Corporation obtaining approval, the name of its representative, and the address of its main office

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Soybean tolerant to imidazolinone herbicides (Modified *csr1-2*, *Glycine max* (L.) Merr.) (CV127, OECD UI: BPS-CV127-9)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	-

Outline of the Biological Diversity Risk Assessment Report

I. Information collected prior to assessing Adverse Effect on Biological Diversity

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

Composition and origins of component elements of donor nucleic acid are shown in Table 1 (p4).

2) Functions of component elements

(a) Functions of individual component elements of donor nucleic acid, including target gene, expression regulatory region, localization signal, and selectable marker

Functions of component elements of donor nucleic acid are shown in Table 1 (p4). Target genes, expression regulatory region and other factors are detailed below.

Modified *csr1-2* gene

Soybean tolerant to imidazolinone herbicides (modified *csr1-2*, *Glycine max* (L.) Merr.) (CV127, OECD UI: BPS-CV127-9) (hereinafter referred to as the CV127 line) was developed jointly by BASF Plant Science Company GmbH and Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA).

The imidazolinone herbicides-tolerant gene (modified *csr1-2* gene derived from *Arabidopsis thaliana*) transferred to the CV127 line encodes the acetohydroxyacid synthase (hereinafter referred to as the AHAS protein) (also known as acetolactate synthase (ALS)), and the serine residue at position 653rd in the amino acid sequence of the AHAS protein, a product of the gene, is substituted by the asparagine residue (hereinafter referred to as the modified AHAS protein) (Reference 11; Reference 12). The AHAS protein is an enzyme required by many plants for their life survival and then it is contained in every plant and microorganism, acting to catalyze the first step in the biosynthesis of the branched chain amino acids (valine, leucine, isoleucine) (Reference 32). In non-transgenic plants, inhibition of the AHAS protein by imidazolinone herbicides leads to a deficiency in branched chain amino acids, this causing inhibition of plant growth. On the other hand, in the case of plants to which the modified *csr1-2* gene is transferred, the

normal biosynthesis functions remain unaffected but they exhibit the tolerance to the herbicides (Reference 25; Figure 1, p5). It is generally known that the amino acid mutation in the modified AHAS protein inhibits the binding of imidazolinone herbicides and as a result the tolerance to the herbicides is conferred without any effects on the normal biosynthesis functions (Reference 23).

Table 1 Origins and functions of component elements of donor nucleic acid

Component elements	Location (bp)	Origin and function
Linear DNA fragment of approx. 6.2 kb (LF-6.2PvuII) used for transfer to the soybean genome		
lacZ promoter	8468-8589	lacZ promoter of *Escherichia coli* (*E. coli*); It promotes transcription of β-galactosidase alpha fragment (lacZ').
lacZ' CDS	8590-8669 5718-5994	Coding sequence of *E. coli* β-galactosidase alpha-fragment; The alpha complementation helps determine whether the target sequence has been transferred in the multi-cloning site through the blue/white screening.
T3 promoter [1]	8632	Transcription initiation site of bacteriophage T3 promoter; The phagemid enables the *in vitro* synthesis of RNA by the T3 RNA polymerase.
Arabidopsis gDNA, unannotated 1	1-1051	A sequence derived from *Arabidopsis thaliana* genome DNA As a result of homology search, no sequence has been identified that has homology with any known gene.
AtSEC61γ 5'UTR	1052-1113	5' untranslated region of *Arabidopsis thaliana SEC61γ* A region involved in transcription and regulation of *SEC61γ* gene
*AtSEC61γ*CDS	1114-1207 1307-1422	Coding region of *Arabidopsis thaliana SEC61γ* *SEC61γ* subunit works jointly with the α and β subunits to constitute a part of transport protein complex.
AtSEC61γ intron 1	1208-1306	First intron of *Arabidopsis thaliana SEC61γ* To be cleaved after transcription and not included in the end RNA product.
AtSEC61γ 3'UTR and terminator	1423-1442 1916-2119	3' untranslated region and terminator of *Arabidopsis thaliana SEC61γ* A region involved in termination and regulation of transcription of *SEC61γ* gene
AtSEC61γ intron 2	1443-1915	Second intron of *Arabidopsis thaliana SEC61γ* To be cleaved after transcription and not included in the end RNA product.
AtAHAS 5'UTR and putative promoter	2120-2483	5' untranslated region and putative promoter of *Arabidopsis thaliana AHAS* A region involved in transcription and regulation of *AHAS* gene
Modified *csr1-2* gene	2484-4496	Modified *csr1-2* gene region of *Arabidopsis thaliana* It expresses the modified acetohydroxyacid synthase (modified AHAS protein), which has the serine residue at position 653rd in the amino acid sequence of AHAS protein replaced by the asparagine residue, and offers the tolerance to imidazolinone herbicides.
AtAHAS 3'UTR and terminator	4497-4714	Modified *AHAS* 3' untranslated region and terminator of *Arabidopsis thaliana* A region involved in termination and regulation of transcription of *AHAS* gene
Arabidopsis gDNA, unannotated 2	4715-5717	A sequence derived from *Arabidopsis thaliana* genome DNA As a result of homology search, no sequence has been identified that has homology with any known gene.
T7 promoter [1]	5805	Bacteriophage T7 promoter transcription initiation site; The phagemid enables the *in vitro* synthesis of RNA by the T7 RNA polymerase.

1. For T3 promoter and T7 promoter, no end point has been determined.

The thick-line box refers to the modified *csr1-2* gene expressed region.

