Utilization of High GABA Tomato via CRISPR/Cas9 for Hybrid Breeding

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Abbreviations

GABA: gamma-amino butyric acid

SN: site-specific nuclease

NHEJ: non-homologous end joining

HR: homologous recombination

GAD glutamate decarboxylase

CaMBD: Ca^{2+}/calmodulin binding domain

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9

Chapter 1. General introduction

GABA and genes responsible for GABA shunt

Gamma-amino butyric acid (GABA) is a non-proteogenic amino acid present in bacteria, fungi, animals and plants. GABA functions as an inhibitory neurotransmitter in animals (Owens and Kriegstein 2002, Bowery, 2004). After the first report on GABA shunt in petunia, biosynthesis and metabolism in many plants have been reported (Baum *et al.*, 1993), but the functional role of GABA is not fully understood. In tomato fruits NMR study using cherry tomato have revealed that GABA is the predominant free amino acid in green fruits, composing up to 50% of total free amino acids, and rapidly decreased during the ripening in the cherry tomato cultivar (Rolin *et al.*, 2000). In plants, GABA is metabolized through a pathway referred to as the "GABA shunt," which bypasses two steps of the tricarboxylic acid (TCA) cycle. It is started with the decarboxylation of glutamate (Glu) into GABA by glutamate decarboxylase (GAD) in cytosol. A secondary pathway of GABA synthesis may be via polyamine (putrescine and spermidine) degradation (Fait *et al.*, 2008, Shelp *et al.*, 2012a). Under oxidative stress, a non-enzymatic conversion from proline has also been reported (Signorelli *et al.*, 2015). GABA is degraded by the action of GABA transaminase (GABA-T) in the mitochondrial matrix. In this reaction, succinic semi-aldehyde (SSA) is produced with the possible participation of several amino acceptors, such as α-ketoglutarate, pyruvate or glyoxylate (Clark *et al.*, 2009, Shelp *et al.*, 2012b). Subsequently, SSA is converted by SSA dehydrogenase (SSADH) to succinate in the mitochondria (Shelp *et al.*, 2012b, Bouché *et al.*, 2003), and then SSA re-enters the TCA cycle. Alternatively, SSA can be converted to gamma-hydroxybutyric acid (GHBA) via the action of GHB dehydrogenase (GHBDH) (Andriamampandry *et al.*, 1998, Breitkreuz *et al.*, 2003).

GAD genes in plant were firstly isolated from petunia (Baum *et al.*, 1993), and subsequently GAD homologs have been identified in other plants. In tomato, three GAD genes were isolated from *Solanum lycopersicum* cv. "Micro-Tom" (Akihiro *et al.*, 2008). Among them, *SlGAD2* and *SlGAD3* showed correlation on GABA accumulation in tomato fruits. In short, as expression of *SlGAD2* and *SlGAD3* increased, GABA was accumulated in tomato fruit. In another study using transgenic engineering, RNA interference lines of *SlGAD1*, *SlGAD2*, *SlGAD3* and all three *SlGAD*s were generated (Takayama *et al.*, 2015). Suppressed *SlGAD2* and *SlGAD3* resulted in severely decreased level of GABA in fruits while *SlGAD1* showing no relationship with the level of GABA. These results indicated that *SlGAD2* and *SlGAD3* are responsible for accumulation of GABA in tomato fruits.

Plant GAD generally has Ca^{2+}/calmodulin binding domain (CaMBD) at C-terminus region, mostly consisting of 30-59 amino acids and functioning as CaMBD. This domain is considered that inhibits GAD activity at physiological pH by folding the active site of GAD. This auto-inhibition is suppressed when the active site is unfolded by conformational changes promoted via an acidic pH and/or by binding of Ca^{2+}/calmodulin to the CaMBD. GAD activity is nearly Ca^{2+}/calmodulin independent under pH6.5, but *in vitro* study under physilological pH reveled that GAD activity is stimulated by increased calcium via Ca^{2+}/calmodulin activation (Snedden *et al.*, 1996, Shelp *et al.*, 1999). C-terminus CaMBD is considered to function as an autoinhibitory domain. Transgenic plants with removed CaMBD showed highly increased GABA accumulation (Akama and Takaiwa 2007). Truncated *OsGAD2* showed higher level of Ca^{2+}/calmodulin-independent activity compared to that of WT *in vitro* and contributes to the high level of GABA in rice. Transgenic tomato with truncated C-terminal of *SlGAD3* (Takayama *et al.*, 2017) also showed the higher accumulation of GABA. Over-expression of *SlGAD3* with truncated C-terminal region results

in 11-12 times higher level of GABA compared to that of WT, while over-expression of full length of *SlGAD3* showed 6-7 folds higher level of GABA than that of WT. The highest level of GABA (about 29 μmol/gFW) caused abnormal pigmentation, not turning to fully red, of fruits. Targeted mutagenesis on *SlGAD3* via CRISPR/Cas9 also revealed the effect of truncation of C-terminus on the level of GABA (Nonaka *et al.*, 2017). Random insertion/deletion at target sequence occurred and caused early stop codon, and resulted in removal of C-terminus region, autoinhibitory domain. Transgene-free T_1 plants showed high-GABA contents in the fruits, which was 17-times higher than the wild type of "Micro-Tom" (WT-MT).

The advantage of GABA ingestion for improving human health

GABA is effective in the prevention of certain life style-related diseases, including hypertension and diabetes (Inoue *et al.*, 2003, Nishimura *et al.*, 2015), and also has anti-stress properties (Abdou *et al.*, 2006). Several studies on humans and experimental animals have shown that administration of exogenous GABA is effective in lowering blood pressure in patients with mild high blood pressure or high normal blood pressure (Eliott *et al.*, 1959, Takahashi *et al.*, 1961, Inoue *et al.*, 2003, Nishimura *et al.*, 2015). GABA also decreases the glucose level in diabetics (Tian *et al.*, 2011). Intake of GABA through the daily diet helps to prevent "life style-related diseases" from becoming serious.

In animals, GABA is widely distributed in central nervous system. Also, GABA is found in spinal fluid and blood as relatively high concentration. The level of plasma GABA concentration is known to be low in patients with Huntington's chorea and depression and to

be high in patients with hepatic encephalopathy (Petty *et al.*, 1984). GABA also involved in the regulation of cardiovascular functions, such as to depress the elevation of systolic blood pressure and to induce mental relaxation (Mody *et al.*, 1994, Abdou *et al.*, 2006). Anti-stress effects were observed by ingestion of GABA on brain wave test (Abdou *et al.*, 2006) that administration of 10mg of GABA induced significantly increased alpha waves related to relaxing and anxiolytic effect. Also, it contributes to immunity enhancement under stressful conditions. Hypertension is one of the most common lifestyle-related diseases. For its effect on decreasing blood pressure, oral intake GABA has been studied in many GABA-enrich foods. The consumption of GABA enriched foods such as milk (Hayakawa *et al.*, 2004), soybene (Shizuka *et al.*, 2004) and Tempeh (Aoki *et al.*, 2003) has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats. GABA-rich tomato cultivar showed a dose-dependent antihypertensive effect on systolic blood pressure (Yoshimura *et al.*, 2010). Although foods mentioned above contain GABA, but the concentration of GABA is generally insufficient for preventing life style-related diseases for human, and thus GABA enrichment of food is necessary to confer a health-promoting effect.

F_1 hybrid breeding

Intake vegetables in daily diet are highly related to human health, especially to reduce of cardiovascular disease like stroke. Vegetables are important for balanced nutrition like vitamins, minerals. For consumer, breeding has been developed to fulfill the objective standard such as quality, appearance, shelf-life, taste. For growers, other traits such as increased yield or less-labor-needed crops being resistant or tolerant against diseases and insects had paid attention. Vegetable breeding for nutritional quality was not mentioned as a

primary goal in plant breeding, but effort for nutritionally improved crops has been carried on through last few decades (Bai and Lindhout, 2007). Consumers are becoming more aware of nutritional traits. A golden tomato can be a good example which can be provide a person`s full daily vitamin requirements (Dias, 2014).

Commercially distributed fresh tomato in market is mainly hybrid like many other vegetable crops for their better marketability based on heterosis (Cheema and Dahliwal 2005). Cross between two elite cultivars, guarantee uniformity of improved traits. Usually, hybrid tomato represents intermediate character between two parental lines. The global vegetable seed market was estimate at US$.41 billion in 2010, and worldwide the share of hybrid seed is increasing at a fast pace of 8-10% annually in most of the vegetables crops (Dias 2014). For hybrid breeding, the most important issue is retaining the good parental cultivars, heirloom or mutant whatever it is, which can be directly used for hybrid crossing. Selection among mutants generated by treatment of ethyl methane sulfonate (EMS) or radiation is widely used methods for cultivar improvement, but the amount of labor and time to inbreed for finding and fixing some valuable traits is tremendous. Another option is genetically modified plants by inserting favorable genes. Although many genetically modified crops show improved traits to give the crops more attractive to consumers, and lessen labor by pesticide and herbicide resistance, genetic engineering with remained inserted construct is still controversial (Wolfenbarger and Phifer, 2000, O'Callaghan *et al.*, 2005). Genome editing via site-spefcific nuclease can be a good method for making precise mutations for crop breeding and may possible to avoid genetically modified organism (GMO) controversy.

Genome editing technology

Manipulation of specific target sequence on genome using site specific nucleases has been reported in many previous researches. It can be easily applied for increasing/decreasing functional materials in plants, which accumulation mechanism is well elucidated. Zinc-Finger Nuclease (ZFN) have been received attention as a candidate having immense possibility for targeted genetic modification in many plants over last two decades (Lioyd *et al.*, 2005, Townsend *et al.*, 2009, Shukla *et al.*, 2009). Transcription Activator Like Effector Nuclease (TALEN) also got attention recently as a tool for genome editing for its possibility of more precise distinguish the target site within genome comparing to ZFN. However, both methods are too labor-intensive and time-wasting to produce the construct which basically based on unit assembly.

Recently emerged clustered regularly interspaced short palindromic repeats/CRISPR-assosiated nuclease 9 (CRISPR/Cas9), a bacterial RNA-guided DNA endonuclease system, becomes clearly taking the center of genome editing. CRISPR/Cas9 has been widely applied to many organisms, mainly due to the simplicity of selecting target site in genome and of relative easiness to make construct. Only 20 nucleotides possessing protospacer-adjacent motif, 5-NGG-3 (PAM) sequence are enough to be a target. Also, the most important forte of CRISPR/Cas9 is its simplicity producing. (Shan *et al.*, 2014, Feng *et al.*, 2014, Jiang *et al.*, 2013).

Every site-specific nuclease has been applied in two ways. Basically, genome editing technology is based on double strand break (DSB) in genomic DNA, formed by designed nuclease cutting its target sequence. There are two strategies to repair the broken DNA ends, homologous recombination (HR) and/or non-homologous end joining (NHEJ) (Ray and Langer, 2002, Britt and May, 2003). HR refers the homologous sequence, so DNA can be

restored to the original information through HR. Repairing via NHEJ, by contrast, broken DNAs are connected without any reference. Therefore, random number of nucleotides are inserted and deleted to fit the ends of DNA before DNA ligating why they usually called error-prone DNA repairing. As a result, mutation occurs at repaired site. Although HR seems favorable strategy to preserve genetic information, actually preferred route for DNA repairing seems NHEJ in eukaryotes, especially in plants (Ray and Langer, 2002). Accordingly, in site-specific nuclease system, NHEJ can be technically used for random mutagenesis when designed nuclease properly induces DSB on targeted loci or used for large size of deletion (reviewed by Chen and Gao, 2013, Shan *et al.*, 2013). On the other hands, HR can be used for site-specific insertion (Li *et al.*, 2011, Bedell *et al.*, 2012, Zhang *et al.*, 2013).

To sum the genome editing methods by site-specific nuclease briefly, the best merit of genome editing via site-specific nuclease seems mutation on target is remained after removing transformation cassette unlike usual GM, losing the effect of over-expression or RNA interference under the cassette removal by segregation. Also, various type of mutation can be obtained via NHEJ, due to the imperfect DNA repair.

Targeted mutagenesis via CRISPR/Cas9 in tomato

Tomato is economically important crop, one of the most produced vegetable in the world (FAOSTAT). It is also fascinating material for research with its relatively small genome size, which was sequenced over (Tomato Genome Consortium 2012). Tomato bears berry-type fruits, so it is also good for the researches on fruit development or maturation. From 2013, research of targeted mutagenesis via CRISPR/Cas9 system has been reported in

plants (Pennisi, 2013, Li *et al.*, 2013, Nekrasov *et al.*, 2013). CRISPR/Cas9 mediated mutagenesis applied to tomato soon for its advantage for research, such as possibility to study of fruit development, easiness for accessing to information of whole genome and availability of efficient transformation methods. Accordingly, tomato was one of the vegetable crops that genome modifications reported, using CRISRP/ Cas9 (Brooks *et al.*, 2014).

Brooks *et al.* (2014) applied CRISPR/Cas9 to tomato successfully using *SlAGO7*, which can be immediately detected its phenotype, the first leaflets without petioles and another leave showing lack of laminae. However, intensive study for finding off-target binding, they found two sites possessing high similarity with target gene were all mutated. Further practical applications of CRISPR/Cas9 to tomato have been reported. Ueta *et al* (2017) targeted *SlIAA9*, which is known a responsible gene for tomato parthenocarpic fruit development (Wang *et al.*, 2005) in “Micro-Tom” and commercial cultivar “Alisa Craig”. Transgenics and their progenies with no transformation cassette possessing disrupted second exon of *SlIAA9* still, showed representative *SlIAA9* knockout phenotype, highly merged leaf and parthenocarpic fruit development.

It is also applied for improved disease-resistance, a powdery mildew resistant tomato was produced (Nekrasov *et al.*, 2017). Tomato line with variety resistant to the powdery mildew fungal pathogen *Oidium neolycopersici* was generated by targeting *SlMlo1*, a major contributor to powdery mildew susceptibility. Transgene-free line, carrying no foreign DNA, was generated and representing resistance to fungal pathogen due to the 48bp deletion of *SlMlo1*.

Application for improved tomato shelf life was studied (Yu *et al.*, 2017). In this study, they targeted *SlALC*, which represent little negative impact on fruit quality, better fruit color

and aroma properties. In this study, HR-mediated gene replacement also tried and proved that gene replacement using CRISPR/Cas9 is possible to use in tomato, although its rate is quietly low than random insertion/deletion caused by NHEJ in tomato. Mutants generated via NHEJ repair and HR-mediated substitution showed highly improved shelf life.

Metabolite engineering was also studied in tomato (Nonaka *et al.*, 2017). Dramatically enhanced GABA content was observed by targeting the responsible gene, *SlGAD3*, affecting on GABA accumulation in tomato. Transgene-free 4 types of mutants showed stably increased GABA contents on red fruits without affecting on the other tissue. The targeted mutagenesis on *SlGAD3* showed 15 times increased GABA contents comparing to the wild type Micro-Tom cultivar (WT-MT).

***SlGAD3*, a good target candidate gene to manipulate GABA contents in tomato**

SlGAD3 is the most effective candidate gene to modify the amount of GABA in tomato fruits. In previous study (Nonaka *et al.*, 2017), *SlGAD3* with truncated C-terminus via CRISPR/Cas9 showed extremely increased GABA contents on the T_1 generation of "Micro-Tom", TG3C37 (Nonaka *et al.*, 2017). Shorten C-terminal functions autoinhibitory domain under the circumstance of increased Ca^{2+}/Calmodulin. TG3C37 (divided into 4 type of mutation) showed stably increased GABA contents on Red ripe fruits without affecting the other traits. The targeted mutagenesis on *SlGAD3* brings 15 times increased GABA contents comparing to WT-MT.

Introgression of precise mutations into an elite variety takes too much time and is very laborious. Some countries, such as the U.S.A., have considered transgene-free genetically

edited crops as non-genetic modified organism (GMO) (Waltz, 2016). Under this circumstance, genome editing can be a well technique for time and labor-saving breeding. In this study, TG3C37 will be evaluated as a breeding material. More specifically, stability of TG3C37 for increasing GABA without affecting on the other traits will be discussed in Chapter 2. Effect of increased GABA on the other free amino acid, vegetative growth and fruit appearance will be discussed. Also, the practical application and evaluation of TG3C37 as a breeding material for hybrid breeding, will be discussed in Chapter 3. Fruit quality and GABA contents of F_1 between TG3C37 and commercial cultivar 'Aichi-First' will be compared to that of F_1 of 'Aichi-First' and WT-MT.

Chapter 2. Genetic stability of mutated *SlGAD3* via CRISPR/Cas9 in the following generation

2.1 Introduction

Vegetables are important for balanced nutrition like vitamins and minerals. Intake vegetables in daily diet are highly related to human health, especially to reduce of cardiovascular disease like stroke. Breeding has been developed to fulfill the objective standard such as labor-reducing crops being strong against diseases and insects had paid attention. Vegetable breeding for nutritional quality was not mentioned as a primary goal in plant breeding, but effort for nutritionally improved crops has been carried on through last few decades (Bai and Lindhout 2007). Consumers are becoming more aware of nutritional traits, and recently developed genome editing technology can be a good answer to get a good breeding material. Genome editing is inducing mutation on specific site on genome to manipulate 'specific site'-related gene's function. For that, mechanism to recognize the 'specific site' and to induce mutation on the site is needed. Plus, it should be easily changed according to the target site. Therefore, concept of genome editing has been developed along with site-specific nuclease. Genome editing technology using site-specifically directed site-specific nuclease, such as ZFN, TALEN and CRISPR/Cas9, has been reported for last few decades. Three platforms allow inducing mutation on intended sequence in genome, leading loss of function of target gene(s).

Genome editing is started with double strand break (DSB), induced by site-specific nuclease. Basically, there are mainly two strategies to repair broken DNA ends, homologous recombination (HR) and non-homologous end joining (NHEJ) (Ray and Langer, 2002, Britt

and May, 2003). HR refers other homologous sequence when repairing, so DNA can stably conserve original information. Therefore, HR applied technically for site-specific insertion (Li *et al.*, 2011, Bedell *et al.*, 2012, Zhang *et al.*, 2013). On the other hands, DSB repairing via NHEJ does not refer any sequence. As a result, random number of nucleotides are inserted and/or deleted to ligate the ends of DNA, thus they usually called error-prone DNA repairing. Although HR seems favorable strategy to preserve genetic information, the frequency is very low in cell cycle. The system mainly works in DNA synthesis term. In contract, NHEJ, error-prone DNA repairing, occurs high frequency all through the cell cycles in eukaryotes, especially in plants (Ray and Langer, 2002). Technically, NHEJ are usually used for random mutagenesis.

