total soluble solids (TSS)

Tomato fruit (RR) homogenate was utilized for measuring TSS content by a hand refractrometer (Model: Fisher, Japan). Results were expressed as ^{o}Brix and were corrected to 20 °C temperature. TSS content was measured in nine replicates in ten independent experiments.

Determination of sugars

To determine the sugars in tomato fruits, the method described by Hortwitz (1960) was followed. Sugar content was measured in nine fruits in three independent experiments.

Estimation of ascorbic acid (AsA) content

AsA content in the red fruits was estimated titrimetrically using 2, 6-dichlorophenol indophenol as the indicator dye (Singh and Pal 2008). AsA standard was prepared by dissolving 100 mg of L-ascorbic acid in 100 mL of 1 % HPO_3 (Singh and Pal 2008). AsA content was measured in nine fruits in three independent experiments.

Quantification of lycopene content

Lycopene fraction of RR fruit pericarp tissue (~2 g) was determined by spectrophotometric method as described by AOAC (2000). Lycopene fractions were estimated in nine fruits in three independent experiments.

Data collection and analysis

For each of the experiment, data from three consecutive generations, with their replicates was collected. The data presented are average (mean) with the standard error from all the experiments. Significant differences were determined by Student's *T*-test ($p<0.05$).

Results and discussion

Generation of homozygous lines of transgenic tomato plants over-expressing h-*SAMDC*

Several tomato transformants over-expressing h-*SAMDC* gene under the control of 2A11-fruit specific promoter were generated. Transgene integration in the transformants was confirmed by PCR with *NPT*-II and h-*SAMDC* gene specific primers, while Southern hybridization detailed the transgene copy number (supplementary Fig. 1a, 1b, 1c). Transgene expression in transgenic plants is correlated with copy number (Matzke et al. 1994) and the site of transgene integration (Hobbs et al. 1990). Therefore, several tomato transformants for h-*SAMDC* over-expression were analyzed for its segregation pattern in three generations. The results revealed Lesam16, Lesam24 and Lesam56 transgenic lines to be homozygous for h-*SAMDC* integration (supplementary Fig. 1d). To negate the differential expression due to variable copy number, the homozygous lines were proceeded for further analysis.

Stable expression of transgene during fruit ripening

The transgene transcript levels were examined in the T_3 fruits from transgenics and WT plants. The stability of transgene expression in the developed transgenics was depicted by the specific presence of h-*SAMDC* transcripts in transgenic tomato fruits (Fig. 2a). The transgene was found to be exclusively expressed across MG to RR stages with higher expression in BR and PR over MG and RR stage. Such trend in expression was concomitant with the onset of upstream promoter i.e., 2A11 activity. This promoter has been very well characterized from tomato plant where the *2A11* gene expresses strictly in a fruit-specific manner (Pear et al. 1989).

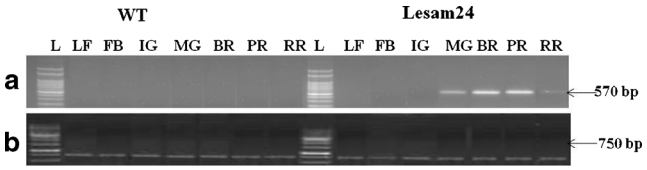

Fig. 2 Fruit specific transgene expression in T_3 generation of Lesam24 fruits. **a** Semi-quantitative RT-PCR analysis showing stable expression of human-*SAMDC* at different stages of fruit ripening in Lesam24; **b** Semi-quantitative RT-PCR analysis for *ACTIN* gene as an internal control, L-1 Kb ladder; LF-Leaf; FB-Flower bud; IG-Immature green; MG-Mature green; BR-Breaker; PR-Pink red; RR-Red ripe

Elevated polyamine levels

Accumulation of h-*SAMDC* transcripts influenced the levels of PAs in transgenic fruits. PA titers were significantly higher in the transgenic tomatoes as compared to WT. In WT, the PAs titers, mainly Put levels were higher in the FB followed by MG and BR fruits, and thereafter declined in the later stages of ripening, yet these levels were significantly higher in transgenic line over WT. As expected with the over expression of h-*SAMDC*, significant increase was observed in the Spd followed by Spm and then Put in the Lesam fruits (Fig. 3). The PA analysis in the transgenic lines revealed increase in all three fractions of Put, Spd and Spm. Put showed about 1.1–2.2 fold increase in the free, 1.1–3.4 fold increase in the conjugated and 1.1–2.3 fold increase in the bound fractions. Free Spd fraction showed ~3 fold increase, whereas the conjugated and bound fractions showed ~1.2–4.1 and 1.1–3 fold increase respectively. Transgenic fruits exhibited ~1.1–3.0 fold increase in free Spm, conjugated fraction ~1.2–3.9 and ~1.1–3 fold increase in bound Spm over WT fruits (Fig. 5a).

