Screening for γ -aminobutyric Acid (GABA)-rich Tomato Varieties

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γ -aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is present in a wide variety of prokaryotic and eukaryotic organisms. Because of its antihypertensive effect on the human body, the demand for naturally occurring GABA has increased recently in the Japanese food industry. In this study, we evaluated the GABA content of tomato fruits of 61 commercial cultivars, wild species, and wild derivatives in 2005 and 2006 to screen for resources to breed a GABA-rich cultivar. GABA contents in tomato fruits greatly varied among the tested varieties and were poorly reproducible between the tested years. GABA-rich candidates selected from the screening were then subjected to salinity-stressed cultivation using the NFT system to assess their suitability for cultivation to produce GABA-rich fruit. Based on the results of two screenings and the salinity-stress cultivation test, ‘DG03-9’ was selected as a GABA-rich cultivar. The accumulation profiles for GABA, glutamine, and glutamic and aspartic acid during fruit development were also investigated in ‘DG03-9’ and ‘House Momotaro’ under salinity stress. The GABA content peaked at 24 days after flowering (DAF) in ‘DG03-9’ and 36 DAF in ‘House Momotaro’, and then declined during ripening. Salinity stress apparently promoted GABA accumulation during the early developing stages, but its effect on GABA decrease was different between the varieties. Although the GABA content in red mature fruits of ‘DG03-9’ was higher than that in fruits of ‘House Momotaro’ under normal and saline conditions, the maximum contents were almost the same in both cultivars. These results suggest that the lower reduction rate of GABA during the ripening stage causes high GABA accumulation in ‘DG03-9’ fruit. This variety will be a useful resource in the breeding of new GABA-rich cultivars. Additionally, we utilized an enzymatic assay with GABase to quantify GABA content in tomato fruit. This method will be a powerful screening tool for breeding.

Key Words: γ -aminobutyric acid (GABA), salinity stress, *Solanum lycopersicum*, tomato.

Introduction

γ -aminobutyric acid (GABA) is a ubiquitous four-carbon non-protein amino acid that is present in a wide range of organisms, including bacteria, vertebrates, and plants. In animals and plants, GABA is mainly metabolized *via* a short three-enzyme pathway termed the “GABA shunt”, a bypass of the tricarboxylic acid (TCA) cycle (Bouché and Fromm, 2004; Shelp et al., 1999). In this shunt, GABA is irreversibly synthesized

from glutamic acid by glutamic acid decarboxylase (GAD) (Chung et al., 1992; Tuin and Shelp, 1994) and reversibly converted to succinic semialdehyde by GABA transaminase (GABA-T) (Bouché and Fromm, 2004; Shelp et al., 1999). Succinic semialdehyde is then irreversibly reduced by succinate semialdehyde dehydrogenase (SSADH), and the resulting succinate eventually flows back into the TCA cycle.

In plants, GABA and the GABA shunt play roles in various physiological processes, including nitrogen storage (Breitkreuz et al., 1999), regulation of cytosolic pH (Shelp et al., 1999), protection against oxidative stresses (Bouché et al., 2003a), defense against insects (MacGregor et al., 2003; McLean et al., 2003), and osmoregulation, and GABA also acts as a signaling molecule (Bouché et al., 2003b; Kinnersley and Turano,

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2000). Furthermore, various abiotic stresses, such as acidosis (Crawford et al., 1994), mechanical damage (Ramputh and Bown, 1996; Wallace et al., 1984), cold (Cholewa et al., 1997; Wallace et al., 1984) and heat (Mayer et al., 1990) stimulation, anoxia (Aurisano et al., 1995; Streeter and Thompson, 1972; Tsushida and Murai, 1987), drought stress (Raggi, 1994; Thompson et al., 1966), and salinity stress (Bolarin et al., 1995) lead to the rapid accumulation of high levels of GABA. This evidence indicates that the component is involved in plant stress responses. In vertebrates, GABA is present at high levels in the brain and central nervous system, and functions mainly as an inhibitory neurotransmitter (Curtis and Johnston, 1974; Jessen et al., 1979). A lack of GABA tends to lead to anxiety disorders and epilepsy. Numerous studies have reported the blood pressure-lowering effect of GABA in experimental animals (Abe et al., 1995; Aoki et al., 2003; Shizuka et al., 2004; Takahashi et al., 1955) and humans (Elliot and Hobbiger, 1959; Inoue et al., 2003).