Manipulation of specific target gene on genome using site-specific nuclease has been reported in many plants. ZFN has been received attention as a candidate having immense possibility for targeted genetic modification in many plants over last two decades (Lioyd *et al.*, 2005, Townsend *et al.*, 2009, Shukla *et al.*, 2009). TALEN also got attention recently as a tool for genome editing for its possibility of more precise distinguish the target site within genome comparing to ZFN. Both methods require much labor and time for construction of unit assembly. Recently emerged CRISPR/Cas9, a bacterial RNA-guided DNA endonuclease system is mainly used for genome editing. The key components of CRISPR/Cas9 system are a Cas9 endonuclease protein and two small RNA molecules, CRISPR RNA (crRNA) and trans-activating crRNA. In type II CRISPR system originated from *Streptococcus pyogenes*, invader's, *i.e.* phage, short DNA fragments integrated into a CRISPR locus are converted into crRNA and tracrRNA. crRNA and tracrRNA binds to target gene and forms chimeric RNA molecule and calls endonuclease Cas to the protospacer motif within the target DNA (Chen and Gao, 2013). Technically used RNA is designed as a fusion form of these two RNA,

usually called guide RNA (gRNA) (Cong *et al.*, 2013, Mali *et al.*, 2013). Only 20 nucleotides possessing PAM sequence are enough to be a target. Targeting is based on the intuitively understandable DNA-RNA interaction, this system is adapted to genome modification immediately. For application, the most important forte of CRISPR/Cas9 is its simplicity of producing and targeting. After determining the optimized Cas9 for some organisms, constructs can be relatively easily produced through cloning technique (Shan *et al.*, 2014, Feng *et al.*, 2014, Jiang *et al.*, 2013).

Tomato is economically important crop, one of the most produced vegetable in the world. It is also good material for research with its relatively small genome size (950Mb), which was sequenced over (Tomato Genome Consortium 2012). Tomato bears berry-type fruits, so it is also good for the researches on fruit developing or maturation. From 2013, research of targeted mutagenesis via CRISPR/Cas9 system was reported in plant (Pennisi, 2013, Li *et al.*, 2013, Nekrasov *et al.*, 2013). CRISPR/Cas9 mediated mutagenesis applied to tomato soon for its advantage for research, such as possibility to study of fruit development, easiness for accessing to information of whole genome and availability of efficient transformation methods. Accordingly, tomato was one of the vegetable crops that genome modifications reported, using CRISRP/ Cas9 (Brooks *et al.*, 2014). Many applications have been reported in tomato, to change phenotype (Brooks *et al.*, 2014, Ueta *et al.*, 2017), to extend fruit shelf life (Yu *et al.*, 2017), to improve disease-resistance (Kekrasov *et al.*, 2017).

Further application was reported in metabolite engineering in tomato (Nonaka *et al.*, 2017). C-terminals of SlGAD3, where possessing CaMBD, function as autoinhibitory domain under the circumstance of increased Ca^{2+}/calmodulin. CRISPR/Cas9 targeting the C-terminus region of SlGAD3 was introduced into dwarf cultivar tomato “Micro-Tom” and 4 types of

insertion/deletion mutation (TG3C37s) were acquired. All mutation causes early stopped proteins which causes truncated C-terminus of SlGAD3. Truncation results in highly increased SlGAD3 enzymatic activity, mainly due to the loss of auto-inhibition ability. As a result, the targeted mutagenesis on SlGAD3 brings 15 times increased GABA contents comparing to WT-MT without affecting on the other tissue. Many previous research showed that the effect of targeted mutagenesis via site-specific nucleases is maintained after generation proceeding. One of the advantages of genome editing can be sum up that maintained mutation after segregation of transformed site-specific nuclease gene cassette. In this chapter, vegetative growth and fruit properties of TG3C37 will be evaluated based on the aspect of stability of mutation. To evaluate breeding materials for increasing GABA contents in tomato fruits, GABA content of T_2 and T_3 generation on Red ripe (RED) stage will be analyzed.

2.2 Materials and methods

2.2.1 Plant materials

Four T_1 generation tomato lines, TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 containing mutations generated via CRISPR/Cas9 at the C-terminus of the *SlGAD3* gene (Figure 1a, b), was self-pollinated to get a stable homozygous mutation without transformation construct. T_2 generation plants also grew as isolated and also self-pollinated. T_2 and T_3 seeds were germinated on wet filter paper and transferred to rock wool at 2 weeks after from germination. All TG3C37s and MT-WT were cultured in a culture room under fluorescent light with a 16 h light (60 μmol/m^2/s)/8h dark photoperiod at 25℃, using standard nutrient solution (Otsuka A; Otsuka Chemical Co., Ltd., Osaka, Japan). For balanced growth of fruits, up to two flowers in one truss were pollinated. The time to breaker was counted during cultivation.

2.2.2 Genetic analysis of T_2 and T_3 lines by dCAPS analysis and genomic DNA sequencing

Mutation on *SlGAD3* on T_2 and T_3 generation was confirmed using the dCAPS method and sequencing. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Kim and Hamada, 2005). Leaf material (200 mg) was ground to a fine powder and pre-warmed CTAB at 65 ℃ was added to each sample. After adding the same volume of chloroform/isoamyl alcohol (24:1, v/v) followed by inverting the contents a

few times, all samples were centrifuged at 13,000 rpm for 15 min at 4ºC. Clear supernatant was transferred to a new tube and the same volume of iso-propanol was added. After maintaining at -20ºC for 2 h, the mixture was centrifuged at 13,000 rpm for 15 min at 4ºC and the supernatant was carefully discarded so as not to lose the DNA pellet. The pellet was rinsed using 70% (v/v) ethanol. After brief drying at room temperature, the pellet was re-suspended in 50 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) containing 40 µg/ml of RNase A (Amresco, texas, US).

For dCAPS analysis, PCR was carried out using *SlGAD3* C-terminal-specific primers (forward 5′-GATGAAGGTATACCCTTGGTGG-3′, reverse 5′- CACTCTCGCCTCTAT CGCTTAT-3′) using 100 ng of genomic DNA. A 50 µg aliquot of the amplicon was incubated with *Bst*XI (NEB, MA, US) for 3 h at 37ºC, and loaded onto a 2% agarose gel. Sequence of *SlGAD3* was read for more precise confirmation. The PCR product was diluted 50 folds with distilled water and used as a template. The forward primer used for gene-specific PCR was used for sequencing.

2.2.3 Analysis of plant and fruit characteristics

Plants height is measured after first florescence observed. Mature green fruits were harvested 30 days after from pollination, and Red ripe fruits were harvested 10 days after from Breaker stage. The diameter, length and weight of harvested fruits at the Mature green (MG) and Red ripe (RED) stages were measured to investigate the effect of the truncated C-terminus of *SlGAD3* on T_2 and T_3 generation. Fruit diameter was measured twice, and length was measured once. The fruits were weighed after removing old sepals and the pedicle. For

fruit size comparison, averages of the diameter, length, and weight of 10 fruits were used. Statically, the results obtained for each line were compared by Tukey's HSD test at $P < 0.05$.

2.2.4 Amino acid extraction

Free amino acid was extracted from 50–70 mg of the fine powder with 500 µl of 8% trichloroacetic acid (w/v). After brief shaking, the mixture was centrifuged at 13,000 rpm for 20 min. Three hundred microliters of the resulting supernatant were transferred to a new tube, to which 400 µl of di-ethyl ether was added. After 10 min of vigorous shaking, the mixture was centrifuged at 12,000 rpm for 10 min. After removing the diethyl ether, this step was repeated once more. Thereafter, the diethyl ether was evaporated in a chamber for 30 min at room temperature.

2.2.5 Measurement of GABA contents by GABase method

For screening GABA content, the GABase method was used as described previously by Saito *et al.* (2008). One hundred and fifty microliters of reaction buffer containing 0.1 M sodium-pyrophosphate (pH 9.0), 50 mM mercaptoethanol, 50 mM NADP, and 60 mM α-keto glutaric acid was added to 20 µl of extracted amino acids. After measuring absorbance at 340 nm, 0.005 units of GABase in a 1 µl volume was added with 24 µl of 0.1 M sodium pyrophosphate (pH 9.0). The mixture was incubated at 37ºC for 90 min and the absorbance at 340 nm measured. To measure the content of GABA, the absorbance was measured after and before incubation, and the differences were calculated. Statically, the results obtained for each line were compared by Tukey's HSD test at $P < 0.05$.

2.2.6 RNA extraction, cDNA synthesis and RT-PCR

To investigate the relative expression of *SlGAD3* in T_2 generation, quantitative RT-PCR was carried out. Three fruits at each stage are harvested and considered as one sample, and three replicates of MG and RED from each line were used. Fruits were quickly frozen after harvest and grounded into a fine powder in liquid nitrogen after removing jelly tissue and seeds. RNA was extracted from 100 mg of fruit powder using RNeasy plant mini kit (Qiagen, Hilden, Germany), and 1.5 ng of total RNA was used for cDNA synthesis. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan) and used as template for qRT PCR. Takara Thermal Cycler Dice Real-Time systemTP800 (Takara-Bio Inc., Shiga, Japan) was used with SYBR premix Ex Taq II (Takara-Bio Inc., Shiga, Japan). The reaction was carried out by 30 sec at 95ºC for pre-denaturation, and by 40 cycle of 2 step PCR at 5 sec at 9 ºC for denature and 30 sec at 60ºC for annealing/extension. Relative quantification of *SlGAD3* was normalized to the expression level of *SAND* gene, an internal control (Rodriguez *et al.*, 2008). Primers for *SlGAD3* were SlGAD3 Forward (5'- AAGGTCCTCCACGAGCTC -3'), SlGAD3 Reverse (5'- CCCTAACAAATAGATGCTTTCC -3'). Statically, the results (*SlGAD3* relative expression level over internal control *SAND*) of each line were compared by Tukey's HSD test at $P < 0.05$.

2.3 Results

2.3.1 Mutation types of each line and their genetic analysis

From many mutations caused by CRISPR/Cas9 on *SlGAD3*, T_1 lines were selected possessing 4 different types of mutations (Figure 1a and b), without CRISPR/Cas9 expression cassette. Mutation was confirmed using dCAPS methods and sequencing (Figure 1c, Figure 2), and mutations were remained as homozygous. TG3C37#21-7 and TG3C37#21-19 have 1 nucleotide inserted mutation and 53 nucleotides deleted mutation on C-terminal of SlGAD3, respectively. TG3C37#3-8 and TG3C37#3-13 have 6 nucleotides deletion with 1 insertion and 4 nucleotides deletion mutation on target site. All mutation causes early stop codon, so C-terminal of SlGAD3 is truncated (Table 3). As results, 34 (TG3C37#21-7), 40 (TG3C37#21-19), 36 (TG3C37#3-8) and 34 (TG3C37#3-13) amino acids of C-terminal are shortened, where is responsible region for auto-inhibition of SlGAD3. Mutation was stably maintained as homozygous on T_2 and T_3 also, as observed as SlGAD3 of T_1.

2.3.2 Plant height and fruit size in T_2 and T_3

Fruit size and plant height in T_2 and T_3 generation was measured to investigate the effect of truncated SlGAD3. Little difference was observed in fruit size. The diameter of fruits, a horizontal length of fruits, of TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 were 15.12±1.66, 14.08±1.36, 16.93±2.21, 17.12±1.91 (mm) respectively, while diameter of WT-MT was 16.76±1.26 mm (Table 1). Statistical differences were found only in two lines, TG3C37#21-7, TG3C37#21-19. Differences on length of fruits, a vertical

length of fruits, were found only TG3C37#21-19 (15.25±1.67 mm in average). The fruit length of TG3C37#21-7, TG3C37#3-8, TG3C37#3-13 and WT-MT were 16.84±1.52, 16.90±1.34, 16.63±1.32 and 16.64±1.02 (mm) respectively, which shares no statistical differences (Table 1). These results indicate that truncated C-terminal of SlGAD3 has no or little effect on the volume of fruits in T_2 generation. In T_3 generation of fruits, difference on fruit size was more decreased. The average diameter (mm) of TG3C37#21-7, TG3C37#21-19, TG3C37#3-8, TG3C37#3-13 and WT was 15.70±0.96, 15.20±1.20, 16.67±1.25, 16.67±0.96 and 16.75±1.18 (mm) respectively, which share no statistical difference (Table 2). The vertical length of fruits also didn't exhibit any difference (Table 2). The average vertical length of TG3C37#21-7, TG3C37#21-19, TG3C37#3-8, TG3C37#3-13 and WT-MT were 16.91±1.35, 16.49±1.18, 16.50±1.03, 17.23±1.72 and 16.56±1.00 (mm), respectively.

Average of one fruit weight in WT-MT, TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 were 2.96±0.62, 2.32±0.62, 1.92±0.54, 2.89±0.88 and 3.18±0.88 (g), respectively in T_2 generation (Table 1). Statistically, two lines TG3C37#21-7, TG3C37#21-19 are slightly lighter than fruits from WT-MT. In T_3 generation, average of fruit weight in WT-MT, TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 were 2.91±0.65, 2.49±0.44, 2.36±0.46, 2.89±0.58, 2.77±0.54 (g), respectively (Table 2). Statistically, fruits from four TG3C37s were slightly lighter than that of WT.

Truncated SlGAD3 did not affect on vegetative growth in T_2 and T_3 generation. The average plant height of WT-MT, TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 were 117.8±22.0, 101.2±13.2, 103.0±12.7, 114.0±18.6, 109.2±19.4 (mm), respectively in T_2 generation (Table 1). There were no statistical differences in TG3C37s and WT-MT. In T_3 generation also, the average of height in WT-MT, TG3C37ΔC#21-7,

TG3C37ΔC#21-19, TG3C37#3-8 and TG3C37#3-13 were 106.6±8.5, 102.2±7.3, 109.0±10.1, 112.4±14.6 and 116.0±18.3 (mm), respectively (Table 2). In summary, no difference was found on vegetative growth in T_2 and T_3 generation.

2.3.3 GABA content of fruits in T_2 and T_3 generations

To evaluate genetic stability of phenotype on mutated *SlGAD3*, GABA contents of T_2 and T_3 generations were measured. At MG stage in T_2 generation, GABA contents of all lines of TG3C37 were 17.03 ± 0.91, 13.64 ±0.92, 9.48 ±0.47, 10.58 ± 1.92 μmol/g FW for TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 respectively, which is highly increased level comparing to that of MT-WT, 4.05 ± 0.32 μmol/g FW (Figure 3a). Those are 4.2-, 3.4-, 2.3- and 2.6-folds higher level compared to that of WT-MT. At RED stage, GABA content showed a tendency of little bit increased (Figure 3b). TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13was 6.98 ± 1.06, 8.70 ± 2.17, 3.96 ± 0.33, 2.36 ±0.10 μmol/g FW respectively while that of WT-MT dropped to 0.99 ± 0.11 μmol/g FW. Finally, GABA content of each TG3C37 was higher than WT-MT almost 7.0, 8.7, 4.4 and 2.4 times at RED stage. GABA was decreased along with fruit development, MG to RED. In average 10.05, 4.94, 5.52, 8.22 and 3.05 of MG stage GABA were decreased TG3C37#21-7, TG3C37#21-19, TG3C37#3-8, TG3C37#3-13and WT-MT respectively. At RED stage almost 41%, 64%, 42%, 22% level of GABA of MG were remained while 25% GABA of WT-MT was remained.

In T_3 generation, highly increased GABA contents were observed in four TG3C37s. GABA contents of TG3C37#21-7 was 8.19 ± 0.28 μmol/g FW, which is 8.2-fold higher level

comparing that of WT (Figure 5). In case of TG3C37#21-19, 9.4-times higher level of GABA was observed, 9.42 ± 0.6 μmol/gFW, which is the highest level of GABA as T_2 generation do. GABA contents of TG3C37#3-8 and TG3C37#3-13 were 5.73±0.76 and 3.45±5.14 μmol/g FW, respectively. This is almost 5.7- and 3.5-folds higher level than that of WT although the GABA content is slightly lower than TG3C37#21-19 or TG3C37#21-7.

2.3.4 Relative gene expression of *SlGAD3* in T_2 generation

Relative *SlGAD3* expression was measured to compare truncated C-terminus SlGAD3 effect on their expression or not at MG stage. *SlGAD3* expression was normalized using *SAND* (Gidsifo *et al.*, 2014). TG3C37#21-19 and TG3C37#21-7 little bit lower level of gene expression comparing to that of WT (Figure 4). Those are almost 92% and 74% level of WT *SlGAD3* expression level. On the other hands, TG3C37#3-8 and TG3C37#3-13 showed clearly decreased expression level. Notably, expression level of TG3C37#3-13 is 40% comparing to that of WT. At the time point of MG, before breaker, *SlGAD3* expression is slightly to largely differed according to lines, which related to GABA content in fruits within TG3C37 (Figure 3, 5).

2.4 Discussion

F_1 hybrids are widely used for its marketability based on heterosis in many crops, and hybrid seed market is getting bigger (Cheema and Dahliwal, 2005). Therefore, it is important to retain a stable mutant material as a parental line to get tomatoes having better traits stably through hybrid breeding. Mutant generated via mutagen treatment has few drawbacks, although it may possible to generate plants that have desirable traits using mutagens, such as radiation or chemicals such as ethyl methane sulphonate (EMS). First, mutations are induced randomly. It means it is impossible to manipulate the site where mutation occurs, a fatal site or a weak one. Moreover, sometimes it is hard to find the genes responsible for the phenotypes. Second, it is time-intensive and labor-intensive to get a certain trait. For isolating a trait-responsible gene(s), it is needed to segregate through many times backcross. It will take at least 2 years to fix a specific mutant in case of “Micro-Tom”, which has relatively short life cycle comparing to other tomato genotypes. Whereas producing a breeding material via traditional genetic transformation is still controversial and remain some concern. Therefore, genome editing via site-specific nuclease is an alternative option to effectively generate tomato having desirable traits.

The best advantage of genome editing via site-specific nuclease can be sum up ‘getting various mutants rapidly without transformation cassette’. Unlike other transformation platform, genome editing allows to maintain its effect after transformation cassette removal. Also, various type of mutation can be obtained via NHEJ, due to the imperfect DNA repair (Chen and Gao, 2013, Shan *et al.*, 2013). In this chapter, TG3C37 was evaluated as a breeding material for high-GABA containing tomato (Table 3). Mutation caused by CRISPR/Cas9 on *SlGAD3* showed high possibility having forte as tomato breeding materials

as aforesaid. First, plants possess no transformation cassette in T_1 generation (Nonaka *et al.*, 2017). This is quite big forte as a breeding material which can avoid argument on GMO controversy. Mutations were confirmed to be fixed through 3 generations (Figure 1, 2). Mutations were stably inherited to the following generations. Next, mutation showed clear effect on intended traits, increasing GABA contents in tomato fruits (Figure 3, 5). Homozygous mutation producing C-terminal truncated *SlGAD3* conferred high-GABA contents of tomato fruits in the mutant comparing to WT-MT. The highest level of GABA in Red stage was observed in line TG3C37#21-19, which is 7 times higher level than that of WT (Figure 3, 5). Also, in line TG3C37#21-7 showed relatively high GABA contents, while TG3C37#3-8 and TG3C37#3-13 showed relatively low GABA contents, comparing TG3C37#21-19. These GABA content are also clearly higher than that of WT, 2-4 times higher at T_2 generation and 3-5 times higher at T_3 generation, but that was lower level comparing to TG3C37#21-19 or TG3C37#21-7.

In vitro assay revealed that C-terminal truncated SlGAD3 has extremely high enzymatic activity than wild type SlGAD3 (Nonaka *et al.*, 2017). Relative *SlGAD3* expression, which can detect SlGAD3 and SlGAD3ΔC both, can explain the differences between TG3C37 lines (Figure 4). Amount of GABA can be simplified ‘accumulated GABA until measured point minus degraded GABA flowing GABA shunt’. GABA content of each line at RED stage fruits showed a good correlation with *SlGAD3* expression (Figure 3, 4). Especially, TG3C37#3-13 showed 44% of *SlGAD3* expression level comparing to TG3C37#21-19, which line showed lowest GABA contents at RED (Figure 3). In T_3 generation, patterns of accumulated GABA contents in RED fruits was almost same with that of T_2 generation, which can convince that *SlGAD3* expression is stably settled by following generation (Figure 3, 5).

