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

Name   Monsanto Japan Limited
Applicant Seiichiro Yamane, President
Address   4-10-10, Ginza, Chuo-ku, Tokyo

Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize tolerant to drought, resistant to Lepidoptera and Coleoptera, and tolerant to glyphosate herbicide (modified *cspB*, *cry1A.105*, modified *cry2Ab2*, modified *cp4 epsps*, modified *cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis) (MON87460 × MON89034 × MON88017, OECD UI: MON-8746Ø-4 × MON-89Ø34-3 × MON-88Ø17-3) (including the progeny lines isolated from the maize lines, MON87460, MON89034, and MON88017, that contain a combination of any of the transferred genes in the individual maize lines [except those already granted approval regarding Type 1 Use Regulation])
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal, and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

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

5   1   Information concerning preparation of living modified organisms

The crossed progeny (OECD UI: MON-8746Ø-4 × MON-89Ø34-3 × MON-88Ø17-3) (hereinafter referred to as "this stack line maize") was developed through the crossing of the following three recombinant maize lines with the use of a traditional crossbreeding method. The three recombinant maize lines are: drought-tolerant maize (modified *cspB*, *Zea mays* subsp. *mays* (L.) Iltis) (MON87460, OECD UI: MON-8746Ø-4) (hereinafter referred to as "MON87460"); maize resistant to Lepidoptera (*cry1A.105*, modified *cry2Ab2*, *Zea mays* subsp. *mays* (L.) Iltis) (MON89034, OECD UI: MON-89Ø34-3) (hereinafter referred to as "MON89034"); and maize resistant to glyphosate herbicide and resistant to Coleoptera (modified *cp4 epsps*, modified *cry3Bb1*, *Zea mays* subsp. *mays* (L.) Iltis.) (MON88017, OECD UI: MON-88Ø17-3) (hereinafter referred to as "MON88017"). Therefore, this stack line maize possesses the characteristics of these three parent recombinant maize lines, MON87460, MON89034, and MON88017. This stack line maize is commercialized as a hybrid variety (F1), and the grain harvested from this stack line maize is composed of combinations of the transferred genes in the individual parent lines of this stack line maize due to the genetic segregation. The information concerning preparation of MON87460, MON89034, and MON88017 is explained individually in the following sections.

25

(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 the parent lines MON87460, MON89034, and MON88017 are shown individually in Figure 1 to Figure 3 (p. 4–6) and   Table 1 to Table 3 (p. 7–13).

2)   Function of component elements
35

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

Functions of individual component elements of donor nucleic acid that were used

2

for the production of MON87460, MON89034, and MON88017 are shown individually in Table 1 to Table 3 (p. 7–13). Detailed information of the target gene, that is the modified cold shock protein B (modified *cspB*) gene, the *cry1A.105* gene, the modified *cry2Ab2* gene, the modified *cp4 epsps* gene, and the modified *cry3Bb1* gene, is shown individually in Table 1 to Table 3 (p. 7–13).
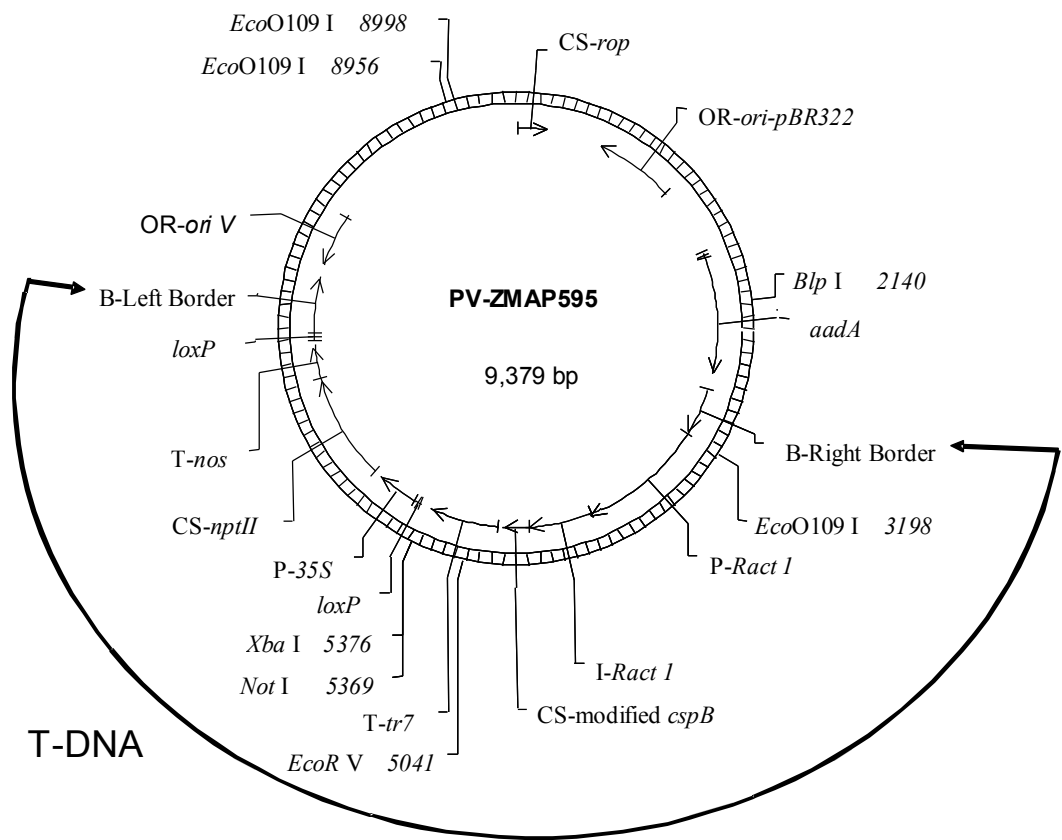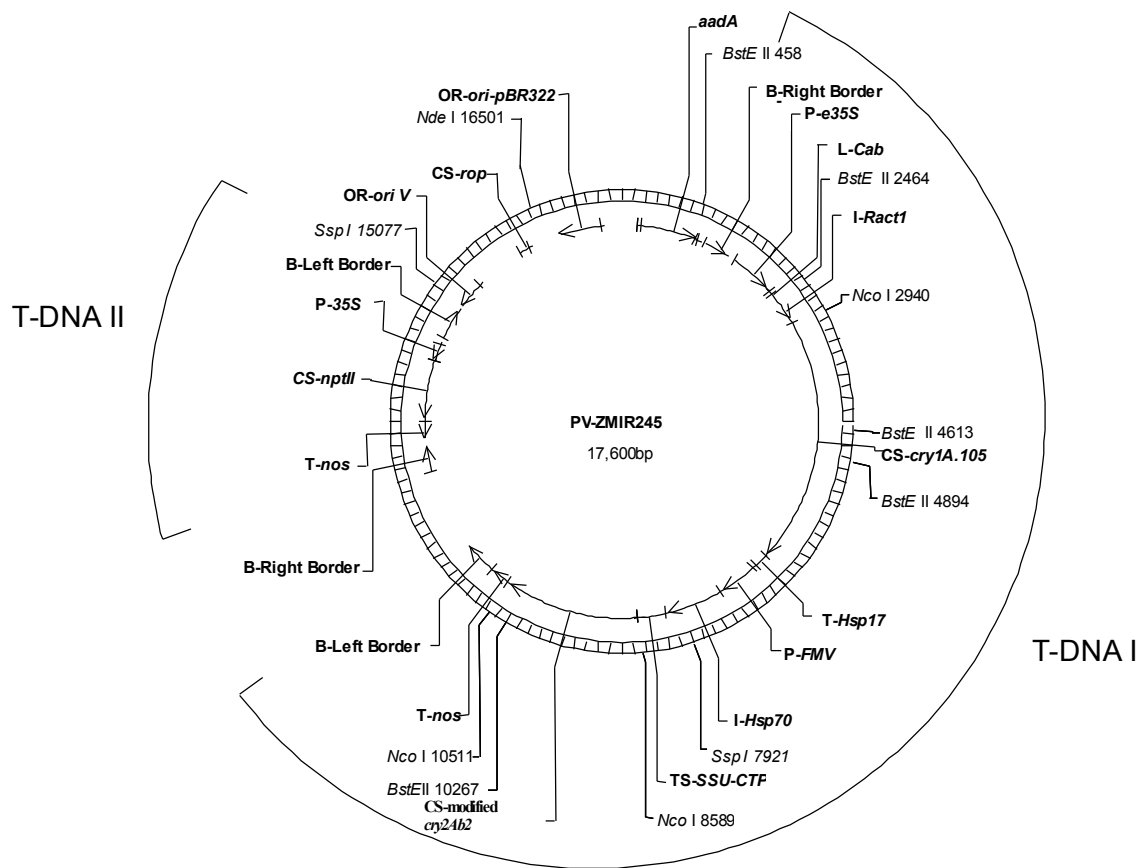
5

5

*Eco*O109 I   *8998*

*Eco*O109 I   *8956*

CS-*rop*

OR-*ori-pBR322*

OR-*ori V*

B-Left Border

Blp I   *2140*

loxP

aadA

PV-ZMAP595

9,379 bp

B-Right Border

T-*nos*

CS-*nptII*

*Eco*O109 I   *3198*

P-*35S*

P-*Ract 1*

loxP

Xba I   *5376*

Not I   *5369*

I-*Ract 1*

T-*tr7*

CS-modified *cspB*

T-DNA

EcoR V   *5041*

10

Figure 1   Map of the plasmid PV-ZMAP595 used for the development of MON87460[1]

Figure 2   Map of the plasmid PV-ZMIR245 used for the development of MON89034[2]

In the process of the rearing of MON89034, those individuals were selected that contain the T-DNA I region shown above but do not contain the T-DNA II region.

---

[2]All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Monsanto Japan Limited.

5

Figure 3   Map of the plasmid PV-ZMIR39 used for the development of MON88017[3]

10

---

[3]All the rights pertinent to the information in the diagram above and the responsibility for the contents rest upon Monsanto Japan Limited.
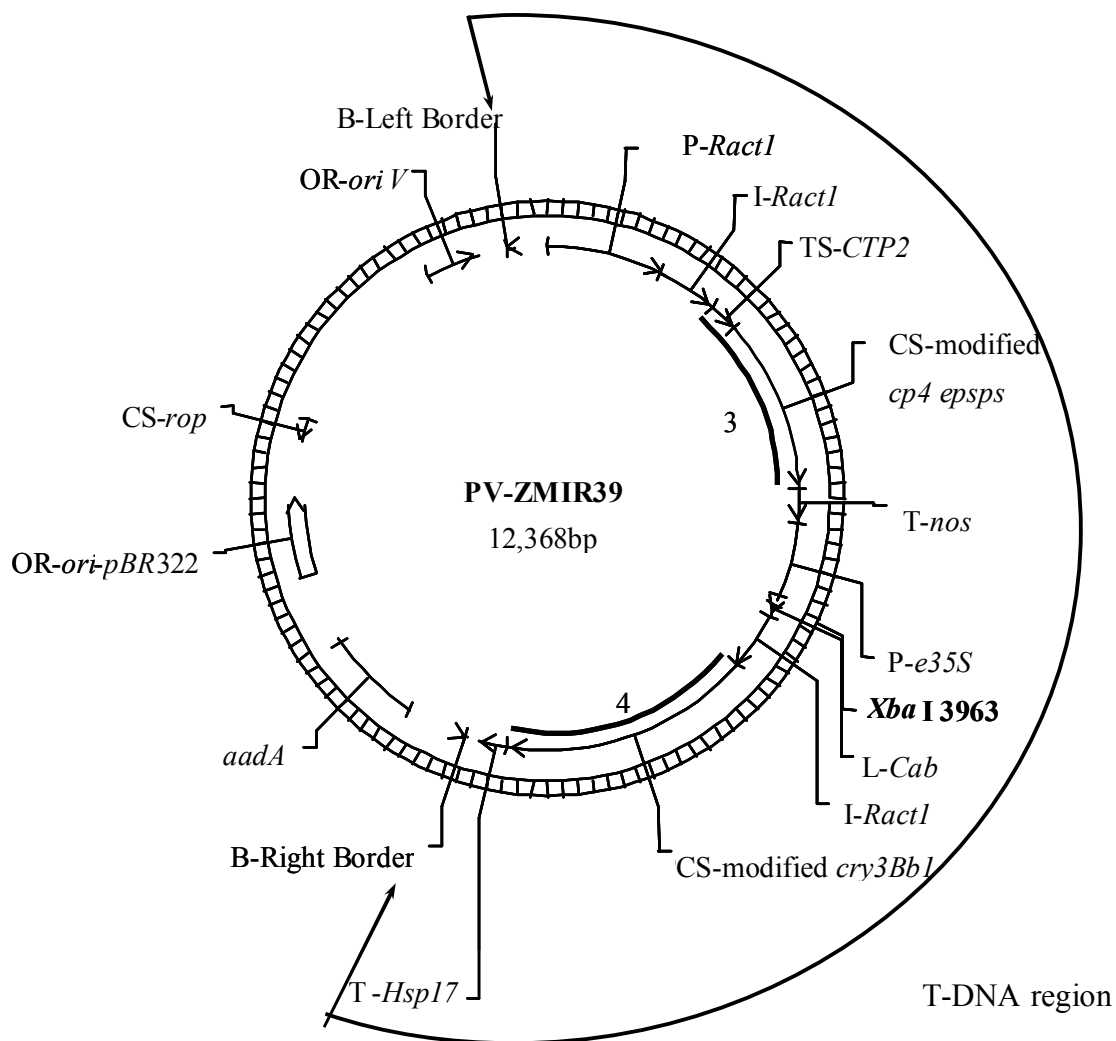
Table 1　Component elements of the plasmid PV-ZMAP595 that were used for the production of MON87460, and their origins and functions[4]