Recently, the Japanese consumer market has begun to focus on GABA as a dietary component with a potential antihypertensive effect (Yamakoshi et al., 2007). Many foodstuffs containing GABA have come on the market, in the form of soft drinks, sweets, alcoholic beverages, and supplements; however, most utilize artificially synthesized GABA as an additive. The demand for naturally occurring GABA is increasing, which will enhance the commercial value of processed and natural food products. Suzuki et al. (1976) reported that tomato (*Solanum lycopersicum* L.) contains one of the highest levels of GABA of all vegetable crops. Furthermore, cultivation under saline or drought stress promotes the accumulation of amino acids, including GABA, in tomato fruit, as well as soluble sugars and organic acids (Adams, 1991; Cuartero and Fernandez-Munoz, 1999; Zushi and Matsuzoe, 1998; Zushi et al., 2005). Nevertheless, no breeding has been carried out for horticultural crops, including tomato, for the fruit GABA content.

In this study, as an initial step in breeding a GABA-rich tomato cultivar, we evaluated the fruit GABA content in 61 commercial varieties, wild species, and derivatives, and then subjected the selected varieties to salinity-stress cultivation to assess its effect on GABA accumulation to produce GABA-rich fruit in each variety.

Materials and Methods

Plant materials and cultivation

In this work, the fruit GABA contents of 61 tomato varieties (38 processing cultivars, 11 fresh market cultivars, six wild species, and six wild derivatives) were evaluated (Table 1). Most of the processing and fresh-market cultivars were bred by Nippon Del Monte Corporation (Numata, Japan), except for ‘BOS3155’ (Orsetti), ‘AB2’ (AB Seed), ‘H9280’, ‘H9780’, ‘H9995’

(Heinz), ‘APT410’ (Seminis), ‘Shasta’, ‘CXD207’, ‘CXD224’ (Campbell Seed Inc.), ‘U941’ (Unilever), and ‘House Momotaro’ (Takii & Co., Ltd.). The cultivars ‘BOS3155’, ‘AB2’, ‘H9280’, ‘H9780’, ‘H9995’, ‘APT410’, ‘Shasta’, ‘CXD207’, ‘CXD224’, and ‘U941’ were obtained from TS&L Seed Company (Woodland, CA, USA). All of the wild species and wild derivatives were provided by the C. M. Rick Tomato Genetics Resource Center (TGRRC), University of California, Davis, USA. The accession numbers for the wild species are as follows: LA722 (*S. pimpinellifolium*), LA927 (*S. cheesmanii*), LA1403 (*S. cheesmanii*), LA1449 (*S. cheesmanii*), LA1627 (*S. cheesmanii*), and LA2076 (*S. lycopersicum* var. *cerasiforme*). The wild derivatives corresponding to LA1063, LA1500, LA1501, LA1502, LA1503, and LA1563 were generated from *S. chmielewskii* as lines with high soluble solid contents.

The results presented in Table 1 are from experiments carried out in 2005 (*Exp. 1*) and 2006 (*Exp. 2*). The first field trial in 2005 (*Exp. 1*) submitted 18 cultivars to screening at the Agricultural and Forestry Research Center, University of Tsukuba, Tsukuba, Japan. Sixteen seeds of each variety were sown in soil in nursery cell trays on May 17, 2005. After germination, the seedlings were transferred to 1-L plastic pots and grown for 30–35 days in a greenhouse. At the end of June, five plants of each variety were transplanted into the field at a density of ten plants per m². For sampling, one red mature fruit was harvested from the second truss of each plant from the end of September through October. The second field trial in 2006 (*Exp. 2*) submitted 61 varieties, including the 18 cultivars tested in *Exp. 1* to screening in a rented field in Tsukuba, Japan. As in *Exp. 1*, 16 seeds of each variety were sown on February 7, 2006 and grown for 50 days in a greenhouse. At the beginning of April, six plants of each variety were transplanted at a density of ten plants per m² in a plastic house. For sampling, one red mature fruit was harvested from the second or third truss of each plant from the end of June through July. In *Exp. 1* and 2, we determined the red stage of fruit by date after flowering, color and firmness of fruit. The harvested fruits were stored at –80°C until GABA levels were measured.

