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

Name of Entity: Monsanto Japan Limited
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Approved Type1 Use Regulation

Names of types of living modified organisms	Coleoptera resistant and herbicide (glyphosate) tolerant maize (*DvSnf7,* modified *cry3Bb1*, modified *cp4 epsps, Zea mays* subsp. *mays* (L.) Iltis) (MON87411, OECD UI: MON-87411-9)
Content of Type 1 Use of living modified organisms	Use for provision as food, animal feed or other purposes, cultivation and other growing, processing, storage, transportation and disposal, and other acts attendant with these.
Method of Type 1 Use of living modified organisms	-

Biological Diversity Risk Assessment Report

Chapter 1 Information collected for Biological Diversity Risk Assessment

1. Information on preparation, etc. of living modified organisms

In the United States, corn fields over 17.6 million hectares are currently damaged by insects, including corn root worms (*Diabrotica* spp.) (hereinafter referred to as "CRW") belonging to the genus Diabrotica. CRWs have caused a significant damage on corns with enormous economic losses, causing over $ 1 billion annually for CRW control (Marra et al., 2012).

Monsanto Company has developed maize with Coleoptera pest resistance using RNA interference (RNAi) technology and modified Cry3Bb1 protein, and with glyphosate herbicide-tolerance using modified CP4 EPSPS protein (*DvSnf7*, modified *cry3Bb1*, modified *cp4 epsps, Zea mays* subsp*. mays* (L.) Iltis) (MON87411, OECD UI: MON-87411-9) (hereinafter referred to as "Genetically Modified Maize"). In the genetically modified maize, *Snf7* gene (hereinafter referred to as "*DvSnf7* gene", which is essential for maintaining the cell function of western corn rootworm (*Diabrotica virgifera virgifera*) (hereinafter referred to as "WCRW") partial sequence of 240 bases of an exon (hereinafter referred to as "*DvSnf 7* gene fragment") are introduced in the form of inverted repeat sequence. The transcript from the inverted repeat sequence forms double-stranded RNA (dsRNA) (hereinafter referred to as "dsRNA of *DvSnf7* gene fragment"). The dsRNA of the *DvSnf7* gene fragment shows insecticidal activity by inducing RNAi and suppressing the expression of the DvSnf7 gene after taken into cells of CRW through oral intake of the genetically modified maize.

In the genetically modified maize, a modified *cry3Bb1* gene has been introduced in addition to the aforementioned RNAi, and resistance to the same Coleoptera pest is ensured by expressing the modified Cry3Bb1 protein. Thus, by providing insecticidal activity against CRW with RNAi and Bt protein having different modes of actions, it is expected that CRW will reduce the risk of acquiring resistance to the genetically modified maize.

(1) Information on donor nucleic acids

A Composition and Origin of Constituent Elements

The composition and origin of constituent elements of the donor nucleic acid used for the production of the genetically modified maize are shown in FIG. 1 (p 4) and Table 1 (p 5 - p8).

The Cry3Bb1 protein expressed from the *cry3Bb1* gene introduced into the genetically modified maize has six amino acid substitutions compared with the wild type Cry3Bb1 protein. One[1] of them was modified for the purpose of adding the cleavage site of a restriction enzyme at the time of cloning and the other 5 sites[2] were modified for the purpose of enhancing insecticidal activity.

In addition, the CP4 EPSPS protein expressed from the *cp4 epsps* gene has the second serine from the N-terminal sequence modified to leucine, compared with the amino acid sequence of the CP4 EPSPS protein derived from the *Agrobacterium* sp. CP4 strain by inserting restriction enzyme cleavage sites in the process of cloning.

Therefore, the *cry3Bb1* gene and the *cp4 epsps* gene introduced into the genetically modified maize are referred to as "modified *cry3Bb1* gene" and "modified *cp4 epsps* gene", respectively. The proteins to be expressed are also referred to as "modified Cry3Bb1 protein" and "modified CP4 EPSPS protein", respectively. The deduced amino acid sequences of the modified Cry3Bb1 protein and the modified CP4 EPSPS protein expressed in the genetically modified maize are shown in Appendix 1 and Appendix 2, respectively.

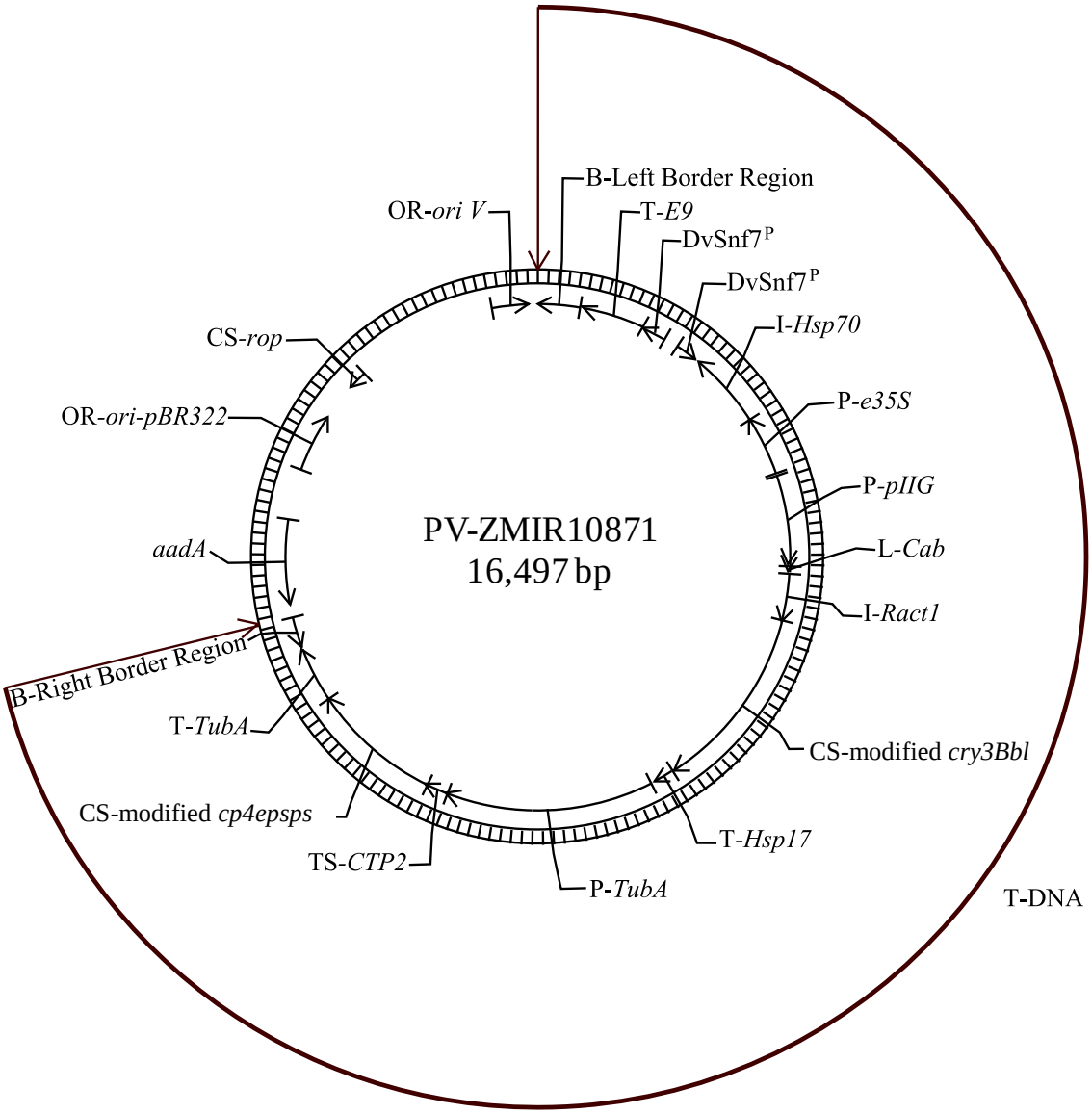
B. Function of Components

① Functions of target genes, expression regulatory regions, localization signals, selected markers and other components of donor nucleic acids

The functions of donor nucleic acids used for the production of the genetically modified maize are shown in Table 1 (p5 - p8).

[1] Alanine is inserted the second from the N terminus.

[2] The amino acid at position 232 from the N terminus is substituted from histidine to arginine, the amino acid at position 312 from the N terminus from serine to leucine, the 314th amino acid from the N terminus from asparagine to threonine, the amino acid at position 318 from glutamic acid to lysine, and the amino acid at position 349 from glutamine to arginine.



P indicates that it is a partial sequence.

Fig. 1 Plasmid map[3] of PV-ZMIR10871 used to produce the genetically modified maize.

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Table 1 Origins and functions of each constituent element of PV-ZMIR10871 used for making the genetically modified maize[4]

Constituent elements	Position in the plasmid	Origins and functions
T DNA region		
B[Note1]-Left Border Region	1-442	A DNA region derived from *Agrobacterium tumefaciens* and containing a left boundary region used for transmitting T-DNA (Barker et al., 1983).
Intervening Sequence	443-485	Sequences used for DNA cloning.
T [Note2]-*E9*	486-1,118	3 'untranslated region derived from the RbcS 2 gene encoding ribulose-1,5-diphosphate carboxylase small subunit of *Pisum sativum* (pea). It induces polyadenylation of mRNA (Coruzzi et al., 1984).
Intervening Sequence	1,119-1,147	Sequence used for DNA cloning.
DvSnf7^{p} [Note3]	1,148-1,387	Partial sequence of Snf7 gene derived from *Diabrotica virgifera virgifera* (Baum et al., 2007; Baum et al., 2011). It encodes a part of the SNF7 subunit of the ESCRT III complex (Babst et al., 2002).
Intervening Sequence	1,388-1537	Sequence used for DNA cloning.
DvSnf7^{p}	1,538-1,777	Partial sequence of *Snf7* gene derived from *D. virgifera virgifera* (Baum et al., 2007; Baum et al., 2011). It encodes a part of the SNF7 subunit of the ESCRT III complex (Babst et al., 2002).
Intervening Sequence	1,778-1,813	Sequence used for DNA cloning.
I [Note4]-*Hsp70*	1,814-2,617	The first intron of the heat shock protein gene (hsp70) of Zea mays (corn) and a part of its neighboring exons (Rochester et al., 1986). Increase the expression activity at the expression site of the target gene (Brown and Santino, 1997)