SlGAD3ΔC showed relatively low effect on vegetative growth and fruit size (Tables 1, 2). There were no lines that showed differences on plant height. Also, fruits from each line share similar fruit size. In previous research, overexpression truncated SlGAD3 showed dramatically increased GABA contents (Takayama *et al.*, 2015). At the same time, extremely increased GABA in the fruits inhibits its size and normal coloration. Therefore, relatively high GABA contents of TG3C37#21-19 can be a reason for setting a little bit smaller fruits (Table 1, 2). It has been reported that ingestion of 10-20 mg of GABA is effective on decreasing many cardiovascular diseases (Inoue *et al.*, 2003, Nishimura *et al.*, 2015, Fukuwatari *et al.*, 2001, Kazami *et al.*, 2002). GABA contents of TG3C37#2-7, TG3C37#2-19, TG3C37#3-8 and TG3C37#3-13 can be calculated to 85, 97, 59 and 36 mg/100g FW in T_3 generation, respectively. In case of TG3C37#2-7, TG3C37#2-19 which weigh approximately over 2 g, almost 10-20 fruits can fit the intake guide.

When considering the hybrid tomato present intermediate traits between two parental lines (Kitagawa *et al.*, 2005, Shalaby, 2013), high GABA containing tomato should be selected for F_1 generating. Two lines, TG3C37#21-19 and TG3C37#21-7, showed relatively high expression level of SlGAD3 in T_2 generation. Also, both lines maintain high GABA contents through whole generations, T_1, T_2 and T_3. It will be worth to make a F_1 plants to evaluate two TG3C37 as a breeding material. Further study will be discussed in Chapter 3.

Table 1. Growth comparison of T_2 generation (TG3C37#21-19, TG3C37#21-7, TG3C37#3-8, TG3C37#3-13) for plant height, and fruits size and weight compared to WT.

	Fruit size			Plant height (mm)
	Diameter (mm)	Length (mm)	Weight (g)	
WT-MT	16.76±1.26 [a]	16.64±1.02 [a]	2.96±0.62 [a]	117.8±22.0 [a]
T_2#21-7	15.12±1.66 [ab]	16.84±1.52 [a]	2.32±0.62 [ab]	101.2±13.2 [a]
T_2#21-19	14.08±1.36 [b]	15.25±1.67 [a]	1.92±0.54 [b]	103.0±12.7 [a]
T_2#3-8	16.93±2.21 [a]	16.90±1.34 [a]	2.89±0.88 [a]	114.0±18.6 [a]
T_2#3-13	17.12±1.91 [a]	16.63±1.32 [a]	3.18±0.88 [a]	109.2±19.4 [a]

The mean values ± Standard deviation is shown. WT-MT, T_2#21-7, T_2#21-19, T_2#3-8, T_2#3-13 indicate Wild-type of "Micro-Tom", T_2 generation of TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13, respectively. 10 fruits used for fruit size and 5 plants were used for measuring plant height. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

Table 2. Growth comparison of T_3 generation (TG3C37#21-19, TG3C37#21-7, TG3C37#3-8, TG3C37#3-13) for plant height, and fruits size and weight compared to WT.

	Fruit size			Plant height (mm)
	Diameter (mm)	Length (mm)	Weight (g)	
WT-MT	16.75±1.18 [a]	16.56±1.00 [a]	2.91±0.65 [a]	106.6±8.5 [a]
ΔC 21-7	15.70±0.96 [a]	16.91±1.35 [a]	2.49±0.44 [ab]	102.2±7.3 [a]
ΔC 21-19	15.20±1.20 [a]	16.49±1.18 [a]	2.36±0.46 [b]	109.0±10.1 [a]
ΔC 3-8	16.67±1.25 [a]	16.50±1.03 [a]	2.89±0.58 [ab]	112.4±14.6 [a]
ΔC 3-13	16.67±0.96 [a]	17.23±1.72 [a]	2.77±0.54 [ab]	116.0±18.3 [a]

The mean values ± Standard deviation is shown. WT-MT, ΔC 21-7, ΔC 21-19, ΔC 3-8, ΔC 3-13 indicate Wild-type of "Micro-Tom", T_3 generation of TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13 respectively. Ten fruits used for measuring of fruit size and 5 plants were for plant height. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

Table 3. Partial amino acid sequence of high GABA-containing tomato.

Line	Parental zygosity	Mutation type	Translated amino acids sequence
WT			…VKVLHELPNAKK*V*EDNLMINNEKKTEIEVQRAIAEFWKKYVLARKASIC*
TG3C37#21-7	Homo T(-)	1 i	…VKVLHELPNAKK*V***GG***
TG3C37#21-19	Homo T(-)	53 d	…VKVLHELPN*
TG3C37#3-8	Homo T(-)	6 d 1 i	…VKVLHELPNA**IGG***
TG3C37#3-13	Homo T(-)	4 d	…VKVLHELPNAK**WRII***
i; insertion, d; dillition, and *; stop. Changed amino acid is written in red bold. T(-) indicates transformation cassette is not inserted.			

Amino acid sequence of C-terminal. The changed sequence by mutation via CRISPR/Cas9 is in red bold. Underlined amino acid is the putative autoinhibitory domain and CaMB domain. The target site of CRISPR/Cas9 is written in Italic. ‘i’, ‘d’ and ‘*’ indicate inserted or deleted nucleotide(s) and stop, respectively. “Homo” in zygosity means mutation type of homozygous. T(-) indicates no transcription cassette is inserted anymore.

(a)

SlGAD3

(b)

WT	AGAGGACTTCTCCCGAACCCTAGCAGATCGTCTTGTCTCTGACATCGTCAAGGTCCTCCACG
TG3C37*Δ*C3-13 (4d)	AGAGGACTTCTCCCGAACCCTAGCAGATCGTCTTGTCTCTGACATCGTCAAGGTCCTCCACG
TG3C37*Δ*C3-8 (6d 1i)	AGAGGACTTCTCCCGAACCCTAGCAGATCGTCTTGTCTCTGACATCGTCAAGGTCCTCCACG
TG3C37*Δ*C21-19 (53d)	AGAGGACTTCTCCCGAACCCTAGCAGATCGTCTTGTCTCTGACATCGTCAAGGTCCTCCACG
TG3C37*Δ*C21-7 (1i)	AGAGGACTTCTCCCGAACCCTAGCAGATCGTCTTGTCTCTGACATCGTCAAGGTCCTCCACG

AGCTCCCGAATG**CCAAAAAAGTGG**AGGATAATTTGATGATCAATAATGAGAAGAAAACAGAAATTGAAGTTCAAAGGGC

AGCTCCCGAATG**CC** **AAGTGG**AGGATAATTTGATGATCAATAATGAGAAGAAAACAGAAATTGAAGTTCAAAGGGC

AGCTCCCGAATG**CCA** T**TGG**AGGATAATTTGATGATCAATAATGAGAAGAAAACAGAAATTGAAGTTCAAAGGGC

AGCTCCCGAAT TGAAGTTCAAAGGGC

AGCTCCCGAATG**CCAAAAAAGTGG**AGGATAATTTGATGATCAATAATGAGAAGAAAACAGAAATTGAAGTTCAAAGGGC

T

(c)

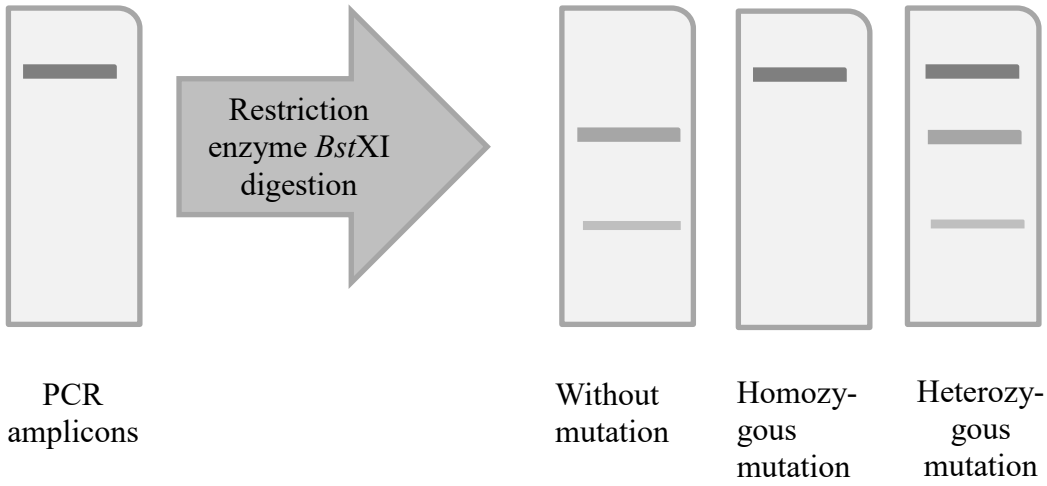


Figure 1. Diagram of *SlGAD3* and partial sequence where mutation occurred.

(a) Diagram of *SlGAD3*. Olive and dark grey boxes indicate untranslated regions and exons, respectively. Green line means targets where expected to be mutated. Sequences in red box are shown in (b). (b) Sequence of wild type of "Micro-Tom" and 4 types of TG3C37s are shown. 'i', 'd' in blank indicate inserted or deleted nucleotide(s). Inserted nucleotide is written in grey, and deleted DNA nucleotides are not written in this sequence. *Bst*XI site is written in black bold, and recognition site of *Bst*XI is dotted lined. *BstXI* site exists 240bp from the forward primers, so the amplicons are not digested by *Bst*XI when mutation occurred. (c) Simplified dCAPs methods. PCR products of WT (or when mutation did not occur), is digested by *Bst*XI treatment. On the other hands, mutated target site modified is not cut by *Bst*XI treatment. After treatment figures showed three cases, no mutation (including WT), homozygous mutation, and heterozygous mutation existing on target site.

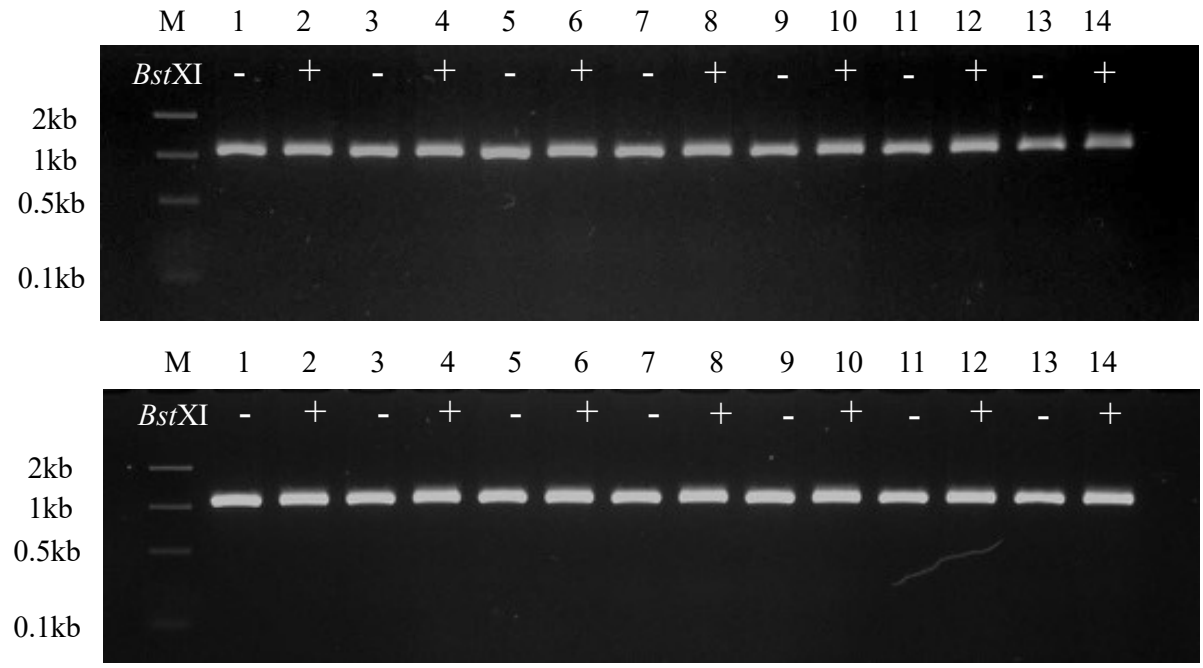


Figure 2. dCAPS analysis of TG3C37 T_2 generation.

(Upper) lane 1; size marker (Nppon gene ladder fast 1), lane 1-10; T_2 of TG3C37#21-7, lane 11-14 ; TG3C37#2-19. (lower) lane 1; size marker (Nippon gene ladder fast 1), lane 1- 6; T_2 of TG3C37#-3-13, lane 7-1 ; TG3C37#3-8. "-" indicates no restriction enzyme *Bst*XI treated and "+" means restriction enzyme treated.

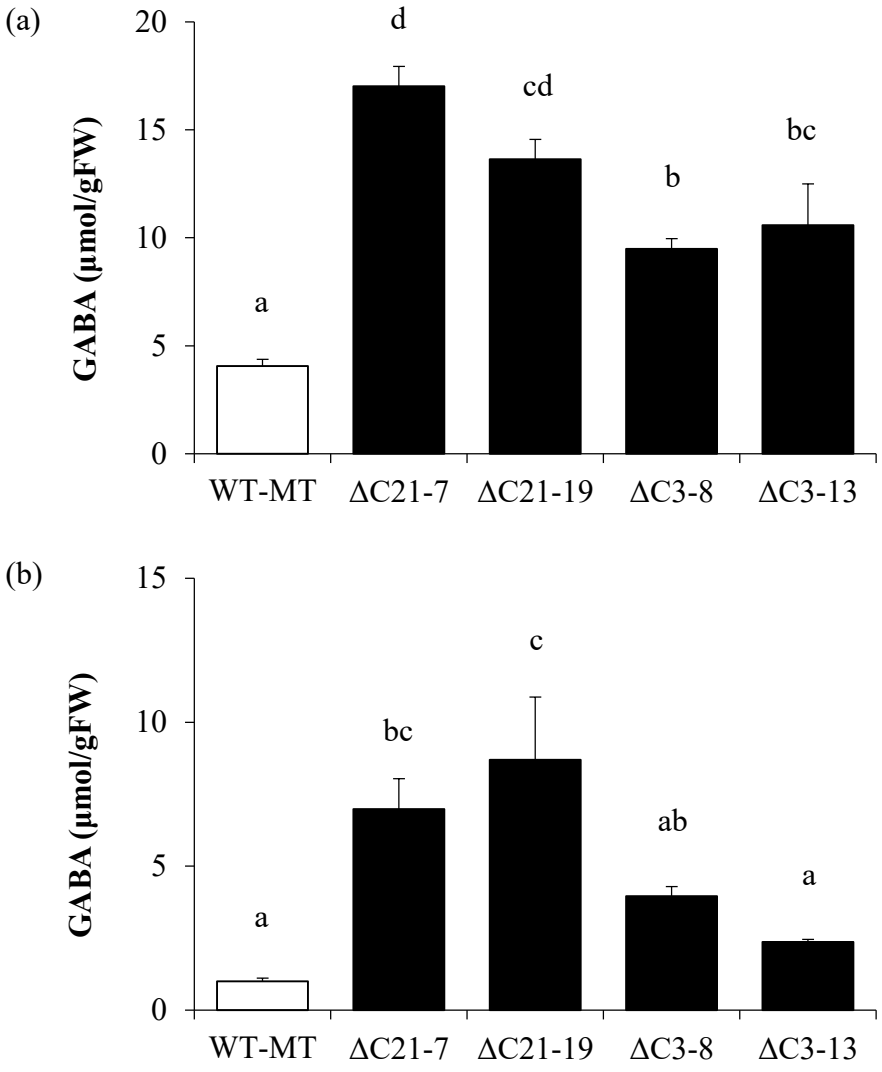


Figure 3. GABA contents of T_2 fruits in MG and RED stages.

(a) GABA content of MG fruits in T_2 generation. (b) GABA contents of RED fruits in T_2 generation. GABA contents was analyzed by GABase methods using three replicates (Three fruits are mixed and considered as one sample). Each data represents the mean ± standard deviation. WT-MT, ΔC21-7, ΔC21-19, ΔC3-8 and Δ3-13 indicate Wild type 'Micro-Tom', TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13, respectively. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

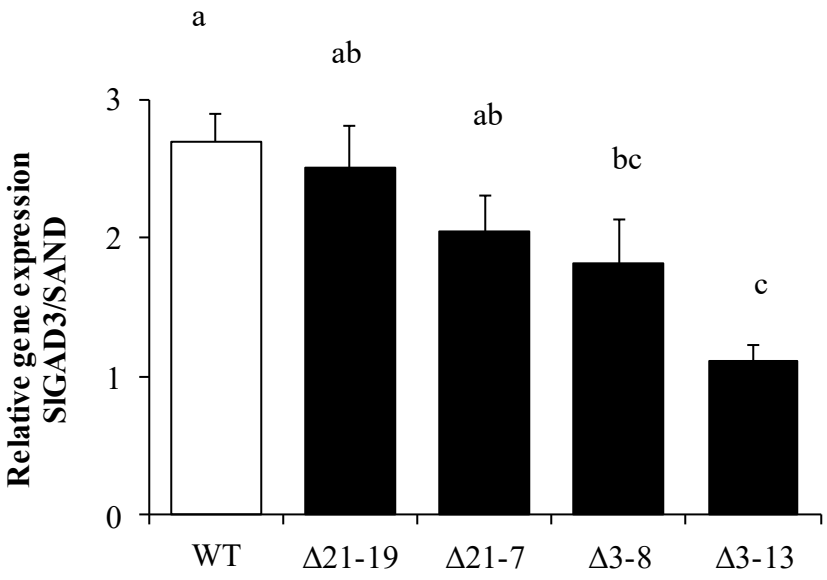


Figure 4. Relative *SlGAD3* expression at MG stage of fruits in T_2 generation.

Relative *SlGAD3* expression was measured using quantitative RT-PCR, using three replicates (Three fruits are mixed and considered as one sample). Expression level is normalized using gene *SAND*, an internal control. Each data represents the mean ± standard deviation. WT-MT, ΔC21-7, ΔC21-19, ΔC3-8 and Δ3-13 mean wild type 'Micro-Tom', TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13, respectively. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

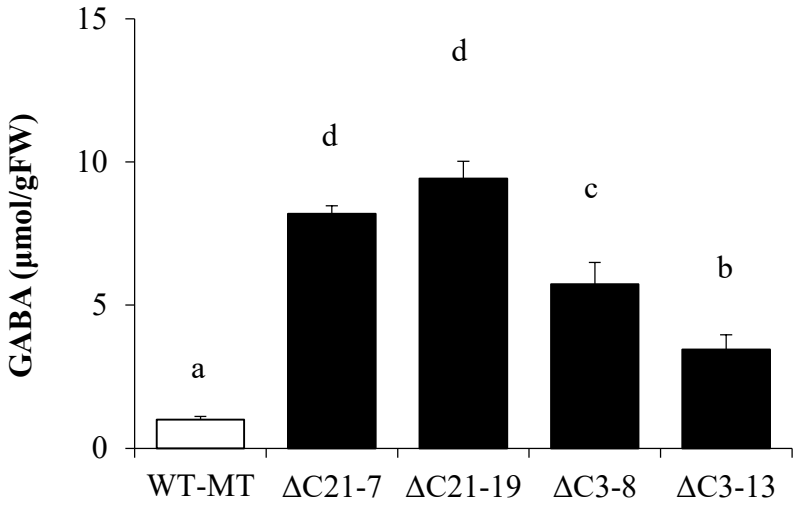


Figure 5. GABA contents of RED stage fruits in T_3 generation.

