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	Maize resistant to Coleoptera (*ecry3.1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (Event 5307, OECD UI: SYN-Ø53Ø7-1)
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 Effects on Biological Diversity

Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of maize resistant to Coleoptera (*ecry3.1Ab*, *Zea mays* subsp. *mays* (L.) Iltis) (Event 5307, OECD UI：SYN-Ø53Ø7-1) (hereinafter referred to as "this recombinant maize") are shown in Table 1 (p. 2–4). The nucleotide sequence of the vector is shown in Annex 1 (Confidential: Not disclosed to unauthorized persons).

Table 1 Origins and functions of the component elements of the donor nucleic acid used for the development of this recombinant maize

Component elements	Size (bp)	Origin and function
ecry3.1Ab gene cassette		
CMP promoter	346	A promoter region derived from Cestrum yellow leaf curling virus and involved in constitutive expression of the target gene (Reference 12). The level of expression induced by this promoter is comparable to that induced by the Cauliflower mosaic virus 35S promoter or the Ubi1 promoter derived from maize Ubiquitin gene (Reference 13). Cestrum yellow leaf curling virus, a member of the *Caulimoviridae* family also including the Cauliflower mosaic virus, is known to infect *Cestrum parqui*, *C. elegans*, and *Nicotiana clevelandii*, but its infectivity in tomato and other plants is unknown (Reference 14). Cestrum yellow leaf curling virus has a circular, double-stranded DNA genome and forms a polycistronic RNA downstream from this promoter (Reference 14).
ecry3.1Ab gene	1,962	The *ecry3.1Ab* gene encodes the eCry3.1Ab protein exhibiting insecticidal activity against pest insects of order Coleoptera for maize. The gene consists of two *cry* gene fragments (modified *cry3Aa2* gene and *cry1Ab* gene) derived from *Bacillus thuringiensis*. Based on the domain structure of known Cry proteins (Reference 15), Domains I and II and some portion of Domain III (459 amino acid residues in total) of the encoded protein are derived from the 5′-terminus of the modified *cry3Aa2* gene, while the rest of Domain III and the 3′-terminus sequence (172 amino acid residues in total) are derived from the *cry1Ab* gene (Figure 1, p. 5). The N-terminus of the eCry3.1Ab protein expressed by this

		gene also contains a synthetic polylinker sequence and a 22-amino acid sequence derived from the 5′-terminus of the modified *cry3Aa2* gene (Figure 2, p.6). Modified *cry3Aa2* gene: A modified version of the *cry3Aa2* gene, which is derived from *B. thuringiensis* subsp. *tenebrionis* (Reference 16). It has some amino acid sequences modified for its optimum expression in maize, the recipient organism (Reference 17), and has been transferred cathepsin G protease recognition sequence to enhance the insecticidal activity against Corn Rootworm (Reference 18). The gene has been introduced into the maize resistant to Coleoptera (MIR604, OECD UI: SYN-IR6Ø4-5), which has been already approved in Japan. *cry1Ab* gene: A modified version of the *cry1Ab* gene, which is derived from *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Reference 19). It has some amino acid sequences modified (Reference 20) for its optimum expression in maize, the recipient organism (Reference 17). The gene has been introduced into the maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Event 176, OECD UI: SYN-EV176-9), in which the modified *cry1Ab* gene, a modified version of the *cry1Ab* gene, has been already approved in Japan.
NOS terminator	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens*. Its function is to terminate transcription of mRNA by polyadenylation (Reference 21).
pmi gene cassette		
ZmUbiInt promoter	1,993	Promoter region from maize polyubiquitin gene, which contains the first intron. It provides constitutive expression of the target gene (Reference 22).
pmi gene	1,176	*manA* gene derived from *Escherichia coli* strain K-12, encoding the phosphomannose isomerase (hereinafter referred to as “PMI protein”). Used as a selectable marker for gene-transferred transformants (Reference 23).
NOS terminator	253	Terminator sequence from the nopaline synthase gene of *A. tumefaciens*. Its function is to terminate transcription of mRNA by polyadenylation (Reference 21).
Other regions		
LB	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 24).

spec	789	Streptomycin adenylyltransferase gene (*aadA*) from *E. coli* transposon Tn7

		(Reference 25). This gene is used as a selective marker for the vector to confer resistance to streptomycin and spectinomycin.
virG	726	A region involved in the transfer of T-DNA derived from pAD1289 derived from *A. tumefaciens*. Partially modified to induce constitutive expression of the *virG* traits (Reference 26).
repA	1,074	Replicon (minimum functional replication unit controlling DNA replication) region derived from *Pseudomonas* bacteria. A gene required for retention of vectors in *A. tumefaciens* (Reference 27).
VS1 ori	405	The replication origin consensus sequence derived from the plasmid pVS1 of *Pseudomonas* bacteria. Functions as the replication origin of plasmid in *A. tumefaciens* (Reference 28).
ColE1 ori	807	The replication origin of the plasmid derived from *E. coli* (Reference 29).
RB	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 30).