[4] Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited

Table 1 Origins and functions of each component of PV-ZMIR10871 used to create the genetically modified maize (continued)

Constituent elements	Position in the plasmid	Origins and functions
P [Note 5]-*e35S*	2,618-3,238	Promoter of 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985). It has a double enhancer region (Kay et al., 1987) and induces transcription in plant cells.
Intervening Sequence	3,239-3,264	Sequence used for DNA cloning.
P-*pIIG*	3,265-4,213	Promoter region of *pIIG* gene sequence coding for *Z. mays* (maize) physical impedance inducing protein (Huang et al., 1998). It induces transcription in plant cells.
Intervening Sequence	4,214-4,219	Sequence used for DNA cloning.
L[Note6]-*Cab*	4,220-4,280	5 'terminal untranslated leader region of chlorophyll a / b binding protein of *Triticum aestivum* (wheat). It activates the expression of the target gene (Lamppa et al., 1985).
Intervening Sequence	4,281-4,296	Sequence used for DNA cloning.
I-*Ract1*	4,297-4,776	Intron of actin gene derived from *Oryza sativa* (rice) (McElroy et al., 1990). It is involved in regulating the expression of the gene in question.
Intervening Sequence	4,777-4,785	Sequence used for DNA cloning.
CS[Note7]-modefied *cry3Bb1*	4,786-6,747	Gene encoding Cry3Bb1 protein derived from *Bacillus thuringiensis* and provides resistance to Coleoptera insects (English et al., 2000).
Intervening Sequence	6,748-6,766	Sequence used for DNA cloning.
T-*Hsp17*	6,767-6,976	3 'untranslated region of Heat Shock Protein 17 of *T. aestivum* (wheat). It terminates transcription and induces polyadenylation (McEwain and Spiker, 1989).
Intervening Sequence	6,977-7,024	Sequence used for DNA cloning.

Table 1 Origin and function of each constituent element of PV-ZMIR10871 used to create the genetically modified maize (continued)

Constituent elements	Position in the plasmid	Origins and functions
P-*TubA*	7,025-9,205	Promoter, 5 'terminal untranslated leader and intron sequence of *OsTubA* gene family encoding *O. sativa* (rice) α tubulin (Qin et al., 1997; Jeon et al., 2000). It induces transcription in plant cells.
Intervening Sequence	9,206-9,209	Sequence used for DNA cloning.
TS [Note8]-*CTP2*	9,210-9,437	Sequence encoding the chloroplast transit peptide of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (*ShkG*) of *Arabidopsis thaliana* (Arabidopsis) (Klee et al., 1987; Herrmann, 1995). It transports the modified CP4 EPSPS protein to the chloroplast.
CS-modified *cp4 epsps*	9,438-10,805	*AroA (epsps)* gene derived from *Agrobacterium* CP4 strain. It encodes 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) and provides herbicide, glyphosate-tolerance (Padgette et al., 1996; Barry et al., 2001).
Intervening Sequence	10,806-10,812	Sequence used for DNA cloning.
T-*TubA*	10,813-11,394	3 'untranslated region of *OsTubA* gene encoding α tubulin of *O. sativa* (rice). It terminates transcription and induces polyadenylation (Qin et al., 1997; Jeon et al., 2000).
Intervening Sequence	11,395-11,412	Sequence used for DNA cloning.
B-Right Border Region	11,413-11,743	DNA fragment containing the right border region derived from *A. tumefaciens.* It is used to transmit T-DNA (Depicker et al., 1982; Zambryski et al., 1982).

Table 1 Origin and function of each constituent element of PV-ZMIR10871 used to create the genetically modified maize (continued)

Constituent elements	Position in the plasmid	Origins and functions
Outer skeletal area (Not present in the genetically modified maize)		
Intervening Sequence	11,744-11,879	Sequence used for DNA cloning.
aadA	11,880-12,768	Bacterial promoter and coding sequence and 3 'terminal untranslated region of 3' '(9) -*O*-nucleotidyl transferase from transposon Tn7 (aminoglycoside modifying enzyme). (Fling et al., 1985) It provides spectinomycin and streptomycin resistance.
Intervening Sequence	12,769-13,298	Sequence used for DNA cloning.
OR[Note9]-*ori-pBR322*	13,299-13,887	Origin of replication isolated from pBR322, providing an autonomous replication ability to a vector in *Escherichia coli* (Sutcliffe, 1979).
Intervening Sequence	13,888-14,314	Sequence used for DNA cloning.
CS-*rop*	14,315-14,506	Coding sequence of the primer protein repressor (Primor protein) derived from the ColE1 plasmid and maintains the plasmid copy number in *E. coli* (Giza and Huang, 1989).
Intervening Sequence	14,507-16,014	Sequence used for DNA cloning.
OR-*ori V*	16,015-16,411	Origin of replication derived from the broad host range plasmid RK2, providing autonomous replication ability to the vector in *Agrobacterium* (Stalker et al., 1981).
Intervening Sequence	16,412-16,497	Sequence used for DNA cloning.

[Note1] B-Border
[Note2]T-Transcription Termination Sequence
[Note3]*P*– Partial sequence
[Note4]I-Intron
[Note5]P-Promoter
[Note6]L-Leader
[Note7]CS-Coding Sequence
[Note8]TS-Targeting Sequence
[Note9]OR-Origin of Replication

② Function of the protein produced by the expression of the target gene and selected markers, and that the protein has homology, if any, with a protein that has been found to have allergic properties

I. 【dsRNA of *DvSnf7* gene fragment】

Regarding the function of dsRNA of the *DvSnf7* gene fragment, we first describe the general mechanism of RNAi as described in "i. General RNAi Mechanism (p 9)". Then, we will describe the action mechanism of dsRNA of the *DvSnf7* gene fragment expressed in the genetically modified maize in the section "ii. Functional Mechanism of the genetically modified maize into which the *DvSnf7* gene fragment is introduced" (p10 - 17) ". Furthermore, the factors that determine the spectrum and specificity of the dsRNA of the *DvSnf7* gene fragment will be explained in "iii. Specificity of dsRNA of DvSnf7 gene fragment for target insect (p 18 - 35)". Regarding the effect on non-target organisms, we will discuss in detail in the section, “iv. Effects of dsRNA of *DvSnf7* gene fragment on soil microorganism (p36 - 38)” and “v. Effects of dsRNA of *DvSnf7* gene fragment on vertebrate (p38 - 42)”.

i. General RNAi Mechanism

RNAi is a mechanism that is commonly seen in eukaryotes for gene expression regulation. In the process where RNAi occurs, dsRNA is first cleaved by an enzyme called Dicer which is classified as ribonuclease III, and small interfering RNA (siRNA) of 21 to 25 bases is formed (Hammond, 2005; Siomi and Siomi, 2009). Then, the siRNA binds to the RNAi-induced silencing complex (RISC) and binds to mRNA having a target complementary sequence. Furthermore, mRNA bound to siRNA is degraded by endonuclease of RISC, which results in inhibition of translation of mRNA into protein (Hammond, 2005; Siomi and Siomi, 2009). Since RNAi has high specificity and can efficiently and stably induce the suppression of gene expression, it is used for imparting specific traits and analyzing gene function (Kusaba, 2004).

In recent years, it has been confirmed that it is possible to suppress the expression of endogenous genes of insects by RNAi by orally ingesting genetically modified plants expressing dsRNA into insects, and it is proposed that RNAi can be used as a promising pest control method. Specifically, specific insecticidal activity can be provided by specifically suppressing the expression of a gene that encodes a protein related to an essential function of a specific insect (Baum et al., 2007; Whyard et Al., 2009).

ii. The Mechanism of Action of the genetically modified maize introduced the inverted repeat sequence of the *DvSnf7* gene fragment

1. Functional Mechanism of Orally Ingested dsRNA Suppressing the Expression of *DvSnf7* Gene

In the genetically modified maize, the DvSnf7 gene fragment, which is indispensable for maintaining the cell function of WCRW, is introduced in the form of inverted repeats. As a result, dsRNA formation of the *DvSnf7* gene fragment was confirmed from this inverted repeat sequence (Appendix 3, Figure 1, p4).

In addition, Real-Time RT-PCR method and western blot analysis found that, when the dsRNA of the *DvSnf7* gene fragment synthesized *in vitro* was orally ingested by WCRW, the dsRNA of the *DvSnf7* gene fragment was recognized by the RNAi mechanism of WCRW, the mRNA expressed from the *DvSnf7* gene was degraded, which suppresses the expression of the DvSNF7 protein (Hereinafter referred to as "suppression of expression of *DvSnf7* gene")(Fig. 2, p12) (Bolognesi et al., (2012) (Attachment 1) Figure 3, p.6).

These findings suggest that dsRNA of the *DvSnf7* gene fragment expressed in the genetically modified maize was ingested by WCRW and then taken up into midgut cells to suppress the expression of the *DvSnf7* gene, thereby resulting in WCRW with insecticidal activity (Fig. 3, p13).

Incidentally, it is also confirmed that another genetically modified maize line expressing only the dsRNA of the *DvSnf7* gene fragment shows resistance to WCRW (Appendix 4, Figure 1, p4).

Also, it is confirmed that the genetically modified maize also produces siRNA derived from the dsRNA in addition to the dsRNA of the *DvSnf7* gene fragment (Table 1 in Appendix 5, p6).

It is acknowledged that there is a selection mechanism of RNA to be taken in by the base length in the midgut cell of WCRW and that only dsRNA of 60 base pairs (bp) or more is efficiently incorporated into the midgut cells (Bolognesi et al., (2012) (Appendix 1)). According to the results of bioassay of WCRW administered high concentration of 21 bp siRNA derived from dsRNA of *DvSnf 7* gene fragment and fluorescence imaging of midgut cells, it has been confirmed that the chances of it being effectively incorporated into midgut cells of WCRW are small, (Bolognesi et al., (2012) (Attachment 1) Figure 2-A., p5) and no insecticidal activity against WCRW has been shown (Bolognesi et al., (2012) (Attachment 1) in Figure 2-B., p5). Based on the facts mentioned above, siRNA

derived from dsRNA of the *DvSnf7* gene fragment produced in the genetically modified maize was not considered to be involved in insecticidal activity of WCRW.

