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Metabolic profiling of tomato over-expressing *SPDS1*

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Title

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.

Keywords: carotenoid biosynthesis, metabolic profiling, polyamines, spermidine synthase, tomato

Abbreviations

DAP, days after pollination; *DXS*, deoxyD-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

Introduction

Polyamines (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). Polyamines 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 serious 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 polyamines 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 polyamines 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 polyamines are involved in the metabolic fluxes that occur during tomato fruit development. Spermidine synthase 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 polyamines, 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 *Bam*HI and *Sac*I 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 *Bam*HI and *Sac*I 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.

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 10 fruits were randomly harvested from 10–15 plants per line. Gas samples were analysed via gas chromatography (GC) (GC-14B gas chromatograph; Shimadzu, Soraku-gun, Japan) using an activated alumina column and a flame-ionisation detection system (Shimadzu). Ethylene was identified by co-migration with an ethylene standard and quantified with reference to a standard curve.

Carotenoid profiling by HPLC-diode array detection (DAD)

The same tissues samples were used for caretenoid profiling and NMR profiling. To focus on polyamine cross-talk and carotenoid metabolism, only the last two stages of development (40 and 50 DAP) were analyzed in the pericarp-columella and placenta tissues. Carotenoid separation, identification, and quantification were performed as described by Télef et al. (2006).

Extraction and ^{1}H-NMR analysis of polar compounds

Tomato fruits were harvested at 10, 20, 30, 40, and 50 DAP from plants grown in a growth chamber at 25°C and 16 h light/8 h dark with approximately 250 µmol photons m^{-2} s^{-1}. The fruits used for the experiment were at similar ripening stages. For metabolomic analysis, four to six fruits of each transgenic line (36A.12 and 43A.12) were pooled. Harvested fruits were cut, the pericarp and the columella were separated from the placenta, and placental tissue was separated from the jelly and seeds. Both the pericarp-columella and placenta were immediately ground into a fine powder in

liquid nitrogen and kept at −80°C. Frozen samples were later lynpholized and stored at -80°C until use for metabolic profiling. Polar metabolites were extracted according to the method of Moing et al. (2004). The solubilized extract was used for chemical shift calibration at 500.162 MHz on a Bruker Avance spectrometer (Bruker, Karlsruhe, Germany) using a 5-mm broadband inverse probe and the ERETIC method (Akoka et al., 1999) for quantification, as described by Moing et al. (2004) and Mounet et al. (2007). We used principal component analysis (PCA) to explore the multidimensional metabolite data set on mean-centred data scaled to the unit variance using MATLAB version 7.4.0 (MathWorks, Inc., Natick MA). The mean comparison between lines for one stage was determined by Student's *t*-test using SAS software version 8.01.

Quantitative real-time PCR

Samples from wild type and transgenic lines harbouring single copy genes were collected from leaves and fruits. Total RNA was extracted as described in "RNA gel blot hybridization". The 0.5 μg of total RNA was subjected to reverse transcription as a template for each sample and converted to double-stranded cDNA using Super Script TM III RNAse H-reverse transcriptase (Invitrogen) following the manufacturer's directions. Quantitative RT-PCR was performed by SYBR Green detection method on Mx 3000P (Stratagene, San Diego, CA, USA). The gene-specific primers for *PSY1*, *PDS*, *DXS*, *LCY-B* and *LCY-E* were previously described by Télef et al. (2006). The tomato ACTIN (*ACT*) gene was used as an internal control (accession number U60482). The reaction cycles were as follows: 95°C for 10 min for initial denaturation, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30s, and 1 cycle of 95°C for 30 s, 55°C for 30 s, and 95°C for 30 s. Specific amplifications were confirmed by single transcript amplification in agarose gel, single dissociation peaks, and calibration curves. Gene expression was calculated in relation to the level of

actin gene expression according to the instructions provided by Stratagene based on the method reported by Pfaffl (2001).

Results

Generation and molecular characterisation of transgenic tomato plants over-expressing apple *SPDS1*

The apple *Md-SPDS1* gene was transformed into tomato cv. "Micro-Tom" via *Agrobacterium*-mediated transformation, and diploid plants harbouring the transgene were recovered. T_0 of the transgenic plants was subjected to genomic DNA gel blot analysis (data not shown), and seven (11A, 14A, 18A, 36A, 43A, 49A, and 70A) were selected as single copy transgenic lines. *Md-SPDS1* mRNA expression in the seven lines of the T_0 generation was evaluated by RNA gel blot analysis. Among the seven lines, five (11A, 18A, 36A, 43A, and 49A) showed a high level of *SPDS1* mRNA expression (Fig. 1B). We proceeded to generate these transgenic lines by self-pollination and obtained homozygous lines for *Md-SPDS1* in the T_2 generation. These homozygous lines showed a high level of *Md-SPDS1* mRNA expression (Fig. 1B) and a 1.5- to 2-fold increase in the levels of free spermidine in fruits than that wild-type plants, suggesting that *Md-SPDS1* was functional and correctly processed in tomato tissues. We randomly selected two lines (36A.12 and 43A.12) for further characterization.

Polyamine content in transgenic lines during fruit development

Polyamine content was measured in the pericarp-columella tissue at 10, 20, 30, 40, and 50 DAP (Fig. 2). Among the free polyamines analysed, spermidine (Fig. 2B) and spermine (Fig. 2C) were present at higher levels in the early stages of tomato fruit development, and tended to decrease

with maturation. Conversely, putrescine content was low in the early stages of fruit development, with the minimum level at 30 DAP, and then increased as fruit matured, peaking at 50 DAP (Fig. 2A). Compared to the wild type, the transgenic lines showed a significant increase in polyamine content, especially of putrescine and spermidine, ranging from 1.5- to 2-fold, at almost all developmental stages. These results demonstrate that transgenic tomato fruits accumulated much higher levels of polyamine than wild-type tomatoes.

PCA of metabolites in transgenic and wild-type tomato fruits

To investigate the possible changes in transgenic tomato fruit metabolites, we compared the pericarp-columella and placenta tissue metabolic profiles of the two independent transgenic tomato lines (36A.12 and 43A.12) homozygous for *Md-SPDS1* with those of the wild type using NMR spectroscopy analysis.

PCA analysis was performed on the 37 metabolites quantified in 61 pericarp-columella samples at five stages of fruit development (Fig. 3). PCA scores (Fig. 3A) revealed that the composition of the samples of all genotypes changed throughout fruit development. Indeed, the first principal component (PC1), explaining 48% of the total variability, clearly separated the early stages (10-30 DAP, immature and mature-green fruit samples) on the negative side from those of 40 and 50 DAP on the positive side. The second principal component (PC2), explaining 15% of the total variability, tended to separate the different genotypes. The transgenic lines mainly separated from the wild-type lines during ripening (40-50 DAP). These data support a stronger stage-driven variation than genotype-driven variation, particularly during early period of fruit development. Observation of the loadings suggested that this separation might be related to differences in quinate and alanine on the negative side and GABA, phenylalanine, glutamine, pyroglutamate, asparagine and two unknown

compounds on the positive side (Fig. 3B). Of the 25 known metabolites analysed by mean comparison, the following showed significant accumulation in the pericarp-columella tissue of the transgenic lines compared to wild type plants during ripening: malate, galactose, inositol, fructose, glutamate, phenylalanine, pyroglutamate, alanine, glutamine, asparagines, tyrosine, and GABA.

PCA analysis was also performed on the 41 metabolites quantified in 50 placenta samples at four stages of fruit development (Fig. 4). The PCA scores (Fig. 4A) showed a pattern similar to that of the pericarp-columella samples. The first two principal components together explained 65% of the total variability. PC1 explained 52% of the total variability, clearly separating the early stages (20 and 30 DAP) on the negative side from of the late stages (40 and 50 DAP) on the positive side. PC2, explaining 13% of the total variability, tended to separate the different genotypes, especially at 40 and 50 DAP. The loading suggested that this separation could be due to differences in glucose, fructose, aspartate and chlorogenate on the negative side, and phenylalanine, isoleucine, glutamate, pyroglutamate, asparagine and glutamine on the positive side (Fig. 4B). Among the 25 known metabolites analysed by mean comparison, the following showed a significant accumulation in the placenta tissue of the transgenic lines compared to wild type during ripening: glucose, sucrose, fructose, galactose chlorogenate, citrate, galacturonate, malate, alanine, asparagine, aspartate, GABA, glutamate, glutamine, isoleucine, phenylalanine, and pyroglutamate.

