

# Marker-assisted Breeding of Tomato to Introduce the High-GABA Trait from *Solanum pennellii* and Remove the Linked Small-fruit Trait

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Increasing the contents of  $\gamma$ -aminobutyric acid (GABA), a non-proteinogenic amino acid neurotransmitter known for its beneficial effects on human health, is an important objective in tomato (*Solanum lycopersicum*) breeding. Improving the nutritional content of fruits remains challenging for breeders, as measuring specific compounds is more labor-intensive than assessing fruit appearance. Therefore, using DNA markers is preferred as an effective, accurate, and simple method for testing nutritional traits. In this study, we identified a tomato line with higher GABA content from progeny derived from a cross with the wild tomato *Solanum pennellii*. Among *S. lycopersicum*  $\times$  *S. pennellii* introgression lines, we initially obtained one line with higher GABA contents, but smaller fruit. To break the linkage between higher GABA contents and the closely linked small-fruit trait, we designed DNA markers around the genomic region conferring these traits and conducted large-scale genotyping of inbred populations over six generations and backcross populations. Finally, we selected two lines with a recombination event between the loci that regulate GABA production and fruit size, resulting in a tomato line with normal-sized fruits and high GABA levels. These lines and the associated DNA markers could be used in breeding to develop additional varieties with high GABA levels.

**Key Words:**  $\gamma$ -aminobutyric acid (GABA), breeding, fruit weight, genetic mapping, tomato.

## Introduction

The non-proteinogenic, four-carbon amino acid  $\gamma$ -aminobutyric acid (GABA) is found in many organisms, including bacteria, animals, and plants. GABA is a major inhibitory neurotransmitter in vertebrates and it lowers blood pressure in animals (Abe et al., 1995; Aoki et al., 2003; Takahashi et al., 1955) and humans (Kajimoto et al., 2004; Takahashi et al., 1961). GABA also has relaxing effects (Abdou et al., 2006). Eating foods high in GABA confers many of these benefits; therefore, the effects of GABA on human health have been the subject of considerable attention in the food industry.

Dietary sources of GABA include rice (*Oryza sativa*) grains, vegetables, tea, chocolate, and fermented foods

(Ding et al., 2016; Inoue et al., 2003; Oh et al., 2003; Tsushida and Murai, 1987). Tomato (*Solanum lycopersicum* L.) fruit contains relatively high levels of GABA compared with other fresh produce (Ohno et al., 2007). Tomatoes are widely consumed as fresh and processed products, such as juices and sauces (Burton-Freeman and Reimers, 2011). Moreover, tomato is an important horticultural crop grown worldwide, with a total production of approximately 187 Mt in 2020 (<http://faostat.fao.org/>). Tomato fruits are a good source of GABA, and their consumption may contribute to the prevention of high blood pressure as well as conferring other health benefits.

GABA biosynthesis in flowering plants occurs through the GABA shunt pathway, mediated by glutamate decarboxylase (GAD) (Bouché and Fromm, 2004). In this pathway, GABA is produced from the precursor glutamate by GAD; GABA can be converted to succinate by GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) in sequential reactions. The tomato genome harbors at least three *GAD* genes (*SIGAD1* to 3), three *GABA-T* genes (*SIGABA-T1* to -T3), and one *SSADH* gene (*SSADH*) (Akihiro et al., 2008); these genes are

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expressed at different stages of tomato fruit development.

Recent advances in gene modification technologies, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) genome editing, now make it possible to modify target genes to enhance the function of their encoded proteins (Jinek et al., 2012). In tomato, successful gene editing at target sites has been performed using the CRISPR/Cas9 system (Brooks et al., 2014; Ito et al., 2015). Among several examples, frameshift mutations resulting in the deletion of the sequence encoding SIGAD3 autoinhibitory domains led to 7- to 15-fold higher GABA levels without significantly affecting plant growth (Nonaka et al., 2017). However, one consideration in developing new varieties of horticultural crops is that conventional breeding methods relying on genetic crosses are generally more acceptable to consumers than those involving transgenic plants or gene editing; conventionally bred plants also likely face less of a regulatory burden. Therefore, finding ways to breed tomato varieties with high GABA contents remains an important consideration for research.

Breeding enhanced tomato varieties requires genetic diversity in the breeding materials, including wild relatives. Indeed, the GABA content of tomato fruits varies widely among different varieties, suggesting the existence of substantial genetic diversity. For example, Saito et al. (2008) reported that the GABA contents in 11 fresh market cultivars ranged from 39.6 to 102.5 mg·100 g<sup>-1</sup> fresh weight (FW), with a similar range of 35.4 to 93.3 mg·100 g<sup>-1</sup> FW in 38 processing cultivars. Notably, GABA levels reached up to 200 mg·100 g<sup>-1</sup> FW in the wild tomato relative *Solanum pennellii* (Schauer et al., 2006), which originated in the arid western slopes of the Andes in central Peru (Torrecillas et al., 1995), where it has evolved adaptations for survival in dry environments (Moyle and Muir, 2010). Therefore, *S. pennellii* constitutes a major genetic resource for tomato breeding.