Component elements	Origin and function
Plasmid backbone region	
Intervening Sequence	Sequence used for DNA cloning
CS [Note 1]-*rop*	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *Escherichia coli* (Giza and Huang, 1989).
Intervening Sequence	Sequence used for DNA cloning
OR [Note 2]-*ori-pBR322*	The replication origin region isolated from pBR322. This confers autonomous replication ability in *E. coli* (Sutcliffe, 1979).
Intervening Sequence	Sequence used for DNA cloning
aadA	Bacterial promoter, coding sequence, and terminator for the 3"(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Fling et al., 1985). (GenBank accession X03043)
Intervening Sequence	Sequence used for DNA cloning
T-DNA region	
B [Note 3]-Right Border	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Depicker et al., 1982; Zambryski et al., 1982).
Intervening Sequence	Sequence used for DNA cloning
P [Note 4]-*Ract1*	Promoter and leader sequence of actin gene, derived from *Oryza sativa* (rice) (McElroy et al., 1990). Constantly induces transcription of the target gene in the entire tissue of plant.
I [Note 5]-*Ract1*	Intron of actin gene, derived from *O. sativa* (rice) (McElroy et al., 1991). Activates the expression of the target gene.
Intervening Sequence	Sequence used for DNA cloning

[4]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 1 Component elements of the plasmid PV-ZMAP595 that were used for the production of MON87460, and their origins and functions (continued)

T-DNA region (continued)	
CS-modified *cspB*	Gene encoding modified cold shock protein B (modified CSPB) derived from *Bacillus subtilis* (Willimsky et al., 1992). Detail is shown in I-2-(1)-2)-(b).
Intervening Sequence	Sequence used for DNA cloning
T [Note 6]-*tr7*	3' untranslated region of transcription 7 gene derived from *A. tumefaciens*. It induces polyadenylation (Dhaese et al., 1983).
Intervening Sequence	Sequence used for DNA cloning
loxP [Note 7]	Recombination site of bacteriophage P1. It functions in pairs. When Cre recombinase (DNA recombination enzyme) recognizes the two *lox P* sites, it removes the DNA region existing between them (Russell et al., 1992).
Intervening Sequence	Sequence used for DNA cloning
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (Odell et al., 1985). Involved in the constant expression of the target gene in the entire tissue of plant body.
Intervening Sequence	Sequence used for DNA cloning
CS-*nptII*	A gene derived from *E. coli* transposon Tn5 (Beck et al., 1982). Encodes neomycin phosphotransferase type II and confers resistance to neomycin and kanamycin on plants. Used as a marker to select the transgenic plant during the gene transfer (Fraley et al., 1983).
Intervening Sequence	Sequence used for DNA cloning
T-*nos*	3' untranslated region of nopaline synthase (nos) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Bevan et al., 1983).
Intervening Sequence	Sequence used for DNA cloning
loxP	Recombination site of bacteriophage P1. It functions in pairs. When Cre recombinase (DNA recombination enzyme) recognizes the two *lox P* sites, it removes the DNA region existing between them (Russell et al., 1992).

Table 1   Component elements of the plasmid PV-ZMAP595 that were used for the production of MON87460, and their origins and functions (continued)

T-DNA region (continued)	
Intervening Sequence	Sequence used for DNA cloning
B-Left Border	A DNA fragment containing the left border sequence (25 bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Barker et al., 1983).
Plasmid backbone region	
Intervening Sequence	Sequence used for DNA cloning
OR-*ori V*	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *Agrobacterium* (Stalker et al., 1981).
Intervening Sequence	Sequence used for DNA cloning

Note [1]CS – Coding Sequence

Note [2]OR – Origin of Replication

Note [3]B – Border

Note [4]P – Promoter

Note [5]I – Intron

Note [6]T – 3' untranslated transcriptional termination sequence and polyadenylation signal sequences

Note [7]*loxP* – The *nptII* gene is used as a marker to select transformants of the MON87460 strain. At the time of the MON87460 strain development, the European Food Safety Authority (EFSA, a safety evaluation authority for genetically modified crops in the EU) and other organizations were promoting the development and use of new selection methods to replace antibiotic resistance markers. In this regard, MON87460 was designed to remove *nptII* gene cassette by using *loxP* modified sites that are recognized by Cre recombinase. Later, EFSA announced that the risk of *nptII* gene in genetically modified crops affecting human and livestock health is very low (EFSA, 2004), and therefore the *nptII* gene cassette was not removed from the MON87460 strain.

Table 2   Component elements of plasmid PV-ZMIR245 that were used for the production of MON89034, and their origins and functions[5]

Component elements	Origin and function
T-DNA I region	
B [Note 1]-Right Border	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Depicker et al., 1982; Zambryski et al., 1982).
P [Note 2]-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Odell et al., 1985) promoter and 9bp leader sequence, containing double enhancer regions (Kay et al., 1987). Involved in the constant expression of the target gene in the entire tissue of plant body.
L [Note 3]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Lamppa et al., 1985).
I [Note 4]-*Ract1*	Rice (*O. sativa*) actin gene intron (McElroy et al., 1991). Activates the expression of the target gene.
CS [Note 5]-*cry1A.105*	A gene that encodes the Cry1A.105 protein. Detail is shown in I-2-(1)-2)-(b).
T [Note 6]-*Hsp17*	3' untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (McElwain and Spiker, 1989).
P-*FMV*	35S promoter derived from Figwort Mosaic Virus (Rogers, 2000). Involved in the constant expression of the target gene in the entire tissue of plant body.
I-*Hsp70*	First intron of maize heat shock protein 70 gene (Brown and Santino, 1995). Activates the expression of the target gene.
TS [Note 7]-*SSU-CTP*	Transit peptide of small subunit of ribulose 1,5-carboxylase diphosphate of maize, including the first intron sequence (Matsuoka et al., 1987). Transfers downstream-connected protein to plastid.
CS-modified *cry2Ab2*	A gene that encodes the modified Cry2Ab2 protein derived from *Bacillus thuringiensis* (Widner and Whitely, 1989). In order to add a restriction enzyme cutting site for cloning purposes, a single aspartic acid is transferred after the methionine at the N-terminal compared to the wild-type Cry2Ab2 protein.
T-*nos*	3' untranslated region of nopaline synthase (nos) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Bevan et al., 1983).
B-Left Border	A DNA fragment containing the left border sequence (25 bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Barker et al., 1983).

Table 2   Component elements of plasmid PV-ZMIR245 that were used for the production of MON89034 (continued)

Component elements	Origin and function
T-DNA II region	
B-Right Border	A DNA fragment containing the right border sequence (24 bp) of nopaline type T-DNA, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Depicker et al., 1982; Zambryski et al., 1982).
T-*nos*	3' untranslated region of nopaline synthase (nos) gene derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Bevan et al., 1983).
CS-*nptII*	A gene derived from *E. coli* transposon Tn5 (Beck et al., 1982). Encodes neomycin phosphotransferase type II and confers resistance to kanamycin on plants. Used as a marker to select the transgenic plant during the gene transfer (Fraley et al., 1983).
P-*35S*	35S promoter region of cauliflower mosaic virus (CaMV) (Odell et al., 1985). Involved in the constant expression of the target gene in the entire tissue of plant body.
B-Left Border	A DNA fragment containing the left border sequence (25 bp) derived from *A. tumefaciens*. It is the termination point of T-DNA transfer from *A. tumefaciens* to plant genome (Barker et al., 1983).
Plasmid backbone region	
OR [Note 8]-*ori V*	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *A. tumefaciens* (Stalker et al., 1981).
CS-*rop*	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Giza and Huang, 1989).
OR-*ori-pBR322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Sutcliffe, 1979).
aadA	Bacterial promoter, coding sequence, and terminator for the 3"(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Fling et al., 1985).

[Note 1] B – border
5   [Note 2] P – promoter
[Note 3] L – leader
[Note 4] I – intron
[Note 5] CS – coding sequence
[Note 6] T – transcript termination sequence
10   [Note 7] TS – targeting sequence
[Note 8] OR – Origin of Replication

Table 3 Component elements of the plasmid PV-ZM IR39 that were used for the production of MON88017, and their origins and functions[6]

Component elements	Origin and function
T-DNA region	
P [Note 1]-*Ract1*	Promoter region of actin 1 gene derived from rice (*O. sativa*). It makes target genes expressed (McElroy et al., 1990).
I [Note 2]-*Ract1*	Rice (*O. sativa*) actin gene intron (McElroy et al., 1991). Activates the expression of the target gene.
TS [Note 3]-*CTP2*	A sequence encoding the N-terminal chloroplast transit peptide of EPSPS protein in the *Arabidopsis thaliana epsps* gene (Klee et al., 1987). Transfers target proteins from cytoplasm to chloroplast.
CS [Note 4]-modified *cp4 epsps*	5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene derived from *Agrobacterium* CP4 strain (Padgette et al., 1996; Barry et al., 1997). To enhance the expression in plants, the second amino acid from the N-terminal in the wild-type CP4 EPSPS protein is modified to leucine, instead of serine.
T [Note 5]-*nos*	3' untranslated region of nopaline synthase (nos) derived from *A. tumefaciens* T-DNA. Terminates transcription of mRNA and induces polyadenylation (Bevan et al., 1983).
P-*e35S*	Cauliflower mosaic virus (CaMV) 35SRNA (Odell et al., 1985) promoter and 9bp leader sequence, containing double enhancer regions (Kay et al., 1987). Involved in the constant expression of the target gene in the entire tissue of plant body.
L [Note 6]-*Cab*	5'-terminal untranslated leader region of wheat chlorophyll a/b binding protein. Activates the expression of target gene (Lamppa et al., 1985).
I-*Ract1*	Rice (*O. sativa*) actin gene intron (McElroy et al., 1991). Activates the expression of target gene.
CS-modified *cry3Bb1*	The gene which encodes Cry3Bb1 protein of *B. thuringiensis* (Donovan et al., 1992). It differs from the modified Cry3Bb1 protein in MON863 at one site in the amino acid sequence: the 166th amino acid is aspartic acid in MON88017 whereas glycine in MON863.
T-*Hsp*17	3'-terminal untranslated region of wheat heat shock protein 17.3. Terminates transcription and induces polyadenylation (McElwain and Spiker, 1989).

Table 3 Component elements of the plasmid PV-ZM IR39 that were used for the production of MON88017 (continued)

Component elements	Origin and function
Plasmid backbone region	
B[Note 7]-Right Border	A DNA fragment containing the right border sequence of nopaline type T-DNA region, derived from *Agrobacterium tumefaciens*. The right border sequence is used as the initiation point of T-DNA transfer from *A. tumefaciens* to plant genome (Depicker et al., 1982; Zambryski et al., 1982).
aadA	Bacterial promoter, coding region, and terminator for the 3"(9)-O-nucleotidyltransferase, the aminoglycoside modified enzyme, derived from transposon Tn7. Confers resistance to spectinomycin or streptomycin (Fling et al., 1985).
OR[Note 8]-*ori-pBR322*	The replication origin region isolated from pBR322. Permits autonomous replication of vector in *E. coli* (Sutcliffe, 1979).
CS-*rop*	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Giza and Huang, 1989).
OR-*ori-V*	The replication origin region isolated from the broad-host range plasmid RK2. Permits autonomous replication of vector in *A. tumefaciens* (Stalker et al., 1981).
B-Left Border	Coding sequence for suppression of primer protein to maintain the number of copies of plasmid in *E. coli* (Giza and Huang, 1989).

