

Nuclear Magnetic Resonance Spectroscopy-Based Metabolite Profiling of Transgenic Tomato Fruit Engineered to Accumulate Spermidine and Spermine Reveals Enhanced Anabolic and Nitrogen-Carbon Interactions^{1[W][OA]}

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Polyamines are ubiquitous aliphatic amines that have been implicated in myriad processes, but their precise biochemical roles are not fully understood. We have carried out metabolite profiling analyses of transgenic tomato (*Solanum lycopersicum*) fruit engineered to accumulate the higher polyamines spermidine (Spd) and spermine (Spm) to bring an insight into the metabolic processes that Spd/Spm regulate in plants. NMR spectroscopic analysis revealed distinct metabolite trends in the transgenic and wild-type/azygous fruits ripened off the vine. Distinct metabolites (glutamine, asparagine, choline, citrate, fumarate, malate, and an unidentified compound A) accumulated in the red transgenic fruit, while the levels of valine, aspartic acid, sucrose, and glucose were significantly lower as compared to the control (wild-type and azygous) red fruit. The levels of isoleucine, glucose, γ -aminobutyrate, phenylalanine, and fructose remained similar in the nontransgenic and transgenic fruits. Statistical treatment of the metabolite variables distinguished the control fruits from the transgenic fruit and provided credence to the pronounced, differential metabolite profiles seen during ripening of the transgenic fruits. The pathways involved in the nitrogen sensing/signaling and carbon metabolism seem preferentially activated in the high Spd/Spm transgenics. The metabolite profiling analysis suggests that Spd and Spm are perceived as nitrogenous metabolites by the fruit cells, which in turn results in the stimulation of carbon sequestration. This is seen manifested in higher respiratory activity and up-regulation of phosphoenolpyruvate carboxylase and NADP-dependent isocitrate dehydrogenase transcripts in the transgenic fruit compared to controls, indicating high metabolic status of the transgenics even late in fruit ripening.

Polyamines represent a class of aliphatic nitrogenous compounds that are ubiquitous in nature with essential functions in living organisms (Tabor and Tabor, 1984; Slocum and Flores, 1991; Cohen, 1998). In eukaryotes, the most prevalent are the diamine putrescine, triamine spermidine (Spd), and the tetra-

mine spermine (Spm). Polyamines have been implicated in myriad biological processes, including cell proliferation, cell division and differentiation, apoptosis, homeostasis, gene expression, and protein and DNA synthesis (Tabor and Tabor, 1984; Slocum and Flores, 1991; Cohen, 1998; Igarashi and Kashiwagi, 2000; Wallace et al., 2003; Janne et al., 2004). The list gets longer when processes implicated in plants are considered: embryogenesis, root formation, floral initiation and development, fruit development and ripening, pollen tube growth and senescence, and biotic and abiotic stress responses (Slocum and Flores, 1991; Cohen, 1998; Cassol and Mattoo, 2003; Kaur-Sawhney et al., 2003). Polyamines have been shown to induce DNA conformational transitions from the right-handed to a left-handed Z-DNA, and to an alternative form of right-handed helix, A-DNA (Ouameur and Tajmir-Riahi, 2004). Based on immunocytochemical studies, it has been reported that Spd and Spm are associated with highly compacted mitotic chromosomes (Igarashi and Kashiwagi, 2000), inducing stabilizing rather than a regulating effect on the chromatin structure during the cell cycle (Sauve et al., 1999). By

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their characteristic property of binding to nucleic acids, polyamines have also been implicated in providing protection to cells from oxidative damage or external agents such as radiation damage (Tabor and Tabor, 1984; Drohlert et al., 1986; Ha et al., 1998; Janne et al., 2004). In vitro studies have indicated that Spd and Spm have the ability to inhibit senescence and ripening in a number of plant tissues (Cassol and Mattoo, 2003). In spite of significant progress made in understanding aspects of polyamine metabolism and transport, we know little about the in vivo role(s) of polyamines in cellular metabolism (Cohen, 1998; Igarashi and Kashiwagi, 2000; Cassol and Mattoo, 2003; Kaur-Sawhney et al., 2003; Uemura et al., 2005). Interestingly, polyamine catabolism has the potential to provide defense against biotic and abiotic stresses, particularly because the oxidation products include signaling molecules such as hydrogen peroxide (Paschalidis and Roubelakis-Angelakis, 2005; Cona et al., 2006).

In an effort to gain insight into the role of polyamines in growth and development, several studies have succeeded in altering endogenous polyamines in animals and plant cells by overexpression and knock-out of genes of polyamine biosynthesis. However, in some cases, the elevated polyamine levels resulted in abnormal phenotypes, including stunting (Noh and Minocha, 1994; Kumar et al., 1996). We developed transgenic tomato (*Solanum lycopersicum*) fruit lines homozygous for the introduced yeast (*Saccharomyces cerevisiae*) S-adenosylmethionine (SAM) decarboxylase (SAMdc) gene fused to a ripening-specific promoter (Mehta et al., 2002). Since a fruit ripening-specific promoter was used to drive the expression of SAMdc, the introduced gene had no significant effect on normal growth and development. Fruits from transgenic plants accumulated higher polyamines in a ripening-specific manner, and the phenotypes showed increased antioxidant capacity and enhanced fruit processing quality. These transgenic tomato plants afford an excellent model system to analyze effects of polyamines, especially Spd/Spm, on metabolism and also to understand how plants regulate cellular processes. Importantly, harvested mature green fruit of these transgenics can be ripened on the bench, thus allowing evaluation of the effects of high levels of Spd/Spm in the absence of any perturbation from the parent plant. Profiling of metabolites and engineering their pathways are avenues to understand how plants regulate cellular processes and to reveal intracellular networks (Ratcliffe and Shachar-Hill, 2001; Fernie et al., 2004; Whitfield et al., 2004; Alba et al., 2005).

NMR spectroscopy is a powerful tool to analyze and quantify metabolite levels in cell extracts, in solution, and/or on semi-wet samples, requiring minimum preparation or handling of the tissue and no derivatization (Ratcliffe and Shachar-Hill, 2001; Sobolev et al., 2003). When high-resolution NMR methods are coupled to multivariate statistical analysis, the resulting partitions give unambiguous information about influences of nutritional and genetic backgrounds (Ratcliffe

and Shachar-Hill, 2001; Mannina et al., 2003; Amato et al., 2004; Krishnan et al., 2005). Metabolite profiling, metabolomics, offers a powerful approach to monitor complexity of genetically modified crops and document any unintended consequences of a modified gene introduced in crops (Kuiper et al., 2001; Sumner et al., 2003; Tretheway, 2004; Mungur et al., 2005). We have used high-resolution NMR methods to generate an analysis of the principal, soluble constituents of wild-type and polyamine-accumulating transgenic tomato. Here, we show that the same metabolites present in wild-type or azygous control tomatoes are also present in the higher polyamine-accumulating, transgenic tomato fruit. However, accumulation of Spd/Spm in transgenic fruit results in differential metabolite content as compared to the controls. The synthesis of the data presented suggests that Spd/Spm are sensed as regulatory amines/organic nitrogen (N), which, in turn, signal carbon (C) metabolism. These results show, to our knowledge, for the first time that Spd and Spm have profound effects on cellular metabolism, likely via regulation of distinct biochemical pathways.

RESULTS

A typical ^1H NMR spectrum of tomato fruit powder in D_2O buffer solution is shown in Figure 1, illustrating the spectral assignment corresponding to a few important compounds. Almost all resonances corresponded to known compounds. The chemical shifts corresponding with known metabolites are listed in Table I. Resonances labeled A, B, and C could not be assigned because they remained unidentified; however, A gave a spectral pattern identical to that of citrate and may therefore be a complex of citrate with a small molecule, B is an unidentified multiplet, and C is a singlet, possibly a choline derivative. These resonances were present in all samples. The values corresponding to the resonances were used to determine the profiles of the identified metabolites in wild-type/azygous controls and the two transgenic lines, 556HO and 579HO.