To explore the genetic basis of GABA levels and identify potentially useful loci, we utilized introgression lines (ILs) derived from *S. pennellii*. These ILs were developed by crossing with tomato cultivar ‘M82’ (Eshed and Zamir, 1995). In ILs, a specific chromosome or sub-chromosomal fragment from the donor parent replaces the equivalent chromosome or sub-chromosomal fragment in the genome of the recurrent parent. ILs are suitable for detailed mapping studies, and the *S. pennellii* IL population has been the subject of extensive research to study the genetic basis of complex traits in tomato, including yield (Eshed and Zamir, 1995; Schauer et al., 2006), fruit quality (Eshed and Zamir, 1995; Fridman et al., 2004; Liu et al., 2003; Schauer et al., 2006; Tieman et al., 2006; Yang et al., 2016), and response to drought stress (Dariva et al., 2020).

In this study, we identified an IL with high GABA levels comparable to those of *S. pennellii* fruits, although these fruits were smaller than the parental tomato cultivar. In the progeny of this line, we obtained lines with improved GABA content and normal-sized fruits by identifying recombinants that segregated the loci responsible for each trait. This study constitutes the first genetic analysis with the aim of improving GABA content in tomato fruits. The DNA markers associated with high GABA levels and desirable fruit weight will provide support for breeders, enabling them to use marker-assisted selection (MAS) to increase the GABA content of tomato fruits without decreasing fruit weight.

## Materials and Methods

### *Plant materials and growth conditions*

The tomato (*S. lycopersicum*) lines used in this study are listed in Table 1. IL3-3 was obtained from the Tomato Genetics Resource Center, University of California, Davis, USA. The tomato variety M82 and breeding inbred line 19F272, a processing tomato, were used to compare GABA contents and fruit weights. 19F272 was used as the parental line to produce inbred mapping populations, and another breeding inbred line, 20K124, a cherry tomato, was used as the recurrent parental line to produce backcrossed mapping populations. All plants were grown and their fruits harvested in a greenhouse in Nasushiobara City or in an experimental field in Ohtawara City, Tochigi Prefecture, Japan. The GABA contents of fruits from IL3-3 and M82 were determined in 2014, 2015, and 2017. The F<sub>2</sub>, F<sub>4</sub>, and F<sub>6</sub> generations were cultivated and evaluated in 2016, 2018, and 2019, respectively, in a field in Ohtawara, Japan. The BC<sub>4</sub>F<sub>3</sub> generation was cultivated and evaluated in 2020 in a greenhouse in Nasushiobara City, Japan.

**Table 1.** Breeding populations.

| Name     | Description                                                 |
|----------|-------------------------------------------------------------|
| M82      | Recurrent parent of IL3-3                                   |
| IL3-3    | Introgression line ( <i>S. pennellii</i> [LA 716])          |
| 19F272   | Breeding inbred line                                        |
| 20K124   | Breeding inbred line, recurrent parent of 20K126            |
| 16F280_A | F <sub>2</sub> generation of IL3-3 × 19F272                 |
| 16F280_B | F <sub>2</sub> generation of IL3-3 × 19F272                 |
| 16F280_C | F <sub>2</sub> generation of IL3-3 × 19F272                 |
| 16F280_D | F <sub>2</sub> generation of IL3-3 × 19F272                 |
| 17F253_A | F <sub>3</sub> generation of IL3-3 × 19F272                 |
| 17F253_B | F <sub>3</sub> generation of IL3-3 × 19F272                 |
| 18F307_A | F <sub>4</sub> generation of IL3-3 × 19F272                 |
| 18F308_A | F <sub>4</sub> generation of IL3-3 × 19F272                 |
| 18F1853  | F <sub>5</sub> generation of IL3-3 × 19F272                 |
| 19F273   | F <sub>6</sub> generation of IL3-3 × 19F272                 |
| 20K126_A | BC <sub>4</sub> F <sub>3</sub> generation of IL3-3 × 20K124 |
| 20K126_B | BC <sub>4</sub> F <sub>3</sub> generation of IL3-3 × 20K124 |
| 20K126_C | BC <sub>4</sub> F <sub>3</sub> generation of IL3-3 × 20K124 |

### Genetic mapping

Genetic mapping was performed using the markers developed in this study. The SL3.0 version of the tomato cultivar ‘Heinz 1706’ reference genome was used to identify the position of the primers on chromosome 3. The genomic DNA of individuals was extracted by grinding leaf discs, which had been punched using a 2-mm-diameter punch, with an extraction buffer (0.1 M Tris HCl, 10 mM EDTA, 1 M NaCl, pH 8.0) using a Geno/Grinder (SPEX SamplePrep, USA) tissue grinder at 1000 rpm for 1 min. PCR was performed using the primer pairs listed in Table 2 and 0.5 µL of the DNA solution described above in a 25-µL reaction mixture using KOD FX DNA polymerase (Toyobo, Japan), with an initial denaturation of 2 min at 94°C, followed by 40 cycles of 10 s at 98°C, 30 s at 55°C, and 30 s at 68°C. The PCR products were analyzed on a Microchip Electrophoresis System MCE-202 MultiNA (Shimadzu, Japan) according to the manufacturer’s instructions using a DNA-500 reagent kit (Shimadzu).