Temporal profiles of metabolites in wild type and transgenic tomato fruits

Organic acids: Citrate content increased throughout fruit development in pericarp-columella and placenta tissues, and no significant difference was detected between the transgenic lines and the wild type in either tissue (Fig. 5A). Malate content increased during the early to middle stages of fruit development and peaked at 30 DAP in both tissue types, and then gradually decreased during

ripening. The malate content in the transgenic lines was significantly lower than that of the wild type in both tissues at all stages except 50 DAP (Fig. 5B).

Soluble sugars and sugar alcohol: Four major soluble sugars (sucrose, glucose, fructose, and galactose) were measured (Fig. 6). The glucose content in placenta tissue in transgenic fruits was similar to wild type fruits through all stages of ripening. At 50 DAP, transgenic fruits exhibited a reduction of 32.5% and 27.5% of glucose in lines 36A.12 and 43A.12, respectively, compared to wild type. A similar significant reduction was also observed in glucose accumulation at 50 DAP in the pericarp-columella tissue of transgenic fruits (Fig. 6A). Sucrose content in placenta tissue peaked at 40 DAP in wild-type fruit, with 1.5-fold increase compared to transgenic fruits, and then decreased at 50 DAP. In both transgenic lines, sucrose levels remained at similar levels at 40 DAP and then dropped by nearly 50% at 50 DAP. However, sucrose contents in pericarp-columella tissue decreased during development of the transgenic fruits, but not in wild type fruits (Fig. 6B). Fructose content was the highest of the soluble sugars measured (Fig. 6C). In wild type fruit, the fructose level increased during development in the both tissue types in columella-pericarp without 20 DAP. Fructose levels were generally lower (30% less) in both placenta and pericarp tissues of transgenic fruits than in wild type fruits, particularly at 50 DAP. Galactose content was the lowest of the soluble sugars measured. Galactose levels rose at 40 DAP in both tissue types, and then dropped in transgenic fruits by 20-40% compared to wild-type at 50 DAP (Fig. 6D). The inositol content in placenta tissue was slightly higher than in the pericarp-columella in most stages, and tended to decrease as ripening progressed (Fig. 6E). Inositol content was significantly higher at most of the stages in transgenic than that in wild-type fruit, particularly in line 43A.12.

Amino acids: Glutamate content showed a marked accumulation between 40 and 50 DAP in the two transgenic lines, with a 10-fold increase compared to the wild type in both tissues (Fig. 7A). At the early stage of fruit development, aspartate content was similar in both the wild type and transgenic

fruits. However, at stages 40 and 50 DAP, a sharp rise in aspartate content was observed in wild type fruits, with a 3- to 3.3-fold increase in pericarp-columella and a 4- to 5-fold increase in placenta tissues compared to transgenic fruits (Fig. 7B). Pyroglutamate, glutamine, tyrosine, phenylananine, isoleucine, alanine, and asparagine contents were higher in transgenic fruits compared the wild-type in late ripening stages, and this trend was most pronounced in pericarp-columella (Figs. 7C-I). The γ-aminobutyric acid (GABA) content peaked at the mature-green stage (30 DAP) in the both tissue types, but was much higher in the placental tissue than in the pericarp-columella (Fig. 7J). GABA accumulation was greater in the transgenic fruits than in the wild type, especially in the placenta after 30 DAP. GABA levels in placenta tissues at 50 DAP was 1.77-fold and 2.33-fold higher in the 36A.12 and 43A.12 lines, respectively, comparatively to wild type fruits.

Unknown metabolites: A number of metabolites appearing in the ^{1}H-NMR signature were not identified: 12 metabolites in the pericarp-columella extracts and 16 metabolites in the placenta extracts (data not shown).

Carotenoid accumulation in transgenic tomato fruits

The total carotenoid content in pericarp-columella and placental tissues of wild type and transgenic tomato fruits at 40 and 50 DAP were quantified by HPLC-DAD. Total carotenoid contents in pericarp-columella of transgenic lines were significantly higher than that of wild type fruits at the both time points (Table 1). A similar trend was also observed for lycopene and lutein contents. The total carotenoid contents in the pericarp-columella tissues were higher in the transgenic lines than in the wild type at both stages, whereas this difference was less pronounced in placenta (Table 2). Although the total carotenoid content in placenta tissue was not significantly different between transgenic lines and wild type, the β-carotene content was significantly higher in

the transgenic lines (Table 2). Overall, we observed enhanced accumulation in several kinds of carotenoids, especially lycopene, lutein, and β-carotene, in the transgenic fruits during ripening.

Ethylene production in transgenic fruits

Ethylene production in transgenic and the wild type fruits at different ripening stages was measured by GC (Fig. 8). Ethylene production in tomato fruits from transgenic lines began to increase at 30 DAP peaked at 40 DAP in line 36A.12, and at 37 DAP in line 43A.12. In the wild-type fruits, ethylene production gradually increased from 20 DAP and peaked at 40 DAP. Levels of ethylene production were significantly higher in the transgenic lines than in the wild type, and the increase to the peak value was steeper in the transgenic lines. These observations suggest that transgenic tomato fruits accumulating higher polyamines produce more ethylene during ripening.

Transcriptional characterization of genes responsible for carotenoid metabolism

To elucidate the mechanism underlying the increase in carotenoid contents in the transgenic fruits, transcriptional levels of several key genes encoding carotenoid metabolic enzymes were analysed by RT-PCR (Fig. 9). *PSY1*, *DXS*, and *PDS* (which encode phytoene synthase, deoxy-D-xylulose 5-phosphate synthase, and phytoene desaturase, respectively) are involved in lycopene biosynthesis, whereas *LCY-E* and *LCY-B* (which encode lycopene-epsilon cyclase and lycopene beta cyclase, respectively) are involved in lycopene cyclisation. The transcriptional levels of *DXS*, *PSY1*, and *PDS* tended to be higher in the two transgenic lines than in the wild type during late ripening stages (40-50 DAP). *PSY1* in the two transgenic lines showed a strong increase compared to wild type fruits, from 1.63-fold (line 43A.12) to 1.91-fold (line 36A.12) at 40 DAP, and 1.7-fold (line

43A.12) to 2.2-fold (line 36A.12) at 50 DAP (Fig. 9B). In contrast, *LCY-E* and *LCY-B* transcriptional levels were significantly lower in the two transgenic lines than in the wild type (Figs. 10D-E). *LCY-E* gene expression showed a significant decline, to 80.2% in line 36A.12 and 69.5% in line 43A.12 at 40 DAP, and to 51.2% in line 36A.12 and 63.1% in line 43A.12 at 50 DAP. These results indicate that carotenoid biosynthetic genes were up-regulated, whereas those involved in carotenoid degradation were suppressed, at the transcriptional level during fruit ripening in transgenic tomato fruits.

Discussion

Polyamine contents in the pericarp-columella

Over-expression of *Md-SPDS1* resulted in an increase in the free spermidine and spermine titres in transformed plants (Figs. 2B, C). Interestingly, over-expression of spermidine synthase also led to increased putrescine levels in the pericarp-columella tissue of tomato fruit (Fig. 2A). Similar results have been reported by Kasukabe et al. (2004), in which arabidopsis over-expressing *SPDS* cDNA from *Cucurbita ficifolia* showed significant increases in spermidine synthase activity and spermidine, spermine and putrescine concentrations. Generally, putrescine is biosynthesised either from ornithine by ornithine decarboxylase (ODC) or from arginine by arginine decarboxylase (ADC). On the other hand, the back-conversion of polyamines from spermidine to putrescine has been reported recently in arabidopsis (Moschou et al. 2008). Thus, it is likely that the increase of putrescine observed in the transgenic fruit in this study was due to spermidine to putrescine back-conversion by an unknown tomato PAO isoform to maintain a spermidine homeostasis, although we do not rule out the possibility that the observed putrescine accumulation could result from an effect on ADC and/or ODC activity related to increased spermidine content. Intracellular polyamine levels

are tightly regulated in all cells, and the changes in their levels result not only from biosynthesis but also from catabolism, transport, and conjugation (Cowley and Walters, 2005).