However, no significant change in mRNA titers of endogenous genes viz., *ODC*, *ADC*, *SAMDC* and *SPDSYN* was seen in transgenic fruits over WT (Fig. 7). Also the expression patterns of these genes were observed to be steady during all the stages which implies the possibility of post-translational or metabolic regulation of PA concentration in Lesam transgenics. The increase in Put in the transgenic lines over-expressing h-*SAMDC* could be attributed to enhanced inter-conversion of Spd/Spm into Put through the acetylation mechanism. The PA acetylating enzyme, Spd/Spm N1-acetyltransferase (SSAT) has the potential to participate in PA pool homeostasis by lowering intracellular PA levels. SSAT can actively respond to PA excess by acetylating Spd and Spm and thereby facilitating their excretion out of the cell and/or promoting their back-

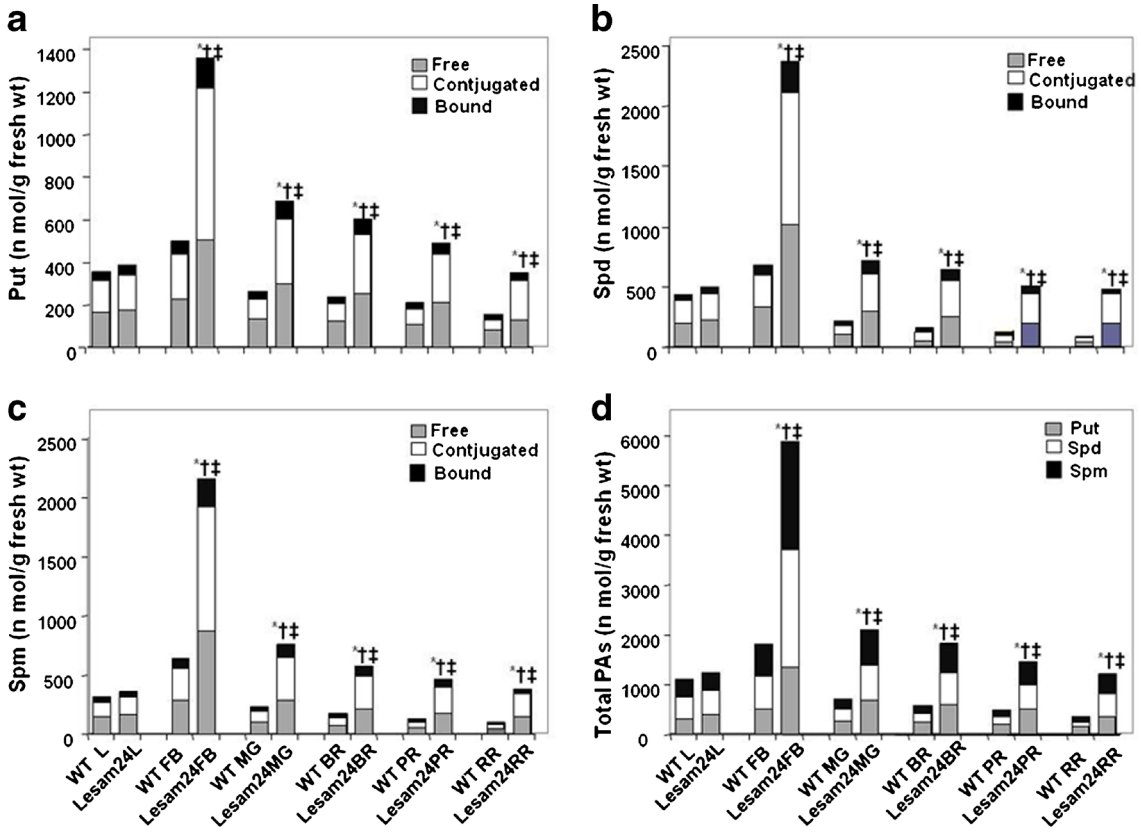

Fig. 3 PA accumulation in Lesam24 fruits over WT fruits. **a** Put levels; **b** Spd levels; **c** Spm levels; **d** total PA levels, Bars represent the standard errors of three independent experiments. *Asterisk*, *dagger* and *double dagger* denote significant differences in free, conjugated and bound fractions respectively from WT at ($P<0.05$)

