Biochemical Mechanism on GABA Accumulation During Fruit Development in Tomato

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A large amount of γ-aminobutyric acid (GABA) was found to accumulate in tomato (Solanum lycopersicum) fruits before the breaker stage. Shortly thereafter, GABA was rapidly catabolized after the breaker stage. We screened the GABA-rich tomato cultivar ‘DG03-9’ which did not show rapid GABA catabolism after the breaker stage. Although GABA hyperaccumulation and rapid catabolism in fruits is well known, the mechanisms are not clearly understood. In order to clarify these mechanisms, we performed comparative studies of ‘Micro-Tom’ and ‘DG03-9’ fruits for the analysis of gene expression levels, protein levels and enzymatic activity levels of GABA biosynthesis- and catabolism-related enzymes. During GABA accumulation, we found positive correlations among GABA contents and expression levels of SlGAD2 and SlGAD3. Both of these genes encode glutamate decarboxylase (GAD) which is a key enzyme of GABA biosynthesis. During GABA catabolism, we found a strong correlation between GABA contents and enzyme activity of α-ketoglutarate-dependent GABA transaminase (GABA-TK). The contents of glutamate and aspartate, which are synthesized from GABA and glutamate, respectively, increased with elevation of GABA-TK enzymatic activity. GABA-TK is the major GABA transaminase form in animals and appears to be a minor form in plants. In ‘DG03-9’ fruits, GAD enzymatic activity was prolonged until the ripening stage, and GABA-TK activity was significantly low. Taken together, our results suggest that GAD and GABA-TK play crucial roles in GABA accumulation and catabolism, respectively, in tomato fruits.

Keywords: GABA — GABA-TK — Glutamate — Micro-Tom.

Abbreviations: AAT, aspartate transaminase; Asp, aspartic acid; CaM, calmodulin; DAF, days after flowering; DIG, digoxigenin; DTT, dithiothreitol; GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GABA-TK, α-ketoglutarate-dependent GABA transaminase; GABA-TP, pyruvate-dependent GABA transaminase; GAD, glutamate decarboxylase; GC–MS, gas chromatography–mass spectrometry; Glu, glutamate; PVDF, polyvinylidene difluoride; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; SSADH, succinic semialdehyde dehydrogenase.

Introduction

γ-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid and is widespread in bacteria, animals and plants. In vertebrates, GABA is known to be a major inhibitory neurotransmitter. GABA has the ability to lower blood pressure in rats (Elliott and Hobbiger 1959, Abe et al. 1995, Aoki et al. 2003, Hayakawa et al. 2004) and humans (Takahashi et al. 1961, Inoue et al. 2003, Kajimoto et al. 2004, Noguchi et al. 2007), and is also capable of reducing stress (Abdou et al. 2006). Therefore, the effects of GABA on human health have been the subject of a substantial amount of attention in food production. Many GABA-containing foods (germinated brown rice, chocolate, wine, etc.) are currently available in the marketplace.

The presence of GABA in plants was first described in potato tubers in 1949 (Steward et al. 1949). To date, the widespread occurrence of GABA has been documented in many plants. GABA is rapidly produced in response to anaerobic conditions (Streeter and Thompson 1972), γ-radiation (Jaarma 1969), low pH (Lane and Stiller 1970), low or high temperatures and darkness, and by mechanical manipulation (Wallace et al. 1984). Various
functions of GABA in plants have been described, including involvement with the regulation of cytosolic pH (Snedden et al. 1995), protection against oxidative stress (Bouché et al. 2003), defense against insects (McLean et al. 2003, MacGregor et al. 2003) and the regulation of pollen tube growth and guidance (Palanivelu et al. 2003). The majority of scientific studies carried out with the aim of deciphering the functional roles of GABA have concentrated on stress-related and signaling roles. In addition to these approaches, multiple roles of GABA functioning as a metabolite have also been clarified (Breitkreuz et al. 1999, Bouché and Fromm 2004, Studart-Guimaraes et al. 2007, Fait et al. 2008). In comparison with other species, GABA is relatively abundant in tomato (Solanum lycopersicum) fruits (Matumoto et al. 1997). GABA and glutamate (Glu) are the most abundant amino acids in tomato fruits (Inaba et al. 1980, Rolin et al. 2000). GABA contents in cv ‘Kyouryoku-toukou’, ‘Cherry tomato’ and ‘Moneymaker’ tomato fruits increased after flowering, reached a maximum level during the mature green stage and rapidly decreased after the breaker stage (Inaba et al. 1980, Rolin et al. 2000, Carrari et al. 2006). Glu levels in tomato fruit increase when the GABA levels decrease (Rolin et al. 2000, Carrari et al. 2006). We have screened the GABA-rich tomato cv ‘DG03-9’, which did not show rapid GABA catabolism after the breaker stage (Inaba et al. 1980, Rolin et al. 2000, Carrari et al. 2006). Glu levels in tomato fruit increase when the GABA levels decrease (Rolin et al. 2000, Carrari et al. 2006). We have screened the GABA-rich tomato cv ‘DG03-9’, which did not show rapid GABA catabolism after the breaker stage, from a total of 61 varieties (Saito et al. 2008). To date, the molecular mechanism of GABA accumulation and catabolism in tomato fruits is not well understood. GABA is synthesized from Glu and this reaction is catalyzed by glutamate decarboxylase (GAD). GABA is catabolized through the GABA shunt in the mitochondria, when it bypasses the first two steps of the tricarboxylic acid cycle. The GABA shunt is composed of three enzymes: (i) GAD (EC:4.1.1.15); (ii) GABA transaminase (GABA-T; EC 2.6.1.19) which converts GABA to succinic semialdehyde; and (iii) succinic semi-aldehyde dehydrogenase (SSADH; EC 1.2.1.16) which catalyzes the oxidation of succinic semi-aldehyde to succinate, which then enters the tricarboxylic acid cycle.

