Tomatoes accumulate γ-aminobutyric acid (GABA) at high levels in the immature fruits. GABA is rapidly converted to succinate during fruit ripening through the activities of GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH). Although three genes encoding GABA-T and both pyruvate- and α-ketoglutarate-dependent GABA-T activities have been detected in tomato fruits, the mechanism underlying the GABA-T-mediated conversion of GABA has not been fully understood. In this work, we conducted loss-of-function analyses utilizing RNA interference (RNAi) transgenic plants with suppressed pyruvate- and glyoxylate-dependent GABA-T gene expression to clarify which GABA-T isoforms are essential for its function. The RNAi plants with suppressed SgGABA-T gene expression, particularly SgGABA-T1, showed severe dwarfism and infertility. SgGABA-T1 expression was inversely associated with GABA levels in the fruit at the red ripe stage. The GABA contents in 35S::SgGABA-T1RNAi lines were 1.3–2.0 times and 6.8–9.2 times higher in mature green and red ripe fruits, respectively, than the contents in wild-type fruits. In addition, SgGABA-T1 expression was strongly suppressed in the GABA-accumulating lines. These results indicate that pyruvate- and glyoxylate-dependent GABA-T is the essential isoform for GABA metabolism in tomato plants and that GABA-T1 primarily contributes to GABA reduction in the ripening fruits.

Keywords: Dwarfism • Fruit • GABA • GABA-TP/TG • Infertility • Tomato.

Introduction

γ-Aminobutyric acid (GABA) is a four-carbon non-protein amino acid commonly found in bacteria, animals and plants. GABA is a major inhibitory neurotransmitter in vertebrates (Zhang and Jackson 1993) and has been identified as a functional component in reducing blood pressure in the human body (Takahashi et al. 1961, Inoue et al. 2003, Kajimoto et al. 2004). GABA is metabolized via a short pathway, called the ‘GABA shunt’ (Fig. 1), which is a bypass of the tricarboxylic acid (TCA) cycle composed of three enzymes (Shelp et al. 1999, Bouché and Fromm 2004). In this shunt, GABA is irreversibly synthesized from glutamate through glutamate decarboxylase (GAD) (Chung et al. 1992, Ling et al. 1994) and reversibly converted to succinic semialdehyde (SSA) through GABA transaminase (GABA-T) (Shelp et al. 1999, Van Cauwenbergh et al. 2002). SSA is subsequently reversibly reduced through succinate semialdehyde dehydrogenase (SSADH) and eventually flows back into the TCA cycle.

In plants, GABA was first described in potato tubers in 1949 (Steward et al. 1949). Early studies showed that various environmental or non-environmental stresses, such as drought, UV irradiation, mechanical damage, low temperature and low O2, promote GABA accumulation (Shelp et al. 1999, Snedden and Fromm 1999, Kinnersley and Turano 2000). Furthermore, GABA has been reported to function in the regulation of cytosolic pH (Bown and Shelp 1997), pollen tube growth (Palanivelu et al. 2003), the expression of nitrate transporter (Beuve et al. 2004) and cell elongation (Renault et al. 2011).
There have been many reports describing GABA synthesis, including the regulation and function of GAD, in various plant species (Chen et al. 1994, Rolin et al. 2000, Bouché et al. 2004, Akama et al. 2009). In contrast, in plants, knowledge of the conversion of GABA to succinate remains limited. Two isoforms of the GABA-T enzyme have been reported: pyruvate-dependent GABA-T (GABA-T) and α-ketoglutarate-dependent GABA-T (GABA-TK) (Bouché and Fromm 2004). These enzymes utilize pyruvate or α-ketoglutarate as amino acid acceptors to produce alanine or glutamate, respectively (Bouché and Fromm 2004). GABA-TP also has glyoxylate-dependent GABA-T (GABA-TG) activity, and utilizes glyoxylate as an amino acid acceptor to produce glycine (Clark et al. 2009a, Clark et al. 2009b). However, although the activities of both enzymes have been detected in plants, such as soybean, tobacco, potato and tomato (Satya-Narayan and Nair 1986, Clark et al. 2009b). However, although the activities of both enzymes have been detected in plants, such as soybean, tobacco, potato and tomato (Satya-Narayan and Nair 1986, Clark et al. 2009b).

The tomato accumulates high levels of GABA in its fruits (Matsumoto et al. 1997), reaching a maximum level at the mature green (MG) stage and rapidly decreasing after the breaker stage (Inaba et al. 1980, Rolin et al. 2000, Carrari and Fernie 2006). In a previous study, we reported a role for GABA in climacteric respiration during fruit ripening, and its reduction ratio during ripening was important for GABA levels in fruit (Akihira et al. 2008, Saito et al. 2008, Yin et al. 2010). However, although GABA-TK activities were much higher than those of GABA-TP in ripening fruits, we could only isolate pyruvate- and glyoxylate-dependent GABA-T (GABA-TP/TG) genes, including SIGABA-T1, SIGABA-T2 and SIGABA-T3 (Akihira et al. 2008). Clark et al. (2009b) showed that these proteins exhibited enzymatic activities and localized to the mitochondrion (SIGABA-T1), cytosol (SIGABA-T2) and plastid (SIGABA-T3). These results suggest that GABA-TP/TGs are major isoforms, even in ripening tomato fruits. Therefore, the aim of this study was to determine which GABA-T isoform is responsible for the conversion of GABA to SSA in fruit using RNA interference (RNAi) transgenic plants with suppressed GABA-TP/TG gene expression (SIGABA-TRNAi). The results show that SIGABA-T1 primarily contributes to the GABA conversion in ripening fruit. In addition, systemic GABA-T suppression caused GABA accumulation and affected vegetative and reproductive growth in tomato plants. These findings shed light on the physiological roles of GABA-T in the tomato.