Incidentally, it is reported that low-molecular RNA such as siRNA is methylated and modified in plants, and stability improves (Ji and Chen, 2012). However, as a result of the same test as above using the methylation-modified siRNA, the methylation-modified siRNA also had a low possibility of being efficiently taken up into midgut cells in WCRW, which did not show any insecticidal activity (Appendix 6, Fig. 1, p6 and Fig. 3, p8).

The dsRNA of the orally ingested *DvSnf7* gene fragment induced suppression of the expression of the *DvSnf7* gene not only in the midgut cells of WCRW but also in other body organisms, with an observed phenomenon of systemic spread throughout the body (Bolognesi et al., (2012) (Attachment 1)). It has been known that there is a mechanism called systemic spread in which cells take dsRNA from outside the cell and diffuse incorporated dsRNA into neighboring cells (Huvenne and Smagghe, 2010).

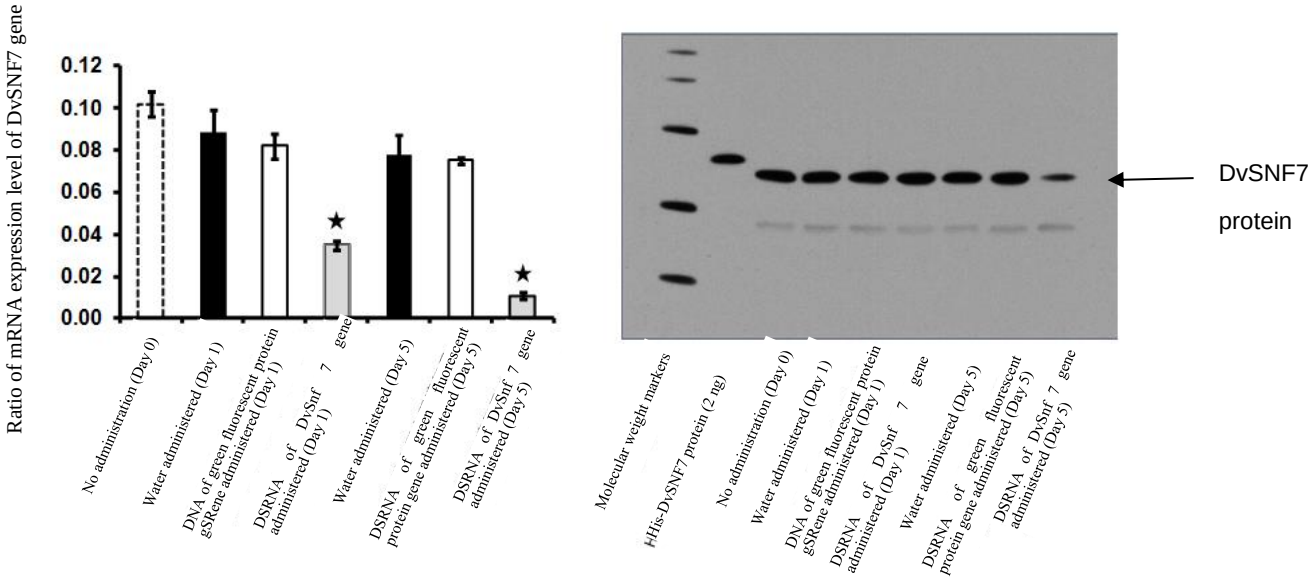
The systemic spread is a mechanism reported in 1998 by *Caenorhabditis elegans* which is a kind of nematode. Besides *C. elegans,* it is reported to be caused by injection of dsRNA into cells or oral ingestion of dsRNA in various insects belonging to the order Coleoptera, Lepidoptera, Diptera and Orthoptera (Bucher et al., 2002; Tomoyasu and Denell, 2004; Dong and Friedrich, 2005; Turner et al., 2006; Tian et al., 2009; Alves et al., 2010; Li et al., 2011b). However, the mechanisms of cellular uptake of orally ingested dsRNA and its systemic spread have yet to be elucidated (Bolognesi et al., (2012) (Appendix 1)).

In addition to the length of dsRNA and the systemic spread of RNAi, it is known that various factors such as selection of the target gene, dsRNA sequence, the amount of dsRNA taken, the mechanism relating to the sustainability of gene expression suppression, insect growth stage, and the like affect the induction of RNAi by orally ingested dsRNA (Huvenne and Smagghe, 2010).

In summary, the dsRNA of the *DvSnf7* gene fragment is expressed in the genetically modified maize. After the target insect, WCRW, ingests the genetically modified maize, the dsRNA of the *DvSnf7* gene fragment is efficiently taken up in midgut cells and the expression of the *DvSnf7* gene, which plays an essential role in maintaining the cell function of WCRW, is inhibited through the mechanism of RNAi. At the same time, dsRNA incorporated into midgut cells may spread to other body organisms by the systemic spread mechanism, suppressing the expression of *DvSnf7* gene through RNAi mechanism. Based on these facts, it is

considered that insecticidal activity is provided to WCRW (Fig. 3, p13).

In addition, it is unlikely that siRNA derived from the dsRNA of the *DvSnf7* gene fragment produced in the genetically modified maize is taken up by the selection mechanism of RNA of WCRW midgut cells, and that it has insecticidal activity against WCRW.



* Ratio of the expression level of *DvSnf7* gene corrected by the expression level of WCRW-derived standard gene (gene encoding tubulin).

★There was a Statistically significant difference (t test; p <0.05) as compared with the controls (a group with water administered and a group with dsRNA of green fluorescent protein gene administered).

The mRNA level of the *DvSnf7* gene decreased from the first day of dsRNA administration of the *DvSnf7* gene fragment (left graph), and the decrease also caused a decrease in the amount of *DvSNF7* protein on the fifth day of administration (right graph).

Fig.2 MRNA level of *DvSnf7* gene in the systemic body organism of WCRW by dsRNA in orally ingested *DvSnf7* gene fragment (left: Real-Time RT-PCR method) and suppression of DvSNF7 protein expression (right: western blot analysis) (Bolognesi et al ., (2012) (Attachement 1))[5]

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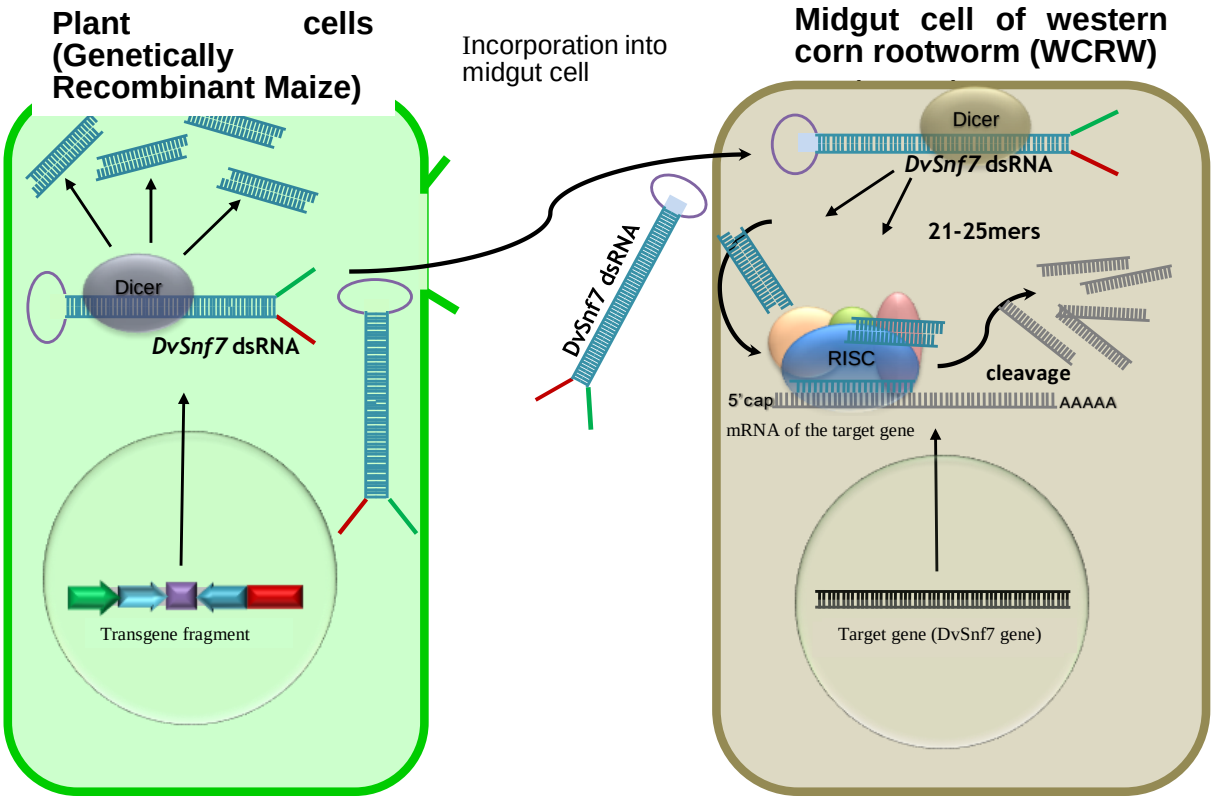

Fig.3 Inhibition of the expression of *DvSnf7* gene in midgut cells of WCRW by dsRNA in orally ingested *DvSnf7* gene fragment (Schematic)[6]

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2. Functional mechanism leading to the death of WCRW by suppressing the expression of *DvSnf7* gene

In general, eukaryotic cells which have autonomic action (autophagy) that decomposes unnecessary organelles and proteins take up unnecessary proteins through endosomes into cells and maintain cell homeostasis by transport, selection and degradation (Fader and Colombo, 2009).

SNF7 protein encoded by the *Snf7* gene, along with the neutral N-terminus and the acidic C-terminus, has a coiled-coil structure domain in which the α helix is coiled like a coil (Peck et al., 2004; Winter and Hauser, 2006). In the above mentioned autophagy pathway, this constituent protein of ESCRT (Endosomal Sorting Complex Required for Transport) -III complex is involved in the selection of whether to decompose the receptor protein etc. in the cell membrane in lysosome (Teis et al., 2008; Vaccari et al., 2009; Kim et al., 2011).