The main purpose of this study was to clarify the mechanism of GABA accumulation and catabolism in tomato fruits during fruit development. We performed a comparative analysis of ‘DG03-9’ (GABA hyperaccumulator) and ‘Micro-Tom’ (control) in terms of GABA and GABA-related metabolites, gene expression, protein and enzymatic activity. Upon completion of this study we have demonstrated that GAD and α-ketoglutarate-dependent GABA transaminase (GABA-TK) might play crucial roles in GABA accumulation and catabolism, respectively, in the tomato fruits.

Results

Data mining, isolation and characterization of genes encoding GAD, GABA-T and SSADH protein, and their respective genomic organization

Although Gallego et al. (1995) isolated tomato ERT D1 (X80840), a gene encoding a putative GAD protein, other GABA shunt-related genes have not been previously isolated. Mining of the tomato expressed sequence tag (EST) database indicated that there are three GADs, three GABA-Ts and one SSADH cDNA available in the database (Table 1). These clones were successfully amplified by reverse transcription–PCR (RT–PCR) with a set of genespecific primer pairs (Supplementary Table S1) according to the available sequence information. Total RNA isolated from ‘Micro-Tom’ fruit at 9 days after flowering (DAF) was used at the template source for amplification of the genes. New accession numbers were assigned to the genes which were isolated from ‘Micro-Tom’ (Table 1).

Since the amino acid sequences of SIGAD1 and ERT D1 are exactly identical (data not shown), it is possible that

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Length (bp)</th>
<th>Amino acids</th>
<th>Mol. wt (KDa)</th>
<th>Accession no. cv. Micro-Tom</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD</td>
<td>SIGAD1</td>
<td>1783</td>
<td>502</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>SIGAD2</td>
<td>1756</td>
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<td></td>
<td>SIGAD3</td>
<td>1606</td>
<td>484</td>
<td>56.7</td>
</tr>
<tr>
<td>GABA-T</td>
<td>SIGA-T1</td>
<td>2005</td>
<td>515</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>SIGA-T2</td>
<td>1586</td>
<td>458</td>
<td>50.5</td>
</tr>
<tr>
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<td>SIGA-T3</td>
<td>1786</td>
<td>520</td>
<td>57.2</td>
</tr>
<tr>
<td>SSADH</td>
<td>SISSADH</td>
<td>1838</td>
<td>522</td>
<td>55.8</td>
</tr>
</tbody>
</table>
SIGAD1 might be an allele of ERT D1. The putative active site of SIGAD protein is strongly conserved (Tanase et al. 1979) and has a putative Ca\(^{2+}\)/calmodulin (CaM)-binding site at its C-terminus (Baum et al. 1993, Arazi et al. 1995) (Supplementary Fig. S1). A comparison of the deduced amino acid sequences of these clones and Arabidopsis AtPOP2 (AT3G22200; GABA transaminase) proteins also showed high homology (Supplementary Fig. S2A, B). SIGABA-T proteins were nearly identical in the consensus pyridoxal phosphate-binding motif (Supplementary Fig. S2A). SISSADH proteins also have strongly conserved putative aldehyde dehydrogenase motifs (Arazi et al. 1995) (Supplementary Fig. S3).

We performed Southern blot analysis in order to estimate the copy numbers of GAD, GABA-T and SSADH genes in the tomato genome. Under highly stringent conditions for hybridization and washing, at least six, three and one band were detected (Supplementary Fig. S4) with GAD, GABA-T and SSADH gene-specific probes (Supplementary Figs. S1, S2 and S3), respectively.