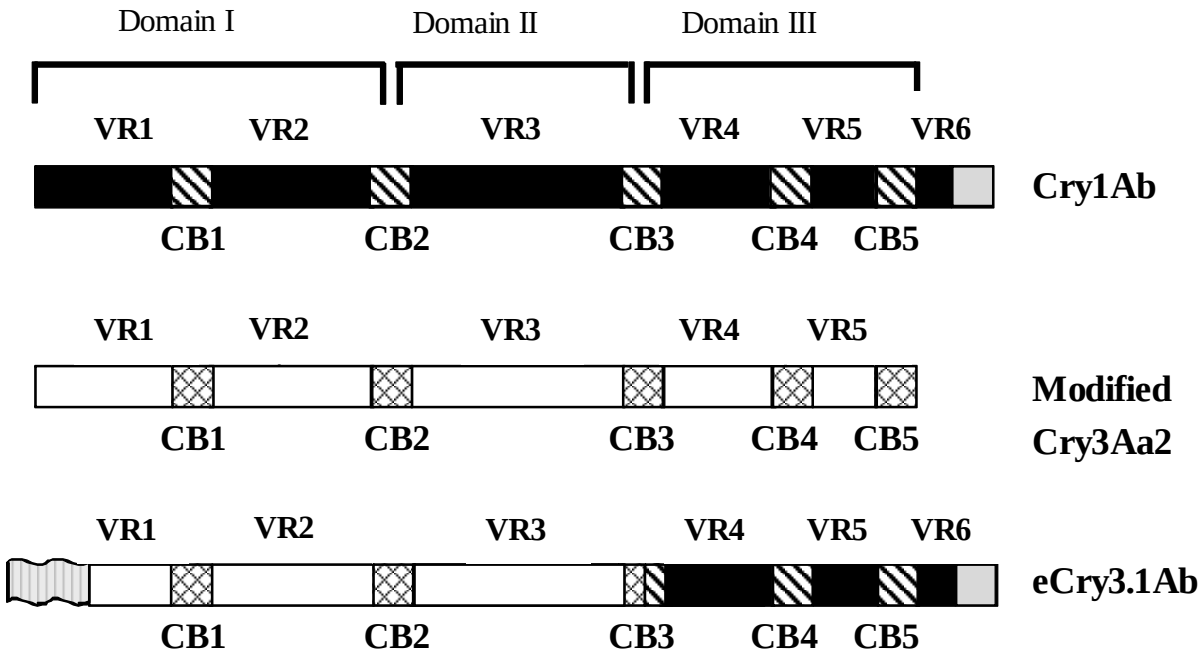

Figure 1 A schematic diagram illustrating the origins of the amino acid residues present in the eCry3.1Ab protein

Cry1Ab: ■ represents the variable regions 1 through 6 (VR1 through VR6) of the Cry1Ab protein. ▨ represents the conserved blocks 1 through 5 (CB1 through CB5) of the Cry1Ab protein. □ represents the tail sequence of the Cry1Ab protein.

Modified Cry3Aa2: □ represents the variable regions 1 through 5 (VR1 through VR5) of the modified Cry3Aa2 protein. ▩ represents the conserved blocks 1 through 5 (CB1 through CB5) of the modified Cry3Aa2 protein.

eCry3.1Ab: ▨ in the eCry3.1Ab protein represents the N-terminal amino acid sequence unique to the eCry3.1Ab protein. In the eCry3.1Ab protein, the region extending from CB1 through CB3 is derived from the modified Cry3Aa2 protein, while the region downstream of CB3 is derived from the Cry1Ab protein.

