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 and tolerant to glufosinate herbicide and glyphosate herbicide (*cry34Ab1*, *cry35Ab1*, *pat*, *cp4 epsps*, *Zea mays* subsp. *mays* (L.) Iltis) (59122×NK603, OECD UI：DAS-59122-7×MON-ØØ6Ø3-6)
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

I. Information concerning preparation of living modified organisms

The stack line 59122×NK603 was developed by Pioneer Hi-Bred International, Inc (USA). The parent line Event DAS-59122-7 was developed jointly by Dow AgroSciences LLC (USA) and Pioneer Hi-Bred International, Inc (USA), and NK603 was developed by Monsanto Company (USA). The stack line 59122×NK603 is a cultivar created by crossbreeding inbred lines of Event DAS-59122-7 and NK603 through the conventional crossbreeding methods.

The following genes are introduced into the stack line 59122×NK603: the *cry34/35Ab1* gene to confer the resistance to the insects of the order Coleoptera derived from the Event DAS-59122-7, the *pat* gene to confer the tolerance to glufosinate herbicide derived from the Event DAS-59122-7, and the *cp4 epsps* gene to confer the tolerance to glyphosate herbicide derived from the NK603.

For the parent lines, Event DAS-59122-7 and NK603, applications have been field already for the Type I Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" enacted in February 2004. The Committee on Biological Diversity Risk Assessment judged that neither Event DAS-59122-7 nor NK603 would result in Adverse Effect on Biological Diversity when used as Type I Use which was described in the application for this stack line 59122×NK603. Consequently, for the Event DAS-59122-7, solicitation of public comments was closed on February 21, 2005, and the NK603 gain approval on November 22, 2004.

To create this evaluation document, we referred to summary documents of Event DAS-59122-7 and NK603, which are both found in the web site of Japan Biosafety Clearing House (J-BCH) provided by the Ministry of the Environment, and the summary documents of NK603 disclosed in the GM database of AGBIOS (Canada). (http://www.bch.biodic.go.jp/download/lmo/public_comment/DAS59122-7ap.pdf, http://www.bch.biodic.go.jp/download/lmo/public_comment/NK603ap.pdf, http://www.agbios.com/docroot/decdocs/02-269-007.pdf.)

1. Information concerning donor nucleic acid

(1) Composition and origins of component elements

Table 1 and Table 2 show the composition and the origins of component elements of the donor nucleic acid used to produce Event DAS-59122-7 and NK603, respectively.

Table 1 Composition and origins of component elements of the donor nucleic acid used for developing Event DAS-59122-7

Component elements	Size (kbp)	Origin and function
cry34Ab1 gene expression cassette		
UBIZM1(2) PRO	1.98	Ubiquitin constitutive promoter[1)] derived from *Zea mays* [including intron and 5' untranslated region（Christensen, *et al*., 1992）]
cry34Ab1	0.37	A gene that encodes Cry34Ab1 protein derived from *Bacillus thuringiensis* PS149B1 strain
PIN Ⅱ TERM	0.32	Protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum*
cry35Ab1 gene expression cassette		
TA Peroxidase PRO	1.30	Peroxidase promoter (base sequence 45-1342 of GenBank X53675) derived from *Triticum aestivum* known to express in roots
cry35Ab1	1.15	A gene that encodes Cry35Ab1 protein derived from *Bacillus thuringiensis* PS149B1 strain
PIN Ⅱ TERM	0.32	Protease inhibitor II terminator to terminate transcription derived from *Solanum tuberosum*
pat gene expression cassette		
CAMV 35S PRO	0.53	35S constitutive promoter[1)] derived from cauliflower mosaic virus (CaMV)（Hohn, *et al*., 1995）
Pat	0.55	A gene that encodes phosphinothricin acetyltransferase (PAT protein) derived from *Streptomyces viridochromogenes* [Optimized to activate the expression in plant body[2)] (Eckes, *et al*., 1989)]
CAMV 35S TERM	0.21	35S terminator to terminate transcription derived from cauliflower mosaic virus (CaMV)（Hohn, *et al*., 1995）

1) Constitutive promoter: A promoter that drives the expression of target genes in all sites in plant body

2） The produced protein has none of its amino acids modified.

Table 2 Composition and origins of component elements of the donor nucleic acid used for developing NK603

Component elements	Size (Kpb)	Origin and function
cp4 epsps gene expression cassette (1)		
P-*ract 1*	0.9	Promoter region of actin 1 gene derived from rice. It makes target genes expressed.
ract 1 intron	0.5	Intron of rice actin gene. It makes target genes expressed by enhancing splicing.
CTP 2	0.2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
cp4 epsps	1.4	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4 strain.
NOS 3'	0.3	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens.* It terminates transcription of mRNA and induces polyadenylation.
cp4 epsps gene expression cassette (2)		
E35S	0.6	Contains 35S promoter and duplicated enhancer from cauliflower mosaic virus (CaMV). Makes target genes expressed in all the tissues constantly.
ZmHsp70 intron	0.8	Intron of heat shock protein gene from maize. ZmHsp70 intron is used to enhance the expression of foreign genes in plants.
CTP 2	0.2	N-terminal chloroplast transit peptide sequence derived from the *Arabidopsis epsps* gene. Transfers target proteins from cytoplasm to chloroplast.
cp4 epsps	1.4	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* CP4 strain.
NOS 3'	0.3	3' untranslated region of nopaline synthase (NOS) gene from T-DNA of *Agrobacterium tumefaciens*. It terminates transcription of mRNA and induces polyadenylation.

(2) Functions of component elements

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

Table 1 and Table 2 show functions of individual component elements of donor nucleic acid, including target genes, expression regulation regions, localization signals, and selective markers, in Event DAS-59122-7 and NK603, respectively.

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

a. Cry34Ab1 protein and Cry35Ab1 protein

Cry34Ab1 protein and Cry35Ab1 protein are ones of *B.t.* protein derived from *Bacillus thuringiensis* PS149B1 strain, having an insecticidal activity against corn rootworm (*Diabrotica spp.*). Since they function in collaboration with each other, they are called binary proteins (Ellis *et al*., 2002).

It is suggested that the Cry34Ab1 protein and Cry35Ab1 protein work in concert to effect, and, like other *B.t.* proteins, destroy midgut cell membrane of target insects (Masson *et al.*, 2004). It is generally known that *B.t.* proteins have extremely high specific insecticidal activity (Shirai, 2003). In addition, Cry34Ab1 protein and Cry35Ab1 protein also show high specific insecticidal activity, and it is confirmed as a result of biological test that they exhibit the insecticidal activity against the larvae of only two kinds of the insects of the order Coleoptera, northern corn rootworm (*Diabrotica barberi*) and western corn rootworm (*Diabrotica virgifera virgifera*) (Poletika, 2003).