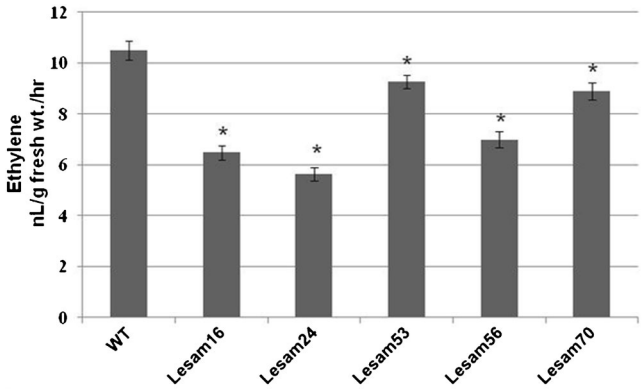

Fig. 4 Reduced ethylene production in Lesam fruits over WT fruits, Bars represent the standard errors of ten independent measurements with three replicates each, in three generations. *Asterisk* values that were determined by the *t*-test to be significantly different ($P<0.05$) from WT

conversion to Put which can be catabolized by various Put-directed oxidases (Seiler 2004).

This result is consistent with the work done on different crop plants such as tobacco (Waie and Rajam 2003), rice (Kumria and Rajam 2002) and sweet potato (Kasukabe et al. 2006) where over-expression of different PA genes under constitutive or tissue-specific promoters have shown an increase in the PA metabolism. Similar observations were noted in the transgenic tomato plants over-expressing heterologous *ODC, ADC* and *SPDS* genes under the control of fruit-specific promoter, 2A11, developed in our Lab (Unpublished data). All these three-types of transgenics showed an up-regulation of PA metabolism with increase seen in all the three PAs.

Reduced ethylene levels in transgenic fruits

Ethylene has been well established as a ripening inducing hormone while it coordinates the fruit maturation and ripening processes by modulating various ripening specific pathways. In the present study transgenic tomato fruits over-expressing h-*SAMDC* evolved ~50 % less ethylene as compared to WT fruits (Fig. 4). Further there was a significant dip in transcript titers of ethylene biosynthesis genes viz., fruit specific homologues of *ACS* and *ACO* in Lesam fruits specifically from BR to RR stages (Fig. 7). The lower accumulation of ethylene biosynthesis gene transcripts in transgenic fruits in comparison to the WT fruits has lead to decreased evolution of ethylene in transgenic fruits. Numerous reports advocate negative correlation between PA levels and *ACS* and *ACO* expression. PAs have been suggested to regulate ethylene biosynthesis at the level of

