Suppression of \( \gamma \)-Aminobutyric Acid (GABA) Transaminases Induces Prominent GABA Accumulation, Dwarfism and Infertility in the Tomato (\textit{Solanum lycopersicum} L.)

Satoshi Koike\textsuperscript{1,2}, Chiaki Matsukura\textsuperscript{1}, Mariko Takayama\textsuperscript{1}, Erika Asamizu\textsuperscript{1} and Hiroshi Ezura\textsuperscript{1,*}

\textsuperscript{1}Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki, 305-8572 Japan
\textsuperscript{2}Research Fellow of the Japan Society for the Promotion of Science

\*Corresponding author: E-mail, ezura@gene.tsukuba.ac.jp; Fax, +81-29-853-7734.

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Tomatoes accumulate \( \gamma \)-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 \( \alpha \)-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 \textit{SlGABA-T} gene expression, particularly \textit{SlGABA-T1}, showed severe dwarfism and infertility. \textit{SlGABA-T1} expression was inversely associated with GABA levels in the fruit at the red ripe stage. The GABA contents in \textit{35S::SlGABA-T1} RNAi 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, \textit{SlGABA-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.

Abbreviations: CaMV, \textit{Cauliflower mosaic virus}; DAF, days after flowering; DTT, dithiothreitol; GABA, \( \gamma \)-aminobutyric acid; GABA-T, GABA transaminase; GABA-TK, \( \alpha \)-ketoglutarate-dependent GABA transaminase; GABA-TP, pyruvate-dependent GABA transaminase; GABA-TG, glyoxylate-dependent GABA transaminase; GABA-TP/TG, pyruvate- and glyoxylate-dependent GABA transaminase; GAD, glutamate decarboxylase; GHB, \( \gamma \)-hydroxy butyric acid; MG, mature green; ORF, open reading frame; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; SSA, succinic semialdehyde; SSADH, succinate semialdehyde dehydrogenase; SSR, succinate semialdehyde reductase; TCA, tricarboxylic acid; UTR, untranslated region; WT, wild type; Yell, yellow.

Introduction

\( \gamma \)-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, Bouche´ 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 irreversibly 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 O\textsubscript{2}, 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-TP) and \( \alpha \)-ketoglutarate-dependent GABA-T (GABA-TK) (Bouché and Fromm 2004). These enzymes utilize pyruvate or \( \alpha \)-ketoglutarate as amino acid acceptors to produce alanine or glutamate, respectively (Bouché and Fromm 2004). GABA-TP also has 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).

![Fig. 1 GABA shunt metabolic pathway. GDH, glutamate dehydrogenase; GAD, glutamate decarboxylase; GABA-TK, \( \alpha \)-ketoglutarate-dependent GABA transaminase; GABA-T1, 2 and 3, pyruvate- and glyoxylate-dependent GABA transaminases; SSADH, succinate semialdehyde dehydrogenase; SSR, succinate semialdehyde reductase.](image)

**Results**

The expression of SIGABA-T genes and GABA contents in the leaves and stems of 35S::SIGABA-T\(^{\text{RNAi}}\) 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-T\(^{\text{RNAi}}\) (35S::SIGABA-T\(^{\text{RNAi}}\)) 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-T1\(^{\text{RNAi}}\) lines was mostly suppressed compared with that in the WT, except for SIGABA-T1\(^{\text{RNAi}}\) line No. 10 (Fig. 2A). The expression levels of the RNAi-targeted genes in the SIGABA-T1\(^{\text{RNAi}}\) 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-T1\(^{\text{RNAi}}\) was decreased in lines No. 2 and 10; however, expression was increased in lines No. 23 and 28. In the SIGABA-T2\(^{\text{RNAi}}\) and SIGABA-T3\(^{\text{RNAi}}\) 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-T2\(^{\text{RNAi}}\) 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-T3\(^{\text{RNAi}}\) lines. The SIGABA-T1 expression was decreased in SIGABA-T2\(^{\text{RNAi}}\) 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 measured in the leaves of the WT and 35S::SIGABA-T\(^{\text{RNAi}}\) lines (Fig. 3A). The GABA content in the leaves of the 35S::SIGABA-T\(^{\text{RNAi}}\) lines was higher than that in the WT plants. The GABA content was dramatically increased in 35S::SIGABA-T\(^{\text{RNAi}}\) lines No. 1 and 23, which corresponded to a 13.3- to 15.9-fold increase, respectively, over that in

(Akihiro 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-T\(^{\text{RNAi}}\)). 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.
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 35S::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.