Characterization of GABA and GABA metabolism-related enzymes during the development of tomato fruits

GABA contents in fruits increased after flowering and reached a maximum at 27 DAF (Fig. 1A). Sixty-seven percent of the total amino acid content was GABA in fruits at 27 DAF (data not shown). After 27 DAF, GABA contents in fruits rapidly decreased, as shown in cv ‘Kyouryoku-Toukou’, ‘Cherry tomato’ and ‘Moneymaker’ (Inaba et al. 1980, Rolin et al. 2000, Carrari et al. 2006). GABA contents in fruits at 45 DAF were approximately 1/9 of the GABA contents in the fruits at 27 DAF (Fig. 1A).

The expression properties of GABA shunt genes in ‘Micro-Tom’ developing fruits were analyzed with semi-quantitative RT–PCR using isoform-specific primer pairs. Although three SIGAD genes were expressed in developing fruits (Fig. 1B), they exhibited differential patterns of gene expression during the various stages of fruit development. The changes in SIGAD2 and SIGAD3 gene expression in fruit were in good accordance with the respective changes of GABA contents in fruits (Fig. 1A, B). The expression patterns of SIGAD2 and SIGAD3 and the ethylene-responsive gene (E8) showed opposite patterns of expression during the stages of fruit development. SIGAD1 gene expression increased after the breaker stage and the expression pattern for this gene was not well correlated with GABA contents during fruit ripening. Three SIGABA-T genes and the SISSADH gene were expressed in fruits; however, they also exhibited differential patterns during fruit maturation (Fig. 1B). The changes in SIGABA-T and SISSADH gene expression in fruits were not correlated with GABA contents in fruits (Fig. 1A, B).

Characterization of the GABA-rich variety ‘DG03-9’

Through a preliminary screening of 61 tomato varieties, we identified ‘DG03-9’ as a GABA-rich cultivar (Saito et al. 2008). The GABA content in ‘DG03-9’ fruits at the ripening stage was the highest among all of the 61 tomato varieties. In order to clarify the time-course of changes in GABA contents in ‘DG03-9’ fruits, we measured GABA contents in ‘DG03-9’ fruits. The breaker stage in ‘DG03-9’ fruits occurred at 27–30 DAF. ‘DG03-9’ fruits
showed GABA accumulation prior to the breaker stage. However, ‘DG03-9’ fruits did not show rapid catabolism of GABA subsequent to the breaker stage (Fig. 2A). These results indicate that the higher accumulation of GABA in ‘DG03-9’ is caused by the slow degradation of GABA after the breaker stage of fruit development.

**Comparison of GABA and other GABA-related components in fruits of ‘Micro-Tom’ and the GABA-rich cultivar ‘DG03-9’**

To characterize the ‘DG03-9’ fruits further, we performed gas chromatography–mass spectrometry (GC–MS) analysis as a means of measuring the GABA shunt and tricarboxylic acid cycle components. We utilized this approach to perform a comparative analysis between ‘Micro-Tom’ and ‘DG03-9’ fruits. GC–MS analysis was unable to identify succinic CoA, oxaloacetate and succinic semialdehyde contents. Therefore, succinic semialdehyde contents were measured by the modified GABase method (Streeter and Thompson 1972). Succinic semialdehyde and succinic acid contents were not significantly different between both cultivars before and after the breaker stage. It is well known that GABA and citrate contents in tomato fruits increase after the breaker stage (Rolin et al. 2000). Conversely, malate, Glu and aspartic acid (Asp) contents increase after the breaker stage (Rolin et al. 2000, Roessner-Tunali et al. 2003, Carrari et al. 2006, Carrari and Fernie 2006, Mattoo et al. 2006, Mounet et al. 2007). Interestingly, Asp and citrate contents did not increase significantly after the breaker stage in ‘DG03-9’ fruits. In addition, Glu contents in ‘DG03-9’ did not increase just after the breaker stage. Although malate contents in ‘Micro-Tom’ were approximately 2–3 times higher in ‘DG03-9’, the tendency towards the reduction of malate at the red stage was the same in both cultivars. These results suggest that ‘DG03-9’ has mutations not only in genes that are implicated in GABA degradation but also in genes that are related to the synthesis or degradation of citrate, Glu and Asp after the breaker stage.