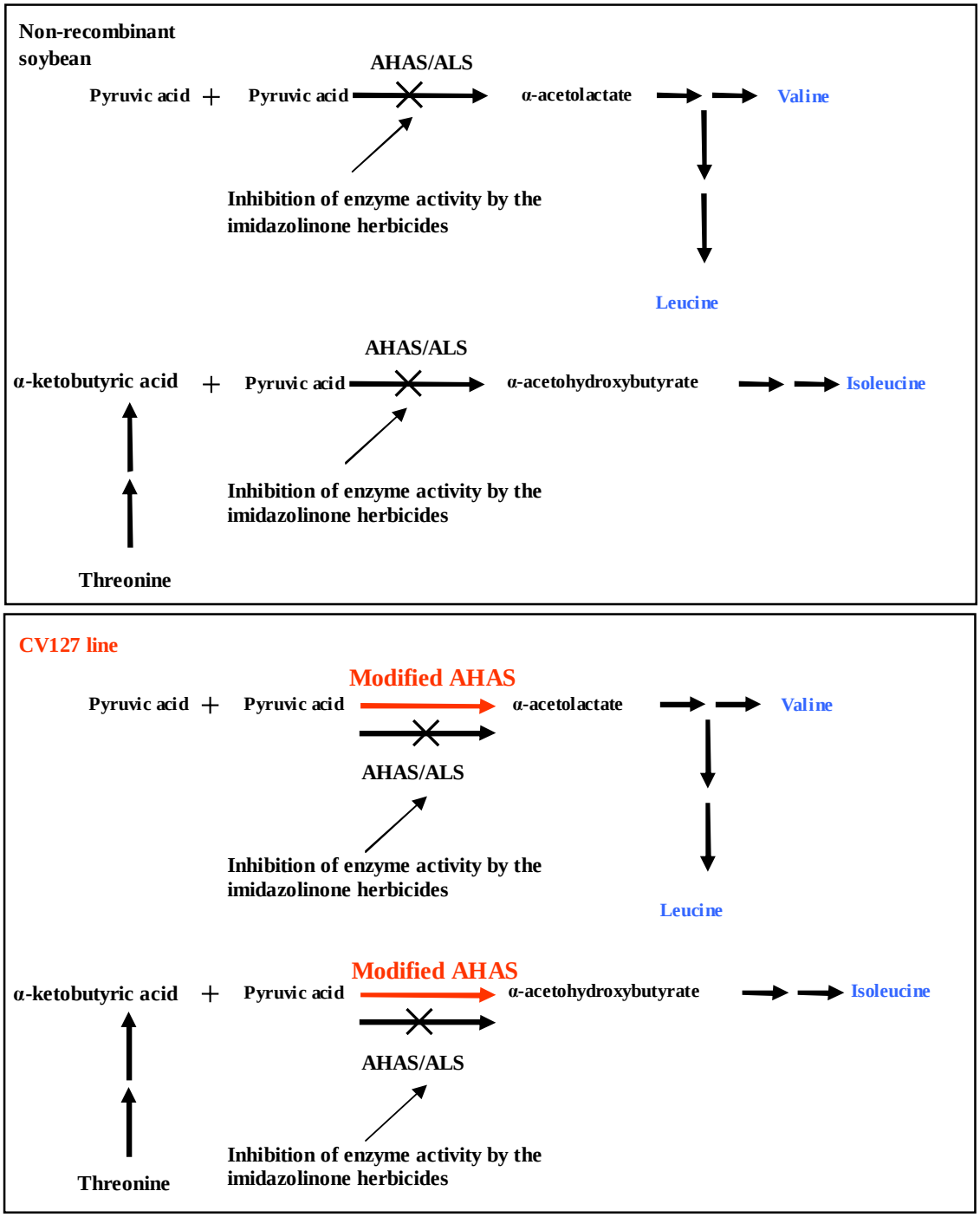

Figure 1 Mechanism of action of the modified AHAS protein

The AHAS/ALS catalyzes the first step in the biosynthesis of the branched chain amino acids (valine, leucine and isoleucine). Typically, the enzyme activity is inhibited by the imidazolinone herbicides, while the modified AHAS protein remains unaffected by the herbicides but it confers the tolerance to the imidazolinone herbicides.

Expression regulatory region

The modified *csr1-2* gene expressed region occurs from the *AHAS* gene promoter region derived from *Arabidopsis thaliana*, containing the 3' untranslated region (UTR) derived from *Arabidopsis thaliana*.

Localization signal

The modified *csr1-2* gene products are considered to form a precursor composed of 670 amino acid sequences containing the chloroplast transport peptide at the N-terminal region (Reference 19). Then, it is considered that from the precursor (670 amino acids), the chloroplast transport peptide (85 amino acids) is cleaved to form the mature modified AHAS protein composed of 585 amino acids. The modified AHAS protein is localized in the chloroplast.

***AtSEC61γ* subunit gene**

At the time of cloning of the modified *csr1-2* gene, there was no information available for promoter of the *AHAS* gene and then, for transformation to soybean, the fragment including up to a length of approx. 2.5 kbp in the upstream from the start codon of the modified *csr1-2* gene was used. Later in 2000, the entire nucleotide sequence of *Arabidopsis thaliana* was identified. As a result of homology search (BLASTn search) based on the identified information, it was confirmed that the complete sequence of the *AtSEC61γ* subunit gene was contained in the length of approx. 2.5 kbp (GenBank International Nucleotide Sequence Databases Accession No. At3g48570, 69 amino acids) (hereinafter referred to as the *AtSEC61γ* gene). The SEC61γ subunit protein encoded by this gene forms transport protein complex jointly with SEC61α and SEC61β subunit proteins, which is highly contained in every plant and eukaryote (Reference 10).

It is generally known that four (4) *SEC61γ* genes are present in the soybean genome. Between the SEC61γ subunit proteins in the *Arabidopsis thaliana* and soybean, amino acid sequence homology (approx. 86%) is confirmed (Figure 2; Table 2). This suggests that the *Arabidopsis thaliana* SEC61γ subunit protein is equivalent to the SEC61γ subunit protein present in the conventional soybean.

Figure 2 **Alignment of SEC61γ subunit proteins in *Arabidopsis thaliana* and soybean (Soy) [Confidential: Not made available or disclosed to unauthorized person]**

Table 2 **Homology of amino acid sequence between SEC61γ subunit proteins in *Arabidopsis thaliana* and soybean (Soy) (%) [Confidential: Not made available or disclosed to unauthorized person]**

(b) Functions of proteins produced by the expression of target gene and selective markers, and the fact, if applicable, that the produced protein is homologous with any protein which is known to possess any allergenicity

In the CV127 line, due to the amino acid mutation in the modified AHAS protein, a product of the transferred modified *csr1-2* gene, binding of imidazolinone herbicides is inhibited. Then the normal biosynthesis functions remain unaffected, allowing the line to possess the tolerance to the herbicides.

In order to check for homology between the modified AHAS protein expressed in the CV127 line and any known allergens, homology search was conducted by downloading the allergen protein database (Version 8.00) of Food Allergy Research and Resource Program (FARRP) (April 26, 2007). As a result, it was found that the modified AHAS protein has no homology with any known allergen.

In addition, as a selection marker, the transferred modified *csr1-2* gene was used.

(c) Contents of any change caused to the metabolic system of recipient organism

The modified AHAS protein is only conferred with the herbicide tolerance due to the mutation of S653N in the AHAS protein (Annex 4), and it has no impact on any other metabolic systems and substrate specificity. Therefore, the modified AHAS protein only catalyzes the first step in the biosynthesis pathway of the branched chain amino acids (valine, leucine, isoleucine) similarly as in the case of the AHAS protein, and it is considered not to have any impact on other metabolic systems except the amino acids.

For the possibility that the modified AHAS protein could affect the metabolic system of amino acids, examination was made by investigating the amino acid composition in the seeds of the CV127 line. For tryptophan, analysis was made based on the method referred to in Reference 31. For the other amino acids, analysis was made based on the method referred to in Reference 30. The results include asparagine for aspartic acid and glutamine for glutamic acid.

As a result of *t*-test, regarding all the amino acids in the soybean seeds analyzed, no statistically significant difference was observed between the BC2-F6 generation of the CV127 line (Figure 5, p14) and the non-recombinant control soybean (hereinafter referred to as the control cultivar) (Table 3).