Also, it has been reported that yeast, Drosophila insect, *C. elegans,* Arabidopsis thaliana, rice and human have such proteins classified as SNF7 (Winter and Hauser, 2006). Regarding the homology of these SNF7 proteins between organisms, it is reported that the homology of SNF7 protein amino acid sequence for plants and humans is in the range of about 30% and 57%, while for yeast and humans is about 50% (Peck et al., 2004; Winter and Hauser, 2006). Also, secondary structure analysis suggests that the basic structure and function of SNF7 protein are conserved among eukaryotes (Peck et al., 2004; Winter and Hauser, 2006). Thus, the SNF7 protein exists in many organisms (Tu et al., 1993; Gao et al., 1999; Peck et al., 2004; Winter and Hauser, 2006; Lee et al., 2007; Kim et al., 2011) and is considered to be indispensable for maintaining cell function.

As described above, the SNF7 protein is known for constituting a part of the ESCRT-III complex involved in the autophagy pathway. Fig. 4, A (p17) shows the autophagic pathway in ordinary insect cells (A in Fig. 4, p17; Ramaseshadri et al., (2013) (Attachment 2) Fig. 5, p 7). As the following, the autophagic pathway in ordinary insect cells will be described according to the numbers in Fig.4 A (p17).

1. Endocytosis of protein in which ubiquitin is bound (ubiquitinated protein) on the cell membrane occurs, transported to the early endosomes through membrane vesicles, (Raiborg and Stenmark, 2009).

2. In the early endosome, the ubiquitinated protein was deubiquitinated by going through the ESCRT pathway (ESCRT-0, I, II and III complex), where the released ubiquitin is transported again to the protein in the cell membrane (Raiborg and Stenmark, 2009).
3. Part of the early endosome containing the deubiquitinated protein germinates, turns into multivesicular (MVB) and subsequently forms of the late endosomes (Raiborg and Stenmark, 2009).
4. When the late endosomes are fused with lysosomes (autolysosomes), lysosomal degradation occurs (Ramaseshadri et al., (2013) (Appendix 2)). In insect cells, autophagocytes (autophagosomes) containing organelles or unnecessary proteins and the like are separately formed (Ramaseshadri et al., (2013) (Attachment 2)).
5. The late endosomes, lysosomes, and autophagosomes are fused to form autolysosomes, thereby inducing autophagy. Autophagy is likely to occur when cells are starved (Ramaseshadri et al., (2013) (Appendix 2)).

For many higher organisms, degradation of unnecessary cellular organelles and proteins by the autophagy pathway as described above is an important process for controlling various physiological and pathological conditions (Ramaseshadri et al., (2013) (Appendix 2)). Also, since receptor proteins generally control signal transduction for eukaryotic cells, to select receptor proteins in the cell membrane by endocytosis is an essential function for maintaining homeostasis. It is also reported that receptors involved in several pathways, including Notch signaling and epidermal growth factor signaling, which control cell proliferation and growth, are screened by the ESCRT pathway (Ramaseshadri et al., (2013) (Appendix 2)).

Like the general SNF7 protein described above, the DvSNF7 protein encoded by the *DvSnf7* gene is a protein constituting a part of the ESCRT-III complex also in the cells of WCRW. The ESCRT-III complex of WCRW is also deemed to be associated with deubiquitination of ubiquitinated protein in early endosome, fusion of the late endosome or autophagosome and lysosome (Ramaseshadri et al., (2013) (Attachment 2)). As shown in B (p17) in Fig. 4, the expression of *DvSnf 7* gene is suppressed due to dsRNA in orally ingested *DvSnf 7* gene fragment, without deubiquitination, as well as without fusion of the late endosome or autophagosome and lysosome, where unnecessary protein which should be otherwise decomposed by autophagy is accumulated in the cell (Ramaseshadri et al., (2013) (Attachment 2) Figure 5, p7).).

In summary, the function of the DvSNF7 protein encoded by the *DvSnf7* gene is essential for maintaining the cellular function of the WCRW, as seen for the general SNF7 protein. It was assumed that, when the expression of the *DvSnf7* gene in WCRW was suppressed with the dsRNA, *DvSnf7* gene fragment, after ingested by the genetically modified maize, cellular homeostasis is damaged, causing the death of WCRW.

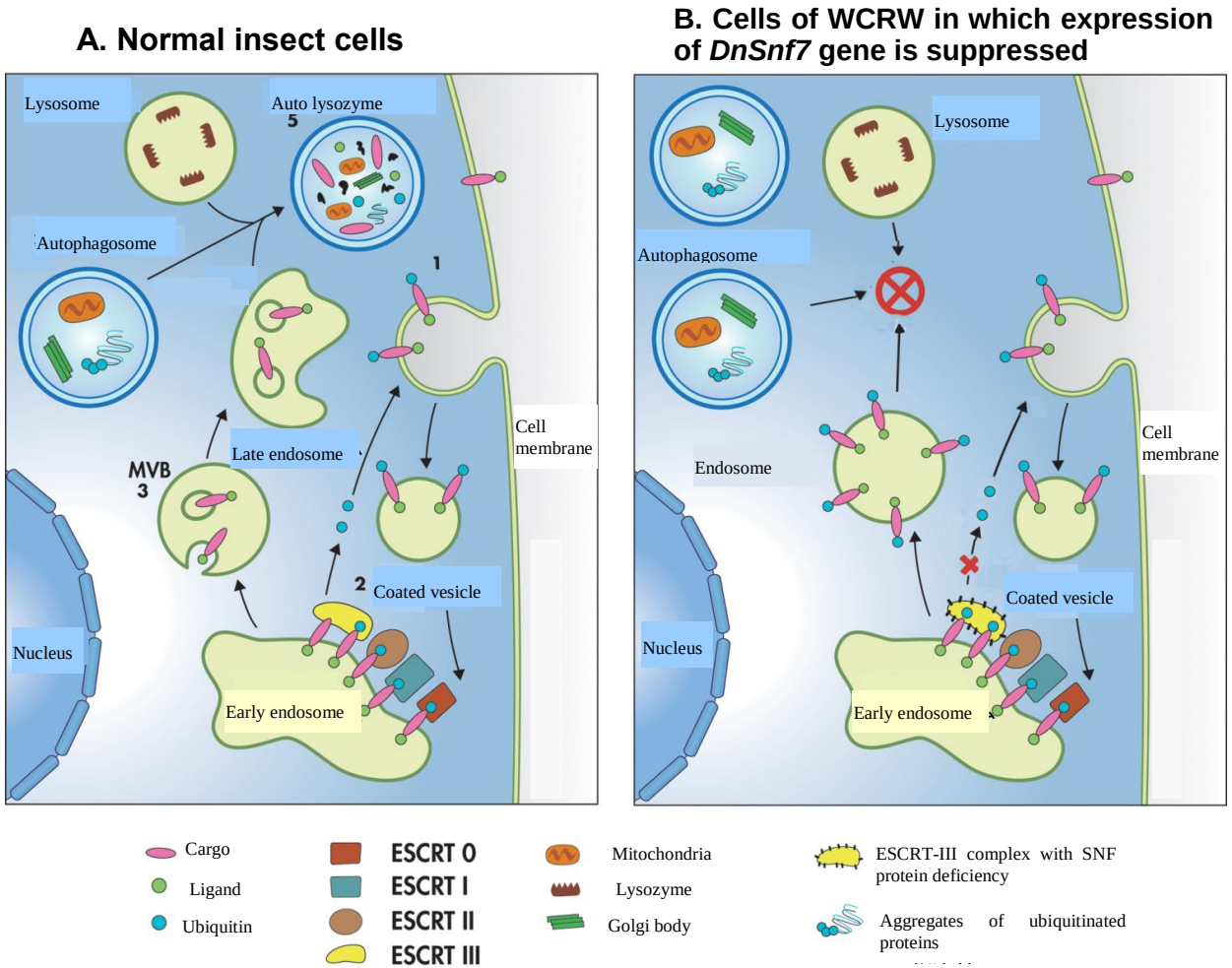

FIG.4 A. Autophagy pathway in cells of normal insect cells and WCRW in which the expression of B. *DvSnf 7* gene is suppressed; modified from Ramaseshadri et. Al., (2013) (Attachment 2) [7]

[7]Responsibility for the rights and contents pertaining to the information described in this figure belongs to Monsanto Japan Limited.

iii. Specificity of dsRNA of *DvSnf7* gene fragment for target insect

1. Insecticidal activity spectrum of dsRNA of *DvSnf7* gene fragment

In order to investigate insecticidal activity spectrum of dsRNA of the *DvSnf7* gene fragment, we studied insecticidal activity against insects selected based on phylogenetic relation with WCRW (Appendix 7).

We conducted bioassey by feeding dsRNA of *DvSnf7* gene fragment to 14 kinds of insects (Table 2, p.20) representative of 10 families of 4 orders, of Coleoptera, Lepidoptera, Hymenoptera and Hemiptera (Table 2, p20). To clarify the specificity of the dsRNA of the *DvSnf7* gene fragment in Coleoptera, seven species belonging to four families were tested for Coleoptera. It also includes southern corn rootworm (*Diabrotica undecimpunctata howardi*) (hereinafter referred to as "SCRW"), species closely related to target pests, WCRW and WCRW. The insecticidal activity against these insects was confirmed by bioassay after feeding for 12 days.

The dsRNA of the *DvSnf7* gene fragment synthesized *in vitro* was tested for dietary administration, and it was tested in the concentration range up to 5,000 ng / mL diet. Compared with the concentration of dsRNA in the *DvSnf7* gene fragment, this concentration is about higher by double to triple digits in this Genetically Modified Maize (Table 10, p. 62-63). Thus, this bioassay was designed to continuously administer dsRNA of *DvSnf7* gene fragment with sufficient administration period to evaluate the possibility if the dsRNA of the DvSnf7 gene fragment affects the growth, development and survival of non-target pests.

As a result, there was no effect on the growth, development and survival of the *DvSnf7* gene fragment by dsRNA for five species of Coleoptera, including Colorado potato beetle (Leptinotarsa decemlineata) (hereinafter referred to as "CPB") as non-target insects, Lepidoptera, Hymenoptera and Hemiptera. It was confirmed that dsRNA of the *DvSnf7* gene fragment exhibits activity only against WCRW and SCRW belonging to the subfamily Galerucinae, Family Chrysomelidae of Coleoptera. LC_{50} (half lethal concentration) levels of WCRW and SCRW were 4.4 ng / mL diet and 1.2 ng / mL diet, respectively (Table 2, p.20; Table 3, p.32 of Appendix 7). Also, there are 12 subfamilies in total, including the subfamily Hymenoptera and the subfamily Chrysomelinae. The subfamily Galerucinae to which WCRW belongs is known to be closest to the subfamily Chrysomelinae to which CPB belongs of all subfamilies of

Phylogenetically, Family Chrysomelidae (Gómez-Zurita et al., 2007).