Profile of Amino Acids

The profiles of nine amino acids and γ -aminobutyrate (GABA) during ripening of wild-type, nontransgenic azygous (556AZ), and two transgenic (556HO and 579HO) tomato fruits are shown in Figure 2 (see also Supplemental Table S1). The levels of Ile, Val, Thr, Ala, and GABA in wild-type and 556AZ control fruit decreased during ripening. For the most part, a similar trend was apparent in 556HO and 579HO fruits except for Val, whose level was significantly lower at the late stages of ripening compared to the control fruits, and so was the case for Ala in the pink transgenic fruit (Fig. 2A). Phe levels declined during later stages of ripening in both nontransgenic controls (wild type, 556AZ) as well as in the 556HO and 579HO transgenics; however, Phe levels in red fruit of 579HO plants were lower than

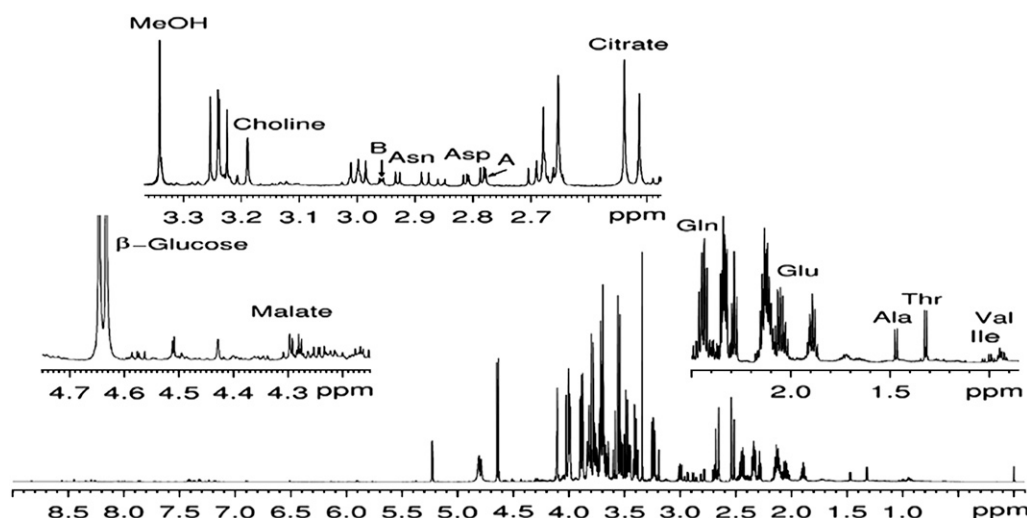


Figure 1. A typical 600-MHz ^1H NMR spectrum of tomato fruit powder in D_2O buffer solution and resonance for some of the indicated metabolites.

the rest of them. The levels of Gln and Asn decreased in the red fruit from wild-type and 556AZ plants. However, in the two high-polyamine transgenics, both Gln and Asn distinctly accumulated in the red fruit as compared to the two controls. Both Asp and Glu dramatically increased as ripening progressed in the wild-type and 556AZ fruit. In the two transgenics, the rise in Asp level was less as compared to the controls, a trend exactly opposite to that seen with Glu (Fig. 2). These profiles suggest that the increased levels of Spd/Spm in the transgenic tomato fruit have a ripening-related effect on the levels of Asp, Asn, Gln, and Val.

Profile of Organic Acids and Sugars

Two major organic acid constituents of fruits are citrate and malate (Mattoo et al., 1975; Tucker, 1993). Profiles of organic acids citrate, fumarate, and malate in the wild-type and 556AZ nontransgenic fruits indicate considerable decrease in their levels after the breaker stage as ripening progressed. However, citrate content remained significantly higher in the red fruit from both the high-polyamine transgenic lines (556HO and 659HO). Although the levels of malate and fumarate declined during ripening of the fruit from both the transgenics, their levels in the red fruit were also significantly higher than in the azygous/wild-type controls (Fig. 3A; Supplemental Table S1).

The contents of Glc and Fru remained more or less similar until breaker stage in fruit from wild-type and 556AZ plants but slightly declined at the pink and red ripening stages. In the transgenic fruits, Glc levels progressively declined from the green to ripe stage, while the Fru level remained similar to the controls (Fig. 3A). The higher levels of citrate, malate, and fumarate were associated with a decreased Glc content in the red ripe transgenic fruits. Suc levels, on the other hand, decreased during ripening in the fruit from all

the genotypes tested but faster decline was apparent in fruit from both the transgenic lines (Fig. 3A; Supplemental Table S1).

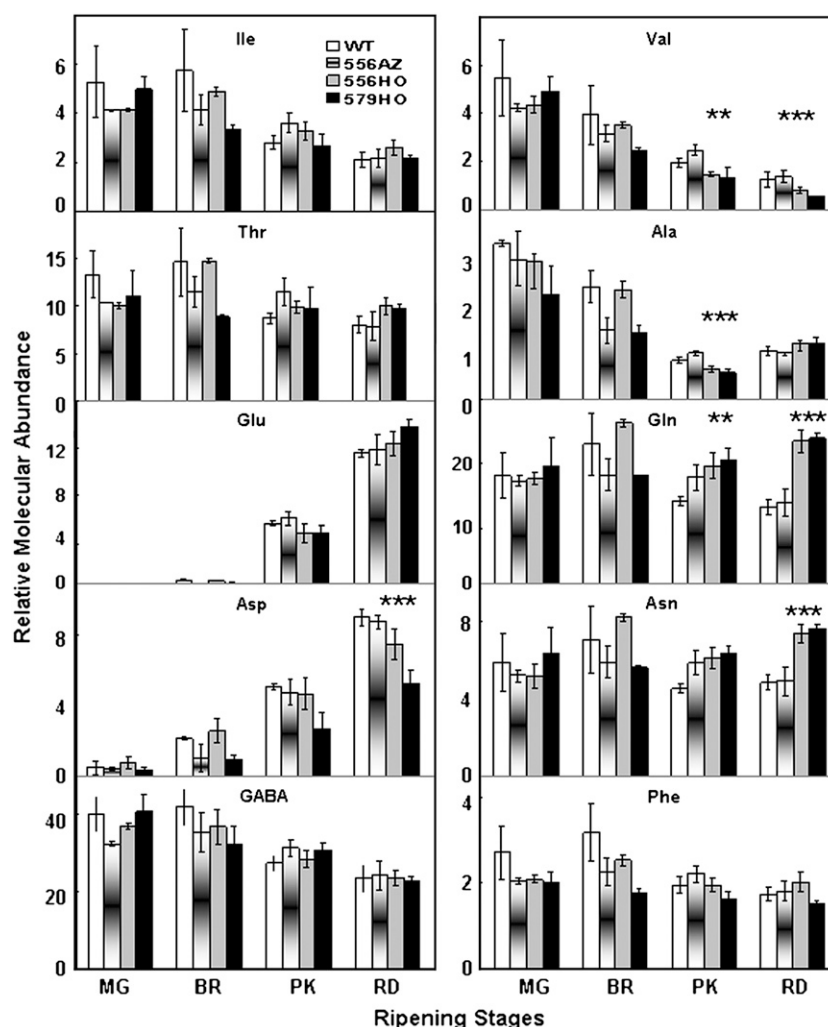
Compound A, likely a citrate complex, increased as ripening progressed in transgenic fruits, while levels decreased in the two nontransgenic control fruits (Fig. 3C). Changes in the levels of compound B were apparent among most of the genotypes examined here, but

Table 1. List of resonances and variables used for the evaluation of the various metabolites

Resonance No.	Chemical Shift (ppm)	Compound	Variable No.
1	1.0046	Ile	1
2	1.0370	Val	2
3	1.3284	Thr	3
4	1.4780	Ala	4
5	2.0687	Glu	5
6	2.2880	GABA	6
7	2.4245	Gln	7
8	2.5397	Citrate	8
9	2.7807	A (citrate pattern)	9
10	2.7903	Asp	10
11	2.8912	Asn	11
12	2.9615	B, doublet, unknown	12
13	3.1920	Choline	13
14	3.2100	C singlet, choline derivative	14
15	3.2564	β -Glc ^a	20
16	3.3432	MeOH	15
17	3.5625	β -Fructofuranose ^a	19
18	3.5830	β -Fructopyranose ^a	19
19	4.2940	Malate	16
20	5.2327	α -Glc ^a	20
21	5.4059	Suc	17
22	7.4195	Phe	18

^aIn the experimental conditions used (uniform concentration, buffered solvent for constant pH, constant temperature), the α and β isomers of Glc and those of fructose are in equilibrium, and, therefore, the isomers were not considered as independent.

