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RESEARCH PAPER

Reduced levels of NADH-dependent glutamate dehydrogenase decrease the glutamate content of ripe tomato fruit but have no effect on green fruit or leaves

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Abstract

Glutamate (Glu) is a taste enhancer that contributes to the characteristic flavour of foods. In fruit of tomato (*Solanum lycopersicum* L.), the Glu content increases dramatically during the ripening process, becoming the most abundant free amino acid when the fruit become red. There is also a concomitant increase in NADH-dependent glutamate dehydrogenase (GDH) activity during the ripening transition. This enzyme is located in the mitochondria and catalyses the reversible amination of 2-oxoglutarate to Glu. To investigate the potential effect of GDH on Glu metabolism, the abundance of GDH was altered by artificial microRNA technology. Efficient silencing of all the endogenous *SlGDH* genes was achieved, leading to a dramatic decrease in total GDH activity. This decrease in GDH activity did not lead to any clear morphological or metabolic phenotype in leaves or green fruit. However, red fruit on the transgenic plants showed markedly reduced levels of Glu and a large increase in aspartate, glucose and fructose content in comparison to wild-type fruit. These results suggest that GDH is involved in the synthesis of Glu in tomato fruit during the ripening processes. This contrasts with the biological role ascribed to GDH in many other tissues and species. Overall, these findings suggest that GDH has a major effect on the control of metabolic composition during tomato fruit ripening, but not at other stages of development.

Key words: Amino acid, artificial microRNA, aspartate, gdh silencing, mature fruit, ripening.

Introduction

The taste of a tomato fruit was previously attributed to the content of sugars (glucose and fructose) and acids (citrate and malate). However, it is now widely accepted that a broader combination of chemicals is needed to provide the characteristic tomato taste and flavour (Tieman *et al.*, 2012). The content of free glutamate (Glu) in red fruit of an elite tomato species (*Solanum lycopersicum* L.) is much higher than in related wild species (Schauer *et al.*, 2005), suggesting that this characteristic was acquired during the domestication process. Moreover, Glu is the main free amino acid that accumulates when fruit ripens in all cultivated varieties of tomato (Boggio *et al.*, 2000). Glutamate is a taste enhancer, and also occupies a central role in the metabolism of amino acids in plants (Forde and Lea, 2007).

Biochemical and genetic approaches have provided some clues to the sources of Glu accumulation in tomato fruit (Carrari and Fernie, 2006; Akihiro *et al.*, 2008; Sorrequieta *et al.*, 2010; Bemer *et al.*, 2012). Based on the enzyme activities that correlate with the increase in Glu level during the tomato ripening transition it was suggested that the glutamate dehydrogenase (GDH), γ-aminobutyrate transaminase (GABA-T), and glutamate decarboxylase (GAD) might be involved in determining Glu levels. GDH catalyses the reversible reductive conversion of 2-oxoglutarate

and ammonium to Glu; GABA-T catalyses the reversible conversion of γ-aminobutyrate (GABA) and 2-oxoglutarate to succinate semialdehyde and Glu, and GAD catalyses the decarboxylation of Glu to GABA and CO_2. Both GDH and GABA-T increase (Sorrequieta *et al.*, 2010) while GAD decreases (Bemer *et al.*, 2012) during fruit ripening. Additionally, the lower content of Glu in red fruit when they are ripened off the vine rather than on the vine is accompanied by an increase in GAD and a decrease in GABA-T activity compared to red fruit ripened on the vine (Sorrequieta *et al.*, 2013).

In a genetic approach designed to uncover the role of enzymes related to Glu metabolism, Koike *et al.* (2013) used an RNA interference technique to suppress GABA-T gene expression. As noted above, this mitochondrial isoform of the enzyme is responsible for the reversible conversion of GABA and 2-oxoglutarate to succinate semialdehyde and Glu. Expression of *GABA-T1* was inversely associated with GABA levels in the transgenic lines at the red fruit stage but the Glu level of transgenic fruit was unaffected (Koike *et al.*, 2013). This contrasts with an earlier study in fruit of GAD antisense tomato, which showed an increase in Glu (Kisaka *et al.*, 2006). In a further study of ethylene-independent ripening regulator (*FUL1* and *FUL2*) mutants, upregulation of the three *GAD* genes was reported alongside an increase in GABA and a decrease in Glu contents in the transgenic tomato fruit (Bemer *et al.*, 2012). Together, these studies point to a role for the Glu degradation pathway in determining Glu levels in red fruit. On the other hand, constitutive overexpression of a tomato gene for NADH-dependent GDH in tomato plants caused a 2-fold increase in the level of Glu in transgenic fruit compared to the control fruit (Kisaka *et al.*, 2007). Recently, another study performed with tomato cv. 'Moneymaker' fruit showed that NAD-GDH activity increased strongly between cell expansion and ripening, corresponding to the time at which Glu started to accumulate (Biais *et al.* 2014). Furthermore, in tomato (cv. 'Micro-Tom') it was recently shown that the GDH activity ratio of aminating- vs deaminating-reaction was significantly higher in red fruit than in other fruit-ripening stages (Sorrequieta *et al.* 2010; Sorrequieta *et al.* 2013).

A better understanding of the biochemical basis of the accumulation of molecules that provide tomato taste is a major challenge in tomato breeding programmes. For this, characterization of genes that control the end products affecting taste is crucial. To this end, four genes in the tomato genome that encode GDH were identified. Of these, three encode the α-subunit (*SlGDH-NAD;A1–3*) and one encodes the β-subunit of GDH (*SlGDH-NAD;B1*). They are located in different tomato chromosomes (Solyc03g094010, Solyc05g052100, Solyc06033860; and Solyc10g078550) (Ferraro *et al.*, 2012). The present study explores the influence of reducing NADH-dependent GDH activity on the Glu accumulation of tomato fruit by expressing artificial microRNAs (amiRNAs) targeted to all four GDH genes, with the goal of silencing all *SlGDH-NAD* genes and studying the role of GDH in determining Glu content.