![Fig. 1 GABA shunt metabolic pathway. GDH, glutamate dehydrogenase; GAD, glutamate decarboxylase; GABA-TK, α-ketoglutarate-dependent GABA transaminase; GABA-T, 2 and 3, pyruvate- and glyoxylate-dependent GABA transaminases; SSADH, succinate semialdehyde dehydrogenase; SSR, succinate semialdehyde reductase. The pathways presented by dotted lines indicate predicted pathways based on the localization of each enzyme reported in Arabidopsis (Hoover et al. 2007, Simpson et al. 2008).](./image)

**Results**

The expression of SIGABA-T genes and GABA contents in the leaves and stems of 35S::SIGABA-TRNAi transgenic lines

First, to evaluate suppression of SIGABA-T gene expression through RNAi, quantitative reverse transcription–PCR (qRT–PCR) was performed using leaves (10 weeks after transplanting) from wild-type (WT) and 35S promoter-driven SIGABA-TRNAi (35S::SIGABA-TRNAi) lines (Fig. 2). The expression levels in each line were calculated relative to that of the SIGABA-T1 gene in the WT, which was established as 100%. In WT leaves, the relative expression levels of SIGABA-T2 and SIGABA-T3 were 2.1% and 169.4% compared with SIGABA-T1 in the WT, respectively (Supplementary Fig. S3). The expression of SIGABA-T1 and SIGABA-T3 genes in SIGABA-T1RNAi lines was mostly suppressed compared with that in the WT, except for SIGABA-T1RNAi line No. 10 (Fig. 2A). The expression levels of the RNAi-targeted genes in the SIGABA-T1RNAi lines were 30.6% (line No. 1), 20.7% (line No. 2), 5.1% (line No. 23) and 3.9% (line No. 28). SIGABA-T2 expression in SIGABA-T1RNAi was decreased in lines No. 2 and 10; however, expression was increased in lines No. 23 and 28. In the SIGABA-T2RNAi and SIGABA-T3RNAi lines, the expression levels of SIGABA-T2 and SIGABA-T3 genes were suppressed in all of the tested lines (Fig. 2B, C). The relative expression levels of the RNAi-targeted genes were 0.02% (line No. 21), 0.003% (line No. 22), 0.01% (line No. 42), 0.32% (line No. 48) and 0.52% (line No. 57) in SIGABA-T2RNAi lines, and 10.2% (line No. 2), 0.2% (line No. 5), 30.2% (line No. 10), 4.7% (line No. 20) and 48.9% (line No. 21) in SIGABA-T3RNAi lines. The SIGABA-T1 expression was decreased in SIGABA-T2RNAi lines No. 22 and 42; however, the expression was similar or higher compared with that of the WT plants in other lines. The GABA content was dramatically increased in 35S::SIGABA-T1RNAi lines No. 1 and 23, which corresponded to a 13.3- to 15.9-fold increase, respectively, over that in wild-type plants. The GABA content was dramatically increased in 35S::SIGABA-T1RNAi lines No. 1 and 23, which corresponded to a 13.3- to 15.9-fold increase, respectively, over that in wild-type plants.
WT plants. The GABA content in 35S::SlGABA-T1 RNAi line No. 2 and 35S::SlGABA-T2 RNAi line No. 22 was 7.3–7.7 times higher than that in the WT plants. The GABA content in the SlGABA-T2 RNAi and SlGABA-T3 RNAi lines, except for SlGABA-T2 RNAi line No. 22, was 2.7- to 3.7-fold higher than that in the WT. Glutamate content in leaves was also measured in the WT and the SlGABA-T RNAi lines (Fig. 3B). The leaf glutamate levels were significantly lower in the 35S::SlGABA-T RNAi lines than that in the WT, except for SlGABA-T1 RNAi line No. 1. The content in 35S::SlGABA-T1 RNAi line No. 2 and 35S::SlGABA-T3 RNAi line No. 10 corresponded to 52.3% and 52.5% compared with the WT, respectively. In the other 35S::SlGABA-T RNAi lines, it was suppressed to 20.5–32.5% compared with the WT.

The GABA and glutamate contents in the stems were measured in the WT and 35S::SlGABA-T RNAi lines (Fig. 3A, B). The GABA content in the stems of the 35S::SlGABA-T1 RNAi lines No. 1, 2 and 23 corresponded to a 10.1-, 8.0- and 11.7-fold increase, respectively, over that in WT plants (Fig. 3A). However, the stem GABA content in SlGABA-T2 RNAi and SlGABA-T3 RNAi lines remained at the level of 1.3–3.0 times compared with that in the WT. The stem glutamate levels were almost similar in the WT and all 35S::SlGABA-T RNAi lines, except for 35S::SlGABA-T3 RNAi lines No. 5 and 21 (Fig. 3B).

**Vegetative growth and flower/fruit setting in the 35S::SlGABA-T RNAi lines**

The vegetative growth of the 10-week-old 35S::SlGABA-T RNAi lines was also evaluated (Fig. 4). As shown in Figs. 4 and 5, transgenic plants with suppressed SlGABA-T gene expression showed severe dwarfish and infertility. The plant heights were 59.0% (No. 1), 41.8% (No. 2), 61.2% (No. 10), 68.7% (No. 23) and 49.3% (No. 28) suppressed in SlGABA-T1 RNAi lines and 61.2% (No. 2), 43.3% (No. 5), 73.1% (No. 10), 61.9% (No. 20) and 71.6%
(No. 21) in SIGABA-T3RNAi lines compared with the WT plants (Fig. 4D). However, there was no clear effect on the plant heights in the SIGABA-T2RNAi lines, although line No. 42 exhibited a level of dwarfism similar to that of the SIGABA-T1RNAi and SIGABA-T3RNAi lines.

