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For Tomorrow's Children and Earth

Sanatech Seed Co., Ltd.
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19th May 2020

Bernadette Juarez
APHIS Deputy Administrator
Biotechnology Regulatory Services
4700 River Road, Unit 98
Riverdale, MD 20737

Confidential Business Information Deleted

Dear Ms. Juarez,

Sanatech Seed Co., Ltd. is Japanese Seed Company that produces and sells seeds that have undergone selective breeding through the use of genome editing.

We respectfully seek a confirmation from the Biotechnology Regulatory Services (BRS) that our high γ-aminobutyric acid (GABA) tomato line (*Solanum lycopersicum,* the name of variety is Sicilian Rouge CF) produced with CRISPR-Cas9 gene editing technology does not meet the definition of a regulated article described in 7 CFR Part 340.

Development purpose and the target gene

γ-aminobutyric acid (GABA) is a non-protein amino acid, which is widely found in bacteria, animals, and plants. In mammalian central nervous system, GABA functions as a major inhibitory neurotransmitter[1] and oral administration of GABA provides various benefits to human health, such as lowering blood pressure[2, 3] and producing relaxation effects[4]. Daily intake of GABA-enriched foods would be an effective way to prevent hypertension.

In higher plants, GABA is mainly metabolized via a short pathway known as the GABA shunt and glutamate decarboxylase (GAD) catalyzes the irreversible decarboxylation of glutamate to produce GABA[5, 6]. GAD possesses an additional C-terminal residue, known as the calmodulin (CaM)-binding domain (CaMBD). *In vitro* studies have shown that GAD activity is stimulated through a low pH or the binding of Ca^{2+}/CaM to the CaMBD[7, 8, 9]. In addition, transgenic studies show that the removal of CaMBD results in higher GABA accumulation in plants[10, 11, 12, 13]. Thus, it is considered that the CaMBD acts as a negative regulator/autoinhibitory domain in the absence of Ca^{2+}/CaM, and the negative regulation is relieved through the binding of Ca^{2+}/CaM to CaMBD. Therefore, we aim to remove CaMBD by the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9

technology to enhance GABA productivity in tomato. The tomato genome contains at least three *GAD* genes *SlGAD1*, *SlGAD2* and *SlGAD3* that function during fruit development[14]. Since the expression pattern of [ ] is positively correlated with the GABA accumulation during fruit development, we targeted [ ] for CRISPR/Cas9 engineering. CBI-deleted CBI-deleted

Genome editing technology used and details of the genetic change

To generate high GABA tomato line, the parental *S. lycopersicum* Sicilian Rouge CF was first transformed with T-DNA containing the coding sequence for Cas9 driven by a *Petroselinum crispum* (parsley) ubiquitin promoter (pcUbi) and the DNA sequences for targeted-gene-specific small guide RNAs (sgRNAs) (**Figure 1**), via *Agrobacterium tumefaciens* mediated transformation method[15, 16]. Genetic elements of the T-DNA region of Agrobacterium plasmid are described in Figure 1. Null segregant lines were obtained by self-crossing and screened for the absence of 10 regions which cover the entire binary vector by Polymerase Chain Reaction (PCR) (**Figure 2 and Table1**). sgRNA-targeted site in these null segregant plants were sequenced by Sanger method (**Table2**). We obtained a line with 1 bp insertion mutation that induces a stop codon immediately upstream of the autoinhibitory domain in C-terminal (**Table2**).

Phenotype of proposed line

The high GABA tomato line showed 4 to 5 times higher GABA accumulation in red-stage tomato fruits (10 days after the Breaker stage), which was 4 to 5 times higher than that in WT, when measured by the GABase assay method[17] (**Figure 3**). The high GABA accumulation trait was found through three generations (T_0, T_1 and T_2), suggesting that this trait is genetically stable.

Such a large amount of GABA did not affect plant growth and fruit ripening. We confirmed that the level of Glutamate that is precursor of GABA was not changed in red fruit so we considered the high GABA line doesn't affect other metabolic product. We also confirmed that a steroidal glycoalkaloid tomatine, that is known as a phytoalexins[18] was not detected in red fruits (not data shown, entrusted to Japan Food Research Laboratories). Therefore, we considered the high GABA line doesn't lead to increase of toxin or allergen productions.