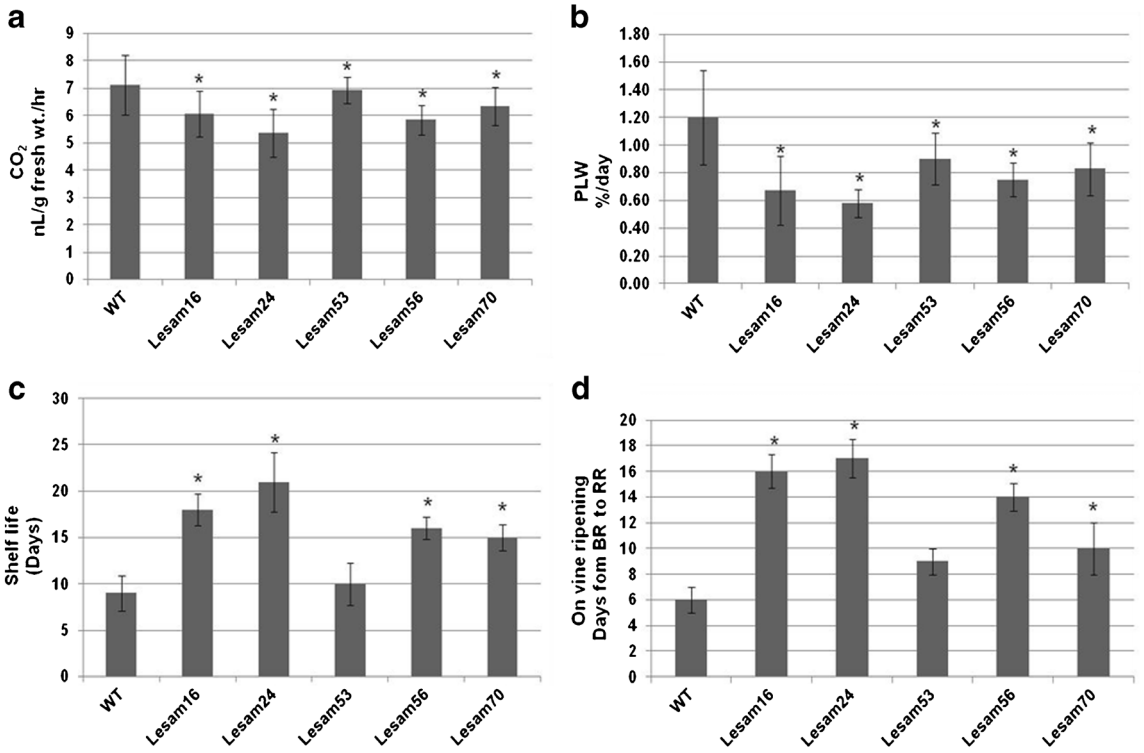

Fig. 5 Storage attributes of transgenic tomato. **a** Reduced rate of respiration in transgenic fruits; **b** Decreased PLW in transgenic fruits over WT fruits kept at room temperature; **c** Enhanced shelf life of Lesam transgenic fruits as compared to WT fruits at room temperature; **d** Transgenic fruits exhibiting delayed fruit ripening (noted as days taken to reach RR stage from BR) in comparison with WT fruits. Bars represent the standard errors of measurements in nine fruits each for ten independent sets carried for three generations. *Asterisk* values that were determined by the *t*-test to be significantly different ($P<0.05$) from WT

ACS transcript accumulation (Li et al. 1992; Handa and Mattoo 2010). Also, PAs and ethylene intersect biosynthetically at a common point for precursor, SAM (Winer and Apelbaum 1986). Therefore, it would not be irrational to argue that the synthesis of PAs and ethylene in Lesam tomatoes may be competitive and increased PA biosynthesis has caused a dip in ethylene levels, thereby strongly supporting that SAM could be a rate limiting factor for both the pathways. The same is supported by the use of PA inhibitors (Kushad and Dumbroff 1991; Parra-Lobato and Gomez-Jimenez 2011). On the contrary Mehta et al. (2002) reported elevated ethylene levels in the tomato transgenic over-expressing yeast *SAMDC*, suggesting the co-existence of these two biosynthetic pathways.

Delayed fruit ripening and enhanced shelf life

The pooled data of three generations revealed that the over expression of h-*SAMDC* in Lesam tomatoes resulted in decreased rate of respiration and reduced physiological loss of water (PLW). A significant decline in CO_2 evolution of around 20–30 % was noted in transgenic fruits over WT (Fig. 5a). Results on PLW showed almost similar trends of reduced PLW percentage among the transgenic tomatoes over WT fruits (Fig. 5b). On-vine ripening period taken for MG fruit to reach BR stage and BR to RR showed minimum of 10 days delay as compared to the WT (Fig. 5d). WT red fruits stored at room temperature rotted after 8–10 days whereas the Lesam red fruits had a prolonged shelf life of about 11 days over WT (Fig. 5c). The reduced respiratory activity and PLW possibly explains the delayed on-vine and off-vine ripening period accompanied by enhanced shelf life in transgenic tomato fruits over WT fruits. The enhanced shelf life can also be attributed to low levels of ethylene in transgenic tomato fruits since the rate of ethylene evolution of harvested tomato fruit is negatively correlated with its shelf life (Guillén et al. 2007). Similarly, in many fruits the pre- and post-harvest applications of Put have shown to reduce their respiration rate by suppressing the climacteric ethylene production thereby, delaying the ripening process (Khan et al. 2008; Torrigiani et al. 2012). These results thus establish that increase in PA decrease the fruit ripening rate.