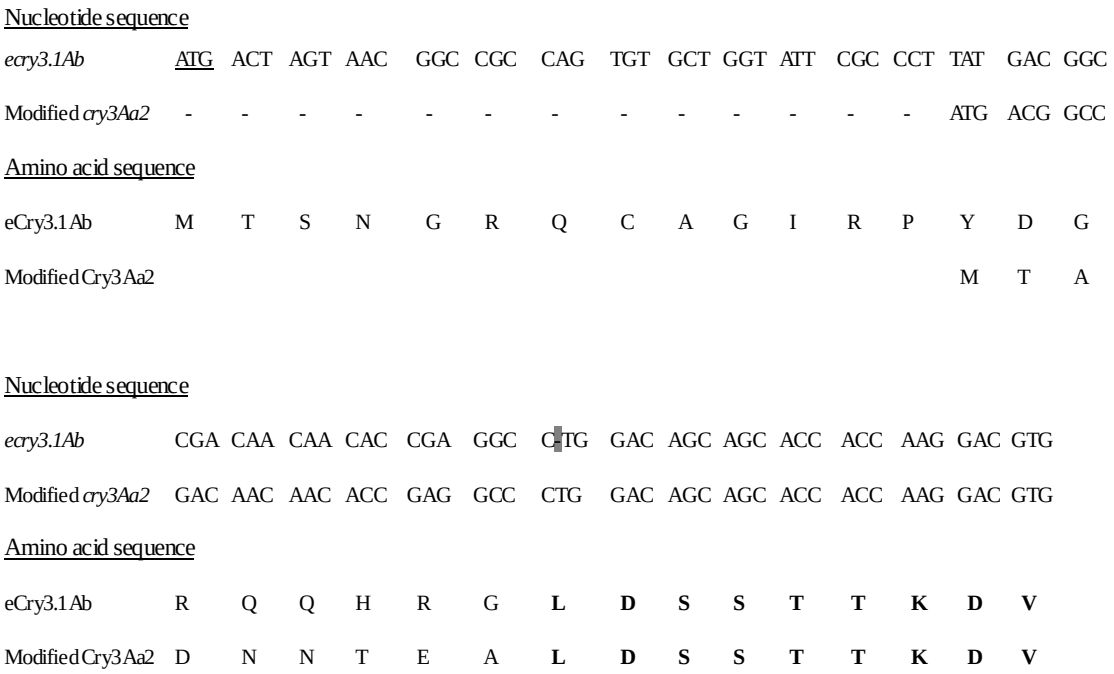

Figure 2 Nucleotide alignment at the 5′-terminus between the *ecry3.1Ab* gene and the modified *cry3Aa2* gene and the corresponding amino acids present at the N-termini of the eCry3.1Ab protein and the Cry3Aa2 protein

The N-terminal amino acid sequence of the eCry3.1Ab protein differs from that of the modified Cry3Aa2 protein, because the translation of the eCry3.1Ab protein starts from a start codon (ATG: underlined text in the above figure) derived from the polylinker sequence, which was added to the 5′-terminus during the construction of the *ecry3.1Ab* gene. However, due to the occurrence of a one-base-pair deletion, indicated by a gray-meshed dash in the above figure, regarding the amino acid sequences downstream the deletion (residues indicated in bold), the sequence of the modified Cry3Aa2 protein is restored in the eCry3.1Ab protein.

2) Function of component elements

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

Functions of component elements of the donor nucleic acid used for the production of this recombinant maize are shown in Table 1 (p. 2–4).

(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 known to possess any allergenicity

eCry3.1Ab protein

1) Purpose of development

A maize resistant to Coleoptera expressing the modified Cry3Aa2 (MIR604, OECD UI: SYN-IR6Ø4-5) (hereinafter referred to as "MIR604") was developed for controlling pest insects of the order Coleoptera in maize cultivation. However, long-term cultivation of MIR604 alone has a risk of inducing Coleopteran insects resistant to the modified Cry3Aa2 protein. Development of a maize variety expressing a different insect-resistant protein is expected to prevent the appearance of resistant insects. A variety expressing a protein that can bind to a midgut receptor other than the one to which the modified Cry3Aa2 protein binds in the target insects would be an effective means for preventing the appearance of such resistant pest insects. From this standpoint, development of a new protein was started, and eCry3.1Ab protein has been selected.

2) Structure

Most Cry proteins, such as Cry1 and Cry3 proteins, have regions called Conserved Blocks (hereinafter referred to as "CB") where the amino acid sequence is highly conserved (Reference 15) and regions called Variable Regions (hereinafter referred to as "VR") where the sequence is highly variable (Figure 1, p. 5). Since the presence of the CB structures seems to be necessary for the Cry proteins to maintain their basic characteristics, chimera proteins possessing these structures were generated by exchanging sequences between the modified Cry3Aa2 protein and the Cry1Ab protein, among which the eCry3.1Ab protein having an insecticidal activity against Coleopteran insects was selected (Reference 31). Since the eCry3.1Ab protein comprises a region encompassing CB1 through CB3 derived from the modified Cry3Aa2 protein and the region downstream from CB3 derived from the Cry1Ab protein (Figure 1, p. 5), its three-dimensional structure and basic characteristics are

considered to be similar to those of the conventionally known Cry proteins.

Moreover, past studies have demonstrated that known Cry proteins share a similar three-dimensional structure comprised of three domains (Reference 32, Reference 33, Reference 34, Reference 35). In the eCry3.1Ab protein, Domain I, Domain II, and a part of Domain III (upstream of CB3) are derived from the modified Cry3Aa2 protein exerting insecticidal activity against Coleopteran pests, and the remaining part of Domain III and the downstream region (downstream from CB3) are derived from the Cry1Ab protein exerting insecticidal activity against Lepidopteran pests, and these domain structures are also maintained (Figure 1, p. 5).

The functions of individual domains are as follows: Domain I is involved in the formation of ion channels, and Domains II and III are involved in receptor binding (Reference 32, Reference 34, Reference 36). In the course of developing the eCry3.1Ab protein, various combinations of domains from the Cry1Ab protein and the modified Cry3Aa2 protein were tested to examine their insecticidal activities. As a result, a protein possessing domains from the Cry1Ab protein and the modified Cry3Aa2 protein in a reverse combination of that of the eCry3.1Ab protein (i.e., regions from VR1 in Domain I through CB3 in Domain III derived from the Cry1Ab protein and regions from CB3 through CB5 in Domain III derived from the modified Cry3Aa2 protein) exhibited insecticidal activity against European corn borer (*Ostrinia nubilalis*) of the order Lepidoptera but not against Western cornroot worm (*Diabrotica virgifera virgifera*) of the order Coleoptera (Reference 31). Thus, it was considered that Domain II and a part of Domain III (upstream of CB3) in the eCry3.1Ab protein are mainly involved in the recognition of and binding to specific receptors in the target pests and, consequently, the eCry3.1Ab protein exhibits specific insecticidal activity against certain insects of the order Coleoptera.

