RESEARCH PAPER

A field-grown transgenic tomato line expressing higher levels of polyamines reveals legume cover crop mulch-specific perturbations in fruit phenotype at the levels of metabolite profiles, gene expression, and agronomic characteristics

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Abstract

Genetic modification of crop plants to introduce desirable traits such as nutritional enhancement, disease and pest resistance, and enhanced crop productivity is increasingly seen as a promising technology for sustainable agriculture and boosting food production in the world. Independently, cultural practices that utilize alternative agriculture strategies including organic cultivation subscribe to sustainable agriculture by limiting chemical usage and reduced tillage. How the two together affect fruit metabolism or plant growth in the field or whether they are compatible has not yet been tested. Fruit-specific yeast *S*-adenosylmethionine decarboxylase (ySAMdc) line 579HO, and a control line 556AZ were grown in leguminous hairy vetch (*Vicia villosa* Roth) (HV) mulch and conventional black polyethylene (BP) mulch, and their fruit analysed. Significant genotype×mulch-dependent interactions on fruit phenotype were exemplified by differential profiles of 20 fruit metabolites such as amino acids, sugars, and organic acids. Expression patterns of the ySAMdc transgene, and tomato SAMdc, E8, PEPC, and ICDHc genes were compared between the two lines as a function of growth on either BP or HV mulch. HV mulch significantly stimulated the accumulation of asparagine, glutamate, glutamine, choline, and citrate concomitant with a decrease in glucose in the 556AZ fruits during ripening as compared to BP. It enables a metabolic system in tomato somewhat akin to the one in higher polyamine-accumulating transgenic fruit that have higher phytonutrient content. Finally, synergism was found between HV mulch and transgenic tomato in up-regulating N:C indicator genes PEPC and ICDHc in the fruit.

Key words: Alternative agriculture, black polyethylene, hairy vetch, metabolite profiles, NMR, tomato fruit ripening, transgene expression.

Introduction

Genetic modification of crop plants to introduce desirable traits such as nutritional enhancement, disease and pest resistance, and enhanced crop productivity has revolutionized agriculture and is increasingly sought as a promising technology to boost food production in the world (Chrispeels *et al.*, 2002). In 2007, 23 countries planted biotech crops and the biotech crop area increased by 12%,

reaching a total of 114.3 million hectares (James, 2007). The increasing global percentages of maize (14%), canola (18%), cotton (28%), and soybean (60%) that are genetically modified (GM) indicate that genetic engineering of crop plants has rapidly taken hold (James, 2005). Environmental impact data obtained from 42 field experiments with Bt cotton and maize have revealed that genetically modified crops can contribute to sustainable agriculture (Marvier *et al.*, 2007). The need for agricultural sustainability is gaining more relevance now than before. Intensive conventional agriculture based on non-renewable resources has led to the depletion of natural resources and the loss of fertile agricultural lands, by soil erosion and contamination of two major natural resources, water and air, thereby raising concerns for human and animal health (National Research Council, 1989; Smil, 1997). It is now perceived that cultural practices that utilize alternative agriculture strategies, including organic cultivation practices, can subscribe to sustainable agriculture by limiting chemical usage and reduced tillage (National Research Council, 1989; Trewavas, 2004).

The most promising among the alternative farming practices for growing solanaceous crops is a reduced-tillage, cover crop based sustainable system (Abdul-Baki and Teasdale, 1993; Lu *et al.*, 2000). The use of cover crop mulches such as the leguminous hairy vetch (*Vicia villosa;* HV) and non-leguminous rye (*Secale cereale*) increases crop productivity in traditionally bred crop plants including tomatoes (Abdul-Baki *et al.*, 1996). In addition, the use of HV mulch is economically beneficial because it limits disease incidence and delays leaf senescence (Teasdale and Abdul-Baki, 1997; Mills *et al.*, 2002). Despite these advantages, there is a lack of information on the integration of genetically engineered crops using such agricultural practices. To date, genetically engineered crops have not been tested in sustainable, alternative agriculture. Nor do we know the nature of changes brought about by cover crops in GM crops. This is of particular interest since the longer foliage life and tolerance to disease of tomato plants grown on HV mulch were correlated to activation of select signalling pathways and cross-talk among plant organs (Kumar *et al.*, 2004, 2005; Mattoo and Abdul-Baki, 2006).

Previously, homozygous transgenic tomato lines were developed following transformation with yeast *S*-adenosylmethionine decarboxylase (ySAMdc) gene, *Spe*2, fused to a ripening-specific E8 promoter (Mehta *et al.*, 2002). The fruit of these transgenic tomatoes accumulate higher polyamines, spermidine (Spd), and spermine (Spm), are longer-lasting on the vine, and richer in juice and nutritional quality. In addition, the high Spd-Spm tomatoes have higher fructose/glucose and sugar/acid ratios (Mattoo *et al.*, 2006). The transgenic fruit have proven to be a good resource to study the role of Spd-Spm in regulating gene expression (Srivastava *et al.*, 2007;

Mattoo *et al.*, 2007) and metabolite profiles (Mattoo *et al.*, 2006) in fruit during ripening. These studies have been reviewed (Mattoo and Handa, 2008). The effect on plant phenotype is reported here, as exemplified by profiles of 20 fruit metabolites and expression patterns of the ySAMdc transgene, and tomato genes SAMdc, E8, PEPC, and cytosolic ICDH (ICDHc), by growing the homozygous transgenic tomato line along with a control azygous line in the field on beds made up of either black polyethylene (BP) or HV. Significant genotype×mulch-dependent interactions were revealed, including the finding that HV mulch enables a metabolic system in untransformed tomato somewhat akin to the one in polyamine-accumulating transgenic fruit.