GABA contents of RED fruits in T_3 generation. GABA contents was analyzed by GABase methods using three replicates (Three fruits are mixed and considered as one sample). Each data represents the mean ± standard deviation. WT-MT, ΔC21-7, ΔC21-19, ΔC3-8 and Δ3-13 mean wild type 'Micro-Tom', TG3C37#21-7, TG3C37#21-19, TG3C37#3-8 and TG3C37#3-13, respectively. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

Chapter 3. Utilization of a genome-edited tomato (*Solanum lycopersicum*) with high gamma aminobutyric acid (GABA) content in hybrid breeding

3.1 Introduction

Gamma-amino butyric acid (GABA) is a non-proteogenic amino acid present in bacteria, fungi, animals, and plants. GABA is also known to be an inhibitory neurotransmitter in the central nervous system (Owens and Kriegstein 2002). GABA is effective in the prevention of certain life style-related diseases, including hypertension and diabetes (Inoue *et al.*, 2003, Nishimura *et al.*, 2015), and also has anti-stress properties (Abdou *et al.*, 2006). In the initial treatment of patients with life style-related diseases, dietary therapy is preferred to medication. Several studies on humans and experimental animals have shown that administration of exogenous GABA is effective in lowering blood pressure in patients with mild high blood pressure or high normal blood pressure (Eliott *et al.*, 1959, Takahashi *et al.*, 1961). GABA also decreases the glucose level in diabetics (Tian *et al.*, 2011). Intake of GABA through the daily diet helps to prevent 'life style-related diseases' from becoming serious. Although several foods, such as tea, rice, and fermented products (i.e., fermented milk, tempeh, yogurt, and soy sauce), contain GABA, the concentration of GABA is generally insufficient for preventing life style-related diseases, and thus GABA enrichment of food is necessary to confer a health-promoting effect (Tsushida *et al.*, 1987, Saikusa *et al.*, 1994, Aoki *et al.*, 2003, Park *et al*,m 2007, Yamakoshi *et al.*, 2007).

Since GABA has the potential function of promoting human health, and also plays important roles in the plant life cycle, many scientists have attempted to clarify the pathways

of GABA synthesis and metabolism. In higher plants, GABA is predominantly metabolized through a pathway referred to as the "GABA shunt," which bypasses two steps of the tricarboxylic acid (TCA) cycle. In this pathway, GABA is mainly produced from glutamate by an irreversible reaction catalyzed by the cytosolic enzyme glutamate decarboxylase (GAD) (Baum *et al.*, 1993, Fait *et al.*, 2008). A secondary pathway of GABA synthesis may be via polyamine (putrescine and spermidine) degradation (Fait *et al.*, 2008, Shelp *et al.*, 2012). Under oxidative stress, a non-enzymatic conversion from proline has also been reported (Signorelli *et al.*, 2015). GABA is degraded by the action of GABA transaminase (GABA-T) in the mitochondrial matrix. In this reaction, succinic semi-aldehyde (SSA) is produced with the possible participation of several amino acceptors, such as α-ketoglutarate, pyruvate, or glyoxylate (Clark *et al.*, 2009, Shelp *et al.*, 2012). Subsequently, SSA is converted by SSA dehydrogenase (SSADH) to succinate in the mitochondria (SHelp *et al.*, 2012, Bouché *et al.*, 2003), and then SSA re-enters the TCA cycle. Alternatively, SSA can be converted to gamma-hydroxybutyric acid (GHBA) via the action of GHB dehydrogenase (GHBDH) (Andriamampandry *et al.*, 1998, Breitkreuz *et al.*, 2003).

Tomato contains relatively higher levels of GABA than other plants and/or crops (Ramesh *et al.*, 2017). In addition, tomato is one of the most extensively produced vegetables in the world and widely consumed in the daily diet. In cultivated tomato, GABA accumulates to a high degree in mature green fruits (comprising up to 50% of the total free amino acids); however, when the fruits ripen, GABA levels decrease to less than 20% of the total free amino acids (Rolin *et al.*, 2000). To understand the molecular mechanisms underlying the changes in GABA accumulation in tomato fruits, our previous study isolated GABA metabolism-related genes from the tomato cultivar "Micro-Tom" (Akihiro *et al.*, 2008). Three *SlGAD* genes (*SlGAD1*, *SlGAD2*, and *SlGAD3*), which are involved in GABA synthesis, three

SlGABA-T genes (*SlGABA-T1*, *SlGABA-T2,* and *SlGABA-T3*) related to GABA degradation, and an *SlSSADH* gene were identified (Akihiro *et al.*, 2008).

Several studies indicated that regulation of *SlGADs* was deeply involved in GABA accumulation. Suppression of *SlGAD2* and/or *SlGAD3* reduced GABA accumulation in MG and RED fruit (Takayama *et al.*, 2015). Conversely, over-expression of *SlGAD3* driven by the E8 promoter (a fruit-specific promoter) increased the GABA content in RED without affecting plant growth (Takayama *et al.*, 2017). In contrast, although inhibition of *SlGABA-T* gene expression increased GABA accumulation, a strong inhibition of plant growth was observed (Koike *et al.*, 2013). These results indicate that upregulation of *SlGAD* gene expression and/or enzymatic activity is more effective in increasing GABA accumulation in the fruit than downregulation of *SlGABA-T*.

Plant GADs generally contain an autoinhibitory domain at the C-terminus. This domain is composed of 30–50 amino acids and it also functions as CaMBD. It is considered that this domain inhibits GAD activity at physiological pH by folding its active site. This autoinhibition is suppressed when the active site is unfolded by conformational changes promoted via an acidic pH and/or by the binding of Ca^{2+}/CaM to the CaMBD (Gut *et al.*, 2009). Thus, it is assumed that removal of the C-terminus containing this autoinhibitory domain allows the enzyme to be constitutively active, resulting in high GABA accumulation. Therefore, modification of the C-terminus of SlGADs would appear to represent an effective approach in breeding tomatoes with high GABA accumulation. Indeed, in our previous study, we used a transgenic approach to truncate the C-terminus of SlGAD3, which resulted in increased GABA levels (Takayama *et al.*, 2017). Furthermore, we succeeded in breed a new high-GABA content tomato line via removal of the C-terminus of SlGAD3 using site-specific

targeted mutagenesis (genome editing; CRISPR/Cas9) technology in the dwarf experimental cultivar “Micro-Tom”, which we named TG3C37#21-19 and TG3C37#21-7 (Nonaka *et al.*, 2017).

Similar to other vegetable crops, the commercially distributed tomatoes sold in markets are mainly hybrid cultivars, because hybrid tomatoes generally combine the good characteristics of both parents and have considerably better marketability as a consequence of their high fruit quality (Bai and Lindhout, 2007). Many trials to improve the productivity and attractive appearance, including color and size, of hybrid tomatoes have been carried out over a number of years; however, the most important consideration for producing hybrids is to identify or develop a valuable parental line for hybrid breeding. Recently, there has been a trend to develop tomatoes with regard not only to the appearance of fruit but also to their nutrient value (Bai and Lindhout, 2007). After removal of the transformation cassette, mutations remained in two TG3C37 lines (TG3C37#21-19 and TG3C37#21-7) that had an effect on GABA content. I decided to cross TG3C37s (TG3C37#21-19 and/or TG3C37#21-7) to the commercial cultivar “Aichi First” to evaluate the effect of TG3C37s (TG3C37#21-19 and TG3C37#21-7) used as a single parent for producing an F_1 hybrid. In the present study, I analyzed two tomato hybrid lines derived from TG3C37 (TG3C37#21-19 and TG3C37#21-7) containing a single allele with a truncated C-terminus and evaluated their fruit quality. Finally, I evaluate the genome of edited lines as parental lines for hybrid breeding.

3.2 Materials and methods

3.2.1. Plant materials

Two T_1 generation tomato lines, TG3C37#21-7 and TG3C37#21-19, containing mutations generated via CRISPR/Cas9 at the C-terminus of the *SlGAD3* gene (Table 4, Nonaka *et al.*, 2017), and the wild-type of WT-MT were cultivated in a culture room under fluorescent light with a 16 h light (60 μmol/m^2/s)/8 h dark photoperiod at 25°C, using standard nutrient solution (Otsuka A; Otsuka Chemical Co., Ltd., Osaka, Japan). For producing F_1 generation plants, pollen from the wild type of the commercial pure line cultivar "Aichi First" was used to pollinate the emasculated pistils of the two TG3C37 lines and WT-MT before flower opening. F_1 seeds were germinated on wet filter paper and transferred to rock wool at 2 weeks after germination. Seedlings were cultured in a culture room as described above for 1 month (to the 5 to 6 true leaf stage), and then transferred to a semi-containment greenhouse in the Gene Research Center of the University of Tsukuba (Ibaraki, Japan). All plants were fertilized using an NFT cultivation system and irrigated with Otsuka A standard nutrient solution at an EC level of 1.8–2.2 dS/m. Cultivation for this experiment was carried out from April to August 2017. Pollination of F_1 plants was carried out up to the 6th truss from the bottom of each plant and additional trusses from the 7th were discarded. After observing enlargement of three fruits per truss, other flowers on the same truss were discarded. Fruits were harvested at three stages: The BR, MG, and RED. The BR stage is that in which the fruit color changes from green to yellow. The MGstage fruits are the fruits pollinated at the same time to -2 days before the BR fruits, but have yet to break. The RED fruits are harvested 10 days after the BR day. The time to BR was counted during cultivation.

3.2.2. Genetic analysis of F_1 lines by dCAPS analysis and genomic DNA sequencing

The F_1 generation was confirmed using the dCAPS method and sequencing. More specifically, genomic DNA was extracted using a modified CTAB method (Kim and Hamada, 2005). Leaf material (200 mg) was ground to a fine powder and pre-warmed CTAB at 65°C was added to each sample. After adding the same volume of chloroform/isoamyl alcohol (24:1, v/v) followed by inverting the contents a few times, all samples were centrifuged at 13,000 rpm for 15 min at 4°C. The resulting clear supernatant was transferred to a new tube and the same volume of iso-propanol was added. After maintaining at -20°C for 2 h, the mixture was centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant was carefully discarded so as not to lose the DNA pellet. The pellet was rinsed using 70% (v/v) ethanol. After briefly drying at room temperature, the pellet was re-suspended in 50 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) containing 40 µg/ml of RNase A (Amresco, Texas, US).

For dCAPS analysis, PCR was carried out using *SlGAD3* C-terminal-specific primers (forward 5′-GATGAAGGTATACCCTTGGTGG-3′, reverse 5′-CACTCTCGCCTCTATCGC TTAT-3′) using 100 ng of gDNA. A 50 µg aliquot of the amplicon was incubated with *Bst*XI for 3 h at 37℃ and loaded onto a 2% agarose gel. Selected F_1 plants were sequenced for more precise confirmation. The PCR product was diluted 50-folds with distilled water and used as a template. The forward primer used for gene-specific PCR was used for sequencing.

3.2.3. Analysis of fruit characteristics

The diameter, length, weight, and color of harvested fruits at the MG, BR. and RED stages were measured to investigate the effect of the mutated *SlGAD3* allele of TG3C37 on F_1

fruit appearance compared with the WT F_1 hybrid. Fruit diameter was measured twice, and length was measured once. The fruits were weighed after removing old sepals and the pedicle. For fruit size comparison, averages of the diameter, length, and weight of 20 fruits were used. Color was measured three times on the surface of the middle of fruits using a Minolta Color Reader CR-10 (Konica Minolta Sensing, Inc., Osaka, Japan). The color parameters assessed were the lightness, indicating the range from black (0) to white (100) (L*), and color direction, indicating the red to green scale (a*) and the yellow to blue scale (b*). For color calculation, the average of the L*, a*, and b* values was used from 20 red fruits of each line. Chroma, the saturation of color, was calculated as $(a^2+b^2)^{1/2}$. Hue classification of red, yellow, blue, and green, was calculated by arctan (b/a). Statically, the results obtained for each F_1 line were compared with those for the WT F_1 hybrid at $P < 0.05$.

3.2.4. Analysis of carotenoid contents

Beta-carotene and lycopene contents were analyzed according to previously described methods (Nagata and Yamashita, 1992). Three milliliters of acetone/hexane (4:6 v/v) was added to 300 mg of frozen fruit powder. After brief vortexing, the clear supernatant was used for analysis. Absorption was measured at 663 nm (A_{663}), 645 nm (A_{645}), 505 nm (A_{505}), and 453 nm (A_{453}) using a Beckman Coulter DU 640 spectrophotometers (Fullerton, CA, USA). Lycopene and β-carotene contents were calculated according to following equations:

$$\text{Lycopene} = -0.04584(A_{663}) + 0.204(A_{645}) + 0.372(A_{505}) - 0.0806(A_{453})$$

$$\beta\text{-carotene} = 0.216(A_{663}) - 1.22(A_{645}) - 0.304(A_{505}) + 0.452(A_{453})$$

The values for lycopene and β-carotene results were compared with those for the WT F_1 hybrid at $P < 0.05$.

3.2.5. Analysis of total soluble solids (TSS) and titratable acidity (TA) analysis

TSS and TA were measured to estimate sugar and organic acid levels, respectively. TSS was measured from 300 mg of frozen fine powder of red-stage fruits using a PAL-J refractometer (Atago, Tokyo, Japan). TA was measured using a pH meter as described previously (Dalal *et al.*, 1965). One gram of red fruit powder was mixed with 10 ml of distilled water, and titrated using 0.1N sodium hydroxide up to pH 8.1. Nine red fruits were used for TSS analysis and five were used for TA analysis. Measured TSS and TA values were compared statistically at $P < 0.05$.

3.2.6. Analysis of GABA and free amino acid contents

Fruits at the MG, BR, and RED stages were harvested, and jelly tissue and seeds were removed. The fruits were immediately frozen and crushed in liquid nitrogen. Free amino acid was extracted from 50–70 mg of the fine powder with 500 µl of 8% trichloroacetic acid (w/v). After brief shaking, the mixture was centrifuged at 12,000 rpm for 20 min. Three hundred microliters of the resulting supernatant were transferred to a new tube, to which 400 µl of diethyl ether was added. After 10 min of vigorous shaking, the mixture was centrifuged at 12,000 rpm for 10 min. After removing the diethyl ether, this step was repeated once more. Thereafter, the diethyl ether was evaporated in a chamber for 30 min at room temperature. Sixty microliters of extracted amino acids (extracted as described above) was purified. The

amino acid solution was dried at 60°C under vacuum. The resulting pellet was dissolved in 150 µl of distilled water and subsequently re-dried. After repeating this step again, the pellet was dissolved in 300 µl of 0.1 N HCl and analyzed using a JLC-500/V2 amino acid analyzer (Japan Electron Optics Laboratory, Tokyo, Japan). Analysis condition followed the instruction of JLC-500/V2: using citric acid lithium buffer, ion exchange resin column, the ninhydrin color development method, and absorbance of 570 nm and 440 nm. Statically, the results of each line at each stage were compared by Tukey's HSD test at $P < 0.05$.

3.2.7. RNA extraction, cDNA synthesis and RT-PCR

To investigate the relative *SlGAD3* expression in F_1 generation, quantative PCR was carried out. Three fruits at each stage were selected for relative gene expression analysis. First, RNA was extracted from 100mg of fruit powder using RNeasy plant mini kit (Qiagen, Hilden, Germany), and removed DNA fragment using RNA clean & concentrator kit (Zymo Research, Freiberg, Germany). RNA was adjusted up to 1.5 mg and used for cDNA synthesis. Takara Thermal Cycler Dice Real-Time systemTP800 (Takara-Bio Inc., Shiga, Japan) was used with SYBR premix Ex Taq II (Takara-Bio Inc., Shiga, Japan). The reaction was carried out by 30 sec at 95ºC for pre-denaturation, and by 40 cycle of 2 step PCR at 5 sec at 95ºC for denature and 30 sec at 60ºC for annealing/extension. Relative quantification of *SlGAD3* was normalized to the expression level of *SAND* gene, an internal control (Rodriguez *et al.*, 2008). Primers for *SlGAD3* were SlGAD3 Forward (5'- AAGGTCCTCCACGAGCTC -3'), SlGAD3 Reverse (5'- CCCTA ACAAATAGATGCTTTCC -3'). Statically, the results (*SlGAD3* relative expression level over internal control *SAND*) of each line were compared by Tukey's HSD test at $P < 0.05$.

3.3 Results

3.3.1. Genetic confirmation of the C-terminal region of *SlGAD3* in F_1 lines

Using the dCAPS method and sequence analysis, I checked the genotypes of the F_1 lines “Micro-Tom” TG3C37#21-19 × “Aichi First” WT (F_1#21-19) and “Micro-Tom” TG3C37#21-7 × “Aichi First” WT (F_1#21-7) (Figure 6, 7). In TG3C37, the C-terminus of *SlGAD3* contains a mutation upstream of the autoinhibitory domain in the region of the CaMDB (Figure 6a), resulting in a loss of the autoinhibitory domain region. TG3C37#21-19 and TG3C37#21-7 have 1-nucleotide and 53-nucleotide deletions and an introduced stop codon upstream of the autoinhibitory domain, respectively (Figure 7c, d (middle), Table 3). The target site of the WT possesses a *Bst*XI site, whereas in TG3C37#21-19 and TG3C37#21-7 this restriction enzyme recognition site is lost. Accordingly, although the WT of “Micro-Tom” and “Aichi First” were clearly digested into two bands, the two TG3C37 lines (T_1) were not. Using dCAPS analysis, F_1#21-19 and F_1#21-7 showed three bands, representing the two digested bands from “Aichi First” WT and an un-digested band from “Micro-Tom” TG3C37 (the parental lines). This result confirmed the heterozygous mutation in F_1#21-19 and F_1#21-7 (Figure 7a, b). For a more precise confirmation, sequences at the target site were also analyzed. In F_1 generation plants, two peaks were detected downstream of the mutation site (Figure 7c, d).