Although there were no visible morphological changes in the flowers in the transgenic plants, a marked flower abscission was observed in the SIGABA-T1RNAi lines (Fig. 5B). The fruit-setting ratio in total flowering was decreased in most of the SIGABA-T1RNAi lines compared with that of the WT, by 27.5% in line No. 1 and 40.4% in line No. 28. Line No. 23 exhibited severe infertility and did not set any fruit (Fig. 5E). To confirm infertility in SIGABA-T1RNAi plants (Supplementary Fig. S5), additional tests were performed with SIGABA-T1RNAi plants (Supplementary Fig. S5). The suppression of SIGABA-T1 gene expression was consistent with the decreased fruit-setting ratio in the additionally tested SIGABA-T1RNAi lines (Supplementary Fig. S5A, B). The fruit-setting ratio was positively correlated with the SIGABA-T1 mRNA levels in SIGABA-T1RNAi plants (Supplementary Fig. S5C). Flowering in the SIGABA-T2RNAi and SIGABA-T3RNAi lines was similar to that in the WT (Fig. 5A, C, D). Although the fruit-setting ratio was slightly or moderately decreased in lines No. 22 and 42 in SIGABA-T2RNAi and lines No. 5 and 21 in SIGABA-T3RNAi, no correlation was observed between the fruit-setting ratio and the expression levels of SIGABA-T genes in both lines (Figs. 2, 5E).

**GABA contents and expression of SIGABA-T genes in the fruit of SIGABA-T RNAi lines**

To determine which isoform is important for GABA metabolism in ripening fruit, the GABA contents and gene expression of SIGABA-T genes were analyzed in the RNAi
lines (Figs. 6–10). The GABA content in 35S::SlGABA-T1RNAi lines No. 2 and 28 and 35S::SlGABA-T2RNAi line No. 22 reached 11.5–18.1 μmol g FW⁻¹ at the MG stage, 12.3–19.9 μmol g FW⁻¹ at the yellow (Yell) stage and 10.3–14.0 μmol g FW⁻¹ at the red (Red) stage, which correspond to 1.3- to 2.0-fold higher than the WT in the MG fruits, 2.0- to 3.3-fold higher in the Yell fruits and 6.8- to 9.2-fold higher in the Red fruits, respectively (Fig. 6). In addition, the reduction of the GABA ratio during ripening was changed in the GABA-accumulating lines. When the GABA content at the MG stage was set as 100%, the ratio was 68.3% at the Yell stage and 16.9% at the Red stage in the WT fruits. In contrast, the ratios were 109.9, 91.9 and 107.3% at the Yell stage and 77.1, 74.4 and 89.8% at the Red stage in 35S::SlGABA-T1RNAi lines No. 2 and 28 and 35S::SlGABA-T2RNAi line No. 22, respectively. However, the GABA contents in the fruits of other RNAi lines were similar or lower than that in the WT. Although the GABA ratios at the Yell stage were different (37.6–83.1%) among these lines, the ratios at the Red stage were 16.4–43.1%, which was reduced compared with that in GABA-accumulating lines. The expression of SlGABA-T1 was strongly suppressed in the GABA-accumulating lines, at 3.6% and 4.7% in 35S::SlGABA-T1RNAi lines No. 2 and No. 28, and 4.7% in 35S::SlGABA-T2RNAi line No. 22 compared with that of the WT (Fig. 7A–C). However, the expression of the SlGABA-T1 gene in other lines was not changed, and the GABA content was not increased in those lines (Figs. 6, 7A–C). The expression of SlGABA-T2 was significantly suppressed in most of the tested lines except 35S::SlGABA-T2RNAi line No. 57 and 35S::SlGABA-T3RNAi line No. 10 (Fig. 7A–C). The expression of SlGABA-T3...
was suppressed in 35S::SIGABA-T1 RNAi line No. 28 and 35S::SIGABA-T3 RNAi lines No. 2 and 5. The expression of RNAi-targeted genes was effectively suppressed in 35S::SIGABA-T2 RNAi line No. 21 and 35S::SIGABA-T3 RNAi lines No. 2 and 5, which corresponds to 14.0, 1.6 and 1.7 % compared with those of the WT, respectively. However, these lines did not show an increase in GABA accumulation (Fig. 6).

To avoid the negative effects of the systemic suppression of SIGABA-T genes through the 35S promoter (see Figs. 4 and 5), we also generated SIGABA-T RNAi lines through the E8 promoter (E8::SIGABA-T RNAi), which is a strong inducible promoter specific to ripening tomato fruit (Deikman et al. 1998). Unlike the 35S::SIGABA-T RNAi lines, the E8::SIGABA-T RNAi lines showed a similar phenotype to WT plants and did not show dwarfism or infertility (data not shown). The fruit GABA content at the MG stage in E8::SIGABA-T RNAi lines was 6.9–8.5 μmol g FW⁻¹, a similar level to that observed in the WT plants (Fig. 8). However, the fruit GABA content rapidly dropped to approximately 2.2 μmol g FW⁻¹ at the Red stage in the WT, E8::SIGABA-T2 RNAi and E8::SIGABA-T3 RNAi lines, and

---

**Fig. 5** Flowering and fruit setting in 35S::SIGABA-T RNAi lines. (A) WT, (B) SIGABA-T1 RNAi, (C) SIGABA-T2 RNAi and (D) SIGABA-T3 RNAi. Scale bars = 1 cm. (E) The fruit-setting ratio in the WT and 35S::SIGABA-T RNAi lines. The labels below the horizontal axis indicate the genotypes of the transgenic lines.