Materials and methods

Plant materials, growth conditions, and fruit yield

The parental tomato cultivar used was Ohio 8245. A homozygous transgenic tomato line with ySAMdc gene (579HO) was generated as previously described by Mehta *et al.* (2002). The azygous control 556AZ line was randomly selected from 10 independent azygous lines generated in the laboratory. None of these azygous lines had exhibited any significant differences in agronomic characteristics during field trials in previous years. Plants were grown in the North Farm of Beltsville Agricultural Research Center, Maryland on leguminous hairy vetch (*Vicia villosa*; HV) beds next to black polyethylene (BP) beds. They were arranged in a randomized complete block design with the mulch being the main block (plot) and the tomato genotypes (lines) as the sub-plots. The tomato lines were then randomized within the main plots. Each treatment was replicated six times using 15 or more plants per replicate. Two plants at each end were used as border plants. Preparation of the field and seeding the HV cover crop with a no-till seeder were done during late summer (September) as previously described by Abdul-Baki *et al.* (1996). Hairy vetch was seeded at the rate of 45 kg ha^{-1} on raised beds of 15 cm high. When HV reached the flowering stage in May of the following year, it was mowed mechanically with a flair mower to 5 cm above the bed surface without disrupting the soil. The residue formed a uniform layer of mulch, several centimetres thick. BP beds were set up with trickle irrigation lines laid at 5 cm deep in the soil and 5 cm away from the plants. Drip irrigation lines were also set up over the surface of HV residue for both irrigation and fertilizer applications (Mills *et al.*, 2002). Five-week-old tomato seedlings grown in the greenhouse were planted using a no-till mechanical transplanter, leaving 45 cm space between plants within a row and 185 cm between each row. Water was delivered through the drip lines as needed. Ammonium nitrate as fertilizer was applied at the rate of 82 kg ha^{-1} for HV (50% less than BP) and 164 kg ha^{-1} for BP beds, respectively, through the drip lines at 3-week intervals and spread over six applications per season, starting a week after transplantation of seedlings. Soil in the field plots was optimum in phosphorus and potassium, therefore no additional fertilizer was applied. Fruit yield, weight, and number were measured five times at weekly intervals and the cumulative data obtained. Yield data for the lines grown on BP and HV mulch were compiled from 4–8 replications each consisting of 10–20 plants. Average fruit weights for BP and HV-grown 556AZ and 579HO lines were calculated from an average of 175–225 fruits from each replication. Fruits were harvested at green (GR), breaker (BR), pink (PK), and red (RD) stages (Mehta *et al.*, 2002) for biochemical and molecular analyses.

NMR spectroscopy-based metabolite profiling

Mature green fruit from the lines field grown on BP and HV mulches were harvested and transported to a temperature-controlled laboratory (at 25 °C), and sampled at different ripening stages. Skin-free pericarp was freeze-dried at –80 °C and the dry powder analysed for metabolite profiles by NMR spectrometry as previously described (Sobolev *et al.*, 2003; Mattoo *et al.*, 2006).

RNA extraction and RNA-DNA hybridizations

Fruit at GR, BR, PK, and RD stages were harvested from field-grown plants and total RNA was isolated (Mehta *et al.*, 2002). Twenty μg of total RNA were electrophoresed in 1% formaldehyde agarose gels and blotted onto Nytran SuperCharge Nylon membranes (Schleicher and Schuell) with $20\times$ SSC. DNA probes were prepared from plasmid clones after digestion with appropriate restriction enzymes followed by gel purification. Radiolabelled probes for hybridization were made with ^{32}P using the High Prime Random Labelling kit (Roche). Yeast SAMdc, LeSAMdc, and E8 exon 1 probes were prepared from respective restriction enzyme-digested plasmid vectors as described by Mehta *et al.* (2002). Northern blots were hybridized in 50% formamide, $6\times$ SSC, $5\times$ Denhardt's reagent, 0.5% SDS, and 100 μg ml^{-1} heterologous DNA at 42 °C. Hybridizations were carried out overnight with probe concentrations of $>1\times10^{6}$ cpm ml^{-1}. Membranes were washed twice in $2\times$ SSC and 0.1% SDS at 55 °C and final washes (two to four) were carried out in $0.2\times$ SSC and 0.1% SDS at 55 °C. Blots were exposed to X-ray films at –70 °C with intensifying screens. Hybridization probes were stripped from RNA blots in a solution of $0.1\times$ SSC and 0.1% SDS and blots rehybridized with 18S ribosomal tomato RNA probe.

Phosphoenolpyruvate carboxylase (PEPC) and cytosolic NADP-dependent isocitrate dehydrogenase (ICDHc) transcript analysis by real-time polymerase chain reaction (PCR)

Analysis of PEPC2 and ICDHc gene transcripts was conducted by real-time PCR as previously described by Mattoo *et al.* (2006). PCR primers were designed for tomato PEPC2 gene (GenBank accession no. AJ313434) (Guillet *et al.*, 2002) and NADP^{+}-ICDH gene (TIGR tomato gene index no. TC164449). The amplicon for PEPC2 gene included 144 bp of the 3′ UTR region and a 39 bp coding sequence; the forward and reverse primers were: 5′-ATGAAAGG-TATTGCTGCTGGA-3′ (LePEPC2RTF) and 5′-TCGAGAAGC-TACTAAACAAAGAGG-3′ (LePEPC2RTR), respectively. The ICDH amplicon included 98 bp of the 3′ UTR region and a 63 bp coding sequence; the forward and reverse primers were: 5′-GGAG-AGTTCATCGATGCTGA-3′ (LeICDHRTF) and 5′-TCTGACAC-CTTAATCCCAAACA-3′ (LeICDHRTR), respectively. Tomato 18S primers were designed from rRNA gene sequence (accession number X51576) and a product of 160 bp in size was amplified. The forward and reverse 18S primers were: 5′-GCCCGGGTA-ATCTTTGAAAT-3′ (Le18SRTF) and 5′-CGGATCATTCAAT-CGGTAGG-3′ (Le18SRTR), respectively. Details on real-time PCR conditions were the same as before (Mattoo *et al.*, 2006) and levels of PEPC and ICDHc transcripts were quantified in control and transgenic fruit during ripening by the comparative C_T method using the $2^{-\Delta\Delta C_T}$ formula as described by Livak and Schmittgen (2001). The amount of the target was normalized to the 18S rRNA reference. Relative efficiency of LePEPC2 or LeICDHc in relation to Le18S amplification with respective primer sets was found to be approximately equal after plotting the log concentrations of serial dilutions of cDNA input amounts against the delta C_T values. The slope of the plot was less than 0.1 for the LePEPC2, LeICDH, and 18S primer sets.