It has not been confirmed that the Cry34Ab1 protein and Cry35Ab1 protein share amino acid structurally sequence homology with any of the known allergenic proteins (Song, 2003).

b. PAT protein

PAT protein (phosphinothricin acetyltransferase) confers the tolerance to glufosinate herbicide. The glufosinate herbicide contains *L*-glufosinate, the active ingredient, which inhibits the activity of glutamine synthase that synthesizes glutamine from glutamic acid and ammonia. As a result, ammonia is accumulated in the plant body, causing the plant to die. The PAT protein acetylates and detoxifies *L*-glufosinate, thereby conferring the glufosinate

tolerance to the plant body. It is reported that the PAT protein shows extremely high substrate specificity against *L*-glufosinate, and that it does not accept other *L*-amino acids or select *D*-glufosinate for substrates (OECD, 1999).

It has not been confirmed that the PAT proteins share amino acid sequence homology with any of the known allergenic proteins (Meyer, 1999).

c. CP4 EPSPS protein

CP4 EPSPS protein confers the tolerance to glyphosate herbicide. The glyphosate herbicide inhibits the activity of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway for aromatic amino acid biosynthesis. As a result, plants treated with glyphosate cannot synthesize amino acids essential for growth and ultimately die. The CP4 EPSPS protein is not inhibited even in the presence of glyphosate and it properly works as an enzyme in the shikimate pathway, thereby conferring the glyphosate tolerance on plants. In addition, it is suggested that the EPSPS protein is the enzyme which specifically reacts with the phosphoenolpyruvate (PEP) and shimimate-3-phosphate (S3P), and that it is not the rate-determining enzyme in the aromatic amino acid biosynthesis pathway.

It is reported that the CP4 EPSPS protein did not share amino acid sequence homology with any of the known allergenic proteins.

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

It is reported that Cry34Ab1 protein and Cry35Ab1 protein never act as enzyme in any plant body similarly as other Cry proteins. It is reported that the PAT protein exhibits high substrate specificity (OECD, 1999). On the other hand, as mentioned above, it is suggested that the EPSPS protein works as the enzyme that specifically reacts with the phosphoenolpyruvate (PEP) and shimimate-3-phosphate (S3P), and that it is not the rate-determining enzyme in the aromatic amino acid biosynthesis pathway. As a result, it is considered that EPSPS protein does not affect the metabolism of plants unintentionally.

Based on the above understanding that Cry34Ab1 protein and Cry35Ab1 protein are considered not to possess any enzyme activity, and that PAT protein and CP4 EPSPS protein possess high substrate specificities and differ from each other in the action mechanism, it is considered that the introduced genes in the stack line 59122 ×NK603 do not affect nor interact unintentionally with the metabolic system of the recipient organism.

2. Information concerning vector

(1) Name and origin

The vector used to produce Event DAS-59122-7 is plasmid PHP17662 derived from the plasmid pSB1 of *Escherichia coli* (Figure 1). On the other hand, the vector used to produce NK603 is plasmid PV-ZMGT32 derived from the plasmid pUC119 of *Escherichia coli* (Figure 2).

(2) Properties

1) The numbers of base pairs and nucleotide sequence of vector

The number of base pairs of the vector used for the production of Event DAS-59122-7 is 50,321bp, and the number of base pairs of the vector used for the production of NK603 is 9,308bp. The nucleotide sequences of the component elements in each vector have become clear.

2) Types of any nucleotide sequence having specific functions

The vector backbone of plasmid PHP17662, it contains antibiotic resistant marker genes (*tet* gene and *spc* gene) to select the microorganisms that contain transformed plasmid while the vector is growing in the microorganisms. The *tet* gene confers the resistance to antibiotics, tetracycline; and the *spc* gene confers the resistance to antibiotics, spectinomycin.

On the other hand, in the region other than the donor nucleic acid of plasmid PV-ZMGT32, it contains antibiotic resistant marker gene (*nptII* gene) to select the microorganisms that contain transformed plasmid. The *nptII* gene confers the resistance to antibiotics, kanamycin.

These antibiotic resistant genes are not introduced to the recipient organism.

3) Presence or absence of infectivity of vector

Neither of these vectors is known to be infectious.

3. Method of preparing living modified organisms

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

As for plasmid PHP17662 used to create Event DAS-59122-7, its T-DNA region consists of the following components; [*UBIZM1(2) Promoter*]-[*cry34Ab1*]-[*PINII Terminator*]-[*TA Peroxidase Promoter*]-[*cry35Ab1*]-[*PINII Terminator*]-[*CAMV35S Promoter*]-[*pat*]-[*CAMV35S Terminator*].

On the other hand, the linear DNA fragment (PV-ZMGT32L) used to create NK603 consists of two (2) *cp4 epsps* gene cassettes; [*P-ract1*]-[*ract1 intron*]-[*CTP2*]-[*cp4 epsps*]-[*NOS 3'*] and [*E35S*]-[*Zmhsp70*]-[*CTP2*]-[*cp4 epsps*]-[*NOS 3'*].

(2) Method of transferring nucleic acid transferred in the recipient organism

Introduction of nucleic acid into recipient organism was based on the *Agrobacterium* method for Event DAS-59122-7 in which the nucleic acid was introduced into the Hi-II callus of the maize variety A188×B73, and the particle gun bombardment method for NK603 in which the nucleic acid was introduced into the maize variety AW×CW.

(3) Processes of rearing of living modified organisms

The parent lines, Event DAS-59122-7 and NK603, were bred based on the conventional F1 cross breeding method. In addition, this stack line 59122×NK603 was bred, as shown in the process of rearing in Figure 3, by Pioneer Hi-Bred International, Inc (USA) based on the conventional cross breeding method.

In Japan, for the Event DAS-59122-7, an applications was filed for the Type I Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" carried into effect in February 2004, and solicitation of public comments was closed on February 21, 2005. In addition, applications were filed for approval for its safety as food with the Ministry of Health, Labour and Welfare in May 2004, and applications for approval for its safety as feed were submitted to the Ministry of Agriculture, Forestry and Fisheries in June 2004.

On the other hand, in accordance with the "Guideline for the Use of Recombinant in Agriculture, Forestry and Fisheries" (hereafter referred to as "Guidelines"), the intended use of the NK603 in any open system in Japan was approved in May 2001 as conforming to the Guideline. Then, also for the NK603, similarly as the Event DAS-59122-7, an application was filed for the Type I Use of Living Modified Organism, in line with Item 2 of Article 4 of the "Law concerning the Conservation and

Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms", and then gained approval on November 22, 2004. In addition, its safeties as food and as feed were confirmed in March 2001 (the safety as feed was re-approved in March 2003 in accordance with the legislation of the examination system).