Table 3 **Amino acid composition in the soybean seeds (mg/100 g of dry weight) [Confidential: Not made available or disclosed to unauthorized person]**

(2) Information concerning vectors

1) Name and origin

The linear DNA fragment of approx. 6.2 kb in length used for the development of the CV127 line (hereinafter referred to as the LF-6.2PvuII) is a fragment cleaved from the plasmid pAC321. The plasmid pAC321 was constructed based on the plasmid pBluescript SK (-) (Reference 27). The plasmid pAC321 is shown in Figure 3A (p10), and the LF-6.2PvuII is shown in Table 1 (p4) and Figure 3B (p10).

2) Properties

(a) The numbers of base pairs and nucleotide sequence of vector

The plasmid pAC321 has the entire length of 8,669 bp. The detail is shown in Figure 3A. In addition, the nucleotide sequence is shown in Annex 1.

(b) Presence or absence of nucleotide sequence having specific functions, and the functions

The plasmid and the DNA fragments used for the construction of the plasmid pAC321 are derived from nonpathogenic *Escherichia coli* (*E. coli*) or *Arabidopsis thaliana*, and they do not contain any known noxious nucleotide sequence.

The LF-6.2PvuII used for transfer to the soybean genome was cleaved from the plasmid pAC321, and this fragment does not contain any

ampicillin-tolerant gene. In addition, the plasmid pAC321 does not contain any sequence allowing transmission.

(c) Presence or absence of infectivity of vector and, if present, the information concerning the host range

The plasmid pAC321 is considered not to possess any infectivity.

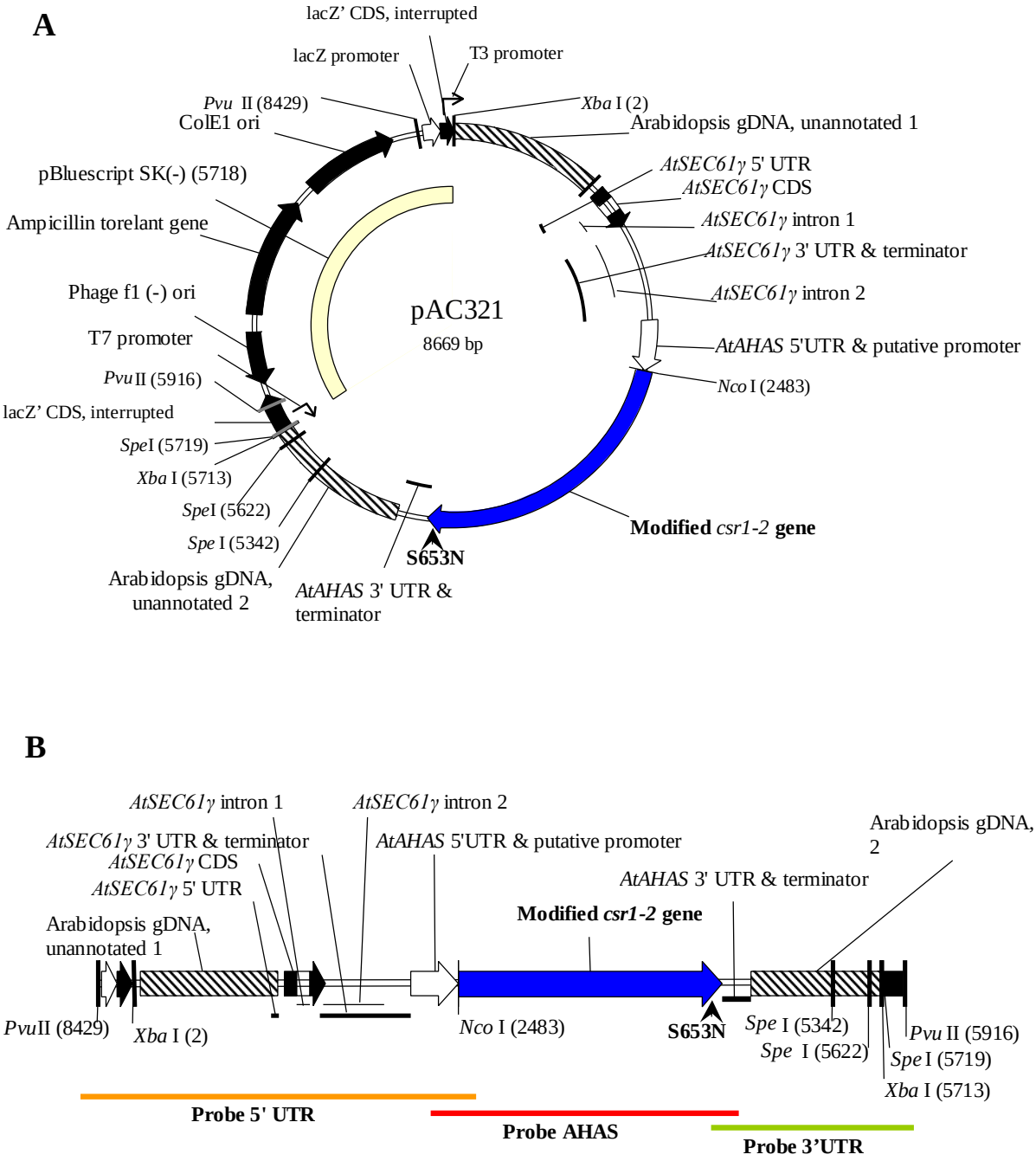

Figure 3 Map of the plasmid pAC321 and the linear DNA fragment of approx. 6.2 kb transferred in the soybean

A. Map of the plasmid pAC321

B. Linear DNA fragment of approx. 6.2 kb transferred in the soybean (LF-6.2PvuII) Number of copies, and the restriction enzyme sites (*Nco*I, *Xba*I, *Spe*I) and probe regions used in the Southern blotting analysis for the stability through multiple generations are also shown.

(3) Method of preparing living modified organisms

1) Structure of the entire nucleic acid transferred in the recipient organism

Locations and directions of component elements in the plasmid pAC321 in the nucleic acid-transferred region are shown in Table 1 (p4) and Figure 3A (p10). In addition, the schematic diagram of the nucleic acid-transferred region is shown in Figure 3B. The transferred sequence is the LF-6.2PvuII formed by the cleavage of the restriction enzyme *Pvu*II.

2) Method of transferring nucleic acid transferred to the recipient organism

Methods of cloning of the modified *csr1-2* gene and transformation to soybean are as described below (Figure 4, p13).