Materials and methods

amiRNA constructs and plant transformation

To design an amiRNA sequence that targeted all *SlGDH* genes at the same time, candidate 21-mer sequences that followed the criteria proposed by Ossowski *et al.* (2008) were sought in the conserved coding region of all the *SlGDH* genes. Candidate sequences were analysed with Web Micro RNA designer (www.wmd3.weigelworld.org). miR319a under the control of the constitutive 35S-promoter from cauliflower mosaic virus (CaMV) was used as backbone for amiRNA expression. Using overlapping PCR (Schwab *et al.*, 2006), the natural miRNA sequences as well as the partially complementary region of the miRNA* were replaced with those of amiRNAs, taking care that both structural and energetic features of the miRNA precursor were retained. Plasmid NB147 was kindly provided by Dr Palatnik and the following primers were used:

I: 5′-GACAGGTGAAATCTGATACCTTCACAGGTCGTGATATGA-3′,
II: 5′-AGGTATCAGATTTCACCTGTCCTACATATATATTCCTA-3′,
III: 5′-TGGTATCTGATTCCACCTTTCCTCTCTTTTGTATTCC-3′,
IV: 5′-GAAAGGTGGAATCAGATACCATCAAAGAGAATCAATGAT-3′,
192: 5′-CATTTCATTTGGAGAGAACACG-3′,
193: 5′-CGAAACCGATGATACGAACG-3′

The amiRNA backbone was cloned in the binary vector pCHF3 and used to transform tomato (*Solanum lycopersicum* L.) cv. Micro-Tom plants via *Agrobacterium tumefaciens* GV2260 according to Ferraro *et al.* (2012). Seeds were collected from kanamycin-resistant transgenic plants, and this first generation was identified as T1. Seeds from following generations were also selected using kanamycin, and T3 progeny were used for further studies.

Plant growth conditions

Tomato (cv. Micro-Tom) seeds were provided by Gulf Coast Research and Education Centre, University of Florida, USA. Tomato plants were grown in a controlled environment cabinet under a light intensity at the top of a fruit-containing plant of 400 μmol m^{-2} s^{-1}. The temperature ranged from 23°C during the light period (14 h) to 18°C during the dark period and the relative humidity was 70%. Plants were grown in soil, maintained under optimal irrigation, and supplied with a standard nutrient medium. Fruit were allowed to ripen naturally on the plant and then selected for uniformity to maximise homogeneity between samples. They were harvested at the mature green stage (when fruit stopped growing), and red stage (5.5 ± 2.1 days after breaker stage), when fruit began to soften (Gonzalez *et al.*, 2015). Pericarp tissue of harvested fruit was obtained by peeling off and removing the locular tissues (placenta) and seeds; the tissue was then immediately processed or frozen in liquid nitrogen and stored at −80°C until use. Fruit was harvested between 6 and 8 h after the lights were switched on.

In-gel staining for NAD-GDH activity

Fruit protein extracts were obtained as previously described (Scarpeci *et al.*, 2007) and immediately used or stored at −80°C until analysis. Total soluble protein concentration in each sample was estimated according to Bradford's assay using bovine serum albumin as the standard (Bradford, 1976). Total NAD-GDH activity was measured spectrophotometrically as described by Sorrequieta *et al.* (2010). In-gel NAD-GDH activity was determined by loading the same amount of total soluble protein onto each lane of a native gel and incubating with the substrate. Staining of non-denaturing 8% (w/v) polyacrylamide gel was performed as previously described (Turano *et al.* 1996). Briefly, the GDH activity-staining solution, containing 100 mM Tris-HCl (pH 9.3), 50 mM sodium glutamate,

0.5 mM NAD, 0.05 mM phenazine methosulphate, and 0.5 mM nitro blue tetrazolium, was poured onto the gel; staining was performed at 37°C in the dark until blue formazan precipitate appeared (20–60 min) and stopped by replacing the staining solution with distilled water. In-gel GDH activity detection was performed at least in triplicate with different fruit samples for each experiment.

Determination of Glu content

Glu measurements were adapted to a microplate format in a coupled enzymatic assay as described by Cross *et al.* (2006). Metabolites were extracted twice with 80% ethanol and once with 50% ethanol. Extracts were randomized across microplates. Standards were included on each microplate as measuring references. The reaction mix contained 45 µl fruit extract or 15 µl leaf extract, 100 mM Tricine (pH 8.0), 0.5 mM MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide],1.5 mM NAD, 0.25 % (w/v) triton, 0.5 mM ADP, and 0.1 units of diaphorase in a total volume of 200 µl. The reaction was triggered with 0.5 units of GDH. Optical density was read at 570 nm.

Enzyme activity measurements

Samples of ~20 mg fresh weight were extracted by addition of 10 mg (w/v) polyvinylpolypyrrolidone and vigorous shaking in 300 ml of ice-cold extraction buffer: 20% (v/v) glycerol, 0.25% (w/v) bovine serum albumine, 2% (v/v) Triton-X100, 50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 10 mM leupeptin, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. The crude extract was centrifuged for 10 min at 20 000*g* and 4°C. Enzyme activities were assayed using a robotized platform as described in Steinhauser *et al.* (2010).

RNA isolation

Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Karlsruhe, Germany) following the manufacturer's instructions. Quality and quantity of RNA were monitored spectrophotometrically at 260 and 280 nm, and RNA integrity was checked by electrophoresis in 1.5% (w/v) agarose gel.