Methods for polyamine analysis and statistics

Putrescine (Put), spermidine (Spd), and spermine (Spm) levels in GR, BR, PK, and RD fruit pericarp were determined as previously described (Minocha *et al.*, 1990). Statistical methods were the same as previously described (Mattoo *et al.*, 2006).

Results

Mulch×genotype interactions coexist in transgenic and non-transgenic tomato genotypes

Transgenic tomato genotype (579HO), along with its azygous non-transgenic control line (556AZ), was evaluated for agronomic performance (fruit yield and fruit weight) when grown in black polyethylene (BP) and reduced-till, alternative hairy vetch (HV) cover crop (Fig. 1). The 556AZ (control) line produced a significantly higher fruit yield ($P \leq 0.05$) when grown in HV mulch as compared to BP (Fig. 1, upper panel), with only a slight but positive effect on average fruit weight (Fig. 1, lower panel). These data are consistent with earlier observations on a fresh market tomato variety (Abdul-Baki *et al.*, 1996). Fruit yield and fruit weight for the 579HO line were also higher when grown on HV compared with BP (Fig. 1). However, the HV-mediated increase in fruit yield for 579HO line was less pronounced than the
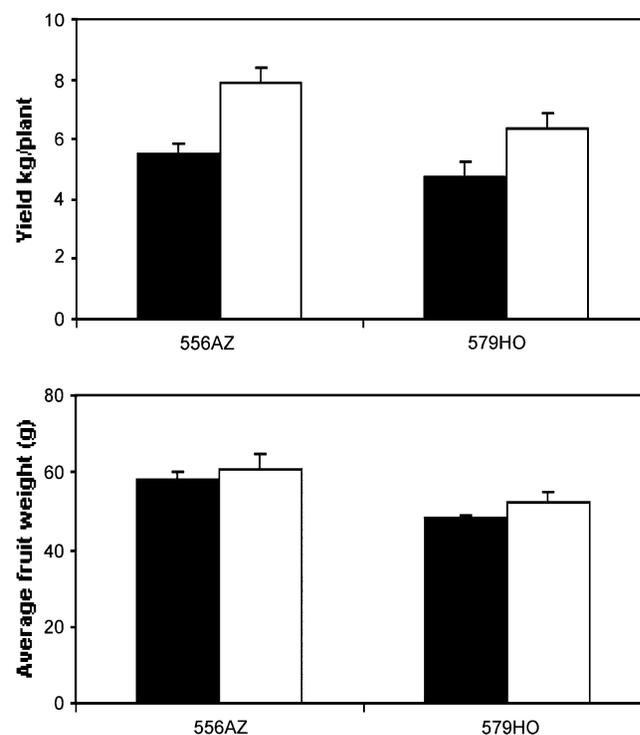

Fig. 1. Field performance, measured as fruit weight and yield, of transgenic 579HO tomato and the non-transgenic azygous (556AZ) control line grown in BP and HV mulches. Data are from independent samples shown as mean ±SE (*n*=4–8). Closed and open bars represent BP-grown and HV-grown plants, respectively.

non-transgenic 556AZ line. Fruits per plant and fruit yield were positively linked in HV-grown 556AZ (control) and 579HO lines. These data show 'environment (cover crop mulches)×genotype (tomato)'-interactions in influencing the agronomic characteristics of both transgenic and non-transgenic tomato plants.

Differential accumulation of polyamines in HV- and BP-grown 556AZ and 579HO fruits during ripening

Fruits of transgenic 579HO line accumulate spermidine (Spd) and spermine (Spm) at the cost of putrescine (Put) during ripening compared with the 556AZ control line (Mehta *et al.*, 2002; Fig. 2). The effect of the production system on the concentrations of Put, Spd, and Spm in the fruits from 556AZ control and 579HO transgenic lines as related to the stage of ripening is shown in Fig. 2. The focus here is particularly on changes occurring at the later stages of ripening since the transgene is induced in a ripening-specific manner (Mehta *et al.*, 2002). BP and HV mulch did not affect the Put content in fruits from 556AZ line, except at the red stage in HV fruit that showed a slightly higher concentration (Fig. 2). In the 579HO transgenic fruit, Put content declined upon fruit ripening and was impervious to the growth condition except at the breaker stage when it was higher in HV fruit compared with BP fruit. Spd content in 556AZ fruit at the different stages of ripeness was similar, irrespective of the

growth conditions. In the transgenic 579HO fruit, Spd content increased as ripening progressed, and was stimulated in breaker and red fruit from HV plants with a slight decrease in pink fruit compared with BP fruit (Fig. 2). Spm content decreased during ripening of 556AZ control fruits from plants grown in BP but significantly increased in HV-grown breaker and pink fruit by 62% and 47%, respectively, versus the corresponding BP-grown fruits (Fig. 2, 556AZ). At the red stage of 556AZ fruit, Spm content in BP- and HV-grown plants was similar. Interestingly, the Spm accumulation pattern during ripening of the transgenic fruit from BP-grown plants paralleled that seen with HV-grown 556AZ control fruit (Fig. 2, 579HO). In both 556AZ and 579HO fruits, the Spm accumulation pattern was similar when grown in HV mulch except that the decrease seen in the 556AZ red fruit did not occur in the corresponding HV- or BP-grown 579HO transgenic red fruit. In other words, the HV-mulch-mediated pattern of Spm accumulation in 556AZ fruit during ripening is similar to the transgene-mediated pattern in BP-grown 579HO fruit. In non-transgenic tomatoes, HV could therefore signal pathways that are similar to those regulated by higher polyamines in the transgenic line.