**Fig. 6** The GABA contents in the fruits of 35S::SIGABA-T RNAi lines. The open, shaded and filled columns indicate the MG, Yell and Red stages, respectively. The labels below the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3). The level of significance compared with the WT at each stage was determined using Student’s t-test (*P < 0.05, **P < 0.01).
remained between 4.4 and 5.8 μmol g FW⁻¹ in the E8::SlGABA-T1 RNAi lines, which was approximately 2.5-fold higher than in the WT and other RNAi lines (Fig. 8). In WT fruits, the GABA ratio was 50.2% at the Yell stage and 27.6% at the Red stage compared with the GABA content at the MG stage. However, the ratios were 97.5, 87.3 and 69.3% at the Yell stage and 67.2, 68.7 and 64.6% at the Red stage in E8::SlGABA-T1 RNAi lines No. 1, 8 and 27, respectively. In the GABA-accumulating lines, SlGABA-T1 expression was suppressed (Fig. 9A), and its relative values were 5.1% (line No. 1), 6.1% (line No. 8) and 6.8% (line No. 27) compared with that in the WT plants. The suppression of the SlGABA-T1 gene was only observed in those lines. Although the expression of SlGABA-T2 and SlGABA-T3 was suppressed in some lines (No. 1 and 8 of E8::SlGABA-T1 RNAi, No. 5 and 39 of E8::SlGABA-T2 RNAi, and No. 7, 18 and 57 of E8::SlGABA-T3 RNAi), there was no correlation between GABA accumulation and gene suppression (Figs. 8, 9A–C).

Finally, correlations between the GABA contents and the mRNA levels of the SlGABA-T genes in SlGABA-T RNAi lines were analyzed (Fig. 10). The expression of SlGABA-T1 was clearly correlated with the fruit GABA contents in the 35S::SlGABA-T RNAi lines (Fig. 10A). In the E8::SlGABA-T RNAi lines, although the coefficient of determination was lower than that in 35S::SlGABA-T RNAi, a correlation between SlGABA-T1 expression and the fruit GABA content was observed. In contrast, there were no correlations between the SlGABA-T2 and SlGABA-T3 expression in the 35S::SlGABA-T RNAi and E8::SlGABA-T RNAi lines (Fig. 10B, C).

**Amino acid contents in WT and 35S::SlGABA-T RNAi fruits**

The profiles of major and minor amino acids in WT and 35S::SlGABA-T RNAi fruits are shown in Table 1 and Supplementary Table S2, respectively. In WT fruit, GABA and glutamine accumulated at the MG stage and decreased...
after the breaker stage (Table 1). In contrast, aspartate contents in the WT increased after the breaker stage. In the GABA-accumulating 3SS::SIGABA-T1 RNAi lines such as No. 2 and 28, and 3SS::SIGABA-T2 RNAi line No. 22 (Fig. 6), the GABA ratio in the total amino acids reached 57.7, 54.3 and 36.9% at the MG stage, respectively (Table 1). Those values did not decrease rapidly even after the breaker stage. On the other hand, the aspartate ratio in the GABA-accumulating lines was lower than that in the WT even after the breaker stage. There was a negative correlation between GABA and aspartate. Although GABA is converted to alanine and glycine by GABA-T reaction, alanine and glycine contents were not associated with GABA contents (Table 1).

Whereas the total amino acid content in the WT decreased at the Yell stage compared with that of the MG stage, it increased again at the Red stage (Table 1). The total amino acid and total protein amino acid levels in the MG stage of 3SS::SIGABA-T2 RNAi lines No. 21 and 57 and 3SS::SIGABA-T3 RNAi line No. 10 were lower than those of the WT. GABA contents in these lines were also lower than that in the WT. Total amino acids in the Red stage were almost constant in the WT and all 3SS::SIGABA-T RNAi lines, except for 3SS::SIGABA-T3 RNAi lines No. 2 and 10.

### Discussion

**The suppression of SIGABA-T gene expression induced the alteration of phenotypes**

To clarify the physiological function of SIGABA-T genes, we conducted loss-of-function analyses utilizing RNAi transgenic lines with suppressed SIGABA-T gene expression. The transgenic plants showed severe abnormal phenotypes, such as dwarfism and infertility. The plant height in the 3SS::SIGABA-T1 RNAi and SIGABA-T3 RNAi lines was half or less than that in the WT plants (Fig. 4A, C, D). On the other hand, no remarkable changes were observed in the SIGABA-T2 RNAi lines except for line No. 42, in which the expression of SIGABA-T1 and SIGABA-T3 was also suppressed (Fig. 2B). Actually, the trigger sequence of each SIGABA-T RNAi was designed in the region lying astride between the 5′-untranslated region (UTR) and the open reading frame (ORF). However, the targeting region for SIGABA-T2 RNAi was mostly included in the ORF region and shared a high similarity to the other two genes because there is no signal peptide in the N-terminal region (Supplementary Table S1). This would cause the unexpected suppression of the other isoforms in 3SS::SIGABA-T2 RNAi lines.