The integrity of cell membrane and cell wall is a major factor determining processing characteristics, firmness, and shelf life of tomato fruits. During ripening, cell wall undergoes

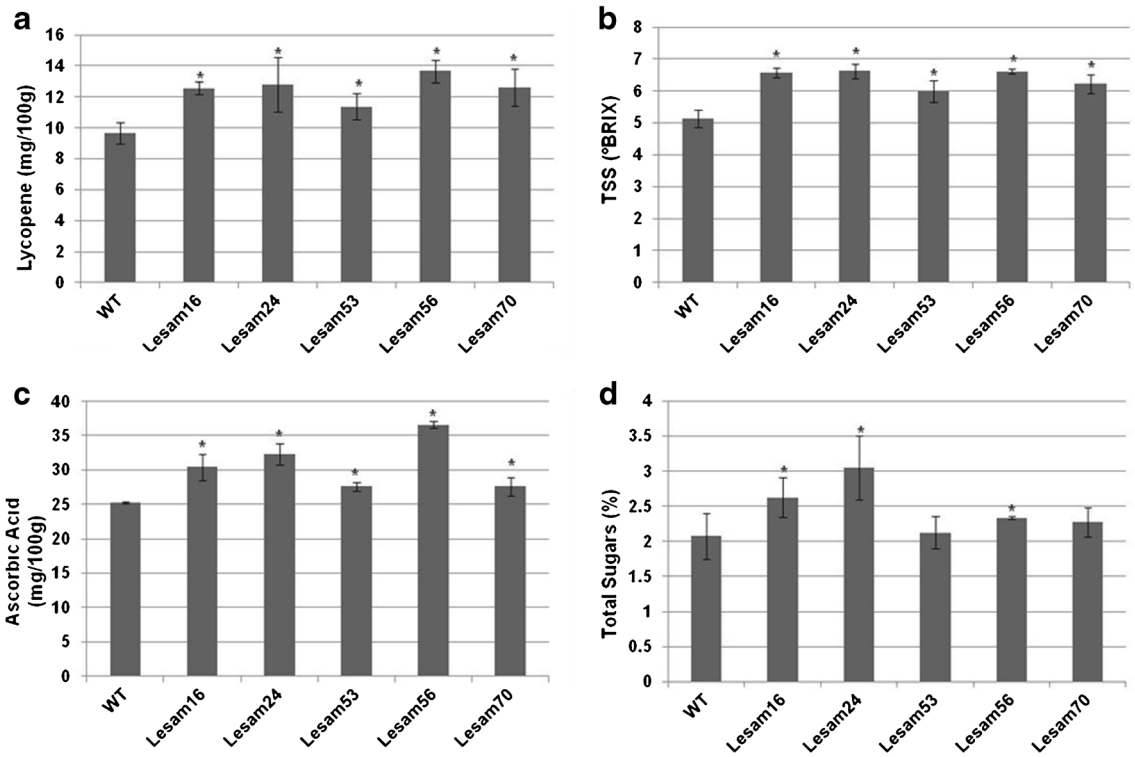

Fig. 6 Improvement in fruit quality traits in transgenic plants **a** Fruits from Lesam lines were enriched with phytonutrient lycopene, Bars represent the standard errors of measurements in nine fruits each for three independent sets carried for three generations; **b** Fruits of Lesam lines exhibited enhanced TSS content, Bars represent the standard errors of measurements in nine fruits each for ten independent sets carried for three generations; **c** Increased AsA contents in Lesam fruits over WT, Bars represent the standard errors of measurements in nine fruits each for three independent sets carried for three generations; **d** Accumulation of total sugars in Lesam fruits over WT, Bars represent the standard errors of measurements in nine fruits each for three independent sets carried for three generations. *Asterisk* values that were determined by the *t*-test to be significantly different ($P< 0.05$) from WT