3.3.2. Effect of the mutated *SlGAD3* allele on fruit size and weight

I further examined whether the mutated *SlGAD3* allele had any effects on fruit size and weight. To evaluate differences in fruit size, RED fruit diameters (horizontal length and vertical length) were measured. The size of all F_1#21-19 and F_1#21-7 fruits was similar to that of F_1-WT. The average horizontal lengths of the fruits of F_1-WT, F_1#21-19, and F_1#21-7 were 52.3 ± 7.3, 52.6 ± 5.5, and 51.5 ± 6.2 mm (mean ± SD, n=20), respectively (Figure 8a). The corresponding vertical lengths of fruit were 43.9 ± 3.8, 45.7 ± 3.1, and 46.2 ± 4.1 mm (mean ± SD, n =20). No statistically significant differences in fruit size were observed among the lines ($P < 0.01$) (Figure 8b). The average weights of RED fruit in F_1-WT, F_1#21-19, and F_1#21-7 were 76.2 ± 29.2, 77.8 ± 22.0, and 75.7 ± 21.4 g (mean ± SD, n=20), respectively. Although there was a relatively large variation in fruit weight within lines, no statistically significant differences were observed in the fruit weight of different lines (Figure 8c). These results indicated that although mutation of SlGAD3 increased the GABA content in RED, the mutation of this allele did not affect fruit size or weight.

3.3.3 Color of the red fruit of F_1 lines

To investigate whether the mutated *SlGAD3* allele and high GABA accumulation were associated with fruit color, coloration was observed during the fruit ripening process (Figure 9a). No differences were observed in coloration among the F_1 lines. To estimate in more detail, color was measured using a color difference meter in 20 red-stage fruits (Breaker + 10 days) of F_1 lines. In the evaluation of fruit color (Table 5), there were no significant differences in any values among F_1 lines. The value of *Chr* (chroma) in F_1#21-7 were slightly higher than F_1#21-19 and F_1-WT. These results indicate that F_1#21-7 had a slightly stronger yellow color than the other F_1 lines. Since a slight difference was found in the color of RED

of F_1#21-7, the levels of lycopene and beta-carotene among F_1 lines were compared. The content of lycopene (red pigment) in F_1-WT, F_1#21-19, and F_1#21-7 was 36.2 ± 8.1, 45.5 ± 10.2, and 38.1 ± 9.0 μg/g FW (mean ± SD, n=5), respectively. Although the lycopene level tended to be higher in F_1#21-19, the difference was not significant (Figure 9b). The contents of beta-carotene (yellow color pigment) were almost the same among the F_1 lines (Figure 9c), with concentrations of 7.7±1.2, 7.1±1.9 and 7.1±1.7 μg/g FW (mean ± SD, n=5) in the F_1-WT, F_1#21-19, and F_1#21-7 lines, respectively. Although F_1#21-7, which contained the highest GABA levels, showed slightly stronger yellow color, beta-carotene concentration was similar in F_1-WT. These results suggest that "the slightly stronger yellow color" in F_1#21-7 is not meaningful differences caused by the yellow color pigment. Therefore, the mutated *SlGAD3* allele with high GABA accumulation seemed not to affect colorization.

3.3.4. Total soluble solids and titratable acid in F_1 lines

Since taste is one of the most important fruit traits, we examined whether higher GABA accumulation affects the flavor of red ripe fruit. Total soluble solids (TSS, Brix) and titratable acid (TA) were measured in the RED (Breaker + 10 days) of F_1 lines. The values of TSS and TA are indicators of sugar and organic acid concentrations, respectively. The Brix values of F_1#21-19 and F_1#21-7 were found to be slightly lower than that of F_1-WT (Figure 10a): 4.9 ± 0.30, 4.4 ± 0.31, and 4.3 ± 0.27 (mean ± SD, n=9) for F_1-WT, F_1#21-19, and F_1#21-7, respectively (Figure 10a). In contrast, the TA levels of F_1#21-19 and F_1#21-7 were increased by 20% and 10%, respectively, compared with that of F_1-WT (Figure 11b): 0.49 ± 0.02, 0.60 ± 0.04 and 0.55 ± 0.02 for F_1-WT, F_1#21-19, and F_1#21-7 (mean ± SD, n=5), respectively. These results suggest that higher GABA accumulation influences fruit taste.

3.3.5. GABA content in F_1 lines

To determine whether the heterozygous mutation of *SlGAD3* was effective in promoting high GABA accumulation in the F_1 generation plants, GABA accumulation in F_1#21-19, F_1#21-7, and F_1-WT were measured by HPLC. Three fruits from each line were used for this analysis at three fruit development stages (MG, BR, and RED). In all of the F_1 lines, the highest GABA accumulation was observed in MG, and decreased during ripening (Figure 11). Compared with the F_1-WT, a higher GABA level was detected throughout the entire fruiting stage of F_1#21-19 and F_1#21-7. In F_1#21-19, the GABA concentration was 33.58 ± 6.35, 26.80 ± 1.66, and 18.50 ± 2.09 μmol/gFW (mean ± SD, n=3) at the MG, BR, and RED stages, respectively. F_1#21-19 contained 2.1, 2.5, and 3.6 times higher GABA than F_1-WT at these stages, respectively. Similarly, the GABA concentration in F_1#21-7 was 31.13 ± 0.24, 21.56 ± 4.24, and 12.76 ± 1.08 μmol/gFW (mean ± SD, n=3) at the MG, BR, and RED stages, respectively, which were 2.3-, 2.1-, and 3.0-fold higher than those in the corresponding stages of F_1-WT. In both F_1#21-19 and F_1#21-7, the reduction ratio of GABA was lower than that of F_1-WT. The reduction ratios (RED/MG) of GABA in F_1-WT, F_1#21-19, and F_1#21-7 were 66.7%, 44.9, and 59%, respectively. These results indicate that the heterozygous mutation in *SlGAD3* was effective in promoting an up-regulation of GABA accumulation in the F_1 generation, and the high GABA phenotype was a dominant trait.

3.3.6. Content of other free amino acids in F_1 lines

The extremely high accumulation of GABA in fruit has previously been shown to affect the levels of other free amino acids (Takayama *et al.*, 2017). Therefore, I investigated

whether the high accumulation of GABA in F_1 generation fruit affects amino acid metabolism (Table 6). Free amino acids were measured by HPLC at three stages of fruit development (MG, BR, and RED). In F_1#21-19 and F_1#21-7, the levels of Ala, Asp, and Glu were increased, whereas GABA, Ser, and Val decreased during the fruit ripening process. Other free amino acids showed the same level from MG to RED. The same tendency was observed in F_1-WT. Although the concentrations of Asp and Glu in F_1#21-19 and F_1#21-7 at the MG stage were lower than those in F_1-WT, in the RED stage, the concentrations of all other free amino acids were similar among F_1-WT, F_1#21-19, and F_1#21-7, and, with the exception of GABA, no statistically significant differences were observed. These results indicate that higher GABA concentrations in F_1#21-19 and F_1#21-7 did not have any significant effects on the levels of other free amino acids in RED tomato fruit.

3.3.7 Relative SlGAD3 expression

To investigate the effect of truncated SlGAD3 on GABA accumulation in fruits, quantitative gene expression was measured (Figure 11). Relative expression of *SlGAD3* level showed peak at MG stage, and drastically decreased after Breaker in three lines. In F_1#21-19, expression level is dropped to under 40% level of MG as F_1-WT did. Notably, *SlGAD3* expression level in F1#21-7 was lower than other two lines at MG, and the amount of decreased was small than other two lines. At BR and RED, almost same *SlGAD3* expression level was observed in three lines. To explain effect of truncated *SlGAD3* on GABA accumulation in fruits, quantitative RT-PCR was carried out using truncated TG3C37#21-19 and TG3C37#21-7 specific primers (Figure 12), and target specific amplification is observed. In F_1#21-19, relatively high level of truncated *SlGAD3* was expressed, over 80%, among total

SlGAD3 expression (Figure 11, Figure 12a). On the other hands, truncated *SlGAD3* in F_1#21-7 expressed under 50 % level of total SlGAD3, at whole stages (Figure 11, Figure 12b).

3.3.8 Enzymatic activity of SlGAD

Enzymatic activity is measured to understand increased GABA content in two F_1 lines. Under pH 7.0, a physiological pH, SlGAD from F_1#21-19 and F_1#21-7 showed higher GAD activity comparing to that of F_1-WT. Average activity of F_1#21-19 and F_1#21-7 were 29 and 26 (nmol/min/mg protein), while that of F_1-WT was 15. These results mean F_1#21-19 and F_1#21-7 have near 2-times higher activity comparing to F_1-WT. Under pH 7.0 with Calmodulin, enzyme activity was little bit higher than without calmodulin in whole lines, 17, 33 and 30 for F_1-WT, F_1#21-19 and F_1#21-7, respectively. Under pH 5.8 also, the optimal pH for GAD protein, SlGAD from F_1#21-19 and F_1#21-7 showed higher GAD activity comparing to that of F1-WT. Average activity of F_1#21-19 and F_1#21-7 were 222, 187 (nmol/min/mg protein) respectively, while that of F_1-WT was 106 in average. Under pH 5.8 with Calmodulin, average SlGAD activity of F_1#21-19, F_1#21-7 and F_1-WT was 213, 193 and 107 (nmol/min/mg protein) respectively.

3.4 Discussion

In this study, I used a dwarf experimental cultivar, “Micro-Tom”, containing a *SlGAD3ΔC* allele mutated using the CRISPR/Cas9 system, and the commercialized pure line cultivar “Aichi First” as parental lines to produce two hybrid F_1 lines (F_1#21-19 and F_1#21-7). These two lines harbor a heterozygous mutated *SlGAD3* allele and showed higher GABA accumulation in RED than F_1-WT (Figure 11). The gene expression level of *SlGAD3* in F_1#21-7 was lower than other two lines at mature green stage. On the other hand, it was almost same among three lines at breaker and red ripe stage (Figure 12). Truncated SlGAD3 elevated GAD activity at MG stage, up to almost 2 folds higher level (Figure 14). Increased enzymatic activity results in increased GABA accumulation in fruits. The amount of accumulated GABA in fruits at RED stage slightly differ between two F_1 lines, due to GAD activity (Figure 14), mainly derived from the amount of truncated SlGAD3 at each stage (Figure 13). *SlGAD3* expression in F1#21-7 is clearly low level three F_1 lines, also truncated SlGAD3 expression is near 30% of total SlGAD3 expression (Figure 12, 13b). On the other hands, near 80% of truncated SlGAD3 allele is expressed in F1#21-19 (Figure 12, 13a), resulting in high level of GABA 18.50 μmol/gFW RED in fruit. Although the expression level is lower, GABA accumulates higher. Therefore, GABA content correlated with SlGAD3 activity produced by truncated SlGAD3.

Compared with the TG3C37 line of the dwarf cultivar “Micro-Tom”, which contains a homozygous *SlGAD3ΔC* (Nonaka *et al.*, 2017), the level of GABA in F_1#21-19 and F_1#21-7 was higher. Moreover, F_1#21-19 and F_1#21-7 showed the same GABA accumulation as the GABA-rich cultivar ‘DG03-9’ (Saito *et al.*, 2008). This indicates that the heterozygous *SlGAD3ΔC* is linked to an increase in GABA accumulation, even in fruit of larger size than

that of “Micro-Tom”. A daily GABA intake of 10 to 20 mg has been demonstrated to effectively reduced blood pressure in adults with mild hypertension (Inoue *et al.*, 2003, Nishimura *et al.*, 2015, Fukuwatari *et al.*, 2001, Kazami *et al.*, 2002). In F_1#21-19 and F_1#21-7, the GABA concentration was up to 18.50 μmol/gFW and 12.76 μmol/gFW, which are equivalent to 190.8 ± 21.6 mg and 131.6 ± 11.1 mg/100 gFW tomato fruit, respectively. Thus, the requisite amount of GABA for health-promoting effects can be obtained in 10 to 20 gFW of F_1#21-19 and F_1#21-7, which corresponds to one-eighth to one-quarter of F_1 fruits, which have an average weight approximately 75g. These results indicate that the elevated accumulation of GABA in the F_1 hybrid lines examined in this study would appear to be sufficient to have a health-promoting function.

Our results indicated that the increased GABA concentration in RED (131.6 mg/100gFW and 190.8 mg/100 gFW in F_1#21-7 and F_1#21-19, respectively) did not influence either free amino acid accumulation (Figure 11, Table 6) or coloration during the ripening process (Figure 9). In contrast, a previously study using the dwarf cultivar “Micro-Tom” grown in a growth room showed that 268 mg/100 gFW of GABA affects free amino acid accumulation and inhibited the development of fruits with a red-ripe coloration from those with an orange color (Takayama *et al.*, 2017). The F_1 lines (“Micro-Tom” × “Aichi First”) produced in the present study were grown in semi-containment green house. Accordingly, taken together, the results of the present and previous studies indicate that the level at which GABA begins to show an effect on fruit colorization and free amino acid accumulation is approximately 190 mg/100 gFW, and/or that the influence of GABA on colorization and amino acid accumulation is dependent on cultivar and growth conditions.

Along with the indices TSS and TA, taste can be estimated from the levels of glutamate (Glu) and aspartate (Asp), since these amino acids are among the taste components responsible for “UMAMI,” and are important amino acids in red ripe fruit. TSS and TA are primarily used to estimate the sugar and organic acid contents in tomato fruits, respectively, which are commonly associated with fruit sweetness and sourness. In the GABA-rich lines F_1#21-19 and F_1#21-7, the levels of Glu and Asp were found to be similar to those in F_1-WT. with the TSS value being slightly lower and the TA value being slightly higher in the red ripe fruits of GABA-rich lines (Fig. 10). These results indicate that only sweetness and sourness were changed in these two lines. Further experiments, including sensory evaluation, are required to determine whether the taste of the fruit of GABA-rich lines is altered. I determined the size and weight of fruits, which are also important factors in fruit quality. No differences were observed with regards to fruit diameter, length, and weight in F_1-WT, F_1#21-19 and F_1#21-7 (Fig. 8). Each of the F_1 lines examined in the present study had a fruit size that was intermediate between that of “Micro-Tom”(WT and TG3C37, average 2–3 cm) and the commercial pure line cultivar “Aichi First”.

Previously, it has been demonstrated that Glu and Asp accumulate as GABA decreases (Rolin *et al.*, 2000, Crrari *et al.*, 2006). Although the same tendency was observed in F_1-WT, F_1#21-19, and F_1#21-7 (Fig. 11, Table 6), there were differences in the stage at which the accumulation of Asp commenced. The BR/MG ratios for Asp in F_1-WT, F_1#21-19, and F_1#21-7 were 2.6, 2.25, and 2.0, respectively, whereas the corresponding RED/BR ratios were 3.3, 6.2, and 4.2 (Table 6). These results indicated that in F_1-WT, Asp increases constantly during fruit development, whereas in F_1#21-19 and F_1#21-7 there is a marked increase in the levels of this amino acid after BR. At the RED stage, however, the concentrations of Asp tended to be similar in the three F_1 lines. In contrast, the pattern of Glu

accumulation during fruit development tended to be similar in the three F_1 lines. It seems reasonable to assume that the Asp/Glu balance could induce differences in Asp accumulation pattern, but not that of Glu, which is a precursor of GABA. Asp and Glu are interconverted by the activity of aspartate aminotransferase (AST) (Metha *et al.*, 1993). The activity of this enzyme should be higher in F_1#21-19 and F_1#21-7, which contain a mutated *SlGAD3ΔC* allele, than in F_1-WT (Nonaka *et al.*, 2017), resulting in a deficiency of Glu in the metabolic system. To compensate for this deficiency, Asp should be converted to Glu via the activity of AST.

The GABA-rich cultivar “DG03-9” was bred in a previous study (Saito *et al.*, 2008, Yoshimura *et al.*, 2010). The mechanism of high GABA accumulation in “DG03-9” appears to differ from that observed in our F_1 lines. Comparative studies of ‘Micro-Tom’ and “DG03-9” have clarified the differences in the behavior of synthesis and degradation enzymes between these two cultivars (Akihiro *et al.*, 2008). “DG03-9” showed higher activity of GABA synthesis enzymes (SlGADs) than “Micro-Tom”, whereas the activity of the GABA degradation enzyme GABA-TK was considerably lower than that in “Micro-Tom” in the breaker and red stages. Since the Asp and citrate contents in “DG03-9” did not increase significantly after the breaker stage, and the Glu content was not elevated immediately after the breaker stage, this previous study also suggests that ‘DG03-9’ has mutations not only in genes that are implicated in GABA degradation but also in genes that are related to the synthesis or degradation of citrate, Glu, and Asp. In contrast, the effect of *SlGAD3ΔC* allele on Asp and Glu accumulation in red ripe fruit appeared to be small. Therefore, if “Micro-Tom” TG3C37#21-19 and/or TG3C37#21-19 were crossed with “DG03-9”, it may be possible to breed a ‘new GABA-rich tomato’ with high Asp and Glu.

In tomato breeding, F_1 hybrids are predominantly used. In this study, I examined the utilization of a tomato line mutated via CRISPR/Cas9 for breeding. We found that F_1 hybrid lines containing a heterozygous mutated *SlGAD3ΔC* allele showed an increase in GABA accumulation in red RED as a dominant trait, without any negative effects on other tomato fruit traits. Either parental line with a truncated GAD C terminus introduced by CRSIRP/Cas9 can be effectively used for breeding tomatoes with a high GABA content. The removal of the C-terminus in GADs has been demonstrated to increase the activity of these enzymes in many plant species, including rice and apple (Akama and Takaiwa, 2007, Trobacher *et al.*, 2013a). Therefore, the results of the present study indicate that the utilization of lines mutated using the CRISPR/Cas9 system as parental lines would be effective in breeding high-GABA accumulation varieties of a wide range of crops.

Table 4. *SlGAD3* genotype and translated SlGAD3 sequence of F_1 lines

Parent / F_1	ID	Zygosity	Genotype	Amino acid sequence
Parent (F)	"Micro-Tom"	Homo-	WT	**…VKVLHELPNAKK*I*EDNLMINNEKKTEIEVQRAIAEFWKKYVLARKASIC***
Parent (F)	TG3C37#21-7	Homo-	1i	…**VKVLHELPNAKK*I***GG*
Parent (F)	TG3C37#21-19	Homo-	53d	**…VKVLHELPN***
Parent (M)	"Aichi First"	Homo	WT	**…VKVLHELPNAKK*I*EDNLMINNEKKTEIEVQRAIAEFWKKYVLARKASIC***
F_1	F_1-WT	Homos	WT	
F_1	F_1#21-7	Hetero	1i WT	
F_1	F_1#21-19	Hetero	53d WT	

Amino acid sequence of C-terminal (F_1 lines). The same sequence as Wild Type is expressed in **Bold**. The changed sequence by mutation via CRISPR/Cas9 is in Regular. **Bold with underline** is the putative autoinhibitory domain and CaMBD. The target site is written in *Italic*. * is

stop. Genotype of WT, 1i, and 53d are wild-type, 1 nucleotide insertion, 53 nucleotides deletion, respectively. “Homo” and “Hetero” in zygosity means homozygous and heterozygous, respectively. F_1 lines: F_1-WT, “Micro-Tom” WT × “Aichi First” WT; F_1#21-7, “Micro-Tom” TG3C37#21-7 × “Aichi First” WT; F_1#21-19, “Micro-Tom” TG3C37#21-19 × “Aichi First”. “F” and “M” means Female and Male as parent, respectively.

Table 5. Fruit color of F_1 lines.