3) Characteristics

For examining the insecticidal activity and the characteristics of the eCry3.1Ab protein, A) bioassays on Coleopteran insects, Lepidopteran insects, and other non-target organisms and B) comparison of affinity to receptors in Coleopteran insects between the eCry3.1Ab protein and conventional Cry proteins were conducted.

A) Bioassays on Coleopteran insects, Lepidopteran insects, and other non-target organisms

Results of a feeding test of the eCry3.1Ab protein demonstrated that the protein exhibits insecticidal activity against Coleopteran insects including Western cornroot worm (*D. virgifera virgifera*), Northern cornroot worm (*D. longicornis barberi*), and Colorado potato beetle (*Leptinotarsa decemlineata*) (Annex 2; Confidential: not made available or disclosed to

unauthorized persons). Field trials conducted in 2008 in the U.S. confirmed that this recombinant maize exhibits resistance to pest insects of the order Coleoptera (Table 2, p. 10) and a strong resistance to Western cornroot worm, as in the case of MIR604 expressing the modified Cry3Aa2 protein (Table 3, p. 11). In addition, the LC_{50} value of the eCry3.1Ab protein against Western cornroot worm, which is a target Coleopteran insect of the eCry3.1Ab protein, was 40 μg/ml (Annex 3; Confidential: not made available or disclosed to unauthorized persons). Meanwhile, the eCry3.1Ab protein did not exhibit any insecticidal activity against Southern cornroot worm (*D. undecimpunctata*), ladybug (*Coleomegilla maculata*), rove beetle (*Aleochara bilineata*), and carabid beetle (*Poecilus cupreus*) belonging to the order Coleoptera (Annex 2; Confidential: not made available or disclosed to unauthorized persons). Moreover, the eCry3.1Ab protein is partially derived from the Cry1Ab protein that exhibits insecticidal activity against Lepidopteran insects, but this recombinant maize did not exhibit any insecticidal activity against Lepidopteran insects including the Cry1A-sensitive species in the order Lepidoptera (Appendix 2; Confidential: not made available or disclosed to unauthorized persons).

Also, it was confirmed that the eCry3.1Ab protein has no toxic effects on non-target organisms including quail, mouse, honeybee, shrimp, and catfish (Annex 2; Confidential: not made available or disclosed to unauthorized persons).

As mentioned above, the protein having a reverse domain combination of that of the eCry3.1Ab protein (i.e., regions from VR1 through CB3 are derived from the Cry1Ab protein and regions from CB3 through CB5 are derived from the modified Cry3Aa2 protein) exhibited insecticidal activity against European corn borer (*Ostrinia nubilalis*) of the order Lepidoptera but not against Western cornroot worm (*Diabrotica virgifera virgifera*) of the order Coleoptera (Reference 31).

These findings demonstrated that the eCry3.1Ab protein exhibits specific insecticidal activity against the order Coleoptera.

B) Comparison of affinity to receptors in Coleopteran insects between the eCry3.1Ab protein and conventional Cry proteins

In target insects, Cry proteins are known to be partially digested and bind to specific receptors located on the midgut surface to form ion channels, which causes damages to the digestive organs of the target insects and leads to their death.

It has been confirmed that the eCry3.1Ab protein is partially digested as with the modified Cry3Aa2 protein or other Cry proteins and binds to the midgut receptors in Coleopteran

insects to provide an insecticidal activity as with the modified Cry3Aa2 protein (Reference 37). It has also been confirmed that the eCry3.1Ab protein binds to a midgut receptor different from the one to which the modified Cry3Aa2 protein binds (Reference 37).

These findings suggest that the eCry3.1Ab protein exhibits specific insecticidal activity against certain species of the order Coleoptera and binds to a receptor different from the one to which the modified Cry3Aa2 protein binds. Thus, it is suggested that the eCry3.1Ab protein is effective for preventing the appearance of pest insects resistant to the modified Cry3Aa2 protein.

Table 2 Severity of feeding damage by Western corn rootworm based on field trials of this recombinant maize conducted in the U.S. (2008)

Test site	This recombinant		Non-recombinant		P-value[2)]
	Mean [1)]	Standard deviation	Mean	Standard deviation	
Stanton, Minnesota	0.04	0.03	2.69	0.37	0.0056*
Goodfield, Illinois	0.06	0.01	2.81	0.10	0.0004*
Bloomington, Illinois	0.06	0.005	2.46	0.18	0.0018*
Shirley, Illinois	0.06	0.01	1.42	0.40	0.026*

The test was conducted for 6 plant bodies and 3 repeats (n = 3). Maize plants were cultivated in fields where Western corn rootworms exist in soil, and the roots were recovered at the time of silking to examine the severity of insect damage.

1) Severity of insect damage was evaluated based on the following scales:

Severity	Description
0.01	No damage, or 1 or 2 minor damages on the root surface
0.02	3 or more minor feeding damages at the root surface and 4 or less moderate damages on the root
0.05	5 or more severe damages but no root destroyed
0.10	1 root eaten to within 3.8 cm
0.25	2 or more roots eaten to within 3.8 cm
0.50	Root corresponding to 0.50 nodes destroyed
～	Severity of damage evaluated at 0.25 intervals
3.00	Root corresponding to 3.00 nodes destroyed

2) F-test was conducted at each test site between this recombinant maize and the non-recombinant. The difference was deemed significant when the P-value was less than 0.05 (indicated by an asterisk).