Quantitative real-time PCR

Total RNA (1.5 µg) was digested with DNAse (Promega) and reverse transcribed with Superscript III reverse transcriptase (Invitrogen) in a reaction volume of 20 µl to generate first-strand cDNA. The quantitative real-time PCR (qPCR) analyses of *SlGDH-NAD;A1–3* and *SlGDH-NAD;B1* transcripts were performed using the following primers: for *SlGDH-NAD;B1* forward 5′-AAGGAGTCACCATCCTACCG-3′ and reverse 5′-TGTGAGT CTTGCACATATCCTTG-3′; for *SlGDH-NAD;A1-3* forward 5′-CCAGACATCTATGCCAATGC-3′ and reverse 5′-ATTCACCCCC AATGTGAATG-3′; for *SlGDH-NAD;A1* forward 5′- GAAAGCT CATCGATTTTGCT-3′ and reverse 5′-TAATGAATTTGGCCTT GACGTT-3′; for *SlGDH-NAD;A2* forward 5′-GGAACCTAATT GATTTCGGC-3′ and reverse 5′-CGATGAACTTGGCATTTACAT G-3′; and for *SlGDH-NAD;A3* forward 5′-AGCTGATGAGGTAC TGTGC-3′ and reverse 5′-CAGGTTATGGAAGGCATTC-3′. *Rpl2* (encoding the ribosomal protein large subunit 2) was chosen as a housekeeping gene and was measured using the primers *Rpl2* forward 5′-CGTGGTGTTGCTATGAATCC-3′ and *Rpl2* reverse 5′-GTCAGCTTTGGCAGCAGTAG-3′. cDNAs were amplified using a Mastercyclerep Realplex2 thermocycler (Eppendorf, Westbury, USA). PCR conditions were 1 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 58°C, and 40 s at 72°C. Following amplification, products were denatured by heating from 60 to 95°C to check amplification specificity. qPCR was performed using a SYBR Green fluorescence-based assay. Gene-specific cDNA amounts were calculated from threshold cycle (Ct) values, expressed relative to controls, and normalized with respect to Rpl2 cDNA, used as an internal reference (Ctr) according to the equation $\Delta Ct = Ct - Ctr$ and quantified as $2^{-\Delta Ct}$. A second normalization by a control (Ctc) $\Delta\Delta Ct = Ct - Ctc$ produces a relative quantification: $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Experiments were carried out in triplicate and data are shown as the means ± SE or SD of the mean of each independent experiment.

NMR spectroscopy

Metabolic profiles by ^{1}H-NMR spectroscopy were performed according to Sorrequieta *et al.* (2013). Briefly, liquid nitrogen powder (1 g) from each sample was rapidly dissolved in 0.3 ml of cold 1 M sodium phosphate buffer (pH 7.4) prepared in D_2O to obtain a mixture containing about 30% by weight of D_2O. The mixtures were centrifuged at 13 500 rpm for 15 min at 4°C and the supernatant filtered to remove any insoluble material. 1 mM internal standard [TSP: 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid] was added to the resulting transparent soluble fraction and the solution was subjected to spectral analysis at 600.13 MHz on a Bruker Avance II spectrometer. Proton spectra were acquired at 298 K by adding 512 transients of 32 K data points with a relaxation delay of 5 s. A 1D-NOESY pulse sequence was utilized to remove the water signal. The 90° flip-angle pulse was always ~10 µs. Proton spectra were referenced to the TSP signal (δ = 0 ppm) and their intensities were scaled to that of TSP. Spectral assignment and identification of specific metabolites was established by fitting the reference ^{1}H-NMR spectra of several compounds using the software Mixtures, developed ad hoc as an alternative to commercial programs (Abriata, 2012). Further confirmation of the assignments for some metabolites was obtained by acquisition of new spectra after addition of authentic standards.

Statistical analysis

Significant difference was determined at $P < 0.05$ using an unpaired two-tailed Student's *t*-test or ANOVA followed by Holm Sidak test analysis ($P < 0.05$). Principal component analysis (PCA) was performed using the software Statistica 7.1 (Statsoft,1984−2005). The metabolites considered to have 'high' correlation to PCs were the four that showed highest (absolute) correlation values for each factor (i.e. PC1 and PC2).

Results

Molecular and phenotypic analyses of tomato plants with reduced levels of GDH by amiRNA silencing

Transgenic tomato plants were generated that constitutively overexpress amiRNAs by *Agrobacterium*-mediated transformation of tomato cotyledons (cv. Micro-Tom). The amiRNA was designed to silence the expression of all *SlGDH-NAD* genes (Ferraro *et al.*, 2012). From here on, *GDH* will be used to refer to the *SlGDH-NAD* genes. To achieve high-level ectopic expression, the amiRNA was placed under the control of the CaMV 35S-promoter. The transgenic tomato lines (amiGDH-all) generated were selected using kanamycin. The presence of amiRNA transgenes was confirmed by PCR and overexpression of 35S-amiR by qPCR.To confirm *GDH* silencing in the amiGDH-all lines, qPCR analysis of *GDHA* and *GDHB* transcripts was performed in leaf and fruit extracts (Fig. 1). All *GDH* genes were silenced in the leaves and fruit (mature green and red). *GDHA3* transcripts were not detected in these organs, including in the wild type (WT) (see also Ferraro *et al.*, 2012).