[Note 1] P – promoter

5   [Note 2] I – intron

[Note 3] L – leader

[Note 4] TS – targeting sequence

[Note 5] CS – coding sequence

[Note 6] T – transcript termination sequence

10   [Note 7] B – border

[Note 8] OR – Origin of Replication

(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 that is known to possess any allergenicity

[Modified cold shock protein B (modified CSPB)]

The modified CSPB expressed in MON87460 is derived from a soil bacterium *Bacillus subtilus*.

Studies have shown that the modified CSPB binds to RNA and disrupts the secondary structure of the RNA. It is also known that the modified CSPB is localized in the nucleus and cytoplasm and is especially abundant in the meristem tissues. These findings are also recognized in proteins containing bacterial cold shock protein (CSP) and plant cold shock domain (CSD) (Fusaro et al., 2007; Sasaki et al., 2007; Chaikam and Karlson, 2008), suggesting that the modified CSPB acts on stress response pathways to confer drought tolerance on MON87460. Photosynthesis rate, stomatal conductance, and quantum efficiency at photosystem II of MON87460 were improved compared with the non-recombinant control maize under water-limited conditions in an environment-controlled greenhouse. The results of the field tests indicated that MON87460 efficiently distributes photosynthates to ears under water-limited conditions, which results in higher ear dry weight, grain count per ear, yield, and harvest index than the non-recombinant control maize. These findings together suggest that the modified CSPB functions as an RNA chaperone under water-limited conditions and hence, maintains the growth and development of MON87460, resulting in a reduction of yield loss.

The modified *cspB* gene has been reported to show tolerance to various environmental stresses (Castiglioni et al., 2008). However, the results of the studies for tolerance of matured plants to drought, low-temperature stress, high-temperature stress, and salt stress conclude that MON87460 obtains drought tolerance through expression of the modified CSPB but does not have tolerance to low temperatures, high temperatures, and salt.

The amino acid sequence of the modified CSPB is identical to that in the wild-type CSPB derived from *B. subtilis* (a bacterium widely distributed in soil), with the exception of a single amino acid change from leucine to valine at the second position from the N-terminal. The change was necessary to add restriction enzyme cutting site for cloning purposes.

[NPTII protein]

The *nptII* gene, an antibiotic tolerance marker gene introduced to select transformants, is derived from an *E. coli* transposon Tn5. The encoded NPT II protein inactivates aminoglycoside antibiotics (e.g., kanamycin) through

phosphorylation, conferring tolerance to these antibiotics on the transformant. As a result, application of kanamycin to media will enable selection of transformed cells (Beck et al., 1982; Nap et al., 1992; Shaw et al., 1993).

5   —Insecticidal proteins[7]—

[Cry1A.105 protein]

The Cry1A.105 protein, which is encoded by the *cry1A.105* gene used for the development of MON89034, is a chimeric Bt protein composed of Domains I and II of the Cry1Ab protein, Domain III of the Cry1F protein, and C-terminal Domain of the Cry1Ac protein, and it has been developed to enhance insecticidal activity against target pest insects by combining the different Domains of the Bt protein.

In order to investigate the insecticidal spectrum of the Cry1A.105 protein, the Cry1A.105 protein was added to artificial feeds, which were given to 15 different species of insects including 5 insects of the order Lepidoptera. As a result, the Cry1A.105 protein exhibited insecticidal activity against the larvae of Corn earworm (*Helicoverpa zea*) (MacRae et al., 2005), Black cutworm (*Agrotis ipsilon*) (MacRae, 2005), Fall armyworm (*Spodoptera frugiperda*) (MacRae, 2005), Southwestern corn borer (*Diatraea grandiosella*) (MacRae, 2005), and European corn borer (*Ostrinia nubilalis*) (MacRae et al., 2006a), which are the major pest insects of order Lepidoptera for maize, though it did not exhibit any insecticidal activity against honeybee (Richards, 2006a; Richards, 2006b), ladybug (Paradise, 2006a), and other beneficial insects except insects of the order Lepidoptera.

Based on the above results, it was confirmed that the Cry1A.105 protein exhibits selective insecticidal activity against only insects of the order Lepidoptera similarly to the Cry1Ab protein, the Cry1F protein, and the Cry1Ac protein, which are the component elements of Cry1A.105, and it does not possess any insecticidal activity against other species of insects.

30   **[Modified Cry2Ab2 protein]**

In order to investigate the insecticidal spectrum of the modified Cry2Ab2 protein,

---

[7]The Bt protein, produced by *Bacillus thuringiensis*, a gram-positive bacterium existing universally in soil, is known to bind to the specific receptors in the midgut epithelium of the target pest insects and form cation-selective pores in the cells and, as a result, inhibit the digestive process, thereby providing insecticidal activity (Hofmann et al., 1988; Slaney et al., 1992; Van Rie et al., 1990). In addition, several studies have shown that the Bt protein is composed of several Domains and what functions individual Domains possess. It has been revealed that the Bt protein is composed of Domains I, II, and III and C-terminal Domain, and Domain I is involved in formation of cation-selective pores to inhibit the digestive process, Domain II is involved in recognition of specific receptors, Domain III is involved in binding to receptors, and C-terminal Domain is involved in the crystal structure of the Bt protein (de Maagd et al., 2001; Masson et al., 2002).

the modified Cry2Ab2 protein was added to artificial feeds, which were given to 15 different species of insects including 4 insects of the order Lepidoptera. As a result, the modified Cry2Ab2 protein exhibited insecticidal activity against the larvae of Corn earworm (MacRae et al., 2006a), Fall armyworm (MacRae et al., 2006b), and European corn borer (MacRae et al., 2006a) among the 4 species of major pest insects of the order Lepidoptera used in the investigation and not against Black cutworm (MacRae et al., 2006b). Also, the modified Cry2Ab2 protein did not exhibit any insecticidal activity against honeybee (Maggi, 2000a; Maggi, 2000b), ladybug (Paradise, 2006b), and other beneficial insects except insects of the order Lepidoptera; therefore, it was confirmed that the modified Cry2Ab2 protein offers specific insecticidal activity against only insects of the order Lepidoptera and not against other species of insects.

The modified Cry2Ab2 protein, which is encoded by the modified *cry2Ab2* gene, has a single aspartic acid introduced after the methionine at the N-terminal compared to the wild-type Cry2Ab2 protein. The change was necessary to add restriction enzyme cutting site for cloning purposes.

[Modified Cry3Bb1 protein]

The modified Cry3Bb1 protein exhibits insecticidal activity against Corn rootworm (*Diabrotica* sp.), which is one of the major pest insects of the order Coleoptera to maize cultivation in the U.S. and eats and damages the roots of maize.

The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow, and the modified Cry3Bb1 protein exhibits insecticidal activity only against the Colorado potato beetle (*Leptinotarsa decimlineata*) and Corn rootworm, which are respectively classified into two genera *Leptinotarsa* and *Diabrotica* of the family Chrysomelidae, among the order Coleoptera. It was indicated by literature search that there was no report that related species of the same genera with these two insects have ever inhabited in Japan (Nakane et al., 1963).

The modified Cry3Bb1 protein has 6 amino acid substitutions compared to the wild-type Cry3Bb1 protein. One of the 6 substitutions was necessary to add a restriction enzyme cutting site for cloning purposes, while the other 5 were modified to enhance the insecticidal activity.

[Modified CP4 EPSPS protein]

Plants treated with glyphosate wither and die, since the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (E.C.2.5.1.19) is inhibited and the synthesis of aromatic amino acids essential for synthesis of proteins is interrupted. The modified *cp4 epsps* gene, the target gene of MON88017, produces the modified CP4 EPSPS protein that has high tolerance to the glyphosate herbicide.

16

The activity of the modified CP4 EPSPS protein produced by the modified *cp4 epsps* gene is not inhibited even under the presence of glyphosate, and thus the recombinant plants that express this protein have normal functions of shikimate synthesis pathway and can grow.

The modified *cp4 epsps* gene has the nucleotide sequence of the wild-type *cp4 epsps* gene modified to enhance the expression level in plants without changing the functional activity of the wild-type CP4 EPSPS protein, with only a single modification introduced to the amino acid sequence: the second amino acid from the N-terminal is modified to leucine, instead of serine.

In order to investigate whether the modified CSPB protein, the NPTII protein, the Cry1A.105 protein, the modified Cry2Ab2 protein, the modified Cry3Bb1 protein, and the modified CP4 EPSPS protein expressed in the parent lines share any functionally important amino acid sequences with known contact allergens, they were compared with the allergens in the database (including AD8, AD 2009, GenBank, EMBL, PIR, and SwissProt). Results showed they did not share structurally related sequences with known allergens.

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

[Modified CSPB]

Bacteria-derived CSPs including CSPB non-specifically bind to RNA and function as RNA chaperones (Herschlag, 1995; Jiang et al., 1997). As a result, CSP plays a role in maintaining translation under conditions where translation is inhibited otherwise (Graumann et al., 1997). CSPB does not have functions to directly induce transcription (Schindler et al., 1999; Weber et al., 2001), and there is no report showing that CSPB has enzymatic activity.

Thus, it is unlikely that the modified CSPB expressed in MON87460 acts as an enzyme to produce new metabolites.

[NPTII protein]

NPTII protein is an enzyme that catalyzes phosphorylation reaction of hydroxyl group in aminoglycosides contained in aminoglycoside antibiotics (Shaw et al., 1993). NPTII protein is reported to have association only in phosphorylation reactions in limited aminoglycoside antibiotics, including neomycin, kanamycin, paromomycin, ribostamycin, and butirosin (Price et al., 1974; Davies and Smith, 1978; Davies, 1986). A study of structural activities of NPTII protein demonstrated that the protein cannot use aminoglycoside antibiotics as a substrate if there are any minute changes (e.g., removal of the hydroxyl group, modification of the amino

17

group) in the aminoglycoside structure in the aminoglycoside antibiotics (Price et al., 1974).

Thus, it is unlikely that expression of NTPII protein in MON87460 results in production of new metabolites.

5

[Cry1A.105 protein, modified Cry2Ab2 protein, modified Cry3Bb1 protein]

The Cry1A.105 protein, the modified Cry2Ab2 protein, and the modified Cry3Bb1 protein are crystalline insecticidal proteins called the Bt proteins derived from *B. thuringiensis*. For the mechanism of insecticidal activity by the Bt proteins, many

10 studies have been made (OECD, 2007), and there is no report available to date that the Bt proteins possess any other functions. Therefore, it is considered unlikely that the Bt proteins possess any enzymatic activity and they would affect the metabolic system of the recipient organism.

15 **[Modified CP4 EPSPS protein]**

The EPSPS, identical functionally to the modified CP4 EPSPS protein, is an enzyme protein that catalyzes the shikimate pathway for the biosynthesis of aromatic amino acid. However, it is not a rate-determining enzyme in the pathway, and as such, it is not considered that enhanced EPSPS activity would increase the

20 concentration of aromatic amino acids, the end products of this pathway. In addition, EPSPS is known to react specifically with its substrates, phosphoenolpyruvic acid (PEP) and shikimate-3-phosphate (S3P) (Gruys et al., 1992). It is also known to react with shikimate, an analogue of SP3, but the reactivity with shikimate is only one–two millionth of the reactivity with S3P, when compared in terms of specificity constant

25 k_{cat}/K_m (Gruys et al., 1992). Thus, it is unlikely that shikimate reacts as the substrate of EPSPS in any living organism. Consequently, it is considered unlikely that the modified CP4 EPSPS protein would affect the metabolic system of the recipient organism.

30 (2) Information concerning vectors

1) Name and origin

The plasmid vectors used for the production of the parent lines are as follows.
35 MON87460: PV-ZMAP595 assembled from plasmids including the vector pBR322 (Sutcliffe, 1979) derived from *E. coli*

MON89034: PV-ZMIR245 assembled from plasmids including the vector pBR322 (Sutcliffe, 1979) derived from *E. coli*

MON88017: PV-ZMIR39 assembled from plasmids including the vector pBR322

(Sutcliffe, 1979) 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 vectors used for the production of parent lines is as follows.
MON87460: PV-ZMAP595; 9,379 bp
MON89034: PV-ZMIR245; 17,600 bp
MON88017: PV-ZMIR39; 12,368 bp

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

The following antibiotic resistance genes were used as selective markers.
MON87460: The *nptII* gene to confer resistance to aminoglycoside antibiotics including kanamycin and neomycin, and the *aadA* gene to confer resistance to spectinomycin and streptomycin
MON89034: The *aadA* gene to confer resistance to spectinomycin and streptomycin
MON88017: The *aadA* gene to confer resistance to spectinomycin and streptomycin
None of these antibiotic resistance genes have been transferred to the recipients, except for *nptII* gene that has been transferred to MON87460.