Metabolic profiling of tomato fruit

To assess the effects of spermidine synthase overexpression on the accumulation of other metabolites in tomato fruit, we used quantitative metabolite profiling (Fig. 2, 5-7, Tables 1, 2). Quantitative metabolic profiling is a method to measure the absolute concentration of unambiguously detected metabolites (Nielsen and Oliver, 2005), which allows increased accuracy in determining metabolite or metabolite/developmental correlation characteristics (Mounet et al., 2007). Obiadalla-Ali et al. (2004) showed that pericarp metabolism is different from that of the placenta; placental tissue acts as a conduit for nutrients going to the developing seeds, whereas the pericarp protects the seeds within the fruit. We therefore used the placenta without seeds and the pericarp-columella for metabolic analysis instead of the whole tomato fruit. A number of morphological, biochemical, and metabolic changes and modifications that affect carbohydrates (Obiadalla-Ali et al., 2004), amino acids (Boggio et al., 2000), and carotenoids (Fraser et al., 1994) occur during tomato fruit development. The parallel differentiation of chloroplasts into chromoplasts and the dominance of carotenoids and lycopene in ripening tomato fruit follow the transition from partially photosynthetic to true heterotrophic metabolism (Carrari and Fernie, 2006).

Accumulation of polyamines and primary metabolites during tomato fruit development

The accumulation profile of soluble sugars in the pericarp-columella and placenta were consistent with the results of Obiadalla-Ali et al. (2004), who performed carbohydrate metabolism

developmental analysis in the tomato (*Solanum lycopersicum* L.) cv. Micro-Tom, the same cultivar used in our study (Fig. 6). Contrary to the results of Mattoo et al. (2006), we found that citrate levels increased throughout fruit development, but observed no significant difference between transgenic and wild type fruits (Fig. 5A). Malate, which decreased during ripening, showed similar accumulation in pericarp-columella as in placenta tissues (Fig. 5B). Citrate and malate accumulation during tomato fruit development seen in this study was similar to that reported by Kortestee et al. (2007).

A dramatic increase in glutamate content during ripening was also observed. The glutamate level in both the pericarp and placenta tissues was about six times higher in the transgenic fruits than in the wild type. Other glutamate-related metabolites, such as glutamine and pyroglutamate, also increased significantly in transgenic fruits during ripening. It is well known that 70% of the total amino acid content in the pericarp of developing tomato fruits belongs to the glutamate family (Valle et al., 1998). Glutamate, glutamine, asparagine, and GABA all showed higher accumulation in transgenic ripened fruits than in wild-type plants. Similar results were reported by Boggio et al. (2000). Generally, GABA is converted to succinate through GABA shunt (Shelp et al., 1999). In tomato fruit, GABA peaked at the mature-green stage and decreased during maturation. In our results, overexpression of the *Md-SPDS1* gene clearly suppressed the GABA decrease during ripening in transgenic fruit (Fig. 7J). Because succinate derived from GABA is thought to be converted to malate, the suppression of GABA degradation was likely responsible for the observed decrease in malate contents (Fig. 5B). These results are consistent with those of Mattoo et al. (2006) in the sense that both metabolite profiling analyses pointed to the influence of polyamines on multiple cellular pathways in ripening tomato fruit. Although a similar accumulation was observed for some metabolites, such as GABA, citrate, glutamate, and glutamine, the amplitude of the change different between the wild type and the transgenic fruits with altered polyamine contents. Mattoo et

al. (2006) generated transgenic tomatoes with high spermidine and spermine levels by introducing the yeast S-adenosylmethionine decarboxylase (*SAM-DC*) gene driven by a ripening-specific E8 promoter. *SAM-DC* is involved in spermidine and spermine synthesis, both of which proceed by the sequential addition of aminopropyl groups to putrescine and spermidine; the amino propyl groups are generated from SAM by SAM decarboxylase. We generated transformants using the 35S promoter and spermdine synthase, which only acts to convert putrescine to spermidine. The discrepancy between our results and those of Mattoo et al. (2006) could also be related to differences in experimental conditions. Indeed, metabolite accumulation and regulation are far more complex in tomato fruit still attached to the plant (our conditions) than in fruits permitted to mature off the vine, due in particular to the influence of plant hormones. For example, Johnson et al. (2003) clearly showed that data gathered from fruits ripened off the vine clustered differently compared to the data for those ripened on the vine. PCA analysis of the metabolic fingerprints clearly discriminated between the treatments, indicating significant differences in fruit biochemistry depending on the method of ripening.

The overall picture that emerges from analyzing the quantitative metabolic profiling strongly suggests that changes in free polyamine content (putrescine, spermidine, and spermine) in transgenic plants affects primary metabolic pathways in tomato fruit, particularly during ripening. It is interesting that constitutive over-expression of the *SPDS* gene enhanced the accumulation of not only spermidine and spermine, but also putrescine (Fig. 2). Despite the considerable increases in spermidine and spermine, little change was observed in metabolite contents during 10–30 DAP (Figs. 2, 5-7). Spermidine and spermine were mainly associated with the cell division phase and minimal levels were observed at ripening, when climacteric ethylene production occurs (Torrigiani et al., 2008). In contrast, the putrescine content was low in early developing fruit and steadily increased with ripening, eventually reaching the highest concentration of all the polyamines (Fig. 2A). The

metabolite profile diverged into different clusters between wild type and transgenic fruit for both tissue types during ripening (Figs. 3 and 4). These results indicate that the enhanced accumulation of spermidine and spermine in early fruit development affects the primary metabolism of ripening fruit, but does not have significant effects during early stages of development. It is still unclear how *SPDS* over-expression caused such a metabolic alteration. One possible explanation is that excessive spermidine and/or spermine accumulation activates putrescine biosynthesis through feedback regulation, and the accumulated putrescine alters primary metabolism in ripening fruit.

Ethylene production in transgenic tomato accumulating high polyamines

In climacteric fruits, such as the tomato, ethylene plays a major role in fruit development and ripening (Alba et al., 2005). The rate-limiting steps in fruit ethylene biosynthesis include the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and the subsequent metabolism of ACC to ethylene by ACC oxidase (Tassoni et al., 2006). SAM is also the substrate for SAM decarboxylase in polyamine biosynthesis. These two pathways are directly connected, as all polyamines share ethylene as the common substrate. Several studies have suggested that there is competition for SAM between biosynthetic pathways (Kumar et al., 1996). Additional support for the existence of competition between the two pathways comes from the observation that transcription of ethylene perception and biosynthesis genes is altered by putrescine and spermidine during ripening in *Prunus persica* (Ziosi et al., 2006). In this work, the two transformed lines over-expressing the *Md-SPDS1* gene showed a considerable and significant increase in ethylene production compared to the wild type during early ripening stages (37 and 40 DAP, Fig. 8). Similar results have been reported for tomatoes ripened off the vine, which showed delayed development and senescence in addition to high polyamine accumulation (Mehta et al., 2002). In harvested apple

fruits, transient antagonistic relationships between polyamine and ethylene were observed soon after 1-methylcyclopropene treatment (Pang et al., 2006). It is noteworthy that over-production of ethylene in transgenic fruits was limited to early ripening stages, and was lower than wild type in other fruit stages (Fig. 8). Because the promotional effect of the transgene on total polyamine contents was lowest at 40 DAP (Fig. 2), lower usage of substrate could transiently slant the antagonistic relationships toward ethylene biosynthesis at this stage.

Enhanced availability of carotenoid precursors and regulation of carotenoid metabolic genes

Ppolyamine contents are under strict homeostasis, and many mechanisms cooperate to maintain their cellular levels within strict limits (Moschou et al. 2008). Polyamine oxidation is part of the regulatory process. The oxidation reaction products of diamines are pyrroline, hydrogen peroxide and ammonia, whereas polyamine oxidation gives rise to pyroline and 1,5-diazabicyclononane, along with diaminopropane and hydogen peroxide. Diaminopropane can be converted into β-alanine, whereas pyrroline can be further catabolised to GABA in a reaction catalysed by pyrroline dehydrogenase (Bouchereau et al., 1999). GABA is a major intermediate of polyamine oxidation. According to Rontein et al. (2002), the accumulation of GABA and alanine may reflect a higher carbon flux through glycolysis leading to higher pyruvate and glyceraldehyde-3-phosphate (GA3P) availability. For this reason, we investigated the contents of these metabolites. We found that GABA and alanine contents were significantly higher in 36A.12 and 43A.12 transgenic fruits at 40 and 50 DAP than in the wild type (Figs. 7H and J). Notably, pyruvate and GA3P are also isoprenoid pathway precursors. The availability of these two precursors was reported as a major factor that can limit isoprenoid production in *E. coli* (Farmer and Liao, 2001) and also determine the level of carotenoid accumulation in tomato fruit tissues (Télef et al., 2006).