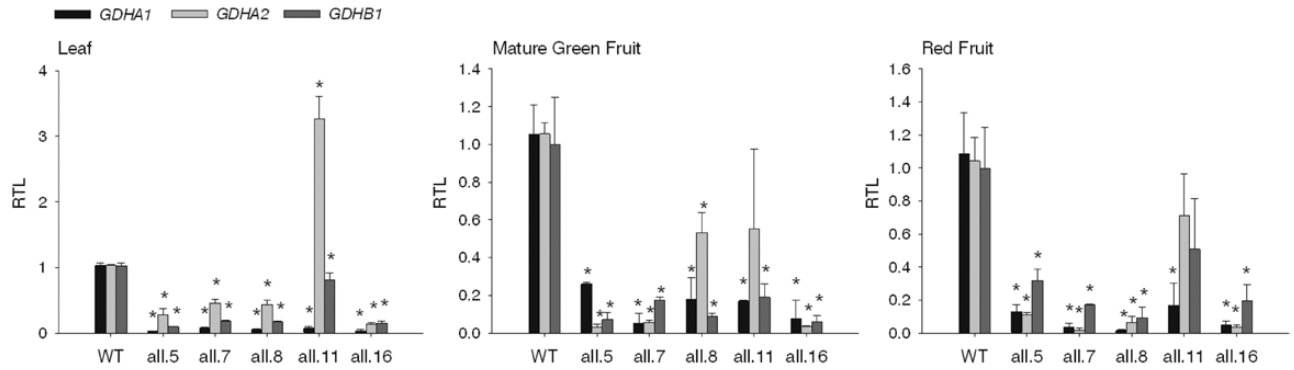

Fig. 1. Molecular characterization of transgenic amiGDH-all tomato fruit. Relative transcript levels (RTLs) for all members of the *SlGDH* gene family in tomato fruit were determined by qRT-PCR. Reactions were performed on total RNA extracted from leaves, mature green fruit, and red fruit of the WT and five mutants. The level of expression of the *SlGDHA1*, *SlGDHA2*, and *SlGDHB*1 genes was quantified relative to the expression of the *SlRpl2* gene. *SlGDHA3* expression was not detected in the organs assayed. Results are presented as mean values for four plants ± SE. Asterisks indicate significant differences with a confidence interval at $P < 0.05$.

The visual phenotypes of transgenic plants of amiGDH-all lines were indistinguishable from the WT at all developmental stages, including fruit development, ripening, and production (data not shown). These data suggest that a large decrease in GDH expression has limited impact on tomato plant growth, survival, and reproduction.

Three independent amiGDH-all lines (T3 progeny) that showed the largest reduction in all *GDH* expression levels were selected for further analysis (termed lines all.7, all.8, and all.16).

To demonstrate that the selected amiGDH-all lines had effective silencing of GDH enzyme activity in fruit, GDH activity was followed spectrophotometrically and in gel using mature green and red fruit extracts from the WT and three amiGDH-all lines. NAD-GDH activity was detected in WT fruit of mature green (0.2 U mg^{-1} protein) and red fruit (0.052 U mg^{-1} protein), although NAD-GDH activity was under the detection limits in the fruit extracts of amiGDH-all plants. Next, the NAD-GDH isoenzyme pattern was assayed in gel using mature green and red fruit extracts from the WT and three amiGDH-all lines. Qualitatively, the same results were obtained for both mature green (Fig. 2A) and red (Fig. 2B) fruit. The GDH pattern in the transgenic lines differed from the pattern in the WT. Whilst WT fruit showed the typical seven GDH isoenzymes, the transgenic fruit showed only a single faint band corresponding to the homohexamer GDH1 (6β) (Fig. 2). A similar GDH pattern was seen in all three amiGDH-all lines. Immunoblot analysis confirmed that the different GDH isoenzyme detected using NAD-GDH enzyme activity staining corresponded to a GDH protein (data not shown) as they were recognized by an antibody against the α and β subunits of tomato GDH (Ferraro *et al.*, 2012). The activities of eight other enzymes (acid invertase, isocitrate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, shikimate dehydrogenase, phosphoenolpyruvate carboxylase, malate dehydrogenase, and fumarase) were investigated in fruit extracts from WT and transgenic plants using a robot-based platform (Gibon *et al.*, 2004). These enzyme activities were not significantly different between WT and transgenic fruit (Supplementary Table S1).

To evaluate the impact of the absence of different GDH isoenzymes on Glu accumulation, the Glu content was measured spectrophotometrically in the pericarp of mature green and red fruit of WT and amiGDH-all plants. The content of free Glu in mature green fruit was similar in WT and transgenic plants (Fig. 3). However, amiGDH-red fruit contained about 5-fold less Glu than WT fruit.

GDH activity and Glu content were similar in leaves of the WT and all amiGDH transgenic lines (Supplementary Figure S1).

Metabolic profiling of ripening fruit

To further explore the metabolism of the mature fruit, we performed metabolic profiling of mature green and red fruit by ^{1}H-NMR. The metabolic composition of WT and transgenic fruit was obtained after assignment of specific individual signals to each metabolite and quantification of each metabolite as described by Sorrequieta *et al.* (2013). Results are shown in Table 1. The metabolic profiles of mature green fruit from WT and amiGDH-all lines were quite similar with no significant differences for individual metabolites except for the glucose and fumarate content. A different picture emerged for the metabolic profile of red tomato fruit from WT and amiGDH-all lines (Table 2). In the red fruit of all transgenic lines a marked increase in the hexose (glucose and fructose; up to 2-fold) and aspartate (up to 10-fold) content was observed in comparison with the WT, and a concomitant 2- to 5-fold decrease in Glu content. The decrease in Glu level in red fruit of amiGDH-all lines compared to the WT was then confirmed by both enzymatic measurement of Glu (Fig. 3) and ^{1}H-NMR measurements of Glu (Tables 1 and 2). In red fruit of two transgenic lines a lower content of valine and a higher content of phenylalanine and tyrosine were also observed (Table 2). The red fruit weight of WT and amiGDH-all lines was similar (data not shown), indicating that the higher sugar content of transgenic plants had no impact on fruit growth. There were no significant changes in the levels of any organic acids.