The abnormal phenotypes have also been reported in transgenic tobacco lines overexpressing the C-terminal-truncated GAD gene, which overaccumulated GABA (Baum et al. 1996, Akama and Takaaki 2007). In those transgenic plants, a decrease of glutamate associated with the GABA increase was observed. In this study, although a similar tendency was observed in leaves of 3SS::SIGABA-T RNAi lines in the glutamate and GABA contents, there was no quantitative correlation to the dwarfism (Figs. 3, 4). In addition, whereas the GABA content was increased, the glutamate content was almost constant in the stem of 3SS::SIGABA-T RNAi lines compared with that in the WT (Fig. 3). Renault et al. (2011) reported that excessive GABA accumulation negatively affected cell elongation in the hypocotyl through the down-regulation of cell wall-related gene expression, such as the genes encoding arabinogalactan, expansin and tonoplast intrinsic proteins. The vertical cell size of stem cortex tissue in the 3SS::SIGABA-T RNAi and 3SS::SIGABA-T3 RNAi lines was obviously smaller than that of the WT, whereas it was similar in the 3SS::SIGABA-T RNAi line (Supplementary Fig. S4). This tendency was consistent with the results regarding plant heights (Fig. 4), indicating that the dwarf phenotype observed in the present study also results from defects in cell elongation, and SIGABA-T2 would not be involved in this event. However, the plant heights were similar between 3SS::SIGABA-T RNAi and 3SS::SIGABA-T3 RNAi individuals, although the GABA contents in 3SS::SIGABA-T3 RNAi leaves were much lower than those in 3SS::SIGABA-T RNAi plants (Figs. 3, 4D), suggesting that other factors are involved in the dwarf phenotype.

A severe abscission of flowers was observed in the 3SS::SIGABA-T RNAi lines (Fig. 5B). Therefore, we compared the fruit-setting ratios between the WT and RNAi transgenic plants (Fig. 5). The fruit-setting ratios in 3SS::SIGABA-T RNAi lines No. 1, 23 and 28 were markedly decreased compared with that of the WT (Fig. 5E). The lower fruit-setting ratio and positive correlation with the SIGABA-T1 mRNA levels were also confirmed in additionally tested SIGABA-T RNAi lines.
Indeed, the subcellular localization was previously shown to be different for each SlGABA-T protein, with SlGABA-T1 localized in the mitochondria (Clark et al. 2009b). However, these phenomena were not observed in E8::SlGABA-T1 RNAi plants in the present study (data not shown). The E8 promoter is a fruit ripening-specific promoter in tomato, and it does not control gene expression in flowers (He et al. 2008). The inhibition of pollen tube growth and a reduction of seed fertility have been reported in a GABA-TP/TG knockdown mutant/transgenic Arabidopsis plant, which also showed that GABA-TP/TG is localized in the mitochondria (Palanivelu et al. 2003, Mirabella et al. 2007, Clark et al. 2009a, Renault et al. 2011). These results indicate that impairment of mitochondrial-localized GABA-T1 would cause aberrant GABA accumulation in the cytosol and result in aberrant plant development.

**Change of amino acid contents in tomato fruits in SlGABA-T RNAi lines**

GABA is the most abundant amino acid in tomato fruits at the MG stage, and the content is related to that of the total amino acids by the Yell stage (Rolin et al. 2000, Akihiro et al. 2008). In the present study, in the GABA-accumulating 35S::SlGABA-T1 RNAi lines No. 2 and 28 and 35S::SlGABA-T2 RNAi line No. 22 (Fig. 6), the GABA ratio in the total amino acids reached 57.7, 54.3 and 36.9%, respectively, at the MG stage (Table 1), and the GABA content did not decrease rapidly, even after the breaker stage. However, the aspartate ratio in the GABA-accumulating lines was lower than that in the WT after the breaker stage (Table 1). Accumulation of glutamate and asparatate after the breaker stage in tomato fruits has been reported in previous studies (Rolin et al. 2000, Roessner-Tunali et al. 2003, Mattoo et al. 2006, Mounet et al. 2007). In this study, there was a negative correlation between the accumulation of...
GABA and aspartate at the Red stage (Table 1). However, the total amino acids accumulated in the Red stage were almost identical between the WT and the GABA-accumulating lines. The reduced aspartate content has been reported in the GABA-rich tomato cultivar ‘DG03-9’ (Akihiro et al. 2008, Saito et al. 2008). These results suggest that both GABA and aspartate are synthesized from glutamate, and the accumulation of GABA after the ripening stage prevents aspartate accumulation.

GABA was converted to alanine and glycine by GABA-T reaction (Clark et al. 2009a, Clark et al. 2009b). In this study, alanine and glycine contents in the GABA-accumulating lines were not changed compared with those of the WT (Table 1). Because the absolute values of these amino acids were lower in the fruits of the WT, they would be rapidly converted to other amino acids in tomato fruit. However, at the Yell stage, all of the GABA, glutamine and total amino acid levels in the WT were decreased compared with the MG stage. Our previous work showed that GABA is converted to organic acids during ripening (Yin et al. 2010). However, it has not been fully understood what those amino acids are converted to during the Yell stage. It would be interesting to perform metabolome analyses focusing on the primary metabolites utilizing the SIGABA-T<sup>RNAi</sup> lines.

The isoform responsible for GABA conversion in tomato plants

It has been accepted that GABA is catabolized in the mitochondria (Bouché and Fromm 2004). However, Clark et al. (2009b) reported three, GABA-T<sub>1</sub>–GABA-T<sub>3</sub>, enzymes in tomato that were localized in the mitochondrion, cytosol and plastid, with each isoform predicted to have unique functions.