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

The infectivity of PV-ZMAP595, PV-ZMIR245, and PV-ZMIR39 is not known.

(3) Method of preparing living modified organisms

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

The component elements of the plasmid vectors PV-ZMAP595, PV-ZMIR245, and PV-ZMIR39 transferred to the recipient organisms for development of MON87460, MON89034, and MON88017, respectively, are listed in Table 1 to Table 3 (p. 7–13), and the positions of the component elements of the donor nucleic acid and sites cleaved by restriction enzymes in the vector are shown in Figure 1 to Figure 3 (p. 4–6).

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

Transferring nucleic acid into the recipient organism was based on the following method.

MON87460: The T-DNA region of the plasmid vector PV-ZMAP595 was transferred by the *Agrobacterium* method.

MON89034: The T-DNA I and T-DNA II regions of the plasmid vector PV-ZMIR245 were transferred by the *Agrobacterium* method. The T-DNA II region was subsequently removed by segregation.

MON88017: The T-DNA region of the plasmid vector PV-ZMIR39 was transferred by the *Agrobacterium* method.

3) Process of rearing of living modified organisms

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

Selection of transformed cells was made on the medium containing the followings.

MON87460: Paromomycin

MON89034: Paromomycin

MON88017: Glyphosate

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

In the development of MON87460, MON89034, and MON88017, *Agrobacterium* was removed by adding carbenicillin to the medium. Absence of remaining *Agrobacterium* in MON87460, MON89034, and MON88017 was confirmed by transferring MON87460, MON89034, or MON88017 to the carbenicillin-free medium and then observing that no colony of *Agrobacterium* is formed on the medium.

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

In the development of MON87460, transformed R_0 (regenerated plants) was bred with a conventional maize line LH59 and then self-crossed. The subsequent R_1 plants were screened for the expression of the modified CSPB, tolerance to kanamycin, and homozygosity of the inserted gene. Only the progeny of the screened plants were subjected to the analysis of the inserted genes and morphological assessments, and the results were used to select MON87460 line as the final commercial line.

In the development of MON89034, R_0 (regenerated plants) plants were bred with a conventional maize line LH172 to obtain LH172BC0F$_1$ plants. Based on the PCR method, the LH172BC0F$_1$ plants were subsequently selected for the presence of the T-DNA I region and the absence of T-DNA II region, and those containing the T-DNA II region were discarded. Regarding the selected individuals, further selection was carried out based on the analysis of transferred genes and the expression levels of the Cry1A.105 protein and the modified Cry2Ab2 protein. Tests in a climate chamber and greenhouse were then carried out, and actual pest insect resistance and agronomic characters (e.g., morphological and growth characteristics, yield-related characteristics, and sensitivity to disease or pest insects) were examined in outdoor field tests. MON89034 was selected upon comprehensive evaluation of these results.

In the development of MON88017, field experiments were carried out at a total of 169 field sites from 2000 to 2001. The strain for the final commercial cultivation was selected, and its environmental safety was evaluated.

[Process of rearing of MON87460 × MON89034 × MON88017]

This stack line maize is an F1 hybrid developed using inbred lines of MON87460, MON89034, and MON88017 as parents (Figure 4, p. 22).

5

[Confidential: Not made available or disclosed to unauthorized person]

10

15

20

25

Figure 4   Process of rearing of this stack line maize

(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

It has been confirmed that the transferred genes in MON87460, MON89034, and MON88017 exist on the maize genome.

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

[MON87460]

A Southern blot analysis of the transferred genes confirmed that one copy of the transferred gene region exists at one site in the genomic DNA of MON87460. It was also confirmed by Southern blot analysis on multiple generations that the transferred genes are inherited stably by offspring.

Analysis of the base sequence of MON87460 revealed that the right border region of the transferred gene region (2,816-3,172 bp of PV-ZMAP595) and the upstream 733 bp of the subsequent P-Ract1 region (3,205-3,937 bp of PV-ZMAP595) were missing.

[MON89034]

A Southern blot analysis of the transferred genes confirmed that one copy of the transferred gene region exists at one site in the genomic DNA of MON89034. It was also confirmed by Southern blot analysis on multiple generations that the transferred genes are inherited stably by offspring.

As a result of analysis on the nucleotide sequence of the transferred genes in MON89034, it was found that the 5'-terminal region of P-*e35S* controlling the expression of the *cry1A.105* gene and the neighboring right border region have been replaced by the left border region in the T-DNA II region and the 5'-terminal region of P-*35S* controlling the expression of the *nptII* gene due to homologous recombination. However, this homologous recombination did not take place in the protein encoding regions, even in the Cry1A.105 protein encoding region, the nearest open reading frame, which has been confirmed by the normal expression of the Cry1A.105 protein in individual tissues. Consequently, it was concluded that this homologous recombination could not cause formation of any new open reading frame.

[MON88017]

A Southern blot analysis of the transferred genes confirmed that one copy of the transferred gene region exists at one site in the genomic DNA of MON88017. It was also confirmed by Southern blot analysis on multiple generations that the transferred genes are inherited stably by offspring.

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

This item is not applicable because there was only one copy each in MON87460, MON89034, and MON88017.

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

The stability of expression was identified as follows:

MON87460: Confirming the expression of proteins by ELISA method

MON89034: Confirming the expression of proteins by Western blot analysis

MON88017: Confirming the expression of proteins by glufosinate herbicide spraying test and ELISA method

(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 nucleic acids transferred to MON87460, MON89034, and MON88017 do not contain any sequence that allows gene transmission, and thus, there is no possibility that the transferred nucleic acids could be transmitted to any other wild animals or plants through virus infection and/or other routes.

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

MON87460, MON89034, and MON88017 can be specifically detected by using the DNA sequences of the transferred genes and the nearby regions of the plant genome as primers. For detection and identification of this stack line maize, the above-mentioned method must be applied to each grain of maize seeds.

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

24

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

This stack line maize is given the traits as described below:

MON87460: Drought resistance due to the modified CSPB which is derived from the transferred gene

MON89034: Resistance to insects of the order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein which are derived from the transferred genes

MON88017: Tolerance to glyphosate herbicide due to the modified CP4 EPSPS protein and resistance to insects of the order Coleoptera due to the modified Cry3Bb1 protein, both of which are derived from the transferred genes

The modified CSPB expressed in MON87460 is derived from *B. subtilis*. Bacteria-derived CSPs including CSPB nonspecifically bind to RNA and function as RNA chaperons (Herschlag, 1995; Jiang et al., 1997). CSPB does not have functions to directly induce transcription (Schindler et al., 1999; Weber et al., 2001), and there is no report showing that CSPB has enzymatic activity. The modified CSPB expressed in MON87460 also functions as a RNA chaperon, and there is no report that the modified CSPB directly induces transcription or has any enzymatic activity.

The NPTII protein introduced into MON87460 as a selective marker has high substance specificity (Price et al., 1974; Davies and Smith, 1978; Davies, 1986).

The Cry1A.105 protein and the modified Cry2Ab2 protein expressed in MON89034 and the modified Cry3Bb1 protein expressed in MON88017 are crystalline insecticidal proteins derived from *B. thuringiensis* (Bt proteins). For the mechanism in which Bt proteins develop insecticidal activity, a number of studies have been made (OECD, 2007), and there is no report published to date that Bt proteins possess any other functions. Based on the above understanding, Bt proteins are considered not to offer any enzymatic activity. The Cry1A.105 protein and the modified Cry2Ab2 protein, which are Bt proteins, exhibit their insecticidal activities by binding to the specific receptors on the cell membranes of midgut epithelium of the insects of the order Lepidoptera such as European corn borer and Fall armyworm, and thus exhibit selective insecticidal activities against insects of the order Lepidoptera. To date, there is no report that combined use of different Bt proteins or Bt formulations showing insecticidal activities against insects of the same order resulted in broadening of their insecticidal spectrum to other orders. Therefore, it is

considered unlikely that the insecticidal activity would be broadened in this stack line maize.

The EPSPS protein expressed in MON88017, which is functionally identical to the modified CP4 EPSPS protein, is one of the enzymes in the shikimate pathway for aromatic amino acid biosynthesis. Since EPSPS has high substrate specificity and is not the rate-determining enzyme in the pathway of shikimate synthesis, the enhanced EPSPS activity due to the expression of the modified CP4 EPSPS protein would not increase the concentrations of aromatic amino acids, the end products of this pathway.

Based on the above results, it was considered that the modified CSPB protein, the NPTII protein, the Bt protein, and the modified CP4 EPSPS protein expressed in this stack line maize function independently from each other through different mechanisms.

As mentioned above, the modified CSPB expressed in this stack line maize does not directly induce transcription, has no enzymatic activity, and binds to RNA in a non-specific manner; these facts suggest that the modified CSPB protein would not enhance or reduce particular traits. Thus, it is unlikely that the modified CSPB protein expressed in this stack line maize would specifically affect the resistance to insects of the order Lepidoptera or Coleoptera or tolerance to glyphosate herbicide. Moreover, since the NPTII protein, the Cry1A.105 protein, the modified Cry2Ab2 protein, the modified Cry3Bb1 protein, and the modified CP4 EPSPS protein expressed in this stack line maize have no enzymatic activity or have high substrate specificity, they would not affect the metabolic pathway of plants. Thus, it is unlikely that the expressed proteins derived from individual parent lines have a new effect on the metabolic pathway of plants.

Based on the above understanding, it is unlikely that the expressed proteins derived from individual parent lines exhibit interaction with each other in this stack line maize.

To actually confirm that the expressed proteins derived from individual parent lines do not interact with each other, bioassays were carried out in 2006–2007, 2007, and 2007 to confirm the lack of differences between this stack line maize and MON87460 regarding yield, this stack line maize and MON89034 regarding resistance to the insects of order Lepidoptera, and this stack line maize and MON88017 regarding resistance to insects of the order Coleoptera and tolerance to glyphosate herbicide, respectively.

26

[Yield]

In order to check whether the yield remained unchanged by crossing MON87460, MON89034, and MON88017 using the traditional crossbreeding method, this stack line maize, MON87460, and non-recombinant control maize (HCL301 × LH59) were grown under two soil moisture conditions (well-watered and water-limited) to compare their yield. This stack line maize and MON87460 used in the assay shared the same genetic background (HCL301 × LH59).

In 2006–2007, four maize varieties, that is, this stack line maize, MON87460, non-recombinant control maize, and a commercial cultivar, were cultivated at four field locations (Calera de Tango [CT], Colina [CL], Lumbreras [LUM], and Quillota [QUI]) in Chile under two soil moisture conditions (well-watered and water-limited). Three replicates were made for each condition. The test plots designated for the well-watered conditions received proper levels of irrigation to achieve optimum yield, while irrigation in the test plots designated for the water-limited conditions was limited in the period from the later vegetative stage (V10) to the early reproductive stage (R2) to impose drought stress to the test plants (Table 4, p.29). Based on the report that moderate drought stress reduces maize yield by 15% or more every year (Barker et al., 2005), a minimum of 15% reduction in yield of the commercial cultivar grown under the water-limited conditions compared to that under the well-watered conditions was used as the index of the presence of drought stress. In addition, days to silking, ear height, and culm length were also examined, since decrease in these items are also reported to serve as drought stress indicators in maize (Campos et al., 2006). Results showed the commercial cultivar grown under the water-limited conditions in fields in CT, CL, and LUM exhibited 15% or more reduction in yield compared to that grown under the well-watered conditions and also suffered growth inhibition in terms of other morphological characteristics. Meanwhile, the commercial cultivar grown under the water-limited conditions in field in QUI did not show 15% or more reduction in yield compared to that grown under the well-watered conditions and did not suffer any growth inhibition in terms of other morphological characteristics (Table 5, p. 30). Therefore, results from the field in QUI were excluded from the statistical processing of the results of all fields. ANOVA by the linear mixed model was carried out using the results obtained from all lines cultivated in the three fields other than QUI, and the least mean square calculated by ANOVA was used to compare the results between the three populations, that is, this stack line maize, MON87460, and the non-recombinant control maize, by Fisher's LSD.