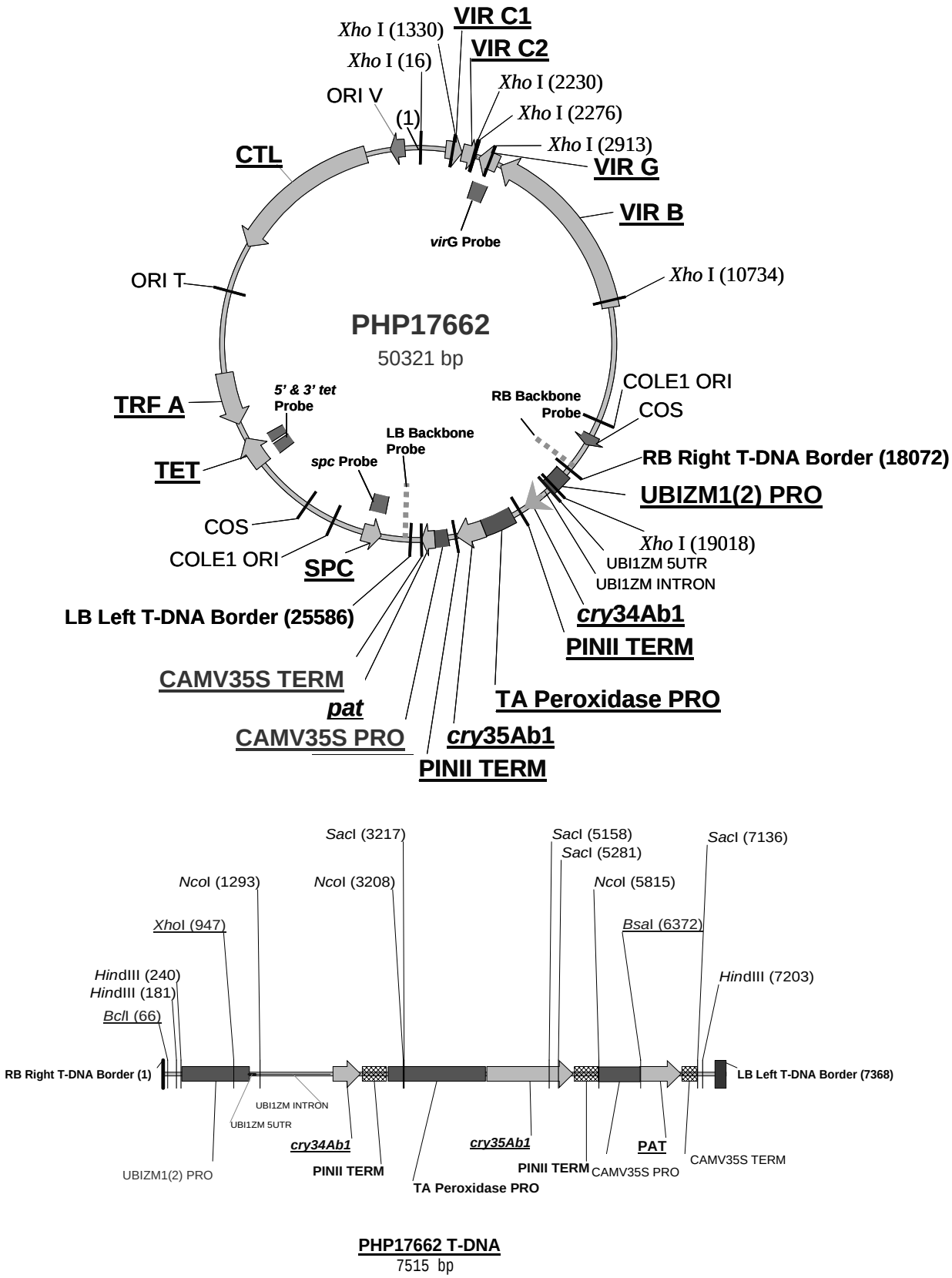

Figure 1 Compositions of plasmid PHP17662* and T-DNA region

*Vector used for the production of Event DAS-59122-7

(All the rights pertinent to the information in the diagram above and the responsibility for the contents lie with Du Pont Kabushiki Kaisha.)

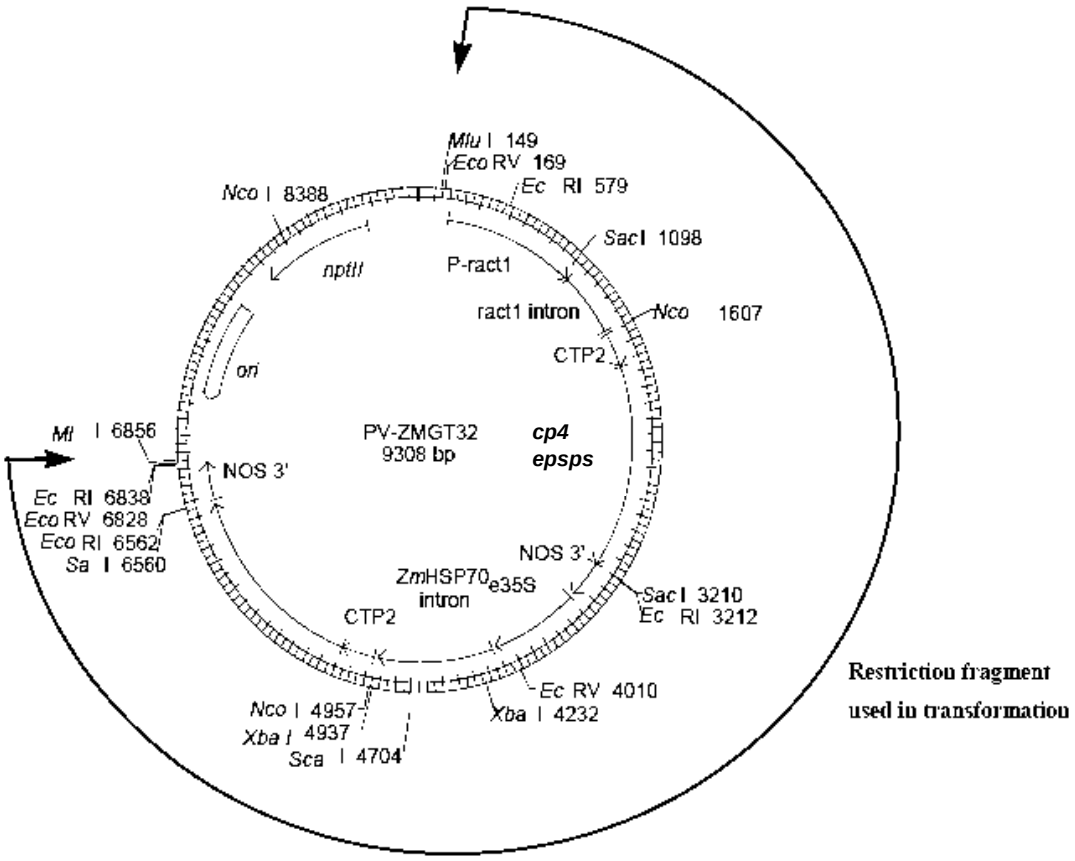

Figure 2　Compositions of plasmid PV-ZMGT32*

* Vector used for the production of NK603

Plasmid PV-ZMGT32 was treated with the restriction enzyme *Mlu*I (cleaved at the two points indicated by arrows in the upper diagram) and the resultantly obtained linear DNA fragment PV-ZMGT32L was used for introduction of genes into the recipient organism.