Figure 2. Profiles of amino acids and GABA in wild-type (WT), azygous (556AZ), and two homozygous, transgenic (556HO, 579HO) tomato fruits at four stages of ripening. Shown is relative molecular abundance based on intensities of ^1H NMR signals from indicated metabolites in tomato fruit at four different stages of ripeness (MG, mature green; BR, breaker; PK, pink; RD, red). Data given are means $\pm$ SEM. ** and ***, Significant differences, respectively, at P values <0.05 and <0.005 in the levels of indicated metabolite between the transgenic fruits and the controls (WT and 556AZ). See also Supplemental Table S1.



differences between the transgenics and nontransgenics were not significant.

One consequence of these data is that the transgenic red fruit have significantly higher Fru to Glc (Fig. 4A) and acid (citrate + malate) to sugar (Glc + Fru + Suc; Fig. 4B) ratios based on integrals (see Supplemental Table S2), consistent with higher fruit juice and nutritional quality reported in the two transgenics (Mehta et al., 2002). These higher ratios of typical fruit metabolites are attributes favorably considered as higher quality in tomato breeding programs.

Polyamines Enhance the Accumulation of Choline

Like Asn and hexose profiles, choline levels mostly remain similar throughout ripening of wild-type and 556AZ fruits (Fig. 3C). However, in the Spd/Spm-accumulating transgenic fruits (556HO and 579HO), choline significantly accumulated as ripening progressed, particularly in the red ripe fruit, as was the case for the profiles of Asn, Gln, and compound A (Figs. 2 and 3). Compound C (Fig. 2C), a singlet and possibly a choline derivative, showed a trend toward

accumulation during ripening of fruit in the four genotypes, but the content at the ripe stage of ripening was slightly higher in the 579HO transgenic (Fig. 3C). These data, together with those described above, suggest that higher Spd/Spm levels affect the Glu/Gln and choline signaling networks.

Statistical Analysis Accentuates the Differences in the Metabolite Variables between the Transgenic and Nontransgenic Tomato Fruits

Tree clustering analysis (TCA), linear discriminant analysis (LDA), principal component analysis (PCA), and ANOVA were used to treat the data to perform a partition to classify them and determine the variables with maximum discrimination for use in cluster analysis. Under the experimental conditions used (uniform concentration, buffered solvent leading to constant pH, constant temperature, etc.), α and β isomeric forms of Glc are in equilibrium, and their concentration was therefore not considered independently. This was also true for β -fructofuranose and β -fructopyranose. The sum of the corresponding line intensities were

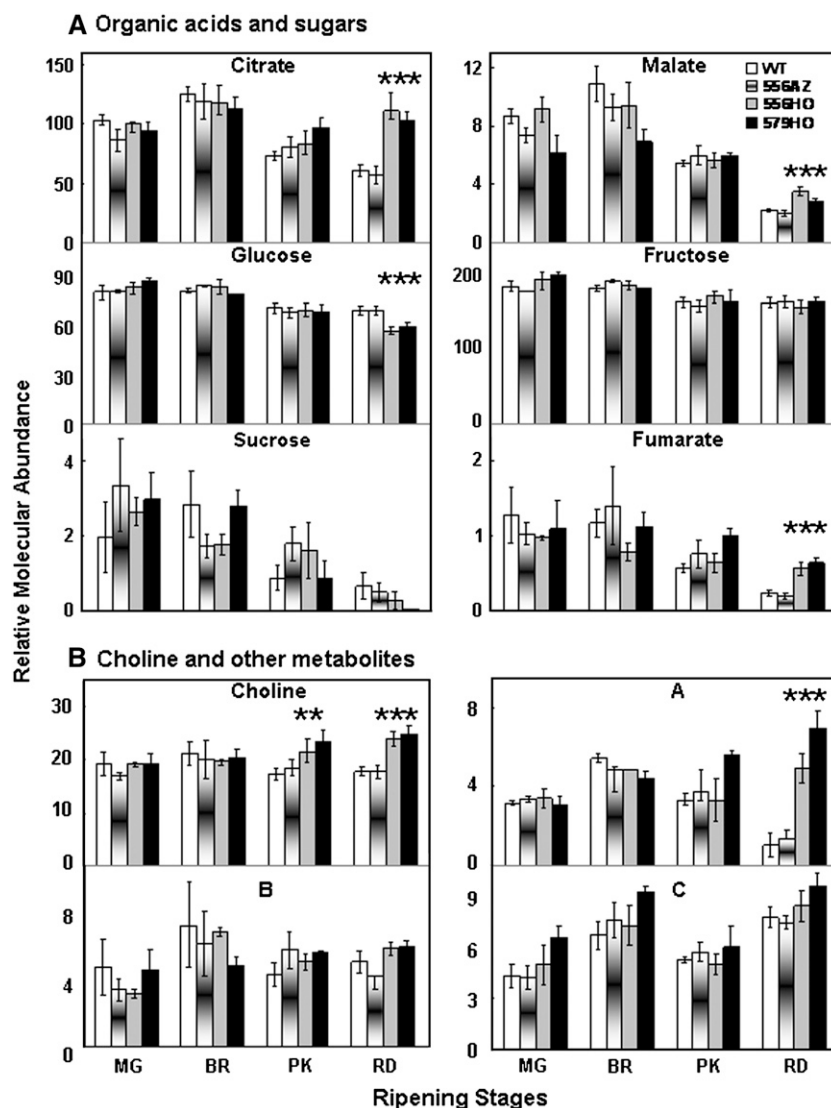


Figure 3. Profiles of organic acids and sugars (A) and choline and other metabolites (B) in wild-type (WT), azygous (556AZ), and two homozygous, transgenic (556HO, 579HO) tomato fruits at four stages of ripening (MG, mature green; BR, breaker; PK, pink; RD, red). The *P* values of significant differences between the transgenics versus nontransgenic controls were as follows: 0.0001 for citrate (**), 0.001 for malate (**), 0.00001 for fumarate (**), 0.015 at PK (**) and 0.002 at RD (**) for choline, and 0.00001 for compound A (**). All other details are the same as given in the Figure 2 legend. See also Supplemental Table S1.

introduced as data in the statistical analysis omitting the values of individual variables, which resulted in a list of variables shown in Table I.

The cluster analysis of NMR data obtained from 16 fruit samples (Supplemental Fig. S1) showed a clear partition between the stages of ripeness. LDA of these data, taking into account three classes, i.e. red, pink, and green + breaker, generated a map shown in Figure 5A, while their PCA map was striking (Fig. 5B). In this map, all green samples were clearly grouped and so were the breaker samples. The pink and ripe samples grouped in a particular manner, with clear distinction between the transgenic (556HO and 579HO) and the controls (wild type and 556AZ). This clear distinction was again observed for pink and ripe fruits when the sample size was doubled to 32 samples (data not shown). It was noteworthy that the first PC (PC1) was responsible for separating samples based on their stage of ripeness, while the second PC (PC2) provided a separation based on the genotypic differences, i.e. con-

trol (wild type and 556AZ) versus transgenic (556HO and 579HO) fruits.

Major variations of the data, due to different ripening stages of tomatoes, associated with PC1 involved variables Ile, Val, Ala, Glu, GABA, Glc, and compound C, while the variation arising from the introduction of the engineered gene represents mostly the variables Gln, Asn, and choline. Some variables that changed both during the ripening as well as a function of the introduced transgene are, for instance, variables Thr and citrate. They contributed to both PC1 and PC2. Thus, changes in the concentration of metabolites during ripening may mask the greater differences due to the transgene. To eliminate this interference and clarify the distinction between control and transgenic tomatoes, we sought statistical analysis on samples from two separate sets, one corresponding to pink and the other to red samples. ANOVA was used to select individual variables able to discriminate between control and transgenic tomatoes (LDA for pink and ripe samples;

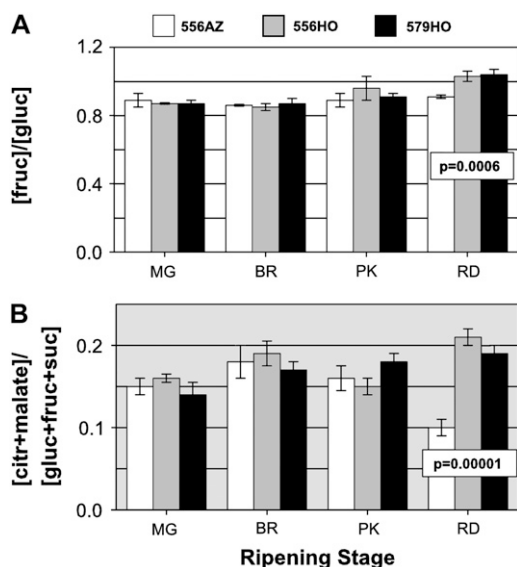


Figure 4. A typical representation of ratios. A and B, Fru (fruc) to Glc (gluc; A) and acid to sugar (B) in transgenic (556HO and 579HO) fruits compared to azygous (556AZ) control fruit. The ratio of acid to sugar (B) is given as the sum of citrate (citr) and malate to that of Glc plus Fru plus Suc. These results were generated from the data given in Figure 2. Azygous556 (white bars), 556HO (gray bars), and 579HO (black bars) fruits at mature green (MG), breaker (BR), pink (PK), and red (RD) stages of ripening are represented. Data given are means + SEM. The *P* values of significant differences between the transgenic versus azygous control are indicated for the red fruits.