### Measurements of GABA content and fruit weight

Red, ripe fruit was harvested from plants at approximately 100 days after planting. Fruit extracts were prepared by grinding at least three red-stage fruits from each harvest for 1 min and then boiling the extract in a sealed glass jar for 30 min for sterilization. After the extracts had cooled to room temperature, the mixture was filtered through quantitative filter paper No. 5A (Advantec, JAPAN). The resulting filtrate was diluted 10-fold with 3% (w/v) sulfosalicylic acid and filtered through a 0.20-µm membrane filter (DISMIC-25CS 0.20 µm 25CS020AN; Advantec). The amount of

GABA was determined using an automatic amino acid analyzer L-8900 (Hitachi, Japan). The GABA concentration was calculated from a calibration curve prepared using a commercially available standard (010-02441; FUJIFILM Wako, Japan). Fruit weight was measured using an electronic balance (ME1002E; Mettler, Germany).

### Statistical analysis of phenotypic values

Significant differences in the mean values among IL3-3, M82, and the progeny generated by crossing IL3-3 with breeding inbred lines were determined using Student’s *t*-test. For comparisons involving three or more groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey–Kramer multiple comparison tests when the ANOVA results were significant ( $P < 0.05$ ).

## Results

### Phenotypic analysis of parental genotypes

To identify lines with elevated GABA content, we initially focused on IL3-3, one of the introgression lines (ILs) derived from a cross between the tomato cultivar M82 and the wild tomato *S. pennellii* (LA 716) (Eshed and Zamir, 1995). Notably, chromosome 3 of IL3-3 carries a chromosomal region from *S. pennellii* corresponding to the region in which *SIGAD1* is located. Phenotypic analysis of the ILs and the parents revealed that the GABA content in IL3-3 was over 3-fold that in M82 (Table 3). The GABA content of IL3-3 was significantly higher ( $P < 0.001$ ), but its fruits weighed less ( $P = 0.020$ ) than those of M82, with fruit weight being about half that of M82. We therefore tried to identify

**Table 2.** Primer sets for mapping.

| Marker No. | Primer | SL3.0 position (bp) | Sequence (5'–3')              | Annealing temperature | Length           |                  |
|------------|--------|---------------------|-------------------------------|-----------------------|------------------|------------------|
|            |        |                     |                               |                       | REF <sup>z</sup> | ALT <sup>y</sup> |
| 1          | F      | 44,332,454          | CATGTTCTTGCTTGCTGC            | 60°C                  | 105 bp           | 115 bp           |
|            | R      | 44,332,545          | AAATGGGGCTCTTGTTGCT           |                       |                  |                  |
| 2          | F      | 62,033,083          | AGCCTAATATAGTCACCTGGCGCTAATG  | 58°C                  | 435 bp           | 390 bp           |
|            | R      | 62,033,500          | CAATTTTCCCAGCAGACC            |                       |                  |                  |
| 31         | F      | 62,187,683          | GCTCTTACAACGCGGACTCT          | 60°C                  | 161 bp           | 141 bp           |
|            | R      | 62,187,824          | GAAATCGCTCGGTATGCTTT          |                       |                  |                  |
| 32         | F      | 62,216,456          | TGTTTATTTATAGAATCATACCAGCTA   | 55°C                  | 97 bp            | 78 bp            |
|            | R      | 62,216,526          | TGTTTAATAAAATAGATATCCAAAAGG   |                       |                  |                  |
| 8          | F      | 62,495,850          | AATTCACAGGTGTACTATAGGTC       | 58°C                  | 80 bp            | 70 bp            |
|            | R      | 62,495,924          | ACCTAGGTGAGTCCCCGAAT          |                       |                  |                  |
| 6          | F      | 63,888,174          | GGAGGTTACCCACCCATAT           | 58°C                  | 145 bp           | 160 bp           |
|            | R      | 63,888,310          | GCAATTGTGTAATGAGTGTTCATCAC    |                       |                  |                  |
| 3          | F      | 64,432,945          | TCTACAAAGTACAGAAGTGACACCTCA   | 60°C                  | 125 bp           | 115 bp           |
|            | R      | 64,433,064          | AAGGTCAGCAGAGGGCTTG           |                       |                  |                  |
| 7          | F      | 64,973,770          | GATCGTAAATTGGGTGGACTT         | 58°C                  | 140 bp           | 130 bp           |
|            | R      | 64,973,894          | CTTAGATATACTATAGCAGGCACAGAACA |                       |                  |                  |

<sup>z</sup> Reference allele from Heinz 1706.