Principal component analysis was performed using the metabolite concentrations obtained for amiGDH-all and

WT fruit (Fig. 4). Whilst there was no separation of WT and transgenic mature green fruit (Fig. 4A), there was a clear difference between WT and transgenic red fruit (Fig. 4B). In the latter, the two first PCs explained 47.3 and 24% of the total variance for PC 1 and PC 2, respectively. Wild type and transgenic fruit were separated along PC 2 (Fig. 4B). For the correlation values between the different metabolites with each factor see Supplementary Table S2. PC 2 was mainly correlated with Glu, valine, and pyruvate in a positive direction, and with fructose in a negative direction. Correspondingly, these metabolites contributed most strongly to explain the differences between transgenic and WT red fruit (Fig. 4C). Also, PCA revealed that transgenic red fruit are scattered along the PC 1 axis. Glutamine, fumarate, aspartate, and succinate are the metabolites with a high (negative) correlation with this PC and are consequently involved in the separation (Fig. 4C; Supplementary Table S2).

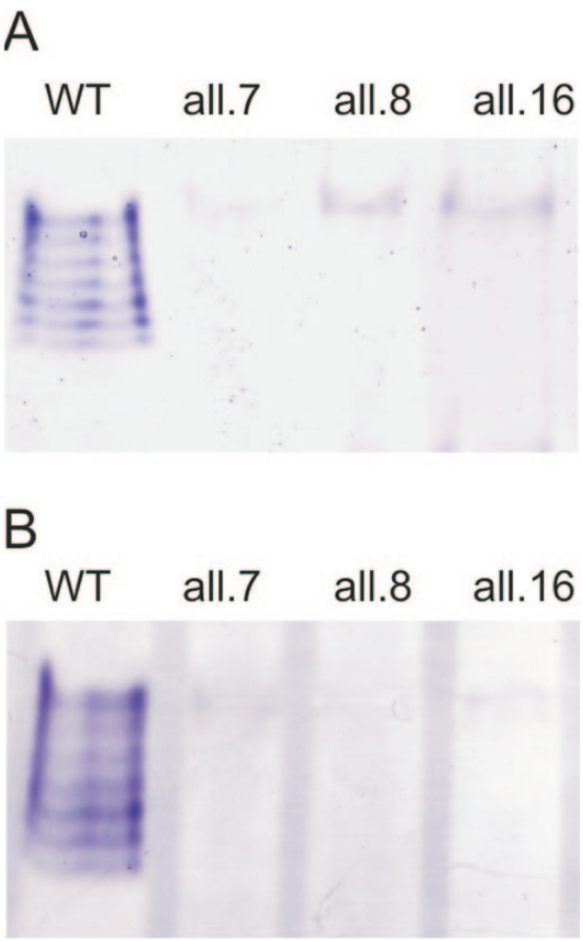

Fig. 2. NAD-GDH isoenzyme patterns of mature green and red tomato fruit of WT and mutants with reduced levels of all *SlGDH* genes. Protein extracts were subjected to native PAGE followed by NAD-GDH in-gel activity staining. An equal amount of total soluble protein was loaded onto each lane. (A) Mature green fruit extracts (15 μg of protein). (B) Red fruit extracts (45 μg of protein). In the WT fruit the seven isoenzymes are present; however, in the amiGDH-all mutants only a faint band of the homohexamer GDH1 (6β) is active. This figure is available in colour at *JXB* online.

Discussion

Tomato is an important species for fresh and industrialized food production. There are many cultivated varieties worldwide with different organoleptic and nutritional qualities in their fruit. This is in part due to breeding programmes selecting lines based on differing fruit characteristics such as form, size, colour, and shelf life (Bai and Lindhout, 2007) and not necessarily their taste. Tasty tomato fruit are in increasing demand by consumers, and intense efforts have been devoted to correlate metabolic traits with organoleptic traits (Zanor *et al.*, 2009*a*). However, there is still a lack of knowledge about what combination of metabolites is required to generate the typical tomato taste. Consequently, identifying candidate genes for breeding programmes or to genetically engineer tomato plants to achieve fruit with valuable taste is a challenging goal.

The work presented in this paper shows that a specific reduction in the levels of GDH expression and activity in transgenic tomato plants has a large impact on the metabolite content of red fruit, particularly hexoses and the main free amino acids, Glu and asparate. The red fruit of transgenic plants contained significantly higher levels of glucose, fructose, and aspartate, and a 5-fold lower Glu content than WT fruit. There was no consistent change in the level of carboxylic acid intermediates of the tricarboxylic acid cycle (Table 2).

The lower Glu in the amiGDH-all transgenic red fruit shows that GDH is involved in the synthesis of Glu by amination of 2-oxoglutarate in this tissue during ripening.

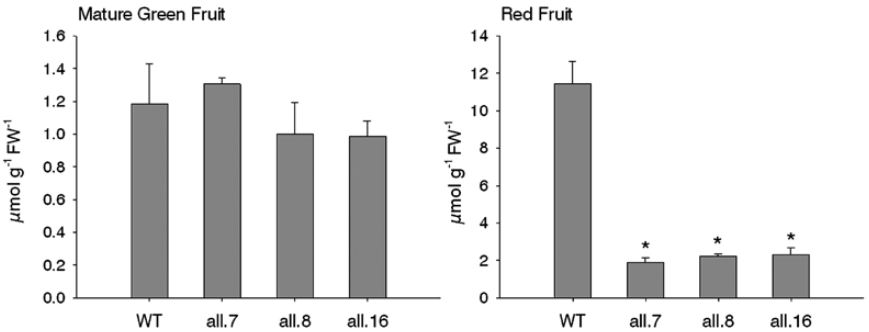

Fig. 3. Glutamate content in mature green and red fruit of WT and amiGDH-all plants. Glutamate content was measured spectrophotometrically in the pericarp of mature green and red fruit. Results are presented as mean values for four plants ± SE. Asterisks indicate significant differences with a confidence interval at $P < 0.05$.