From these results, dsRNA of the *DvSnf7* gene fragment has an extremely narrow insecticidal activity spectrum; among Coleoptera species, it is supposed to be limited to insects belonging to the subfamily Galerucinae, Family Chrysomelidae. Out of typical 14 insect species belonging to 10 families, 4 orders, Coleoptera, Lepidoptera, Hymenoptera and Hemiptera, bioassayed this time, 10 homogeneous or congeneric species inhabit in Japan (Table 2, p20); however, a target insect, CRW, does not inhabit Japan (Nakane et al., 1963).

Table 2. Insecticidal spectrum of dsRNA of *DvSnf7* gene fragment (susceptibility of various insects to the relevant dsRNA) [8]

Order	**Family**	**The subfamily**	**Academic name**	**LC_{50}[a] or maximum concentration[b] (ng/mL diet)**	Insecticidal activity*	**Inhabitance in Japan**
Hemiptera	Anthocoridae	The subfamily Anthocorinae	*Orius insidiosus*	5,000[b]	No	Congeneric species available
Hymenoptera	Eulophidae	The subfamily Entedoninae	*Pediobius foveolatus*	3,000[b]	No	Congeneric species available
	Pteromalidae	The subfamily Pteromalinae	*Nasonia vitripennis*	5,000[b]	No	Available
Lepidoptera	Noctuidae	The subfamily Noctuinae	*Spodoptera frugiperda*	500[b]	No	Congeneric species available
		The subfamily Heliothinae	*Helicoverpa zea*	5,000[b]	No	Congeneric species available
	Crambidae	The subfamily Pyraustinae	*Ostrinia nubilalis*	5,000[b]	No	Congeneric species available
	Bombycidae	The subfamily Bombycinae	*Bombyx mori*	5,000[b]	No	Available
Coleoptera	Carabidae	The subfamily Harpalinae	*Poecilus chalcites*	5,000[b]	No	Congeneric species available
	Coccinellidae	The subfamily Coccinellidae (Coccinellinae)	*Coleomegilla maculate* (A kind of ladybird))	3,000[b]	No	×
		The subfamily Epilachninae	*Epilachna varivestis*	3,000[b]	No	Congeneric species available
	Tenebrionidae	The subfamily Tenebrioninae	*Tribolium castaneum*	5,000[b]	No	Available
	Family Chrysomelidae	The subfamily Chrysomelinae	**CPB** (*Leptinotarsa decemlineata*)	5,000[b]	No	×
		The subfamily Galerucinae	**SCRW** (*Diabrotica undecimpunctata howardi)*	1.2[a]	Yes	×
			WCRW (*Diabrotica virgifera virgifera)*	4.4[a]	Yes	×

* We used bioassay to study sensitivity of each insect against the dsRNA through diet administration for 12 days. It showed insecticidal activity only for species belonging to the subfamily Galerucinae, Family Chrysomelidae of Coleoptera (Appendix 7).

[a] LC_{50}= half lethal concentration; [b] maximum dose administered

In addition to the 14 kinds of insects bioassayed by direct feeding of dsRNA of the *DvSnf7* gene fragment as described above, a more detailed insecticidal spectrum was examined by indirectly studying the insecticidal activity of the dsRNA of the *DvSnf7* gene fragment against 5 species (*Acalymma vittatum, Ceratoma trifurcate, Galerucella calamriensis, Microtheca ochroloma* and *Chrysolina quadrigemina*; Table 3, p.23) belonging to The subfamily Galerucinae and The subfamily Chrysomelinae, close to WCRW(Bachman et al., (2013) (Attachment 3))。

Because the breeding method for bioassay has not been established for the above mentioned five insects, it is impossible to carry out the bioassay by directly feeding diet. Instead of giving the dsRNA of the *DvSnf7* gene fragment directly to these five species, we gave WCRW whose breeding method has been already established to the dsRNA of the *Snf7* gene fragment and observed insecticidal activity on WCRW. This aims to examine the possibility of insecticidal activity of dsRNA of *DvSnf7* gene fragment against these five species.

In regards to the indirect feeding administration test method for predicting insecticidal activity against insect species for which breeding methods, there are some studies already reported in the literature (Baum et al., 2007; Whyard et al., 2009; Burand and Hunter, 2013). We provided the indirect diet administration test, by giving dsRNA of Snf7 gene fragment of each other to WCRW and CPB to examine this method (Bachman et al., (2013) (Attachment 3)). As shown in Table 2 (p.20), the direct feeding test has demonstrated that the dsRNA of the *DvSnf7* gene fragment has insecticidal activity against WCRW, while it has no insecticidal activity against CPB. As a result of the indirect feeding test, the insecticidal activity was observed when the dsRNA of the *DvSnf7* gene fragment was given to WCRW. However, insecticidal activity was not observed when dsRNA of *Snf7* gene fragment derived from CPB (*LdSnf7* gene fragment) was given (Bachman et al., (2013) (Fig. 1) (Attachment 3)). On the other hand, insecticidal activity was observed when dsRNA of LdSnf7 gene fragment was given to CPB, while no insecticidal activity was found when the dsRNA of the *DvSnf7* gene fragment was given (Bachman et al., (2013) (Fig. 1 of Attachment 3)). Since this result was consistent with the results of the direct feeding test, it was assumed that insectidal activity by dsRNA of the *DvSnf7* gene fragment in insects whose breeding method has not been established might be studied using this indirect feeding test.

In this study, among the *Snf7* genes of *A. vittatum*、*C. trifurcate*、*M. ochroloma*、*G. calamriensis and C. quadrigemina,* we identified the sequence corresponding to the *DvSnf7* gene fragment derived from WCRW using nucleotide sequence analysis. Next, we prepared dsRNA with the sequence most highly homologous to the *DvSnf7* gene fragment in vitro, and provided the dsRNA to the WCRW at a concentration of up to 5,000 ng / mL diet for 12 days.

As a result of the test, when we provided dsRNA of *Snf7* gene fragment derived from *A. vittatum, C. trifurcate*, and *G. calamriensisbe,* belonging to The subfamily Galerucinae to WCRW, the survival rates were 8%, 15% and 13 %, respectively, exhibiting insecticidal activity against WCRW (Table 3, p.23; Table 3 of Bachman et al., (2013) (Attachment 3)). On the other hand, when dsRNA of *Snf7* gene fragment derived from M. ochroloma and C. quadrigemina belonging to The subfamily Chrysomelinae was given to WCRW, their survival rates were 72% and 88%, showing no insecticidal activity against WCRW (Table 3, p.23; Table 3 of Bachman et al., (2013) (Appendix 3)). From these results, the dsRNA of the *DvSnf7* gene fragment was thought to exhibit insecticidal activity to *A. vittatum, C. trifurcate* and *G. calamriensis*, belonging to the same The subfamily Galerucinae (Family Chrysomelidae) as WCRW. However, even belonging the same Family Chrysomelidae, it was thought to have no insecticidal activity against *M. ochroloma* and *C. quadrigemina* belonging to the subfamily Chrysomelinae.

The above result also agrees with the result from the direct feeding test (Table 2, p.20; Table 3, p.39 of Appendix 7) on the insecticidal spectrum of the dsRNA of the *DvSnf 7* gene fragment, supporting the idea that the insecticidal spectrum of the dsRNA of the *DvSnf 7* gene fragment is limited to the subfamily Galerucinae, Family Chrysomelidae of Coleoptera.

Table 3 Results of dietary feeding test of WSWW with dsRNA of five types of Snf7 gene fragments belonging to The subfamily Galerucinae and The subfamily Chrysomelinae for 12 days[9]

Name of insect used for synthesis of Snf7 gene fragment (The subfamily name)	**Administration concentration (ng/mL)**	**Control test number**	**Control survival population**	**Control [1]survival rate (%)**	**Number of dsRNA treatment assay**	**Number of dsRNA treated survival populations**	**DsRNA treated [2] survival rate (%)**	**P value[3]**	**Insecticidal activity**	**Length of 21 base compatible/incompatible[4]**
Acalymma vittatum (The subfamily Galerucinae)	1,000	96	86	90	101	8	8	<0.0001	Active	Compatible
Ceratoma trifurcata (The subfamily Galerucinae)	500	106	81	76	108	16	15	<0.0001	Active	Compatible
Galerucella calamriensis (The subfamily Galerucinae)	5,000	89	79	89	93	12	13	<0.0001	Active	Compatible
Microtheca ochroloma (The subfamily Chrysomelinae)	500	106	81	76	106	76	72	0.5311	Not Active	Incompatible
Chrysolina quadrigemina (The subfamily Chrysomelinae)	5,000	103	97	94	103	91	88	0.2165	Not Active	Incompatible

[1] WCRW fed with daRNA-free feed

[2] WCRW fed with dsRNA of various *Snf7* gene fragments

[3] Statistical treatment was performed on the numbers of surviving individuals and non-viable individuals in the dsRNA treated group of *Snf7* gene fragment and the control group using Fisher's exact ratio test (significant at $p < 0.05$).

[4] Existence/non-existence of a sequence consistent with the length of 21 bases from the sequence of the *DvSnf 7* gene fragment in the *Snf7* gene fragment sequence derived from five species shown in the table.

[9] Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

2. Factors that determine specific insecticidal activity of the dsRNA of the *DvSnf7* gene fragment against the target insect

a. Sequence specificity of the *DvSnf 7* gene fragment to the gene in the target insect

As described in the previous section, it was revealed that the dsRNA of the *DvSnf7* gene fragment exhibits insecticidal activity only to insects belonging to the subfamily Galerucinae (Family Chrysomelidae, Coleoptera). One of the factors determining the specificity of the dsRNA of the *DvSnf7* gene fragment to the target insect is the specificity to the endogenous gene sequence within the target insect. The *Snf7* gene, a targeted endogenous gene for the present study, is widely kept among eukaryotes (Tu et al., 1993; Gao et al., 1999; Peck et al., 2004; Winter and Hauser, 2006; Lee et al., 2007; Kim et al., 2011). It has been reported that, even when targeting genes that are kept among many species, specific insecticidal activity against target insects will be available by designing dsRNA sequences focusing on diversity of endogenous gene sequences (Whyard et al., 2009).