Differential accumulation of select metabolites in non-transgenic and transgenic fruit when grown on HV compared to BP mulch

The unexpected finding of differential effects of the growth conditions on polyamine accumulation in 556AZ and 579HO fruits prompted us to obtain profiles of select metabolites to determine which of them are sensitive to G×E interactions. Twenty metabolites, including three unverified compounds A, B, and C, at defined stages of ripening of 556AZ and 579HO fruits were quantified using NMR spectroscopy as described previously (Sobolev *et al.*, 2003; Mattoo *et al.*, 2006). Compounds labelled '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' a singlet, possibly a choline derivative.

Interactions of HV mulch with the 556AZ line resulted in its fruit having significantly higher concentrations of Thr, Gln, Asn, Phe, Ile, GABA, and Val at the later stages of ripening (Fig. 3). The concentration of Glu was significantly different in 556AZ HV fruit only at the red stage compared with the fruit from the same line grown in BP; Ala levels remained more or less similar at each ripening stage (Fig. 3). In the red fruit from control plants grown in either BP or HV mulch, concentrations of Asp, Ala, and compound C were similar (Figs 3, 4, 556AZ). Similarly, the concentrations of the organic acids citrate
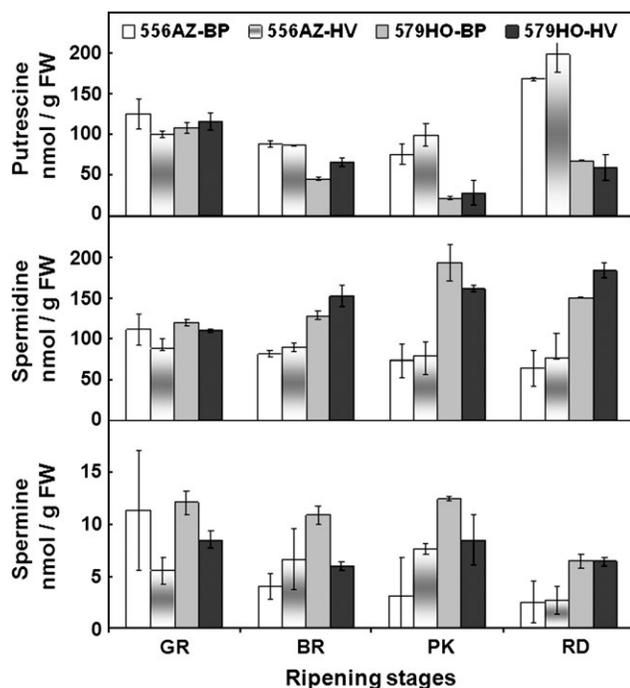
Fig. 2. Pattern of polyamines in ripening fruit of transgenic (579H0) and non-transgenic (556AZ) plants grown in BP and HV mulches. Data shown are for putrescine, spermidine, and spermine at different stages of ripening from independent samples, as mean ±SE.
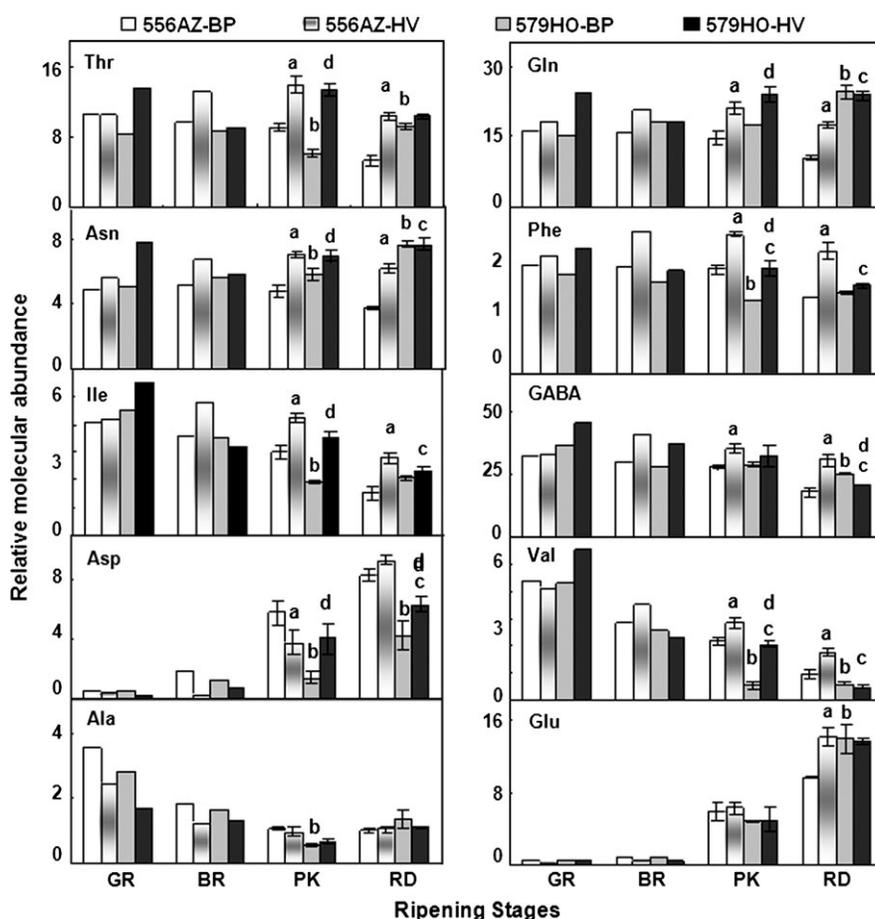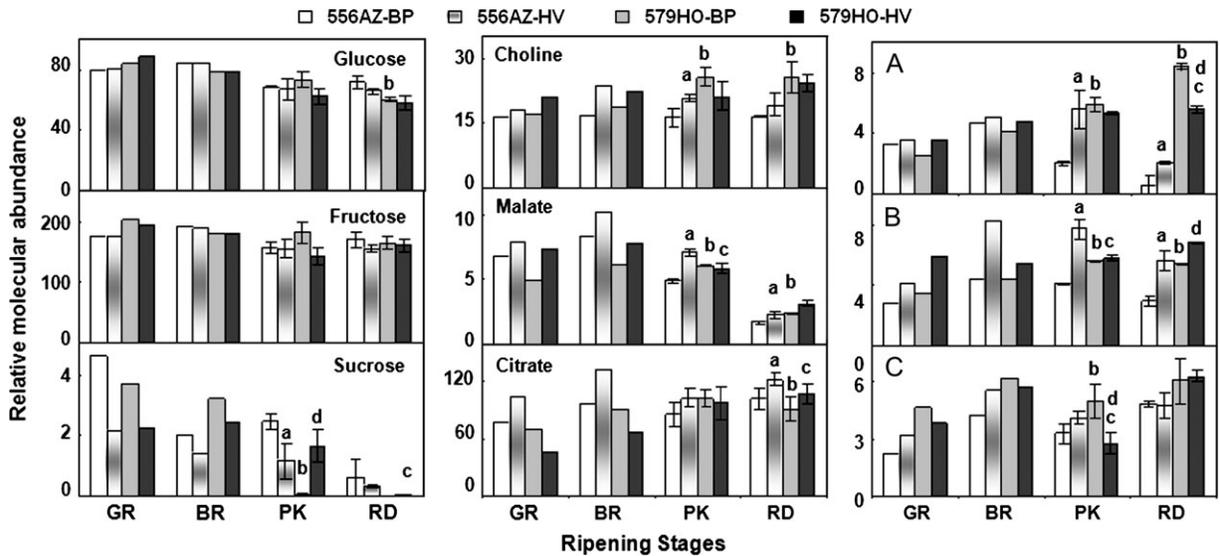
Fig. 3. NMR spectroscopic analysis of amino acids in ripening fruit of transgenic (579HO) and non-transgenic (556AZ) plants grown in BP and HV mulch systems. Letters a, b, c, and d marked above the bars indicate significant differences, $P < 0.05$, respectively, between 556AZ-BP and 556AZ-HV, 556AZ-BP and 579HO-BP, 556AZ-HV and 579HO-HV, and 579HO-BP and 579HO-HV. Data shown are means $\pm$SE ($n=3$–5). Only significant differences during late ripening stages are highlighted. Other details are as in Fig. 2.