The yields of this stack line maize and MON87460 were 13.8 MT/ha and 13.9

MT/ha, respectively, under the well-watered conditions and were 6.5 MT/ha and 7.2 MT/ha, respectively, under the water-limited conditions (Table 6, p. 31). In either soil moisture condition, no statistically significant difference was observed in yield between this stack line maize and MON87460 ($p > 0.05$) (Table 7, p.31).

Under the water-limited conditions, a statistically significant difference was observed between MON87460 and the non-recombinant control maize ($p \leqq 0.05$), while no statistically significant difference was observed between this stack line maize and the non-recombinant control maize ($p > 0.05$). However, the yields of this stack line maize and MON87460 were both higher than that of the non-recombinant control, and no statistically significant difference was observed between this stack line maize and MON87460.

In addition, the yield of a stack line different from this stack line maize, which was developed by crossing MON87460, MON89034, and the glyphosate-tolerant NK603, showed a statistically significant difference from that of the non-recombinant control maize under the water-limited conditions. The only difference between the above-mentioned stack line and this stack line maize is that one of their parents is either NK603 or MON88017, and it is unlikely that this difference affects the drought resistance performance derived from MON87460.

Based on the above understanding, the absence of statistically significant difference between this stack line maize and the non-recombinant control maize under water-limited conditions was not attributed to interactions between the individual events.

Table 4　Cumulative irrigation volume during the cultivation period under well-watered and water-limited conditions (2006–2007, Chile)[8]

Month	Irrigation volume (inch)							
	CL[1]		CT[1]		LUM[1]		QUI[1]	
	Well-watered	Water-limited	Well-watered	Water-limited	Well-watered	Water-limited	Well-watered	Water-limited
December	0.9	0.9	2.8	3.8	2.8	2.8	1.9	1.9
January	10.3	10.3	9.4	9.4	9.4	9.4	10.3	10.3
February	8.5	4.7	8.5	2.8	8.5	2.8	8.5	1.9
March	9.4	5.6	10.3	6.6	10.3	6.6	9.4	5.6
April	2.8	2.8	2.8	2.8	1.9	2.8	1.9	1.9
May	0	0	0	0	0	0	0	0
Total irrigation volume	31.9	24.3	33.8	25.4	32.9	24.4	32.0	21.6

No rainfall was observed during the cultivation.

[1] Test fields in Chile: CL = Colina; CT = Calera de Tango; LUM = Lumbreras; QUI = Quillota

---

[8]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 5 Morphological and growth characteristics of a commercial variety under well-watered and water-limited conditions (2006–2007, Chile)[9]

Morphological and growth characteristics	CL[1]		CT[1]		LUM[1]		QUI[1]	
	Well-watered	Water-limited	Well-watered	Water-limited	Well-watered	Water-limited	Well-watered	Water-limited
Days to 50% silking	63.1	63.8	66.2	67.3	70.3	73.7*	67.7	67.1
Ear height (inch)	63.4	50.9*	55.0	46.0	50.4	41.8*	63.5	63.4
Culm length (inch)	110.7	79.7*	105.9	92.1	97.9	75.0*	112.0	112.8
Yield (bushel/acre)	185.5	82.3*	236.5	152.3*	213.9	94.4*	203.1	196.3
Loss of yield (%)		56%		36%		56%		3%

Statistical analysis was carried out by Fisher's LSD using the least mean square calculated by ANOVA by the linear mixed model (n = 3, 3 replicates/field) (significant at $p \leqq 0.05$).
* Indicates statistically significant differences between well-watered and water-limited conditions in each field ($p \leqq 0.05$).
[1] Test fields in Chile: CL = Colina; CT = Calera de Tango; LUM = Lumbreras; QUI = Quillota

---

[9]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 6　Mean values and standard errors of yields of this stack line maize, MON87460, and non-recombinant control maize (2006–2007, Chile) [1] [10]

Soil moisture condition	Test plant	Mean ± Standard error (bu/acre)	Mean ± Standard error (MT/ha)
Well-watered	This stack line maize	220.1±7.28	13.8±0.46
	MON87460	220.7± 7.87	13.9±0.49
	Non-recombinant control maize	220.0±10.19	13.8±0.64
Water-limited	This stack line maize	102.8±11.79	6.5±0.74
	MON87460	114.5±16.04	7.2±1.01
	Non-recombinant control maize	86.7±14.17	5.4±0.89

[1]Results from fields in CT, CL, and LUM

5

Table 7　Paired comparison of yields among this stack line maize, MON87460, and non-recombinant control maize[11]

Comparison	Soil moisture condition	P-value
This stack line maize vs. MON87460	Well-watered	0.961
	Water-limited	0.378
This stack line maize vs. non-recombinant control maize	Well-watered	0.998
	Water-limited	0.231
MON87460 vs. non-recombinant control maize	Well-watered	0.958
	Water-limited	0.048

Statistical analysis was carried out by Fisher's LSD using the least mean square calculated by ANOVA by the linear mixed model ($n = 9$, 3 replicates/field, 3 fields) (significant at $p \leqq 0.05$).

10

---

[10]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[11]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[Resistance to insects of the order Lepidoptera]

In order to check whether the resistance to the insects of the order Lepidoptera, a trait derived from MON89034, remained unchanged by crossing MON87460, MON89034, and MON88017 using the traditional crossbreeding method, feeding damage by Fall armyworm (*Spodoptera frugiperda*), a major pest insect of order Lepidoptera for maize, was evaluated to compare the resistance to insects of order Lepidoptera in this stack line maize, MON89034, MON88017, and non-recombinant control maize. In this assay, experiments were conducted twice under the same condition, and the data obtained from the two experiments were analyzed together. The assay was conducted using 12 plants each (3 plants/plot × 2 replicates × 2 experiments) of this stack line maize, MON89034, MON88017, and non-recombinant control maize (HCL301 × LH59). This stack line maize, MON89034, and MON88017 used in the assay shared the same genetic background (HCL301 × LH59).

At the 5th leaf stage, the first instar larvae of Fall armyworm were inoculated (25 larvae/plant). On the 7th day after inoculation of the Fall armyworm, the severity of feeding damage was determined based on the leaf damage rate (LDR) on a 10-step scale from 0 (no damage) to 9 (serious damage) proposed by Davis et al. (1992). On this scale, 0 indicates that no feeding damage was observed, while 9 indicates that the leaf was seriously damaged, having thin scratches or holes of various sizes. Due to the unevenness of variance, the data were subjected to rank transformation prior to statistical processing. The transformed data were used to perform ANOVA by the linear mixed model, and multiple comparison was performed by Fisher's protected LSD.

Results showed the severity of feeding damage by pest insects of order Lepidoptera in this stack line maize and MON89034 was 0.42 and 0.33, respectively (Table 8, p.33), and no statistically significant difference was observed ($p > 0.05$) (Table 9, p.33).

Table 8　Mean values and standard errors of the leaf damage rate (LDR) in this stack line maize, MON89034, MON88017, and non-recombinant control maize (SEM)[12]

Samples tested	Mean value ± standard error of leaf damage rate (LDR)[1]
This stack line maize	0.42 ± 0.08
MON89034	0.33 ± 0.14
MON88017	5.33 ± 0.68
Non-recombinant control maize	6.17 ± 0.29

[1] On the 7th day after inoculation, the leaf damage rate was determined on a scale proposed by Davis et al. (1992). On this scale, 0 indicates that no feeding damage was observed, while 9 indicates that the leaf was seriously damaged, having thin scratches or holes of various sizes.

Table 9　Paired comparison of leaf damage rate among this stack line maize, MON89034, MON88017, and non-recombinant control maize[13]

Comparison	P-value
This stack line maize vs. MON89034	0.758
This stack line maize vs. MON88017	0.002
This stack line maize vs. non-recombinant control maize	<0.001
MON89034 vs. non-recombinant control maize	<0.001
MON89034 vs. MON88017	<0.001
MON88017 vs. non-recombinant control maize	0.400

After carrying out ANOVA by the linear mixed mode, multiple comparison test was performed by Fisher's protected LSD (n = 4, 3 individuals/plot, 4 replicates) (significant at $p \leqq 0.05$).

---

[12]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[13]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[Resistance to insects of the order Coleoptera]

In order to check whether the resistance to the insects of the order Coleoptera, a trait derived from MON88017, remained unchanged by crossing MON87460, MON89034, and MON88017 using the traditional crossbreeding method, feeding damage by Western corn rootworm (*Diabrotica virgifera virgifera*), a major pest insect of order Coleoptera for maize, was evaluated to compare the resistance to insects of order Coleoptera of this stack line maize with those of MON88017, MON89034, and non-recombinant control maize (HCL301 × LH59). The experiments were conducted using 12 plants each (3 plants/plot × 4 replicates) of this stack line maize, MON88017, MON89034, and non-recombinant control maize. This stack line maize, MON88017, and MON89034 used in the assay shared the same genetic background (HCL301 × LH59).

At the 3rd leaf stage, eggs of Western corn rootworm were inoculated (810 eggs/pot). Four weeks after inoculation of Western corn rootworm, the severity of feeding damage was determined based on the nodal injury score (NIS) proposed by Oleson et al. (2005) on a scale from 0 to 3. On this scale, 0 indicates that no feeding damage was observed, while 3 indicates that feeding damage was observed in 3 or more nodes. In this method, the severity of feeding damage is indicated using values from 0.00 (no damage) to 3.00 (serious damage). The obtained data were used to perform ANOVA by the linear mixed mode, and multiple comparison was performed by Fisher's protected LSD.

Results showed the severity of feeding damage by pest insects of order Coleoptera in this stack line maize and MON88017 was 0.23 and 0.22, respectively (Table 10, p. 35), and no statistically significant difference was observed ($p > 0.05$) (Table 11, p.35).

Table 10 Mean values and standard errors of the nodal injury score (NIS) in this stack line maize, MON88017, MON89034, and non-recombinant control maize (SEM)[14]

Samples tested	Mean value ± standard error of nodal injury score (NIS)[1]
This stack line maize	0.23 ± 0.09
MON88017	0.22 ± 0.04
MON89034	1.54 ± 0.08
Non-recombinant control maize	1.44 ± 0.13

[1] The feeding damage of roots was evaluated using the nodal injury score proposed by Oleson et al. (2005) on a scale from 0 to 3. On this scale, 0 indicates that no feeding damage was observed, while 3 indicates that feeding damage was observed in 3 or more nodes.

Table 11 Paired comparison of nodal injury score among this stack line maize, MON88017, MON89034, and non-recombinant control maize[15]

Comparison	P-value
This stack line maize vs. MON88017	0.882
This stack line maize vs. MON89034	<0.001
This stack line maize vs. non-recombinant control maize	<0.001
MON89034 vs. non-recombinant control maize	0.407
MON89034 vs. MON88017	<0.001
MON88017 vs. non-recombinant control maize	<0.001

After carrying out ANOVA by the linear mixed mode, multiple comparison test was performed by Fisher's protected LSD (n = 4, 3 individuals/plot, 4 replicates) (significant at $p \leqq 0.05$).

---

[14]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[15]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

[Tolerance to glyphosate herbicide]

In order to check whether the tolerance to glyphosate herbicide, a trait derived from MON88017, remained unchanged by crossing MON87460, MON89034, and MON88017 using the traditional crossbreeding method, glyphosate herbicide spraying test was performed. The test was conducted using 5 plants each (1 plant/plot × 5 replicates) of this stack line maize, MON88017, and the non-recombinant control plant (HCL301 × LH59) that were cultivated in pots in a greenhouse. This stack line maize and MON88017 used in the test shared the same genetic background (HCL301 × LH59).

At the 4th leaf stage, glyphosate herbicide (Product name: Roundup WeatherMAX) was either not sprayed or sprayed at two different concentrations (0.84 kg acid equivalent[16] (a.e.)/ha (normal dosage) and 27 kg a.e./ha (32-time higher dosage than the normal dosage)). On the 7th and 14th days after spraying glyphosate, the severity of injury by spraying of the herbicide to plant bodies was evaluated based on a 11-step scale from 0 (no injury) to 10 (nearly the entire plant withered and died due to the injury). The obtained data were used to perform ANOVA by the linear mixed mode, and multiple comparison was performed by Fisher's protected LSD. The data obtained on the 14th day after spraying showed no variation and were thus excluded from the statistical processing.