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 dwarfism 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 35S::SlGABA-T RNAi lines and 61.2% (No. 2), 43.3% (No. 5), 73.1% (No. 10), 61.9% (No. 20) and 71.6%
However, there was no clear effect on the plant heights in the SlGABA-T2 RNAi lines, although line No. 42 exhibited a level of dwarfism similar to that of the SlGABA-T1 RNAi and SlGABA-T3 RNAi lines. Although there were no visible morphological changes in the flowers in the transgenic plants, a marked flower abscission was observed in the SlGABA-T1 RNAi lines (Fig. 5B). The fruit-setting ratio in total flowering was decreased in most of the SlGABA-T1 RNAi 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 SlGABA-T1 RNAi, additional tests were performed with SlGABA-T1 RNAi plants (Supplementary Fig. S5). The suppression of SlGABA-T1 gene expression was consistent with the decreased fruit-setting ratio in the additionally tested SlGABA-T1 RNAi lines (Supplementary Fig. S5A, B). The fruit-setting ratio was positively correlated with the SlGABA-T1 mRNA levels in SlGABA-T1 RNAi plants (Supplementary Fig. S5C). Flowering in the SlGABA-T2 RNAi and SlGABA-T3 RNAi 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 SlGABA-T2 RNAi and lines No. 5 and 21 in SlGABA-T3 RNAi, no correlation was observed between the fruit-setting ratio and the expression levels of SlGABA-T genes in both lines (Figs. 2, 5E).

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

To determine which isoform is important for GABA metabolism in ripening fruit, the GABA contents and gene expression of SlGABA-T genes were analyzed in the RNAi
The GABA content in 35S::SlGABA-T1 RNAi lines No. 2 and 28 and 35S::SlGABA-T2 RNAi 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-T1 RNAi lines No. 2 and 28 and 35S::SlGABA-T2 RNAi 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-T1 RNAi lines No. 2 and No. 28, and 4.7% in 35S::SlGABA-T2 RNAi 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-T2 RNAi line No. 57 and 35S::SlGABA-T3 RNAi line No. 10 (Fig. 7A–C). The expression of SlGABA-T3
was suppressed in 35S::SlGABA-T1 RNAi line No. 28 and 35S::SlGABA-T3 RNAi lines No. 2 and 5. The expression of RNAi-targeted genes was effectively suppressed in 35S::SlGABA-T2 RNAi line No. 21 and 35S::SlGABA-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 SlGABA-T genes through the 35S promoter (see Figs. 4 and 5), we also generated SlGABA-T RNAi lines through the E8 promoter (E8::SlGABA-T RNAi), which is a strong inducible promoter specific to ripening tomato fruit (Deikman et al. 1998). Unlike the 35S::SlGABA-T1 RNAi lines, the E8::SlGABA-T1 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::SlGABA-T1 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::SlGABA-T2 RNAi and E8::SlGABA-T3 RNAi lines, and

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

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**Fig. 6** The GABA contents in the fruits of 35S::SlGABA-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 35S::SlGABA-T1 RNAi lines such as 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% 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 aspartate. 

Table 1 contains the amino acid contents in the WT and RNAi lines. In all cases, the GABA contents were almost constant in the WT, whereas they were lower or much lower in the RNAi lines. The GABA contents in the RNAi lines were lower than those of the WT. GABA contents in the Red stage were much lower than those in the WT. Total amino acids in the Red stage were almost constant in the WT and all 35S::SlGABA-T RNAi lines, except for 35S::SlGABA-T3 RNAi lines No. 2 and 10.