The cDNA of the mutation of imidazolinone herbicide-tolerant *Arabidopsis thaliana* developed through the ethyl methanesulfonate (EMS) treatment was cloned in the vector λ sep6 and transformed in *E. coli* of the LE392 strain to construct the cDNA genome library (Reference 17). From the genome library, a clone containing the *AHAS* gene sequence was selected, using the *AHAS* gene sequence of yeast (*Saccharomyces cerevisiae*) as a probe (Reference 19), and a fragment of approx. 5.7 kb cleaved by the restriction enzyme *Xba*I was cloned in the plasmid pBluescript SK (-) (Stratagene Corporation). Then, the blue/white screening with the alpha complementation of β-galactosidase alpha fragment was conducted and the approx. 5.7 kb fragment-transferred plasmid was subjected to the sequence analysis. Confirming that the modified *csr1-2* gene-transferred region was cloned in the plasmid pBluescript SK (-), the plasmid of approx. 8.7 kb was selected as the plasmid pAC321. From this plasmid, approx. 6.2 kb of linear DNA fragment LF-6.2PvuII was cleaved by the restriction enzyme *Pvu*II, separated through the agarose gel electrophoresis and then purified, and transformed in the soybean.

From a single grain of mature seed of soybean, the embryonic axis including the apical meristem was taken out (Reference 1) and placed on the MS agar medium containing 44.3μM of benzylaminopurine and 3% sucrose (Reference 21), and the LF-6.2PvuII was transferred in the recipient organism based on the particle bombardment (Reference 14; Reference 16; Reference 26). The CV127 line was developed through the single transformation and the selection method discussed later.

3) Processes of rearing living modified organisms

(a) Mode of selecting the cells containing the transferred nucleic acid

Selection was made based on the selective media of 0.5 to 1.0 μM of imidazolinone herbicide Imazapyr.

(b) Presence or absence of remaining *Agrobacterium* in case of using *Agrobacterium* method for transferring nucleic acid

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(c) Process of rearing and pedigree trees of the following lines; cells to which the nucleic acid was transferred, the line in which the state of existence of replication products of transferred nucleic acid was confirmed, the line subjected to isolated field tests; and the line used for collection of other necessary information for assessment of Adverse Effect on Biological Diversity

The pedigree tree of the lines used for the sequence analysis including soybean flanking sequences, Southern blotting analysis for examination of inter-generational expression stability and collection of other necessary information for assessment of Adverse Effect on Biological Diversity is shown in Figure 5 (p14).

1. The cDNA of the mutation of imidazolinone herbicide-tolerant *Arabidopsis thaliana* developed through the ethyl methanesulfonate (EMS) treatment was cloned in the vector λ sep6 and transformed in *E. coli* of the LE392 strain to construct the cDNA genome library.
2. Using the *AHAS* gene sequence of yeast as a probe, a clone containing the *AHAS* gene sequence was selected.

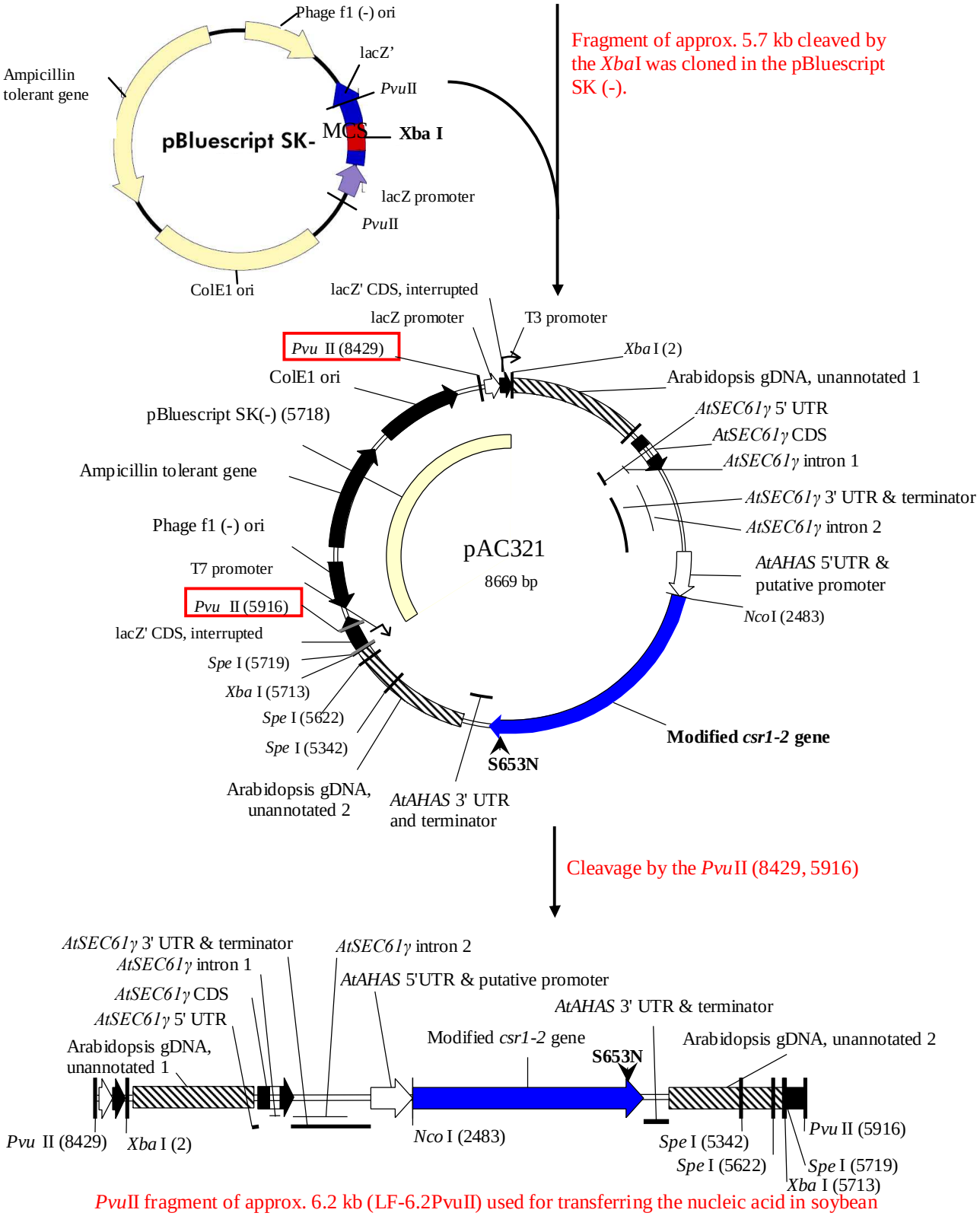

Figure 4 Flow chart of cloning of the modified *csr1-2* gene and transformation to soybean

(All the rights pertinent to the information in the diagrams above and the responsibility for the content rest upon BASF Japan Ltd.)

Figure 5 **The pedigree tree of the CV127 line [Confidential: Not made available or disclosed to unauthorized person]**

Table 4 **A list of generations used for safety assessment [Confidential: Not made available or disclosed to unauthorized person]**

(4) State of existence of nucleic acid transferred in cells and stability of expression of traits caused by the nucleic acid

1) Place where the replication product of transferred nucleic acid exists

Based on the genetic analysis described below and the Southern blotting analysis discussed later, it was confirmed that the transferred modified *csr1-2* gene expressed region exists on the chromosome.