From the statistical processing of the data obtained on the 7th day after spraying, no statistically significant difference was observed between this stack line maize and MON88017 regarding the severity of injury by spraying of the herbicide at either dosage at any time of observation ($p > 0.05$) (Table 12 to Table 13, p.37).

---

[16] Acid equivalent. Herbicide active ingredients are formulated either as the active ingredients themselves or in the form of their salts. In active ingredients formulated as their salts, the acid moieties serve as the active components, and the base moieties vary between formulations. If the dosage of a herbicide is expressed as the amount of the salt of the active ingredient formulated, the amount of the active component cannot be precisely compared between formulations with different base moieties. Thus, acid equivalent was used as the unit for the active component.

Table 12   Levels of herbicide injury to this stack line maize, MON88017, and non-recombinant control maize by spraying of glyphosate herbicide (normal dosage[1] and 32-times higher dosage[2])[3] [17]

	Normal dosage			32-times higher dosage than the normal dosage		
Observation day	This stack line maize	MON88017	Non-recombinant maize	This stack line maize	MON88017	Non-recombinant maize
7th day after spraying	0.0 ± 0.00	0.0 ± 0.00	8.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	8.2 ± 0.20
14th day after spraying	0.0 ± 0.00	0.0 ± 0.00	10.0 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	10.0 ± 0.00

The values represent mean value ± standard error.
[1] Normal dosage is 0.84 kg a.e./ha.
[2] 32-times higher dosage is 27 kg a.e./ha.
[3] Evaluation was made on a scale from 0 (no injury) to 10 (nearly the entire plant withered and died due to the injury).

Table 13   Paired comparison of levels of herbicide injury to this stack line maize, MON88017, and non-recombinant control maize on 7th day after spraying[18]

Dosage	Comparison	P-value
Normal dosage	This stack line maize vs. MON 88017	1.000
	This stack line maize vs. non-recombinant control maize	<0.001
	MON 88017 vs. non-recombinant control maize	<0.001
32-times higher dosage	This stack line maize vs. MON 88017	1.000
	This stack line maize vs. non-recombinant control maize	<0.001
	MON 88017 vs. non-recombinant control maize	<0.001

After carrying out ANOVA by the linear mixed mode, multiple comparison test was performed by Fisher's protected LSD (n = 5, 1 individual/plot, 5 replicates) (significant at $p \leqq 0.05$).

---

[17] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.
[18] All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

Based on the above findings, it was concluded that the individual proteins expressed in the relevant parent lines do not interact with each other and that the traits acquired through the transferred genes remain unchanged in this stack line maize.

Consequently, with regard to the differences in physiological or ecological characteristics between this stack line maize and maize, the taxonomic species to which the recipient organism belongs, the evaluation was conducted based on the results of individual examinations of the parent lines (MON87460, MON89034, and MON88017) at the times of their approval.

(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[19]

The evaluation results of adverse effect on biological diversity in Japan of the parent lines (MON87460, MON89034, and MON88017) of this stack line maize are provided below.

i.   Test conducted under typical cultivation conditions with irrigation

a   Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between the parent lines (MON87460, MON89034, and MON88017) and their respective non-recombinant controls regarding the items listed in Table 14 (p. 39).

In the results, a difference was observed between MON87460 and its non-recombinant control maize in the flowering period (Table 3 of Annex 1, p. 12). In addition, a statistically significant difference was observed between MON88017 and its non-recombinant control maize in culm length (Table 2-1 to Table 2-2 of Annex 4, p. 30–31). No statistically significant difference was observed between MON89034 and its non-recombinant control maize (Table 2 of Annex 3, p. 8).

---

[19]All the rights pertinent to the information in a-g under i, a-b under ii, and a-b under iii below and the responsibility for the contents rest upon Monsanto Japan Limited.

Table 14   Investigational results of morphological and growth characteristics in MON87460, MON89034, and MON88017[20]

	MON87460	MON89034	MON88017
Uniformity of germination	○	○	○
Number of plants germinated	－	○	－
Germination rate	○	○	○
Time to tasseling	○	○	○
Time to silking	○	○	○
Start of flowering	○	－	－
Flowering period	○*	○	－
Culm length	○	○	○*
Culm diameter	－	○	－
Plant shape	○	○	○
Tiller number	○	○	○
Ear height	○	○	○
Maturation time	○	○	○
Weight of above-ground parts at harvest	○	○	○
Grain shape	○	○	○
Grain color	○	○	○

○ : Examined

－ : Not examined

* A statistically significant difference or a difference was observed.

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

Cold tolerance of MON87460 was evaluated under four different temperature conditions (optimum temperature: 30ºC/22ºC (day/night), mildly low temperature: 20ºC/15ºC, moderately low temperature: 15ºC/10ºC, severely low temperature: 4ºC/4ºC). Results showed MON87460 withered due to the low temperature treatment at the early stage of growth similarly to its non-recombinant control (Table 2 of Annex 2, p. 17). Under the optimum temperature condition, statistically significant differences were observed in growth stages and dry weights at the 4th and 8th day after transfer to a climate chamber (Table 2 of Annex 2, p. 17).

MON89034 and MON88017 both withered and died due to the low temperature treatment at the early stage of growth similarly to their non-recombinant control plants (Figure 6-2 of Annex 3, p. 14; Table 4 of Annex 4, p. 42).

---

[20]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

c   Wintering ability and summer survival of the mature plant

Maize is a summer type annual plant, and after ripening, it usually dies out in winter, and it does not re-grow and propagate vegetatively, or produce seeds. Actually, at the end of isolated field tests, start of withering and death after ripening was observed.

d   Fertility and size of the pollen

MON87460, MON89034, and MON88017 all exhibited high fertility of pollen, similarly to their non-recombinant control plants, and no difference was observed regarding the shape and size of pollens (Figure 6 of Annex 1, p. 15; Figure 8-1 to Figure 8-2 of Annex 3, p. 19; Table 3 and Photo 4-1 to Photo 4-2 of Annex 4, p. 38–40).

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

Regarding seed production, comparison was conducted between MON87460, MON89034, and MON88017 and their non-recombinant control plants for the characteristics listed in Table 15 (p. 40).

Results showed statistically significant differences between MON89034 and its non-recombinant control plant regarding ear diameter and grain number per ear, and between MON88017 and its non-recombinant control plant regarding ear diameter (Table 2, p. 8 and Table 6, p. 20 of Annex 3; Table 2-1 to Table 2-2 of Annex 4, p. 30–31). Meanwhile, no statistically significant difference was observed between MON87460 and its non-recombinant control plant (Table 4 of Annex 1, p. 17).

Table 15   Investigational results of seed production in MON87460, MON89034, and MON88017[21]

	MON87460	MON89034	MON88017
Number of ears	○	○	○
Number of effective ears/Total number of effective ears	○	○	—
Ear length	○	○	○
Ear diameter	○	○*	○*
Number of grains	○	○*	○

---

[21]All the rights pertinent to the information in the table above and the responsibility for the contents rest upon Monsanto Japan Limited.

per ear			
Row number per ear	○	○	○
Grain number per row	－	○	○
100-kernel weight	○	○	○

○ : Examined

－ : Not examined

* A statistically significant difference was observed

Regarding shedding habit of the seed, it was not observed in natural environment, since the ears of MON87460, MON89034, and MON88017 and their non-recombinant control plants were covered with bracts at the time of harvesting.

Regarding germination rate, in order to identify the dormancy of harvested seeds, germination test was carried out for the seeds harvested from MON87460, MON89034, and MON88017 and their non-transgenic control plants. Results showed no difference, and no dormancy of the seed was identified (Table 4 and Table 5 of Annex 1, p. 17; Table 3 to Table 4 of Annex 3, p. 16; Table 4 of Annex 4, p. 42).

f   Crossability

Crossability test was not performed for the parent lines MON87460, MON89034, and MON88017, since no wild relatives that can be crossed grow in Japan.

g   Productivity of harmful substances

Soil microflora tests, plow-in tests, and succeeding crop tests were performed for MON87460, MON89034, and MON88017. Statistically significant difference was observed in the fresh weight of radishes in the plow-in tests for MON88017 (Table 5 to Table 7 of Annex 4, p. 44–45, p. 47).

ii.   Test conducted under typical cultivation conditions without irrigation

In order to evaluate the drought tolerance of MON87460 under environmental conditions in Japan, MON87460 and its non-recombinant control maize were cultivated under normal conditions without irrigation, and comparison was made regarding (a) morphological and growth characteristics and (b) production and shedding habit of the seed.

41

a   Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between MON87460 and the non-recombinant control maize regarding 14 items (uniformity of germination [date], germination rate [%], time to tasseling [date], time to silking [date], start of flowering [date], flowering period [date], culm length [cm], ear height [cm], tiller number, plant shape, maturation time [rate], weight of above-ground parts at harvest [kg], grain shape, and grain color). Among the statistically analyzed items (germination rate, culm length, ear height, tiller number, and weight of above-ground parts at harvest), a statistically significant difference was observed between MON87460 and its non-recombinant control plant in ear height (Table 9 of Annex 1, p. 25). Among the items not statistically analyzed (uniformity of germination, time to tasseling, time to silking, start of flowering, flowering period, plant shape, maturation time, grain shape, and grain color), a difference was observed in start of flowering between MON87460 and the non-recombinant control maize (Table 9 of Annex 1, p. 25).

b   Production and shedding habit of the seed

Regarding seed production, comparison was made between MON87460 and its non-recombinant control plant for the following items: total number of effective ears, ear length (cm), ear diameter (cm), row number per ear, number of grains per ear, and 100-kernel weight (g). Results showed statistically significant differences between MON87460 and its non-recombinant control plant regarding total number of effective ear, ear length and number of grains per ear (Table 10 of Annex 1, p. 28).

Regarding shedding habit of the seed, it was not observed in natural environment, since the ears of MON87460 and its non-recombinant control plant were covered with bracts at the time of harvest.

iii.   Test conducted with no agronomical treatment

In order to evaluate the self-seeding ability of MON87460, MON87460, and its non-recombinant control plant were cultivated without any agronomical treatment such as irrigation, fertilization, disease, and pest insect control or weeding, and comparison was made regarding (a) morphological and growth characteristics and (b) production and shedding habit of the seed. In response to abundance of weeds, nutrient deficiency due to the lack of fertilization, drought stress, feeding damage by

42

pest insects, and other types of stresses, the survival rate was extremely low; 18 out of 33 MON87460 plants tested and 26 out of 33 non-recombinant control plants tested withered and died (Table 13 of Annex 1, p. 35). Moreover, the total numbers of effective ears recovered from 33 MON87460 plants and 33 non-recombinant control plants were 9 and 2, respectively (Table 13 of Annex 1, p. 35). Because of the low values, the test results were not statistically processed.

a   Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between MON87460 and the non-recombinant control maize regarding five items (uniformity of germination [date], germination rate [%], culm length [cm], maturation time [date], and weight of above-ground parts at harvest [kg]). The results suggested that there is no difference between MON87460 and the non-recombinant control maize (Table 14 of Annex 1, p. 36).

b   Production and shedding habit of the seed

Regarding seed production, comparison was made between MON87460 and its non-recombinant control plant for the following items: total number of effective ears, ear length (cm), ear diameter (cm), row number per ear, and number of grains per ear. Results showed differences between MON87460 and its non-recombinant control plant regarding total number of effective ears and number of grains per ear (Table 15 of Annex 1, p. 38).

Regarding shedding habit of the seed, it was not observed in natural environment, since the ears of MON87460 and its non-recombinant control plant were covered with bracts at the time of harvest.

43

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 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
Maize tolerant to drought, resistant to Lepidoptera and Coleoptera and tolerant to glyphosate herbicide (including the progeny lines isolated from the maize lines, MON87460, MON89034, and MON88017, that contain a combination of any of the transferred genes in the individual maize lines [except those already granted an approval regarding Type 1 Use Regulation]) (hereinafter referred to as this stack line maize) was developed by crossing the following three recombinant maize lines, using the traditional crossbreeding method:

(a) drought-tolerant maize (MON87460) due to the modified *cspB* gene that encodes the modified CSPB protein (modified cold shock protein B) and the *nptII* gene that encodes the NPTII protein (neomycin phosphotransferase type II);

(b) maize resistant to Lepidoptera (MON89034) due to the *cry1A.105* gene that encodes the Cry1A.105 protein and the modified *cry2Ab2* gene that encodes the modified Cry2Ab2 protein;

(c) maize tolerant to glyphosate herbicide and resistant to Coleoptera (MON88017) due to the modified *cp4 epsps* gene that encodes the modified CP4 EPSPS protein (5-enol-pyruvylshikimate-3-phosphate synthase) and the modified *cry3Bb1* gene that encodes the modified Cry3Bb1 protein.