Table 3 Number of adult-stage Western corn rootworms based on field trials of this recombinant maize and MIR604 conducted in the U.S. (2008)

	Total number of adults (worms)		Relative number of surviving worms[1)]	Significant or non-significant[2)]
	Mean	Standard deviation		
This recombinant	0.6	0.89	0.06	b
MIR604	14.0	7.62	1.45	b
Non-recombinant	963.6	329.78	100	a

The test was conducted for 64 plant bodies and 5 repeats (n = 5). Eggs of Western corn rootworm were inoculated into soil along the ridge (1,380 eggs/0.3 m) during the 1st to 2nd leaf stages of maize, and the number of adult-stage worms was counted 7 weeks to about 1 month after the inoculation.

1) The relative number of surviving adult-stage Western corn rootworms per 100 adult-stage Western-corn rootworms in the non-recombinant maize plot
2) LSD test was conducted among this recombinant maize, MIR604, and the non-recombinant maize. The difference was deemed significant when the P-value was less than 0.05 (indicated by different letters).

Based on the homology search using the publicly available databases (FARRP Allergen Database (2010) and NCBI Entrez Protein database (2010)), it has been confirmed that the amino acid sequence in the eCry3.1Ab protein does not have any homology with known allergens and toxins other than Cry proteins.

PMI protein

The *pmi* gene is a gene derived from *E. coli*, which encodes the PMI protein (Phosphomannose isomerase), and the PMI protein has the capability of catalyzing the reversible isomerization of mannose-6-phosphate and fructose-6-phosphate. Generally, maize and many other plants cannot utilize mannose as a carbon source, though the cells containing the *pmi* gene can use mannose for their growth. For this reason, with transferring the *pmi* gene into plant cells as a selective marker together with the target gene and subsequent incubation in the mannose-containing medium, transformed cells including the target gene can be selected (Reference 23).

Based on the homology search using the publicly available databases (FARRP Allergen Database (2010) and NCBI Entrez Protein database (2010)), it has been confirmed that the amino acid sequence in the PMI protein does not have any homology with known allergens and toxins.

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

The eCry3.1Ab protein expressed by the *ecry3.1Ab* gene comprises the modified Cry3Aa2 protein and the Cry1Ab protein and is considered to have a structure and function similar to those of conventionally known Cry proteins. As with the conventional Cry proteins, the eCry3.1Ab protein is unlikely to possess any enzymatic activity and is thus considered to function independently from the metabolic system of the recipient organism. In addition, the PMI protein is an enzyme catalyzing the reversible isomerization of mannose-6-phosphate and fructose-6-phosphate. The PMI protein reacts specifically with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein (Reference 38). Consequently, it is considered very unlikely that the PMI protein affects the metabolic pathway of the recipient organism.

Based on the above understanding, it is considered unlikely that the transferred genes could affect the metabolic system of the recipient organism.

(2) Information concerning vectors

1) Name and origin

The plasmid pSYN12274 used for the production of this recombinant maize was constructed based on pUC19, etc., derived from *E. coli*.

2) Properties

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

The total number of base pairs in the plasmid vector is 11,769 bp. Its nucleotide sequence has been disclosed (Annex 1; Confidential: Not made available or disclosed to unauthorized persons).

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

The vector pSYN12274 contains the *spec* gene, which expresses resistance to streptomycin and spectinomycin as a selective marker for growth of the vector in microorganisms, though the gene is not transferred to this recombinant maize.

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

There is no data that the plasmid pSYN12274 contain any sequence showing infectivity.

(3) Method of preparing living modified organisms

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

The positions and directions of the component elements of the plasmid vector pSYN12274 used for the development of this recombinant maize and the sites cleaved by restriction enzymes are shown in Figure 3 (p. 14).

