Prior Evaluation Form (PEF) for Products of Plant Breeding Innovation (PBI)

Part I. Background Information	
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Part II. Description of the PBI Product	
1. Name of the PBI Product	High GABA Sicilian Rouge tomato
2. Identification of the PBI Product (organism)	Scientific Name: *Solanum lycopersicum*
	Common Name: Tomato
3. Phenotypic feature before and after genetic change (Explain in detail.)	The PBI product is the tomato containing high levels of gamma-aminobutyric acid (GABA), an amino acid that has been associated with health benefits, for example, lowering blood pressure with mild hypertensive people, improving sleep quality and decreasing in the immediate stress score. In higher plants, GABA is mainly metabolized via a short pathway known as the GABA shunt and glutamic acid decarboxylase (GAD) catalyzes the irreversible decarboxylation of glutamic acid to produce GABA. GABA is produced at 190 to 600 ug/gFW in the unedited tomato. The *Solanum lycopersicon* GAD was targeted for CRISPR/Cas9 engineering. To generate high GABA tomato line, the parental S. lycopersicum Sicilian Rouge was first transformed with T-DNA containing the coding sequence for Cas9, via Agrobacterium-mediated transformation method. We obtained a line with 1 bp insertion mutation that induces a stop codon immediately upstream of the autoinhibitory domain (AID) in C-terminal. This insertion was induced by DBS and was not intentionally inserted. The high GABA tomato line showed 5 to 6 times higher GABA accumulation in red-stage tomato fruits (10 days after the Breaker stage) than the wild type. The high GABA tomato has no unintended changes in other phenotypes.

Part III. Description of the Plant Breeding Innovation (PBI) Procedure Used	
1. Purpose of the PBI	Develop tomato *(S. lycopersicum)* with high levels of γ-Aminobutyric acid (GABA)
2. PBI procedure	SDN 1
3. Genetic change in the organism	
a. Name of the molecular tools used	CRISPR / Cas9
b. Description and nucleotide sequence ofthe molecular tools used i. Nuclease ii. Vectors	 Cas9 pPZP200
c. Delivery system	Agrobacterium-mediated
d. Nature of DNA changes	1-base insertion
4. Experimental evidence showing the final PBI product has no new combination of genetic material in the form of foreign DNA insert or sequences from gene editing tool construct usingappropriate molecular techniques.	Null segregant lines were obtained by self-crossing (Figure 1) and screened for the absence of 10 regions which cover the entire binary vector by Polymerase Chain Reaction (PCR), and Southern blot analysis with vector sequences. The transformant plant heterozygous for the T-DNA insertion was grown to maturity. The T1 seeds were grown to maturity and screened for T-DNA. The null segregants homozygous for T-DNA absence were selected and advanced to T2 seeds. PCR results showed that no bands were detected in the T1 generation or WT in any of the 10 regions of the plasmid vector. On the other hand, bands were detected in vector regions 4 and 5 in the positive control T0 generation (Figure 2). Southern blot analysis indicated partial absence of vector elements in T0 and complete absence in T1(Figure 3). Probes 1-6 were used to check for residual T-DNA regions, and bands were detected in only T0 generation for regions 3-6. In addition, the presence or absence of the binary vector region was confirmed using probes 7-10, and no bands were detected in any lines. This indicates that T-DNA fragments in the T0 generation were removed in the T1 lines by self-crossing. <u>Summary:</u> The product was derived by self-crossing the initial transformants with one copy of the editing vector and with high GABA content 4.5 x higher than the wild type, #87. The progeny, #87-17, with no copy of the vector from segregation and expressing high GABA level 5 to 6 x higher was selected. The absence of the vector sequences in the selected T1and T2 plants was confirmed by PCR screening and Southern blot analysis using primers and probes that amplify or detect 10 locations in the T-DNA and vector regions. No PCR amplification products and no Southern hybridization bands that correspond to T-DNA and vector regions were obtained in the final gene-edited high-GABA tomato line.
5. Any existing regulatory precedence on the PBI Product in the issuing country and purpose of thedecision (if applicable).	1. Determined as non-GMO by Japan MAFF 2. Determined as gene-edited food by Japan MHLW 3. Deregulated by the US Department of Agriculture being not a pest nor containing pest sequences in its genome