In the present study, the leaf GABA contents in all 35S::SIGABA-T<sup>RNAi</sup> lines were higher than that in the WT plants (Fig. 3). The expression of SIGABA-T<sub>1</sub> was decreased in SIGABA-T<sub>1</sub><sup>RNAi</sup> plants and SIGABA-T<sub>2</sub><sup>RNAi</sup> lines No. 22 and
metabolism in tomato fruits. Unexpectedly, GABA-TP/TG indicate that enzymatic activity of SlGABA-T1 is highest among the three Figs. 8–10. In these lines, the expression of SlGABA-T1 accumulation was not observed in other SlGABA-T2 genes was suppressed (Fig. 6). A clear correlation between fruit GABA contents in SlGABA-T3 expression level in the Fig. 2A–C). For example, the 35S::SlGABA-T3RNAi lines showed severe dwarfism (Fig. 4). GABA reduction through 35S::SlGABA-T2 and SlGABA-T3 was observed in the tomato leaves (Figs. 2, 3), but it was not observed in the fruits (Figs. 6, 7, 10). The expression of SlGABA-T1 was correlated with the fruit-setting ratio and GABA accumulation in tomato fruits (Fig. 10A; Supplementary Fig. S5). Thus, SlGABA-T1 is probably the predominant isofrom in tomato flowers and fruits. These results also show that the three GABA-T genes cooperatively function during the vegetative phase, and GABA reduction occurs through SlGABA-T1 in the reproductive phase.

| Table 1 Amino acid contents (μmol g FW−1) in WT and 35S::SlGABA-T3RNAi fruits |
|---------------------------------|---------------------|---------------------|---------------------|
| WT                             | 35S::SlGABA-T1RNAi | 35S::SlGABA-T2RNAi | 35S::SlGABA-T3RNAi |
| Asp                            | 1.01 ± 0.05        | 0.40 ± 0.03         | 0.88 ± 0.02         |
| Asn                            | 3.03 ± 0.18        | 0.74 ± 0.03         | 3.44 ± 0.15         |
| Glu                            | 1.20 ± 0.12        | 0.63 ± 0.05         | 1.36 ± 0.06         |
| Gin                            | 5.49 ± 0.27        | 1.53 ± 0.07         | 7.60 ± 0.35         |
| Glic                           | 0.14 ± 0.01        | 0.05 ± 0.00         | 0.11 ± 0.00         |
| Ala                            | 0.28 ± 0.03        | 0.16 ± 0.01         | 0.20 ± 0.00         |
| GABA                           | 7.75 ± 1.12        | 5.34 ± 0.38         | 5.98 ± 0.04         |
| Total                          | 35S::SlGABA-T1RNAi | 35S::SlGABA-T2RNAi | 35S::SlGABA-T3RNAi |
| Red                            | 15.82 ± 0.80       | 12.41 ± 0.95        | 24.13 ± 0.89        |
| Asp                            | 5.29 ± 0.11        | 1.55 ± 0.02         | 3.35 ± 0.13         |
| Asn                            | 1.22 ± 0.08        | 0.79 ± 0.01         | 2.50 ± 0.11         |
| Glu                            | 0.75 ± 0.03        | 0.56 ± 0.02         | 0.96 ± 0.06         |
| Gin                            | 3.04 ± 0.21        | 1.37 ± 0.14         | 5.81 ± 0.33         |
| Glic                           | 0.60 ± 0.01        | 0.04 ± 0.00         | 0.07 ± 0.00         |
| Ala                            | 0.20 ± 0.00        | 0.16 ± 0.00         | 0.20 ± 0.01         |
| GABA                           | 5.68 ± 0.59        | 4.20 ± 0.07         | 1.91 ± 0.06         |
| Total                          | 35S::SlGABA-T1RNAi | 35S::SlGABA-T2RNAi | 35S::SlGABA-T3RNAi |
| Yell                           | 33.66 ± 1.31       | 4.30 ± 0.22         | 15.27 ± 0.57        |
| Asp                            | 1.65 ± 0.52        | 1.55 ± 0.06         | 7.92 ± 0.65         |
| Asn                            | 1.48 ± 0.25        | 1.39 ± 0.11         | 2.76 ± 0.87         |
| Glu                            | 0.88 ± 0.04        | 1.01 ± 0.02         | 1.30 ± 0.10         |
| Gin                            | 4.62 ± 0.25        | 2.57 ± 0.16         | 1.25 ± 0.15         |
| Glic                           | 0.14 ± 0.00        | 0.07 ± 0.00         | 0.02 ± 0.00         |
| Ala                            | 0.27 ± 0.01        | 0.02 ± 0.00         | 0.03 ± 0.01         |
| GABA                           | 35S::SlGABA-T1RNAi | 11.43 ± 0.31        | 35S::SlGABA-T2RNAi |
| Total                          | 10.69 ± 0.23       | 5.15 ± 0.04         | 35S::SlGABA-T3RNAi |
| Total                          | 13.66 ± 1.31       | 5.15 ± 0.04         | 13.99 ± 0.66        |

The labels above the horizontal axis indicate the genotypes of the transgenic lines. The values indicate the mean and standard deviation (n = 3).
In previous work, we reported that SlGABA-T1 was expressed in fruit at all developmental stages whereas SlGABA-T2 and SlGABA-T3 expression was decreased at the Red stage (Akihiro et al. 2008). However, Clark et al. (2009b) reported that the expression levels of all three GABA-T genes were low at the MG stage, then only SlGABA-T1 expression significantly increased after the breaker stage. In the present study, the accumulation of GABA in 35S::SlGABA-T1RNAi lines was observed not only at the Yell and Red stages, but also at the MG stage (Fig. 6). The reduction ratio of GABA in those lines during ripening was also lower than that in the WT, although SIGADs were already down-regulated after the breaker stage (Akihiro et al. 2008). Those results suggest that the conversion of GABA by SlGABA-T1 has proceeded at least to the MG stage and increases during ripening. The expression pattern of SlGABA-T2 and SlGABA-T3 was not correlated with GABA accumulation during fruit developmental stages (Akihiro et al. 2008). In the present study, from the point of view of transcription levels, an essential role for SlGABA-T1, but not SlGABA-T2 and SlGABA-T3, in the fruit GABA level was demonstrated through the loss-of-function analyses (Fig. 6, 7, 10). On the other hand, the expression level of SlGABA-T3 was higher than that of SlGABA-T1 in WT leaves (Supplementary Fig. S3). It is likely that SlGABA-T3, as well as SlGABA-T1, is involved in the regulation of the GABA level in leaf and stem tissues.