Table II). In all these sets, wild-type and transgenic samples did not superimpose, showing no ambiguity of separation. The degree of separation was evaluated using the squared Mahalanobis distance (SMD; Brereton, 2003). The probability that a sample belongs to a particular group is basically proportional to the SMD from each centroid group. Therefore, the larger the SMD between the centroids of two groups, the better is the separation. SMD values thus obtained were: pink samples = 29; red samples = 709. Thus, the degree of separation obtained between transgenics and wild-type samples followed the sequence: red > pink. This is not surprising because the transgene is activated in a fruit ripening-specific promoter, and, therefore, major changes in the metabolite content would be significant only at and/or after the breaker stage of ripening, i.e. after transcription, translation, and post-translational processes are coordinated.

Sustained, Higher Respiration Rate in Transgenic Fruit during Ripening

The analysis of the NMR profiles of amino acids, sugars, and organic acids in the Spd/Spm-accumulating, transgenic fruits indicated that polyamines may regulate respiratory and C metabolism in fruits. We therefore monitored CO₂ evolution in the fruits harvested at the mature green stage and during their ripening development for a period of 2 weeks at 25°C (Fig. 6). As surmised, higher rates of respiration were

found in both the transgenic fruit, particularly at the red stage and thereafter as compared to nontransgenic, control fruit. Higher rates of respiration in the transgenic fruits throughout the ripening phase is indicative of enhanced carbohydrate catabolism for generating energy, signifying enhanced metabolic activity in these fruit. This conclusion is consistent with the differential trends in the profiles of amino acids, citrate, and Glc/Suc, as shown above.

Higher Transcript Levels for Phosphoenolpyruvate Carboxylase2 and NADP⁺-Cytosolic Isocitrate Dehydrogenase Genes

Several key enzymes link C metabolism and N sensing. Integration of glycolysis with N assimilation is likely linked with phosphoenolpyruvate carboxylase (PEPC), which provides C skeletons (Huppe and Turpin, 1994; Foyer and Noctor, 2002; Rademacher et al., 2002), and cytosolic isocitrate dehydrogenase

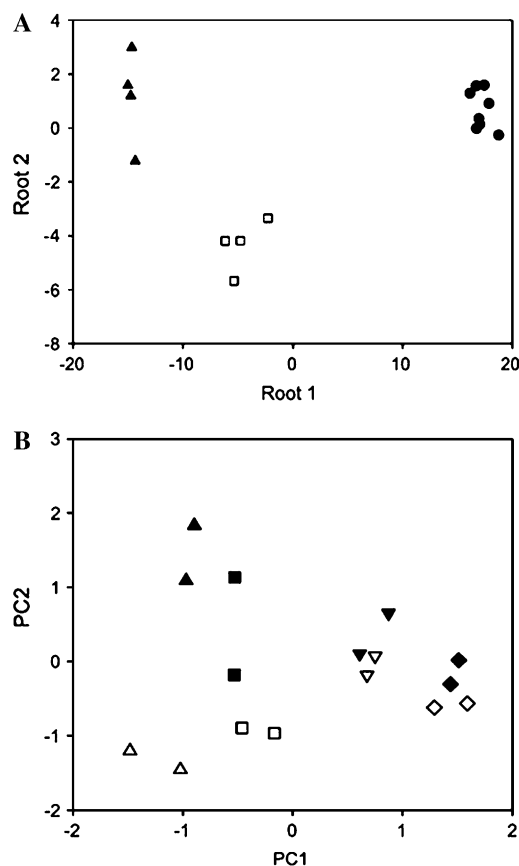


Figure 5. A, LDA of 16 tomato fruit samples. Symbols: ●, mature green and breaker tomatoes; □, pink; ▲, red tomatoes. B, PCA map of 16 tomato fruit samples. Symbols: ◆, mature green wild type and 556AZ; ◆, mature green 556HO and 579HO; ▽, breaker wild type and 556AZ; ▼, breaker 556HO and 579HO; ◻, pink wild type and 556AZ; ■, pink 556HO and 579HO; △, red wild type and 556AZ; ▲, red 556HO and 579HO. The following variables were included in the analysis: Glu, Val, compound A, Glc, Asp, compound C, citrate, Asn, GABA, Ala, Phe, Thr, and Suc. ANOVA analysis is provided in Table II.

Table II. ANOVA results (*F* and *P* level) for red (RD) and pink (PK) tomato samples divided into two groups (wild type + AZ, 556HO + 579HO)

The variables with *P* level higher than 0.05 (5%) are not significantly different in two groups, and their *F* values are not shown.

Compound	RD		PK	
	<i>F</i>	<i>P</i> Level	<i>F</i>	<i>P</i> Level
Ile				
Val	11	0.005	9.4	0.008
Thr				
Ala			22.3	0.0003
Glu				
GABA				
Gln	49.9	0.000006	5.6	0.032
Citrate	28.9	0.0001		
A	42.4	0.000014		
Asp	11.5	0.004		
Asn	33.2	0.00005		
B	5	0.04		
Choline	24.8	0.0002	7.6	0.015
C				
Glc	23.4	0.0003		
Fru				
Malate	17.4	0.0009		
Suc				
Phe				
Fumarate	45.2	0.00001		

(ICDHc), which obviously functions in supplying NADPH for reductive biogenesis. We therefore quantified transcript levels of PEPC and ICDHc genes during ripening of azygous (556AZ) and transgenic (556HO) fruit using real-time reverse transcription-PCR. The data on transcript levels are summarized in Figure 7. In the control (556AZ) fruit, both PEPC and ICDHc levels increased early during fruit ripening and precipitously decreased after reaching highest levels at the breaker stage (Fig. 7). A similar trend was apparent for PEPC transcripts in the transgenic (556HO) fruit, but, in contrast to the control fruit, the decreases in the transcript levels at pink and red stages were less dramatic (Fig. 7A). This is more apparent when the ratio of PEPC transcripts in the transgenic fruit to

azygous fruit (i.e. 556HO:556AZ) is compared at pink and red stages, which were 168.9 and 3.7, respectively (see also inset in Fig. 7A). The ICDHc transcripts accumulated throughout ripening in the transgenic (556HO) fruit, in contrast to the azygous control fruit, reaching highest level at the red stage (Fig. 7B). The ICDHc transcript levels at pink and red stages of the fruit were, respectively, 12.7- and 6.7-fold higher in transgenic fruit compared to the azygous fruit.

DISCUSSION

Higher Polyamines May Control Multiple Sites in Cellular Metabolism

Metabolite profiling analysis presented here indicates that Spd/Spm influence multiple cellular pathways in tomato fruit during ripening. Significant changes were found in the levels of choline, Gln, Asp, Asn, Val, citrate, fumarate, malate, Glc, and unidentified compound A in the transgenic tomatoes during ripening compared to the wild-type/azygous controls. Because these metabolites are known to be synthesized in specific subcellular compartments as well as transported across cells, these data indicate that Spd/Spm accumulation in the tomato fruit is associated with previously unknown facets such as N:C interdependent signaling involving processes that take place in diverse subcellular compartments, reminiscent of multiple processes that are associated with another plant growth regulator, the plant hormone ethylene (Mattoo and Suttle, 1991; Abeles et al., 1992; Alba et al., 2005). These results provide a basic framework to further investigate how these processes are interlinked, consequences thereof on the nutritional attributes and the ripening behavior of fruit. The observed changes in the cellular metabolism in the transgenic tomato fruit are obviously not a result of a single mode of action. However, it is likely that transcription/translation of a number of genes is coordinated with Spd/Spm-mediated changes in the metabolite profiles in the transgenic tomato fruit. In animal cells, higher polyamines regulate transcription by acting as a switch