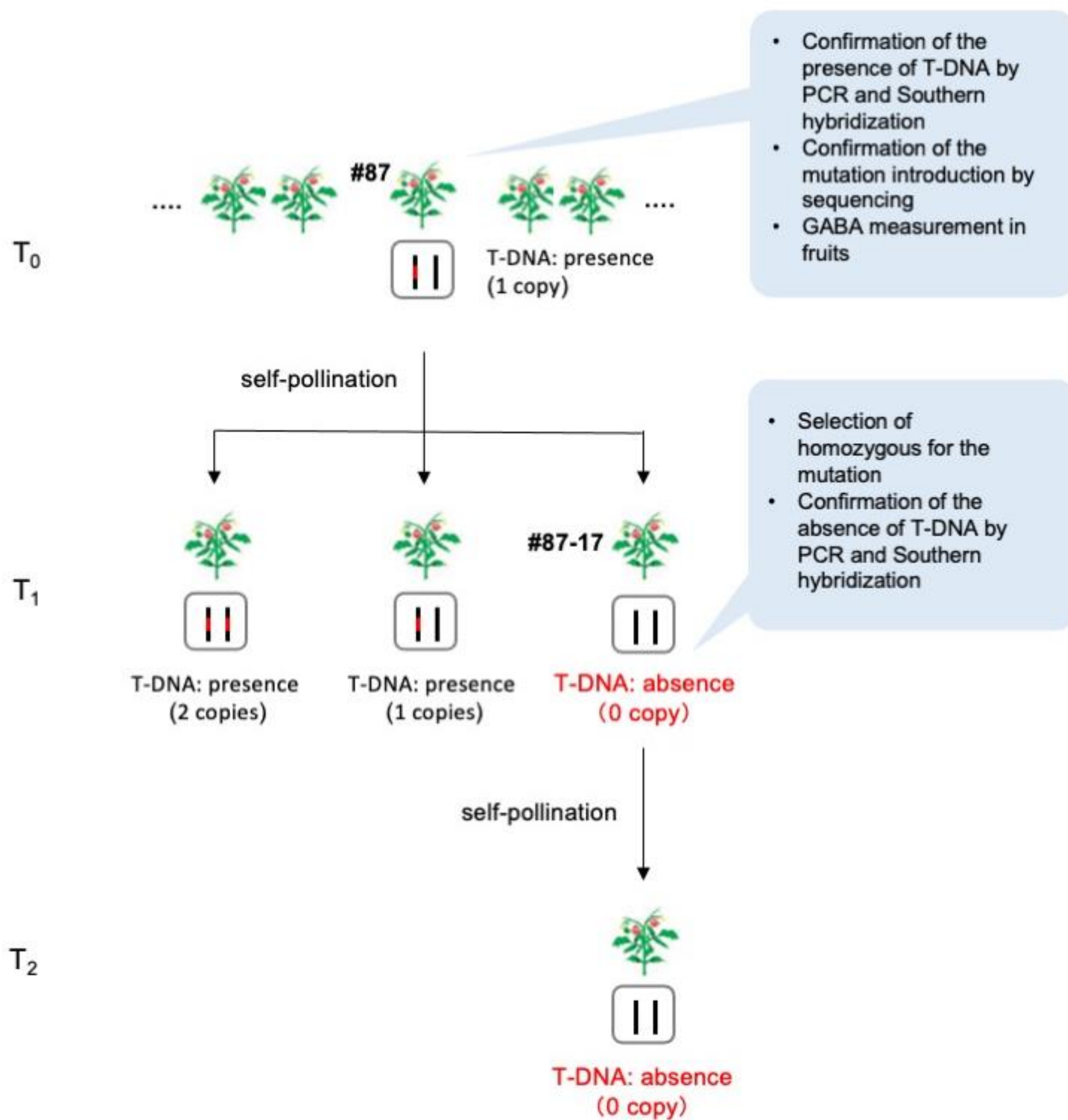

Figure 1. Identification of edited lines, breeding scheme, and screening for null segregants devoid of vector sequence