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Summary

In summary, the high GABA tomato line shows no remarkable changes other than GABA amount, compared with conventional tomatoes. GABA is widely found in bacteria, animals, and plants. Tomato is not a plant pest itself within the meaning of the Plant Protection Act and does not pose a weed potential and there are no Agrobacterium or other plant pest sequences inserted in the final plant line. For all of the reasons provided above, we respectfully ask for APHIS confirmation that the high GABA tomato line is not a regulated article subject to APHIS oversight under 7 C.F.R. Part 340.

We thank APHIS in advance for your consideration of this request. If you have any questions, we would welcome the opportunity to meet with you at a convenient time to further discuss the high GABA tomato line.

Sincerely,

住吉美奈子

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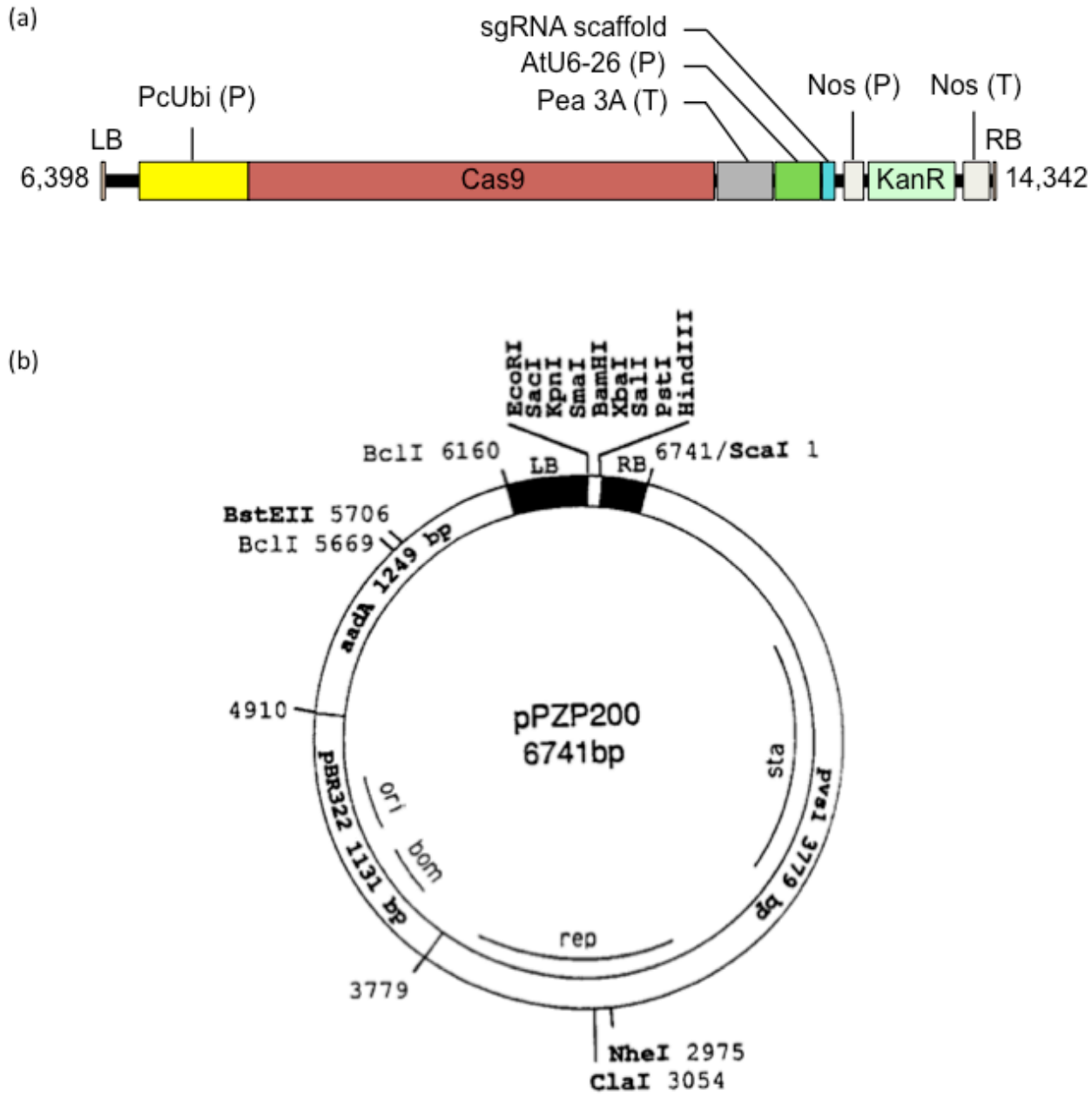