Genetic analysis for transferred genes based on the PCR method and herbicide spraying

The heterozygous self-propagated progeny (BC2-F4 generation) of the BC2-F3 generation of the CV127 line was separated into two replicates (Replicate 1 containing 57 plants and Replicate 2 containing 52 plants), and the genetic analysis was conducted for the transferred genes based on the PCR method specific to this event, using the primer "Event PCR3" (Table 10, p21) (Table 5-1). The PCR method is a quantitative PCR analysis to allow quantitative detection of homozygous dominant, heterozygous and homozygous recessive individuals through the fluorescent labeling of primer. As a result, no statistically significant difference was observed between the number of individuals observed and expected value ($p<0.05$).

Similarly, using two replicates of the BC2-F4 generation of the CV127 line, tolerance and sensitivity to the imidazolinone herbicide Imazapyr (100g a.i. (active ingredient)/ha) were evaluated (Table 5-2). As a result, no statistically significant difference was observed between the number of individuals observed and expected value ($p<0.05$) but the individuals were found segregated following the Mendel's law. Consequently, it is considered that the modified *csr1-2* gene expressed region exists on the chromosome.

Table 5-1 Genetic analysis of the modified *csr1-2* gene in the BC2-F4 generation of the CV127 line based on the PCR method [Confidential: Not made available or disclosed to unauthorized person]

Table 5-2 Genetic analysis of the modified *csr1-2* gene in the BC2-F4 generation of the CV127 line based on spraying of herbicides [Confidential: Not made available or disclosed to unauthorized person]

2) The number of copies of replication products of transferred nucleic acid and stability of its inheritance through multiple generations

Results of sequence analysis on the nucleic acid transferred region

The transferred sequence in the genome of soybean and flanking sequences including the modified *csr1-2* gene expressed region are shown in Annex 2. Results of structure analysis of all the transferred regions and flanking sequences are shown in Figure 7 (p17).

As a result of sequence analysis of the CV127 line, it was found that the amino acid substitution of a serine with an asparagine at residue 653rd (S653N) that results in tolerance to imidazolinone herbicides is retained even after transferring the nucleic acid into the soybean genome. In addition, after transformation, a point mutation from G to A in the nucleotide sequence of the modified *csr1-2* gene was newly identified at one site. As a result, it was confirmed that the arginine residue at position 272nd is substituted by the lysine residue (R272K). The amino acid sequence of the AHAS protein including the both amino acid substitutions R272K and S653N is shown in Annex 3. It is suggested that the AHAS protein containing the R272K and S653N possesses the catalytic activity and the tolerance to imidazolinone herbicides, which are comparable to those of the AHAS protein containing only the amino acid substitution S653N (Annex 4).

Number of copies and the integrity of the transferred sequences

To identify the number of copies of the transferred sequences and the inter-generational expression stability, Southern blotting analysis was conducted. The pedigree tree of the CV127 line used is shown in Figure 5 (p14). The primer sequence used for preparation of probes for the Southern blotting analysis is shown in Table 6 (p17), and estimated and observed band sizes by the Southern blotting analysis are listed in Table 7 (p17).

The number of copies of the transferred sequences in the CV127 line was examined based on the Southern blotting analysis by digesting the genome DNA of the BC1-F8 generation of the CV127 line with the restriction enzymes *Nco*I, *Spe*I, and *Xba*I. As probes, *AHAS* 5' UTR region, *AHAS* region, and *AHAS* 3' UTR region were used (Figure 3B, p10). As a result, it was indicated that in the genome of the CV127 line, one copy of the modified *csr1-2* gene expressed region is transferred in the intact form (Lanes 4, 8, and 12 in Figure 8, p17).

Inter-generational expression stability based on the Southern blotting analysis

In order to examine the stability of the modified *csr1-2* gene expressed region transferred in the genome of the CV127 line, Southern blotting analysis was conducted using the genome DNA of 4 generations (T4, BC1-F4, BC1-F8, and BC1-F9). As probes, *AHAS* 5' UTR region, *AHAS* region, and *AHAS* 3' UTR region were used (Figure 3B, p10). It was confirmed that one copy of the transferred modified *csr1-2* gene expressed region exists in the genome of BC1-F4, BC1-F8, and BC1-F9 generations (Lanes 5 to 7, and 12 to 14 in Figure 9, p17). On the other hand, in the genome of T4 generation (Lanes 4 and 11) before backcross, multiple copies are present fragmentarily, resulting in higher lane backgrounds on the whole. Based on the results, it was confirmed that in the BC1-F4 generation of the CV127 line, a hybrid progeny of the cultivar Conquista after backcross, the number of copies of the modified *csr1-2* gene expressed region became one and that the one copy is stably inherited to the subsequent BC1-F8 and BC1-F9 generations of the CV127 line.

Absence of backbone region of the plasmid pAC321

For production of the CV127 line, approx. 6.2 kb of the LF-6.2PvuII was cleaved from the plasmid pAC321 by the restriction enzyme *Pvu*II, separated through the agarose gel electrophoresis and purified and then transferred in the soybean genome. Therefore, it is considered unlikely that the nucleotide sequence of backbone region of the plasmid pAC321 would be transferred in the soybean genome.

In order to confirm the absence of the backbone region of the plasmid pAC321 in the genome of the CV127 line, Southern blotting analysis was conducted using the BC1-F8 generation of the CV127 line. As probes, the VP1 and VP2 probes, which specifically recognize the backbone region of the pAC321, were used (Table 6 and Table 7, p17; Figure 10C, p18).

As a result, it was confirmed that the DNA fragment corresponding to the backbone region of the plasmid pAC321 is not detected in the genome of the CV127 line and that the backbone region of the plasmid pAC321 has not been transferred in the CV127 line (Lanes 4, 8, and 12 in Figure 10, p18).

Amino acid sequence of putative 501 bp of ORF produced at 3'-terminal and RT-PCR analysis

As a result of sequence analysis of the transferred gene, it was confirmed that 376 bp, a part of the modified *csr1-2* gene expressed region, are transferred at 3'-terminal in the form of sense sequence (Figure 7; Annex 2). As a result, it was considered likely that it would create an open reading frame (ORF) of 501 bp (Figure 6). Potential transcription of this ORF was investigated by RT-PCR analysis, using young leaf tissue of the BC1-F8 generation of the CV127 line, which indicated that the putative ORF was not detected (Figure 11, p18).