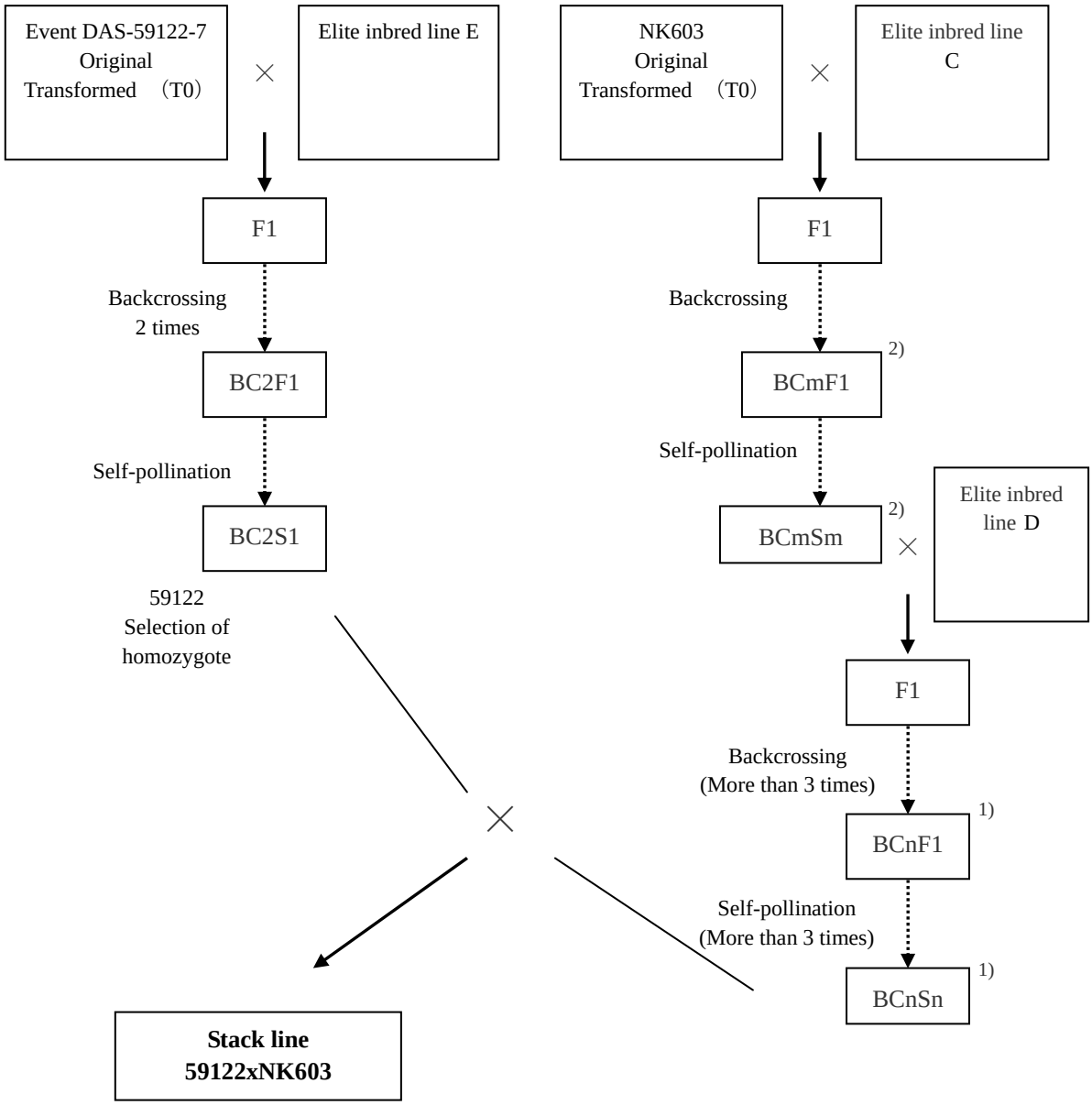

Figure 3 Outline of the process of rearing the stack line 59122×NK603

1) The parameter n means n≧3, indicating that backcrossing (BC) or self-pollination (S) has been carried out more than 3 times.
2) BCmSm, provided from Monsanto Company (USA) to Pioneer Hi-Bred International, Inc (USA), is a variety crossed based on the breeding program of Monsanto Company (USA). The suffix "m" in BCmF1 and BCmSm refers to the number of times of backcrossing or self-pollination.

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

(1) Location of the copy of transferred nucleic acid

It was confirmed based on the result of Southern blotting analysis that a copy of transferred nucleic acid was introduced into the maize genome in both Event DAS-59122-7 and NK603.

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

For Event DAS-59122-7, as a result of Southern blotting analysis, it was confirmed that one copy of each of *cry34Ab1* gene expression cassette and *cry35Ab1* gene expression cassette to confer the resistance to insects of the order Coleoptera including corn root worm, and *pat* gene expression cassette to confer the tolerance to glufosinate herbicide is inserted in the maize genome in the intact form. It was also confirmed that and that the introduced genes are all inherited stably in offspring.

On the other hand, for NK603, it was confirmed as a result of Southern blotting analysis that one copy of linear DNA fragment (PV-ZMGT32L, composed of two *cp4 epsps* gene expression cassettes) to confer the tolerance to glyphosate herbicide is inserted in the maize genome, and that the introduced genes are all inherited stably in offspring. It was confirmed that 217bp fragment of *P-ract1* exists in the reverse direction near the 3'-terminal of the introduced gene, though this fragment never causes any additional production of protein. In addition, it was also confirmed that the bases of *cp4 epsps* gene induced by *E35S* were changed during production of this recombinant maize and consequently one of the amino acids constituting the CP4 EPSPS protein was changed, though the function of the CP4 EPSPS protein remains unchanged.

(3) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid

It was confirmed based on the results of herbicide-spraying test and the biological test using the target insects that the traits inherited in the stack line 59122×NK603 from Event DAS-59122-7 and NK603 are stably expressed. The results of the tests are described below.

Herbicide-spraying test

To identify whether the stack line 59122×NK603 is comparable to the parent lines Event DAS-59122-7 and NK603 in possession of the tolerance to the both herbicides glufosinate and glyphosate, the spraying tests of the herbicides were conducted in March 2005 in a greenhouse at Pioneer Hi-Bred International, Inc (USA) (Appendix 1).