	L*	**a***	**b***	***Chr***	***hue***
WT	38.22 ± 1.89 [a]	27.42 ± 3.13 [a]	28.99 ± 3.25 [a]	39.93 ± 4.30 [a]	0.841 ± 0.068 [a]
F21-19	38.68 ± 2.25 [a]	27.68 ± 1.97 [a]	29.61 ± 3.04 [a]	40.56 ± 3.27 [ab]	0.849 ± 0.077 [a]
F21-7	39.09 ± 1.92 [a]	29.20 ± 2.47 [a]	31.34 ± 3.29 [ab]	42.88 ± 3.53 [b]	0.852 ± 0.096 [a]

The mean values ± standard deviation of of L*, a*, b* parameters, *Chr* and *hue* functions from 20 replicates are shown. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

Table 6. Free amino acid levels (μmol/gFW) in MG, BR, and RED fruits.

	MG			BR			RED		
	F_1-WT	**F_1#21-19**	**F_1#21-7**	**F_1-WT**	**F_1#21-19**	**F_1#21-7**	**F_1-WT**	**F_1#21-19**	**F_1#21-7**
Ala	0.57 ± 0.08[a] (2.1)	0.44 ± 0.08[a] (1.0)	0.58 ± 0.13[a] (1.3)	0.32 ± 0.09[a] (1.2)	0.49 ± 0.11[a] (1.2)	0.42 ± 0.07[a] (1.2)	1.14 ± 0.20[a] (2.5)	1.57 ± 0.26[a] (2.8)	1.31 ± 0.32[a] (2.8)
Arg	0.26 ± 0.06[a] (0.9)	0.30 ± 0.13[a] (0.7)	0.46 ± 0.04[a] (1.0)	0.28 ± 0.10[a] (1.0)	0.29 ± 0.06[a] (0.7)	0.31 ± 0.03[a] (0.9)	0.50 ± 0.14[a] (1.1)	0.41 ± 0.02[a] (0.7)	0.41 ± 0.07[a] (0.9)
Asn	1.65 ± 0.24[a] (5.6)	0.83 ± 0.28[a] (1.8)	1.45 ± 0.49[a] (2.9)	1.29 ± 0.23[a] (4.4)	1.15 ± 0.26[a] (2.6)	1.14 ± 0.23[a] (2.9)	1.60 ± 0.67[a] (3.2)	1.04 ± 0.12[a] (1.7)	1.38 ± 0.42[a] (2.7)
Asp	2.22 ± 0.03[a] (8.1)	1.18 ± 0.19[b] (2.8)	1.79 ± 0.21[b] (3.9)	5.84 ± 2.85[a] (21.6)	2.66 ± 0.38[a] (6.7)	3.59 ± 0.88[a] (10.0)	19.47 ± 1.52[a] (42.3)	16.67 ± 0.65[a] (29.8)	15.06 ± 2.04[a] (32.1)
GABA	12.57 ± 1.71[a] (46.6)	33.58 ± 6.53[b] (79.5)	31.13±0.24[b] (68.4)	9.59 ± 1.30[a] (35.5)	26.80 ± 1.66[b] (66.9)	21.56 ± 4.24[b] (60.1)	4.19 ± 0.71[a] (9.1)	18.50 ± 2.09[c] (33.1)	12.76 ± 1.08[b] (27.2)
Gln	2.38 ± 0.40[a] (8.8)	1.17 ± 0.47[a] (2.8)	1.90 ± 0.73 (4.2) [a]	2.01 ± 0.50[a] (7.4)	1.61 ± 0.40[a] (3.9)	1.72 ± 0.29[a] (4.8)	2.05 ± 0.52[a] (4.4)	1.41 ± 0.22[a] (2.5)	1.82 ± 0.37[a] (3.9)
Glu	2.08 ± 0.39[a] (7.7)	1.16 ± 0.28[a] (2.7)	2.06 ± 0.48[a] (4.5)	3.51 ± 0.98[a] (13.0)	2.42 ± 0.25[a] (6.0)	2.94 ± 0.26[a] (8.2)	12.72 ± 2.19[a] (27.6)	11.92 ± 1.00[a] (21.3)	10.17 ± 1.82[a] (21.7)
Gly	0.26 ± 0.07[a] (1.0)	0.25 ± 0.10[a] (0.6)	0.29 ± 0.05[a] (0.6)	0.13 ± 0.02[a] (0.5)	0.19 ± 0.01[a] (0.5)	0.15 ± 0.04[a] (0.4)	0.18 ± 0.05[a] (0.4)	0.16 ± 0.00[a] (0.3)	0.16 ± 0.04[a] (0.3)
His	0.23 ± 0.05[a] (0.8)	0.19 ± 0.06[a] (0.4)	0.28 ± 0.05[a] (0.6)	0.22 ± 0.05[a] (0.8)	0.22 ± 0.01[a] (0.6)	0.21 ± 0.04[a] (0.6)	0.36 ± 0.02[a] (0.8)	0.33 ± 0.02[a] (0.6)	0.33 ± 0.04 [a] (0.7)
Ile	0.32 ± 0.08[a] (1.2)	0.33 ± 0.09[a] (0.8)	0.46 ± 0.06[a] (1.0)	0.25 ± 0.07[a] (0.9)	0.29 ± 0.04[a] (0.7)	0.29 ± 0.05[a] (0.8)	0.29 ± 0.07[a] (0.6)	0.24 ± 0.02[a] (0.4)	0.21 ± 0.06[a] (0.5)
Leu	0.22 ± 0.06[a]	0.18 ± 0.06[a]	0.30 ± 0.05[a]	0.27 ± 0.05[a]	0.25 ± 0.04[a]	0.26 ± 0.03[a]	0.27 ± 0.04[a]	0.23 ± 0.01 [a]	0.20 ± 0.02[a]

	(0.8)	(0.4)	(0.7)	(1.0)	(0.6)	(0.7)	(0.6)	(0.4)	(0.4)
Lys	0.28 ± 0.08[a] (1.0)	0.31 ± 0.12[a] (0.7)	0.47 ± 0.07[a] (1.0)	0.28 ± 0.04[a] (1.0)	0.33 ± 0.07[a] (0.8)	0.33 ± 0.05[a] (0.9)	0.65 ± 0.17[a] (1.4)	0.44 ± 0.02[a] (0.8)	0.47 ± 0.09[a] (1.0)
Phe	0.62 ± 0.28[a] (2.3)	0.65 ± 0.18[a] (1.5)	0.91 ± 0.18[a] (2.0)	0.64 ± 0.17[a] (2.4)	0.84 ± 0.30 [a] (2.1)	0.72 ± 0.17[a] (2.0)	0.71 ± 0.11 [a] (1.5)	0.88 ± 0.01[a] (1.6)	0.69 ± 0.07[a] (1.5)
Ser	1.89 ± 0.38[a] (7.0)	1.44 ± 0.54[a] (3.4)	1.69 ± 0.17[a] (3.7)	1.24 ± 0.28[a] (4.6)	1.30 ± 0.15[a] (3.2)	1.09 ± 0.23[a] (3.0)	0.95 ± 0.31[a] (2.1)	0.97 ± 0.10[a] (1.7)	0.96 ± 0.15 [a] (2.1)
Thr	0.72 ± 0.18[a] (2.7)	0.48 ± 0.12[a] (1.1)	0.80 ± 0.21[a] (1.8)	0.66 ± 0.28[a] (2.4)	0.59 ± 0.05[a] (1.5)	0.48 ± 0.05[a] (1.4)	0.59 ± 0.16[a] (1.3)	0.67 ± 0.01[a] (1.2)	0.52 ± 0.04 (1.1)
Tyr	0.24 ± 0.08[a] (0.9)	0.24 ± 0.07[a] (0.6)	0.32 ± 0.08[a] (0.7)	0.12 ± 0.03[a] (0.4)	0.21 ± 0.07[a] (0.5)	0.18 ± 0.05[a] (0.5)	0.11 ± 0.04[a] (0.2)	0.11 ± 0.01[a] (0.2)	0.09 ± 0.04[a] (0.2)
Val	0.65 ± 0.15[a] (2.4)	0.60 ± 0.17 [a] (1.4)	0.78 ± 0.14[a] (1.7)	0.51 ± 0.06 (1.9) [a]	0.62 ± 0.12[a] (1.5)	0.57 ± 0.06[a] (1.6)	0.40 ± 0.05[a] (0.9)	0.41 ± 0.03[a] (0.7)	0.41 ± 0.05[a] (0.9)
Total	27.14 ± 4.35 (100)	43.31 ± 9.49 (100)	45.67±3.40 (100)	27.16 ± 7.10 (100)	40.27 ± 4.00 (100)	35.95 ± 6.74 (100)	46.18 ± 6.96 (100)	55.97 ± 4.60 (100)	46.95 ± 6.72 (100)

Each data represents the mean ± S.D. (n=3). Percentage of each amino acid is shown in bracket. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$).

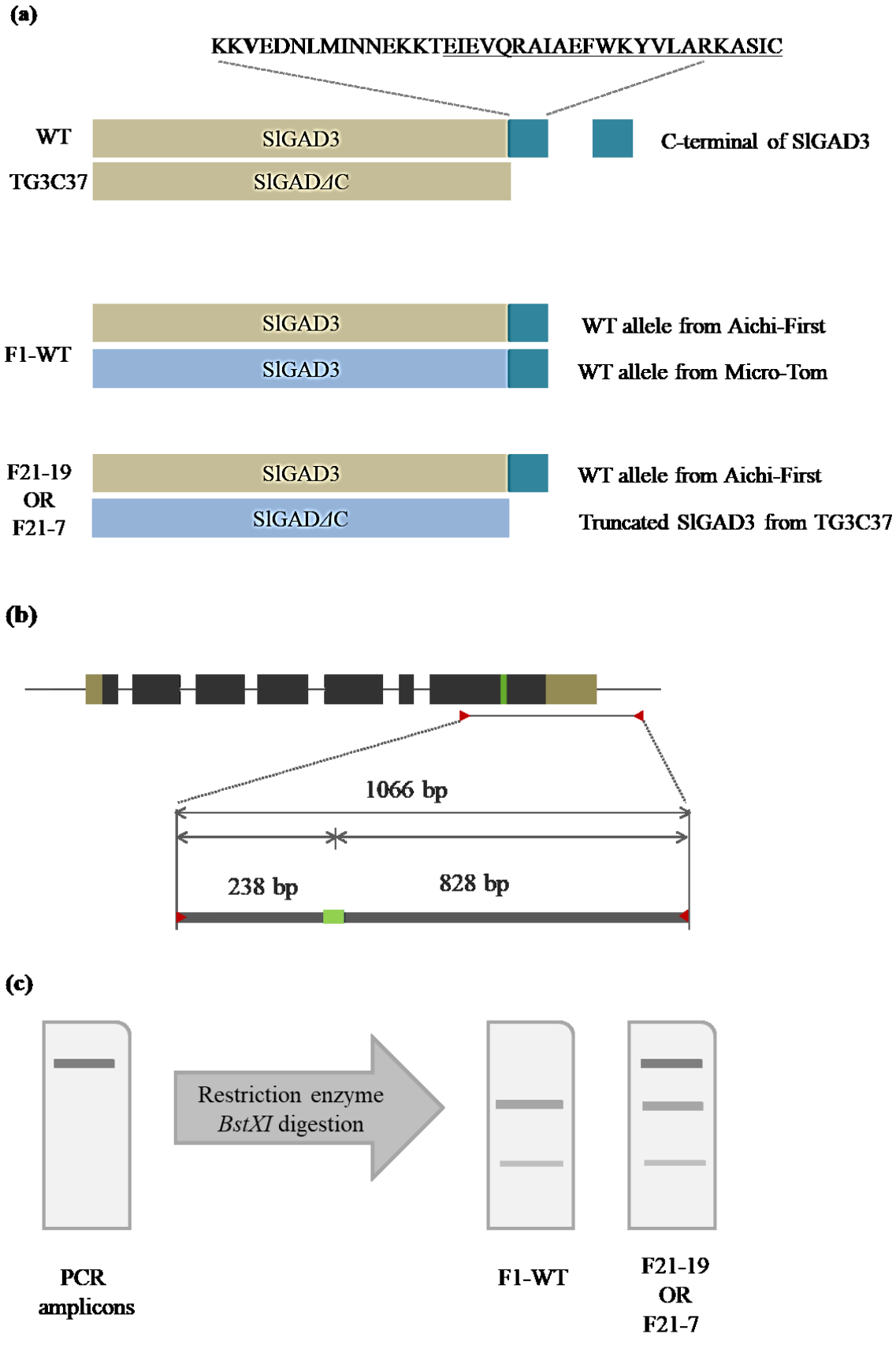
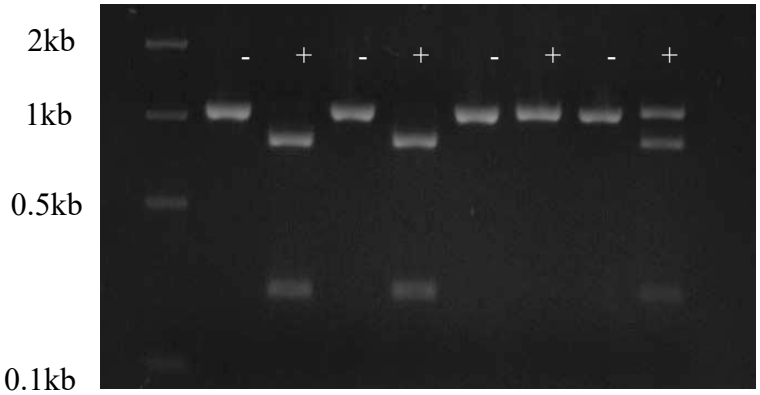

(a)
KKVEDNLMINNEKKTEIEVQRAIAEFWKYVLARKASIC
WT
TG3C37
SlGAD3
SlGADΔC
C-terminal of SlGAD3
F1-WT
SlGAD3
SlGAD3
WT allele from Aichi-First
WT allele from Micro-Tom
F21-19
OR
F21-7
SlGAD3
SlGADΔC
WT allele from Aichi-First
Truncated SlGAD3 from TG3C37
(b)
1066 bp
238 bp
828 bp
(c)
Restriction enzyme
BstXI digestion
PCR
amplicons
F1-WT
F21-19
OR
F21-7

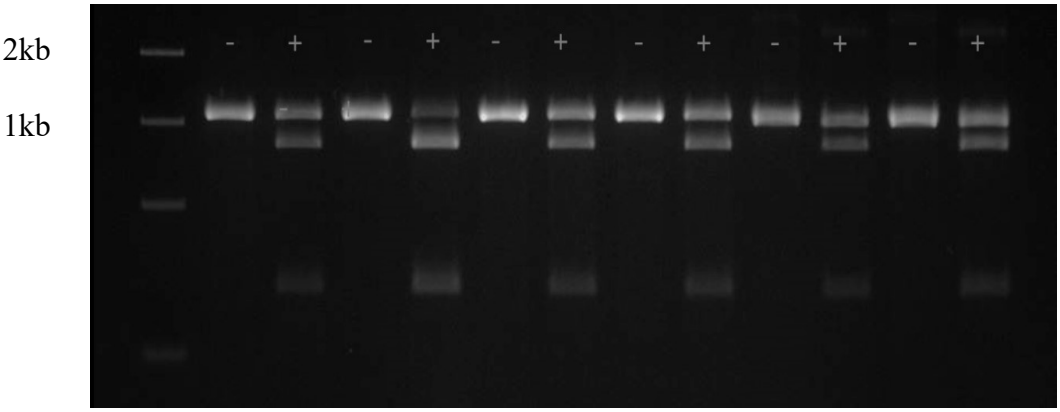
Figure 6. Diagram of SlGAD3 of WT and TG3C37 and simplified dCAPS analysis.

Diagram of SlGAD3 of SlGAD3. Upper and lower are diagram of WT and truncated C-terminal (TG3C37#21-7 and TG3C37#21-19). Blue box indicates autoinhibitory domain in C-terminal, and beige box and sky-blue box indicate parental allele derived from "Aichi First" or materal one from "Micro-Tom"/TG3C37. (b) Structure of *SlGAD3*. Olive and dark grey box indicates untranslated regions and exons respectively. Green line means targets where expected to be mutated. Green line means the target site of CRISPR/Cas9 include *Bst*XI site. Red triangles indicate PCR primers. The amplicons were digested by *Bst*XI into two fragments, 238 bp and 828 bp. c) Simplified dCAPS analysis result. PCR amplicons after digestion using *Bst*XI is divided into two, that F_1-WT shows 2 bands (WT alleles from "Aichi First" and "Micro-Tom") and F_1#21-19 and F_1#21-7 show 3 band (digested two band from WT allele from "Aichi First" and un-digested one from TG3C37).

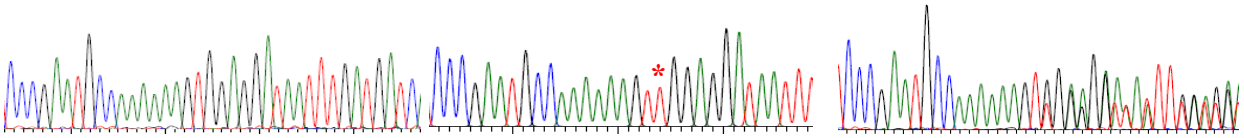
(a)



(b)

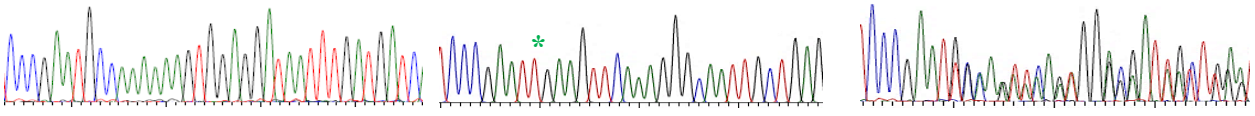


(c)



"Aichi First" WT　　TG3CΔ37#21-7(T_1)　　"Aichi Fisrt" X TG3CΔ37#21-7 (F_1)

(d)



"Aichi First" WT　　TG3CΔ37#21-19 (T_1)　　"Aichi Fisrt" X TG3CΔ37#21-19 (F_1)

Figure 7. dCAPS assay and sequencing chromatograph of *SlGAD3* sequence.

(a) A representative result of dCAPS method analysis. From left, Lane 1: marker (nippon gene ladder fast 1), lane 2,3: Micro-Tom WT, lane 4,5: Aichi first WT, lane 6,7: TG3CΔ37#21-7 (T_1 generation) and lane 8,9: F1 between Aichi first (WT) and TG3CΔ37#21-19. "-" indicates no restriction enzyme treated and "+" means restriction enzyme treated. (b) dCAPS analysis of the F_1 generation. From the left, lane 1: marker (Nippon gene ladder fast 1), lanes 2–9: F_1#21-7, and lanes 10–13: F_1#21-19. "-" indicates no restriction enzyme treatment and "+" indicates restriction enzyme treated. (c) Sequence chromatogram of wild type "Aichi First" (left), T_1 of TG3C37#21-7 (middle, red asterisk indicates the point that one nucleotide inserted) and F_1 of "Aichi First" (WT) and TG3CΔ37#21-7 (F_1#21-7). (d) Sequence chromatogram of wild type "Aichi First" (left), TG3CΔ37#21-19 (middle, green asterisk indicates the point where 53 nucleotides deletion started) and F_1 (right) between of them (F_1#21-19).