Figure 6 **Amino acid sequence of putative ORF of 501 bp [Confidential: Not made available or disclosed to unauthorized person]**

Figure 7 **Comparison between LF-6.2PvuII of approx. 6.2 kb transformed in the soybean and sequence of 4,758 bp transferred in the soybean [Confidential: Not made available or disclosed to unauthorized person]**

Table 6 **Sequence of primers used for preparation of probes for Southern blotting analysis [Confidential: Not made available or disclosed to unauthorized person]**

Table 7 **Estimated and observed band sizes in the Southern blotting analysis [Confidential: Not made available or disclosed to unauthorized person]**

Figure 8 **Southern blotting analysis for investigation of the number of copies of the transferred regions [Confidential: Not made available or disclosed to unauthorized person]**

Figure 9 **Southern blotting analysis for investigation of the inter-generational expression stability [Confidential: Not made available or disclosed to unauthorized person]**

Figure 10 **Southern blotting analysis for investigation of the backbone region of the plasmid pAC321 [Confidential: Not made available or disclosed to unauthorized person]**

Figure 11 **RT-PCR analysis for putative ORF of 501 bp due to the transferred partial sequence (376 bp) of the modified *csr1-2* gene [Confidential: Not made available or disclosed to unauthorized person]**

3) Nearby or separate location of multiple copies, if present, on the chromosome

This item is not applicable due to the one copy confirmed by the Southern blotting analysis.

4) Inter-individual or inter-generational expression stability under a natural environment with respect to the characteristics referred to specifically in (6)-1)

In order to investigate the expression level and stability of the modified AHAS protein in the CV127 line, measurement was made based on the enzyme-linked immunosorbent assay (ELISA). Since the antibody used in the assay recognizes both of the AHAS protein derived from soybean and the modified AHAS protein, the total amount of AHAS proteins was measured for the CV127 line and the control cultivar.

From October 2006 to March 2007 (Season 1), assay samples were collected from the BC2-F5 generation of the CV127 line, leaves from five (5) field test sites in Brazil and seeds from four (4) field test sites. In addition, from February 2007 to July 2007 (Season 2), leaf and seed samples were collected from the progeny BC2-F6 generation in four (4) field test sites for each sample and subjected to the assay (Table 8, p19).

The growth stages subjected to the assay are as follows.
V2 stage: Seedling period (Plants are 15 to 20 cm tall and have three nodes with two unfolded leaflets.)
R8 stage: Maturation period

From the CV127 line and the control cultivar, leaves and seeds were collected in the V2 stage and R8 stage and subjected to the analysis. For the leaves, the primary leaves were collected from 6 plants each in two plots in each test field. The leaves collected from 6 plants in each plot were crushed to provide a sample, and the total amount of AHAS proteins was measured for a total of 2 samples (n=2). Similarly for the seeds, 500 g of seeds each from 2 plots in each test field were collected. The seeds from each plot were crushed to

provide a sample, and the total amount of AHAS proteins was measured for a total of 2 samples (n=2).

It is generally known that the enzyme activity of total AHAS protein becomes highest in the leaves in the vegetative stage where the need for branched chain amino acids (valine, leucine, isoleucine) and other amino acids is greatest (Reference 5). This assay has also revealed that the expression of the total amount of AHAS proteins was highest in the leaves in the V2 stage of the CV127 line in the both Seasons (Table 8). In the leaves, the expression of total amount of AHAS protein in the CV127 line is found higher compared to the control cultivar. This may be explained by the fact that the modified AHAS protein and the AHAS protein derived from soybean are both expressed due to the transferred modified *csr1-2* gene, which confers the tolerance to imidazolinone herbicides. In addition, it is confirmed that the AHAS protein is expressed at a very low level in the other tissues than the leaves. For the seeds subjected to the assay, the expression of total AHAS protein was found at or below the limit of determination for both CV127 line and control cultivar.

Table 8 **Total amount of AHAS protein in the leaves (V2 stage) and seeds (R8 stage) of the CV127 line and the control cultivar cultivated in the field test sites in Brazil in Season 1 and Season 2 [Confidential: Not made available or disclosed to unauthorized person]**

In addition, based on the tolerance tests of the W-F5 generation of the CV127 line to the imidazolinone herbicide Imazapyr conducted in 2008 in the isolated field at National Institute for Agro-Environmental Sciences in Tsukuba City, Ibaraki Prefecture, the expression of the tolerance to imidazolinone herbicides in the CV127 line was investigated. Two plant individuals each of the CV127 line and control cultivar (Williams) were raised in pots. A total of 8 pots for each plot were subjected to the tests. Twenty (20) days after sowing, the herbicide Imazapyr was sprayed to the leaves and stems in the amount of 70 g a.i./ha (the standard dose level in Brazil). Two (2), 4, 7, 11, and 14 days after the treatment, herbicide injury was visually estimated and the severity of herbicide injury was evaluated on the scale of 0 to 5. The CV127 line exhibited tolerance to the imidazolinone herbicide in the concentration of 70 g a.i./ha (Table 9, p20). On the other hand, the control cultivar almost died in this concentration. As a result, it was confirmed that the CV127 line possesses the tolerance to the imidazolinone herbicide. The results of evaluation of the tolerance to imidazolinone herbicide in two test fields in Brazil are shown in Annex 5.

Based on the above results, it was indicated that the modified *csr1-2* gene transferred in the CV127 line is stably inherited to the progeny and that the modified AHAS protein is expressed also in the progeny.

Table 9 Test results for herbicide injury by the treatment of the herbicide Imazapyr

Date of investigation	No. of days after treatment (Day)	CV127 line [1,2] W-F5	Control cultivar [1,2] Williams
Aug. 20, 2008	2	**0**	**3**
Aug. 22	4	**0**	**4**
Aug. 25	7	**0**	**4**
Aug. 29	11	**0**	**4**
Sep. 1	14	**0**	**5**

1. Eight (8) pots each ×2 plant individuals were investigated. Twenty (20) days after sowing, the herbicide Imazapyr of 70 g a.i./ha (the standard dose level in Brazil) was sprayed to the leaves and stems from above soybean.
2. Herbicide injury was visually estimated two days after treatment and the severity of injury was evaluated based on the following scale.
   0: No injury
   1: Slightly delayed growth and temporary chlorosis (bleached to yellow-white condition) identified on the leaves.
   2: Leaves significantly turned yellow, which continues for several days.
   3: Significantly delayed growth and chlorosis and/or purple-turned leaves.
   4: Significantly inhibited growing point and severe leaf damage found extending over a wide area.
   5: Greater part of plants found damaged severely or dead.

5) Presence or absence, and if present, degree of transmission of nucleic acid transferred through virus infection and/or other routes to wild animals and wild plants

The nucleotide sequence of the transferred nucleic acid possesses no transmissibility to any wild animals and wild plants.

(5) Methods of detection and identification of living modified organisms and their sensitivity and reliability

<u>Development of an event-specific PCR method</u>

Based on the information obtained from the flanking sequence and the transferred sequence, an event-specific PCR method was developed. The designed four (4) sets of primers are located in the 5'-terminal flanking sequence region at one end

and the modified *csr1-2* gene expressed region at the other end. The information about the primers is shown in Table 10 (p21). Expected PCR products are 212 to 447 bp in length.

Based on the findings that the event-specific expected band size was amplified in all the PCR analyses, it was confirmed that the transferred gene could be identified by use of the four sets of primers, which have been applied for subsequent analyses (Figure 12A, p21). Furthermore, for the PCR products using the event-specific primer set "Event PCR3," additional verification was conducted using the samples of the CV127 line and the control cultivar collected from six different test sites (Figures 12 B and C). As a result, a total of 24 samples of the CV127 line exhibited specific amplification of expected band size, while the samples of the control cultivar did not show any amplification. Based on the results, it is considered that this event-specific PCR method using the "Event PCR3" in particular is highly sensitive and reliable.