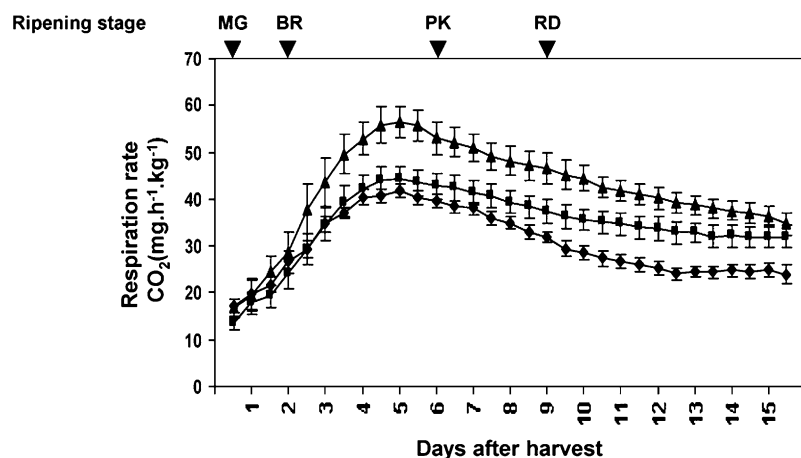


Figure 6. Rates of respiration in azygous and two transgenic fruits. CO_2 evolution from nontransgenic azygous (556AZ, $\blacklozenge$) and transgenic tomato fruit (556HO, $\blacksquare$; 579HO, $\blacktriangle$) during ripening was measured in a flow-through system by gas chromatography. Ripening stages were recorded based on color development. Data are represented as means $\pm$ SEM; $n = 8$.

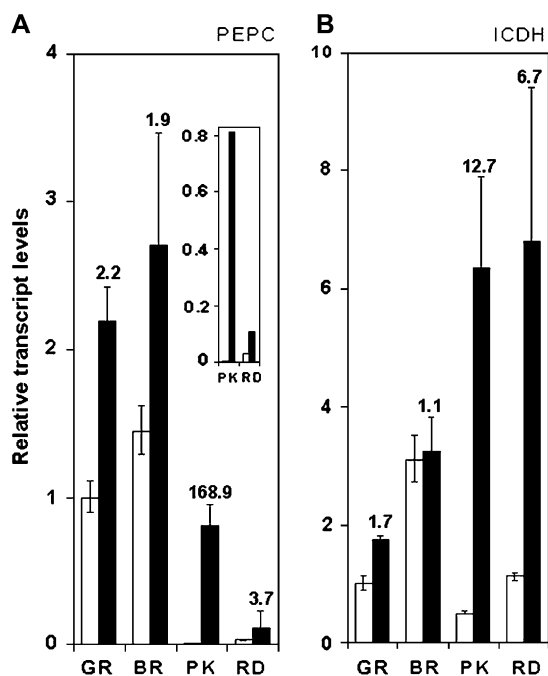


Figure 7. Real-time PCR analysis of LePEPC2 (A) and LeICDH (B) transcript levels. The levels of PEPC2 and ICDHc transcripts were determined relative to the calibrator azygous (556AZ, □) at green (GR) stage. The ratio of transcript levels in transgenic fruit (556HO, black bars) to those in the azygous, control fruit (556AZ, white bars) at different stages (GR, BR, PK, and RD) of ripening is indicated on top of the black bars. The range in variation is shown as error bars, which was determined by evaluating the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T = s$ and $\Delta\Delta C_T - s$, where s is the SD of the $\Delta\Delta C_T$ value ($n = 3$). The inset in A shows an enlarged view of the PEPC transcript levels at PK and RD stages of 556AZ (white bars) and 556HO (black bars) fruit.

between different coactivators without altering the state of the chromatin (Maeda et al., 2002) or by affecting histone acetylation/deacetylation (Hobbs and Gilmour, 2000). Also, a posttranscriptional role for polyamines has been shown in down-regulating animal genes (Li et al., 2001), while a certain group of *Escherichia coli* genes is controlled by the diamine putrescine at the translational level (Yoshida et al., 2004). In the latter case, it was suggested that putrescine enhances transcription of a group of genes by increasing the level of transcription factors. Polyamine-mediated modulation of protein synthesis may involve, among other things, structural changes in RNA, stimulation of 30S ribosomal assembly, or tRNA formation (Igarashi and Kashiwagi, 2000). Regulation of gene expression by polyamines can occur via an effect on the structure of eIF5A or oligomerization of nucleosomal arrays.

Interestingly, NMR spectroscopic analysis presented here shows that resonances present in the genetically engineered tomato fruit samples were not different than those in the fruit from wild-type/azygous plants. Thus, the introduction of the yeast SAMdc gene into tomato plants, which subsequently leads to the accumulation of Spd/Spm at the cost of putrescine (Mehta et al., 2002), does not lead to unusual resonances.

Statistical analysis validated the changes in the levels of the indicated metabolites, and certain variables were active at particular stage while others were active at another phase of fruit ripening. For example, Asn, Gln, and choline were active throughout ripening, whereas citrate, fumarate, and Glc, likely indicators of a changing source of C, were active only during the later phase of fruit ripening. The trends in some of the metabolites in tomato fruit during ripening seen here are similar to previous reports, including fruits other than tomatoes (Mattoo et al., 1975; Biale and Young, 1981; Tucker, 1993). Therefore, it will be of future interest to test if Spd/Spm similarly affect the metabolism of other fruits as shown here with tomatoes.

Are Polyamines Sensed as Organic N That Signals C Metabolism in the Fruit?

Figure 8 illustrates the metabolic pathways linking the identified metabolites that show regulated expression and pathways linked through SAM. Specific changes in cellular metabolites emphasize polyamine-mediated effects on N and C metabolism in a ripening fruit. How the polyamine circuitry integrates with the N:C signaling pathway is unclear. It is noted here that these data provide only a steady-state picture, but, because they were followed at four ripening stages, these studies offer a foundation for future experiments dedicated to metabolite fluxes. Nonetheless, we provide a synthesis of this information vis a vis other data presented here as well as the findings in the literature.

Glu, Gln, and Asn are the major N forms in plant leaves (Corruzi and Zhou, 2001; Glass et al., 2002; Stitt et al., 2002). The Asn to Gln ratio has been predicted as a sensor of N status in maize (*Zea mays*), part of a signal transduction pathway involving Asp aminotransferase, Gln synthetase, and Asn synthetase as indicators of plant N status during kernel development (Seebauer et al., 2004). Gln (Glass et al., 2002) and other amino acids (Stitt et al., 2002) have been suggested as sensors of N status. From these observations, it is possible that the transgenic fruit senses Spd and Spm as N forms, which correspondingly leads to increases in the other N forms such as Gln and Asn. Such a response to higher polyamines is reminiscent of how plant roots and leaves sense and signal N availability (Foyer and Noctor, 2002), as is also the case with the response of trees to exogenous chronic additions of N to a formerly N-limited forest (Rennenberg et al., 1998; Bauer et al., 2004). Thus, a reproductive organ, such as the transgenic fruit in this study, appears to house, and likely maintains, an organic N (here Spd/Spm)-sensing machinery at the late ripening phase. The N:C interactions revealed imply that the skeletons and moieties for most of the building blocks of biomolecules are more available in the transgenic fruit as compared to the control fruits, indicating a higher anabolic activity in these transgenics. It is particularly important to note that these metabolic shifts occur in a fruit-specific manner without any interaction with the