Salinity-stress cultivation tests

Salinity-stress cultivation tests were conducted twice, in 2006 (*Exp. 3*) and 2007 (*Exp. 4*), in a greenhouse in the Agricultural and Forestry Research Center, University of Tsukuba. In *Exp. 3*, ‘NDM051TM’ (used as a standard cultivar in this work) and the GABA-rich candidate cultivars selected in *Exp. 1* (‘Shasta’, ‘NDM1185’, ‘DG03-9’, ‘Fruit Yellow’, and ‘House Momotaro’) and *Exp. 4* (‘DG03-9’ and ‘House Momotaro’) were tested. Tomato seeds were sown on moist vermiculite in a greenhouse on February 7, 2006 (*Exp. 3*) and September 1, 2006 (*Exp. 4*). After the cotyledons had fully opened, the seedlings were

Table 1. Fruit GABA content among 61 tomato cultivars, wild species and wild derivatives.

Cultivar	Type ^z	GABA content ^y (mg/100 g FW)		Cultivar	Type ^z	GABA content ^y (mg/100 g FW)	
		Exp. 1 (2005)	Exp. 2 (2006)			Exp. 1 (2005)	Exp. 2 (2006)
NDM051TM	P	23.4 ± 1.3	46.9 ± 5.7	S397	P	46.2 ± 5.8	35.3 ± 3.7
NDM055	P		62.0 ± 6.3	S398	P		54.7 ± 9.2
NDM87-5-1	P		74.2 ± 11.9	S399	P		35.8 ± 3.5
NDM447	P		85.4 ± 10.0	U941	P		71.2 ± 10.6
NDM736	P		63.7 ± 9.4	22333	P		39.5 ± 4.9
NDM958	P	21.5 ± 4.2	84.3 ± 13.6	24189	P		72.5 ± 9.4
NDM1185	P	70.3 ± 8.4	38.0 ± 6.0				
NDM1213	P		42.3 ± 2.5	NDM79	F	44.2 ± 6.7	61.7 ± 6.7
NDM1224	P		82.1 ± 12.2	NDM362	F	33.2 ± 3.0	59.2 ± 7.6
NDM2260	P	69.2 ± 22.3	52.9 ± 12.7	DG03-9	F	189.7 ± 30.0	102.5 ± 9.9
NDM2280	P		52.9 ± 7.3	Fruit Yellow	F	149.4 ± 34.5	39.6 ± 2.7
NDM3366	P		70.7 ± 5.0	Little Summer Kiss	F	50.6 ± 2.9	71.5 ± 5.9
NDM3374	P		90.0 ± 8.8	L03-103	F	37.7 ± 5.1	79.8 ± 11.5
NDM3379	P		67.1 ± 5.4	L03-291	F	69.8 ± 5.1	80.6 ± 3.4
NDM4460	P		60.1 ± 4.8	L04-498	F	37.2 ± 39.5	58.0 ± 4.5
NDM4461	P		80.7 ± 4.4	Twinkle	F	54.5 ± 10.4	78.5 ± 4.1
NDM4462	P		93.3 ± 10.8	Vitamin Ace	F	48.9 ± 3.6	83.0 ± 6.7
NDM4463	P		77.3 ± 8.5	House Momotaro	F	68.3 ± 13.5	90.5 ± 5.8
NDM5562	P		92.2 ± 9.0				
NDM5565	P		45.3 ± 5.4	LA722	W		25.2 ± 2.7
NDM5600	P		38.3 ± 4.0	LA927	W		53.0 ± 46.9
NDM5602	P		39.4 ± 21.4	LA1403	W		30.9 ± 0.6
AB2	P	30.6 ± 5.2	60.5 ± 6.3	LA1449	W		8.8 ± 0.4
APT410	P		60.0 ± 9.0	LA1627	W		35.6 ± 2.7
BOS3155	P		60.7 ± 10.2	LA2076	W		11.0 ± 1.8
CXD207	P		70.2 ± 7.3				
CXD224	P		52.1 ± 5.9	LA1063	WD		55.3 ± 3.4
DR736TM	P		57.6 ± 2.3	LA1500	WD		106.4 ± 15.2
H9280	P		45.6 ± 6.1	LA1501	WD		107.2 ± 8.6
H9780	P		82.6 ± 9.0	LA1502	WD		57.4 ± 6.7
H9995	P		59.1 ± 10.8	LA1503	WD		114.7 ± 12.1
Shasta	P	98.8 ± 6.8	58.3 ± 6.0	LA1563	WD		55.3 ± 3.4

^z P, F, W, and WD indicate processing cultivar, fresh market cultivar, wild species and wild derivative, respectively.