The *DvSnf7* gene is composed of 4 exons and 3 introns, and its open reading frame has a length of 663 bp (Appendix 8, Figure 1). The nucleotide sequence constituting the dsRNA of the *DvSnf7* gene fragment expressed in this Genetically Modified Maize is a sequence from exons 1 to 3 of this *DvSnf7* gene, corresponding to the 151st to 390th sequence of the open reading frame (Appendix 8 in Figure 1)

This sequence has high homology among *Snf7* genes of insects belonging to the subfamily Galerucinae (Family Chrysomelidae, Coleoptera); while selecting a site with low homology with the *Snf7* gene of other organisms.

We performed bioinformatics analysis in order to study the degree of homology between the sequence of the *DvSnf7* gene fragment and the sequence of the *Snf7* gene of the same Coleoptera insect as WCRW (Table 4, p.26; Table 5, p.29; Appendix 10) (Bachman et al., (2013) (Attachment 3)). Details are described below.

We selected nine species of insects (Coleoptera) which are

phylogenetically related to WCRW and identified the sequence corresponding to the WCRW-derived *DvSnf7* gene fragment in the *Snf7* gene inherent in these insects using nucleotide sequence analysis (Table 2 in Bachman et al., (2013) (Attachment 3)). Next, we studied the homology between these sequences and the sequence of the *DvSnf7* gene fragment (Bachman et al., (2013) (Attachment 3)). As a result, the sequence identified from nine closely related insects has a lower homology with the sequence of the *DvSnf7* gene fragment as it becomes phylogenetically distant from WCRW. Even insects belonging to Family Chrysomelidae (Chrysomelidae) revealed clear differences between subfamilies (Table 4, p. 26) (Bachman et al., (2013) (Attachment 3), Table 2). It has been also reported that sequence homology of more than 21 consecutive bases is required for inducing RNAi by dsRNA ingested orally in insects belonging to The subfamily Galerucinae (Bolognesi et al., (2012) (Appendix 1); Bachman et al., (2013) (Attachment 3)). Even the *Snf7* gene of insects of The subfamily Chrysomelinae, closest to The subfamily Galerucinae, does not have a sequence that is 21 bases length compatible to the *DvSnf7* gene fragment (Table 4, p.33) (Bachman et al., (2013) (Attachment 3)). These results are consistent with the results of the bioassay to examine the insecticidal activity of the dsRNA of the above mentioned *DvSnf7* gene fragment (Table 4, p. 26; Appendix 7) (Bachman et al., (2013) (Appendix 3)).

In the sequence of *Snf7* gene of insect species closest to the subfamily Galerucinae (Table 4, p 26) studied by Bachman et al. (2013), there were sequences consistent with the *DvSnf7* gene fragment up to 19 base length, but sequences that coincided consecutively up to 20 base length were not confirmed. Therefore, in order to examine the insecticidal activity of the *DvSnf7* gene fragment against the insect having the *Snf7* gene sequence which is consistent with the *DvSnf7* gene fragment up to 20 base length, we studied insecticidal activity by indirect feeding test using the dsRNA synthesized from the *Snf7* gene of *Drosophila pseudoobscura* (Table 5, p 29) of the fly (Diptera) having one match with the *DvSnf7* gene fragment by 20 bases, (Appendix 9). After the 12-day indirect feeding test where survival rate and body weight of WCRW were investigated, no influence on WCRW survival rate and weight was observed (Appendix 9).

Thus, it was inferred that homology of sequences of 21 or more

consecutive bases is required for dsRNA of the *DvSnf 7* gene fragment to induce RNAi.

The classification of subfamilies belonging to Family Chrysomelidae has been determined using molecular phylogenetic analysis comparing RNA sequences of small ribosome units that are highly kept among most organisms (FIG. 5, p 27) (Clark et al., 2001; Gomez-Zurita et al., 2007; Hunt et al., 2007; Gillespie et al., 2008).Because the classification determined using this molecular phylogenetic analysis was also consistent with the result of the homology search with the sequence of the *DvSnf7* gene fragment shown in Table 4, it was supposed that, systematically, the closer it is to WCRW, the lower the homology with the *DvSnf7* gene of the *Snf7* gene, less likely to show consecutive matching of more than 21 bases (Fig. 5, p. 27).

Table 4 Results of comparing the sequence of the *DvSnf7* gene fragment and the sequence of the Snf7 gene fragment internal to the insect species belonging to Family Chrysomelidae (Coleoptera) and Family Tenebrionidae, and their availability of insecticidal activity by bioassay[10]

Name of order, family and the subfamily	**Name of insect species that identified the sequence of *Snf7* gene fragment**	**Homology (%) to *DvSnf7* gene fragment sequence**	**Compatible/incompatible of 21 base length[1]**	**Insecticidal activity [2]**
Coleoptera Family Chrysomelidae Subfamily Galerucinae	**WCRW**	100.0	221	Yes
	SCRW	98.8	186	Yes
	A. vittatum	95.0	69	Yes[4]
	C. trifurcata	90.8	18	Yes[4]
	G. calamriensis	90.8	3	Yes[4]
Coleoptera Family Chrysomelidae Subfamily Chrysomelinae	**CPB** (*Leptinotarsa decemlineata*)	78.3	0, (14 base)[3]	None
	C. quadrigemina	82.1	0, (19 base)[3]	None[4]
	M. ochroloma	79.6	0, (19 base)[3]	None[4]
Coleoptera Family Tenebrionidae Subfamily Tenebrionidae	*Tribolium castaneum*	72.1	0, (11 base)[3]	None

[1] Number of sequences matching the DvSnf7 gene fragment sequence by the length of 21 base in the Snf7 gene fragment sequence of various origin in the table

[2] For insects whose insecticidal activity of the dsRNA of the DvSnf7 gene fragment was examined in the bioassay (Table 2, p.20; Appendix 7), the presence or absence of insecticidal activity was also indicated as a reference.

[3] The length of the longest sequence obtained by bioinformatics analysis was described when the species Snf7 gene sequence in the table did not contain an exact match of 21 bases in length with the DvSnf7 gene fragment sequence. Although Bachman et al. (2013) did not verify whether the 20 base length match shows insecticidal activity against insects, there was no 20 base length match in the species Snf7 gene sequence in the table.

[4]For these insect species, since the rearing method in the laboratory has not been established, it is difficult to perform bioassay by feeding dietary dsRNA. Therefore, the presence or absence of susceptibility of these insects to the relevant dsRNA was examined by indirect feeding test (Table 3, p23) (Bachman et al., (2013) (attached document 3), Table 3).

[10]Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

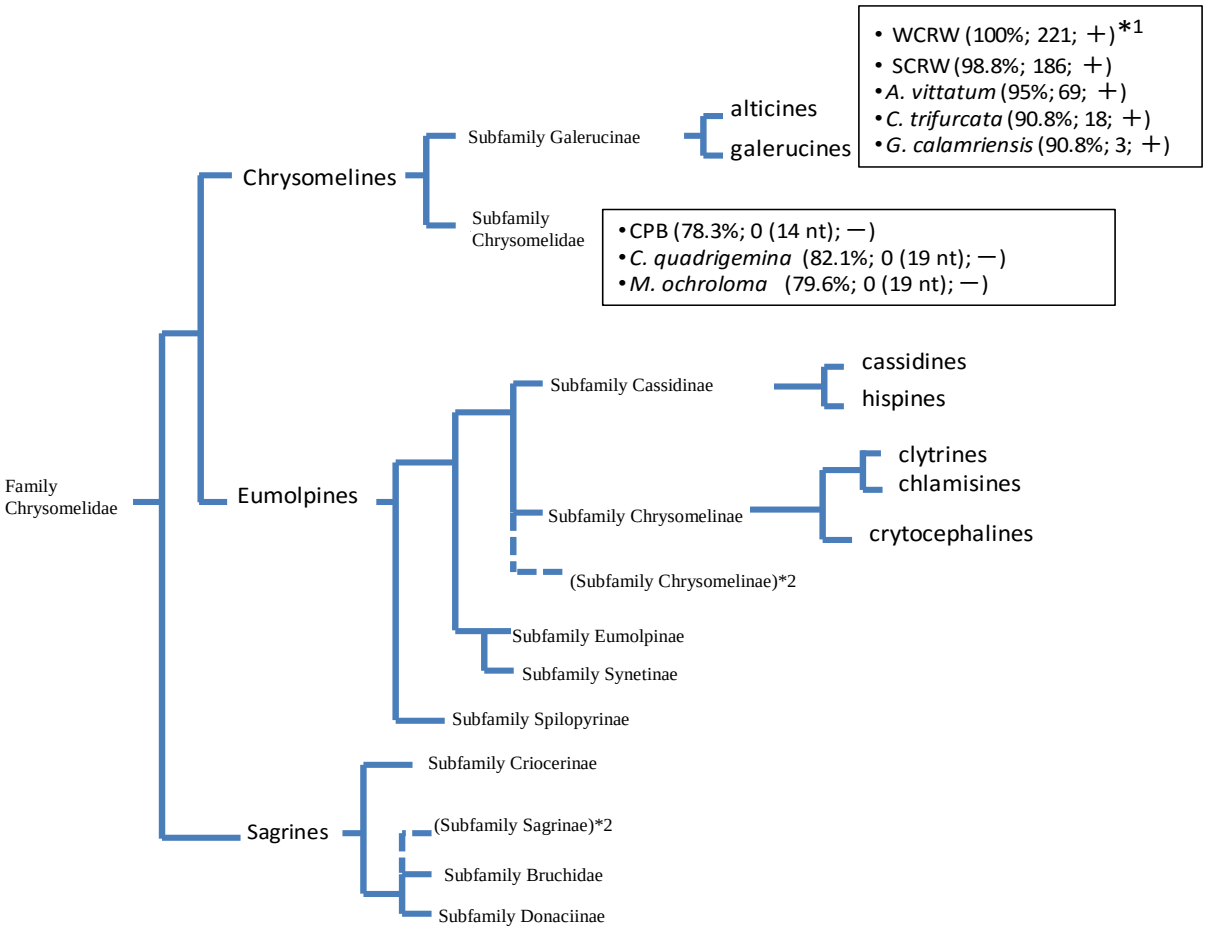

FIG.5 Strain diagram of the subfamily belonging to the Family Chrysomelidae based on Gomez-Zurita et al. (2007)[11]

*1 In parentheses, from the left, the homology (%) with the *DvSnf7* gene fragment, the number of the 21 base length match (the longest number of bases in the case of 0) and the insecticidal activity (+ or -) are described.