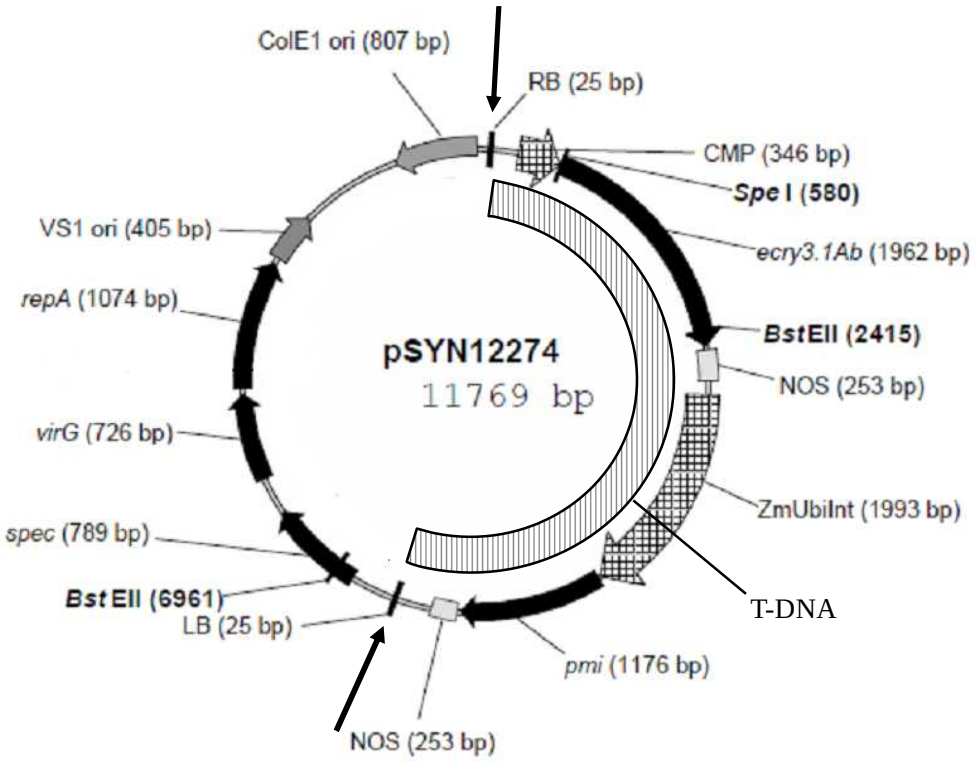

Figure 3 Schematic diagram of the plasmid pSYN12274 used for the development of this recombinant maize

Two gene expression cassettes (*ecry3.1Ab* gene cassette and *pmi* gene cassette) between RB and LB of the T-DNA region were transferred to this recombinant maize.

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

The T-DNA region was transferred based on the *Agrobacterium* method.

3) Process of rearing of living modified organisms

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

Agrobacterium containing the plasmid pSYN12274 was co-cultivated with immature embryos of maize, which were subsequently incubated on the medium containing mannose to select transformed cells.

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

After transferring of genes, the antibiotic Cefotaxime was added to the culture cell medium to remove any residual *Agrobacterium* used for transformation, and then, regarding the regenerated individuals, PCR analysis was conducted for the *spec* gene present in the backbone region of the vector pSYN12274. As a result, no *spec* gene was observed, and thus, it was considered that there was no remaining *Agrobacterium*.

(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 Effects on Biological Diversity

Plants were regenerated from the selected cells, conditioned, and then cultivated in a greenhouse. Then, the plants were analyzed based on the TaqMan PCR (Reference 39) to determine the presence or absence of the *ecry3.1Ab* gene and the *pmi* gene, and T0 plants were selected.

This recombinant maize was approved in June 2010 by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment for Type I Use Regulation (Cultivation in isolated field, storage, transportation and disposal, and acts incidental to them) in accordance with the "Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms." In addition, in July 2011, an application for the safety evaluation as food was submitted to the Ministry of

Health, Labour and Welfare and an application for the safety evaluation as feed was submitted to the Ministry of Agriculture, Forestry and Fisheries.

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

(a) Place where the replication product of transferred nucleic acid exists

As a result of assessment of stability based on the genetic segregation ratio, it was found that the *ecry3.1Ab* gene and the *pmi* gene, the transferred genes in this recombinant maize, were both inherited across multiple generations in accordance with the law of Mendelian inheritance. Consequently, the *ecry3.1Ab* gene and the *pmi* gene are considered to exist on the maize chromosome (Annex 4; Confidential: not made available or disclosed to unauthorized persons).

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

The genomic DNAs were extracted from multiple generations of progenies of this recombinant maize and treated with digestion enzymes to be subjected to Southern blot analysis using the T-DNA region and the backbone region of the plasmid vector SYN12274 as probes (Annex 5; Confidential: not made available or disclosed to unauthorized persons).

When the T-DNA region was used as the probe, an identical band representing one copy of the T-DNA was detected across multiple generations. Thus, it was demonstrated that one copy each of the *ecry3.1Ab* gene cassette and the *pmi* gene cassette were stably inherited through multiple generations and that same genes were transferred into all the generations examined. Meanwhile, when the backbone region was used as the probe, no band was detected in any of the generations examined.

Based on these findings, it was confirmed that one copy of the T-DNA of the plasmid pSYN12274 is present at one site on the genome of this recombinant maize and that the transferred genes are inherited stably by offspring.

(c) The position relationship in the case of multiple copies existing in the chromosome

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

Multiple generations of this recombinant maize were cultivated in a greenhouse of Syngenta Corporation in the U.S. Samples were collected from various tissues at various growth stages, and the expression levels of the eCry3.1Ab protein and the PMI protein were determined by ELISA. As a result, mean values of the expression levels of the eCry3.1Ab protein in multiple generations ranged from 83.40 to 93.67 µg/g dry weight, from 23.88 to 35.39 µg/g dry weight, and below the detection limit (0.08 µg/g dry weight) in leaves, roots, and pollens, respectively (Annex 6; Confidential: not made available or disclosed to unauthorized persons). Meanwhile, mean values of the expression levels of the PMI protein in multiple generations ranged from 1.77 to 1.95 µg/g dry weight, from 1.05 to 1.19 µg/g dry weight, and from 18.96 to 25.58 µg/g dry weight in leaves, roots, and pollens, respectively (Annex 6; Confidential: not made available or disclosed to unauthorized persons).

Based on the above results, it has been confirmed that the eCry3.1Ab protein and the PMI protein in this recombinant maize are stably expressed across individuals and through generations.