Fifteen seeds from each of the stack line 59122×NK603, the parent lines Event DAS-59122-7 and NK603, and non-recombinant maize were sowed on a tray (tray size: length×width×depth = approximately 35 cm×50 cm×10 cm). These plants were thinned out on the eighth day after the sowing, whereby ten plants were left in each tray. Three test fields were provided for spraying of glufosinate herbicide alone, glyphosate herbicide alone, and glyphosate herbicide followed by glufosinate herbicide. As listed in Table 3, the glufosinate herbicide-spraying field was planted with the stack line 59122 ×NK603, Event DAS-59122-7 and the non-recombinant maize, the glyphosate herbicide-spraying field was planted with the stack line 59122×NK603, NK603 and the non-recombinant maize, and the glyphosate herbicide followed by glufosinate herbicide-spraying field was planted with the stack line 59122×NK603 and the non-recombinant maize. The herbicides were sprayed in higher dosage than approved in the registration of agricultural chemicals (standard dosage), and also 16-times and 32-times higher ones than standard dosage. On the 12th day after seeding, the typical timing of herbicide spraying by farmers, glyphosate herbicide was sprayed, and on the 15th day after seeding, glufosinate herbicide was sprayed. On the 23rd day after seeding, visual inspection was carried out on the individual trays to identify the level of pesticide-induced damage (growth inhibition, color fading, spots, etc.), and evaluation was made with the score from 0% to 100% (0%＝no pesticide-induced damage, 100% ＝plant death). The test was performed in triplicate.

As a result of the tests, no statistically significant difference was observed in the level of pesticide-induced damage due to the treatment with spraying of glufosinate herbicide and glyphosate herbicide between the stack line 59122×NK603 and the parent lines Event DAS-59122-7 and NK603 (Table 3). Based on the above understanding, it was confirmed that the stack line 59122×NK603 is comparable to the parent lines Event DAS-59122-7 and NK603 in possession of the tolerance to the both herbicides glufosinate and glyphosate.

In actuality, standard dosage assures sufficient herbicidal effects and thus the actual farming fields never be exposed to such 16-times or 32-times higher dosages of glyphosate herbicide or glufosinate herbicide than standard dosage as applied in the tests which were sprayed specifically for the purpose of the tests. In addition, glyphosate herbicide and glufosinate herbicide were sprayed in succession to certain group in the tests, though such use of herbicides is not approved in the registration of

agricultural chemicals that was applied specifically for the purpose of the tests.

Table 3　Levels of pesticide-induced damage by spraying of the herbicides

Levels of pesticide-induced damage due to spraying of glufosinate herbicide				
Tested plant	Levels of pesticide-induced damage (%)			
	No spraying	Normal dosage (0.5kg ai/ha)	16 times (8.0kg ai/ha)	32 times (16.0kg ai/ha)
Stack line 59122×NK603	0.0±0.0 b	10.0±5.8 ab	10.0±0.0 ab	20.0±0.0 a
Event DAS-59122-7	0.0±0.0 b	6.7±3.3 b	6.7±3.3 b	10.0±0.0 ab
Non-recombinant maize	0.0±0.0 b	40.0±0.0 c		
Levels of pesticide-induced damage due to spraying of glyphosate herbicide				
Tested plant	Levels of pesticide-induced damage (%)			
	No spraying	Normal dosage (1.1kg ai/ha)	16 times (17.6kg ai/ha)	32 times (35.2kg ai/ha)
Stack line 59122×NK603	0.0±0.0 b	3.3±3.3 b	10.0±5.8 b	10.0±5.8 b
NK603	0.0±0.0 b	0.0±0.0 b	3.3±3.3 b	3.3±3.3 b
Non-recombinant maize	0.0±0.0 b	63.3±3.3 a		
Levels of pesticide-induced damage due to spraying of glufosinate herbicide+glyphosate herbicide				
Tested plant	Levels of pesticide-induced damage (%)			
	No spraying	Normal dosage (0.5kg ai/ha, 1.1kg ai/ha)	16 times (8.0kg ai/ha, 17.6kg ai/ha)	32 times (16.0kg ai/ha, 35.2kg ai/ha)
Stack line 59122×NK603	0.0±0.0 b	3.3±3.3 ab	6.7±3.3 ab	23.3±6.7 a
Non-recombinant maize	0.0±0.0 b	73.3±3.3 c		

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For each maize type, the levels of pesticide-induced damage were compared with data from a non-spraying control plot. Symbols written with damage levels indicate that damage levels showed no statistically significant differences within the same group (a/b/c) (Tukey's multiple test, $P<0.05$). Standard deviation is given to the right of ±. Herbicide dosage are expressed in the amount of active ingredient per hectare (kg ai/ha).

Biological test using Western corn rootworm

In order to identify whether the stack line 59122×NK603 is comparable to the parent line Event DAS-59122-7 in possession of the resistance to Coleoptera, biological test was conducted in March 2005 in a greenhouse at Pioneer Hi-Bred International, Inc (USA) using the target insect of Western corn rootworm (Appendix 2).

The stack line 59122 × NK603, the parent line Event DAS-59122-7, and non-recombinant maize were cultivated (one maize individual per pot, pot size: 24 cm in diameter, 22 cm in height, and 6.4 L in volume), and at the V4 stage (the 4th foliage leaf stage), 100 eggs of Western corn rootworm were inoculated at the roots of each plant every other day a total of 4 times (400 eggs in total). Then, at the point of time when the hatched larvae grew to the pupal stage (approximately 2 weeks after inoculation), observation was made to identify the degree of root damage by the western corn rootworm. The test was repeated 5 times in total with 4 maize individuals subjected to each test.

As a result, no statistically significant difference was observed in the level of damage by western corn rootworm between the stack line 59122×NK603 and Event DAS-59122-7 and then, it was confirmed that the obtained trait of resistance to Coleoptera is stably expressed in the stack line 59122×NK603 in the comparable level as in the parent line Event DAS-59122-7 (Table 4) (Appendix 2).

Table 4 Resistance to Western corn rootworm

Tested plant	Score of feeding damage at roots *
Stack line 59122×NK603	0.10±0.02
Event DAS-59122-7	0.09±0.01
Non-recombinant maize	2.64±0.08

* Node-Injury Scale developed by Iowa State University in the US
(0-3: 0 = no feeding damage, 3 = three or more root nodes eaten)
(http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html)
Standard deviation is given to the right of ±.

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Conclusion

Based on the above understanding, it was confirmed that the obtained traits derived from Event DAS-59122-7 and NK603 are stably expressed in the stack line 59122× NK603.

(4) 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

Transmission of this item is absence, because the transferred nucleic acid does not contain any sequence allowing transmission.

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

ELISA method using the polyclonal antibodies respectively for PAT protein and CP4 EPSPS protein have been developed and the analysis kits for detection of the individual proteins are commercially available. The analysis kits for detection of Cry34Ab1 protein and Cry35Ab1 protein are now under development and expected commercially available in August 2005. This stack line 59122×NK603 can be detected by applying these three methods to each grain of maize seeds.