and malate were higher in the fruit from the HV-grown 556AZ line than those grown on BP, whereas the micronutrient choline was only slightly higher in HV fruit compared with BP-grown fruit from 556AZ line (Fig. 4). Concentrations of compounds A and B were significantly higher in pink and red 556AZ fruits from HV-grown plants compared with those grown on BP. In contrast, sucrose concentration was higher in the BP-grown control fruit throughout ripening compared with HV fruit (Fig. 4). The red 556AZ fruit from BP-grown plant had an apparent trend towards slightly higher levels of Glc and Fru compared with the fruit from the HV-grown 556AZ plant.

The interactions between the production system and the transgenic 579HO line were different from that seen with the 556AZ control in regard to the metabolite profiles (Figs 3, 4, 579HO). In the HV-grown transgenic (579HO) pink (PK) fruit, the concentrations of Thr, Gln, Asn, Phe, Ile, Asp, Val, and sucrose were higher while those of compound C were lower compared to the same stage BP-

grown fruit (Figs 3, 4, 579HO). These differences become less pronounced as the transgenic fruit from the BP-mulch-grown plants turned red. The concentrations of GABA and compound A were lower in the red transgenic fruit grown in HV compared with BP (Figs 3, 4, 579HO, RD). Although HV-grown 556AZ and 579HO fruit responded to a large extent similarly, the magnitude of response varied and the pattern of changes in the class of metabolites identified were distinct. For instance, in the BP-grown azygous (control) fruit, Asn and Gln levels remained constant until the pink stage and declined thereafter, while choline levels remained constant. In the red transgenic fruit, Ile, Val, Ala, Asn, Glu, Gln, choline, Glc, Fru, and compound C levels were similar, irrespective of the growth conditions (BP or HV) of the plant. Together, these data suggest that a part of the HV-mulch-mediated differential pattern of metabolites in 556AZ fruit during ripening may be linked to the higher accumulation of polyamines, as shown here by spermine, at the penultimate stages of ripening.

Fig. 4. NMR spectroscopic analysis of sugars, organic acids, choline, and three unidentified compounds (A, B, C) in ripening fruit of transgenic (579HO) and non-transgenic (556AZ) plants grown in BP and HV mulches. Only significant differences during late ripening stages are highlighted. Other details are the same as in the legends to Figs 2 and 3.