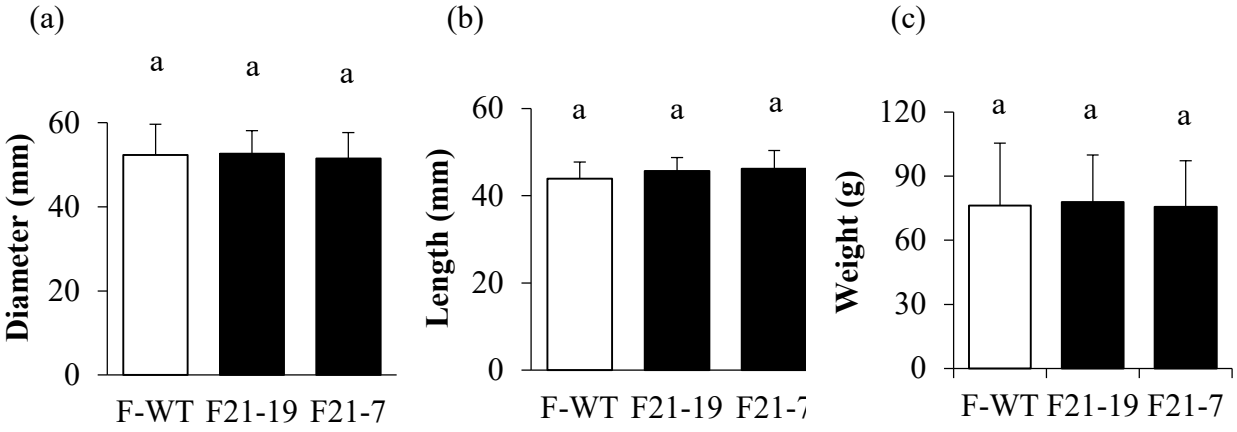


Figure 8. Fruits characteristics of F_1#21-19, F_1#21-7 and F_1-WT lines.

Fruit characteristics are represented by (a) diameter, (b) length and (c) weight. The mean values ± standard deviation of 20 biological replicates are shown. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$). F-WT, F21-19 and F21-7 mean F_1-WT, F_1#21-19 and F_1#21-7, respectively.

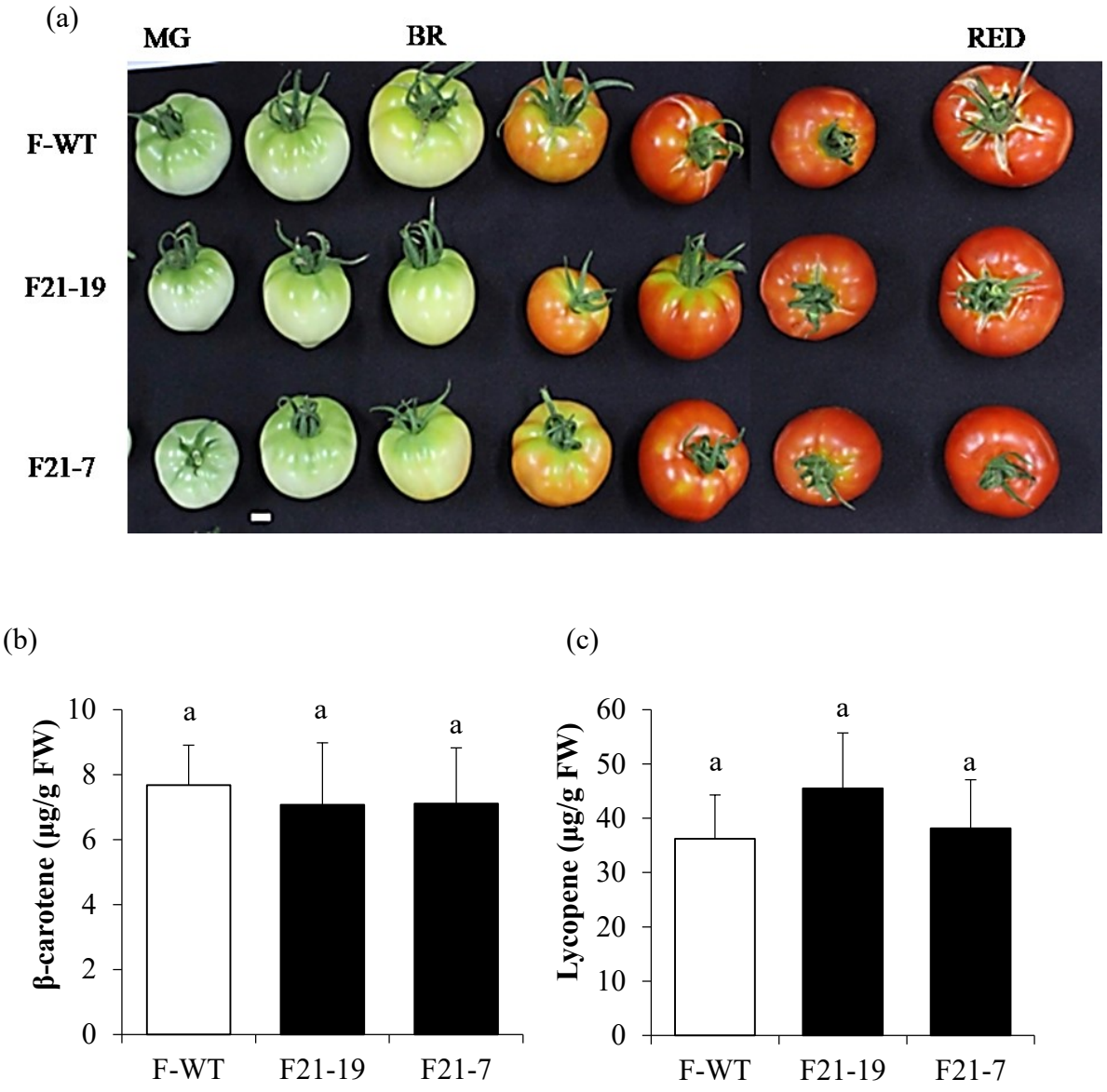


Figure 9. Coloration, and carotenoid contents at red stage of fruits.

(a) Coloration during fruit ripening (b) Lycopene content (c) beta-carotene content at red stage Each data represents the mean value ± standard deviation. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$, n=5). MG, BR and RED indicate Mature Green stage, Breaker stage, and Red ripe (Breaker + 10 days), respectively. F-WT, F21-19 and F21-7 mean F_1-WT, F_1#21-19 and F_1#21-7, respectively.

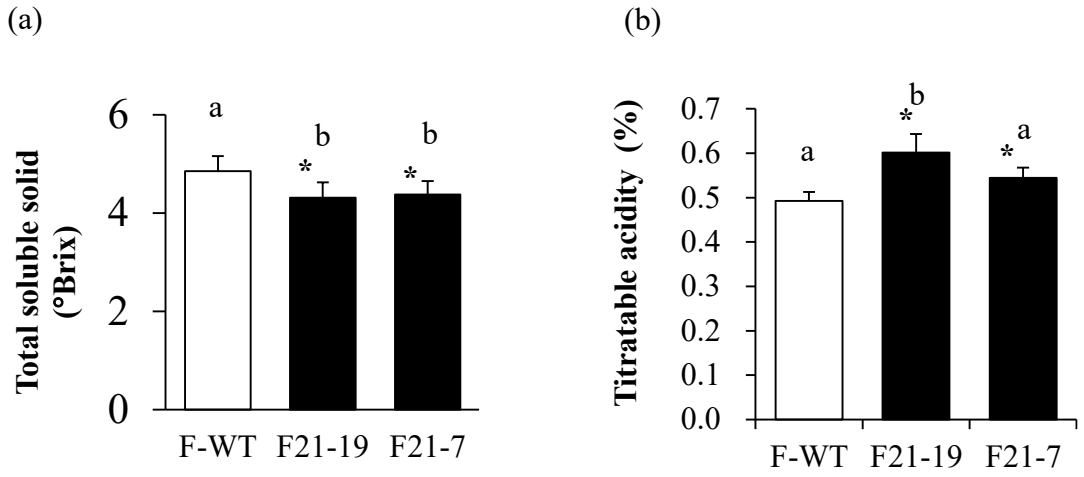


Figure 10. TSS and TA at RED stage of fruits.

(a) Total soluble solid content at red stage (n=9) (b) titratable acidity (n=5). Each data represents the mean value ± standard deviation. Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$). F-WT, F21-19 and F21-7 mean F_1-WT, F_1#21-19 and F_1#21-7, respectively.

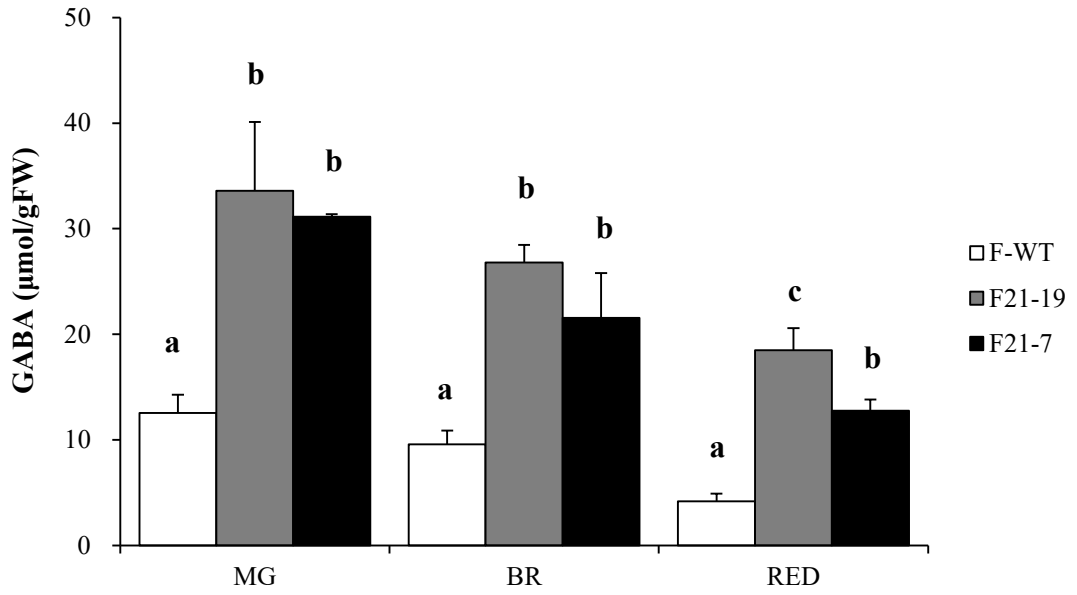


Figure 11. Fruit GABA contents of F_1#21-19, F_1#21-7 and F_1-WT at three stages of fruits.

GABA contents were measured at three stages by HPLC. 'MG' is mature green, 'BR' is breaker and 'RED' is red stage, 10 days after from breaker. Each data represents the mean value ± standard deviation (n=3). Different lower-case letters indicate statistically significant differences by Tukey's HSD test ($P < 0.05$). F-WT, F21-19 and F21-7 mean F_1-WT, F_1#21-19 and F_1#21-7, respectively.

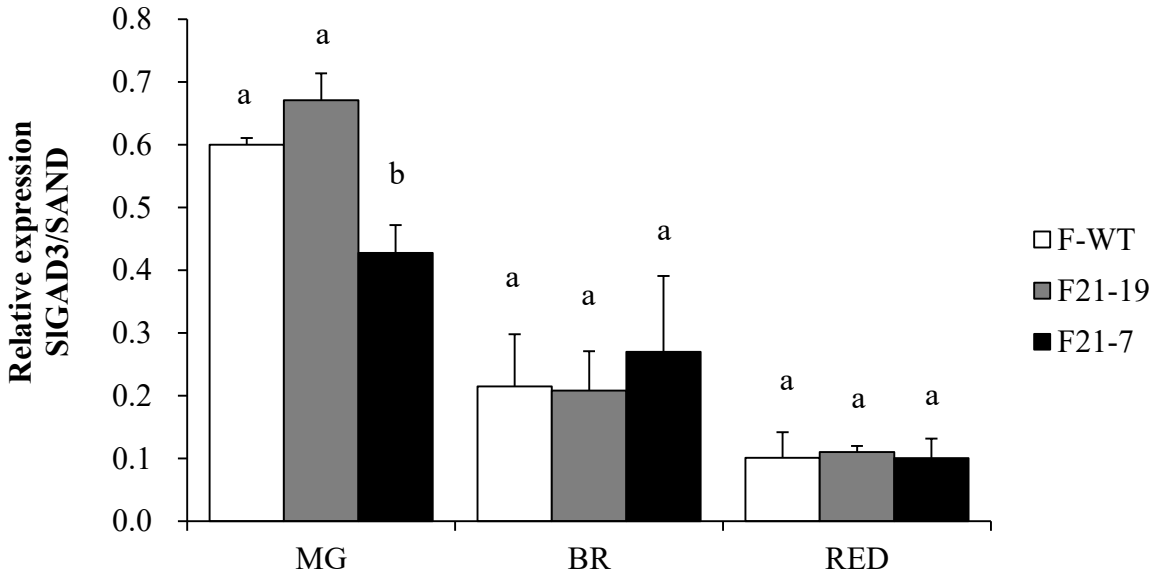


Figure 12. Relative expression of *SlGAD3* at each stage of fruit development.

Relative *SlGAD3* expression was measured over *SAND* gene, an internal control. Bars indicate standard deviation (n=3). F-WT, F21-19, and F21-7 denote F1-WT, F1#21-19, and F1#21-7, respectively. MG, BR, and RED indicate the Mature green stage, Breaker stage, and Red ripe stage (Breaker stage+ 10 days), respectively. Different lower-case letters indicate statistically significant differences by Tukey's HSD ($P < 0.05$).

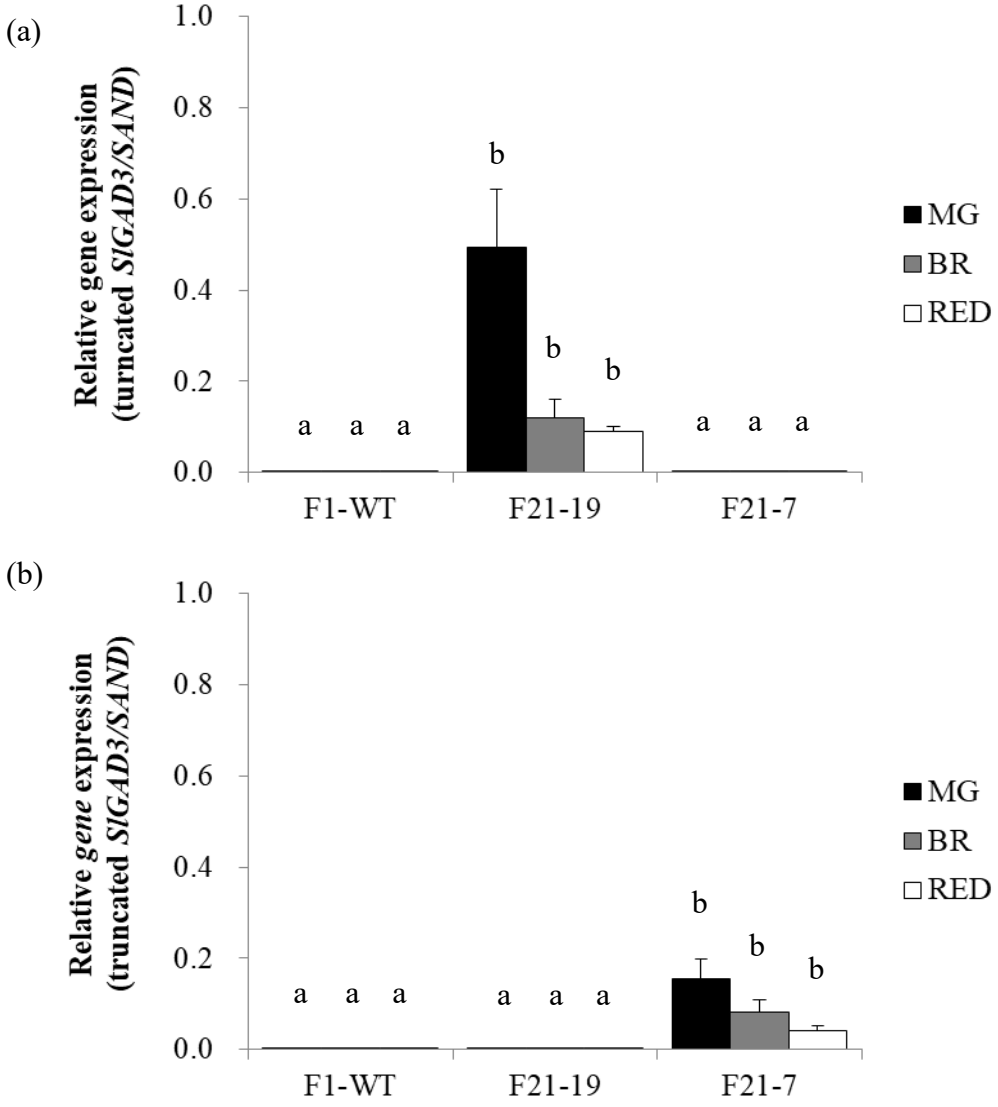


Figure 13. Relative gene expression of truncated *SlGAD3*

To investigate the effect of truncated *SlGAD3* on GABA accumulation, (a) TG3C37#21-19 specific and (b) TG3C37#21-7 specific gene expression was observed. Bars indicate standard deviation (n=3). F-WT, F21-19, and F21-7 denote F1-WT, F1#21-19, and F1#21-7, respectively. MG, BR, and RED indicate the Mature green stage, Breaker stage, and Red ripe stage (Breaker stage+ 10 days), respectively. Different lower-case letters indicate statistically significant differences by Tukey's HSD ($P < 0.05$).

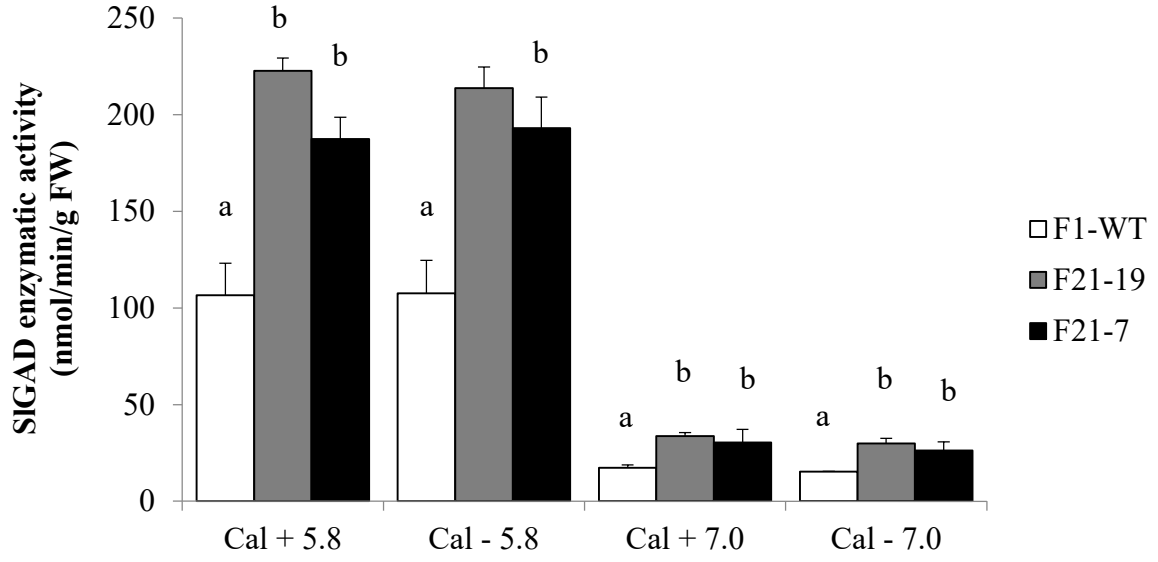


Figure 14. SlGAD enzyme activity at MG stage of fruits.