^y GABA contents of the fruit were measured by LC-MS in *Exp. 1* and enzymatic assay with GABase in *Exp. 2*, respectively.

The values indicate the mean ± SE (*Exp. 1*, n = 5; *Exp. 2*, n = 6).

transplanted into 125-cm³ rockwool cubes (Nittobo Co., Ltd., Fukushima, Japan) and grown in an ebb-and-flow system supplying Otsuka-A nutrient solution (Otsuka Chemical Co., Ltd., Osaka, Japan) with the electrical conductivity (EC) adjusted to 1.2 dS/m and the pH to 6.5–7.0. After two weeks, six (*Exp. 3*) and four (*Exp. 4*) seedlings of each variety were transplanted to a nutrient film-technique (NFT) system in the greenhouse. Otsuka-B nutrient solution adjusted to an EC of 1.6 dS/m and a pH of 6.5–7.0 was supplied until the anthesis of the first truss. After flowering, the EC of the nutrient solution was adjusted to 8.0 dS/m by the addition of NaCl (corresponding to approximately 50 mM NaCl) to the salinity-stress medium, whereas the EC for the control medium was gradually increased from 1.6 dS/m, reaching 2.5 dS/m by the time of harvest. The fresh-market varieties were grown by single-stem training, and

the processing varieties were grown by double-stem training with a lateral shoot just under the first truss. During the winter season, the temperature in the greenhouse was maintained above 15°C by steam heating (*Exp. 4*). In *Exp. 3*, one red mature fruit was harvested from the second or third truss of each plant. In *Exp. 4*, three fruits were harvested at 12, 24, 36, and 48 days after flowering (DAF) and red stage from the fourth to sixth trusses of each plant. These samples were stored at –80°C until GABA and amino-acid analyses were performed.

Fruit yield

In *Exp. 4*, all red mature fruits from the first and second trusses of each plant were harvested separately. The total weight and number of fruits in each truss were evaluated and the fruit yields were calculated.

Amino-acid assays

Measurements of amino acid contents were conducted using liquid chromatography mass spectrometry (LC-MS) in *Exp. 1*, an enzymatic assay with GABase (Akama, unpublished) in *Exp. 2* and Figure 2, and a high-performance liquid chromatography (HPLC) amino-acid analyzer in *Exp. 3* and *Exp. 4*.

Frozen fruits were ground to a powder and homogenized in liquid nitrogen using a mortar and pestle. For LC-MS analysis, 250 μ L each of methanol and CHCl_3 were added to 50 mg of powdered fruit. After vortexing, 25 μ L of 1 mM methionine sulfone as an internal standard and 175 μ L of sterilized water were added to the sample, followed by stirring. After centrifugation at 15000 rpm for 10 min, the aqueous layer was transferred into a new spin-column tube (Amicon Ultrafree-MC 5000, Millipore, MA, USA) and centrifuged for 30 min at 15,000 rpm. The flow-through was dried using a centrifugal evaporator (CVE-3100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for 2 h at 43°C. The dried residue was dissolved in 30 μ L of sterilized water. The sample solution was diluted 5–20 times with sterilized water and analyzed using an Agilent 1100 Series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a Unison UK-Silica column (250 $\times$ 2 mm; Imtakt Co., Kyoto, Japan) and coupled to a mass spectrometer (Finnigan LTQ, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mobile phase was 20 mM ammonium formate:acetonitrile 1:1 (v/v) at a flow rate of 200 μ L $\cdot$ min $^{-1}$. The column was heated to 30°C during the measurement.