The PAT protein detection kit can detect one PAT protein-containing grain per 500 maize grains. The CP4 EPSPS protein detection kit can detect one CP4 EPSPS protein-containing grain per 800 maize grains. These detection kits have been all certified as reliable enough by a variety of tests.

6. Difference from the recipient organism or the taxonomic species to which the recipient organism belongs

(1) Specific contents of physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acids

1) Resistance to Coleoptera

The stack line 59122×NK603 is conferred the resistance to corn root worm that feeds and damages maize with the production of Cry34Ab1 protein and Cry35Ab1 protein due to the introduction of *cry34Ab1* gene and *cry35Ab1* gene derived from *B.t.* PS149B1 strain introduced in Event DAS-59122-7.

2) Tolerance to glufosinate herbicide

The stack line 59122×NK603 is given the tolerance to glufosinate by the function of *pat* gene derived from *Streptomyces viridochromogenes* introduced into Event DAS-59122-7. The PAT protein produced by the expression of *pat* gene acetylates the glufosinate herbicide and transforms it to nontoxic acetylglufosinate, thereby conferring on the plant body the tolerance to glufosinate.

3) Tolerance to glyphosate herbicide

The stack line 59122×NK603 is given the tolerance to glyphosate herbicide by the function of *cp4 epsps* gene derived from *Agrobacterium* CP4 strain introduced in NK603. The CP4 EPSPS protein produced by the expression of *cp4 epsps* gene is not inhibited even in the presence of glyphosate and it properly works as an

enzyme in the shikimate pathway, thereby conferring the glyphosate tolerance on plant body.

(2) Presence or absence of difference between recombinant plant and the species to which recipient organism belongs, and the degree of difference, if any

The stack line 59122×NK603 is a hybrid obtained from the crossing of the two inbred lines Event DAS-59122-7 and NK603 with the use of conventional cross-breeding method and thus, it obtained traits derived from the Event DAS-59122-7 and NK603. Based on the findings that the Cry34Ab1 protein and Cry35Ab1 protein expressed in this stack line are considered not to possess any enzyme activity similarly as the other Cry proteins, and that both PAT protein and CP4 EPSPS protein possess high substrate specificities and differ from each other in the action mechanism, it is considered that these proteins conferring to the traits in this stack line will not interact with each other. In fact, as mentioned in 4-I. "(3) The stability of the expression among individuals and generations under natural conditions with respect to the physiological or ecological characteristics that were accompanied by the expression of copies of transferred nucleic acid", it was confirmed as a result of herbicide-spraying test and biological test that the traits inherited from the both parent lines were stably expressed in the stack line 59122 ×NK603.

Therefore, the difference between this stack line and the taxonomic species of maize to which the recipient organism belongs was described with the use of the results of individual examinations for the various characteristics conducted for Event DAS-59122-7 and NK603. The individual examinations were conducted in isolated fields in Japan in 2003 for Event DAS-59122-7 and in 2000 for NK603.

1) Morphological and growth characteristics

For the morphological and growth characteristics of Event DAS-59122-7, evaluation was conducted regarding germination rate, uniformity of germination, time of tasseling, time of silking, time of flower initiation, time of flower completion, flowering period, maturation time, plant type, tiller number, number of productive ears, grain color and grain shape, culm length, height of ear, ear length, ear diameter, fresh weight of above-ground part, and shape of flower organ. As a result, in all evaluation items except culm length, no difference was observed between the Event DAS-59122-7 and the non-recombinant maize. For the culm length, the significant difference was observed only in one of the two recombinant varieties tested (Event DAS-59122-7: 192.0 cm, non-recombinant: 212.3 cm), though no difference was observed in the other variety.

For the morphological and growth characteristics of NK603, evaluation was also

conducted regarding uniformity of germination, germination rate, time of tasseling, time of silking, culm length, plant shape or plant type, tiller number, height of ear, maturation time, number of ears, and weight of plant at harvesting time. As a result, in all evaluation items, no statistically significant difference was observed between the recombinant and the non-recombinant control maize.

Consequently, it is considered that there is no difference in morphological and growth characteristics between this stack line 59122×NK603 and the taxonomic species of maize to which the recipient organism belongs.

As a result of germination test conducted in US on this stack line in practice, the germination rate of this stack line was 99%, which was equivalent to 96% in the case of Event DAS-59122-7 and 98% in NK603. In addition, the germination rates measured in the test are all found higher than 90% which is the standard germination rate determined by OECD (NEBRASKA OECD GUIDELINES, 1995).

2) Cold-tolerance at the early stage of growth

Cold tolerance of the seedlings of Event DAS-59122-7 was evaluated. As a result, all the plant bodies tested withered and died when the minimum temperature dropped down to 1.5℃, and no difference was observed in the sensitivity to low temperatures between Event DAS-59122-7 and the non-recombinant maize.

Cold tolerance of the seedlings of NK603 was also evaluated. As a result, almost all the plant bodies tested withered and died on the 14th day after exposure to the low temperature (ambient temperature of 4℃), and no difference was observed between NK603 and the non-recombinant control maize.

Consequently, regarding cold tolerance of this stack line 59122×NK603, it is considered that there is no difference between this stack line 59122×NK603 and the taxonomic species of maize to which the recipient organism belongs.

3) Wintering ability and summer survival

It is well known that maize is a summer type annual plant, and after ripening it usually dies out in winter and it can overwinter. In addition, after harvesting, it does not regrow, propagate vegetatively, or produce seeds. Based on the above, an overwintering test for the matured plant of Event DAS-59122-7 and NK603 was not carried out. However, in fact, it was confirmed that, in the US field where cultivation test of Event DAS-59122-7 was conducted in the previous year, there was no plant body observed in the following year which remained alive through the

winter. It was also observed at the end of the isolated field test for NK603 that plant bodies started withering after ripening.

Consequently, regarding wintering ability of this stack line 59122×NK603, it is considered that there is no difference between this stack line maize and the taxonomic species of maize to which the recipient organism belongs.

4) Fertility and size of the pollen

To examine the fertility (maturity) and its size of Event DAS-59122-7, pollens were stained with potassium iodine solution and observed under a microscope. As a result, regarding the fertility and its size of the pollens, no difference was observed in the evaluation items between the Event DAS-59122-7 and the non-recombinant control maize.

Also for NK603, in the fertility and its size of pollens, no difference was observed between the NK603 and the non-recombinant control maize as a result of examination of the stained pollens under a microscope.

Consequently, regarding fertility and size of the pollens, it is considered that there is no difference between this stack line 59122×NK603 and the taxonomic species of maize to which the recipient organism belongs.

