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Enhanced polyamine accumulation alters carotenoid metabolism at the transcriptional level in tomato fruit over-expressing spermidine synthase

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ABSTRACT

Polyamines are involved in crucial plant physiological events, but their roles in fruit development remain unclear. We generated transgenic tomato plants that show a 1.5- to 2-fold increase in polyamine content by over-expressing the spermidine synthase gene, which encodes a key enzyme for polyamine biosynthesis. Pericarp-columella and placental tissue from transgenic tomato fruits were subjected to ^{1}H-nuclear magnetic resonance (NMR) for untargeted metabolic profiling and high-performance liquid chromatography-diode array detection for carotenoid profiling to determine the effects of high levels of polyamine accumulation on tomato fruit metabolism. A principal component analysis of the quantitative ^{1}H NMR data from immature green to red ripe fruit showed a clear discrimination between developmental stages, especially during ripening. Quantification of 37 metabolites in pericarp-columella and 41 metabolites in placenta tissues revealed distinct metabolic profiles between the wild type and transgenic lines, particularly at the late ripening stages. Notably, the transgenic tomato fruits also showed an increase in carotenoid accumulation, especially in lycopene (1.3- to 2.2-fold), and increased ethylene production (1.2- to 1.6-fold) compared to wild-type fruits. Genes responsible for lycopene biosynthesis, including phytoene synthase, phytoene desaturase, and deoxy-D-xylulose 5-phosphate synthase, were significantly up-regulated in ripe transgenic fruits, whereas genes involved in lycopene degradation, including lycopene-epsilon cyclase and lycopene beta cyclase, were down-regulated in the transgenic fruits compared to the wild type. These results suggest that a high level of accumulation of polyamines in the tomato regulates the steady-state level of transcription of genes responsible for the lycopene metabolic pathway, which results in a higher accumulation of lycopene in the fruit.

Introduction

Polyamines (PA) (putrescine, spermidine, and spermine), which are present in all plant cells, are involved in crucial physiological events. Cumulative evidence has shown that these cationic substances are involved in a wide range of plant growth and developmental processes (Bouchereau et al., 1999; Kusano et al., 2008). PA have also been proposed to play protective roles against a broad spectrum of environmental stresses (Alcázar et al., 2010).

Solanaceae is a large family that includes many commercially and/or nutritionally significant species, such as the potato, tomato, tobacco, pepper, eggplant, and petunia (Moco et al., 2007). Solanaceous plants comprise the third most economically important plant taxon, the most valuable vegetable crops, and the most variable crop species in terms of agricultural utility (Mueller et al., 2005). One member of this family, the tomato (*Solanum lycopersicum*), is considered to be the important vegetable crop in the world. The cultivated tomato is a staple crop, known for its remarkable nutritional value and as a source of health-promoting antioxidants in the human diet. Tomato is a major source of the antioxidant lycopene; in fact, ripe tomato fruit and its products provide 85% of lycopene found in the human diet (Canene-Adams et al., 2005). This carotenoid is an essential nutrient for the prevention of seri-

Abbreviations: DAP, days after pollination; *DXS*, deoxy-D-xylulose 5-phosphate synthase; *LCY-E*, lycopene-epsilon cyclase; *LCY-B*, lycopene beta cyclase; PA, polyamines; PCA, principal component analysis; *PDS*, phytoene desaturase; *PSY*, phytoene synthase; SPDS, spermidine synthase; WT, wild type.

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ous chronic human diseases, including cancer and cardiovascular disease (Rao and Rao, 2007).

Despite tremendous research efforts, the mechanisms and modes of action underlying the physiological functions of PA are not clearly understood (Paschalidis and Roubelakis-Angelakis, 2005). Manipulation of the polyamine metabolic pathway through molecular genetics approaches has become a valuable tool for studying their physiological roles. Mehta et al. (2002) showed that expression of the yeast S-adenosylmethionine decarboxylase gene (*SAMDC*) under control of the ripening-specific E8 promoter significantly increases lycopene accumulation in ripe tomato fruit. Metabolite profiling of these transgenic tomatoes using high-resolution nuclear magnetic resonance spectroscopy (NMR) revealed a high accumulation of PA and the influence of multiple cellular pathways that led to specific metabolic fluxes resulting in enhanced nitrogen (N) and carbon (C) metabolism (Mattoo et al., 2006).

In the present study, we generated transgenic tomato plants (cv. Micro-Tom) constitutively expressing the apple spermidine synthase gene (*Md-SPDS1*, Zhang et al., 2003) (accession number: AB072915) to investigate how PA are involved in the metabolic fluxes that occur during tomato fruit development. Spermidine synthase (SPDS) is a key enzyme in polyamine biosynthesis and is responsible for spermidine accumulation. These transgenic tomato plants were subjected to proton-NMR (^{1}H NMR) and high-performance liquid chromatography (HPLC) metabolic profiling. High-resolution ^{1}H NMR can be used to identify and quantify a large number of compounds (Krishnan et al., 2005), which has made it a valuable technique for metabolomics. We found that the transgenic tomato fruits expressing *Md-SPDS1* showed a higher accumulation of PA, some primary metabolites, and carotenoids. Furthermore, transcriptional analysis showed that the changes in polyamine content in the transgenic tomato fruits affected steady state levels of transcription of the genes involved in carotenoid metabolism, resulting in an increase in carotenoid content.