For measurements using the GABase assay and the amino acid analyzer, amino acids were extracted using trichloroacetic acid (TCA). First, 50 mg of frozen fruit were suspended in 500 μ L of 8% TCA. After vortexing for 30 seconds, the sample was centrifuged for 20 min at 4°C, and then 300 μ L of the supernatant were transferred into a new tube. The sample was extracted twice with diethylether to remove the TCA. For the GABase assay, the extracted sample solution was mixed with reaction buffer (85 mM potassium pyrophosphate pH 9.0, 1 mM 2-mercaptoethanol, 500 μ M NADP, and 620 μ M α -ketoglutarate) and GABase solution (diluted with sterilized water to 0.005 units/mL, Sigma-Aldrich Co., MO, USA) at a ratio of 20:174:1. The reaction mixture was incubated for 1 h at 37°C, and absorbance was measured at 340 nm (A^{340}). For measurements with the HPLC amino acid analyzer (JLC-500/V2, JEOL), the sample extracted with TCA was evaporated and then dissolved in sterilized water twice for purification. The purified sample was evaporated again and dissolved in 0.01 N HCl to adjust pH within 1.0–2.0, and then subjected to amino-acid analysis. Measurements with the HPLC amino acid analyzer were carried out in Chemical Analysis Division, Research Facility Center for Science and Technology, University of Tsukuba.

Results

Evaluation of fruit GABA contents in 61 commercial varieties, wild species and wild derivatives

In this work, we evaluated GABA contents in red mature fruits of 61 tomato varieties (Table 1). We used ‘NDM051TM’ as a standard variety, because it is a typical processing cultivar of the Nippon Del Monte Corporation. It was difficult to define a criterion for evaluation throughout this work because of the wide variation in GABA contents among the tested varieties and also between *Exp. 1* and *Exp. 2*. The average fruit GABA contents in the reevaluated 18 varieties were 50.3 mg/100 g FW in *Exp. 1* and 66.8 mg/100 g FW in *Exp. 2*, respectively. There was no correlation among these varieties in the two tests ($R=0.17$); therefore, we set the selection criterion as the GABA content in ‘NDM051TM’ in each experiment plus 40 mg. In *Exp. 1*, 18 cultivars were screened and five varieties (‘NDM1185’, ‘Shasta’, ‘DG03-9’, ‘Fruit Yellow’, and ‘House Momotaro’) were selected as candidate GABA-rich varieties. These varieties were subjected to cultivation tests to assess the effect of salinity stress on their GABA levels (Fig. 1). Although ‘NDM2260’ (69.2 mg/100 g FW) and ‘L03-291’ (69.8 mg/100 g FW) showed higher GABA contents than the criterion, we did not select these varieties because of the substantial SEs of ‘NDM2260’ data (Table 1) and the irreproducibility of ‘L03-291’ data (unpublished data).

In *Exp. 2*, the GABA contents of the 61 cultivars, wild species, and wild derivatives including the 18 cultivars tested in *Exp. 1*, were evaluated. Although ‘NDM1185’, ‘Shasta’, and ‘Fruit Yellow’ showed high GABA contents (70.3, 98.8, and 149.4 mg/100 g FW, respectively) in *Exp. 1*, these cultivars did not show high GABA contents in *Exp. 2*. Only ‘DG03-9’ and ‘House Momotaro’ had GABA contents greater than the criterion in both experiments. Several cultivars and wild derivatives, such as ‘NDM3374’, ‘NDM4462’, ‘NDM5562’, LA1500, LA1501, and LA1503, also showed high GABA contents in *Exp. 2*; however, additional tests will be needed to confirm the reproducibility of these data.

Effect of salinity on amino-acid contents in fruits of the selected cultivars

We investigated the effect of salinity stress on the accumulation of GABA, glutamic acid (Glu), glutamine (Gln), and aspartic acid (Asp) in red mature fruits in the five varieties selected in *Exp. 1* and the standard variety (‘NDM051TM’). These are major amino acids in tomato fruit and closely related to GABA metabolism. This experiment was designated *Exp. 3*. Salinity stress was provided by hydroponic medium containing 50 mM NaCl. It was not possible to quantify the amounts of Glu and Gln separately, since Gln is unstable and easily converted to Glu during measurement; therefore, the

Fig. 1. Effect of salinity stress on amino acid contents in red tomato fruits (Exp. 3). Open and closed columns indicate control and salinity treatments, respectively. GABA, γ -aminobutyric acid; Glu, glutamic acid; Gln, glutamine; Asp, aspartic acid. Values are means $\pm$ SE ($n=5$) except NDM1185 (control; $n=3$, salinity treatment; $n=2$). Different letters indicate significant differences by Student's t -test, $P=0.05$.

contents of both amino acids are presented as a combined value in Figures 1B and 3B. Salinity treatment promoted the accumulation of the four amino acids in most of the cultivars, except for aspartic acid in 'DG03-9' and 'Fruit Yellow' (Fig. 1C). The effect of salinity stress on promoting amino acid accumulation differed by the variety. The average GABA levels in 'DG03-9' were high in all of the six tested cultivars under both control and saline conditions, although the promotion effect of salinity stress on GABA accumulation was smallest in the six tested varieties (Fig. 1A). On the other hand, salinity stress enhanced GABA accumulation almost two times under salinity stress as compared to the control in 'House Momotaro' and 'Fruit Yellow'; however, the GABA level in both varieties was lower than 'DG03-9'.