A large body of work has shown that carotenoid accumulation is regulated by diverse and complex mechanisms during tomato fruit ripening (Cunningham, 2002; Carrari and Fernie, 2006). In the 36A.12 and 43A.12 transgenic lines, the carotenoid biosynthetic genes *DXS, PSY1 and PDS* were up-regulated during ripening (Fig. 9A-C), and transcriptional levels of lycopene cyclisation genes were reduced in the wild type compared to the transgenic lines (Fig. 9D-E). This accounts for the observed increase in lycopene content in the transgenic tomato fruits, as lycopene content is regulated by these enzymes. Previous studies on the regulation of carotenoid biosynthesis have focused on determining transcript levels and patterns. These studies strongly suggest that the mechanism for regulating carotenoid formation occurs at the transcription level. In abiotic stress studies, spermidine has been shown to play a role as a signalling molecule in plant responses to stress through the up-regulation of genes encoding DREB transcription factors (Kasukabe et al., 2004). It is possible that polyamines exert their influence at the transcriptional level and to regulate the genes responsible for lycopene accumulation. However, it is unlikely that transcriptional regulation is the sole mechanism by which carotenoid biosynthesis is regulated (Fraser and Bramley, 2004). Regulation at the enzymatic level has been predicted to account for the high lycopene concentration seen in tomato fruits (reviewed by Hirschberg, 2001). The molecular and metabolic characterization analysis of transgenic tomatoes over-expressing *Md-SPDS1* indicates that polyamines can regulate carotenoid biosynthesis at least in part though modifying steady state levels of gene transcripts involved in these pathways. The increased availability of carotenoid pathway precursors in the transgenic fruit would cooperate with these processes. Taken together, the high levels of polyamines exert complex regulatory effects on the lycopene biosynthesis and metabolism.

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Figure Legends

Figure 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 wild type 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.

Figure 2 Free polyamine accumulation in the pericarp and columella tissues of tomato fruits from wild type and 36A.12 and 43A.12 fixed homozygous transgenic lines. A, B, and C represent free putrescine, free spermidine, and free spermine content, respectively. Values (nmol/gfw) are averages of independent fruit data from each line (n = 3) and are shown as means ± SDs. Asterisks indicate significant differences compared to the wild type: $*P < 0.05$, $**P < 0.01$.

Figure 3 Analysis of the absolute concentration of 37 metabolites from transgenic and wild type tomato fruits sampled at different stages (10, 20, 30, 40, and 50 DAP) quantified using ^{1}H-nuclear magnetic resonance analysis of pericarp-columella tissue. A: scores plot; B: loading plot for each score. Loadings are indexed with the corresponding metabolite names. adeno, adenosine like compound; ala, alanine; asn, asparagine; asp, aspartate; chlor, chlorgenate; chol, choline; cit, citrate;

fru, fructose; GABA, γ-aminobutyric acid; gal, galactose; galact, galacturonate; gln, glutamine; glu, glutamate; gluc, glucose; ileu, isoleucine; inos, inositol; mal, malate; naring, naringenin; phe, phenylalanine; pyroglu, pyroglutamate; quin, quinate; suc, sucrose; trig, trigonelline; tryp, tryptophane; unk, unknown; val, valine.

Figure 4 Analysis of the absolute concentrations of 41 metabolites in placental tissue from transgenic and control tomato fruits quantified using ^{1}H-nuclear magnetic resonance analysis at different stages (20, 30, 40, and 50 days after pollination). A: scores plot; B: loading plot for each score.

Figure 5 Organic acid accumulation during fruit development in the pericarp-columella and placenta of wild type and 36A.12 and 43A.12 transgenic line tomatoes. Values are means ± SDs of 4-5 independent measurements. For each tissue and stage of development, the mean comparison between each transgenic line (36A.12 or 43A.12) and wild type was performed using Student's t test (*P < 0.05, **P < 0.01). A, citrate; B, malate.

Figure 6 Changes in soluble sugar and sugar alcohol content in the pericarp-columella and placenta during tomato fruit development in wild type and 36A.12 and 43A.12 transgenic lines. Values are means ± SDs of 4-5 independent measurements. For each tissue and stage of development, the mean comparison between each transgenic line (36A.12 or 43A.12) and wild type was done using Student's *t*-test (*P < 0.05, **P < 0.01). A, glucose; B, sucrose; C, fructose; D, galactose; E, inositol.

Figure 7 Amino acid content in the pericarp-columella and placenta during tomato fruit development in wild type and the 36A.12 and 43A.12 transgenic line tomatoes. Values are means ± SDs of 4-5 independent measurements. For each tissue and stage of development, the mean comparison between each transgenic line (36A.12 or 43A.12) and wild type was done using Student's *t*-test (**P* < 0.05, ***P* < 0.01). A, glutamate; B, aspartate; C, pyroglutamate; D, glutamine; E, tyrosine; F, phenylalanine; G, isoleucine; H, alanine; I, asparagine; J, GABA.

Figure 8 Rates of ethylene production during fruit ripening in wild type and 36A.12 and 43A.12 transgenic tomato lines. Values represent averages obtained from independent fruits of each line (n = 8–10) and are shown as means ± SDs. Asterisks indicate significant differences compared to the control: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test).

Figure 9 Regulation of carotenoid biosynthesis gene expression during tomato fruit ripening in wild type and transgenic plants. Relative expression levels of DXS (A), PSY1 (B), PDS (C), LCY-E (D), and LCY-B (E) in tomato fruits harvested at different developmental stages. Expression of each gene was analysed in duplicate and repeated three times (n = 6). Asterisks indicate significant differences compared to the control: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test).

Table 1
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Table 1 Carotenoid content (µg/g of dry weight) measured in tomato pericarp-columella.

Line	Fruit stage	Phytoene	Lycopene	ß-Carotene	Lutein	Total
WT	40DAP	133.9 ± 6.8	318.1 ± 20.9	33.4 ± 0.8	17.0 ± 0.8	502.4 ± 29.3
36A.12	40DAP	158.1 ± 32.3	641.9 ± 49.4*	43.1 ± 6.1	24.1 ± 2.2	867.2 ± 90.0*
43A.12	40DAP	141.7 ± 10.4	702.0 ± 56.0	31.5 ± 4.2	25.1 ± 1.5*	900.3 ± 72.8*
WT	50DAP	240.4 ± 16.7	1058.5 ± 178.0	28.3 ± 2.6	10.2 ± 0.7	1337.4 ± 198.0
36A.12	50DAP	252.9 ± 7.6	1720.0 ± 35.2*	41.4 ± 2.4*	16.8 ± 1.2*	2031.2 ± 363.2*
43A.12	50DAP	234.6 ± 12.4	1661.0 ± 51.6*	44.1 ± 3.4*	21.0 ± 0.5*	1960.8 ± 67.8*

* indicates significant difference between the wild type and transgenic lines (Student's test, $P < 0.05$). Values are means ± SEs of four independent determinations.

Table 2
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Table 2 Carotenoid content (µg/g of dry weight) measured in tomato placenta.

Line	Fruit stage	Phytoene	Lycopene	ß-Carotene	Lutein	Total
WT	40DAP	60.0 ± 7.1	181.3 ± 14.8	55.6 ± 4.0	35.1 ± 1.2	331.9 ± 27.1
36A.12	40DAP	69.3 ± 12.8	312.6 ± 13.9*	64.6 ± 7.7	45.6 ± 3.5*	492.0 ± 58.5
43A.12	40DAP	58.3 ± 12.8	235.8 ± 17.0	47.4 ± 3.2	37.7 ± 0.9	379.2 ± 120.0
WT	50DAP	167.0 ± 65.1	484.1 ± 138.1	40.1 ± 15.1	26.2 ± 3.6	717.5 ± 244.6
36A.12	50DAP	155.6 ± 7.4	481.0 ± 87.1	65.0 ± 2.8*	33.0 ± 0.7	734.5 ± 98.0
43A.12	50DAP	117.0 ± 5.2	660.0 ± 78.3	66.7 ± 2.5*	37.7 ± 1.3 *	881.3 ± 87.3

* indicates significant difference between the wild type and transgenic lines (Student's test, $P < 0.05$). Values are means ± SEs of four independent determinations.