These three 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 used in line with Type 1 Use described in the application for this stack line maize.

The modified CSPB protein is known to disrupt the secondary structure of the RNA, but it does not induce transcription, has no enzymatic activity, and binds to RNA in a non-specific manner. These facts suggest that the modified CSPB protein expressed in this stack line maize would not specifically affect the resistance to insects of the order Lepidoptera or Coleoptera or the tolerance to glyphosate herbicide. Moreover, since the NPTII protein and the modified CP4 EPSPS protein expressed in this stack line maize have high substrate specificity, and since the Cry1A.105 protein, the modified Cry2Ab2 protein, and the modified Cry3Bb1 protein have no enzymatic activity, they would not affect the metabolic pathway of plants. Thus, it is unlikely that these proteins expressed in this stack line maize would interact with each other to change the metabolic pathway of the recipient and result

in production of new metabolites.

In addition, based on the bioassays, the tolerance to drought, the resistance to Lepidoptera and Coleoptera and the tolerance to glyphosate herbicide in this stack line maize were found at similar levels as exhibited by the individual parent lines.

Consequently, it is considered unlikely that the proteins expressed in this stack line derived from individual parent lines would cause functional interaction in the plant body of this stack maize line, and it is considered unlikely that notable changes in traits have occurred in this stack maize line except for the traits that it received from the parent lines.

1) Competitiveness

Maize, 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.

From investigation for various characteristics referring to competitiveness of MON87460, MON89034, and MON88017, the parent lines of this stack line maize, statistically significant differences were observed between the parent lines of this stack line maize and the non-recombinant maize in some of the items examined. However, the differences were judged not to be so large as enhancing the competitiveness of this stack maize line.

This stack line maize is given the tolerance to drought due to the modified CSPB protein. However, it is considered that this trait would not enhance the competitiveness of this stack line maize compared to conventional maize cultivars. In addition, this stack line maize is given the traits to be resistant to insects of the order Lepidoptera due to the Cry1A.105 protein and the modified Cry2Ab2 protein and to be resistant to insects of the order Coleoptera due to the modified Cry3Bb1 protein. However, it is not generally considered that insect damage by Lepidopteran and Coleopteran insects is the major factor to inhibit the growth of maize under the natural environment in Japan. Moreover, this stack line maize is also given the tolerance to glyphosate herbicide due to the modified CP4 EPSPS protein. However, it is considered unlikely that, in the natural environment less expected to suffer spraying of herbicides, the tolerance to glyphosate herbicide would increase the competitiveness of this stack line maize.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: Regarding this stack line maize, there are no specific wild animals and wild plants that are possibly affected by this stack line maize, and it would pose no risk of Adverse Effect 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.

45

It has been confirmed that the proteins expressed in this stack maize line (the modified CSPB protein, the Cry1A.105 protein, the modified Cry2Ab2 protein, the modified Cry3Bb1 protein, and the modified CP4 EPSPS protein) do not have any homology with any of the known allergens. In addition, for examining the ability of the parent lines of this stack line maize to produce any harmful substances (the substances secreted from the roots that can affect other plants and microorganisms in soil, the substances existing in the plant body that can affect other plants after dying), plow-in tests, succeeding crop tests, and the soil microflora tests were conducted. In all tests, there was no difference suggesting increase in productivity of harmful substances. Consequently, it is considered unlikely that this stack line maize possesses productivity of unintended harmful substances.

The Cry1A.105 protein and the modified Cry2Ab2 protein expressed in this stack line maize exhibit insecticidal activity against insects of the order Lepidoptera, and the modified Cry3Bb1 protein exhibits insecticidal activity against insects of the order Coleoptera. Therefore, the Lepidopteran and Coleopteran insects were specified as wild animals and wild plants that are possibly affected by this recombinant maize. Then, there is a concern about possible effects on the specified species of Lepidopteran and Coleopteran insects that could directly eat this stack line maize or eat pollens dispersed from this stack maize line and attached to their food plants. However, it is considered unlikely that the Lepidopteran and Coleopteran insects inhabit locally near the fields for cultivation of this stack line maize and thus, it is considered very unlikely that they could be affected in the level of population.

Based on the above understanding, it was judged that the following conclusion made by the applicant is valid: This stack line maize would pose no risk of Adverse Effect on Biological Diversity that is attributable to productivity of harmful substances.

3) Crossability

In the Japanese natural environment, there are no wild plants that can cross with maize. Therefore, it was judged that there are no specific wild plants that are possibly affected by this stack line maize, and that the use of such maize poses no risk of Adverse Effect 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 stack line 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.

References

Barker, R.F., K.B. Idler, D.V. Thompson and J.D. Kemp. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Molecular Biology 2: 335–350.

Barker, T., H. Campos, M. Cooper, D. Dolan, G. Edmeades, J. Habben, J. Schussler, D. Wright and C. Zinselmeier. 2005. Improving Drought Tolerance in Maize. Pages 173-253 in Plant Breeding Reviews. Volume 25. J. Janick (ed.). John Wiley and Sons, Inc., Hoboken, New Jersey.

Barry, G.F., G.M. Kishore, S.R. Padgette and W.C. Stallings. 1997. Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. Patent 5,633,435, U.S. Patent Office, Washington, D.C.

Beck, E., G. Ludwig, E.A. Auerswald, B. Reiss and H. Schaller. 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn*5*. Gene 19: 327–336.

Bevan, M., W.M. Barnes and M.-D. Chilton. 1983. Structure and transcription of the nopaline synthase gene region of T- DNA. Nucleic Acids Research 11: 369–385.

Brown, S.M. and C.G. Santino. 1995. Enhanced expression in plants. Patent 5,424,412, U.S. Patent Office, Washington, D.C.

Campos, H., M. Cooper, G.O. Edmeades, C. Löffler, J.R. Schussler and M. Ibañez. 2006. Changes in drought tolerance in maize associated with fifty years of breeding for yield in the U.S. Corn Belt. Maydica 51: 369–381.

Castiglioni, P., D. Warner, R.J. Bensen, D.C. Anstrom, J. Harrison, M. Stoecker, M. Abad, G. Kumar, S. Salvador, R. D'Ordine, S. Navarro, S. Back, M. Fernandes, J. Targolli, S. Dasgupta, C. Bonin, M.H. Luethy and J.E. Heard. 2008. Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. Plant Physiology 147: 446–455.

Chaikam, V. and D. Karlson. 2008. Functional characterization of two cold shock domain proteins from *Oryza sativa*. Plant, Cell and Environment 31: 995–1006.

Davies, J. 1986. Aminoglycoside-aminocyclitol antibiotics and their modifying

enzymes. Pages 474–489 in Antibiotics in Laboratory Medicine. V. Lorian (ed.). Williams and Wilkins, Baltimore, Maryland.

Davies, J. and D.I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents.
5   Annual Review of Microbiology 32: 469–518.

Davis, F.M., S.S. Ng and W.P. Williams. 1992. Visual rating scales for screening whorl-stage corn for resistance to fall armyworm. Mississippi Agricultural and Forestry Experiment Station Technical Bulletin 186: 1–9.
10

de Maagd, R.A., A. Bravo and N. Crickmore. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends in Genetics 17: 193–199.

15 Depicker, A., S. Stachel, P. Dhaese, P. Zambryski and H.M. Goodman. 1982. Nopaline synthase: Transcript mapping and DNA sequence. Journal of Molecular and Applied Genetics 1: 561–573.

Dhaese, P., H. De Greve, J. Gielen, J. Seurinck, M. Van Montagu and J. Schell. 1983.
20   Identification of sequences involved in polyadenylation of higher plant nuclear transcripts using *Agrobacterium* T-DNA genes as models. The EMBO Journal 2: 419–426.

Donovan, W.P., M.J. Rupar, A.C. Slaney, T. Malvar, M.C. Gawron-Burke and T.B.
25   Johnson. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to *Coleoptera* species. Applied and Environmental Microbiology 58: 3921–3927.

EFSA. 2004. Opinion of the Scientific Panel on genetically modified organisms on the
30   use of antibiotic resistance genes as marker genes in genetically modified plants (Question N° EFSA-Q-2003-109). The EFSA Journal 48: 1–18. http://www.efsa.europa.eu/EFSA/Scientific_Opinion/opinion_gmo_05_en1,0.pd f.

35 FAOSTAT. 2010. Production, crops, maize and maize green. Area harvested, 2009. Food and Agriculture Organization of the United Nations, Rome, Italy. http://faostat.fao.org/default.aspx [Accessed October 16, 2010].

Fling, M.E., J. Kopf and C. Richards. 1985. Nucleotide sequence of the transposon Tn*7*

gene encoding an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase. Nucleic Acids Research 13: 7095–7106.

Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R. Galluppi, S.B. Goldberg, N.L. Hoffman and S.C. Woo. 1983. Expression of bacterial genes in plant cells. Proceedings of the National Academy of Sciences of the United States of America 80: 4803–4807.

Fusaro, A.F., S.N. Bocca, R.L.B. Ramos, R.M. Barrôco, C. Magioli, V.C. Jorge, T.C. Coutinho, C.M. Rangel-Lima, R. De Rycke, D. Inzé, G. Engler and G. Sachetto-Martins. 2007. AtGRP2, a cold-induced nucleo-cytoplasmic RNA-binding protein, has a role in flower and seed development. Planta 225: 1339–1351.

Giza, P.E. and R.C.C. Huang. 1989. A self-inducing runaway-replication plasmid expression system utilizing the Rop protein. Gene 78: 73–84.

Graumann, P., T.M. Wendrich, M.H.W. Weber, K. Schröder and M.A. Marahiel. 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. Molecular Microbiology 25: 741–756.

Gruys, K.J., M.C. Walker and J.A. Sikorski. 1992. Substrate synergism and the steady-state kinetic reaction mechanism for EPSP synthase from *Escherichia coli*. Biochemistry 31: 5534–5544.

Herschlag, D. 1995. RNA chaperones and the RNA folding problem. The Journal of Biological Chemistry 270: 20871–20874.

Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansens and H. Van Mellaert. 1988. Specificity of *Bacillus thuringiensis* d-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Biochemistry 85: 7844–7848.

Jiang, W., Y. Hou and M. Inouye. 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. The Journal of Biological Chemistry 272: 196–202.

Kay, R., A. Chan, M. Daly and J. McPherson. 1987. Duplication of CaMV 35*S* promoter sequences creates a strong enhancer for plant genes. Science 236: 1299–1302.

Klee, H.J., Y.M. Muskopf and C.S. Gasser. 1987. Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: Sequence analysis and manipulation to obtain glyphosate-tolerant plants. Molecular and General Genetics 210: 437–442.

Lamppa, G.K., G. Morelli and N.-H. Chua. 1985. Structure and developmental regulation of a wheat gene encoding the major chlorophyll a/b-binding polypeptide. Molecular and Cellular Biology 5: 1370–1378.

MacRae, T.C. 2005. Insecticidal activity of the Cry1A.105 *Bacillus thuringiensis* protein against five lepidopteran pests of corn. Monsanto Technical Report MSL-20056. St Louis, Missouri.

MacRae, T.C., C.R. Brown and S.L. Levine. 2005. Evaluation of the potential for interactions between the *Bacillus thuringiensis* proteins Cry1A.105 and Cry2Ab2. Monsanto Technical Report MSL-19859. St Louis, Missouri.

MacRae, T.C., C.R. Brown and S.L. Levine. 2006a. Evaluation of potential for interactions between the *Bacillus thuringiensis* proteins Cry1A.105, Cry2Ab2, and Cry3Bb1. Monsanto Technical Report MSL-20270. St. Louis, Missouri.

MacRae, T.C., C.R. Brown and S.L. Levine. 2006b. Spectrum of insecticidal activity of *Bacillus thuringiensis* Cry2Ab2 protein. Monsanto Technical Report MSL-20229. St. Louis, Missouri.

Maggi, V.L. 2000a. Evaluation of the dietary effect(s) of insect protection protein 2 on adult honey bees (*Apis mellifera* L.). Monsanto Technical Report MSL-16176. St Louis, Missouri.

Maggi, V.L. 2000b. Evaluation of the dietary effect(s) of insect protection protein 2 on honey bee larvae. Monsanto Technical Report MSL-16175. St Louis, Missouri.