<sup>y</sup> Alternate allele from *S. pennellii* LA 716.

**Table 3.** GABA contents of IL3-3 and M82 fruits.

| Name  | GABA content<br>(mg·100 g <sup>-1</sup> FW) | Fruit weight (g) | Number of<br>individuals |
|-------|---------------------------------------------|------------------|--------------------------|
| IL3-3 | 165.3±9.2                                   | 37.7±2.4         | 3                        |
| M82   | 51.2±3.0                                    | 61.7±0.5         | 3                        |

Values are presented as means±standard error. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  (Student's *t*-test).

genes involved in GABA metabolism present in the *S. pennellii* chromosomal substitution in IL3-3.

#### Designing markers to distinguish parental genotypes

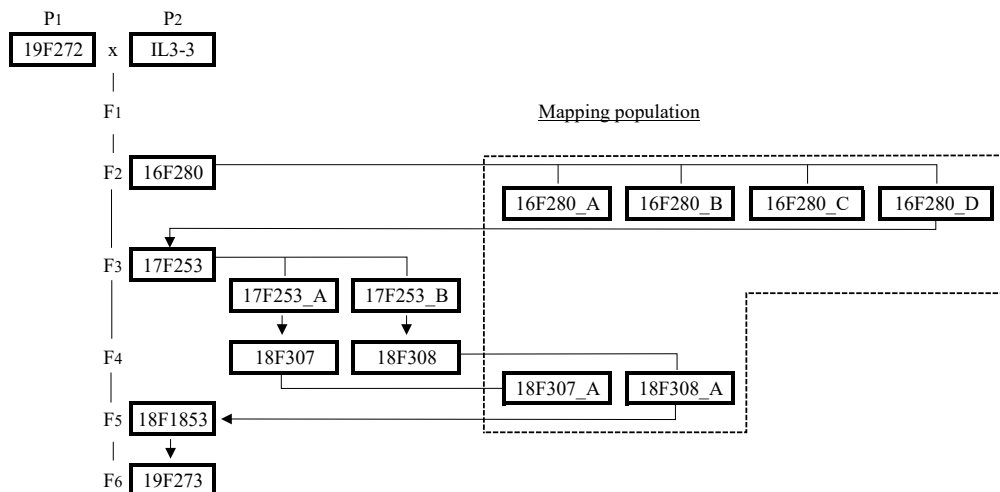
We designed primers to amplify eight insertion–deletion (InDel) markers capable of distinguishing between the parental genotypes. These were designed using the published nucleotide sequence information for *S. pennellii* LA 716 and the tomato cultivar Heinz 1706, the latter having been used to sequence and assemble the reference genome (Table 2). Specifically, we explored the sequence information in public databases (Sol Genomics Network [SGN], <https://solgenomics.net/>; DDBJ, <https://www.ddbj.nig.ac.jp/index-e.html>) and visualized it in the Integrative Genomics Viewer (Robinson et al., 2011). We designed marker No.2 within the *SIGAD1* locus itself. Following confirmation that all eight markers could clearly distinguish between the reference (REF) allele in line 19F272 and the LA 716 (ALT) allele. These markers were used for subsequent mapping studies.

#### Genetic mapping of the progeny

We conducted genetic mapping using the lines developed in this study (Fig. 1). First, we generated an  $F_2$  population, designated 16F280, for primary mapping, by crossing the breeding inbred line 19F272 as the female with IL3-3 as the male parent. After allowing

the  $F_1$  hybrids to self-pollinate, we genotyped 400  $F_2$  plants from 16F280 using markers 1, 2, 6, 3, and 7. We identified five plants homozygous for the REF alleles at all markers tested and designated these plants 16F280\_A (Fig. 2). We also obtained three plants heterozygous at all markers and designated these plants 16F280\_D. Finally, we identified two groups of plants with one recombination event between consecutive markers: four plants (designated as 16F280\_B group) switched from being homozygous for the ALT allele to heterozygous between markers No.3 and 7, whereas another five plants (designated as 16F280\_C group) switched from being heterozygous to homozygous for the ALT allele between markers No.1 and 2. After genotyping, we cultivated all plants from these groups and determined the GABA content and weight of their fruits. The GABA content of plants from the 16F280\_B and 16F280\_C groups was significantly higher ( $P < 0.001$ ), but their fruits were lighter ( $P = 0.0374$  and  $P = 0.003$ , respectively) than those of plants from the 16F280\_A group (Fig. 2). However, we detected no significant difference between the GABA contents of plants from the 16F280\_A and 16F280\_D groups ( $P = 0.972$ ). Despite the greater GABA content of IL3-3, this trait was accompanied by lower fruit weight, which can diminish fruit marketability.