Materials and methods

Plant material

The tomato (*Solanum lycopersicum* L.) cv. 'Micro-Tom' was used to generate transgenic plants in this study. In this genotype, the fruit expansion phase occurs 10–30 days after pollination (DAP), the mature stage at 30 DAP, and ripening at 40–50 DAP, with red ripe fruit at 50 DAP.

Tomato transformation and generation of homozygous transgenic lines

The full-length cDNA of the apple spermidine synthase cDNA (*Md-SPDS1*, GenBank accession number AB072915) (Zhang et al., 2003) was amplified by polymerase chain reaction (PCR) to introduce the BamHI and SacI restrictions sites at the 5′- and 3′-ends of the *SPDS1* cDNA fragment with the following gene-specific primers: 5′-AATGGATCCATGGCGGACGAGAGTGTGGC-3′ and 5′-TGCGAGCTCTCACTTTGCTTTTGCGTCAA-3′. The *SPDS1* cDNA fragment was subcloned into the pBI121 binary vector in which the GUS reporter gene had been excised at the BamHI and SacI restriction sites (Fig. 1A). The construct was transformed into *Rhizobium radiobactor*, strain GV2260, by electroporation. Tomato transformation was performed using the highly efficient protocol established by Sun et al. (2006). Homozygous lines were obtained at the T_2 generation from lines harbouring a single copy of the transgene, which were confirmed by genomic DNA gel blot analyses and PCR at the T_0 and T_1 generations. T_2 lines showing kanamycin resistance in all of the siblings were selected as homozygous lines.

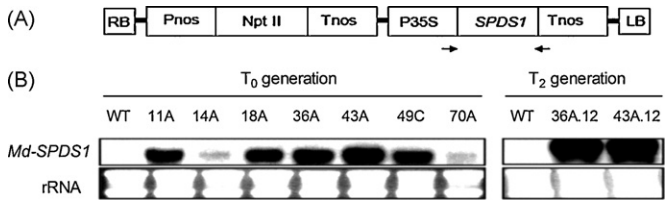

Fig. 1. (A) T-DNA map of the vector construct containing the *Md-SPDS1* gene. LB and RB, left and right T-DNA borders; P35S, 35S promoter; Pnos, nopaline synthase gene promoter; Tnos, nopaline synthase gene terminator; NptII, neomycin phosphotransferase; *SPDS1*, spermidine synthase 1. Arrows indicate the PCR primers used to check T_0 generation. (B) RNA gel blot analysis of RNA isolated from red ripe fruit of WT and transgenic lines at T_0 and T_2 generations. Each lane contained 8 μg of total RNA. The blot was probed with the 1 kb of *Md-SPDS1* fragment.

Plant growth and fruit harvest conditions

Seeds were germinated on filter paper saturated with distilled water on Petri dishes at room temperature before being transplanted to rock wool (Toyobo, Osaka, Japan). Individual flowers were tagged at anthesis to accurately follow fruit ages through development. Tomato fruits were harvested from 10 to 55 days DAP, which encompassed the transition from green to fully ripe fruit, to measure ethylene contents and to determine the polyamine or metabolic profile. For metabolomics, HPLC and polyamine-quantifiable harvested fruits were separated into pericarp-columella and placenta without seeds, immediately frozen in liquid nitrogen and maintained at −80 °C. Entire fruits were used to measure ethylene emission and carotenoid gene expression.

RNA gel blot hybridisation

RNA gel blot hybridisation was performed to confirm expression of the transgene using the 1 kb *Md-SPDSI* fragment as a probe. Wild-type plants and transgenic lines harbouring a single copy of the transgene were used. Leaf samples were frozen in liquid nitrogen and total RNA was prepared from plant material using the RNeasy Plant Minikit (Qiagen, Valencia, CA, USA). RNA samples were electrophoresed on 1.2% formaldehyde gels and transferred to nitrocellulose membranes. Probe labelling, hybridisation, washing and signal detection were carried out with a PCR DIG Probe Synthesis Kit (Roche Diagnostics) according to a procedure described by Saito et al. (2008).

Polyamine quantification

Free polyamine content was quantified by homogenising the fruit pericarp-columella samples with 5% (w/v) perchloric acid. The same samples used for metabolomic analysis were also used to measure polyamine content. After centrifugation, the supernatant was preserved. After dansylation, the polyamines in the supernatant were quantified with HPLC as described by Burtin et al. (1989). 1,6-Hexanediamine was used as the internal standard.

Ethylene measurement

Fruits from wild type and the two selected transgenic lines were ripened on plants and harvested at 20, 30, 37, 40, 50, and 55 DAP to measure ethylene content. Ethylene production was assayed by placing each individual fruit into a sealed Mason jar (0.05 L) for 1–2 h and then withdrawing 1 ml gas samples. Seven to ten fruits were randomly harvested from 10 to 15 plants per line. Gas samples