**Comparison between ‘Micro-Tom’ and ‘DG03-9’ fruits in gene expression levels and enzymatic activities of GABA shunt enzymes and in GAD protein levels**

We analyzed SgGAD, SgGABA-T and SgSSADH gene expression levels as a means to identify and characterize differences in GABA shunt enzymes between ‘Micro-Tom’ and ‘DG03-9’ fruits. SgGAD2 and SgGAD3 gene expression levels in ‘Micro-Tom’ fruits were dramatically decreased after the breaker stage (Fig. 1A). In sharp contrast, SgGAD2 and SgGAD3 transcripts were detected after the breaker stage in ‘DG03-9’ fruits (Fig. 3A). Similar results were obtained for the levels of GAD protein (Fig. 3B) and its respective enzymatic activity (Fig. 3C). These results clearly indicated that GAD enzymatic activity in ‘DG03-9’ fruits was prolonged after the breaker stage.

mRNA levels of SgGABA-T genes did not correlate with GABA contents in ‘Micro-Tom’ fruits (Fig. 1A, B). Similar results were also obtained with measurements in ‘DG03-9’ fruits (Fig. 4A). In both varieties, the expression properties of GABA-T genes were similar (Fig. 4A), and pyruvate-dependent GABA transaminase (GABA-TP) enzymatic activity did not correlate with GABA contents (Fig. 4B). GABA-TK enzymatic activity in ‘Micro-Tom’ fruits dramatically increased after the breaker stage; however, in contrast, GABA-TK enzymatic activity did not increase in ‘DG03-9’ fruits until after the breaker stage (Fig. 4C). A direct correlation between the increase of GABA-TK enzyme activity and the decrease of GABA contents in ‘Micro-Tom’ fruits was detected after the breaker stage. GABA-TK enzymatic activity was significantly higher (>200 times) than GABA-TP enzymatic activity (Fig. 4B, 4C). In contrast, GABA-TP enzymatic activity in ‘Micro-Tom’ leaves was about five times higher than GABA-TK enzymatic activity (Supplementary Fig. S6). These results indicated that GABA-TK plays an important role in the degradation of GABA in tomato fruits.

**SgSSADH** mRNA levels in ‘Micro-Tom’ fruits at mature green, yellow and red stages were approximately 20% higher than in ‘DG03-9’ fruits (Fig. 4A). However, SSADH enzymatic activities (Fig. 5B) and succinic semialdehyde contents (Fig. 2C) in both varieties were not significantly different.

**Discussion**

In comparison with other plants, tomato accumulates a large amount of GABA during the process of fruit development. In tomato fruits, GABA contents reach a maximum level prior to the breaker stage and decrease rapidly thereafter. However, the process by which tomato can regulate dramatic changes in the GABA contents of fruits is not well known. In order to elucidate the mechanism of regulation of GABA contents in tomato fruits, we performed a comparative characterization between GABA-rich and GABA-standard tomato varieties with respect to changes in gene expression, protein levels and enzymatic activities.

There are two types of GABA-Ts which have been studied in various model systems. GABA-Ts use either α-ketoglutarate or pyruvate as amino acid acceptors in order to produce Glu or alanine (Bouché and Fromm 2004). In mammals, it appears that only GABA-TK is present to produce Glu or alanine. In plants, it appears that only GABA-TK is present in tomato fruits, whereas in plants both GABA-TP and GABA-TK activities have been detected (Streeter and Thompson 1972, Reggiani et al. 1988, 2006, Mattoo et al. 2007). Interestingly, Asp and citrate contents did not increase significantly after the breaker stage. Although malate contents in ‘Micro-Tom’ were approximately 2–3 times higher in ‘DG03-9’, the tendency towards the reduction of malate at the red stage was the same in both cultivars. These results suggest that ‘DG03-9’ has mutations not only in genes that are implicated in GABA degradation but also in genes that are related to the synthesis or degradation of citrate, Glu and Asp after the breaker stage.
Fig. 2  Comparison of GABA and GABA-related components in ‘Micro-Tom’ and ‘DG03-9’ fruits. (A) Time-course changes of GABA contents in ‘Micro-Tom’ (filled box) and ‘DG03-9’ (open box) fruits. The presented values for GABA contents represent the means from three replications. Vertical bars represent the standard deviation. The arrow indicates the day when the breaker stage initiated. (B) Development of ‘Micro-Tom’ and ‘DG03-9’ fruits (scale bar = 1 cm). (C) Comparison of GABA and GABA-related components in ‘Micro-Tom’ and ‘DG03-9’ fruits during the stages of fruit development. The presented values for each component represent the means from three replications. Vertical bars represent the standard error. The level of significance was determined using the Student’s t-test (*P<0.05; **P<0.01). (D) Tricarboxylic acid cycle and GABA shunt pathway. IMG, immature green stage; MG, mature green stage; Yellow stage; Red, red stage. GAD, glutamate decarboxylase; GABA-TK, α-ketoglutarate-dependent GABA aminotransferase; GABA-TP, pyruvate-dependent GABA aminotransferase; SSADH, succinic semialdehyde dehydrogenase; AAT, aspartate transaminase.
Wallace et al. 1984, Shelp et al. 1995, Cauwenberge et al. 2002). In Arabidopsis, only genes encoding GABA-TP have been isolated (Cauwenberge et al. 2002). In an in vitro study, recombinant Arabidopsis GABA-TP used pyruvate but not α-ketoglutarate as an amine donor (Cauwenberge et al. 2002). The GABA contents in flowers of an Arabidopsis GABA-TP-disrupted mutant (pop2) were 100-fold higher than those measured in wild-type plants.