Table 1. *Metabolic composition of Micro-Tom mature green WT and amiGDH-all fruit determined by ^{1}H-NMR*

Metabolite	Mature green fruit (μmol g^{-1} FW)			
	WT	all.7	all.8	all.16
Sugars				
Glucose	101.79 ± 11.95	144.48[a] ± 26.02	71.31[a] ± 10.45	128.74 ± 25.90
Fructose	148.84 ± 27.80	182.44 ± 39.82	175.01 ± 12.82	208.90 ± 50.31
Sucrose	8.46 ± 2.81	6.54 ± 2.69	6.24 ± 2.58	6.70 ± 3.73
Organic acids				
2-oxoglutarate	14.40 ± 2.60	14.60 ± 7.40	13.90 ± 10.42	13.14 ± 3.76
Citrate	42.15 ± 12.24	50.62 ± 8.40	41.58 ± 7.24	49.62 ± 5.38
Fumarate	1.08 ± 0.44	0.45 ± 0.37	0.67 ± 0.38	0.30[a] ± 0.23
Malate	18.45 ± 3.12	27.60 ± 6.95	19.84 ± 0.69	22.48 ± 6.55
Succinate	9.96 ± 4.79	9.35 ± 0.66	12.13 ± 4.02	10.08 ± 3.19
Pyruvate	9.20 ± 4.24	9.57 ± 1.59	11.24 ± 2.80	10.37 ± 3.02
Amino acids				
Alanine	1.44 ± 0.86	1.07 ± 0.22	1.46 ± 0.82	1.34 ± 0.55
Asparagine	4.66 ± 3.42	5.70 ± 0.88	2.33 ± 0.57	3.76 ± 1.67
Aspartate	3.21 ± 2.34	2.46 ± 0.59	1.76 ± 0.19	2.65 ± 0.83
GABA	9.50 ± 3.46	11.33 ± 2.78	14.72 ± 10.99	13.65 ± 3.60
Glu	3.20 ± 1.49	3.07 ± 0.85	2.94 ± 1.59	2.33 ± 0.18
Glutamine	9.33 ± 4.82	11.40 ± 2.71	11.67 ± 7.38	7.66 ± 1.89
Isoleucine	0.36 ± 0.16	0.61 ± 0.22	0.57 ± 0.45	0.54 ± 0.22
Phenylalanine	0.50 ± 0.37	0.50 ± 0.07	0.49 ± 0.37	0.45 ± 0.06
Threonine	0.80 ± 0.31	1.03 ± 0.29	0.45 ± 0.07	0.67 ± 0.25
Tryptophan	0.10 ± 0.05	0.08 ± 0.03	0.16 ± 0.09	0.13 ± 0.08
Valine	1.07 ± 1.05	0.92 ± 0.43	0.86 ± 0.65	0.80 ± 0.29
Tyrosine	2.11 ± 1.28	2.49 ± 1.19	0.99 ± 0.07	3.37 ± 2.55
Others				
Methanol	14.13 ± 6.31	11.60 ± 0.95	15.19 ± 4.90	16.55 ± 4.26
Ethanol	3.74 ± 3.94	6.01 ± 4.57	2.39 ± 1.42	1.27 ± 0.27
Trigonelline	3.56 ± 1.97	2.60 ± 0.65	3.03 ± 0.73	2.33 ± 0.55

Results are presented as means of four independent experiments ± SE.
[a] Means of WT and transgenic plants significantly different ($P < 0.05$).

Table 2. *Metabolic composition of Micro-Tom red WT and amiGDH-all fruit determined by ^{1}H NMR*

Metabolite	Red Fruit (μmol g^{-1} FW)			
	WT	all.7	all.8	all.16
Sugars				
Glucose	98.09 ± 9.31	217.81[a] ± 27.41	144.86[a] ± 12.16	144.88[a] ± 24.11
Fructose	155.69 ± 22.15	289.61[a] ± 30.86	211.97[a] ± 15.01	213.59[a] ± 32.67
Sucrose	0.32 ± 0.34	0.55 ± 0.18	0.11 ± 0.04	0.16 ± 0.07
Organic acids				
2-Oxoglutarate	4.21 ± 1.74	5.21 ± 1.08	1.77[a] ± 0.24	3.41 ± 1.25
Citrate	66.37 ± 10.65	83.41 ± 14.43	57.71 ± 0.73	70.47 ± 11.91
Fumarate	0.02 ± 0.00	0.06[a] ± 0.02	0.03 ± 0.02	0.03 ± 0.02
Malate	9.41 ± 3.61	8.86 ± 1.62	6.32 ± 1.05	8.96 ± 2.12
Succinate	4.45 ± 1.54	5.62 ± 0.85	2.17[a] ± 0.43	3.97 ± 0.45
Pyruvate	4.57 ± 1.74	4.53 ± 0.49	2.09[a] ± 0.27	3.65 ± 0.53
Amino acids				
Alanine	0.74 ± 0.14	1.20[a] ± 0.32	0.63 ± 0.10	0.72 ± 0.13
Asparagine	2.01 ± 0.78	3.49[a] ± 0.77	1.15 ± 0.77	2.77 ± 0.63
Aspartate	4.14 ± 1.17	45.66[a] ± 4.33	19.96[a] ± 2.70	27.84[a] ± 6.31
GABA	5.85 ± 2.43	6.43 ± 1.40	2.96 ± 0.24	4.22 ± 1.53
Glu	12.42 ± 1.84	5.64[a] ± 0.28	2.25[a] ± 0.72	3.34[a] ± 1.15
Glutamine	5.57 ± 2.37	9.59[a] ± 1.11	3.56 ± 0.25	5.40 ± 0.54
Isoleucine	0.23 ± 0.08	0.26 ± 0.08	0.10[a] ± 0.03	0.15 ± 0.03
Phenylalanine	0.15 ± 0.03	0.36[a] ± 0.06	0.20 ± 0.06	0.20[a] ± 0.01
Threonine	0.30 ± 0.09	0.51 ± 0.21	0.31 ± 0.07	0.48 ± 0.14
Tryptophan	0.10 ± 0.02	0.12 ± 0.03	0.12 ± 0.02	0.18 ± 0.02
Valine	0.26 ± 0.09	0.20 ± 0.04	0.10[a] ± 0.01	0.13[a] ± 0.02
Tyrosine	1.43 ± 0.35	2.50[a] ± 0.60	1.71 ± 0.36	2.49[a] ± 0.62
Others				
Methanol	10.94 ± 1.27	13.45 ± 2.43	7.37[a] ± 1.16	13.15[a] ± 3.72
Ethanol	1.25 ± 0.14	1.20 ± 0.05	1.14 ± 0.30	1.69 ± 0.67
Trigonelline	2.80 ± 0.63	2.36 ± 0.28	2.42 ± 0.74	2.23 ± 0.23

Results are presented as means of four independent experiments ± SE.
[a] Means of control and transgenic plants significantly different ($P < 0.05$).