Figure 1 The Plasmid for transformation

(a) Structure of T-DNA region. LB: Left Border, pcUbi(P): *Petroselinum crispum* (parsley) ubiquitin promoter, Cas9: *Streptococcus pyogenes* Cas9, Pea3A(T): *Pisum sativum* (Pea) 3A terminator, AtU6-26(P): *Arabidopsis thariana* U6-26 promoter, sgRNA scaffold: synthetic DNA with target sequences, Nos(p): Agrobacterium tumefaciens nopaline synthase promoter, KanR: *Escherichia coli* Kanamycin resistance protein , Nos(T): Agrobacterium tumefaciens nopaline synthase terminator , RB: Right Border

(b) Structure of the binary vector pPZP.
T-DNA region(a) inserted between LB and RB. aada: *Enterrococcus faecails* spectinomycin adenylyltransferase

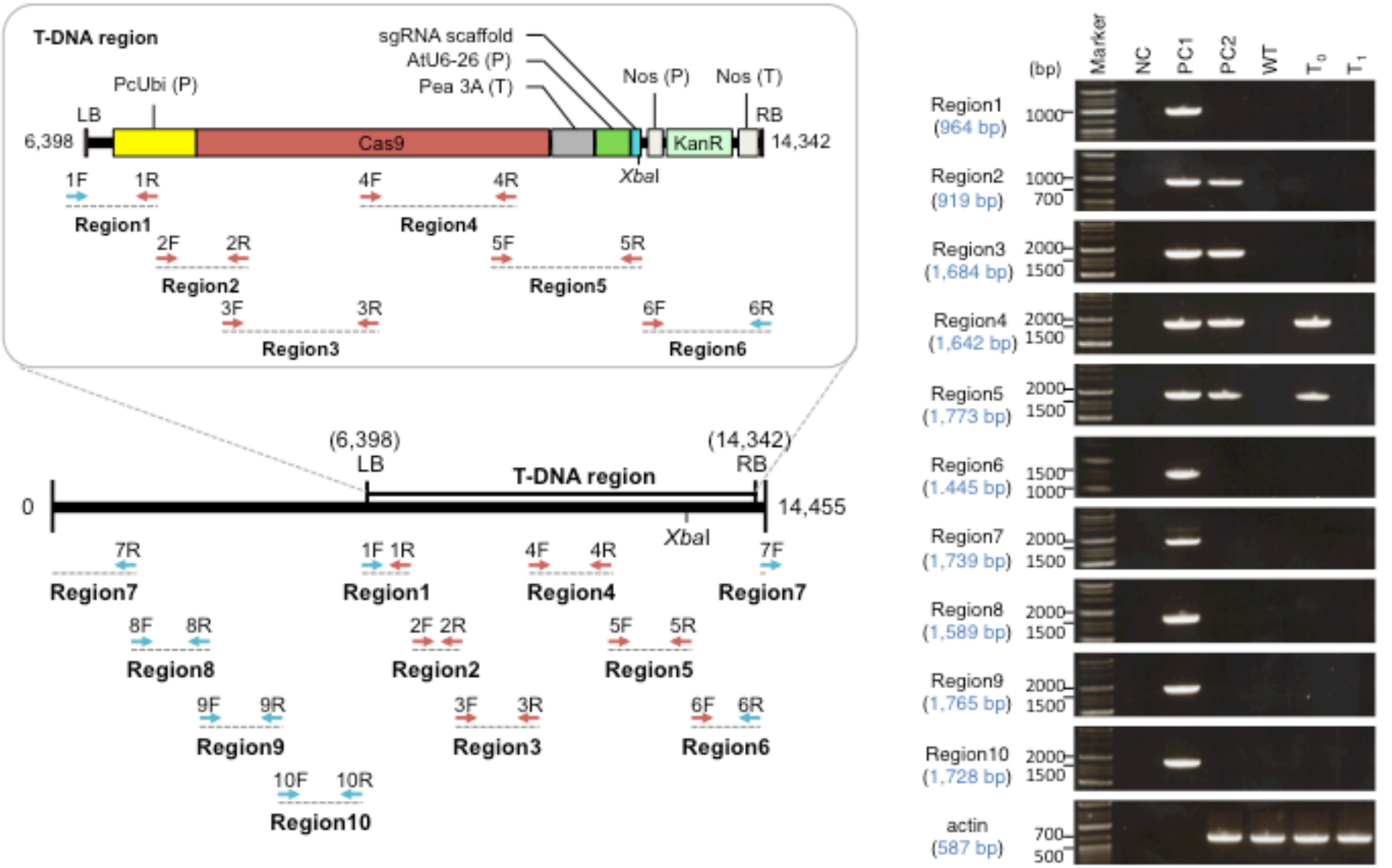

Figure 2 Confirmation of absence of Agrobacterium plasmid DNA by PCR

Positions of PCR primer pairs used to detect the presence of T-DNA outside –DNA region are indicated.Presence of the PCR product indicates the existence of transgene sequence in transgenic plants. NC:negative control, PC1:positive control(binary vector). PC2:positive control2 (T0, the other event).WT: a plant before transformation , T1: high GABA tomato (T1 generation)

Table 1 Primers used in transgenic plant analysis

PCR amplified region	Primer name	Primer (5'→3')	Position (from 5')	Amplified product size (bp)
Region1	Primer_1F	GATGGGCTGCCTGTATCGAG	6,330	964
	Primer_1R	AACATAACCACGTGTAGATACACAGT	7,293	