SlGAD enzymatic activity measured. Each data represent mean ± standard deviation (n=3). F-WT, F21-19, and F21-7 denote F_1-WT, F1#21-19, and F_1#21-7, respectively. Cal + or Cal – indicates the presence or absence of calmodulin, and 5.8 and 7.0 means reacted pH, pH 5.8 (optical pH) and pH 7.0 (physiological pH). Different lower-case letters indicate statistically significant differences by Tukey's HSD ($P < 0.05$).

Chapter 4. General Discussion

Tomato is an economically important crop, and one of the most produced vegetables in the world. Tomato bears berry-type fruits, and it is a good research material for studying fruit development and fruit maturation. It has easy access to information of whole genome sequences (Tomato Genome Consortium 2012). A well-established transformation method is also available. For those reasons, tomato was one of the crops that genome editing technology was initially applied (Brooks *et al.*, 2014, Lor *et al.*, 2014). CRISPR/Cas9 has got attention as a tool for genome editing among many platforms for its simplicity. In plants, research on targeted mutagenesis via CRISPR/Cas9 system was reported in 2013 (Pennisi, 2013, Li *et al.*, 2013, Nekrasov *et al.*, 2013), and CRISPR/Cas9 mediated mutagenesis rapidly applied for tomato improvement (Brooks *et al.*, 2014, Ueta *et al.*, 2017, Yu *et al.*, 2017, Kekrasov *et al.*, 2017).

Commercially distributed tomato in market is mainly hybrid like many other crops. For hybrid breeding, finding and developing a parental line with excellent traits is important to improve many breeding traits related to productivity, marketability and nutritional value. There were many trials to find them, including inducible mutagenesis using mutagens like EMS and radiation treatments. However, to find and fix some traits from those random mutant populations are labor-intensive and time-consuming works. Mutations induced via site-specific nuclease, including CRISPR/Cas9, can be a good alternative option due to not labor-intensive and not time-consuming practices, which allows producing many type of mutation via non-homologous end joining (NHEJ).

In the previous research, in order to show the effectiveness of CRISPR/Cas9 system to

increase GABA contents in tomato fruits, mutations on C-terminal of SlGAD3 via CRISPR/Cas9 (TG3C37) were generated in dwarf cultivar tomato, “Micro-Tom” and elevated for fruit GABA contents (Nonaka *et al.*, 2017). SlGAD3 possess auto-inhibitory domain at C-terminus region, where truncated by the mutation. To evaluate the performance of the induced mutants as a breeding material, the genetic stability of mutations and the effect of mutation must be confirmed. In addition, analysis of practical F_1 plants and fruits is also needed. Therefore, genetic stability of mutations and traits in the induced mutants was confirmed in Chapter 2, and practical application of the mutants generated by CRISPR/Cas9 technology on F_1 hybrid tomato breeding was demonstrated in Chapter 3.

One of advantages of genome editing is to generate mutants with mutation of gene of interests without transformation cassette. CRISPR/Cas9-induced mutants, TG3C37s having 4 types of mutation were evaluated for the genetic stability as a breeding material. Mutations observed in the T_1 generation were confirmed in T_2 generation (Table 4, Figure 7). Truncated SlGAD3 showed an effect on increasing GABA contents at RED stage of fruits, ranging from 2- to over 8-times higher than that of WT in T_2 generation. In T_3 generation, GABA contents showed same pattern of T_2 generation, and TG3C37#21-19 and TG3C37#21-7 showed relatively high level of GABA contents among TG3C37s. Mutations were homozygous in T_2 and T_3 generations. Considering extremely high enzymatic activity of truncated SlGAD3 *in vitro* (Nonaka *et al.*, 2017), GABA content in fruits of each line seems related to the relative *SlGAD3* expression, which has good accordance with GABA contents (Figure 3, 5). In addition, the increased GABA contents showed little effect on the fruit development and vegetative growth (Table 1, 2). Although further study will be needed to investigate the effect of increased GABA on other free amino acids in T_2 and T_3 generations, the highly increased GABA contents in TG3C37 lines showed little effect on fruit size and plant growth. This

result indicates that CRISPR/Cas9 system was very effective as a rapid breeding technology for high-GABA tomato cultivars.

For evaluation as a breeding material, TG3C37#21-19 and TG3C37#21-7 lines are used for F_1 hybrid production with commercial cultivar “Aichi First”. Two lines were selected because they possess relatively high level of GABA contents among TG3C37s, The F_1 generation showed intermediate GABA contents between two parental lines, indicating that the high-GABA is a semi-dominant trait. GABA and glutamate (Glu) are the most abundant amino acids in tomato fruits, and Glu in tomato fruits increased when GABA decreased (Rolin *et al.*, 2000, Carrari *et al.*, 2006). All F_1 lines showed the peak level of GABA at MG stage and decreased after BR stage (Figure 11), but Glu at all stages was not significantly different (Table 6). In F_1#21-7, relatively low truncated *SlGAD3* expression level was observed (Figure 12, 13b), under 30 % of total *SlGAD3*, which was related to GAD activity (Figure 14), and finally resulted in relatively low level of GABA comparing to F_1#21-19 (Figure11, Table 6). Truncated SlGAD3 comprised up to 80% of total expressed *SlGAD3* at MG stage in F_1#21-19, and seemed to be related to extremely high level of GABA 18.5 μmol/gFW at RED stage of fruits (Figure12, 13a, Table 6).

The increased GABA levels in this study did not influence significantly to other traits linked to fruit quality, including fruit size, coloration, and amino acid contents. In previous research, extremely increased GABA (approximately 25 μmol/gFW) by over-expression of truncated SlGAD3 inhibited fruit coloration and maldistribution of amino acid related to GABA metabolism (Takayama *et al.*, 2017). Increased GABA did not influence on the fruit size clearly (Figure 8), and on beta-carotene and lycopene which are responsible pigments for red tomato fruits (Figure 9). Yellow to blue scale (b*) is slightly different from that of WT in

F_1#21-7, but it seems not resulted from increased GABA contents because F_1#21-19, possessing higher GABA contents with similar mutation on *SlGAD3* did not show similar phenotype (Figure 11, Table 5). Although slight changes were detected in acidity and sweetness of fruits (Figure 10), further study is needed whether those slight changes have significant effects on the fruit marketability. In summary, TG3C37#21-19 and TG3C37#21-7 showed a high potential as tomato breeding materials for high-GABA hybrid cultivars.

Based on those results of F_1 generation, TG3C37#21-19 and TG3C37#21-7 lines can be evaluated for few aspects. First, mutations generated by CRISPR/Cas9 system inherited stably to the following generations. Effect of TG3C37s continued at least three generations and the mutation can increase GABA contents in tomato fruits, which conferred similar increased patterns in T_2 and T_3 generations. Second, gene expression in T_2 generation seemed to be correlated with the expression levels in the F_1 generation. Relative expression of truncated *SlGAD3* in F_1#21-19 and F_1#21-7 over total expressed *SlGAD3* is clearly different although the difference seems quite small in T_2 generation (Figure 4, 12 and 13). Therefore, it might better to check the expression level of mutated gene before generating F_1. Third, mutation showing influence on other unintended traits is easily excluded, but the effect caused by mutated gene is weakened at F_1 generation. Size differences found in T_2 and T_3 generations (Table 1, Table 2) were neutralized at F_1 (Figure 8). Therefore, side-effect can be ignored when it is not severe for selecting breeding materials. In addition, the expected effect by mutation should be strongly maintained after generation proceeding, due to the neutralization (Kitagawa *et al.*, 2005, Shalaby, 2013). Totally, mutation caused by CRISPR/Cas9 on *SlGAD3* showed high possibility having forte as tomato breeding materials.

Targeted mutagenesis via site-specific nuclease has many advantages comparing to other methods to create crops for breeding. First, it takes relatively short time to create plants having traits interested in, like usual transformation methods using agrobacterium. From transformation of 'Micro-Tom' using CRISPR/Cas9, it took ca. 10 months to get F_1 generation. Subsequently cultivation and analysis of F_1 plants took 4 months. Then totally it took 14 months for evaluation of F_1 plant in this study (Fig. 15). It is quite rapid to generate a new cultivar comparing to traditional random mutagenesis. On the other hand, crops created by mutagen treatment should be self-pollinated at least for 1-2 times to distinguish dominant/recessive traits by segregation. It also should be backcrossed at least 3 times to isolate responsible gene (Fig. 15). Therefore, it takes relatively long times comparing to targeted mutagenesis. Second, site-specific nuclease can be used for various plants including polyploidy plants such as *Triticum aestivum* (2n=6x=42) and *Gossypium hirsutum* (2n=4x=52). One expressed construct can modify target site in all alleles. Therefore, many type of mutation can be acquired after generation proceeding. Third, it is possible to avoid GMO (genetically modified organisms) controversy with few extra experiments. For example, whole-genome sequencing can be a good option to prove absence of transformation cassette before practical application as breeding materials. Transformed crops possess transformation cassette necessarily. One of the merits of site-directed mutagenesis is 'remaining effects after removing transformation cassette'.

There are shortcomings of site-specific nucleases. First, genetic resources are restricted unlike 'genetically modified plants' via transformation. A gene derived from different kingdom can be used for transformation like bioinsecticidal δ-endotoxin gene (*Bt.* Gene). Expressed exogenous *Bt.* genes from *Bacillus thuringiensis* in tomato showed pest-resistance against *Spodoptera litura* and *Heliothis virescens* (Fischhoff *et al.*, 1987). Site-specific

mutagenesis can target gene(s) that plants have, while there is no barrier for transformation. Second, it is essential to know information of target sequence. Genome sequenced crops gains ascendancy over crops which hasn't understood yet. Genes should be isolated first for those crops. It is also harder to predict off-target binding to cause unintended mutation on unintended site. For genome sequenced plants, it is better to design gRNA carefully to avoid off-target concern using genome information. Few strategies to avoid off-target were suggested, like using partially inactivated CRISPR/Cas9 system (Barabaschi *et al.*, 2016) or regulation construct expression due to highly expressed construct may one of the reasons of off-targeting (Bortesi and Fischer, 2015). In addition, it may be better to optimize promoter and codon for target crops to supplement targeted mutagenesis via CRISPR/Cas9, to compensate the possibility of low efficiency *in vivo* (Lor *et al.*, 2014).

Tomato breeding has focused on many traits including increased yield, taste, shelf-life and nutritional value (Bai and Lindhout, 2007). In this study, highly increased GABA containing tomato was evaluated as a breeding material. GAD, a responsible gene to synthesize GABA, is widely found in plants (Satya-Narayan and Nair 1990) and they generally possess an additional C-terminal residue where works auto-inhibition domain. Many health-prompting effects by GABA ingestion were reported (Inoue *et al.*, 2003, Nishimura *et al.*, 2015, Fukuwatari *et al.*, 2001, Kazami *et al.*, 2002, Abdou *et al.*, 2006), but daily ingested amount GABA is relatively low (Diana *et al.*, 2014). So, it is necessary to develop high-GABA containing crops to supply enough GABA. Therefore, the same mutagenesis strategy via CRISPR/Cas9 can be used for many edible crops such as rice (Akama and Takaiwa, 2007), soybean (Snedden *et al.*, 1995), faba bean (Ling *et al.*, 1994), apple (Trobacher *et al.*, 2013) and potato (Kinnersley and Lin, 2000) to increase GABA contents.

The most important issue in crop breeding is to maintain a wide gene pool. Random mutation via mutagen treatments has a lot of advantages, but has drawbacks at the same time. Therefore, targeted mutagenesis will be broadly used for generation of breeding material. There are many aims to be pursued for tomato hybrid breeding, such as resistance against pest or various environmental stresses, and increased efficiency of nitrogen uptake. Nutritionally improved traits are also important target traits to manipulate. For example, tomato is one of the most important sources of lycopene, possibly associated with decreasing some kind of cancers (Miller *et al.*, 2002).

CRISPR/Cas9 is a recently developed targeted-mutagenesis platform and has started appling in tomato breeding for many traits (Brooks *et al.*, 2014, Nekrasov *et al.*, 2017, Yu *et al.*, 2017, Nonaka *et al.*, 2017). To my knowledge, this study is the first trial to apply mutants via targeted mutagenesis to hybrid breeding. In this study, high-GABA containing tomato via CRISPR/Cas9 were evaluated and showed good performances as a breeding material. F_1 hybrid lines containing a heterozygous truncated SlGAD3 allele showed an increase in GABA accumulation in red fruit as a dominant trait, without any negative effects on other tomato fruit traits. F_1 fruits possessed maximum 18.50 μmol/gFW of GABA. Intake 10 to 20 mg of GABA in daily diet is reported to be effective for decreasing cardiovascular disease (Inoue *et al.*, 2003, Nishimura *et al.*, 2015, Fukuwatari *et al.*, 2001, Kazami *et al.*, 2002). This level of GABA converted to 190.8 ± 21.6 mg/100gFW, suggesting that one out of eight pieces of tomato fruit can satisfy the guideline. Again, the results of this study suggest that mutagenesis via CRISPR/Cas9 is effective methods to generate mutant lines for hybrid breeding. In my opinion, mutagenesis using site-specific nuclease must be broad for crop breeding in near future.

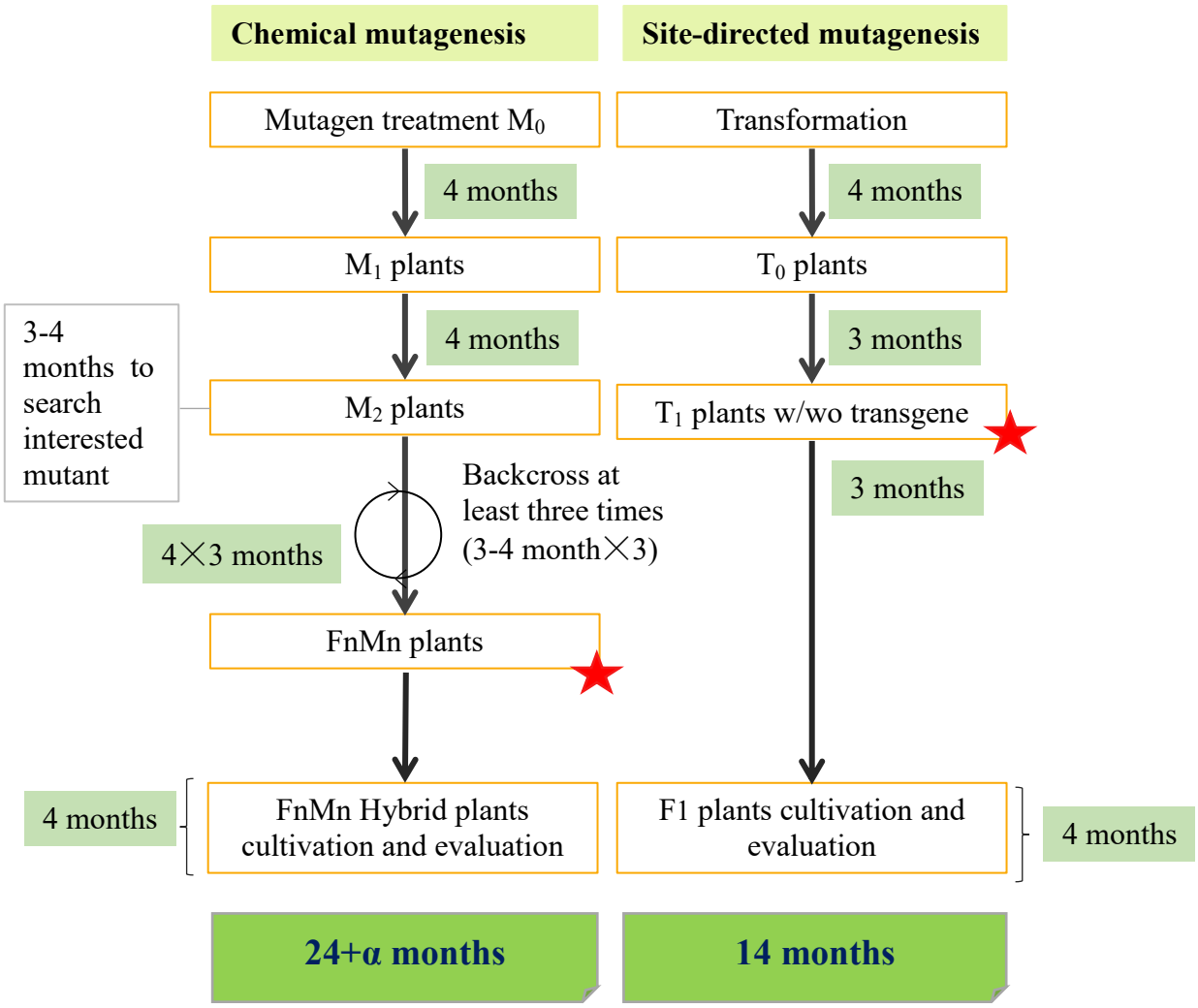


Figure 15. Hybrid breeding in tomato by site-specific mutagenesis comparing to traditional chemical mutagenesis

Time flow for generation F_1 tomatoes using mutants via chemical mutagen treatment and site-specific nuclease is shown. Periods taking cultivation for generation proceeding is written in orange box. Red star indicates the point when is possible to make F_1. The shortest total period to generate hybrid tomato is calculated in green box.

Summary

Tomato is one of the most produced vegetable in the world and economically important crop. Commercially distributed tomato in market is mainly hybrid cultivars like many other vegetable crops. For hybrid breeding, a rapid establishment of parental lines with excellent traits is important. Recently emerged CRISPR/Cas9 is getting attention as a genome editing platform due to its advantages to other genome editing technologies. Previously tomato mutants generated via CRISPR/Cas9 system targeting *SlGAD3*, a responsible gene for GABA accumulation in tomato fruits, exhibited highly increased GABA contents. In the mutants, a mutation were induced at the c-terminal region of *SlGAD3*, resulting in a truncation of auto-inhibitory domain of SlGAD3 protein. In this study, I evaluated those mutants as a parental line of hybrid breeding, especially for genetic stability of mutations and its effect on GABA contents and the other traits of hybrid lines. Two mutant lines (TG3C37#21-7 and TG3C37#21-19) were selected for making hybrids with commercial pure line cultivar "Aichi first". The mutations in the mutants were inherited with genetically stable manner to the following generations. The F_1 hybrid fruits showed increased GABA contents which was a half amount of mutants, indicating the high-GABA is a semi-dominant trait. The GABA contents increased up to 18 μmol/gFW, which is still enough to expect the health-prompting function as a food. On the other hands, the increased GABA did not confer negative effects on other traits including fruit color, size and contents of other free amino acid. In summary, mutants generated via CRISPR/Cas9 system are likely to be useful for producing hybrid cultivars with high-GABA contents in tomato. This study also suggest that mutagenesis via CRISPR/Cas9 system can be useful for establishing a rapid breeding system for a trait which the molecular mechanism is well elucidated.

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