(e) 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 transferred nucleic acid does not contain any sequence allowing transmission. Therefore, it is considered unlikely that the nucleic acid transferred to this recombinant maize could be transmitted to any other wild animals and wild plants.

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

A method for detecting 0.1 ng of DNA of this recombinant maize based on PCR using the neighboring sequences of the transferred DNA as primers has been developed (Annex 7, Annex 8; Confidential: not made available or disclosed to unauthorized persons). This method enables specific detection of individual lines using primers that amplify the 3′-region of the transferred gene and the following maize genome sequence, and its reliability has been confirmed by our laboratory as well as by Eurofins GeneScan USA.

(6) Difference from the recipient organism or the species to which the recipient organism belongs

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

This recombinant maize possesses the *ecry3.1Ab* gene and the *pmi* gene, which express the eCry3.1Ab protein and the PMI protein, respectively. The eCry3.1Ab protein imparts resistance to Coleoptera, and this recombinant maize has been confirmed to exhibit resistance to Western corn rootworm, which is a pest insect of the order Coleopteran, in a bioassay conducted in fields in the U.S. (Tables 2–3, p. 10–11). Meanwhile, the PMI protein serves as a selective marker for transformation of this recombinant maize, and this recombinant maize is capable of using mannose as a carbon source.

(b) 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 2010, isolated field tests were carried out at the Kanza Site of Central Research Station, R&D Division, Syngenta Japan K.K., using this recombinant maize and the non-recombinant control maize. The detailed descriptions of the trials are provided in Annex 9 (Confidential: not made available or disclosed to unauthorized persons).

a Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between this recombinant maize and the non-recombinant control maize regarding the progress of germination, germination rate, time of tassel exertion, time of silking, culm length, ear height, plant type, maturation time, fresh weight at the time of harvesting, tiller number, number of productive ears, ear length, ear diameter, row number per ear, grain number per row, grain number per ear, 100-kernel weight, grain color, and grain shape. As a result, no significant difference or difference was observed between this recombinant maize and the non-recombinant control maize (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

b Cold tolerance and heat tolerance at the early stage of growth

Cold tolerance at the early stage of growth was evaluated for this recombinant maize and the non-recombinant control maize. For the plants at the 2nd leaf stage, evaluation was made on the growth under the low-temperature conditions representing the winter season. As a result, this recombinant maize and the non-recombinant control maize both exhibited yellowing and/or necrotic lesions on leaves caused by low temperature, and their growth was markedly interrupted (Annex 9; Confidential: not made available or disclosed to unauthorized persons). Based on these findings, it was judged that there was no difference in cold tolerance between this recombinant maize and the non-recombinant control maize.

c Wintering ability of the mature plant

Maize is a summer-type annual plant and it usually dies out after ripening. There is no report that, after maturity, maize has further propagated vegetatively or produced and set kernels again. In addition, it was observed in the isolated field tests that this recombinant maize died after maturation similarly as the non-recombinant control maize (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

d Fertility and size of the pollen

As a result of the microscopic observation of pollens stained with an acetocarmine solution, no significant difference or difference was observed in the fertility and size of the pollen between this recombinant maize and the non-recombinant control maize (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

e Production, shedding habit, dormancy, and germination rate of the seed

Seed production:

Regarding seed production, no significant difference was observed between this recombinant maize and the non-recombinant control maize in the number of productive ears, row number per ear, grain number per row, grain number per ear, and 100-kernel weight (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

Shedding habit:

The seeds of maize never shed spontaneously, since they adhere to ears and the ears are covered with husk (Reference 2). In this recombinant maize, similar to the non-recombinant control maize, the ears were found covered with husk, and no shedding was observed under natural conditions (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

Dormancy and germination rate:

Regarding the germination rate of the harvested seeds, no significant difference was observed between this recombinant maize and the non-recombinant control maize (Annex 9; Confidential: not made available or disclosed to unauthorized persons). Thus, it is considered unlikely that the dormancy of this recombinant maize is substantially different from that of the non-recombinant maize.

f Crossability

Crossability test was not performed since there is no report that any wild relatives that can be crossed with maize are growing voluntarily in Japan.

g Productivity of harmful substances

Regarding the productivity of harmful substances under the natural environment in Japan, a plow-in test, a succeeding crop test, and a soil microflora test were carried out. Effects on soil fauna were also investigated.

Plow-in test:

Five grams of dried powder of leaves and stems collected at the harvest time was mixed with 850 g of granular soil and filled into pots, to which seeds of radish were sown (1 seed per pot). The germination rate was determined 9 days after sowing, and the radish plants were harvested after 2 weeks to determine the dry weight. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the germination rate and the dry weight of radish (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

Succeeding crop test:

Soils in the root zones of maize collected from individual experimental plots were mixed with each other and filled into pots, to which seeds of radish were sown (1 seed per pot). The germination rate was determined 10 days after sowing, and the radish plants were harvested after 2 weeks to determine the dry weight. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the germination rate and the dry weight of radish (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

Soil microflora test:

At the time of harvesting of this recombinant maize and the non-recombinant control maize, soil was sampled from the cultivation field to determine the number of colonies of filamentous fungi, bacteria, and actinomycetes based on the dilution plate technique. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the number of colonies of filamentous fungi, bacteria, and actinomycetes (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

Soil fauna test:

The fauna was investigated by the pitfall trapping method during the vegetative stage through the time of harvesting of this recombinant maize and the non-recombinant control maize. In addition, soils in the root zones of maize were collected during the vegetative stage and the time of harvesting to invest the fauna. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize (Annex 9; Confidential: not made available or disclosed to unauthorized persons).