To genetically separate these two traits, we developed an advanced generation. To this end, we collected seeds from a single  $F_2$  plant belonging to the 16F280\_D group that was heterozygous across the entire chromosomal substitution segment, with a low GABA content in its normal-sized fruits, to obtain the corresponding  $F_3$  generation, designated 17F253. We genotyped 400  $F_3$  plants from 17F253 using markers No. 2, 8, 6, and 3. We identified five plants with a heterozygous genotype at markers No. 2, 8, and 6, and homozygous for the REF allele at marker No. 3, forming group 17F253\_A. We also obtained five plants with a heterozygous

**Fig. 1.** Pedigree of the *S. lycopersicum* lines and breeding history in this study.

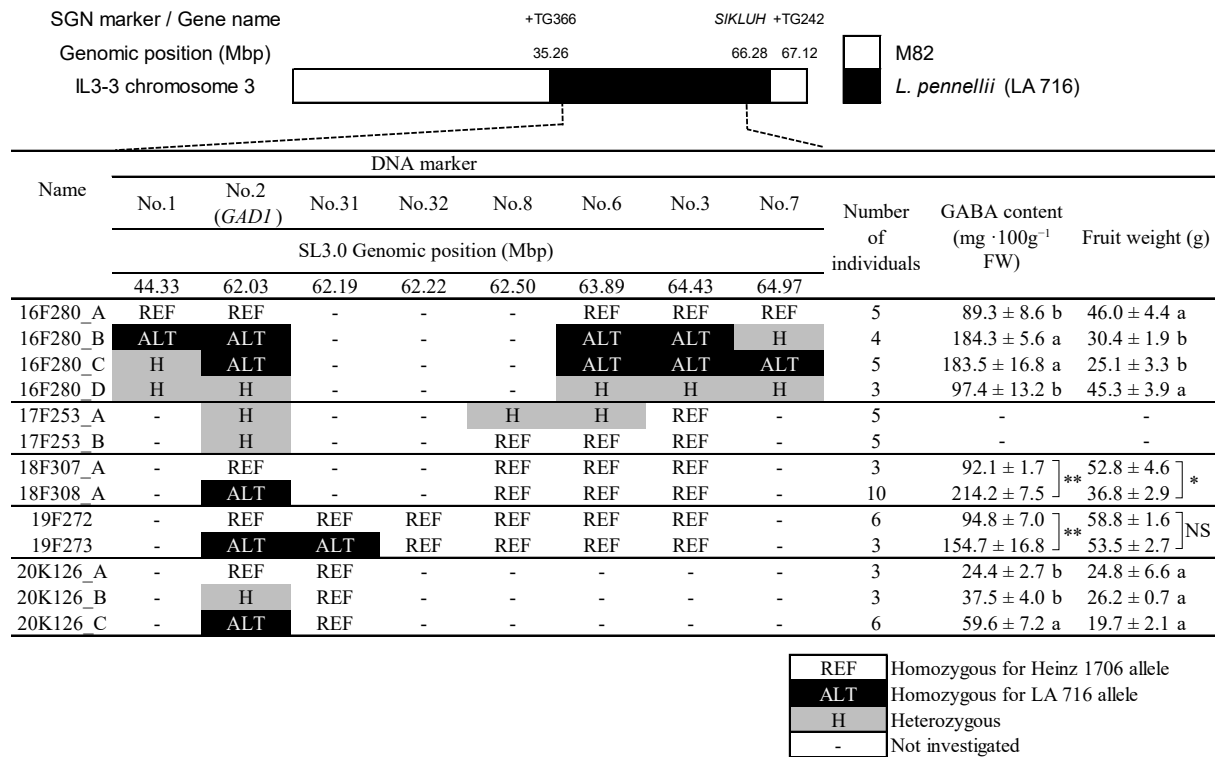


**Fig. 2.** Genetic mapping for GABA content and fruit size. Values are presented as means ± standard error. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; NS, not significant (Student's *t*-test). Different lowercase letters denote significant differences, as determined by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests. The +TG terms are SGN markers located in the outermost region of IL3-3, which has been confirmed to be from the ALT allele.

genotype at marker No. 2 and homozygous for the REF allele at markers No. 8, 6, and 3, comprising group 17F253\_B. As above, we cultivated these plants and collected their  $F_4$  generation seeds, designated 18F307 and 18F308, respectively. We genotyped 100  $F_4$  plants each for 18F307 and 18F308 using markers No. 2, 8, 6, and 3. Since marker No. 3 was homozygous for the REF allele in 17F253\_A and 17F253\_B populations, we did not test marker No. 7, which is closer to the end of the chromosomal substitution segment than marker No. 3. Three plants homozygous for the REF allele at all markers were designated 18F307\_A. We also identified 10 plants homozygous for the ALT allele at marker No. 2 and homozygous for the REF allele at markers No. 8, 6, and 3, forming group 18F308\_A. Plants from group 18F308\_A had a higher GABA content ( $P < 0.001$ ) and lower fruit weight ( $P = 0.021$ ) than those from group 18F307\_A (Fig. 2). Importantly, the GABA content of the plants from group 18F308\_A was similar to that of IL3-3, indicating that these plants carry the causal gene responsible for high GABA levels. The plants from group 18F308\_A had a smaller average fruit size than those of the parental cultivar 19F272, suggesting that the small-fruit trait remained linked to the high-GABA trait in these plants.