Fig. 3 Comparison of mRNA levels, protein levels and enzymatic activity of GAD in ‘Micro-Tom’ and ‘DG03-9’ fruit. (A) Semi-quantitative RT-PCR analysis of SIGAD gene expression. Total RNA was isolated from tomato fruits and used as the template for synthesis of single-stranded cDNA. Gene-specific amplification was accomplished with PCR isoform-specific primer sets (Supplementary Table S1). In order to increase sensitivity, samples were fractionated by electrophoresis on a 1% agarose gel, transferred to a membrane, and subsequently probed with the same fragments that were labeled with [α-32P]dCTP. The intensity of expression levels was quantified by using the ‘QuantityOne’ software (PDI, Inc.) with reference to the intensity of the band of ‘Micro-Tom’ at the immature green stage (IMG). (B) Western blot analysis of GAD protein levels. Anti-OsGAD1 and OsGAD2 (1:1 mixed) primary antibodies (Akama and Takaiwa 2007) were hybridized with blotted total protein extracts (5 μg) from ‘Micro-Tom’ and ‘DG03-9’ fruits at the IMG, mature green (MG), yellow (Yell) and red (Red) stages. Rice seed proteins (15 μg) at 15 DAF were used as a positive control antigen. (C) Time-course changes of GAD enzymatic activity. Total proteins were extracted from ‘Micro-Tom’ (filled box) and ‘DG03-9’ (open box) fruits at the IMG, MG, Yell and Red stages. The presented values represent the means from three replications. The vertical bars represent the standard deviation.
GABA-TK is crucial for GABA catabolism in tomato fruits

(Palanivelu et al. 2003). GABA-TK activity has also been detected in soybean seeds, rice roots, tobacco leaves, radish mature leaves, tomato leaves and potato tubers (Streeter and Thompson 1972, Reggiani et al. 1988, Wallace et al. 1984, Shelp et al. 1995). In these cases, GABA-TP activities were 1.2–19 times higher than GABA-TK activities. The gene encoding GABA-TP of plants was identified; however, GABA-TK of plants remains to be identified. In this study,
we have detected a significantly higher level of GABA-TK activity in tomato fruits after the breaker stage (Fig. 4C). Since GABA-TK enzymatic activity was also detected in a different tomato variety, cv ‘House Momotaro’ (data not shown), these data demonstrate that high GABA-TK enzymatic activity is common in tomato fruits. The observed increase in GABA-TK enzymatic activity in ‘Micro-Tom’ fruits was well correlated with the reduction of GABA content (Figs. 2A, 4C). GABA-TK enzymatic activity in the GABA-rich cultivar ‘DG03-9’ was very low (Fig. 4C). Collectively, these data indicate that GABA-TK might play a crucial role in the catabolism of GABA in tomato fruits.

GABA contents in tomato fruits were in good agreement with the levels of *SlGAD2* and *SlGAD3* gene expression, GAD protein level and GAD enzymatic activity. In ‘Micro-Tom’ fruits, *SlGAD2* and *SlGAD3* mRNA levels, GAD protein level and GAD enzymatic activity were dramatically reduced after the breaker stage (Fig. 3). In contrast, GAD enzymatic activity was detected in ‘DG03-9’ fruits even after the breaker stage (Fig. 3). GAD enzymatic activity in ‘Micro-Tom’ fruits was regulated in an opposite manner to GABA-TK during different developmental stages. In contrast, GAD enzymatic activity in ‘DG03-9’ fruits did not decrease after the breaker stage and GABA-TK enzymatic activity did not increase after the breaker stage (Fig. 3). These results indicated that maintaining GAD enzymatic activity after the breaker stage resulted in the hyperaccumulation of GABA in ‘DG03-9’ fruits. Taken together, these data support the conclusion that GAD and GABA-TK enzymatic activity play a crucial role in GABA biosynthesis and degradation in tomato fruits.