**GABA-TP/TG plays an important role for GABA metabolism in tomato fruits**

In our previous work, we reported a negative correlation between GABA contents and GABA-TK activity in tomato fruits through a comparison between ordinary and GABA-rich cultivars (Akihiro et al. 2008). A recent study suggested that decreased GABA-TK activity causes GABA accumulation in tomatoes stored under low O2 conditions (Mae et al. 2012). However, Clark et al. (2009b) reported that all three SlGABA-T-encoded proteins showed only GABA-TP/TG activities and pointed out the possibility that the above research had detected artificial GABA-TK activity. Deewatthanawong et al. (2010) suggested that higher GABA concentrations in CO2-treated fruits were due to a decreased GABA-TP activity. In the present study, we demonstrated that SlGABA-T1 is important for GABA metabolism in the tomato fruit. Although we previously reported the importance of GABA-TK in the tomato fruit, the gene encoding this protein has not yet been identified in tomato. Therefore, based on the loss-of-function experiments performed in the present study, we now conclude that GABA-TP/TG is an essential factor for GABA metabolism in tomato plants.

In contrast to SlGABA-T1, the physiological functions of SlGABA-T2 and SlGABA-T3 remain unclear. In the tomato and other species, the possibility of an alternative pathway for the breakdown of SSA via γ-hydroxy butyric acid (GHB) production has been reported (Clark et al. 2009b). Although GABA-derived SSA is primarily reduced through SSADH activity in the mitochondria, SSA is also converted to GHB through the activity of succinate semialdehyde reductase (SSR) (Bouché and Fromm 2004). In Arabidopsis, glyoxylate reductase, which is identical to SSR (Shelp et al. 2012), is localized in the cytosol and plastids (Hoover et al. 2007, Simpson et al. 2008). Two SISSR genes have been isolated in the tomato (Akihiro et al. 2008); however, the localization of these genes has not been analyzed. If SISSR1 and SISSR2 are localized to the cytosol and plastids, SlGABA-T2 and SlGABA-T3 participate in an additional route for SSA metabolism (Fig. 1). However, further characterization of the RNAi transgenic plants will be required to clarify SSA metabolism.

In the present study, we successfully generated GABA-overaccumulating tomato plants through the suppression of GABA-T genes and demonstrated that SlGABA-T1 is the most essential isoform for GABA metabolism in tomato fruits. The results of this study will be available for screening GABA-rich mutants, which will be an excellent bioresource for breeding a new GABA-rich tomato cultivar.

**Materials and Methods**

**Plant materials and growth conditions**

The tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom was used in this study. Germinated seedlings were transplanted into rockwool and grown in a culture room at 25 °C under 16 h light/8 h dark conditions. For SlGABA-T1RNAi lines, the shoots derived from calii were transplanted. The plants were fed a standard nutrient solution (Otsuka House. No. 1 and 2, Otsuka Chemical Co.). The plant height measurements and leaf samples were obtained at 10 weeks after transplantation. The fruit-setting ratio was calculated from the number of total fruits set and total flowering. The fruits were sampled at 24–27 days after flowering (DAF), 28–33 DAF and 42–45 DAF to obtain three development stages: MG, Yell and Red, respectively. In this study, only the T0 generation plants were analyzed because SlGABA-T suppression caused severe infertility in transgenic plants.

**Vector construction and transformation**

The RNAi constructs used to suppress the mRNA expression of each SlGABA-T gene were created under the control of the constitutive Cauliflower mosaic virus (CaMV) 35S promoter or the fruit-specific E8 promoter (Supplementary Fig. S1). To create RNAi constructs targeted towards SlGABA-T1 suppression, the RNAi-targeted region of SlGABA-T1 was amplified using gene-specific primers (shown in Supplementary Table S1). The RNAi-targeted region of about 300 bp was designed at the 5′ side of the SlGABA-T gene and contained a UTR and an ORF (Supplementary Fig. S2). The PCR fragment was directly cloned into the entry vector pCR8/GW/TOPO (Invitrogen) and transferred into the Gateway vector pBl sense-antisense GW (Invitrogen) using the Gateway LR Clonase enzyme (Invitrogen). This construct was...
designated SIGABA-T\(^{T}\)\(^{RNAI}\). The same strategy was used to create RNAi constructs for the suppression of other SIGABA-T genes using specific primers (shown in Supplementary Table S1). To create RNAi constructs under the control of the E8 promotor, this region (accession No. AF15784) was amplified using specific primers containing Bln1 and Xho1 sites. The fragment was cloned in place of the CaMV 35S promotor in the pBI sense-antisense GW vector. Subsequent procedures were performed using this same strategy. These constructs were then transformed into Agrobacterium tumefaciens GV2260 using the electroporation method. The constructs were transformed into WT ‘Micro-Tom’ using the Agrobacterium method (Sun et al. 2006). The transgenic plants were selected on Murashige and Skoog (MS) agar plates containing kanamycin (100 mg l\(^{-1}\)).