Table 10 Combination of primers used for the event-specific PCR [Confidential: Not made available or disclosed to unauthorized person]

Figure 12 CV127 line-specific PCR method [Confidential: Not made available or disclosed to unauthorized person]

(6) Difference between the modified organism and the recipient organism or the species to which the recipient organism belongs

1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of replication products of transferred nucleic acid

In non-transgenic plants, inhibition of the AHAS protein by the imidazolinone herbicides causes the loss of the branched chain amino acids, thus leading to inhibited growth. On the other hand, in the CV127 line, to which the modified *csr1-2* gene has been transferred, the normal biosynthesis functions remain unaffected, resulting in the mutation causing failure of binding of imidazolinone herbicides to the AHAS protein. As a result, the CV127 line exhibits the tolerance to imidazolinone herbicides and possesses the characters of normal growth (Figure 1, p5).

2) With respect to the physiological or ecological characteristics listed below, presence or absence of difference between genetically modified agricultural products and the taxonomic species to which the recipient organism belongs, and the degree of difference, if present

In 2008, isolated field tests of the CV127 line were conducted in the isolated field at National Institute for Agro-Environmental Sciences in Tsukuba City, Ibaraki Prefecture. In the tests, BC1 and BC2 of the CV127 line in the Conquista cultivar in the maturity group (MG) VIII, were expected fail to grow in Japan, and the W-F5 generation of the CV127 line was created by crossing with the Williams cultivar in the MG IV, suited the environment in Japan, was used in the trial (Figure 5, p14). For the non-recombinant control soybean (hereinafter referred to as the control cultivar), the Williams cultivar used for the creation of the CV127 line was used. Details on the tests are discussed in Annex 6.

(a) Morphological and growth characteristics

For comparison of morphological and growth characteristics, differences in the following 12 items of morphological and growth characteristics were examined between the CV127 line and the control cultivar (Williams): (the germination rate, the shape of leaflet, the quantity of trichoma, time of 50% flowering, flower color, elongation type, time of 80% maturity, main stem length, the number of main stem nodes, the number of branches, the lowest podding node height, and weight of shoot). As a result, regarding the lowest podding node height, a statistically significant difference was observed between the CV127 line and the control cultivar (Table 11).

Table 11 Morphological and growth characteristics [Confidential: Not made available or disclosed to unauthorized person]

(b) Cold-tolerance or heat-tolerance at the early stage of growth

Possible effects of low-temperature treatment on the CV127 line at the early stage of growth were examined. Seedlings at the 1 leaf stage raised in the conditions (12-hours day length at 25°C and 12-hours night length at 20°C) were cultivated under the low-temperature conditions (12-hours

day length at 10°C and 12-hours night length at 5°C). Then, 14 days and 28 days later, the severity of low-temperature injury was investigated.

As a result, the CV127 line and the control cultivar (Williams) both exhibited retardation of growth on the 14th day after start of the low-temperature treatment, and at the both points of time of 14 days and 28 days later, no statistically significant difference was observed between the CV127 line and the control cultivar in the severity of low-temperature injury (Table 12). Consequently, it was indicated that the CV127 line and the control cultivar are equivalent to each other with respect to the cold-tolerance.

Table 12 **Severity of low-temperature injury at the early stage of growth [Confidential: Not made available or disclosed to unauthorized person]**

(c) Wintering ability or summer survival of the mature plant

The CV127 line and the control cultivar (Williams) raised in the isolated field were left to grow even after the time of maturity (November 25, 2008) and the growth in the winter season in Japan was observed. As a result of observation of growth on March 5, 2009 after passing the winter, the CV127 line and the control cultivar were both found dead.

(d) Fertility and size of the pollen

To compare the fertility of the pollens, pollens collected from the CV127 line and the control cultivar (Williams) were stained with acetocarmine solution. As a result, the CV127 line and the control cultivar are both found to possess high pollen fertilities, and no statistically significant difference was observed between the both plants in the pollen fertility (Table 13). The size of the pollen was found approx. 30 μm for the both plants and no difference was observed in the shape of the pollen.

Table 13 **Fertility of the pollen [Confidential: Not made available or disclosed to unauthorized person]**

(e) Production, pod shattering habit , dormancy and germination rate of the seed

Regarding seed production, the differences between the CV127 line and the control cultivar (Williams) were examined in the survey at the harvest time in the isolated field tests for the number of mature pods per plant, the weight of mature seeds per plant, the total weight of seeds per plant, and weight of 100 seeds. As a result, in all the items examined, no statistically significant difference was observed between the CV127 line and the control cultivar (Table 14, p24).

Regarding pod shattering habit, the CV127 line and the control cultivar (Williams) were harvested at the time of maturity, and the plant body was naturally dried and then exposed to hot air at 60°C to identify difficulty in pod shattering habit. As a result, the CV127 line and the control cultivar both exhibited the difficult pod shattering habit and no difference was observed between them (Table 15).

Regarding germination rate, immediately after harvesting of the CV127 line and the control cultivar (Williams), their seeds were sown and investigated for the germination rate 10 days after the sowing. As a result, no statistically significant difference was observed between the CV127 line and the control cultivar, both of which exhibited high germination rates (Table 16). Due to the high germination rate confirmed for the seeds of both plants, the CV127 line and the control cultivar, it was considered that dormancy of the seed is low for the both plants.

Table 14 Production of the seeds [Confidential: Not made available or disclosed to unauthorized person]

Table 15 Pod shattering habit [Confidential: Not made available or disclosed to unauthorized person]

Table 16 Germination rate of the seeds on 10th day from sowing [Confidential: Not made available or disclosed to unauthorized person]

(f) Crossability

In the isolated field, the control cultivar (Williams), a recombinant soybean, was cultivated adjacent to the CV127 line to investigate the natural crossing of the recombinant soybean. Crossability was evaluated by amplifying the modified *csr1-2* gene region transferred in the CV127 line of pollen parent from the seeds of the non-recombinant soybean based on the PCR method. A total of 13,726 soybean seeds harvested from 100 individuals of the non-recombinant soybean were examined based on the PCR method and as a result, one seed was found from the gene modified individual. Therefore, the natural crossing rate between the CV127 line and the recombinant soybean is found 0.007%.

(g) Productivity of harmful substances

For soybean, the production of any harmful substances, which inhibits the growth of other plants, has not been reported.

To confirm the productivity of any harmful substances of the CV127 line, plow-in test and succeeding crop test were conducted. After cultivation of the CV127 line and the control cultivar (Williams) in the isolated field in 2008, using the soil plowed with the plants or the soil used in the cultivation, the seeds of radish *Raphanus sativus* were sown in a closed greenhouse and raised under the usual management to investigate the germination rate of the seeds, plant height, fresh weight and dry weight. In addition, possible impacts on the soil microflora were also investigated.