Further, the absence of any difference in mature green fruit suggests that this function of GDH in Glu accumulation is closely related to ripening processes. The ammonia needed for the GDH amination reaction during red fruit ripening could be released by the asparaginase reaction (Fig. 5A). In fact, cherry fruit and seeds contain asparaginase, an enzyme that converts asparagine into aspartate and ammonia for further utilization, but not always at the same time or even in the same tissue (Sieciechowicz *et al.*, 1988). In Micro-Tom fruit, high expression levels of a gene encoding an asparaginase was identified (Solyc04g078460) in the Sol Genomics Network (http://solgenomics.net/). Induction of aminating GDH activity was also observed during salt stress in tobacco leaves and grapevine cells (Skopelitis *et al.*, 2006). However, the proposed role of GDH in the synthesis of Glu in the ripening process of tomato fruit contrasts with the biological role ascribed to the enzyme in many other tissues and species. GDH is typically induced during C-starvation (Melo-Oliviera *et al.*, 1996; Gibon *et al.*, 2004; Miyashita and Good, 2008), when it is thought to play a role in amino acid catabolism (Lam *et al.*, 1996; Forde and Lea, 2007), possible via a cycle in which 2-oxoglutarate acts as an acceptor for amino groups from other amino acids, forming Glu, which is then converted back to 2-oxoglutarate with loss of the amino group as ammonium.

The higher levels of aspartate in the transgenic amiGDH-all red fruit could be the result of two different oxaloacetate-dependent transaminase pathways (see Fig. 5B), (i) through the mitochondrial GABA shunt (Fait *et al.*, 2008), where GABA is transaminated to succinic semialdehyde, which is then oxidized to succinate, bypassing two steps of the tricarboxylic acid cycle (Clark *et al.*, 2009) followed by conversion of succinate to oxaloacetate and transamination of oxaloacetate to aspartate; and (ii) through the aspartate and prephenate aminotransferases present in plastids of nonphotosynthetic plant cells (Torres *et al.*, 2014). The former route might explain why the level of 2-oxoglutarate was similar in amiGDH-all and WT fruit, rather than rising as might have been expected. The latter route would explain the observation that tyrosine and phenylalanine are significantly increased in the red fruit from amiGDH-all plants (Table 2); the product of prephenate aminotransferase is arogenate, which is the

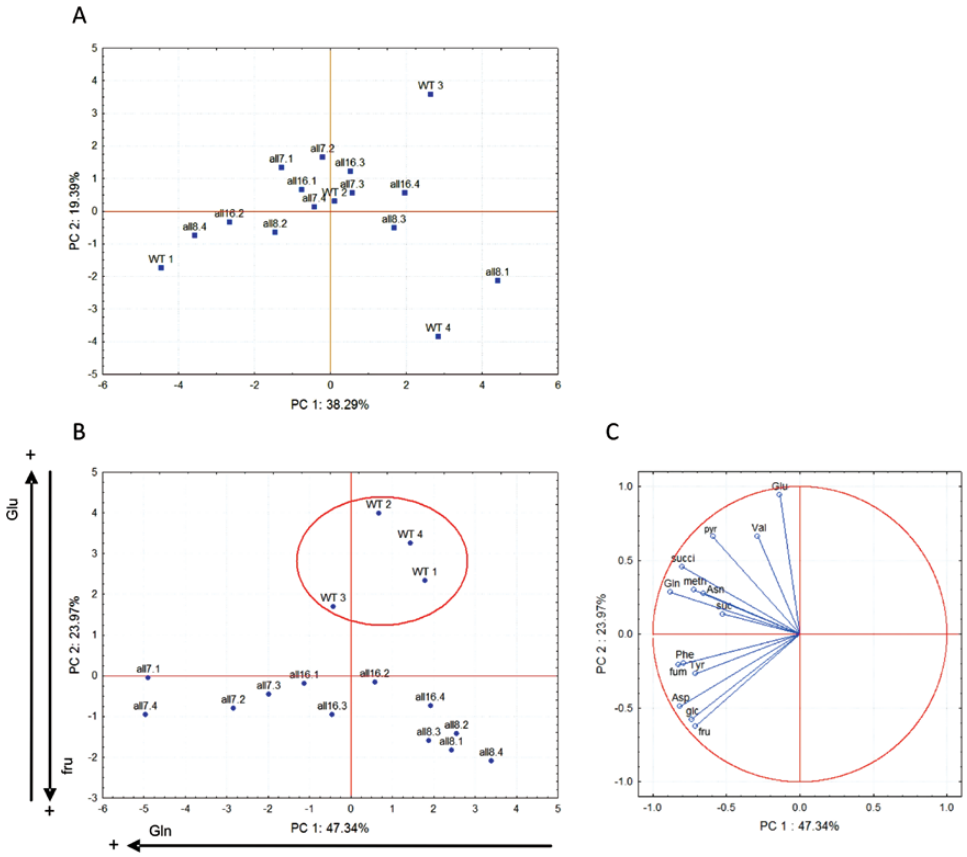

Fig. 4. Principal component analysis performed on metabolite concentrations of all fruit samples analysed. Panels show the score plots in which each point corresponds to one sample of WT and transgenic (all) fruit. (A) Projections of the mature green fruit samples for PC 1 vs PC 2. (B) Projections of the red fruit samples for PC 1 vs PC 2. The WT red fruit samples are marked with a circle. (C) Correlation circle in the plane formed by the first two PCs showing the contribution of metabolites for group separation in red fruit. Asn, asparagine; Asp, aspartate; Fum, fumarate; fru, fructose; glc, glucose; Gln, glutamine; Glu, glutamate; Phe, phenylalanine; suc, sucrose; succi, succinate; meth, methanol; pyr, pyruvate; Tyr, tyrosine.