‘DG03-9’ is the Beta (*B*) mutant which contains a mutation in the locus encoding lycopene β-cyclase (Ronen et al. 2000). The *B* mutant in tomato has the phenotype of orange fruits due to the accumulation of β-carotene (Ronen et al. 2000). Therefore, the color of ‘DG03-9’ fruits remained orange during the maturation stage (Fig. 2B) and the colors of ‘Micro-Tom’ and ‘DG03-9’ fruits during the red stage were significantly different from each other (Fig. 2B). The breaker stage occurred normally in ‘DG03-9’ at 28–34 DAF, and fruit softening also occurred (data not show). These results indicated that the difference between ‘Micro-Tom’ and ‘DG03-9’ in GABA catabolism at the ripening stage was not caused by a difference in the period of fruit development. In a previous study, we evaluated fruit GABA content of seven β-carotene-rich cultivars harboring the *B* locus (Saito et al. 2008). However, the fruit GABA levels of those cultivars were almost similar to or low
compared with other ordinary cultivars, suggesting that the GABA-rich trait of 'DG03-9' is not genetically linked to the B locus.

Increases in Glu and Asp contents after the breaker stage have been well characterized in previous studies (Roline et al. 2000, Roessner-Tunali et al. 2003, Carrari et al. 2006, Carrari and Fernie 2006, Mattoo et al. 2006, Mounet et al. 2007). However, GC–MS analysis indicated that the content of Glu and Asp in 'DG03-9' fruits did not increase after the breaker stage. Asp was synthesized from Glu catalyzed by aspartate transaminase (AAT, EC 2.6.1.1). AAT catalyzes the reversible reaction of transamination between Asp and 2-oxoglutarate to generate Glu and oxaloacetate. AAT plays a key role in the metabolic regulation of carbon and nitrogen metabolism in all organisms (Torre et al. 2006). It is possible that the lower Glu and Asp contents in 'DG03-9' fruits relative to 'Micro-Tom' fruits after the breaker stage may result from lower GABA-TK enzymatic activity in 'DG03-9' fruits. In turn, this would result in decreased Glu accumulation, and eventually a reduction in the accumulation of Asp in 'DG03-9' fruits. To clarify the detailed roles of GABA-TK in GABA catabolism and Glu accumulation in fruits will require further work including the isolation and characterization of gene(s) encoding GABA-TK and the characterization of mutants deficient in this gene.

Materials and Methods

Plant materials and growth conditions

The S. lycopersicum L. cv. Micro-Tom germinated tomato seedlings were grown in a growth chamber with a day/night photoperiod of 16/8 h at 60.5 μmol photons m⁻² s⁻¹ at 25°C. Seedlings were supplied with a standard nutrient solution (Otsuka House Nos. 1 and 2, Otsuka Chemical Co., Osaka Japan). The S. lycopersicum L. cv. 'DG03-9' germinated tomato seedlings were grown in a glass house at the Gene Research Center, the University of Tsukuba, Japan, in May 2007. Developing fruits of 'Micro-Tom' and 'DG03-9' were collected at 12, 24, 36 and 45 DAF. 'Micro-Tom' tomato fruit at 14–18 DAF and 'DG03-9' fruit at 10–14 DAF which were not fully expanded and still green were defined as 'immature green'. 'Micro-Tom' and 'DG03-9' fruit at 22–26 DAF which were fully expanded and green were defined as 'mature green'. 'Micro-Tom' and 'DG03-9' fruit at 27–34 DAF which were fully expanded and green were defined as 'red'. At this stage, there is yellowish plaque on the 'DG03-9' pericarp. 'Micro-Tom' fruit at 42–45 DAF and 'DG03-9' fruit at 43–47 DAF which were fully expanded were defined as 'red'. At this stage, the 'DG03-9' pericarp was orange.

**Extraction and measurement of GABA and succinic semialdehyde contents**

A 50 mg aliquot of fresh fruits was homogenized in 8% (w/v) trichloroacetic acid with a Tissuelyzer (Qiagen, Valencia, CA, USA) at 2,000 r.p.m. for 1 min at room temperature. Samples were then centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was transferred to a new tube and 400 μl of pure diethyl ether was added and vigorously mixed for 10 min. Samples were centrifuged again at 10,000 × g for 20 min at 4°C. The supernatant from this centrifugation step was removed and then vigorously mixed for 10 min and centrifuged at 10,000 r.p.m. for 10 min at 4°C. The supernatant from this centrifugation step was removed and left to stand under a draft of air for 30 min for complete evaporation of ether. The GABA assay for GABA was performed using the method described by Streeter and Thompson (1972).
16 h at 45°C. Hybridization, stringency washes and detection were performed following the instructions outlined in the DIG DNA labeling kit (Roche Diagnostics, Vienna, Austria), with a final wash in 1× SSC, 1% SDS at 60°C. Membranes were then exposed to X-ray film for 5–180 min.