### Extraction and measurement of GABA and amino acid contents

Approximately 50 mg of fresh sample was homogenized in liquid nitrogen using a mortar and pestle, and, subsequently, 500 l of 8% (w/v) trichloroacetic acid was added. The samples were centrifuged at 10,000 \(\times\) g for 20 min at 4°C. The supernatant was transferred into a fresh tube, 400 l of pure diethyl ether was added and the tube was mixed vigorously for 10 min. The samples were centrifuged again at 10,000 \(\times\) g for 10 min at 4°C. The supernatant was removed, and 400 l of diethyl ether was added. The samples were mixed vigorously for 10 min and centrifuged at 10,000 \(\times\) g for 10 min at 4°C. The supernatant was removed, and 400 l of diethyl ether was added. The samples were mixed vigorously for 10 min and centrifuged at 10,000 \(\times\) g for 10 min at 4°C. The supernatant from this centrifugation step was removed and incubated under a draft of air for 30 min for the complete evaporation of diethyl ether. The samples for amino acid analysis were evaporated using an evaporator the complete evaporation of diethyl ether. The samples for amino acid analysis were evaporated using an evaporator and incubated under a draft of air for 30 min for the complete evaporation of diethyl ether. The samples for amino acid analysis were evaporated using an evaporator (CVE3100, TOKYO RIKAKIKAI), and 300 l of water was added. This procedure was repeated twice. The samples were dissolved in 0.1 N HCl for the amino acid analysis (JLC-500/V2, Japan Electron Optics Laboratory). The GBase’ assay for GABA was performed using the method described by Jakoby (1962) with slight modifications. In the ‘GABase’ assay for GABA was performed using the method described by Jakoby (1962) with slight modifications. In the ‘GABase’ assay, the reduction of NADP to NADPH was monitored spectrophotometrically at 340 nm, pH 8.6 at 37°C, as a function of time using GABA as a substrate.

### Quantitative expression analysis

Total RNAs were extracted from tomato plants using the RNeasy Plant Mini kit (Qiagen) and digested using DNase I (NipponGene) according to the manufacturer’s instructions. Approximately 1 \(\mu\)g of total RNA was used to synthesize single-stranded cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen). The mRNA expression of each SIGABA-T gene was analyzed using qRT–PCR. The qRT–PCR experiments were performed using a Takara Thermal Cycler Dice Real-Time System with SYBR Premix Ex Taq II (TAKARA). The qRT–PCR was performed with gene-specific primers (Supplementary Table S1). For the PCR amplification, the cDNA was denatured at 94°C for 30 s in the first cycle, followed by 45 cycles of denaturing for 5 s, primer annealing at 55°C for 10 s and extension at 72°C for 15 s. The mRNA levels of each SIGABA-T were determined relative to the control ubiquitin (UBQ) (accession No. X58253) mRNA according to the methods of Kim et al. (2010).

### Histological analysis

The plants grown from cuttings of the T0 generation were used for histological analysis. Longitudinal sections of stem tissue were obtained by hand-cutting with a razor blade. The sections were immediately stained with 0.1% toluidine blue for 15 min and then rinsed with distilled water. The samples were mounted on a slide glass and observed by an optical microscope (BX53, OLYMPUS).

### GABA-T enzymatic assay

GABA-T enzymatic assay was performed according to the procedure described by Clark et al. (2009a, 2009b). A 5 g aliquot of fresh tomato fruit obtained from T0 35S::SIGABA-T\(^{T}\)\(^{RNAI}\) plants was homogenized with a mortar and pestle in a 5-fold volume of ice-cold extraction buffer [50 mM Tris–HCl (pH 8.2), 3 mM dithiothreitol (DTT), 1.25 mM EDTA, 2.5 mM MgCl\(_2\), 10% (v/v) glycerol, 6 mM CHAPS, 1 mM phenylmethyl-sulfonl fluoride, 2.5 \(\mu\)g ml\(^{-1}\) leupeptin and pepstatin A, 2% (w/v) polyvinylpyrrolidone and 2 \(\mu\)g ml\(^{-1}\) pyridoxal-5-phosphate]. The homogenates were centrifuged at 10,000 \(\times\) g for 15 min at 4°C, and the pellet was discarded. The supernatant was concentrated using Amicon ultra-4 (10 kDa, Millipore). The extract was desalted using PD-10 columns (GE Healthcare) that were equilibrated in the extraction buffer before use. GABA-TP and GABA-TG activities were measured as GABA-dependent alanine and glycine production, respectively. For the assay, 100 l of the crude protein was used in the total 500 l reaction mixture [50 mM N-Tris(hydroxymethyl)methyl-4-amino butanesulfonic acid (TABS, pH 9.0), 1.5 mM DTT, 0.625 mM EDTA, 0.1 mM pyridoxal-5-phosphate, 10% (v/v) glycerol, 1 mM GABA and 1 mM pyruvate or glyoxylate]. The reaction solution was incubated at 30°C for 6 h and then terminated by the addition of ice-cold sulfosalicylic acid to a final concentration of 60 mM (Van Cauwenberge and Shelp 1999). The supernatant was neutralized with NaOH, and the resultant alanine and glycine were measured by the HPLC amino acid analyzer (JLC-500/V2, JEOL).

### Funding

This work was supported by the Japan Society for the Promotion of Science (JSPS) [a Grant-in-Aid for Fellows (No. 225336); the ‘Japan–France Joint Laboratory Project’; the ‘Japan–France Joint Laboratory Project’; the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; the ‘Research and Development Program for New Bio-industry Initiatives (BRAIN)’.
Acknowledgments

The authors would like to thank all laboratory members for their help, advice and discussions. ‘Micro-Tom’ seeds (accession No.TOMIPF00001) were obtained from the Gene Research Center, University of Tsukuba, through the National Bio-Resource Project (NBRP) of MEXT, Japan.

References


Akama, K. and Takaiwa, F. (2007) C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in plant cells. J. Exp. Bot. 58: 2699–2707.