Figure 1

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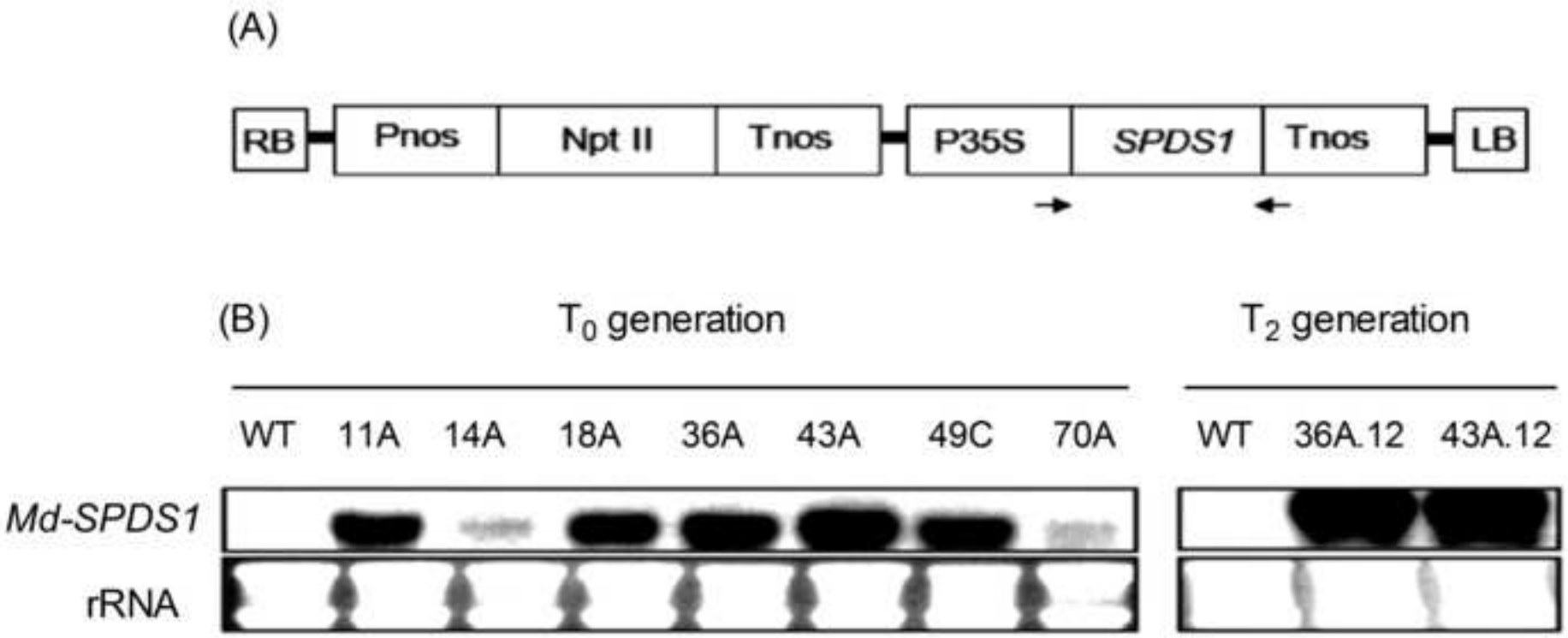

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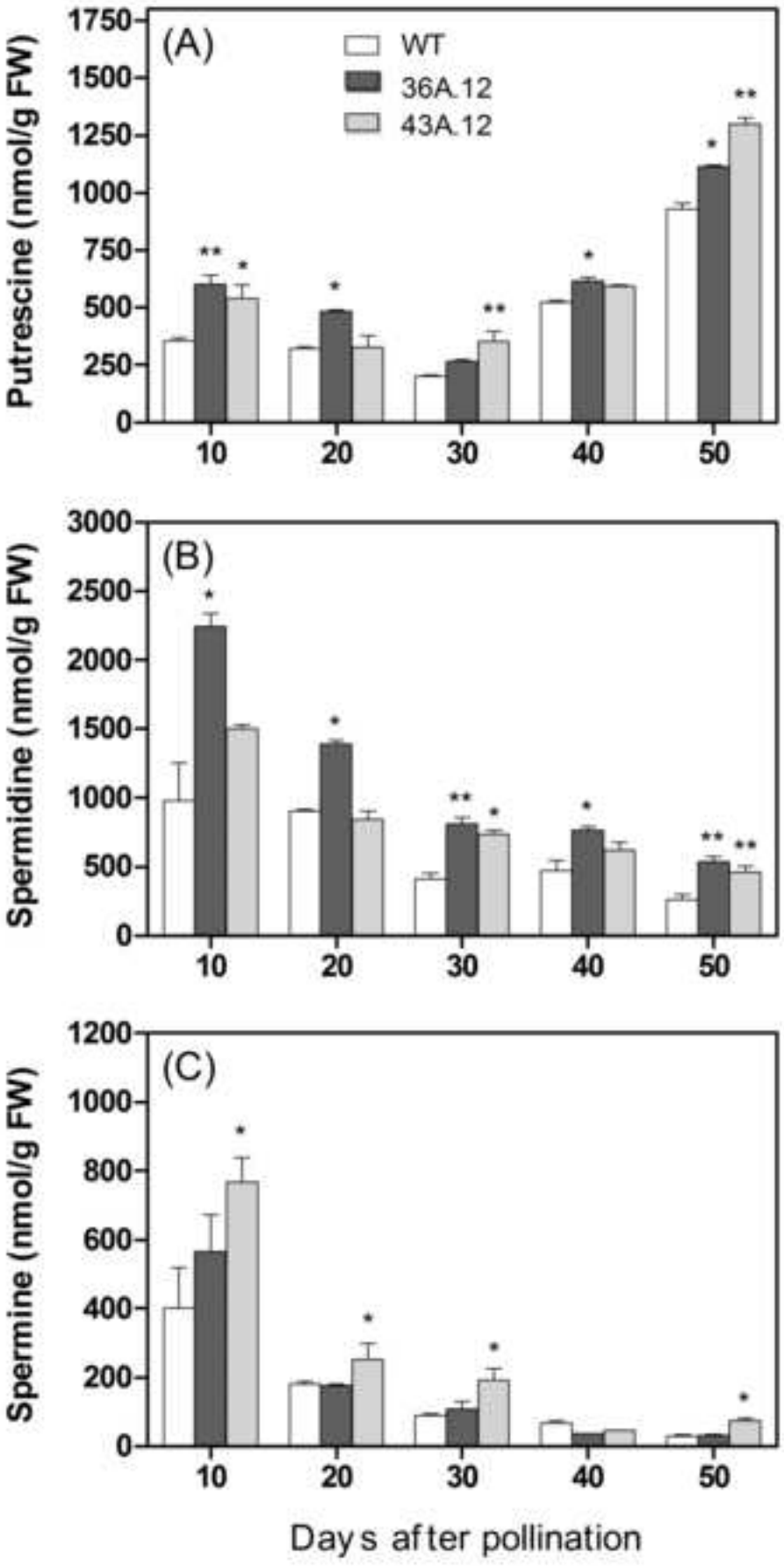