**Expression analysis of GABA shunt enzyme genes by semi-quantitative RT–PCR**

Semi-quantitative RT–PCR was carried out essentially as previously described by Akihiro et al. (2005, 2006). Total RNAs were extracted from tomato plants using the RNeasy Maxi kit (Qiagen, Valencia, CA, USA) and were digested with DNase I (NipponGene, Chiyodaku, Tokyo, Japan) according to the manufacturer’s instructions. The quality of total RNAs was evaluated by the Agilent 2100 bioanalyzer (Agilent, Santa Clara, California, USA). Since the RNA integrity number (Schroeder et al. 2006) of all samples exceeded 7.9, these RNAs were subsequently used for cDNA synthesis. A 15 µg aliquot of total RNAs was used to synthesize single-stranded cDNA using the First strand cDNA synthesis kit (TAKARA SHUZO CO. LTD., Otsu, Otsu, Shiga, Japan) according to the manufacturer’s instructions. RT–PCR was performed semi-quantitatively with sets of gene-specific primers (Supplementary Table S1). For PCR amplification, the cDNA was denatured at 94°C for 2 min in the first cycle, and then for 1 min in subsequent cycles. Primer annealing and extension reactions were carried out at 50°C (SIGAD2 and SISR1), 59°C (SISDH), 60°C (SIGAD3, SIGABA-T2 and SIGABA-T3), 66°C (SIGAD1 and SIGABA-T3) or 72°C for 30 s each. The cycle numbers for SIGAD1, SIGAD2, SIGAD3, SIGABA-T1, SIGABA-T2, SIGABA-T3, SSAD, SSSR1, SSSR2 and SIE8 were: 20, 20, 18, 26, 23, 26, 18, 20, and 20, respectively. The PCR products were fractionated on a 1% (w/v) agarose gel, transferred onto a Hybond N+ membrane (GE Healthcare, Buckinghamshire, UK) and probed with [α-32P]dCTP-labeled DNA (Muromachi Chemicals Inc., Nerima, Tokyo, Japan) using the Bea Best labeling kit (TAKARA SHUZO CO. LTD., Otsu, Shiga, Japan) according to the manufacturer’s instructions.

**GC–MS analysis**

Tomato fruits (50 mg) were homogenized in liquid nitrogen using a mortar and pestle. Each sample was dissolved with methanol and chloroform (250 µl each). After 225 µl of 0.295 mM ribitol solution was added to the samples as an internal standard, they were vigorously mixed. These extracts were centrifuged at 15,000 rpm for 10 min at room temperature, and 200 µl of the supernatant was filtered using Amicon Ultrafree-MC (Millipore, Danvers, Massachusetts, USA). A part of the flow-through fraction (80 µl) was evaporated to dryness using a centrifuge evaporator (EYELA centrifugal evaporator CVE-3100, EYELA, Chuo, Tokyo, Japan). For methylation, 40 µl of methoxylamine hydrochloride (20 mg ml−1 pyridine) was added to the samples and incubated for 90 min at 37°C. Trimethylsilylation was performed by addition of 50 µl of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) solution for 30 min at 37°C. A GC 6890 (Agilent Technologies, Santa Clara, California, USA) was operated under electronic pressure control and equipped with a split/splitless capillary inlet. A 1 µl aliquot of each sample was injected in the splitless mode with the injection temperature set to 250°C. A 30 m DB-17ms column (J&W Scientific, Santa Clara, California, 0.25 mm ID, 0.25 µm film thickness) was used. Helium was used as a flow gas at 1.0 ml min−1. Separation was achieved with a temperature program of 70°C for 5 min, then ramped at 15°C min−1 to 310°C. The transfer line to the mass spectrometer was set to 200°C. The time of flight mass spectrometer was a Pegasus III MS system (Leco, Michigan, USA) with an electron impact ionization source set to 250°C. Mass spectra were monitored with an acquisition rate of 20 spectra s⁻¹ in the mass range m/z = 82–500. The contents of most metabolites were quantified with the standard calibration curves for each compound. The peak area ratios of standard compounds at various concentrations to the internal standard (ribitol) were used for the calibration curves.

**Enzyme extraction and assays of GABA shunt-related enzymes**

A 50 g aliquot of fresh tomato fruit was homogenized with a Waring blender in a 5-fold volume of ice-cold extraction buffer [0.1 M Tris–HCl pH 7.0, 10 mM dithiothreitol (DTT), 5 mM EDTA, 1 mM pyridoxal-5-phosphate and 1% (w/v) insoluble polyvinylpyrrolidone] (Rolin et al. 2000) for measurement of GAD and GABA-TK activities, the homogenates were centrifuged at 10,000×g for 10 min at 4°C, and the pellet was discarded. The extract was desalted using Sephadex G-50 (GE Healthcare, UK) that was previously equilibrated in the extraction buffer. A 500 µl aliquot of effluent was added to microconcentrators (Millipore, Danvers, Massachusetts, USA) and reduced in volume to 100 µl (hereafter, this solution is referred as the ‘crude protein’).