*2 According to the report of Gomez-Zurita et al. (2007), although the subfamily Chrysomelinae and the subfamily Sagrinae were not actually investigated, it is thought that they are related to the subfamily Chrysomelinae and the subfamily Bruchidae, respectively.

[11]Responsibility for the rights and contents pertaining to the information described in this figure belongs to Monsanto Japan Limited.

In fact, besides Family Chrysomelidae (Coleoptera), we chose Family Tenebrionidae (Coleoptera), and Lepidoptera which are closely related to Coleoptera, and Hymenoptera, Diptera, Hemiptera and Orthoptera (Savard et al., 2006), as well as 18 representative arthropods of each order in Order Daphnia, and identified sequence corresponding to the *DvSnf7* gene fragment derived from WCRW in *Snf7* gene inherent in these species based on publicly available genome database information[12] (Appendix 10). Then, the homology between these sequences and the sequence of the *DvSnf7* gene fragment was examined (Appendix 10).

As a result, the sequence of *Snf7* gene fragments of these species was at the most 73% homologous to the sequence of the *DvSnf7* gene fragment, and no compatibility was found between the *DvSnf7* gene fragment and the 21-base length (Table 5, p. 29; Table 1, p 7 of Appendix 10).

In addition to the representative three species (*Tribolium castaneum, Bombyx mori* and *Nasonia vitripennis*) of each of the 18 species used for this study, Coleoptera, Lepidoptera and Hymenoptera, it has been confirmed that *Orius insidiosus,* which is a representative species of the order Hemiptera, does not show the sensitivity of the *DvSnf7* gene fragment to dsRNA by the aforementioned bioassay (Table 2, p.20; Table 5, p. 29).

From the results of the above two bioinformatics analyzes (Table 4, p.26; Table 5, p.29), the sequence of the *DvSnf7* gene fragment derived from WCRW has extremely high homology among *Snf7* genes of insects belonging to the subfamily Galerucinae (Family Chrysomelidae, Coleoptera). It was confirmed that homology was low even for the same Family Chrysomelidae as the *Snf7* gene of other insects. Furthermore, the results of these bioinformatics analyzes supported the result that, as indicated in the aforementioned bioassay, "the dsRNA of the *DvSnf7* gene fragment shows insecticidal activity specific only to insects belonging to the same The subfamily Galerucinae (Family Chrysomelidae) as the target insect WCRW."

[12] As of November 2012, this is the base sequence information obtained from the following sequence database; the Whole Genome Shotgun (WGS) (WGS) (http://www.ncbi.nlm.nih.gov/Traces/wgs/) published by National Center for Biotechnology Information (NCBI) and UniGene (ftp : //ftp.ncbi.nih.gov/repository/UniGene/).

Table 5. Comparison of sequences of the *Snf7* gene fragment of the endogenous *Snf7* gene fragment of the seventeen insects belonging to the seven species except Family Chrysomelidae (Coleoptera) and the presence or absence of insecticidal activity by bioassay[13]

Order names	Species name that identified the sequence of *Snf7* gene fragment	Homology with the sequence of the *DvSnf7* gene fragment (%)	Compatibility of 21 base length[1]	Insecticidal activity [2]
Coleoptera	*Tribolium castaneum*[3]	72.4[4]	0, (11 base)	None
Lepidoptera	*Bombyx mori*[3]	66.0	0, (15 base)	None
Lepidoptera	*Danaus plexippus*	65.7	0, (10 base)	-
Lepidoptera	*Manduca sexta*	61.9	0, (8 base)	-
Hymenoptera	*Apis mellifera*[3]	72.5	0, (13 base)	-
Hymenoptera	*Bombus terrestris*	72.9	0, (12 base)	-
Hymenoptera	*Megachile rotundata*	65.1	0, (11 base)	-
Hymenoptera	*Nasonia vitripennis*[3]	71.2	0, (14 base)	None
Hymenoptera	*Solenopsis invicta*	66.9	0, (18 base)	-
Diptera	*Aedes aegypti*	68.4	0, (11 base)	- [5]
Diptera	*Anopheles gambiae*[3]	67.5	0, (12 base)	- [5]
Diptera	*Drosophila melanogaster*[3]	72.6	0, (9 base)	- [5]
Diptera	*Drosophila pseudoobscura*	70.5	0, (20 base)	None[5, 6]
Diptera	*Drosophila sechellia*	70.4	0, (12 base)	- [5]
Diptera	*Drosophila yakuba*	59.1	0, (8 base)	- [5]
Hemiptera	*Acyrthosiphon pisum*[3]	72.5	0, (12 base)	-
Orthoptera	*Locusta migratoria*	70.9	0, (12 base)	-
Cladocera	*Daphnia pulex* (Cladocera)	67.5	0, (14 base)	-

[1] In the *Snf7* gene fragment sequences of various kinds in the table, the number of sequences coinciding with the length of the *DvSnf7* gene fragment sequence by 21 bases was shown. In addition, when the *Snf7* gene sequence of the species in the table does not contain an exact match of 21 base length with the *DvSnf7* gene fragment sequence, the longest sequence length is described.

[2] Regarding insects whose insecticidal activity of the dsRNA of the *DvSnf7* gene fragment was examined in the bioassay (Table 2, p.20; Appendix 7), the presence or absence of insecticidal activity was also indicated as a reference. In addition, for those not subjected to bioassay, they were written as "-".

[3] The model insect species (Savard et al., 2006) used by Savard et al. (2006) to study phylogenetic relationships

[4] Due to the homology calculated using publicly available databases, there are slight differences from the values described in Table 4 (p.33) (Table 2 of Bachman et al., (2013) (Appendix 3)).

[5]Drosophila spp. (Diptera) do not show sensitivity to dsRNA when ingesting only dsRNA. However, it is known that when larvae are immersed in a solution containing dsRNA encapsulated in cationic liposomes, they are sensitive to dsRNA (Whyard et al., 2009).

[6] With respect to *D. pseudoobscura,* it is difficult to perform bioassay by feeding dsRNA, so the presence or absence of susceptibility to the dsRNA was examined using indirect feeding test (Appendix 9).

[13]Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

b. Other conditions for orally ingested dsRNA to show insecticidal activity against insects

In addition to sequence specificity, there are physical and biochemical barriers preventing the insecticidal activity of orally ingested dsRNA against insects. There are various barriers depending on the order to which the insect belongs. For example, such barriers include degradation of dsRNA, mechanism of uptake of dsRNA into cells due to diet by oral or midgut nucleolytic enzymes, and the degree of sensitivity to orally ingested dsRNA (Furusawa et al., 1993; Arimatsu et al., 2007a; Arimatsu et al., 2007b; Rodriguez-Cabrera et al., 2010; Terenius et al., 2011; Liu et al., 2013; Luo et al., 2013; Christiaens et al., 2014; Wynant et al., 2014). With these barriers, not all insects are sensitive to orally ingested dsRNA (Huvenne and Smagghe, 2010).

Coleoptera insects

It has been reported that Coleoptera insects exhibit relatively high sensitivity to orally ingested dsRNA (Huvenne and Smagghe, 2010) (Baum and Roberts, (2014) (Appendix 5)). Baum et al. conducted experiments to suppress specific genes against insects of the order Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera using dsRNA. It showed that gene expression is suppressed with much lower concentration of dsRNA for insects of Order Coleoptera compared with other orders (FIG. 6, p.33) (Baum and Roberts, (2014) (Attachment 5)). With respect to CRW in particular, it has been confirmed that dsRNA is not degraded in the midgut extract, which is presumed to be one of the factors showing strong susceptibility to orally ingested dsRNA (Baum and Roberts , (2014) (Attachment 5)). Furthermore, it was confirmed that dsRNA ingested orally ingested in WCRW reached the midgut without being degraded (Ivashuta et al., 2015). On the other hand, in order to suppress gene expression through oral ingestion, insects belonging to orders other than Coleoptera it required approximately 1,000 times more dsRNA than the concentration administered to Coleoptera (Baum and Roberts, (2014) (Appendix 5)). However, not all insects belonging to Coleoptera are highly susceptible to orally ingested dsRNA, even Coleoptera insects have low susceptibility to dsRNA ingested like *T.*

castaneum (Whyard et al., 2009) or species such as *Anthonomus grandis* aerialmost insensitive (Baum et al., 2007; Whyard et al., 2009). Also, Baum et al. suggested that the midgut intestinal environment (particularly the midgut intracellular degrading enzyme and pH) is important for the stability of orally ingested dsRNA and is also associated with high sensitivity, When they performed an experiment in which an extract from WCRW midgut was added to the dsRNA (400 bp) of the gene encoding V-ATPase[14] under two conditions of pH 7.4 and pH 10.5[15], it showed that dsRNA under these two pH conditions remained unresolved (Baum and Roberts, (2014) (Attachment 5)).

Lepidopteran insects

The sensitivity of Lepidopteran insects to dsRNA varies, and it is reported that it is necessary to ingest higher concentrations of dsRNA compared to Coleoptera insects in order to show insecticidal activity in Lepidoptera insects (Huvenne and Smagghe, 2010; Terenius et al., 2011) (Baum and Roberts, (2014) (Attachment 5)). Baum et al. also reported that dsRNA (400 bp) of the gene encoding V-ATPase, an essential protein, was supplemented with an extract from the midgut of the fall armyworm in two species of pH 7.4 and pH 10.5 (Baum and Roberts, (2014) (Attachment 5)). However, they reported that, in the case of the extract from the midgut of the fall armyworm, the dsRNA was degraded under two pH conditions and was rapidly degraded particularly at alkaline pH 10.5, which was a general pH in the midgut of Lepidoptera insects (Baum and Roberts, (2014) (Attachment 5)). Also, a nucleolytic enzyme which degrades dsRNA in the blood lymph of Lepidoptera tinctora (*Manduca sexta*) has been found (Garbutt et al., 2013).