substantial disassembly. Expansins (EXP) have been shown to play an important role in fruit softening (Rose et al. 1997), where they are involved in cell wall degradation and simultaneously increasing the accessibility of other cell wall modifying proteins such as polygalaturonase (PG) to cell wall polymers (Rose and Bennett 1999). β-galactosidase (TBG) is reported to be associated with ripening related firmness loss (Smith et al. 2002). Expression analysis of both *EXP1* and *TBG4* revealed elevated transcript levels, at PR and RR stages of fruit development in Lesam fruit over WT fruits (Fig. 7). On the other hand, *PG* which is involved in pectin metabolism and thus has been associated with cell wall break down, fruit softening and loss of tissue integrity, showed no difference in transcript abundance at any of the ripening stage in Lesam tomato from WT fruits (Fig. 7). Although our findings on expression of cell wall loosening genes are in conflict with the results of enhanced shelf life, increased PA content in the Lesam fruits seems to justify the enhanced shelf life possibly by stabilizing the membranes. PAs have been reported as powerful modulator of supra-molecular conformation of pectin (Messiaen et al. 1997). PAs can also bind covalently with cell wall and inhibition of PA biosynthesis interferes with the cell wall formation making it amorphous and its exogenous application reverses the changes (Berta et al. 1997). Comparable results for delayed fruit softening has been reported in PA treated peach (Ziosi et al. 2006) and plum fruits (Khan et al. 2008).

Enhanced lycopene content

Lesam fruits are noticeably deeper red in color than the WT fruits. This can be associated with the phytonutrient lycopene in the fruits. The study of the lycopene content of the transgenic showed its significant higher level ranging from