Region2	Primer_2F	TGACTGTGTATCTACACGTGGTTATG	7,266	919
	Primer_2R	AGCGAGGTAGATGAGCCTGA	8,184	
Region3	Primer_3F	ATCTACCTCGCTCTCGCTCA	8,173	1,684
	Primer_3R	CTGAAACCTGAGCCTTCTGG	9,856	
Region4	Primer_4F	AACGGAATCAGGGATAAGCA	9,715	1,642
	Primer_4R	CGAGCATCCTCTTTCTACCG	11,356	
Region5	Primer_5F	TCCTCGAGGCTAAGGGATACA	11,255	1,773
	Primer_5R	CTGAGTGGCTCCTTCAACGT	13,027	
Region6	Primer_6F	CGAACCGCAACGTTGAAGGAGC	12,999	1,445
	Primer_6R	GGAACCCTGTGGTTGGCATGCAC	14,443	
Region7	Primer_7F	TCCGTTCGTCCATTTGTATGTGCA	14,402	1,739
	Primer_7R	GGGACTCAAGAATGGGCAGCTC	1,685	
Region8	Primer_8F	ATTGAGGTCACGGATGGAAG	1,562	1,589
	Primer_8R	TGCGTTCGTAGATCGTCTTG	3,150	
Region9	Primer_9F	CCGGAGTACATCGAGATCGAGC	2,955	1,765
	Primer_9R	GCTTCAGCAGAGCGCAGATACC	4,719	
Region10	Primer_10F	GCAGAGCGAGGTATGTAGGC	4,618	1,728
	Primer_10R	ATACAGGCAGCCCATCAGTC	6,345	

Table 2 Mutation pattern of high GABA tomato line
(a) DNA sequence (b) amino acid sequence

(a)

WT	[	]	CBI-deleted
high GABA line	[	]	CBI-deleted

(b)

WT	[	]	CBI-deleted
high GABA line	[	]	CBI-deleted

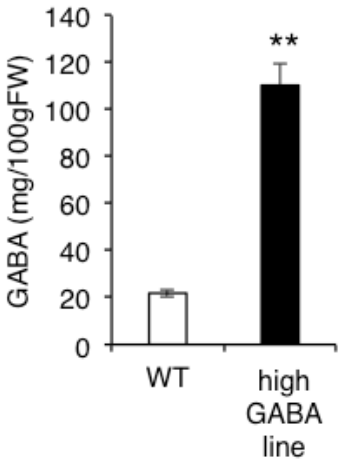

Figure 3 GABA content in high GABA tomato line (T1 generation)
GABA content of red-stage tomato fruits.
Bars indicate standard error (n ≥ 3) and asterisks indicate statistical differences according to the Student's *t*-test between high GABA line and WT (*$P < 0.05$ and **$P < 0.01$).

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