Plow-in test

The soil plowed with 1% by weight of the stems and leaves of the CV127 line and the control cultivar cultivated in the isolated field after being dried sufficiently and cut into pieces was used. The test was conducted with 50 seeds of radish each sown in 2 repeats in each plot.

As a result, regarding the germination rate, plant height, fresh weight and dry weight, no statistically significant difference was observed between the plots of the CV127 line and the control cultivar (Table 17).

Succeeding crop test:

The soil collected from individual cultivation plots following the completion of cultivation of the CV127 line and the control cultivar in the isolated field was used after removing plant residues. The test was conducted with 50 seeds of radish each sown in 2 repeats in each plot.

As a result, regarding the germination rate, plant height, fresh weight and dry weight, no statistically significant difference was observed between the plots of the CV127 line and the control cultivar (Table 18).

Possible impacts on the soil microflora

After harvesting the CV127 line and the control cultivar cultivated in the isolated field, the soil around the roots was collected and used for the test. As a result of investigation for filamentous fungi, bacteria and actinomycete based on the conventional method, in all the items examined, no statistically significant difference was observed between the CV127 line and the control cultivar (Table 19).

Table 17 **Plow-in test [Confidential: Not made available or disclosed to unauthorized person]**

Table 18 **Succeeding crop test [Confidential: Not made available or disclosed to unauthorized person]**

Table 19 **Possible impacts on the soil microflora [Confidential: Not made available or disclosed to unauthorized person]**

II. Review by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms. Results of the review are listed below.

(1) Item-by-item assessment of Adverse Effect on Biological Diversity

1) Competitiveness

The plant of soybean (*Glycine. max* (L.) Merr.), to which the recipient organism belongs, has been cultivated for a long time in Japan, but there is no report that it grows voluntarily in Japan.

In the isolated fields in Japan, morphological and growth characteristics, cold-tolerance at the early stage of growth, wintering ability of the mature plant, fertility and size of the pollen, and production, pod shattering habit, dormancy and germination rate of the seed were examined. As a result, in all the items but the lowest podding node height, no difference or statistically significant difference was observed between this recombinant soybean line and the control cultivar.

Regarding the lowest podding node height for which a statistically significant difference was observed, it is generally known that the height varies greatly according to the cultivation condition and species as demonstrated by the fact that the height is increased due to dense planting or cultivation by early sowing. In fact, the isolated field included a ill-drained plot, thus this aspect of cultivation environment may have lowest podding node height. In addition, the lowest podding node height has low correlation with production of the seed and then, even if this trait alone varies, it is considered unlikely to cause any increase in competitiveness. Based on the above understanding, it is considered unlikely that the competitiveness of this recombinant soybean would be increased even if this trait alone varies.

This recombinant soybean possesses the tolerance to imidazolinone herbicides, though it is considered unlikely that this herbicide tolerance could increase the competitiveness in the natural condition in which spraying of the herbicides is not expected.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant soybean poses no significant risk of Adverse Effect on Biological Diversity attributable to competitiveness is reasonable.

2) Productivity of harmful substances

Regarding the plant species of soybean to which the recipient organism belongs, there is no report that it produces a harmful substance to wild animals and wild plants.

This recombinant soybean produces the modified AHAS protein which

exhibits the tolerance to imidazolinone herbicides, but there is no report that the protein is a harmful substance. This protein catalyzes the first step in the biosynthesis pathway of the branched chain amino acids (valine, leucine, isoleucine), though no statistically significant difference was observed between this recombinant soybean and the control cultivar in the amino acid composition in the soybean seeds.

In 2009 in the isolated field in Japan, succeeding crop test and plow-in test were conducted to examine the possibility of this recombinant soybean of producing any harmful substances (substances secreted from roots to affect the other plants and microorganisms in soil, and substances in the plant body to affect the other plants after dying out). As a result, no difference was observed between this recombinant soybean and the non-recombinant soybean.

As a result of searching for homology with amino acid sequences, it has been confirmed that the modified AHAS protein has no sequence which is structurally homologous with any known allergens.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected, if cannot be specified and that the use of this recombinant soybean poses no significant risk of Adverse Effect on Biological Diversity attributable to productivity of harmful substances is reasonable.

3) Crossability

It is known that *Glycine soya* is closely related to soybean and the both plants have the same chromosome number 2n=40 and thus they can cross with each other. Then, *Glycine soya* was specified as a wild plant likely to be affected, and the following examination was performed.

Since there is no specific obstacle identified to the growth of any hybrid produced by artificial crossing between soybean and *Glycine soya*, there is a possibility that the hybrid, if produced by crossing between this recombinant soybean and *Glycine soya* in the natural environment in Japan, would grow and that the gene transferred in this recombinant soybean through backcross of the hybrid with *Glycine soya* would spread without remaining in a low content in the population of *Glycine soya*.

In addition, since *Glycine soya* ranges widely throughout the country and grows voluntarily in river beaches, banks, in the vicinity of farmlands, orchards and other places, it can cross with this recombinant soybean when it is raised adjacent to the recombinant soybean.

However,

(a) It is known that the flowering time of soybean and *Glycine soya* is unlikely to coincide with each other in general. In addition, there is a report that crossing rate is 0.73% even when flowering time of the both plants is artificially set to coincide with each other and the both plants are cultivated alternately at a planting distance of 50 cm.

(b) There is a report that any genetic marker, which suggests the crossing between soybean and *Glycine soya*, has not been detected.

(c) There is a report that in the crossing test in which the flowering time of herbicide glyphosate-tolerant recombinant soybean 40-3-2 line and *Glycine soya* was set to coincide with each other and the both plants were cultivated adjacent to each other and raised with the *Glycine soya* winding around the soybean, one of the total number of 32,502 seeds of harvested *Glycine soya* was found to have crossed with the soybean.

Furthermore, in the isolated field in Japan, this recombinant soybean and the non-recombinant control soybean (the control cultivar) were cultivated adjacent to each other in the experimental plot to investigate the natural crossing with the non-recombinant soybean. As a result, the crossing rate was found 0.007%. This is considered not to exceed the natural crossing rate of conventional soybean which has been based on the report that cross pollination rate is typically less than 1%. In addition, in the isolated field in Japan, investigation was conducted for the traits relating to reproduction (pollen fertility, pollen shape, production of the seed). As a result, no significant difference was observed between this recombinant soybean and the non-recombinant control soybean. Consequently, it was estimated that the crossability between this recombinant soybean and *Glycine soya* is extremely low as the crossability between the conventional soybean and *Glycine soya*.

Based on the above understanding, it was judged that the conclusion by the applicant that the wild animals and wild plants likely to be affected cannot be specified and that the use of this recombinant soybean poses no significant risk of Adverse Effect on Biological Diversity attributable to crossability is reasonable.

(2) Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant soybean in accordance with Type I Use Regulation causes Adverse Effect on Biological Diversity.