5) Production, shedding habit, dormancy, and germination rate of the seed

The row number per ear, grain number per row and 100-kernel weight and other characteristics were examined during the isolated field test as the characteristics referring to the production of seeds. As a result, no difference was observed between Event DAS-59122-7 and the non-recombinant maize in all of the characteristics examined. In addition, for both Event DAS-59122-7 and the non-recombinant maize, no dormancy of the seeds was observed. Also in the germination rate and shedding habit of second generation hybrid (F2) seeds, no difference was observed between Event DAS-59122-7 and the non-recombinant maize.

Also for NK603, examination was conducted on row number per ear, grain number per row and 100-kernel weight and other characteristics. As a result, no difference was observed between the NK603 and the non-recombinant maize in any of the characteristics examined except in 100-kernel weight. Regarding 100-kernel weight, significant difference was observed between one samples of the two recombinant maize NK603 samples examined (33.6 g for NK603 and 35.1 g for the non-recombinant), though no difference was found between the other sample of the

two recombinant maize NK603 samples examined and the non-recombinant maize. In addition, regarding the germination rate of harvested seeds (F2), no difference was observed between the NK603 and the non-recombinant maize and then no dormancy of the seeds was observed.

The ears of both Event DAS-59122-7 and NK603 were covered with bracts at the time of harvesting and thus, shedding habit was not observed in the natural condition similarly as their non-recombinant control maize samples.

Based on the above understanding, regarding the production of the seed and other characteristics of this stack line 59122×NK603, it is considered that there is no difference between this stack maize and the taxonomic species of maize to which the recipient organism belongs.

6) Crossability

In Japan, the growth of wild relatives (teosinte) that can be crossed with the recipient organism, maize in natural environment has not been reported.

7) Productivity of harmful substances

It is not known that maize secretes any harmful substances that could have adverse effects on the surrounding plants and/or microorganisms in soil. Also it is not known that maize produces any allelochemicals after dying that could affect other plants.

In the Event DAS-59122-7, Cry34Ab1 protein, Cry35Ab1 protein and PAT protein are newly produced due to the introduction of *cry34Ab1* gene, *cry35Ab1* gene and *pat* gene. On the other hand, in the NK603, CP4 EPSPS protein is newly produced due to the introduction of *cp4 epsps* gene. It is suggested that Cry34Ab1 protein and Cry35Ab1 protein do not work as enzyme in plant body similarly as other Cry proteins in *B.t.*. In addition, it is reported that PAT protein possesses extremely high substrate specificity (OECD, 1999). It is also known that CP4 EPSPS protein possesses high substrate specificity similarly as PAT protein. Consequently, it is considered that these proteins could not affect the metabolic pathway of the maize of recipient organism and produce any unexpected harmful substances.

For the Event DAS-59122-7, analysis on the weeds growing in the areas for isolated field test for composition of species (DCA score), total number of individuals and dry weight, and soil microflora tests and plow-in test in the specific screen-house test were performed. In addition, soil microflora tests were additionally conducted in the test field (soil condition of volcanic ashes) in the State

of Hawaii in the USA. Based on the above results, it was confirmed that there was no difference between the Event DAS-59122-7 and the non-recombinant control maize in the productivity of any unexpected harmful substances.

For the NK603, succeeding crop test, soil microflora tests and plow-in test were performed in the isolated field test. As a result, it was confirmed that there was no difference between the recombinant maize examined and the non-recombinant control maize in all evaluation items.

Consequently, regarding the productivity of any unexpected harmful substances, it is considered that there is no difference between the stack line 59122×NK603 and the taxonomic species of maize to which the recipient organism belongs.

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 applied 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.

This stack line maize was produced by conventional cross-breeding method of maize resistant to Coleoptera and tolerant to glufosinate herbicide (DAS-59122-7) and maize tolerant to glyphosate herbicide (MON-00603-6), and the parent lines were individually judged at the Committee for Review on the Biological Diversity Risk Assessment as causing no Adverse Effect on Biological Diversity when applied for the Type I Use same as the stack line maize.

It is reported that the Cry34Ab1 protein and Cry35Ab1 protein (hereafter referred to as “Cry34Ab1/Cry35Ab1 protein” since the two proteins work jointly as a binary protein to exhibit insecticidal activity) which are encoded by the (*cry34Ab1* and *cry35Ab1*) genes resistant to Coleoptera derived from DAS-59122-7 do not possess enzyme activity. It is also reported that the PAT protein which is encoded by the (*pat*) gene tolerant to glufosinate derived from DAS-59122-7, and the CP4 EPSPS protein which is encoded by the (*cp4 epsps*) gene tolerant to glyphosate derived from MON-00603-6 possess high substrate specificities. Therefore, it is considered that the characteristics conferred by *pat*, *cry34Ab1*, *cry35Ab1* and *cp4 epsps* do not interact with each other.

It was confirmed based on the various herbicide-spraying tests and the biological test using the western corn rootworm (*Diabrotica virgifera virgifera*) respectively that the tolerance to glufosinate herbicide and glyphosate herbicide, and the resistance to Coleoptera are properly expressed in this stack line maize.

Based on the above understanding, it is considered that there is no specific change in the characteristics in this stack line maize except it possesses the same characteristics as the parent lines do.

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

1) Competitiveness

This stack line maize possesses the Coleoptera resistance and glufosinate herbicide tolerance derived from DAS-59122-7 and the glyphosate herbicide tolerance derived from MON-00603-6. However, it is considered that the insect damage by Coleoptera is not the major cause to make the maize difficult to grow in the natural environment in Japan, and that the glufosinate and glyphosate tolerances do not exert pressure for selection under a natural environment. Consequently, it is considered that these characteristics do not increase the competitiveness and thus this stack line maize is not predominant over the parent lines in the competitiveness. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to competitiveness is valid.

2) Productivity of harmful substances

This stack line maize possesses the productivity of Cry34Ab1/Cry35Ab1 proteins and PAT protein derived from DAS-59122-7 and the productivity of CP4 EPSPS protein derived from MON-00603-6. It is confirmed that the Cry34Ab1/Cry35Ab1 proteins possesse the insecticidal activity against insects of the order Coleoptera, though PAT protein and CP4 EPSPS protein are not harmful substances to animals and plants. Thus, it is considered that the productivity of harmful substances of this stack line maize would not become higher than that of parent lines even if this stack line maize contains these proteins. Based on the above understanding, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to the productivity of harmful substances is valid.

3) Crossability

In Japan, the wild relatives (teosinte) that can be crossed with maize in natural environment has not been reported.

Based on the above understanding, no wild species can be specified as having some effects, it is judged that the conclusion made by the applicant that there is no risk of Adverse Effect on Biological Diversity attributable to crossability 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 the stack line 59122×NK603 in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is valid.

Bibliography

Brooks, K. J. and DeWidt, P. M. 2000a. PS149B1 14 KDA and 44 KDA Proteins: Acute Oral Toxicity study in CD-1 Mice. The Dow Chemical Company unpublished report. Study ID: 001128.