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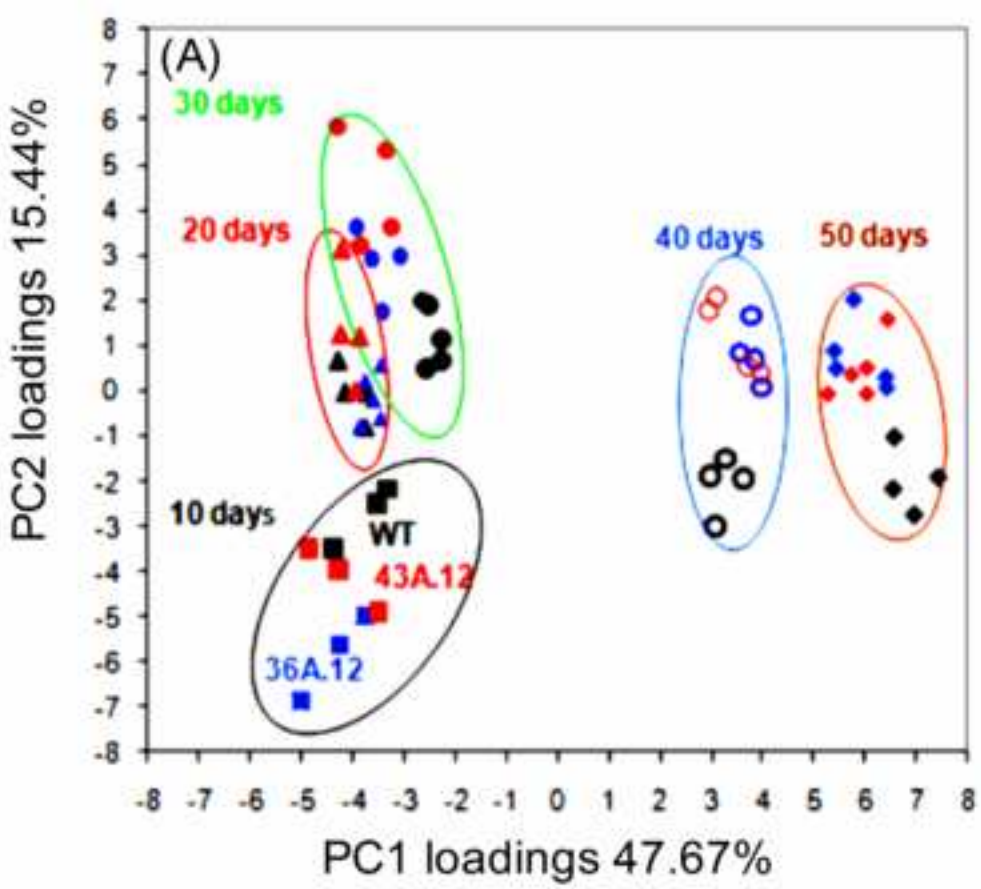

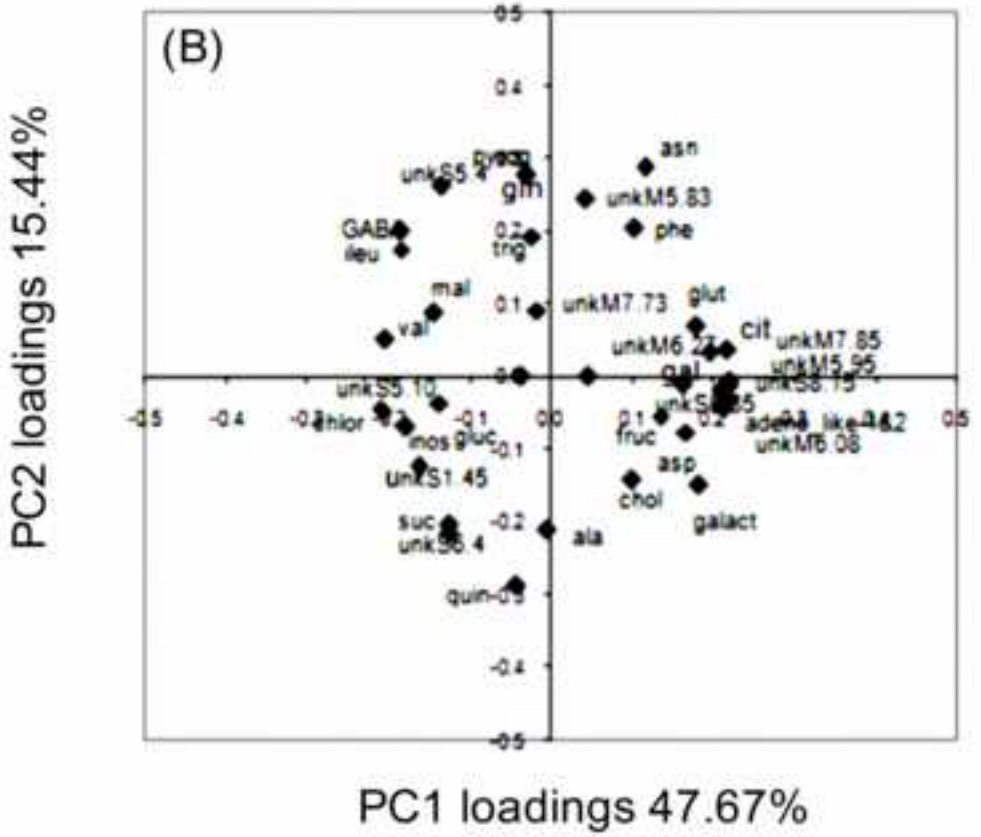

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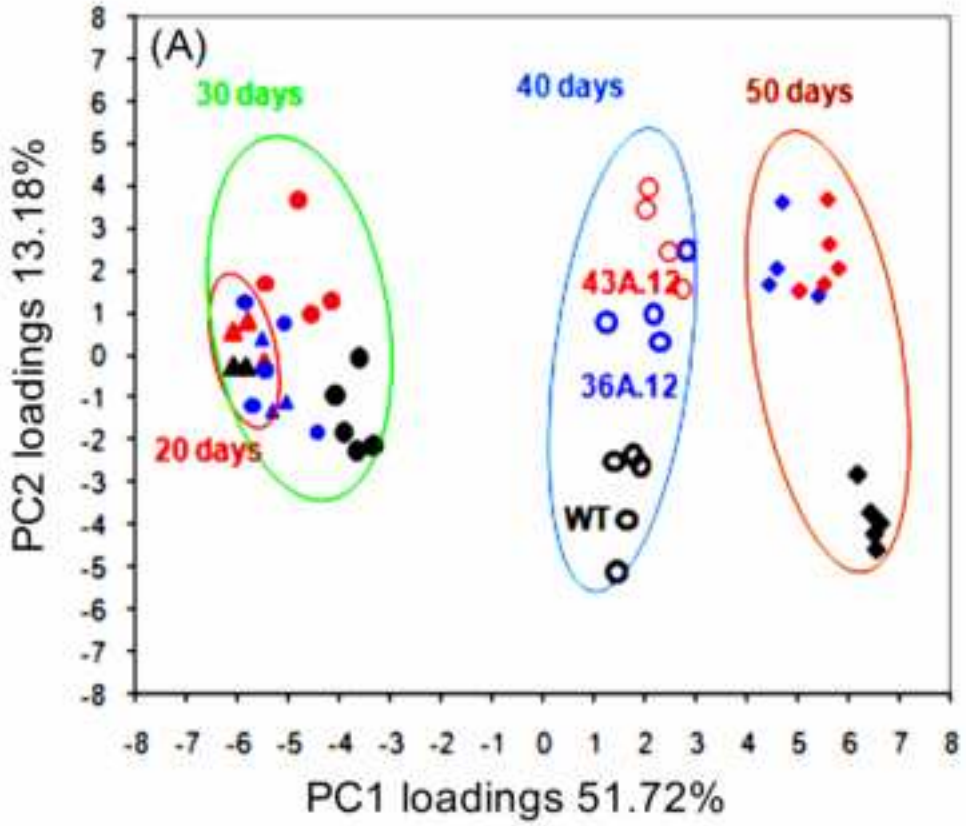