Despite the lower average fruit weight of 18F308\_A compared to 18F307\_A, one individual showed a relatively high fruit weight of 48.7 g. We collected  $F_5$  seeds

from the 18F308\_A plant with the heaviest fruit weight, and defined this population 18F1853. Of 15 plants grown from 18F1853, we chose a single plant with the heaviest fruits and allowed self-pollination to obtain the  $F_6$  generation, designated 19F273. In this  $F_6$  generation, the GABA content of 19F273 plants was significantly higher than that of parental line 19F272 plants ( $P = 0.005$ ) (Fig. 2). The weight of fruits from 19F273 plants was not significantly different from that of 19F272 plants ( $P = 0.117$ ). Thus, 19F273 plants had high GABA levels without a concomitant drop in fruit weight, suggesting a break in the tight linkage between the two causal loci. DNA marker analysis revealed that 19F273 plants were homozygous for the ALT allele at markers No. 2 and 31, and they were homozygous for the REF allele at markers No. 32, 8, 6, and 3 (Fig. 2). The recovery of fruit weight in the individual from the 18F308\_A population suggests that the traits of high-GABA content and low fruit weight became genetically separated at this stage, and that this separation was inherited by subsequent generations.

#### Genetic mapping of the high-GABA trait in backcrossed lines

To independently confirm that the causal gene is located in the genomic region between markers No. 1 and 32, additional genetic mapping was performed using backcrossed lines. We generated the  $BC_4F_3$

tomato population 20K126, which was derived from an interspecific cross between IL3-3 and the cherry-type breeding inbred line 20K124, followed by four backcrosses with 20K124 and self-pollination. At each generation, we identified plants heterozygous for marker No. 2. After these four backcrosses, we genotyped about 400 plants from a BC<sub>4</sub>F<sub>2</sub> segregating population derived by self-pollination of BC<sub>4</sub> heterozygous plants to look for recombination events using markers No. 2, 31, 32, 8, and 7. We obtained five plants with a recombination event within this set of markers, with one plant being homozygous for the REF allele at markers No. 31, 32, 8, and 7, as well as heterozygous at marker No. 2. We allowed this latter plant to self-pollinate and collected seeds for the BC<sub>4</sub>F<sub>3</sub> generation. We genotyped the resulting BC<sub>4</sub>F<sub>3</sub> plants and grouped them based on their genotypes at markers No. 2 and 31. As we confirmed that all plants were homozygous for the REF allele at marker No. 31, we focused on the genotype at marker No. 2. Group 20K126\_A comprised three plants homozygous for the REF allele at marker No. 2, and group 20K126\_B consisted of three plants heterozygous at this marker. Six plants homozygous for the ALT allele were defined as group 20K126\_C. We cultivated all plants and determined their fruit phenotypes. Although the GABA contents of plants from group 20K126\_C were more than two-fold higher and significantly greater ( $P = 0.013$ ) than those for plants from group 20K126\_A (Fig. 2), no statistically significant difference in fruit weight was observed between the two groups ( $P = 0.550$ ). Similarly, no statistically significant difference in fruit weight was observed between groups 20K126\_B and 20K126\_C ( $P = 0.391$ ). Therefore, plants from group 20K126\_C showed high GABA levels without a reduction in fruit weight (Fig. 2), suggesting that the high-GABA trait and the low-fruit-weight trait separated from each other in the backcrossed lines.

### Sequence analysis of GADs in cultivated and wild tomatoes

We investigated the genome sequences of *SIGAD1* in the tomato cultivar Heinz 1706 and *SpGAD1* in the wild tomato line LA 716. We obtained the nucleotide and amino acid sequence information for *SIGAD1*, along with the same information for *SIGAD2* and *SIGAD3* from Heinz 1706, from the SGN database. We detected a 69-bp deletion in intron 1 and a 61-bp deletion in intron 2 of *SpGAD1* in the wild tomato accession relative to cultivated tomatoes (Fig. 3). No insertions or deletions of amino acids between *SIGAD1* and *SpGAD1* were observed, and there were no frameshift mutations. We identified five nonsynonymous polymorphisms between *SIGAD1* and *SpGAD1*: one in the first exon and four in the fourth exon (Fig. 3). Plant GADs contain a calmodulin (CaM)-binding domain (CaMBD) at their C termini (Yap et al., 2003) that regulates GAD activity (Snedden et al., 1996). Conserved tryptophan (W) residues and lysine (K) clusters contribute to CaM binding within the C-terminal regions of GADs (Akama and Takaiwa, 2007; Arazi et al., 1995). Glu (E) and Asp (D) residues act as pseudosubstrates and exert an autoinhibitory role, also within the C terminus of GADs (Yap et al., 2003). However, none of the polymorphisms between *SIGAD1* and *SpGAD1* were included within the critical residues of the putative autoinhibitory domain or CaMBD (Fig. 3).