Brooks, K. J. and DeWidt, P. M. 2000b. PS149B1 44 KDA Protein: Acute Oral Toxicity Study in CD-1 Mice. The Dow Chemical Company unpublished report. (Not made available or disclosed to unauthorized persons)

Brooks, K. J. and DeWidt, P. M. 2000c. PS149B1 14 KDA Protein: Acute Oral Toxicity Study in CD-1 Mice. The Dow Chemical Company unpublished report. (Not made available or disclosed to unauthorized persons)

CFIA (Canadian Food Inspection Agency). 1994.
(http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml#A12).

National Institute for Agro-Environmental Sciences (NIAES). FY 2003 Agro-Environmental Survey & Research Results/Project Outline (Research subject: Evaluation of safety of maize modified with the gene resistant to harmful insects of the order of Lepidoptera).

Ellis, R.T., Stockhoff, B.A., Stamp, L., Schnepf, H.E., Schwab, G.E., Knuth, M., Russell, J., Cardineau, G.A. and Narva, K.E. 2002. Novel Bacillus thuringiensis Binary Insecticidal Crystal Protein Active on Western Corn Rootworm, Diabrotica virgifera virgifera LeConte. Applied Environmental Microbiology 68: 1137-1145.
(http://aem.asm.org/cgi/content/full/68/3/1137).

EPA (U.S. Environmental Protection Agency), 2001a. Biopesticide Registration Action Document.
(http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006481.pdf).

EPA. 2001b. Pesticide Fact Sheet for Cry1F protein.
(http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_006481.pdf).

FAO Statistical Database. 2004. (http://apps.fao.org/page/collections).

Hansen-Jesse, L., and J.J. Obrycki. 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. Oecologia, 125:241-248.

Herman, R.A. 2000. Microbial PS149B1 Binary Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study. Dow AgroSciences LLC unpublished report. (Not made available or disclosed to unauthorized persons)

Herman, R. A., Scherer, P. N. and Wolt, J. D. 2002. Rapid Degradation of a Binary, PS149B1, δ-Endotoxin of Bacillus thuringiensis in Soil, and a Novel Mathematical Model for Fitting Curve-Linear Decay. Environ. Entomol. 31: 208-214.

Higgins, L. S., and D. Wright. 2003. Impact of corm rootworm control strategie on non-target arthropods. Pioneer Hi-Bred International, Inc. unpublished report. (Not made available or disclosed to unauthorized persons)

Red List of Threatened Species by the Ministry of the Environment (List of endangered species of wild fauna and flora in Japan), Revised edition in 2000
（http://www.biodic.go.jp/rdb/rdb_f.htm).

Kawashima, S., K. Matsuo, M. Du, Y. Takahashi, S. Inoue, and S. Yonemura. 2004. An algorithm for estimating potential deposition of corn pollen for environmental assessment. Environ. Biosafety Res. 3: 197-207.

Kazunori Kikuchi. 1987. Production and utilization of maize: 227-243. Korin Publishing Co., Ltd.

Marino, T. A. and Yaroch, A. M. 2002. PS149B1 Binary Insecticidal Crystal Protein: An 8-Day Dietary Study with the Rainbow Trout, Oncorhynchus mykiss, Waldbaum. The Dow Chemical Company unpublished report. (Not made available or disclosed to unauthorized persons)

Masson, L., G. Schwab, A. Mazza, R. Brousseau, L. Potvin, and J.-L. Schwartz. 2004. A novel Bacillus thuringiensis (PS149B1) containing a Cry34Ab1/Cry35Ab1 binary toxin specific for the western corn rootworm Diabrotica virgifera virgifera LeConte forms ion channels in lipid membranes. Biochem. 43(38):12349-57.

Meyer, T. 1999. Comparison of Amino Acid Sequence Similarity of Cry1F and PAT Proteins to Known Allergen Proteins. Pioneer Hi-Bred International, Inc. unpublished report. (Not made available or disclosed to unauthorized persons)

NEBRASKA OECD GUIDELINES. 1995. HYBRID CORN. Seed Certification Standards.

Encyclopedia of Agriculture. Second enlarged and revised edition. Supervised by Yakichi Noguchi and Shinichiro Kawada. 1994: 537-541.
Published by Yokendo Co., Ltd.

OECD. 1999. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 11: Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. ENV/JM/MONO(99)13. (http://www.olis.oecd.org/olis/1999doc.nsf/LinkTo/env-jm-mono(99)13).

OECD. 2003. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 27: Consensus Document of the Biology of Zea mays subsp. mays (Maize). (http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/env-jm-mono(2003)11).

Pleasants, J. M., R. L. Hellmich, G. P. Dively, M. K. Sears, D. E. Stanley-Horn, H. R. Mattila, J. E. Foster, T. L. Clark, and G. D. Jones. 2001. Corn pollen deposition on milkweeds in and near cornfields. PNAS, 98(21):11919-24.

Poletika, N. N. 2003. Non-target Invertebrate Ecological Risk Assessment for Field Corn Expressing Cry34Ab1 and Cry35Ab1 Insecticidal Crystal Proteins in Event DAS-59122-7. Dow AgroScience LLC. unpublished report. (Not made available or disclosed to unauthorized persons)

Yoichi Shirai. 2003. Influence of Transgenic Insecticidal Crops on Non-target Insects: Overview from the Data to Date. Journal of the Japanese Society of Applied Entomology and Zoology Vol. 47, pp.1-11.

Smith, B., McNaughton, J. and Hinds, M. 2003. Nutritional Equivalency Study of Maize Containing Cry34Ab1 and Cry35AB1: Poultry Feeding Study. Solution BioSciences, Inc. unpublished report. (Not made available or disclosed to unauthorized persons)

Song, P. 2003. Comparison of the Amino Acid Sequences of Bacillus thuringiensis Strain PS149B1 Cry34Ab1 and Cry35Ab1 Insecticidal Crystal Proteins as Expressed in Maize to Known Proteins. Dow AgroScience LLC. unpublished report. (Not made available or disclosed to unauthorized persons)

The International Plant Names Index. 2004. (http://www.ipni.org/index.html).

FY 2002 Trade Statistics by the Ministry of Finance
(http://www.customs.go.jp/toukei/info/index.htm).

List of Appendices

Appendix 1 Reference data: Test Report PHI-2005-027

Report summary adapted from study: PHI-2005-027. 2005. Evaluation of herbicide tolerance of hybrid maize lines containing events DAS-59122-7, MON-ØØ6Ø3-6, and the combined trait product DAS-59122-7x MON-ØØ6Ø3-6. Pioneer Hi-Bred International, Inc. Confidential Report.

Not made available or disclosed to unauthorized persons

Appendix 2 Reference data: Test Report PHI-2005-035

Pascual, M. A. 2005. Insect efficacy evaluation of maize expressing the Cry34Ab1, Cry35Ab1, and Cry1F proteins. Pioneer Hi-Bred International, Inc. Confidential Report.

Not made available or disclosed to unauthorized persons