The GAD activity was measured as glutamate-dependent GABA production (as described by Akama et al 2007). A 100 µl aliquot of the crude protein was used for the assay of GAD activity in a 500 µl reaction mixture [100 mM Bis-Tris–HCl (pH 7.0), 0.5 mM pyridoxal-5-phosphate, 1 mM DTT, 5 mM Glu, 0.1 mM bovine CaM (Sigma-Aldrich, Missouri) and 0.5 mM CaCl2]. GABA contents were determined enzymatically using a commercial ‘GABase’ preparation (Sigma-Aldrich, Missouri, USA) (Jakoby 1962).

For measurement of GABA-TP and SSADH activity the protein extraction and concentration were carried out using the above-mentioned method. However, GABA-TP and SSADH enzymatic activities were quite low or undetectable. Therefore, the amount of starting material and the methods of protein concentration were changed as follows: 50 g samples of fresh tomato fruits were homogenized with a Waring blender in a 5-fold volume of ice-cold extraction buffer. The homogenate was centrifuged at 10,000×g for 10 min at 4°C, and the pellet was discarded. Ammonium sulfate was added [final concentration of 60% (v/v)] to the supernatant. The sample was subsequently mixed for 30 min at 4°C and then centrifuged at 10,000×g for 30 min at 4°C. The pellet was dissolved in 1 ml of the extraction buffer. The buffer of the solution was changed to SSADH reaction buffer [100 mM sodium pyrophosphate buffer (pH 9.0), 14 mM 2-mercaptoethanol, 0.5 mM NAD, 0.5 mM succinic semialdehyde (modified from Shelp et al. (1995)], which is the same as the GABA-TK reaction buffer or the GABA-TP reaction buffer [100 mM Tris–HCl buffer (pH 9.0), 20 µM pyridoxal-5-phosphate, 2 mM GABA and 10 mM z-ketoglutarate or 10 mM pyruvate (Shelp et al. 1995)], with Sephadex G-50 (GE Healthcare, Buckinghamshire, UK) which had been equilibrated with new buffer.

The GABA-TK activity was measured as GABA-dependent Glu production (Shelp et al. 1995). The buffer-exchanged solution was incubated at 37°C for 12 h and then boiled for 3 min. Glu contents were measured using the t-glutamate assay kit (Yamasa Co., Chyosho, Chiba, Japan) (Kusakabe et al. 1983) according to the manufacturer’s instructions. GABA-TK activity was measured as for GABA-dependent succinic semialdehyde production.
(Shelp et al. 1995). SSADH activity was measured as succinic semialdehyde-dependent succinate production (Beutler 1989). The succinate content was measured using the succinate assay kit (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions.

**Western blot analysis**

A 15μg aliquot of tomato fruit proteins was separated by SDS–PAGE on polyacrylamide gels [10–20% (w/v) gradient gel]; e-PAGE (Atto Co., Bunkyo, Tokyo, Japan). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, GE Healthcare, Buckinghamshire, UK). The membrane was initially blocked for 1 h at room temperature with 5% Blocking One-P solution (Nakarai Tesque, Inc., Chyu-kyo, Kyoto, Japan) in TBS-T [10 mM Tris-buffered saline with 0.05% (v/w) Tween-20]. The membrane was then incubated for 1 h at 4°C with the primary antibody. Subsequent to rinsing with TBS-T, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Roche Diagnostics, Vienna, Austria) diluted 1:2,000. The immunoblots were developed using chemiluminescence (ChemilumiOne; Nakarai Tesque, Inc., Chyu-kyo, Kyoto, Japan) and visualized with the aid of a digital imaging system (LAS-1000; Fujifilm, Minatoku, Tokyo, Japan). The primary antibodies that were used in the study were mouse anti-rice OsGAD1 and OsGAD2 (Akama and Takaiwa 2007).

**Supplementary material**

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

**Funding**

The Research and Development Program for New Bio-industry Initiatives (BRAIN).

**Acknowledgments**

The authors thank Mr. Syuji Inai of Nippon Del Monte Corporation for providing the ‘DG03-9’ seeds. We also thank Dr. Christophe Rothan (INRA), Ms. Izumi Oshima, Mr. Kenji Higuchi, Mr. Noriyuki Hirooka and all laboratory members for help, advice and discussion. ‘Micro-Tom’ seeds (ID: TOMJP00001) were provided by the Gene Research Center, for help, advice and discussion. ‘Micro-Tom’ seeds (ID: TOMJP00001) were provided by the Gene Research Center, for help, advice and discussion. ‘Micro-Tom’ seeds (ID: TOMJP00001) were provided by the Gene Research Center, for help, advice and discussion.

**References**


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(Rceived May 17, 2008; Accepted August 6, 2008)