### Discussion

Although we observed a wide range of GABA content in tomato fruits, possibly reflecting cultivation environmental factors or the fruit maturity stage, the IL3-3 line reached a high GABA content of at least 145.1 mg·100 g<sup>-1</sup> FW over three years, whereas the tomato cultivar M82 only achieved values about 31% those of IL3-3 (Table 3). Thus, IL3-3 has high potential

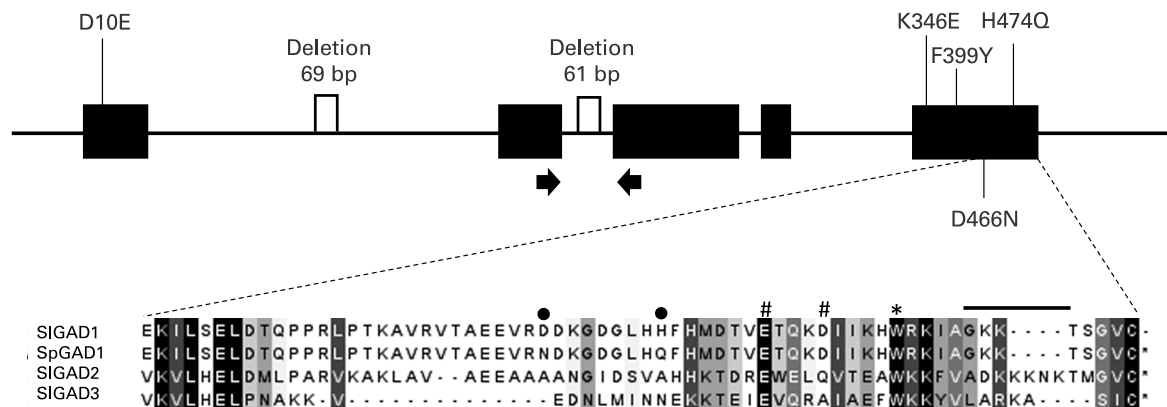


**Fig. 3.** Sequence polymorphisms in *SIGAD1* between *S. lycopersicum* and *S. pennellii*. Top, diagram of the *SIGAD1* locus, showing the polymorphisms between *S. lycopersicum* Heinz 1706 and *S. pennellii* LA 716. For each amino acid substitution, the amino acid in *S. lycopersicum* Heinz 1706 is listed first. The deletions are present in the first and second introns. Bottom, multiple sequence alignment of C-terminal ends of *SIGAD1*, *SIGAD2*, and *SIGAD3* from *S. lycopersicum* Heinz 1706 and *SpGAD1*. Identical and similar amino acids are indicated by black and gray boxes, respectively. The positions of Trp (W), the Lys (K) cluster, and two pseudosubstrate residues are indicated by an asterisk, a black line, and pound signs, respectively. Substitutions in *SIGAD1* compared to *SpGAD1* are indicated by black dots.

as a line that confers high GABA levels. Unfortunately, this trait co-segregates with a small-fruit trait caused by a tightly linked locus. To isolate the GABA-rich trait, we developed several segregating lines in this study to narrow down the size of the chromosomal segment originating from *S. pennellii*, with the added hope of breaking the linkage between the two co-segregating traits. We confirmed the presence of a chromosomal region derived from *S. pennellii* using DNA markers. Mapping efforts with  $F_2$  segregating plants located the causal locus for the high-GABA trait to between markers No. 1 and 7, suggesting the presence of a GABA metabolism-related gene within this interval (Fig. 2). After four more generations of selection from  $F_2$  plants to  $F_6$  plants, we anchored the locus responsible for the high-GABA trait between markers No. 1 and 32 (Fig. 2).

GAD catalyzes the biosynthesis of GABA from glutamate. Notably, one of the three *GAD* genes in the tomato genome, *SIGAD1*, is located within a region introgressed from *S. pennellii* into IL3-3, offering a high-confidence candidate gene for the high-GABA trait. A sequence comparison between *SIGAD1* (using the Heinz 1706 reference genome) and *SpGAD1* from *S. pennellii* revealed several nonsynonymous polymorphisms, as well as two deletions within introns (Fig. 3). However, it has not been previously determined whether the resulting amino acid substitutions affect enzyme activity. If *SIGAD1* and *SpGAD1* exhibit comparable enzymatic activities, another possible explanation for the greater GABA content that may be conferred by *SpGAD1* is that this gene is more highly expressed than *SIGAD1*. *SIGAD2* and *SIGAD3* are highly expressed during immature fruit stages and play significant roles in GABA biosynthesis (Takayama et al., 2015). Although *SIGAD1* expression increases after the breaker stage in fruits (Gallego et al., 1995), research on the specific effects of increasing *SIGAD1* expression on GABA content is limited. An experimental exploration of this idea is necessary to elucidate the precise effects of polymorphisms on protein function or expression levels and their associated GABA levels.