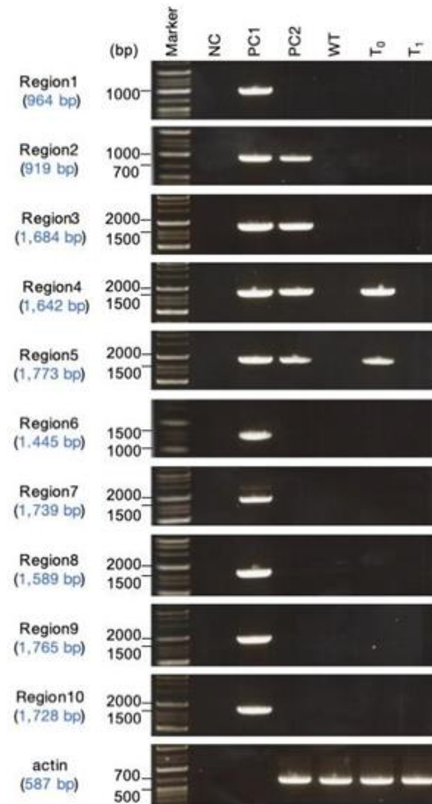

Figure 2. Gel analysis of PCR products from edited lines screened for vector sequences sNC: negative control, PC1: positive control (binary vector), PC2: positive control 2 (Line #21-16 containing partial T-DNA as positive control for deleted region), WT: a plant before transformation, T0: high GABA line #87 (T0 generation), T1: high GABA tomato line #87-17 (T1 generation).

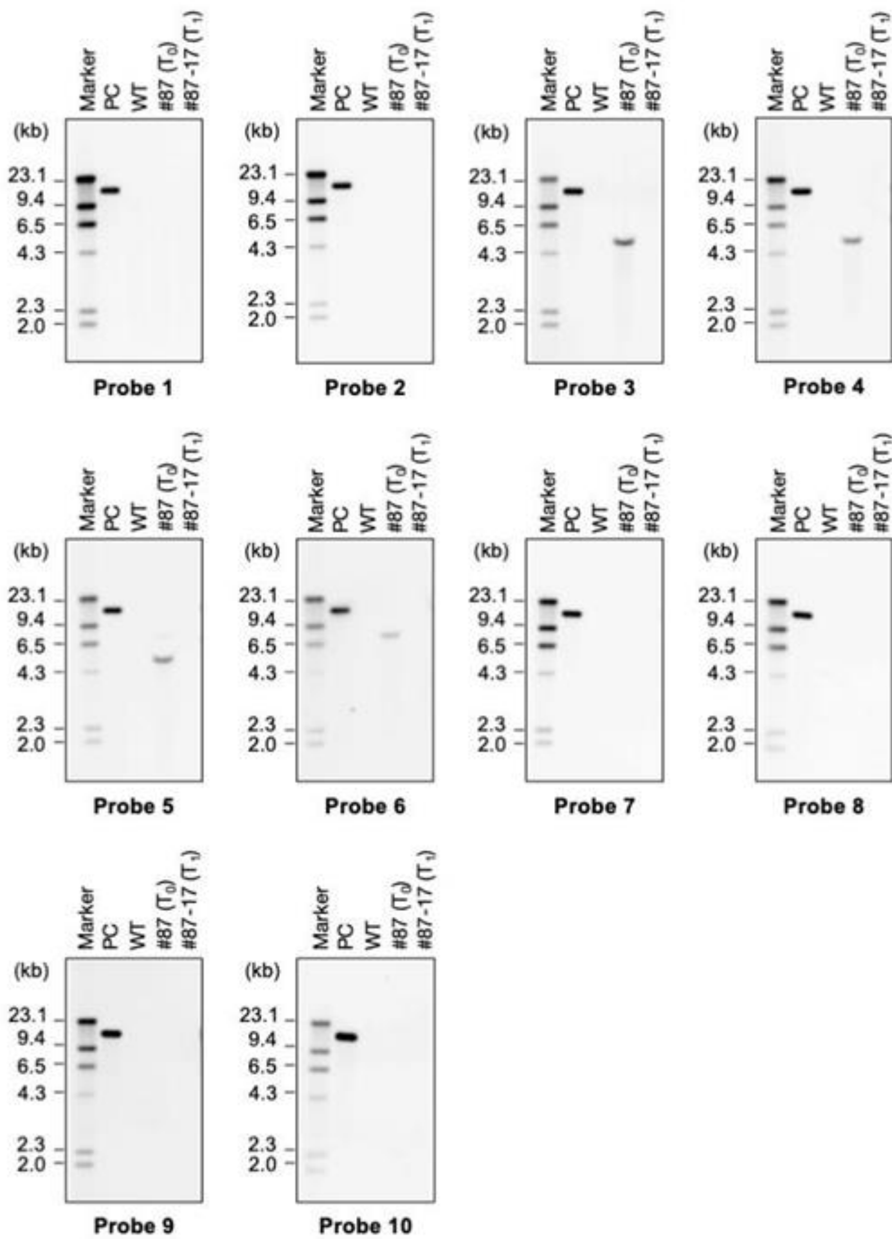

Figure 3. Southern blot analysis of edited lines hybridized with vector probes.