HV mulch influences accumulation of transgene and endogenous gene transcripts during fruit ripening

The effects seen with the type of mulch on differential accumulation of metabolites in 556AZ and 579HO fruits provided a good indication that gene expression may also be differentially influenced. To test this, transcript levels of the yeast SAMdc (ySAMdc), endogenous tomato SAMdc and E8 genes were analysed by northern blots. As expected, no ySAMdc (transgene) transcripts were apparent in azygous fruit from plants grown in either mulch (Fig. 5, 556AZ). In the 579HO fruit from BP-grown plants, ySAMdc transcripts accumulated early in the breaker fruit, declined precipitously at the pink stage and remained at that level in the red fruit (Fig. 5, 579HO, BP). By contrast, the accumulation of ySAMdc transcripts in 579HO fruit from HV-grown plants occurred at the breaker stage, as in the BP-grown transgenic line, but peaked at the pink stage of ripening (579HO, HV).

HV mulch promoted the accumulation of the endogenous SAMdc gene both in the 556AZ and the transgenic 579HO fruit for a longer duration than did the BP mulch, but the effect was longer lasting in the 579HO fruit (Fig. 5, LeSAMdc). In BP-grown 556AZ and 579HO fruit, LeSAMdc gene expression was evident during the early stages of fruit ripening (mature green and breaker), but decreased as ripening progressed (Fig. 5, see 556AZ PK, and RD).

The E8 transcript accumulation pattern during ripening of the transgenic fruits mirrored that of the ySAMdc expression profile whether the plants were grown on BP or HV (Fig. 5, compare E8 Exon1 with ySAMdc). HV mulch notably enhanced the expression of the E8 gene in
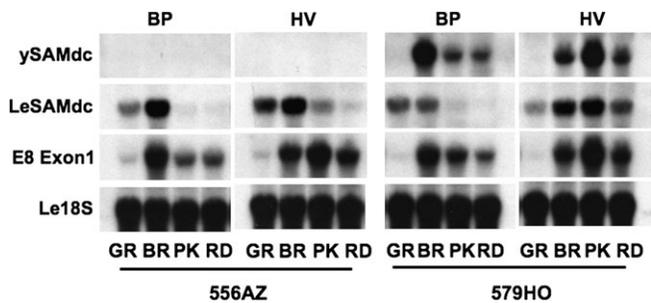
Fig. 5. RNA gel blot analysis of ySAMdc, endogenous SAMdc (LeSAMdc), and E8 (E8 Exon1) gene transcripts at different ripening stages of fruit from non-transgenic (556AZ) and transgenic (579HO) plants grown in BP and HV mulches. Le18S RNA signals indicate loading levels of total RNA in each lane.
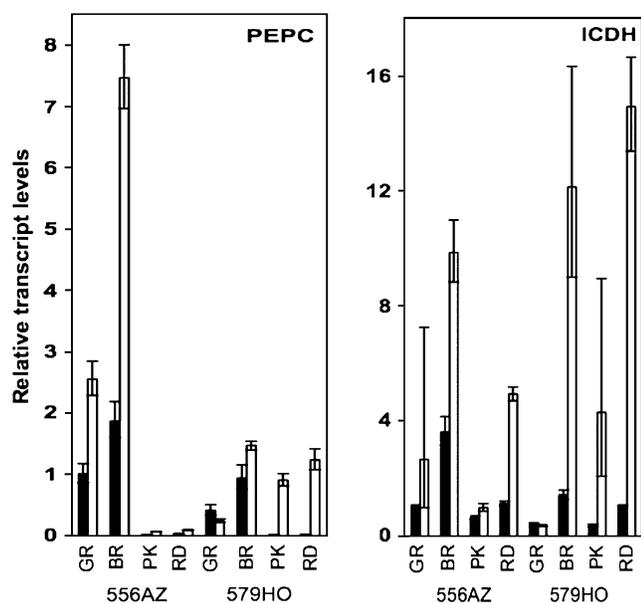
both the non-transgenic control and the transgenic 579HO fruits as compared to the fruits from BP-grown plants. Even though the expression of the E8 gene was enhanced in HV-grown tomatoes, the patterns of appearance and accumulation were specific to each growth condition, BP or HV, independent of the genotype tested (compare 556AZ and 579HO in BP versus HV).

Synergism between HV mulch and transgenic tomato in up-regulating N:C indicator genes PEPC and ICDHc in the fruit

Nitrogen and carbon interactions heralded by the HV system (Kumar *et al.*, 2004) are, to some extent, reflected in the metabolic changes observed in the polyamine (Spd and Spm), accumulating 579HO transgenic fruit (Mattoo *et al.*, 2006). Carbon metabolism linkage with N sensing is known in leaves and roots to be largely achieved