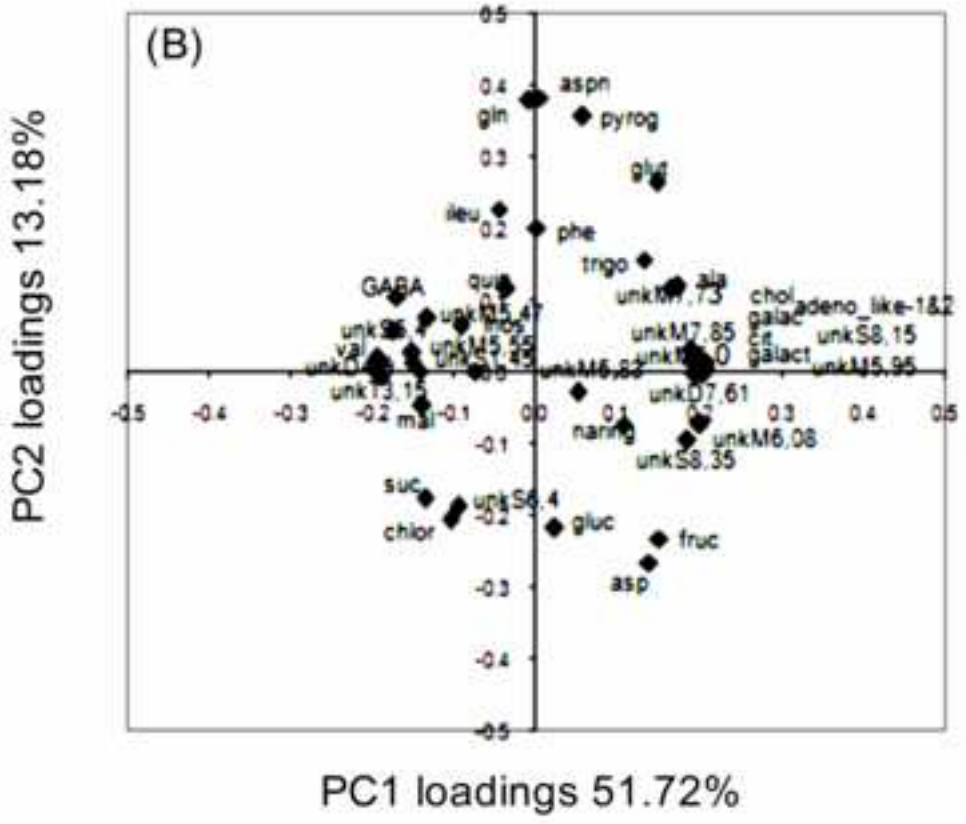

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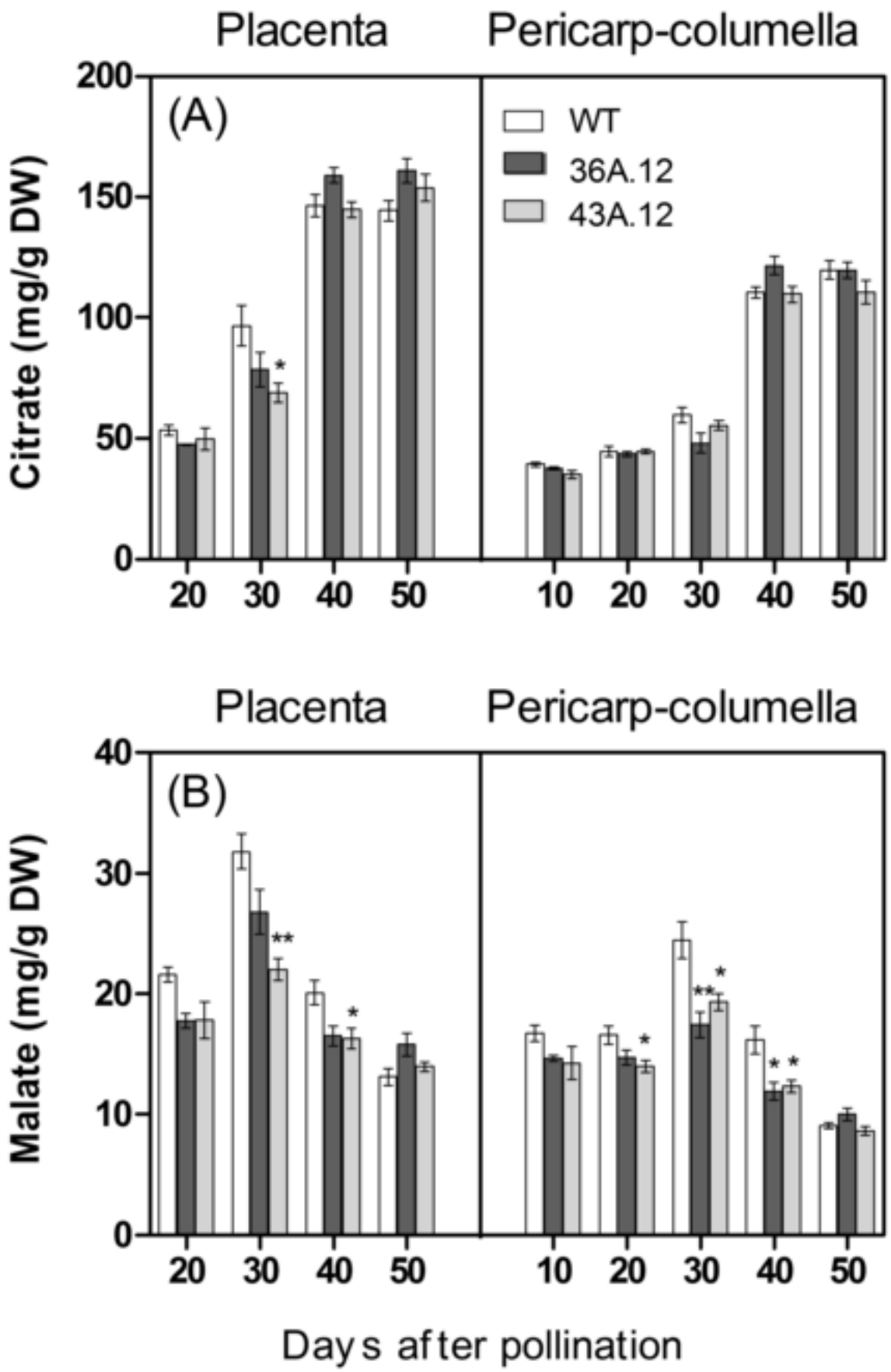

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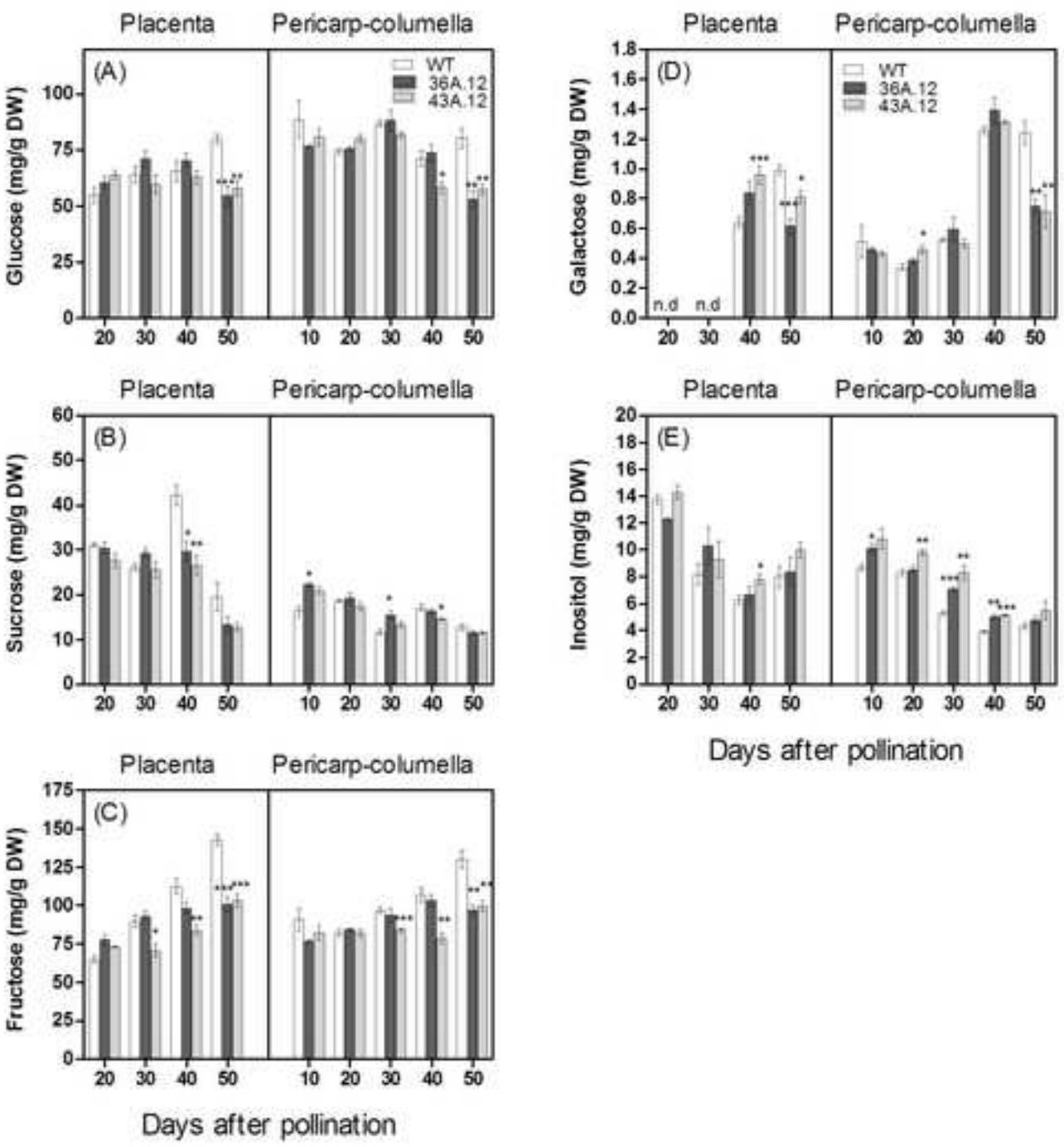