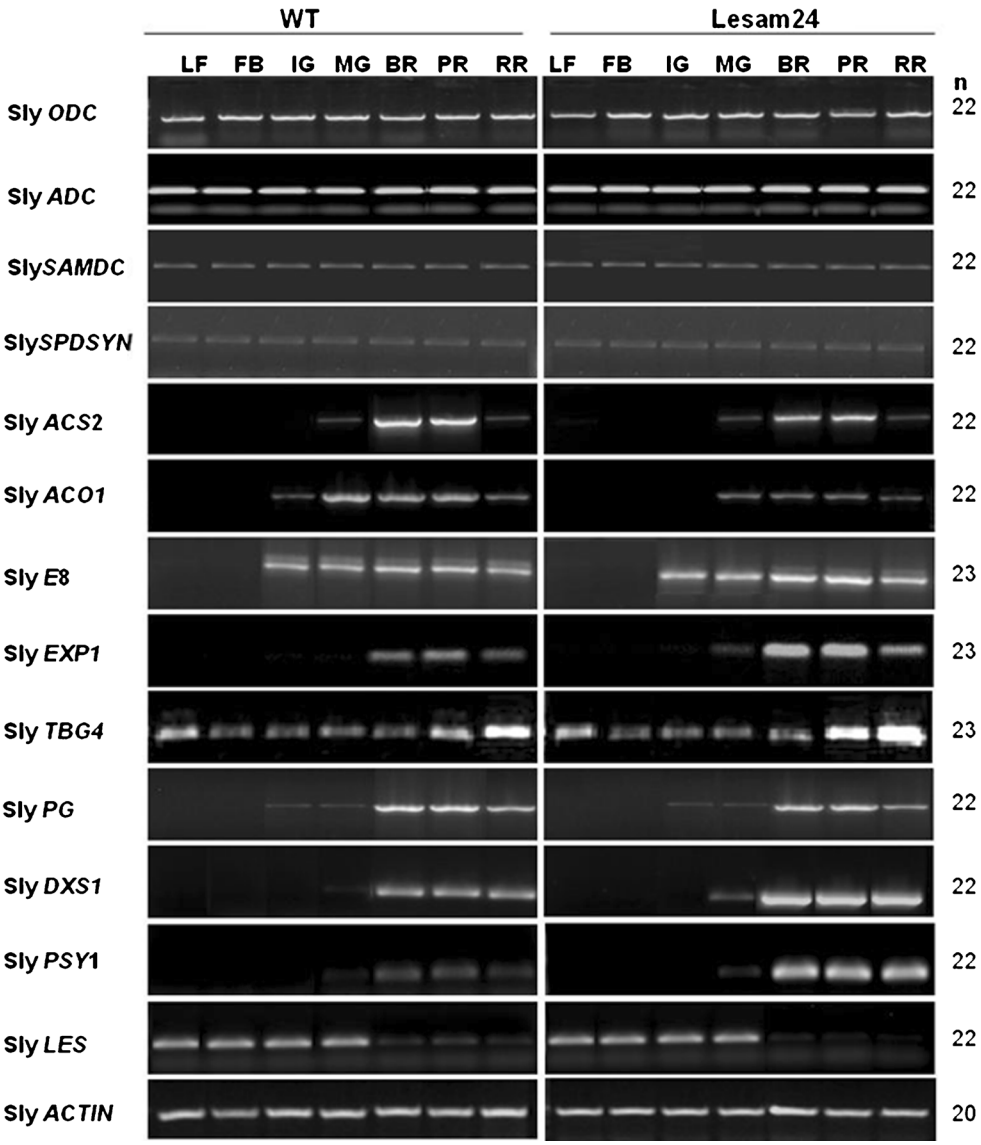

Fig. 7 Semi-quantitative RT-PCR analysis for the expression pattern of various fruit ripening specific genes, at different stages of ripening in Lesam24 line and WT fruits. *n* represents no. of cycles for RT-PCR reaction for respective gene

10 to 40 % in transgenic fruits over WT (Fig. 6a). Lesam56 was found to be significantly superior followed by Lesam24 and Lesam16 transgenic lines with 22–40 % increase in lycopene content. Expression pattern of lycopene biosynthesis genes viz., *DXS*1 and *PSY*1 revealed higher mRNA titres throughout fruit ripening viz., MG, BR, PR and RR while *LES* which is involved in catabolism of lycopene (Ronen et al. 1999) did not show any significant difference from WT (Fig. 7). The up-regulated expression of these transcripts thus accounts for the enhanced lycopene content in transgenic fruits over WT fruits. The elevated levels of lycopene biosynthesis gene transcripts in Lesam tomatoes with increased PAs, suggest the role of PAs in regulation or at least stabilization of mRNA turnover of lycopene biosynthesis genes. In has been studied that reduced degradation of membrane phospholipids in fruits tend to reduce the turnover of phospholipids and free fatty acids. Since fatty acid biosynthesis and carotenoid biosynthesis share the common precursor (Acetyl Coenzyme A), reduced demand for acetyl CoA for fatty acid biosynthesis in transgenic fruits may tend to enhance their availability for carotene and lycopene biosynthesis (Oke et al. 2003). This could also explain the enhanced lycopene in Lesam transgenic fruits. Both these arguments are in agreement with the reports by Neily et al. (2011), in transgenic tomato over-expressing *SPDSYN*. Thus, increase in the PA levels appears to cause an increase in the phytonutrients in fruits.

Improved fruit quality

Total soluble solid (TSS) content as depicted by ratio of total sugars v/s organic acid, is the key parameter of the tomato fruit quality. Sugars and acids are responsible for the taste of fresh tomatoes and play an important role in determining the overall sensory quality of tomatoes. The Lesam transgenics exhibited up to 35 % increase in TSS with the highest of 6.62 ^{o}brix in Lesam24 as against only 5.14 ^{o}brix in WT tomatoes (Fig. 5b). The result seems to be another manifestation of enhanced PA accumulation in Lesam fruits and is supported by various other reports (Costa and Bagni 1983; Mitra and Sanyal 1990). Increase in total sugar was observed to be 1.5 times more in Lesam24 over WT (Fig. 5d). There was a considerable rise in reducing sugar percentage in Lesam16 and Lesam56 among the Lesam lines over WT (data not shown). Lesam24 fruits showed remarkably higher levels of AsA as compared to WT (Fig. 5c). In general the AsA levels decline during ripening and senescence and has been correlated with its getting used up in ethylene biosynthesis pathway while acting as a co-factor for ACO (Watada et al. 1976). Therefore, in transgenic fruits with the reduction in ethylene evolution, the accumulation in AsA is apparent. Yahia et al. (2001) have associated the endogenous concentration of Put with higher ascorbic acid in fruits. Asghar et al. (2010) demonstrated increase in ascorbic acid content on Spd application in pomegranate fruits. Thus PAs have a role in influencing AsA content with the direct mechanism still unknown.

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