Although IL3-3 showed higher GABA levels than the tomato cultivar M82, its fruits were about half the weight of those from M82 (Table 3). IL3-3 and its derived  $F_2$  mapping lines accumulated high levels of GABA in their fruits, which all had a low weight, negatively affecting breeding potential and outcomes. This co-segregation suggested that the loci responsible for the high GABA content and the lower fruit weight were closely linked. Considering the fruit weights measured in the  $F_2$  mapping population, we hypothesize that the small-fruit trait is controlled by a recessive gene(s) located between markers No. 1 and 7 (Fig. 2). Incorporating an unwanted trait along with a desirable trait during introgression of a gene of interest from a donor into an elite cultivar is known as linkage drag. For example,

the *Ty-1* gene, which originated from the wild tomato species *S. chilense*, confers resistance against tomato yellow leaf curl virus, but is closely linked to undesirable traits such as reduced marketable yield and an increased incidence of foliar diseases (Hutton et al., 2015). Between the  $F_2$  and  $F_6$  generations, we succeeded in separating the loci associated with each of the two traits, as plants from the  $F_6$  generation population had fruits of normal size and weight, suggesting that the causative locus related to low fruit weight is located closer to the end of the chromosomal substitution segment than marker No. 31, near the locus responsible for the high GABA content but independent of that locus. In the  $BC_4F_3$  population, although the difference in fruit weight between 20K126\_A and 20K126\_C was not statistically significant, the substantial variation among lines carrying the genotype from the cultivated parent may have masked potential differences. Nevertheless, despite a more than two-fold increase in GABA content derived from *S. pennellii*, the observed change in fruit weight remained within the expected phenotypic variation of cultivated genotypes. This result also supports the feasibility of introducing the high-GABA trait without causing a substantial reduction in fruit size. Notably, the quantitative trait locus (QTL) for fruit weight, *fw3.2*, which controls tomato fruit weight and is a key feature discussed in the context of domestication (Grandillo et al., 1999), is located within the introgressed segment carried by IL3-3. Plants with alleles having a lower copy number of cytochrome P450 *SIKLUH*, the causal gene of *fw3.2*, produce lighter fruits (Alonge et al., 2020). We speculate that *SIKLUH* may affect fruit weight in IL3-3 and its derived lines with smaller fruits. However, *SIKLUH* is located closer to the end of the chromosomal substitution segment than marker No. 7 and  $F_4$  mapping lines homozygous for the REF allele at No. 3, which is closer to the start of the chromosomal substitution segment than marker No. 7, bore small fruits (Fig. 2), suggesting that the low fruit weight trait derived from IL3-3 may not be caused by variation at *SIKLUH*. We propose that currently unknown genes related to the low-fruit-weight trait are located close to the gene responsible for the high-GABA trait.

Based on the mapping results of the  $F_2$  and  $BC_4F_3$  populations, we detected no significant difference in GABA content between plants heterozygous or homozygous for the REF allele across the presumptive location of the locus responsible for the high-GABA trait. This result suggests that this locus is recessive or partially dominant. To achieve higher GABA content, it will be necessary to obtain plants homozygous for the allele derived from the wild tomato *S. pennellii*. When the goal is to collect fruits from  $F_1$  hybrid tomatoes, the causal locus will need to be introduced into both parental lines and segregants homozygous for the *S. pennellii* locus identified, making the breeding

program more complex. To help with genotyping via MAS, we developed DNA markers to select the high-GABA trait, which should significantly improve breeding efficiency and help develop tomato varieties with high GABA content.

In this study, we noticed substantial variation in GABA content among mapping populations, with the F<sub>2</sub> group 16F280\_A and the breeding inbred line 19F272 accumulating higher GABA levels than the tomato cultivar M82 and the BC<sub>4</sub>F<sub>3</sub> lines 20K126\_A, all of which were homozygous for the REF allele at all markers across the mapping interval (Fig. 2). This finding suggests the presence of additional genes related to GABA content. The introduction of high GABA-related genes derived from IL3-3 resulted in higher GABA contents in all lines of the tested cultivated varieties, M82, 19F272, and 20K124, suggesting the potential to further enhance GABA content by combining this gene with other GABA-related genes.

In conclusion, we obtained two tomato lines producing fruits with high GABA content due to a small chromosomal segment surrounding the *SIGAD1* gene originating from the wild tomato *S. pennellii*. In our efforts to narrow down the location of the locus responsible for the high-GABA trait, we identified plants with recombination events that broke the tight linkage between the high-GABA trait and the small-fruit trait typically conferred by this *S. pennellii* chromosomal substitution. It will now be possible to develop high-GABA tomato varieties using the markers developed in this study, making a further contribution to improving human health.

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