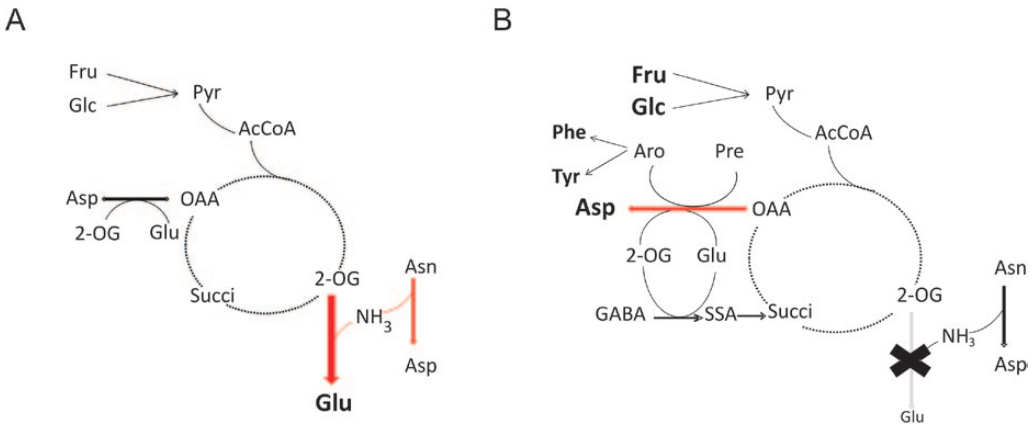

Fig. 5. Proposed metabolic alteration in red tomato fruit by GDH silencing. The main reactions involved in Glu and aspartate metabolism of ripe fruit are shown in WT (A) or amiGDH-all (B) lines. Names in bold indicate the more-abundant metabolites. Red arrows show the preferred direction for the reaction. Metabolite abbreviations used: 2-OG, 2-oxoglutarate; AcCoA, acetyl CoA; Aro, arogenate; GABA, γ-aminobutyrate; OAA, oxaloacetate; Pre, prephenate; SSA, succinic semialdehyde. Others abbreviations are as in Fig. 4.

precursor for the synthesis of tyrosine and phenylalanine (Tzin and Galili, 2010; Maeda and Dudareva, 2012).

As mentioned above, silencing *GDH* resulted in a large increase in the hexose (glucose and fructose) level of ripe amiGDH-all fruit in comparison to the WT fruit (Table 2). Accumulation of glucose and fructose was also reported in roots of the *Arabidopsis gdh1-2–3* mutant, presumably due to a lower rate of glycolysis (Fontaine *et al.*, 2012). Previous studies have used transgenic manipulation of various enzymes to study carbohydrate metabolism in tomato fruit (Beckles *et al.*, 2012). This resulted in fruit with higher or lower sucrose content and decreased hexose content. Specifically, silencing

apoplastic invertase in tomato resulted in reduced levels of glucose and fructose in red fruit (Zanor *et al.*, 2009*b*); and overexpression of hexokinase 1 resulted in fruit with lower levels of hexoses (Menu *et al.*, 2004). In the present study, the higher fructose and glucose content of amiGDH-all ripe fruit did not correlate with soluble invertase activity, which was similar in transgenic and WT fruit (Supplementary Table S1). However, it is possible that a cell wall invertase activity, which was not investigated in our study, is affected and that this alters the hexose level of fruit as suggested in a previous study (Zanor *et al.*, 2009*b*). Apart from the proximal reason for the increase in reducing sugars, it is intriguing that a decrease in *GDH* expression leads to an increase in hexose sugars. As already mentioned, *GDH* is induced during C-starvation (Melo-Olivera *et al.*, 1996; Gibon *et al.*, 2004; Miyashita and Good, 2008). The results presented here point to a reciprocal interaction, in which *GDH* or its products or substrates regulate sugar metabolism.

Previous studies using other tomato mutants showed that Glu accumulation was not affected during tomato fruit ripening in mature *green flesh* fruit (Bortolotti *et al.*, 2003), a tomato mutant which exhibits altered degradation of chloroplast components, indicating that this mechanism is independent of the chloroplast to chromoplast transition. Nevertheless, Glu accumulation was impaired in tomato fruit of the ethylene-independent *FUL1* and *FUL2* mutants, which showed upregulation of all GAD genes (Bemer *et al.*, 2012). A recent study reported that developmental changes in enzyme activities during tomato fruit development were robust across a range of environments, that each developmental stage was characterized by a minimal set of enzyme activities, and that GDH activity increased strongly in tomato fruit before ripening, corresponding with the time when Glu started to accumulate (Biais *et al.* 2014). This is important for understanding how metabolism participates in fruit quality.

Altogether these data suggest that genes involved in Glu synthesis and degradation pathways operate during tomato fruit ripening and modulate the level of free Glu in red tomato fruit.

Supplementary material

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Enzyme activities of Micro-Tom WT and amiGDH-all fruit.

Supplementary Table S2. Correlation values between the PCs and metabolites of red fruit.

Supplementary Figure S1. Glutamate content and GDH activity in the leaves of WT and amiGDH-all lines.

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