Discussion

The suppression of SlGABA-T gene expression induced the alteration of phenotypes

To clarify the physiological function of SlGABA-T genes, we conducted loss-of-function analyses utilizing RNAi transgenic lines with suppressed SlGABA-T gene expression. The transgenic plants showed severe abnormal phenotypes, such as dwarfism and infertility. The plant height in the 35S::SlGABA-T1 RNAi and SlGABA-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 SlGABA-T2 RNAi lines except for line No. 42, in which the expression of SlGABA-T1 and SlGABA-T3 was also suppressed (Fig. 2B). Actually, the trigger sequence of each SlGABA-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 SlGABA-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 35S::SlGABA-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 Takaiwa 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 35S::SlGABA-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 35S::SlGABA-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 35S::SlGABA-T RNAi and 35S::SlGABA-T3 RNAi lines was obviously smaller than that of the WT, whereas it was similar in the 35S::SlGABA-T2 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 SlGABA-T2 would not be involved in this event. However, the plant heights were similar between 35S::SlGABA-T1 RNAi and 35S::SlGABA-T3 RNAi individuals, although the GABA contents in 35S::SlGABA-T3 RNAi leaves were much lower than those in 35S::SlGABA-T1 RNAi plants (Figs. 3, 4D), suggesting that other factors are involved in the dwarf phenotype.

A severe abscission of flowers was observed in the 35S::SlGABA-T1 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 35S::SlGABA-T1 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 SlGABA-T1 mRNA levels were also confirmed in additionally tested SlGABA-T1 RNAi lines.
plants (Supplementary Fig. S5). 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 Ye 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 aspartate 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-TRNAI 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-T1–GABA-T3, 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-TTRNAI lines were higher than that in the WT plants (Fig. 3). The expression of SIGABA-T1 was decreased in SIGABA-T1TRNAI plants and SIGABA-T2TRNAI lines No. 22 and
42, but not in 35S::SlGABA-T1RNAi line No. 10 (Fig. 2A–C). However, SlGABA-T1 expression was similar or higher compared with that of the WT plants in other lines. In those lines, the expression of SlGABA-T2 and SlGABA-T3 genes was suppressed (Fig. 2B, C). These results suggest that SlGABA-T2 or SlGABA-T3 is involved in GABA metabolism in tomato leaves. In tomato fruits, the GABA contents in 35S::SlGABA-T1RNAi lines No. 2 and 28 and 35S::SlGABA-T2RNAi line No. 22 were 1.3–2.0 times higher in MG fruit and 6.8–9.2 times higher in Red fruit, respectively, compared with those of the WT (Fig. 6). In these lines, the expression of SlGABA-T1 and SlGABA-T2 genes was suppressed (Fig. 7A, B). However, excessive GABA accumulation was not observed in other SlGABA-T2 and SlGABA-T3 suppression lines (Fig. 6). A clear correlation between fruit GABA contents and the SlGABA-T1 expression level was observed (Fig. 10A), whereas there was almost no correlation with SlGABA-T2 and SlGABA-T3 genes (Fig. 10B, C). The same results were obtained through the analyses of G8 promoter-driven transgenic lines (Figs. 8–10). Indeed, the enzymatic activity of SlGABA-T1 is highest among the three isoforms in the tomato (Clark et al. 2009b). These results clearly indicate that SlGABA-T1 is primarily responsible for GABA metabolism in tomato fruits. Unexpectedly, GABA-TP/TG activities in fruit were not significantly different between the 35S::SlGABA-T1RNAi lines and the WT (Supplementary Fig. S6B), although fruit GABA contents in the RNAi lines clearly increased (Supplementary Fig. S6A). Because the enzyme assay was performed utilizing crude protein extracted from fruits, the GABA-TP/TG activity corresponding to each isoform could not be separately evaluated. Considering the increase in the SlGABA-T3 expression level in the 35S::SlGABA-T1RNAi line No. 2 (Fig. 7), these results would be the outcome of masking by other isoforms.