To evaluate the correlation between HPLC analyses and enzymatic assays with GABase, the data in Figure 1A were verified by GABase assays and compared (Fig. 2). The correlation coefficient between

Fig. 2. Comparison of GABA contents quantified by HPLC analyses and enzymatic method for 55 tomato fruit samples. Open and closed squares indicate control and salinity treatments, respectively.

the quantification methods was 0.94 and was highly significant ($P<0.001$). The slope of the linear regression was 0.607. GABA contents quantified by the enzymatic assay with GABase tended to be overestimated when compared with those by HPLC analysis.

Accumulation profiles of GABA, glutamic acid, glutamine, and aspartic acid during fruit development under salinity stress

To determine the accumulation profiles of GABA, Glu + Gln, and Asp during fruit development, the contents of these molecules in the fruit of 'DG03-9' and 'House Momotaro' were assayed at 12, 24, 36, and 48 DAF and in the red stage (about 60 DAF) (Exp. 4). Salinity treatment promoted the accumulation of GABA throughout fruit development in both cultivars without changes in the GABA accumulation pattern (Fig. 3A and Table 2). Although the GABA contents in 'DG03-9' in the red stage were higher than those in 'House Momotaro' under either condition, the maximum contents of both cultivars were almost the same under both conditions (104.3–109.7 mg/100 g FW in the control; 151.9–153.8 mg/100 g FW under saline stress). GABA accumulation peaked earlier in 'DG03-9' (24 DAF) than in 'House Momotaro' (36 DAF). These fruit corresponded to mature green in 'DG03-9' and several days before breakers in 'House Momotaro', respectively. After peaking, GABA levels decreased under both conditions and in both varieties. The rates of reduction from the maximum to minimum contents were -25% (control conditions) and -36% (saline conditions) in 'DG03-9' and -72.3% (control conditions) and -58.3% (saline conditions) in 'House Momotaro', showing that the decrease was much greater in 'House Momotaro' than in 'DG03-9'. The rate of reduction was intensified in 'DG03-9' by saline stress, but suppressed in 'House Momotaro'. The contents of Glu + Gln and Asp in both cultivars were not significantly affected by saline conditions through fruit development (Fig. 3B, C and Table 2), although their contents in red mature fruit tended to increase under saline stress without Asp in

Fig. 3. Effect of salinity treatment on the amino acid content of two tomato cultivars during the fruit development period (*Exp. 4*). GABA, γ -aminobutyric acid; Glu, glutamic acid; Gln, glutamine; Asp, aspartic acid. ◇, House Momotaro, control; ◆, House Momotaro, salinity treatment; ○, DG03-9, control; ●, DG03-9, salinity treatment. DAF, days after flowering. R indicates red stage. Values are the means $\pm$ SE ($n=3$).

‘DG03-9’ (Fig. 1B, C).

In the results of two-way repeated measures ANOVA, fruit GABA levels were affected by cultivar and date after flowering as well as salinity treatment. Additionally, there was an interaction effect between cultivar and date after flowering. In contrast to GABA, Glu + Gln and Asp contents were affected only by date after flowering because of the drastic increases in the red stage, but not cultivar and salinity treatment (Table 2).

Simulation model for GABA yield per plant

GABA yield per truss in ‘House Momotaro’ and ‘DG03-9’ was calculated based on the GABA content and fruit yield obtained in *Exp. 4* (Table 3). Salinity stress decreased the fruit weight of both cultivars, but had little effect on the fruit number. Under salinity stress, the average fruit yield per truss decreased to 69.5% and 58.8% of the control in ‘House Momotaro’ and ‘DG03-9’, respectively, whereas the GABA content increased to 210.9% and 132.9%. In spite of the large differences among the mean values, the fruit yield per truss did not show significant differences between the control and salinity conditions in each cultivar because of large variations among trusses. GABA content per fruit increased under salinity stress in both varieties, but GABA yield per truss decreased in ‘DG03-9’. Overall, ‘DG03-9’ grown under control conditions showed the highest GABA yield per truss.