through the up-regulation of phospho*enol*pyruvate carboxylase (PEPC) and cytosolic NADP-dependent isocitrate dehydrogenase (ICDHc) (Scheible *et al.*, 2000; Foyer and Noctor, 2002; Rademacher *et al.*, 2002). Therefore, the effects of BP and HV on the transcripts of these two N:C indicator genes in the fruit from 556AZ and 579HO lines was determined by real-time RT-PCR. Relative transcript levels were calculated using the $2^{-\Delta\Delta C_T}$ method, using the transcript levels at the green (GR) stage of azygous non-transgenic fruit from the BP mulch as a reference.
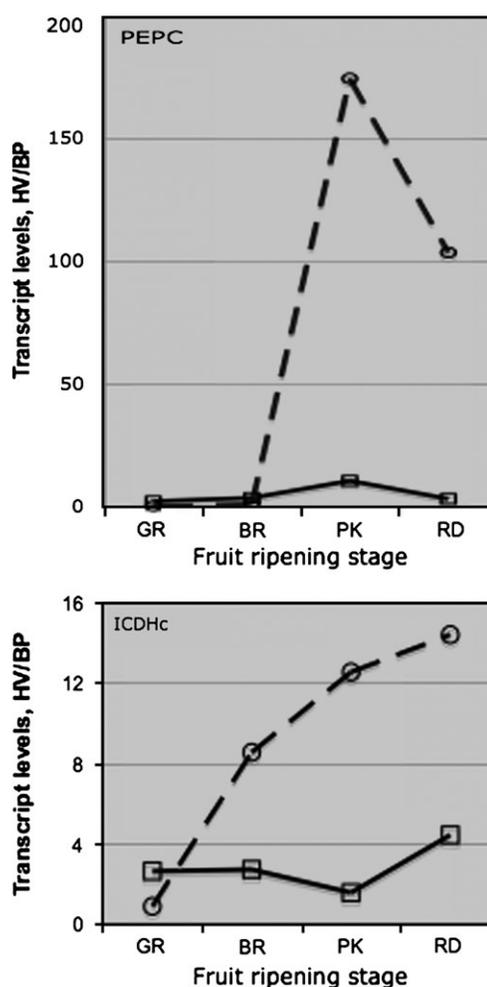
The PEPC and ICDHc transcript levels peaked at the breaker (BR) stage in the fruit from both non-transgenic (556AZ) and transgenic (579HO) lines grown in BP mulch, but decreased thereafter (Fig. 6); however, at each ripening stage, the levels in 579HO fruit were relatively lower compared with the 556AZ control fruit. In contrast to fruit from BP-grown plants, fruit from HV-grown plants contained much higher levels of PEPC and ICDHc transcript levels at each stage of fruit ripening. Early during ripening, in GR and BR stage fruit, PEPC transcript levels were higher in the 556AZ control than the 579HO transgenic line in both the mulch systems, but markedly higher PEPC transcripts were apparent in HV-grown transgenic fruit during the later stages, PK and RD, of ripening (Fig. 6, left panel). In fact, PEPC transcripts in the HV-grown 579HO transgenic fruit at the PK and RD

stages of ripening were, respectively, 174-fold and 103-fold higher than the corresponding fruit from transgenic plants grown on BP (Fig. 7). It is noted here that PEPC transcript levels in the non-transgenic 556AZ fruit grown on HV were only 9.9 at pink and 2.6 at the red (RD) stages compared with BP (Fig. 7).

The patterns for ICDHc transcripts were different from the PEPC transcripts in HV-grown 556AZ control and transgenic 579HO lines (Fig. 6, right panel). At the BR, PK, and RD stages of fruit ripening, ICDHc transcripts showed a relatively higher increase in HV-grown transgenic (579HO) fruit than the 556AZ control line. Relatively, increases in ICDHc transcripts at the PK and RD stages were several-fold higher in HV-grown transgenic fruit compared with BP-grown fruit, a much larger effect than seen in HV-grown non-transgenic fruit compared with BP grown fruits (Fig. 7).

Fig. 6. Real-time PCR analysis of LePEPC2 (A) and LeICDH (B) transcripts at different ripening stages of fruit from control 556AZ line and the transgenic 579HO line grown in BP (filled bars) and HV (open bars) mulches. The levels of PEPC2 and ICDHc transcripts were determined relative to the calibrator azygous (556AZ) at green (GR) stage from BP-grown plants. 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 standard deviation of the $\Delta\Delta C_T$ value (*n*=3). Closed and open bars represent BP-grown and HV-grown fruits, respectively.

Fig. 7. Ratio of PEPC and ICDHc transcript levels, respectively, in HV mulch to that in BP in 556AZ (open rectangles) and 579HO (open circles with dotted line) fruit at different stages (GR, BR, PK, RD) of ripening. Data from Fig. 6 were used to generate this figure. Solid and broken lines represent fruits from 556AZ and 579HO genotypes, respectively.

Discussion

It has been demonstrated here that growth environments created by using cover crop mulch (HV) or black polyethylene (BP) interact with the genetic make-up of a plant (tomato genotypes) in a unique manner. The levels and profile of metabolite accumulation at different stages of fruit ripening were distinct among transgenic and non-transgenic tomatoes, metabolite signatures distinguishing the transgenic from the control fruit whether grown on BP or HV. Notably, while HV-grown 556AZ and 579HO fruit responded to a large extent similarly, albeit to varying magnitudes, their patterns of change in the class of metabolites identified were more revealing. A summary showing the sequence of changes in the content of particular metabolites affected by the transgene introduction, thereby attributed to the accumulation of Spd-Spm in the transgenic fruit from plants grown on BP, and those in fruit from 556AZ control line grown on HV mulch is illustrated in Fig. 8. Following the sequence of changes in the metabolites with the progression of ripening, it is evident that transgene-mediated accumulation of Spd-Spm produced metabolite profiles in the fruit (grown on BP) similar to the one seen in control fruits grown on HV mulch. Interestingly, the influence and effect of the HV system on fruit metabolism are manifested as ripening progressed, as is enabled in the transgenic fruit by the ripening-regulated promoter E8 (Fig. 8). These data unearth a linkage between polyamines and metabolite content in tomato fruit, revealing a surprising finding that HV activates the same metabolic pathways and genes as are activated by the introduction of the ySAMdc transgene. HV also modulates transgene expression and seemingly affects the pattern and quantity of the indicated metabolites during fruit ripening.

Sensing of polyamines as organic-N could be the underlying cause for increases in the other N forms such as Glu, Gln, and Asn in the fruit, a response similar to N availability seen in plant roots and leaves (Foyer *et al.*, 2003) as well as trees (Rennenberg *et al.*, 1998; Bauer *et al.*, 2004). Polyamine accumulation correlates with the expression of ySAMdc transgene in transgenic fruit or the expression pattern of the endogenous SAMdc in the non-transgenic fruit, both genes showing HV-mediated enhancement. Similarly, the E8 gene expression pattern was identical within a mulch system both in non-transgenic and transgenic fruit and enhanced in the fruit from HV-grown plants.