A previous study demonstrated that GABA-TP/TG was present in the cytosol and plastids (Clark et al. 2009b). Although the physiological functions of these genes are unclear, our results show that these genes function in vivo (Figs. 2, 3). For example, the 35S::SlGABA-T3RNAi lines showed severe dwarfism (Fig. 4). GABA reduction through 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 isoform in tomato flowers and fruits. These results suggest 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-T1RNAi fruits

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>2</th>
<th>10</th>
<th>28</th>
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<tr>
<td>Asp</td>
<td>1.01±0.05</td>
<td>1.26±0.12</td>
<td>1.09±0.04</td>
<td>0.93±0.05</td>
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<td>2.12±0.20</td>
<td>2.71±0.17</td>
<td>1.74±0.11</td>
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<tr>
<td>Gln</td>
<td>1.20±0.12</td>
<td>1.10±0.11</td>
<td>1.06±0.04</td>
<td>0.95±0.06</td>
</tr>
<tr>
<td>Gly</td>
<td>0.54±0.27</td>
<td>3.22±0.65</td>
<td>3.61±0.21</td>
<td>4.06±0.34</td>
</tr>
<tr>
<td>Ala</td>
<td>0.14±0.01</td>
<td>0.14±0.00</td>
<td>0.08±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>GABA</td>
<td>0.28±0.03</td>
<td>0.22±0.02</td>
<td>0.29±0.03</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>Total</td>
<td>13.36±0.71</td>
<td>12.66±1.31</td>
<td>10.65±0.53</td>
<td>10.52±0.70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>35S::SlGABA-T1RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>40.03±0.03</td>
</tr>
<tr>
<td>10</td>
<td>0.74±0.03</td>
</tr>
<tr>
<td>28</td>
<td>0.63±0.05</td>
</tr>
<tr>
<td>Total</td>
<td>3.54±0.15</td>
</tr>
</tbody>
</table>

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 SIGABA-T1 was expressed in fruit at all developmental stages whereas SIGABA-T2 and SIGABA-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 SIGABA-T1 expression significantly increased after the breaker stage. In the present study, the accumulation of GABA in 35S::SIGABA-T1 RNAi 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 SIGABA-T1 has proceeded at least to the MG stage and increases during ripening. The expression pattern of SIGABA-T2 and SIGABA-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 SIGABA-T1, but not SIGABA-T2 and SIGABA-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 SIGABA-T3 was higher than that of SIGABA-T1 in WT leaves (Supplementary Fig. S3). It is likely that SIGABA-T3, as well as SIGABA-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 SIGABA-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 SIGABA-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 SIGABA-T1, the physiological functions of SIGABA-T2 and SIGABA-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 SISRR genes have been isolated in the tomato (Akihiro et al. 2008); however, the localization of these genes has not been analyzed. If SISRR1 and SISRR2 are localized to the cytosol and plastids, SIGABA-T2 and SIGABA-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 SIGABA-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 SIGABA-T RNAi lines, the shoots derived from calli 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 SIGABA-T suppression caused severe infertility in transgenic plants.

**Vector construction and transformation**

The RNAi constructs used to suppress the mRNA expression of each SIGABA-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 SIGABA-T1 suppression, the RNAi-targeted region of SIGABA-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 SIGABA-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 (Inplanta Innovations) using the Gateway LR Clonase enzyme (Invitrogen). This construct was
designated SIGABA-T\textsuperscript{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 promoter, this region (accession No. AF15784) was amplified using specific primers containing Bln and XhoI sites. The fragment was cloned in place of the CaMV 35S promoter 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\textsuperscript{-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 \(\mu\)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 \(\mu\)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 \(\mu\)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 GABA enzymatic assay were centrifuged at 10,000 \(\times\) g for 20 min at 4°C and the pellet was discarded. The supernatant was transferred into a fresh tube, 400 \(\mu\)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 \(\mu\)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 (CVE3100, TOKYO RIKAKIKAI), and 300 \(\mu\)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 ‘GABA’ assay for GABA was performed using the method described by Jakoby (1962) with slight modifications. In the ‘GABA’ 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 T\(0\) 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, OLMPUS).

**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 T\(0\) 35S::SIGABA-T\textsuperscript{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 phenylmethylsulfonyl fluoride, 2.5 \(\mu\)g ml\textsuperscript{-1} leupeptin and pepstatin A, 2% (w/v) polyvinylpyrrolidone and 2 \(\mu\)g ml\textsuperscript{-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 \(\mu\)l of the crude protein was used in the total 500 \(\mu\)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 sulfoalicylic acid to a final concentration of 60 mM (Van Cauwenbergue 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).

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