Table 2. Effect of cultivar, salinity treatment and date after flowering on amino acid contents in tomato fruit.

		Amino acid content (mg/100 g FW)		
		GABA	Glu + Gln	Asp
Cultivar	House Momotaro	85.2	100.2	22.2
	DG03-9	98.7	94.2	19.8
Treatment ^z	Control	73.8	92.6	20.5
	Salinity	110.0	101.8	21.4
DAF ^y	12	77.4	61.4	10.4
	24	115.3	33.3	9.0
	36	123.1	26.3	10.2
	48	86.1	32.6	15.5
	R	66.3	273.6	50.0
Significance ^x				
Cultivar		***	NS	NS
Treatment		***	NS	NS
DAF		***	***	***
Cultivar $\times$ Treatment		NS	NS	*
Cultivar $\times$ DAF		***	NS	NS
Treatment $\times$ DAF		NS	*	NS
Cultivar $\times$ Treatment $\times$ DAF		NS	NS	NS

^z Treatment: Control, EC2.5 dS/m, Salinity, 8.0 dS/m.

^y DAF indicates days after flowering. R indicates red stage.

^x NS, * or *** denote non-significant or significant at $P=0.05$ or 0.001, respectively.

Table 3. Effect of salinity treatment on fruit and GABA yield in tomato cv. 'House Momotaro' and 'DG03-9'.

	'House Momotaro'		'DG03-9'	
	Control	Salinity	Control	Salinity
Fruit weight (g)	129.7 ± 9.8 a ^z	84.9 ± 6.1 bc	99.5 ± 11.1 b	77.3 ± 4.1 c
Fruit number ^z (number/truss)	4.0 ± 0.5 a	4.3 ± 0.5 a	4.1 ± 0.7 a	3.1 ± 0.3 a
Fruit yield ^z (g/truss)	518.9 ± 64.5 a	360.8 ± 36.6 ab	410.6 ± 77.7 a	241.5 ± 25.9 b
GABA content (mg/100 g FW)	30.4 ± 0.9 c	64.1 ± 9.4 b	73.2 ± 14.4 ab	97.3 ± 4.0 a
GABA content ^y (mg/fruit)	39.4	54.4	72.8	75.2
GABA yield ^x (mg/truss)	157.7	231.3	300.6	235.0

^z Fruits were harvested from first and second trusses of four plants per condition.

Values indicate the mean ± SE (n = 8).

^y Values indicate the mean ± SE (n = 4).

^x GABA content per fruit was calculated from fruit weight and GABA per 100 g FW.

^w GABA yield was calculated from fruit yield and GABA content per 100 g FW.

^v Different letters indicate significant differences by Student's t-test, $P = 0.05$.

Discussion

Despite increasing interest in GABA in the Japanese consumer market, no screening for GABA-rich varieties of horticultural crops has been conducted to date. In this study, we evaluated GABA content in fruits of various commercial varieties, wild species, and wild derivatives of tomato and then investigated the promotion of GABA accumulation by salinity stress in the selected varieties. As shown in Table 1, fruit GABA contents varied greatly in the tested varieties as well as between the two tested years. Most of the varieties selected as GABA-rich varieties based on the results of *Exp. 1* showed poor reproducibility in *Exp. 2* and *Exp. 3*, except 'DG03-9' (Table 1 and Fig. 1). Similarly, four varieties ('NDM958', 'L03-103', 'Twinkle', and 'Vitamin Ace') that were not selected in *Exp. 1* showed high GABA contents in *Exp. 2* (Table 1). These results indicate that GABA accumulation is highly influenced by the cultivation conditions, suggesting that this trait is controlled by quantitative trait loci (QTL). Only 'DG03-9' showed a high level of GABA accumulation in fruits throughout the study. An additional test carried out on 'DG03-9' at the Nippon Del Monte research farm in Numata produced similar results (data not shown); therefore, we conclude that the variety 'DG03-9' has a genetically stable GABA-rich fruit trait even under varying cultivation conditions. 'DG03-9' is an F₁ hybrid variety that was bred by the Nippon Del Monte Corporation for the fresh market. It will be a useful resource in the breeding of new GABA-rich varieties using the parent lines as parental strains. In this work, 'House Momotaro' also showed a high level of GABA content; therefore, we investigated the GABA content of commercial 'House Momotaro' fruits obtained from markets with the enzymatic assay with GABase. In the results, most of these fruits showed GABA contents below 50 mg/100 g FW. We assume that this cultivar has high potential to accumulate GABA in its fruit, but the characteristic is not stable; however, LA1500, LA1501, and LA1503 also showed high GABA contents