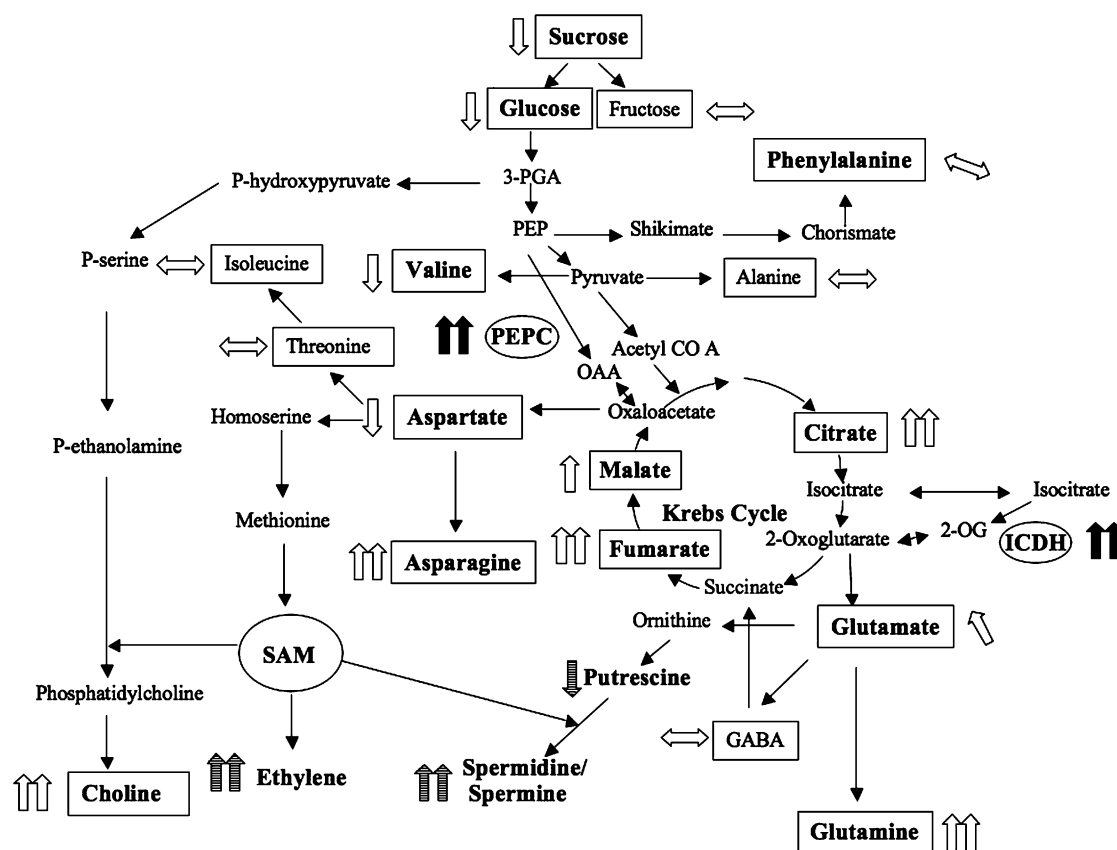


Figure 8. An illustration of metabolic pathways for the biosynthesis of the identified metabolites pinpointing linkages between N and C metabolism in the transgenic tomato fruits. White arrows represent high (single arrow straight up), higher (double arrows straight up), lower (downward single arrow), or no change (parallel) in the indicated metabolite levels in the transgenic, higher polyamines accumulating red fruit compared to wild-type/azygous fruit. Phenylalanine level showed a downward. Light-dark, striped arrows indicate metabolites Spd, Spm, and ethylene, which were higher, and putrescine, which was lower, in the transgenics than the controls (from Mehta et al., 2002). Dark arrows (next to PEPC and ICDH) indicate the sites of the reaction of the corresponding transcripts of PEPC and ICDHc whose levels were higher in the transgenic fruit than the control fruit.

rest of the parent plant because the fruits analyzed here were ripened off the vine and on the shelf, therefore emphasizing transgene-activated responses.

If the transgenic fruit follows N regulatory aspects, similar to roots or leaves (Corruzi and Zhou, 2001; Foyer and Noctor, 2002), it would demand a coordinated signaling of the C metabolism in such fruit to optimize C-N budgets. Indeed, this is precisely indicated by other data in this study, namely, the level of Glc decreased while those of citrate, malate, and fumarate remained higher in the red ripe transgenic fruit (Figs. 3 and 8). Glc metabolism through the Krebs cycle would yield more citrate, which can generate more 2-oxoglutarate that, in turn, would become a substrate to produce the Glu family of amino acids (Fig. 8). Accumulation of citrate, malate, and fumarate in red ripe transgenic tomatoes in comparison to nontransgenic fruit is suggestive of a more active metabolic status of the transgenic fruit. This, together with the overall trend in metabolites, is consistent with the increased respiratory activity in the transgenic tomatoes as compared to wild-type/azygous tomatoes

(Fig. 6). In fruits, organic acid metabolism and respiration is highly dynamic and fruit-type dependent (Mattoo et al., 1975; Biale and Young, 1981). The differential carbohydrate accumulation may also indicate that cell wall metabolism is altered to sustain the supply of C for respiration, which is consistent with the higher juice quality of these transgenic fruit (Mehta et al., 2002). The high respiratory activity in the transgenics reveals an *in vivo* role of polyamines in mitochondrial metabolic regulation, consistent with a previous proposal for Spm function in rat liver mitochondria (Chaffee et al., 1979).

Are genes that encode enzymes implicated in linking C metabolism with N sensing up-regulated in the Spd/Spm-accumulating, transgenic tomatoes? In leaves, C skeletons in response to N assimilation are provided through the activity of PEPC and Krebs cycle enzymes or cytosolic forms of aconitase and ICDH (Foyer and Noctor, 2002). In tobacco (*Nicotiana tabacum*) leaves, C₃-type PEPC and ICDHc are activated for organic acid metabolism in response to N assimilation (Scheible et al., 1997, 2000). Studies on overexpression

of PEPC in transgenic potato (*Solanum tuberosum*) plants showed that the flux of soluble sugars and starch is directed to organic acids such as malate and the amino acids Glu and Gln (Rademacher et al., 2002). We surmised that a fruit such as tomato may also use a similar mechanism, i.e. to up-regulate PEPC and ICDHc transcripts in response to Spd/Spm sensing in the transgenic fruit. Indeed, real-time PCR quantification of the transcripts for the two genes showed them to be markedly higher in the transgenic tomato fruit in comparison to the control fruit (Fig. 7). These data point out the conservation of the sensing/signaling mechanism and gene players involved in N assimilation and C metabolism in different organs of a plant. Importantly, by inference, we suggest that N assimilation signaling in plants may also be linked to a certain threshold of higher polyamines, Spd/Spm.

The steady accumulation of choline during ripening of the transgenic fruit would imply that flux toward choline biosynthesis is enhanced in the transgenics. This is of particular significance in the case of tomato because this plant is not able to effectively metabolize choline to Gly betaine (Weretilnyk et al., 1989). Although we have not studied the particular biosynthesis pathway of choline in tomato fruit, one of the reasons for choline accumulation may be the release of choline from membrane phospholipids by phospholipase D. In support of the latter possibility, our preliminary data show that phospholipase D transcripts are indeed up-regulated in the transgenic fruits, which may be a consequence of higher lipid turnover in the high polyamine-accumulating fruits. The accumulation of choline in these transgenic tomato fruit during ripening is significant. Choline has been classified as a vital amine for human health and has an essential role as a micro-nutrient required for brain development (Blusztajn, 1998; Zeisel, 2000). It is the precursor of membrane phospholipids, intracellular messengers diacylglycerol and ceramide, signaling lipids platelet-activating factor and sphingosylphosphorylcholine, and neurotransmitter acetylcholine (Kent, 1995; Blusztajn, 1998). Also, another methyl donor betaine and an osmoprotectant Gly betaine are synthesized from choline (Rhodes and Hanson, 1993; McNeil et al., 2001). In plants, Gly betaine has been reported to confer tolerance to environmental stresses such as salinity and drought (Rhodes and Hanson, 1993; Sakamoto and Murata, 2000). Interestingly, in transgenic rice, activation of SAMdc results in higher levels of Spd/Spm and the plants exhibit drought-stress response (Capell et al., 2004). Taken together, these results suggest that polyamine-regulated stress responses may occur via an effect on the biosynthesis of choline (McNeil et al., 2001).

MATERIALS AND METHODS

Plant Materials

A chimeric gene construct containing the yeast (*Saccharomyces cerevisiae*) SAMdc gene fused to the ripening-regulated E8 promoter was introduced into

tomato (*Solanum lycopersicum*) plants through Agrobacterium-based transformation as previously described (Mehta et al., 2002). Transgenic (homozygous, 556HO and 579HO), azygous (556AZ), and the parental processing cultivar (Ohio 8245; wild type) of tomato were grown in the fields at the Beltsville Agricultural Research Center Farms, Maryland, as described previously (Mehta et al., 2002). Fruits were harvested at mature green (full size and firm) stage and ripened at 25°C in the laboratory. Ripening stages of fruit were selected as: mature green (full size, firm and green), breaker (orange color on <10% of blossom end of fruit), pink (pink on 30%–70% of fruit surface), and red (red color on >90% of fruit surface). They were rinsed with distilled water, air-dried, deseeded, weighed, and pericarp tissue without the skin immediately frozen in liquid N and freeze-dried. Each sample represents an analysis of a minimum of three fruits at each stage of ripening.