Masson, L., B.E. Tabashnik, A. Mazza, G. Préfontaine, L. Potvin, R. Brousseau and J.-L. Schwartz. 2002. Mutagenic analysis of a conserved region of domain III in the Cry1Ac toxin of *Bacillus thuringiensis*. Applied and Environmental

Microbiology 68: 194–200.

Matsuoka, M., Y. Kano-Murakami, Y. Tanaka, Y. Ozeki and N. Yamamoto. 1987. Nucleotide sequence of cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase from maize. Journal of Biochemistry 102: 673–676.

McElroy, D., W. Zhang, J. Cao and R. Wu. 1990. Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell 2: 163–171.

McElroy, D., A.D. Blowers, B. Jenes and R. Wu. 1991. Construction of expression vectors based on the rice actin 1 (*Act1*) 5' region for use in monocot transformation. Molecular and General Genetics 231: 150–160.

McElwain, E.F. and S. Spiker. 1989. A wheat cDNA clone which is homologous to the 17 kd heat-shock protein gene family of soybean. Nucleic Acids Research 17: 1764.

Nap, J.-P., J. Bijvoet and W.J. Stiekema. 1992. Biosafety of kanamycin-resistant transgenic plants. Transgenic Research 1: 239–249.

Odell, J.T., F. Nagy and N.-H. Chua. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

OECD. 2003. Consensus document on the biology of *Zea mays* subsp. *mays* (Maize). ENV/JM/MONO(2003)11. Organisation of Economic Co-operation and Development, Paris, France.

OECD. 2007. Consensus document on safety information on transgenic plants expressing *Bacillus thuringiensis*-derived insect control proteins. ENV/JM/MONO(2007)14. Series on Harmonisation of Regulatory Oversight in Biotechnology No. 42. Organisation of Economic Co-operation and Development, Paris, France.

Oleson, J.D., Y.-L. Park, T.M. Nowatzki and J.J. Tollefson. 2005. Node-injury scale to evaluate root injury by corn rootworms (Coleoptera: Chrysomelidae). Journal of Economic Entomology 98: 1–8.

Padgette, S.R., D.B. Re, G.F. Barry, D.E. Eichholtz, X. Delannay, R.L. Fuchs, G.M.

Kishore and R.T. Fraley. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready™ gene. Pages 53–84 in Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. S.O. Duke (ed.). CRC Press, Inc., Boca Raton, Florida.

Paradise, M.S. 2006a. Evaluation of potential dietary effects of Cry1A.105 protein on the ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). Monsanto Technical Report MSL-20150. St Louis, Missouri.

Paradise, M.S. 2006b. Evaluation of potential dietary effects of Cry2Ab2 protein on the ladybird beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae). Monsanto Technical Report MSL-20151. St Louis, Missouri.

Pleasants, J.M., R.L. Hellmich, G.P. Dively, M.K. Sears, D.E. Stanley-Horn, H.R. Mattila, J.E. Foster, P. Clark and G.D. Jones. 2001. Corn pollen deposition on milkweeds in and near cornfields. Proceedings of the National Academy of Sciences of the United States 98: 11919–11924.

Price, K.E., J.C. Godfrey and H. Kawaguchi. 1974. Effect of structural modifications on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine. Pages 191–307 in Advances in Applied Microbiology. Volume 18. D. Perlman (ed.). Academic Press, New York, New York.

Raynor, G.S., E.C. Ogden and J.V. Hayes. 1972. Dispersion and deposition of corn pollen from experimental sources. Agronomy Journal 64: 420–427.

Richards, K.B. 2006a. Evaluation of the dietary effect(s) of a Cry1A.105 protein on adult honeybees (*Apis mellifera* L.). Monsanto Technical Report MSL-20354. St Louis, Missouri.

Richards, K.B. 2006b. Evaluation of the dietary effect(s) of a Cry1A.105 protein on honeybee larvae (*Apis mellifera* L.). Monsanto Technical Report MSL-20249. St Louis, Missouri.

Rogers, S.G. 2000. Promoter for transgenic plants. Patent 6,018,100, U.S. Patent Office, Washington, D.C.

Russell, S.H., J.L. Hoopes and J.T. Odell. 1992. Directed excision of a transgene from the plant genome. Molecular and General Genetics 234: 49–59.

Sasaki, K., M.-H. Kim and R. Imai. 2007. *Arabidopsis* COLD SHOCK DOMAIN PROTEIN2 is a RNA chaperone that is regulated by cold and developmental signals. Biochemical and Biophysical Research Communications 364: 633–638.

Schindler, T., P.L. Graumann, D. Perl, S. Ma, F.X. Schmid and M.A. Marahiel. 1999. The family of cold shock proteins of *Bacillus subtilis* - Stability and dynamics *in vitro* and *in vivo*. The Journal of Biological Chemistry 274: 3407–3413.

Sears, M.K., D.E. Stanley-Horn and H.R. Matilla. 2000. Preliminary report on the ecological impact of Bt corn pollen on the Monarch butterfly in Ontario. Plant Biotechnology Office, Canadian Food Inspection Agency, Ottawa, Ontario.

Shaw, K.J., P.N. Rather, R.S. Hare and G.H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiological Reviews 57: 138–163.

Shirai, Y. and M. Takahashi. 2005. Effects of transgenic Bt corn pollen on a non-target lycaenid butterfly, *Pseudozizeeria maha*. Applied Entomology and Zoology 40: 151–159.

Slaney, A.C., H.L. Robbins and L. English. 1992. Mode of action of *Bacillus thuringiensis* toxin CryIIIA: An analysis of toxicity in *Leptinotarsa decemlineata* (Say) an *Diabrotica undecimpunctata howardi* Barber. Insect Biochemistry and Molecular Biology 22: 9–18.

Stalker, D.M., C.M. Thomas and D.R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Molecular and General Genetics 181: 8–12.

Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Pages 77–90 in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, New York.

Van Rie, J., S. Jansens, H. Höfte, D. Degheele and H. Van Mellaert. 1990. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Applied and Environmental Microbiology 56: 1378–1385.

Weber, M.H.W., A.V. Volkov, E. Fricke, M.A. Marahiel and P.L. Graumann. 2001. Localization of cold shock proteins to cytosolic spaces surrounding nucleoids in *Bacillus subtilis* depends on active transcription. Journal of Bacteriology 183: 6435–6443.

Widner, W.R. and H.R. Whitely. 1989. Two highly related insecticidal crystal proteins of *Bacillus thuringiensis* subsp. *kurstaki* possess different host range specificities. Journal of Bacteriology 171: 965–974.

Willimsky, G., H. Bang, G. Fischer and M.A. Marahiel. 1992. Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. Journal of Bacteriology 174: 6326–6335.

Zambryski, P., A. Depicker, K. Kruger and H.M. Goodman. 1982. Tumor induction by *Agrobacterium tumefaciens*: Analysis of the boundaries of T-DNA. Journal of Molecular and Applied Genetics 1: 361–370.

Kakimoto, Y. 1981. Toumorokoshi no kigen to tokusei (Origin and characteristics of maize). I Shokubutsu toshiteno bunrui, ruien kankei (Classification and taxonomy as a plant). Hatasaku Zensho (Encyclopedia of upland farming). Volume 7 Zakkoku-hen (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Kakimoto, Y., Yamada, M. 2001. Toumorokoshi (Maize). Toumorokoshi no kigen to tokusei (Origin and characteristics of maize). III Shokubutsu toshite no tokusei (Characteristics as a plant). Pages 34–38 in Tensaku Zensho (Encyclopedia of crop conversion). Volume 3 Zakkoku (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Wildlife Division, Nature Conservation Bureau, Ministry of the Environment. 2006. Kaitei Nihon no zetsumetsu no osore no aru yasei seibutsu (Endangered wild species in Japan, Revised) – Red Data Book – Japan Wildlife Research Center, Tokyo.

Kikuchi, K. 1987. Toumorokoshi no seisan to riyou (Production and use of maize). Korin Publishing Co. Ltd, Tokyo.

Ministry of Finance. 2011. Trade Statics of Japan. Ministry of Finance. http://www.customs.go.jp/toukei/info/index.htm [Accessed May 2, 2011].

Sendo, S. 2001. Toumorokoshi (Maize). Toumorokoshi no hinshu seitai (Ecology of maize varieties). IV Saishu (Collecting). Tensaku Zensho (Encyclopedia of crop conversion). Volume 3. Zakkoku (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Takizawa, Y. 1981. Shijitsu you toumorokoshi no saibai (Cultivation of grain corn). II Saibai no jissai (Cultivation in practice). Pages 124–143 in Hatasaku Zensho (Encyclopedia of upland farming). Volume 7 Zakkoku-hen (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Chiba, K. 1980. Zushu・ Sakumotsu saibai no kiso chishiki (Illustrated book of basic knowledge on crop cultivation). Kurihara, H., Rural Culture Association Japan, Tokyo.

Tozawa, H. 2005. Toumorokoshi-Rekishi, Bunka, Tokusei, Saibai, kakou, Riyou- (Maize – History, Culture, Property, Cultivation, Processing and Use). Rural Culture Association Japan, Tokyo.

Nakane, T., Oobayashi, K. Nomura, S., Kurosawa, Y. 1963. Genshoku Konchuu Daizukan (Colored illustrations of insects). Hokuryukan Co. Ltd., Tokyo.

Nakamura, S. 2001a. Toumorokoshi (Maize). Seiiku no stage to seiri, seitai (Growth stage, physiology, and ecology). I Shushi to hatsuga (Seed and germination). Pages 41–43 in Tensaku Zensho (Encyclopedia of crop conversion). Volume 3. Zakkoku (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Nakamura, S. 2001b. Toumorokoshi (Maize). Seiiku no stage to seiri, seitai (Growth stage, physiology, and ecology). III Seishoku seichouki no seiri, seitai (Physiology and ecology at reproductive stage). Pages 50–53 in Tensaku Zensho (Encyclopedia of crop conversion). Volume 3. Zakkoku (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.

Nougaku Daijiten Editorial Committee. 1987. 13. Shokuyo sakumotsu (Food crops). Toumorokoshi (Maize). Pages 536–541 in Dainiji zoutei kaihan Nougaku Daijiten (Newly revised second edition of Encyclopedia of Agriculture). Noguchi et al. (eds.). Yokendo Co. Ltd., Tokyo.

Statistics Department, Ministry of Agriculture, Forestry and Fisheries. 2010. Heisei 21-nen san akifuyu yasai, shitei yasai ni junzuru yasai tou no sakutsuke menseki, shuukakuryo oyobi shukkaryo (Planted area, yield and shipping volume of vegetables listed under fall and winter vegetables, designated vegetables in 2009). Ministry of Agriculture, Forestry and Fisheries. http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_yasai/pdf/yasai_syutou09.pdf [Accessed October 16, 2010].

Statistics Department, Ministry of Agriculture, Forestry and Fisheries. 2011. Heisei 22-nen san shiryou sakumotsu no shuukakuryo (Bokusou, Aogari toumorokoshi oyobi sorgo) (Yield of forage crops in 2010 (grass, soiling maize and sorgo)). Ministry of Agriculture, Forestry and Fisheries. http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_kome/pdf/syukaku_siryou_10.pdf [Accessed May 2, 2011].

Matsui, M., Saito, O. 2003. III Nougyou Kankyou Gijutsu Kenkyuujo ni okeru Bt toumorokoshi no kinkyuu chousa (Urgent study on Bt maize at National Institute for Agro-Environmental Sciences). 4. Bt kumikae toumorokoshi kafun chuu no Bt toxin no kenshutsu (Detection of Bt toxins from pollens of Bt recombinant maize) 2. Seibutsu kentei ni yoru kenshutsu (Detection by bioassays). Pages 55-62 in Nougyou kankyou kenkyuu sousho (Agro-environmental study report series) Vol. 14. Idenshi kumikae sakumotsu no seitaikei heno eikyou hyouka (Evaluation of ecological impact of genetically modified crops). National Institute for Agro-Environmental Sciences (ed.). National Institute of Agro-Environmental Sciences, Tsukuba.

Maruyama, K. 1981. Toumorokoshi no hinshu seitai (Ecology of maize varieties). I Hinshu no kihon tokusei (Basic characteristics of varieties). Pages 83–89 in Hatasaku Zensho (Encyclopedia of upland farming). Volume 7 Zakkoku-hen (Cereals). Rural Culture Association Japan (ed.). Rural Culture Association Japan, Tokyo.