of over 100 mg/100 g FW in *Exp. 2*. All three varieties are derivatives of *S. chmielewskii*. Furthermore, Schauer et al. (2005) reported that the GABA content of *S. pennellii* fruit was 3.5 times higher than that of *S. lycopersicum*. Isogenic *S. pennellii* lines in a *S. lycopersicum* 'M82' background have been generated by Eshed and Zamir (1994) as an open resource. These wild species and their derivatives may be useful genetic resources in future GABA-focused breeding.

Because we quantified the fruit GABA content by LC-MS, enzymatic assay with GABase, and HPLC in this work, the correlation of data between HPLC analyses and enzymatic assays was verified. As shown in Figure 2, both methods showed a highly significant correlation; however, enzymatic quantification tended to be overestimated compared to HPLC quantification. We consider that data obtained from the enzymatic assay are not appropriate to make a direct comparison with HPLC data; therefore, we set the selection criterion in each experiment as shown in Table 1. On the other hand, this assay will be a powerful and useful method for high-throughput screening for GABA content because of its ease and speed.

It has been reported that salinity stress promotes GABA accumulation in tomato leaves, roots, and fruits (Bolarin et al., 1995; Zushi et al., 2005). In the present study, GABA-promoting effects by stress were also observed in all six tested varieties (Fig. 1A). There were two responses to salinity, high sensitivity ('House Momotaro', 'Fruit Yellow') and low sensitivity ('NDM051TM', 'NDM1185', and 'DG03-9'). In 'NDM051TM' and 'NDM1185', blossom-end rot frequently arose under increased-salinity conditions (data not shown); therefore, the low sensitivity in these varieties is probably due to forms of physiological damage caused by stress. In contrast, 'DG03-9' fruits did not show this symptom, suggesting that the salinity tolerance of this variety is higher than that of the other two varieties. Although the GABA-promoting effect by stress was smallest in 'DG03-9', the GABA content was high under both conditions (Fig. 1A). From the point of

view of GABA yield per truss (Table 3), ‘DG03-9’ cultivated under normal conditions was most effective for GABA production. We consider that this property of ‘DG03-9’ will be a great advantage in commercial cultivation.

Rolin et al. (2000) reported that GABA content in the fruit of cherry tomato peaked before starting maturation and then declined during ripening. A similar pattern was observed during fruit development in ‘Micro-Tom’ (Akihiro et al., unpublished data). The GABA accumulation profile of ‘House Momotaro’ roughly corresponded to those data; however, GABA levels in ‘DG03-9’ peaked at mature green (24 DAF), which was earlier than in ‘House Momotaro’ (36 DAF) and the report on cherry tomato. Interestingly, the maximum GABA contents in both varieties were almost the same under each condition, and the higher accumulation of GABA in ‘DG03-9’ in the red stage was due to the lower reduction rate during the ripening stages (Fig. 3A). These results suggest that fruit GABA in ‘DG03-9’ decreased in a different manner from that in other varieties, including ‘House Momotaro’. The effect of salinity stress on GABA accumulation was apparent during the early developing stages, but its effect on GABA decrease was different between the varieties (Fig. 3A).

Numerous reports have described the stress regulation of GABA accumulation in plants (reviewed by Kinnersley and Turano, 2000), including tomato (Bolarin et al., 1995; Zushi et al., 2005). There have also been reports of a crucial role for Ca^{2+} and calmodulin in the regulation of GAD activity in many plants (Baum et al., 1996; Snedden et al., 1995; Turano and Fang, 1998). It is possible that an increase in intracellular Ca^{2+} caused by salinity stress stimulates GAD activity, resulting in increased fruit GABA levels. In contrast to its biosynthesis, the regulation of GABA degradation has not been fully understood in plants. To clarify the detailed mechanisms of GABA accumulation in fruits, the identification and characterization of all GABA shunt genes, and consequent post-translational and metabolome studies, will be indispensable.

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