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

A review was made by persons with specialized knowledge and experience concerning Adverse Effects on Biological Diversity (called Experts) for possible Adverse Effects on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organisms 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 Effects on Biological Diversity

This recombinant maize was developed by transferring the T-DNA region of the plasmid pSYN12274, constructed based on the plasmid pUC19, etc., derived from *E. coli*, by the *Agrobacterium* method.

Based on the segregation ratio of the transferred genes and the Southern blot analysis, it has been confirmed that one copy of the T-DNA, which contains the *ecry3.1Ab* gene encoding the eCry3.1Ab protein derived from *Bacillus thuringiensis* and the *pmi* gene encoding the PMI protein (phosphomannose-isomerase) derived from *E. coli* strain K-12, is present on the chromosome of this recombinant maize and is inherited stably through multiple generations. In addition, it has been confirmed by ELISA that these genes are stably expressed through multiple generations.

(1) Competitiveness

Maize (*Zea mays* subsp. *mays* (L.) Iltis), the taxonomical species to which the recipient organism belongs, has been long used in Japan, though there is no report that it has become self-seeding in the natural environment in Japan.

As a result of investigation for various characteristics referring to competitiveness of this recombinant maize, which was carried out in 2010 in isolated fields in Japan, no significant difference, etc., was observed between this recombinant and the non-recombinant control maize.

This recombinant maize is given resistance to Coleoptera due to the expression of the eCry3.1Ab protein. However, it is considered that the insect damage by Coleopteran insects is not the major cause making the maize difficult to grow in the natural environment in Japan. Consequently, it is considered unlikely that this trait would enhance the competitiveness of

this recombinant maize. In addition, this recombinant maize is given the trait to be able to use mannose as a carbon source due to the expression of the PMI protein. However, it is not considered that this recombinant maize uses mannose as a carbon source in the natural environment in Japan. Therefore, it is considered unlikely that this trait enhances the competitiveness of this recombinant maize.

Based on the above understandings, it was judged that the following conclusion made by the applicant is valid: regarding this recombinant maize, there are no specific wild animals and wild plants that are possibly affected by this recombinant maize, and it would pose no risk of Adverse Effects on Biological Diversity that is attributable to competitiveness.

(2) Productivity of harmful substances

Maize, the taxonomical species to which the recipient organism belongs, has been long used in Japan, though it is not generally known to produce any harmful substances that could affect wild animals and wild plants.

It has been confirmed that the eCry3.1Ab protein and the PMI protein expressed in this recombinant maize do not have any structural similarity with known allergens. The eCry3.1Ab protein is considered to have a structure and a function similar to those of conventionally known Cry proteins. Therefore, as with the conventional Cry proteins, the eCry3.1Ab protein is unlikely to possess any enzymatic activity. In addition, the PMI protein reacts specifically with mannose-6-phosphate and fructose-6-phosphate, and there is no other natural substrate known for the PMI protein. Consequently, it is considered very unlikely that these proteins would affect the metabolic pathway of the recipient organism and produce any harmful substances.

In addition, as a result of a soil microflora test, a plow-in test and a succeeding crop test carried out in the isolated field in Japan to examine the production of harmful substances by this recombinant maize (the substances secreted from the roots, which can affect other plants and microorganisms in soil; the substances existing in the plant body, which can affect other plants after dying), no statistically significant difference was observed between this recombinant maize and the non-recombinant control maize in the production of harmful substances.

The eCry3.1Ab protein expressed in this recombinant maize exhibits insecticidal activity against the insects of the order Coleoptera. Therefore, the Coleopteran insects were specified as wild animals and wild plants that are possibly affected by this recombinant maize. There is a concern about possible effects on the specified species of Coleopteran insects, which could

directly eat this recombinant maize or eat pollen dispersed from this recombinant maize together with their food plants. However, it is considered unlikely that the Coleopteran insects inhabit locally near the fields of cultivation of this recombinant maize, and thus, it is considered very unlikely that they could be affected in the level of population.

Based on the above understandings, it was judged that the following conclusion made by the applicant is valid: This recombinant maize would pose no risk of Adverse Effects on Biological Diversity that is attributable to the production of harmful substances.

(3) Crossability

In the Japanese natural environment, there are no reports that maize has adapted to the wild environment and that wild relatives (Teosinte) capable of naturally crossing with maize are growing voluntarily in Japan.

Therefore, it was judged that there are no specific wild plants that are possibly affected by this recombinant maize, and that the use of such maize poses no risk of Adverse Effects on Biological Diversity that is attributable to crossability. It was judged that the conclusion above made by the applicant is valid.

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 maize, in accordance with the Type 1 Use Regulation, causes Adverse Effects on Biological Diversity in Japan. It was judged that the conclusion above made by the applicant is valid.

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