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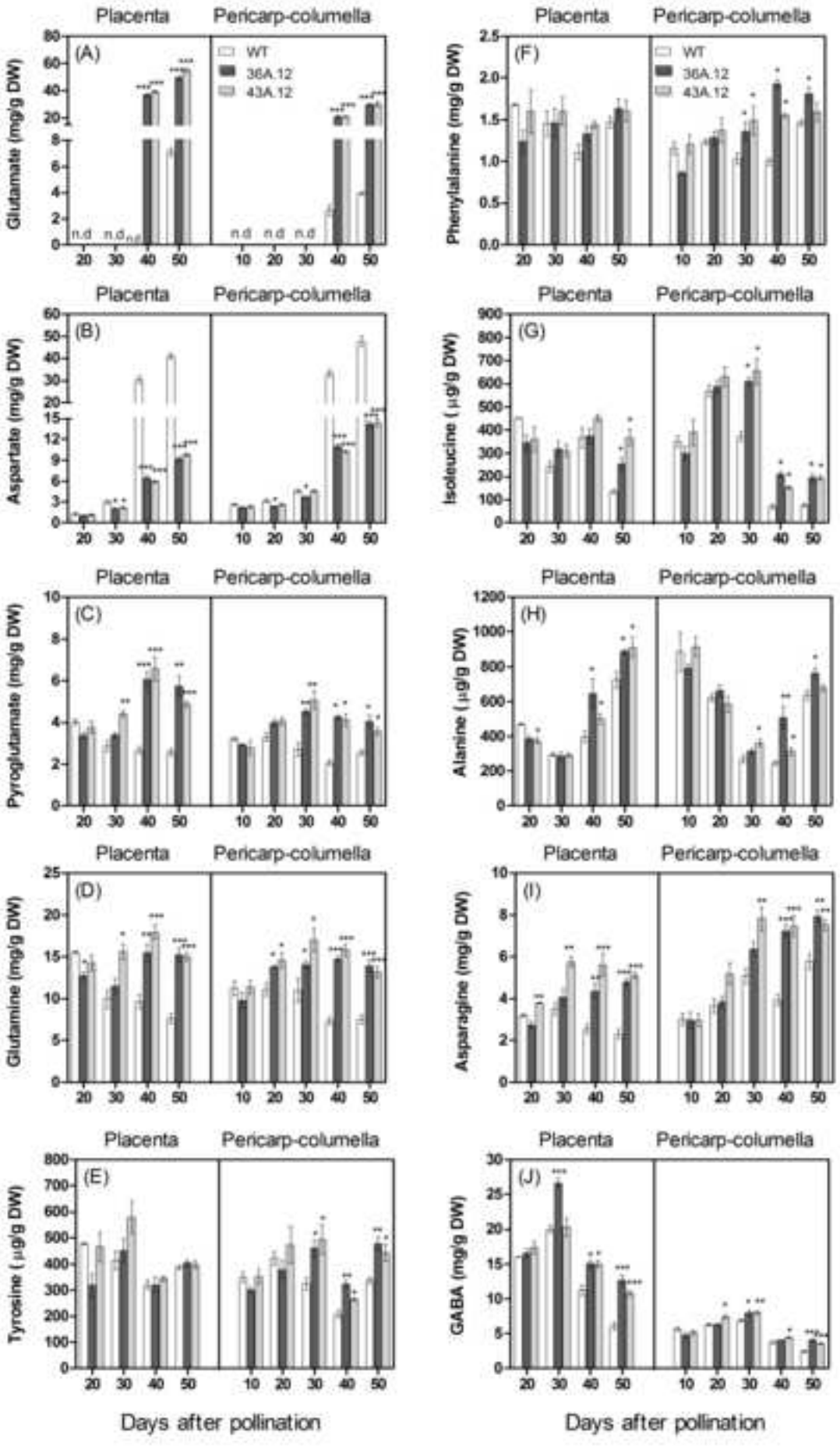

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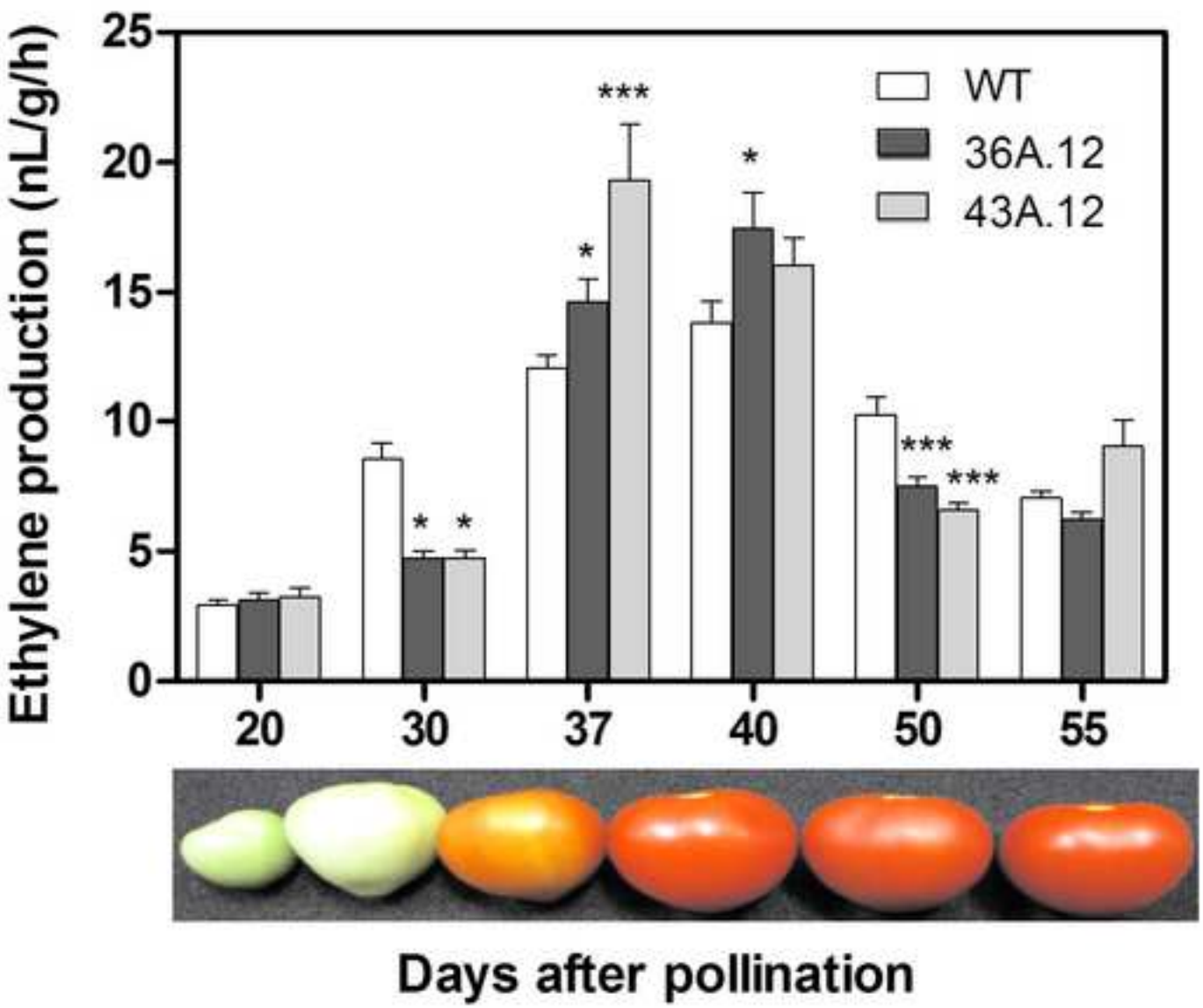

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