NMR Spectroscopy

Spectral assignment and identification of specific metabolites was established using several two-dimensional NMR techniques (COSY, TOCSY, 1H-13C HSQC, and HMB). Taking advantage of the spread of complex resonances in the extra dimension, an unambiguous assignment was accomplished as previously described (Sobolev et al., 2003). Further confirmation was obtained by addition of an authentic standard to each sample. Dry powder (25 mg) from each sample was rapidly dissolved in 1 mL of 0.4 M sodium phosphate buffer prepared in D₂O containing known amounts of an authentic standard, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TSP), pH 6.5, and EDTA (10⁻⁵ M; to avoid citric acid from complexing). The solution was centrifuged at 10,000 rpm for 7 min and the supernatant filtered to remove any insoluble material. The resulting transparent soluble fraction was subjected to spectral analysis at 600.13 MHz on a Bruker Avance 600 AQS spectrometer as described previously (Mannina et al., 2003). Proton spectra were referenced to the TSP signal (δ = 0.00 ppm). Proton signals were acquired at 300 K by co-adding 512 transients with a recycle delay of 3 s. The strong water signal was suppressed by using a NOESY-presat scheme with solvent presaturation during relaxation delay and mixing time. The one-dimensional spectra were run using 90° flip angle pulses of 10.5 μ s, 32K data points, and a 2-s relaxation delay. ¹H, ¹H gradient selected COSY-45 (Braun et al., 1998) was acquired in the following conditions: water presaturation during relaxation delay, spectral width 6,000 Hz in both dimensions, 1-k data points in f_2 , 512 increments in f_1 , unshifted sinusoidal window function in both dimensions. ¹H, ¹H TOCSY (Croasmun and Carlson, 1994) was registered in TPPI phase-sensitive mode, with water presaturation during relaxation delay, a spectral width of 6,000 Hz in both dimensions, 2 s of relaxation delay, 80 ms of mixing time, 1-k data points in f_2 , and 512 increments in f_1 . Zero filling in f_1 to 1-k real data points and unshifted sinusoidal window functions in both dimensions were applied before Fourier transformation. The ¹H-¹³C gradient-selected HSQC spectrum (Schleucher et al., 1994) was registered in the echo-antiecho phase selective mode with the following parameters: 12 μ s 90° ¹³C hard pulse and 80 μ s for GARP ¹³C-decoupling, 6,000-Hz and 18,000-Hz spectral widths in proton and C dimensions, respectively, 1-k data points in f_2 , and 256 increments in f_1 . Linear prediction up to 512 point followed by zero filling to 1-k real data points was applied in f_1 dimension before Fourier transformation. Unshifted squared cosine window functions were also applied in both dimensions. The ¹H-¹³C HMB spectrum (Schleucher et al., 1994) was obtained with 2-s relaxation delay, 90° pulse of 10.5 μ s for ¹H and 12.6 μ s for ¹³C, 6,000-Hz and 30,000-Hz spectral widths in proton and C dimensions, respectively, 2-k data points in f_2 , 256 increments in f_1 , linear prediction up to 512 points, and zero filling in f_1 to 1-k real data points, processed with use of unshifted sinusoidal window functions in both dimensions. The baseline correction was performed as described previously (Mannina and Segre, 2002). The intensity of all signals was referenced to the intensity of an internal standard, i.e. the methyl group resonance of TSP at 0.00 ppm whose intensity was set equal to 10.0.

Measurement of Respiration

Mature, green fruits, harvested from field-grown azygous (556AZ) and transgenic (556HO and 579HO) plants, were transferred to a temperature-controlled room at 25°C. Respiration (CO₂ evolution) was measured with eight fruits, with six replications, in glass jars attached to a gas chromatograph (Corel) housed with alumina column and equipped with a computer control. CO₂ levels were automatically measured at 12-h intervals for 14 d while the fruits ripened. Visual assessments of changes in fruit color as fruits ripened were recorded on a daily basis. The system was programmed to inject and quantify authentic CO₂ prior to each measurement of the experimental samples. CO₂ levels were automatically computed from the standards.

RNA Extraction and Quantification of Transcripts by Real-Time PCR

Total RNA was extracted from pericarps of azygous (556AZ) and transgenic (556HO) fruit at different stages of fruit ripening (green, breaker, pink, and red) as described (Mehta et al., 2002). Transcript expression analysis of PEPC2 and NADP⁺-dependent, ICDHc genes was conducted by real-time PCR analysis. PCR primers for tomato PEPC2 gene were designed (GenBank accession no. AJ313434; Guillet et al., 2002) and the amplicon included 144 bp of 3' untranslated region and a 39-bp coding sequence; the forward and reverse primers were 5'-atgaaaggtattgctgtgga-3' (LePEPC2RTF) and 5'-tcgagaagctactaacaagagg-3' (LePEPC2RTR), respectively. PCR primers for tomato NADP⁺-ICDH gene were designed (TIGR tomato gene index no. TC164449), and the ICDH amplicon included 98 bp of 3' untranslated region and 63 bp of coding sequence; the forward and reverse primers were 5'-ggagaggtcatcgatgctga-3' (LeICDHRTF) and 5'-tctgacacctaatcccaaca-3' (LeICDHRTTR), respectively. Tomato 18S primers were designed from rRNA gene sequence (accession no. X51576) to amplify a product 160 bp in size. The forward and reverse 18S primers were 5'-gccgggtaattctgaaat-3' (Le18SRTF) and 5'-cggtatcattcaatcgtagg-3' (Le18SRTTR), respectively. First-strand cDNA was synthesized from total RNA (100 ng) samples using ImProm II reverse transcriptase enzyme at 42°C according to manufacturer's protocol (Promega). Aliquots of first strand cDNA samples were subjected to real-time PCR with 10 pmol each of either LePEPC2 or LeICDHc and with Le18S primers in iCycler with iQ SYBR green supermix (Bio-Rad) and the fluorescence was detected at 490 nm. Real-time PCR conditions were as follows: 95°C for 30 s, 65°C for 20 s, and 72°C for 10 s. A comparative C_T method using 2^{-ΔΔC_T} formula was used to obtain relative quantitative levels of PEPC and ICDHc transcripts in control and transgenic fruit as described (Livak and Schmittgen, 2001). The amount of the target was normalized to 18S rRNA reference. The relative efficiency of LePEPC2 or LeICDHc in relation to Le18S amplification with respective primer sets was demonstrated to be approximately equal after plotting the log concentrations of serial dilutions of cDNA input amounts against the ΔC_T values. The slope of the plot was determined to be less than 0.1 for the LePEPC2, LeICDH, and 18S primer sets.

Statistical Methods

Data of ¹H-NMR in solution were subjected to statistical analysis by using Statistica software package for Windows (1997; edition by Statsoft) to determine if and to what extent the selected variables were able to distinguish between the different tomatoes and their ripening stages. ANOVA, PCA, TCA, and LDA were used to treat the data (Martens and Martens, 2001). ANOVA was applied to each data set with the aim of selecting variables with a significant discriminant power. The *F* and *P* level parameters were used to perform the selection. In particular, the *F* value is defined as the ratio of the "between-groups variability" and "within-group variability." The larger the ratio, the larger the discriminating power of the corresponding variable. The *P* level gave the probability of error involved in accepting the result: a low *P* level corresponds to a high probability that the difference between groups is significant. Application of PCA results in a linear combination of the original variables to yield a few principal components with the highest variance and so drastically reduces the number of variables to analyze. The contribution of original variables to a particular principal component is given by its factor loading. For every sample, PC scores were used instead of the original variables. TCA, applied to the selected variables, classified samples without any a priori hypothesis, to check how they naturally group according to an amalgamation rule. In this work, the complete linkage method was used to determine when two clusters were sufficiently similar to be joined together. LDA allowed determination if the model is capable of distinguishing between a priori defined groups, i.e. if the formed clusters are significantly different. Moreover, it is possible to establish by LDA which variables have significantly different means across the groups. The discriminating power, given by the Wilks' Lambda factor, ranges from 0.0, which corresponds to a perfect discriminating power, to 1.0, with no discriminating power for the model. This technique, applied to the selected variables, was used to rank all the samples.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Tree cluster analysis of 16 samples (G = mature green and breaker; O = pink; R = red).

Supplemental Table S1. Mean values with ses of tomato metabolites.

Supplemental Table S2. Ratios of selected metabolites during tomato fruit ripening.