The observed molecular changes translated into effects on the agronomic characteristics of azygous and transgenic 579HO lines grown on BP and HV. Fruit number and yield per plant were positively linked in HV-grown 556AZ (control) as well as transgenic line 579HO. The increase in yield of non-transgenic tomatoes on HV is a reflection of increased weight of these fruit and increased number of fruit on each plant, an observation
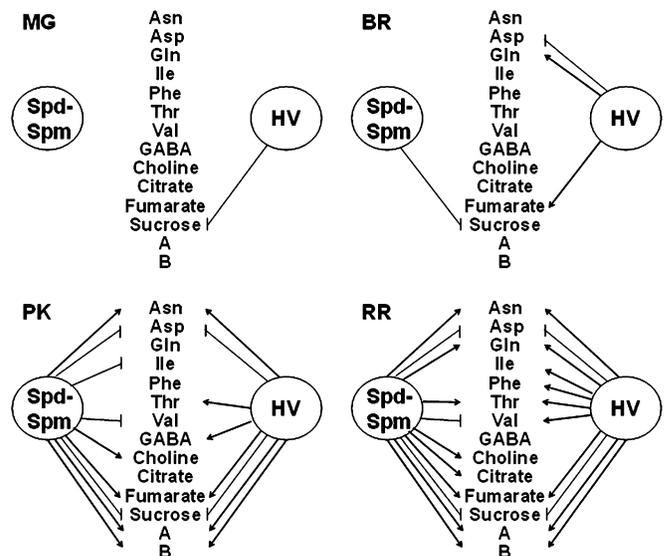
Fig. 8. Illustration of ripening stage-specific modulation of metabolites in the Spd-Spm accumulating transgenic fruit from BP-grown plants and in the azygous, control fruit from HV mulch-grown plants. Metabolites whose levels increased (arrow head) or decreased (vertical bar) at least by 50% in 556AZ-HV or 579HO-BP compared with 556AZ-BP at the indicated stages of ripening were recorded are shown. Profiles of Ala, Glu, compound C, glucose, fructose, and malic acid which were more or less similar in the two genotypes under both growth conditions are not shown. MG, BR, PK, and RR represent mature green, breaker, pink, and red-ripe fruits, respectively.

previously attributed to increased fruit set, photosynthetic activity, and physiological fitness of HV-grown plants compared with those grown on BP (Teasdale and Abdul-Baki, 1997). However, transgenic line 579HO, that accumulates higher polyamines, showed a slight reduction in the average weight of the fruits compared to non-transgenic tomatoes, which was independent of the BP or HV mulch. The reduction in fruit weight is probably due to high levels of metabolism and decreased sugar content in the transgenic fruit (Mattoo *et al.*, 2006, 2007). Potatoes engineered to over-express the SAMdc gene produced smaller tubers (Pedros *et al.*, 1999) while over-expression of hexokinase in tomato fruit, which altered carbon metabolism, also resulted in reduced fruit and seed size (Menu *et al.*, 2003).

An interesting outcome of these studies relates to the nutritionally superior 579HO red fruit (Mehta *et al.*, 2002) over the non-transgenic in terms of the relative content of Phe. Phe accumulates in the HV-grown 556AZ control red fruit, but is significantly lower in the corresponding HV-grown 579HO fruit. Higher dietary levels of Phe are not desirable for patients suffering from phenylketonuria, a genetic disorder caused by the deficiency of Phe-metabolizing enzyme, Phe hydroxylase (Levy, 1999). Thus, the transgenic 579HO tomato fruit in which Phe is down-regulated when grown in HV provides a resource to investigate the mechanism of this down-regulation and thereby find clues to fine-tune this process.

Our investigation into the transcript accumulation of two key genes in carbon metabolism and nitrogen assimilation, previously found to be critical in maintaining a balance of carbon and nitrogen in leaves and roots (Scheible *et al.*, 2000; Foyer and Noctor, 2002; Rademacher *et al.*, 2002; Stitt *et al.*, 2002), PEPC and ICDH, showed that HV mulch promotes their gene activity in both transgenic and non-transgenic fruit during ripening. Notably, the HV system is synergistic with transgenic, SAMdc expressing fruit in up-regulating PEPC and ICDHc genes in tomato fruit. Over-expression of PEPC in transgenic potato plants was shown to increase plant productivity through the direction of starch and sugars to organic acids, and amino acids such as malate and glutamine (Rademacher *et al.*, 2002). Timing of N availability and cytokinins may activate C:N metabolism in a fruit as shown for maize (Sugiharto *et al.*, 1992) and tomato leaves (Kumar *et al.*, 2004, 2005). HV system preferentially affects gene activity related to N-sensing and cytokinin signalling (Kumar *et al.*, 2004; Mattoo and Abdul-Baki, 2006). Here it is shown that HV-mediated increase in the expression of SAMdc in fruit parallels increased expression of PEPC and ICDHc transcripts. It was also found that HV increases cytokinin receptor kinase (Kumar *et al.*, 2004) transcripts in the fruit (data not shown). These correlative data suggest that SAMdc, in concert with N and cytokinins, orchestrates the C:N metabolism in tomato fruit. Thus, specific pathways and genes are activated in tomato plants in response to the HV mulch (Mattoo and Abdul-Baki, 2006). A co-ordination exists between the environmental (mulch type) cues and differential gene expression and metabolism, revealing environment-dependent and transgene-dependent changes in fruit metabolism, but without any apparent qualitative deviation from normal fruit metabolites. This observation bodes well for the incorporation of genetically engineered (transgenic) crops into alternative agriculture practices.

Acknowledgements

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