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Legends

Supplemental Figure 1. Tree cluster analysis of sixteen tomato samples. The dendrogram was obtained using 20 variables, complete linkage method and Euclidean distances. Symbols: G, mature green and breaker; O, pink; R, red.

Supplemental Figure 2. Linear discriminant analysis of thirtytwo pink and red tomato samples. The canonical root scores are plotted for each sample indicating separation between transgenic (T) (556HO and 579HO) and wild-type/azygous (C) (WT and 556AZ) lines. The variables used in the model for analysis are: Val, Ala, Gln, choline (pink tomatoes); Val, Gln, citrate, Asp, compounds A and B, choline, malate, glucose (red tomatoes).

Supplemental Table I. Mean values (signal intensities) and standard errors (SE) of tomato metabolites analysed by NMR spectroscopy.

	WT								556AZ							
	MG		BR		PK		RD		MG		BR		PK		RD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ile	5.28	1.43	5.76	1.65	2.81	0.26	2.12	0.29	4.13	0.04	4.15	0.61	3.62	0.38	2.16	0.38
Val	5.49	1.57	3.94	1.27	1.94	0.20	1.25	0.31	4.26	0.14	3.18	0.33	2.48	0.22	1.36	0.24
Thr	13.3	2.50	14.6	3.6	8.77	0.55	8.08	0.86	10.4	0.01	11.5	1.6	11.5	1.4	7.88	1.49
Ala	3.30	0.06	2.40	0.32	0.87	0.05	1.08	0.09	2.98	0.57	1.50	0.28	1.02	0.06	1.02	0.03
Glu	0.15	0.15	1.02	0.05	5.66	0.21	11.6	0.34	0.31	0.01	0.59	0.14	6.09	0.52	11.9	1.3
GABA	40.2	4.5	42.0	4.8	27.4	1.8	23.4	3.8	32.5	0.6	35.4	5.2	31.4	2.3	24.2	3.8
Gln	18.2	3.6	23.1	4.8	14.1	0.6	13.3	1.2	17.4	0.9	18.3	2.5	17.9	2.0	13.9	2.1
Citrate	103.3	4.5	125.2	6.1	73.3	3.9	61.0	5.2	86.8	9.6	119.2	14.7	80.6	8.7	56.7	7.5
A	3.16	0.14	5.47	0.21	3.32	0.30	1.04	0.60	3.37	0.11	4.86	0.14	3.78	1.13	1.31	0.48
Asp	0.50	0.38	2.17	0.06	5.08	0.18	9.06	0.45	0.42	0.09	1.04	0.78	4.76	0.74	8.8	0.36
Asn	5.94	1.50	7.11	1.74	4.54	0.23	4.88	0.38	5.22	0.30	5.90	0.82	5.89	0.66	4.94	0.72
B	4.87	1.71	7.37	2.55	4.40	0.68	5.14	0.68	3.48	0.68	6.25	1.95	5.89	1.09	4.27	0.82
Choline	19.2	2.30	21.2	2.2	17.3	1.0	17.9	0.9	17.0	0.7	20.1	3.5	18.5	1.6	17.8	1.3
C	2.81	0.44	4.34	0.51	3.41	0.10	5.02	0.40	2.75	0.46	4.91	0.66	3.72	0.34	4.82	0.28
Glucose	79.8	4.3	80.4	1.2	70.0	2.6	68.9	2.2	79.9	0.8	83.8	0.2	67.9	2.8	68.5	2.5
Fumarate	1.28	0.38	1.17	0.19	0.57	0.06	0.24	0.04	1.03	0.16	1.40	0.52	0.76	0.18	0.19	0.04
Fructose	180.8	7.4	179.4	3.7	160.9	5.9	160.7	7.2	175.5	0.03	188.7	1.4	155.7	7.8	162.0	7.5
Malate	8.68	0.49	10.9	1.25	5.40	0.21	2.21	0.10	7.34	0.52	9.30	0.94	5.99	0.63	2.02	0.21
Sucrose	1.95	0.95	2.86	0.90	0.86	0.34	0.66	0.36	3.36	1.23	1.72	0.33	1.80	0.45	0.49	0.27
Phe	2.72	0.64	3.18	0.67	1.96	0.19	1.75	0.16	2.06	0.08	2.28	0.31	2.22	0.19	1.82	0.24

Supplemental Table 1, continued

	556HO								579HO							
	MG		BR		PK		RD		MG		BR		PK		RD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ile	4.14	0.08	4.91	0.20	3.29	0.36	2.62	0.31	4.99	0.51	3.37	0.14	2.70	0.47	2.21	0.11
Val	4.37	0.34	3.50	0.11	1.45	0.10	0.77	0.12	4.90	0.61	2.42	0.13	1.29	0.45	0.53	0.06
Thr	10.1	0.3	14.7	0.2	9.9	0.6	10.0	0.9	11.0	2.7	8.95	0.12	9.78	2.08	9.81	0.35
Ala	2.93	0.14	2.34	0.19	0.68	0.07	1.21	0.09	2.25	0.57	1.44	0.17	0.62	0.05	1.22	0.14
Glu	0.44	0.08	0.93	0.03	4.89	0.72	12.4	1.0	0.37	0.01	0.65	0.15	4.90	0.55	13.8	0.7
GABA	37.1	0.6	36.9	4.4	28.4	2.2	23.7	2.1	40.8	4.6	32.4	4.7	30.6	2.0	22.8	1.3
Gln	17.7	0.9	26.4	0.5	19.8	2.0	23.6	1.8	19.7	4.6	18.1	0.03	20.6	1.9	24.1	0.7
Citrate	100.7	1.4	118.3	13.9	83.1	10.6	111.5	14.9	94.5	7.6	112.9	9.7	97.2	8.8	103.0	7.5
A	3.40	0.45	4.90	0.01	3.29	1.07	4.94	0.74	3.01	0.54	4.40	0.34	5.61	0.26	6.99	0.82
Asp	0.77	0.32	2.59	0.70	4.73	0.90	7.55	0.89	0.37	0.13	0.96	0.27	2.73	0.90	5.29	0.75
Asn	5.18	0.67	8.22	0.18	6.12	0.57	7.41	0.51	6.36	1.34	5.65	0.12	6.37	0.38	7.65	0.19
B	3.26	0.25	6.95	0.21	5.18	0.47	5.95	0.39	4.66	1.24	4.93	0.52	5.72	0.09	6.08	0.41
Choline	19.2	0.4	19.7	0.6	21.7	2.2	23.9	1.5	19.2	2.0	20.5	1.7	23.4	2.1	24.8	1.7
C	3.24	0.75	4.72	0.72	3.26	0.42	5.46	0.56	4.28	0.40	5.98	0.22	3.91	0.79	6.20	0.52
Glucose	82.1	3.4	83.1	4.4	69.1	3.7	56.6	2.7	86.3	2.2	78.6	0.1	67.6	4.4	59.2	1.89
Fumarate	0.98	0.03	0.79	0.11	0.64	0.14	0.56	0.08	1.10	0.38	1.12	0.20	1.00	0.10	0.64	0.06
Fructose	189.5	11.3	183.6	5.5	168.7	8.1	154.1	8.7	197.1	4.5	179.7	0.2	162.6	14.4	161.7	6.0
Malate	9.13	0.90	9.38	1.58	5.66	0.51	3.50	0.27	6.13	1.20	6.94	0.77	5.96	0.19	2.78	0.24
Sucrose	2.66	0.36	1.78	0.27	1.62	0.76	0.27	0.23	2.97	0.73	2.81	0.42	0.85	0.51	0.03	0.02
Phe	2.09	0.08	2.54	0.12	1.96	0.15	2.04	0.21	2.04	0.24	1.76	0.10	1.62	0.18	1.53	0.05

Supplemental Table II. Ratios of fructose/glucose and organic acids/sugars during ripening of azygous and 556HO and 579HO transgenic fruits

Ripening stage	Fructose/Glucose						(Citrate+Malate)/(Glucose+Fructose+Sucrose)					
	556AZ		556HO		579HO		556AZ		556HO		579HO	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
MG	0.89	0.04	0.87	0.005	0.87	0.02	0.15	0.01	0.16	0.005	0.14	0.015
BR	0.86	0.005	0.85	0.02	0.87	0.03	0.18	0.02	0.19	0.015	0.17	0.01
PK	0.89	0.04	0.96	0.07	0.91	0.02	0.16	0.015	0.15	0.01	0.18	0.01
RD	0.91	0.01	1.03	0.03	1.04	0.03	0.10	0.01	0.21	0.01	0.19	0.01