Diptera and Hymenoptera insects

There are also variations in susceptibility to dsRNA of insects belonging to Diptera and Hymenoptera. It has been reported that higher concentrations of dsRNA should be ingested compared with Coleoptera insects in order to show insecticidal activity in the insects of Diptera and

[14] It exists in many biological membranes of eukaryotes and is an enzyme that regulates the pH inside and outside the membrane by transporting hydrogen ions (Whyard et al., 2009).
[15] It is known that pH 7.4 and pH 10.5 are typical pH values in the midgut of Coleoptera insects and Lepidoptera insects (Terra, 1990).

Hymenoptera (Figure 6, p.33) (Coy et al., 2012; Singh et al., 2013; Vander Meer and Choi, 2013; Zhao and Chen, 2013). In addition, *Drosophila spp*. of Diptera do not show sensitivity to dsRNA when oral ingestion of dsRNA alone, and it is known that when larvae are immersed in a solution containing dsRNA encapsulated in cationic liposomes, they are sensitive to dsRNA (Whyard et al., 2009).

Hemiptera insects

The susceptibility of insects to dsRNA also varies, and in order to show insecticidal activity in the order of the Hemiptera insects, it is necessary to ingest higher concentrations of dsRNA compared to the insects belonging to Coleoptera (Figure 6, p.33) (Chen et al., 2010; Li et al., 2011 a; Wuriyanghan et al., 2011; Mao and Zeng, 2012) (Baum and Roberts, (2014) (Appendix 5)). In a recent study on *Lygus lineolaris*, Hemiptera, it has been reported that gene expression is not suppressed even when Lygus lineolaris orally ingest dsRNA as nucleolytic enzymes present in saliva decompose dsRNA in a short time (Allen and Walker, 2012). Also, Christiaens et al. (2014) reports that there are nucleolytic enzymes that degrade dsRNA not only in saliva but also in hemolymph for an insect belonging to Aphididae spp. (Hemiptera).

Other insects

Insects belonging to Orthoptera have a nucleolytic enzyme which degrades dsRNA in the midgut and this nucleolytic enzyme is thought to function as a barrier to the insecticidal activity of orally ingested dsRNA. However, it has been reported that dsRNA directly injected into the body causes gene suppression or death to insects belonging to Orthoptera by (Wynant et al., 2012; Kwon et al., 2013; Luo et al., 2013).

From the above findings, it was considered rare that, as seen in insects like Coleoptera, orally ingested dsRNA is efficiently taken up in midgut cells of without being degraded. Furthermore, even among insects of Coleoptera, it has been revealed that some species show low or no susceptibility to orally ingested dsRNA.

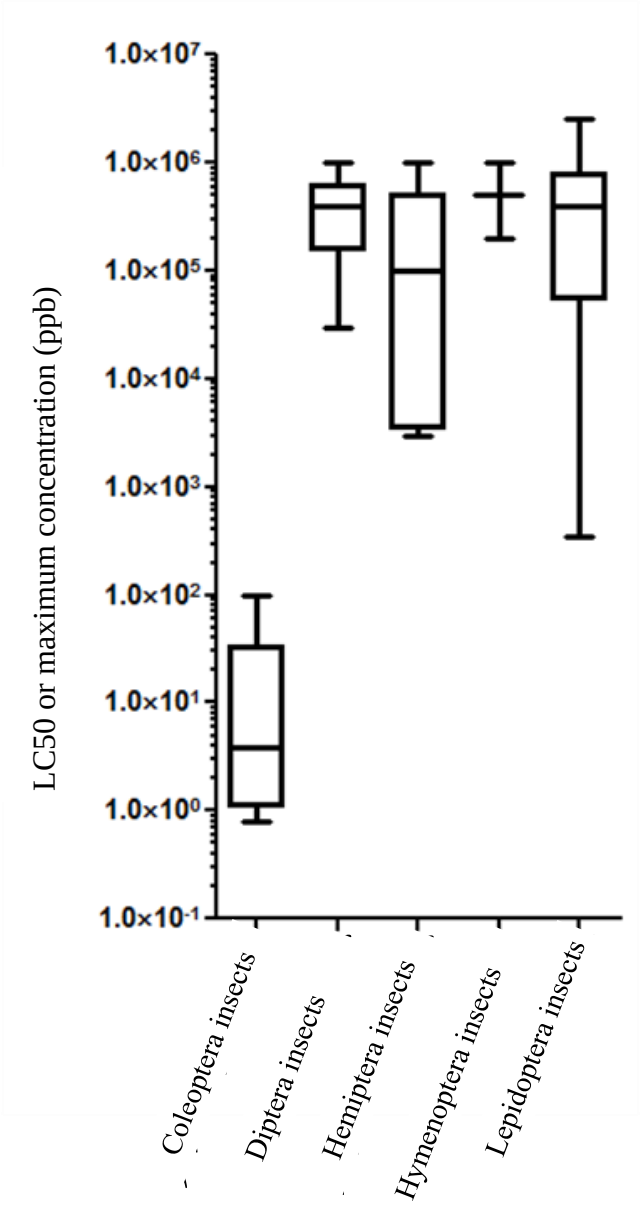

FIG. 6 Comparison of susceptibility to orally ingested dsRNA among Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera insects (Baum and Roberts, (2014) (Appendix 5)) [16]

Each box represents the LC_{50} or the first quartile (25% quantile) of the administered concentration up to the third quartile (75% quantile), and the line in the box represents the median. Each plot extending from the box extends to a minimum or maximum value.

[16] Responsibility for the rights and contents pertaining to the information described in this figure belongs to Monsanto Japan Limited.

3. Summary of specificity of dsRNA for *DvSnf7* gene fragment to target insect

In order to investigate the insecticidal activity spectrum of the dsRNA of the *DvSnf7* gene fragment, we examined the insecticidal activity against 14 kinds of insects selected on the basis of the taxonomic relationship with WCRW. As a result, it was confirmed that the dsRNA of the *DvSnf7* gene fragment was active only in insects belonging to The subfamily Galerucina, Family Chrysomelidae of Coleoptera (1-2 - (1) - B - ii - iii - 1, p.18 - 23).

The following summary shows routes for the dsRNA of the orally ingested *DvSnf7* gene fragment which demonstrates insecticidal activity specific to WCRW.

The dsRNA of the *DvSnf7* gene fragment:

① Through oral ingestion of the genetically modified maize, enters the digestive organs of WCRW and reaches the midgut (1 - 2 - (1)-B-ii, p.9 - 17).

② Is efficiently incorporated into midgut cells of WCRW (1 - 2- (1) - B - 2 - ii, p.9 - 17).

③ Inhibits the expression of the target DvSnf7 gene through the sequence-specific RNAi mechanism and shows insecticidal activity against WCRW (1- 2 - (1) - B - 2 - iii - 2 - a, p.24 - 29).

At this stage ③, as the sequence of the dsRNA of the *DvSnf7* gene fragment has a match of 21 bases or more contiguous with the *Snf7* gene of the target insect belonging to The subfamily Galerucinae, Chrysomelidae, It suppresses the expression of these *Snf7* genes through the RNAi mechanism (1 - 2- (1) - B - 2 - iii - 2 - a, p.24 - 29).

Regarding non-target insects other than Family Chrysomelidae, the dsRNA of the *DvSnf7* gene fragment decomposes before it reaches midgut cells or it is degraded in midgut cells (1 - 2 - (1) - B- 2 - iii - 2 - b, p.30 - 33) at the stage of ① and ②, due to not being efficiently incorporated into the *DvSnf7* gene fragment. Thus, it is considered that dsRNA has low possibility to show insecticidal activity.

Moreover, even if it meets the conditions of ① and ② and is taken in midgut cells, at the stage of ③, it is unlikely that the dsRNA has enough homology to exhibit insecticidal activity with the *Snf7* gene sequence of non-

target insects other than the family Vermillaceae (Family Chrysomelidae) (1-2-(1) - B - 2 - Iii - 2 - a, p,24 - 29). It is also unlikely that *Snf7* gene expression will be suppressed.

Also, various factors are assumed to affect the induction of RNAi by orally ingested dsRNA, such as the length of dsRNA, systemic spread of RNAi, selection of a targeted gene, dsRNA sequence, the amount of dsRNA taken, the mechanism relating to the sustainability of gene expression suppression, the growth stage of insects, etc.(1 - 2 - (1) - B - 2 - ii, p.9 - 17).

In addition to the above points, as described in the Section 2, it is necessary to be exposed to the dsRNA through oral ingestion of the genetically modified maize for non-target insects other than those belonging to Chrysomelidae to be affected by the dsRNA of the *DvSnf7* gene fragment expressed in this Genetically Modified Maize.

From the above fact, the insecticidal activity spectrum of the dsRNA of the *DvSnf7* gene fragment expressed in this Genetically Modified Maize is extremely narrow. We concluded that it is limited to some insects belonging to Chrysomelidae among all insects of Coleoptera.

iv. Effects of dsRNA of *DvSnf7* gene fragment on soil microorganism

As mentioned above, the insecticidal activity spectrum of the dsRNA of the *DvSnf7* gene fragment expressed in the genetically modified maize is extremely narrow, and it was concluded that it was limited to, among the insects of Coleoptera, some insects belonging to Family Chrysomelidae.

In order to confirm that the dsRNA of the *DvSnf7* gene fragment does not show unintentional activity against the organisms living in the soil, we conducted 1. homology search between the sequence of the DvSnf7 gene fragment and the seven eukaryotic gene sequences living in the soil, and 2. soil microbial phase test on soil cultivated the genetically modified maize.

1. Homology search between the sequence of the *DvSnf7* gene fragment and the seven eukaryotic gene sequences in the soil

In conducting this analysis, we selected 7 species of eukaryotes that live in the soil (Table 6, p.38; Table 1, p.4 of Appendix 11) based on the taxonomy and the role in the soil ecosystem. Each of these eukaryotic organisms is systematically distant and is typical species of ameba, algae or fungi, which accounts for the majority of eukaryotes inhabiting the soil. In addition, these seven species play important and diverse roles in soil ecosystems as described in Table 6 (p.38).

In this analysis, we first identified the sequence corresponding to the *DvSnf7* gene fragment in the endogenous *Snf7* gene of seven eukaryotic organisms using publicly available Genome Database Information [1]. Then, we examined homology between these sequences and the sequence of the *DvSnf7* gene fragment (Appendix 11). As a result, the sequences of these seven eukaryotic *Snf7* gene fragments did not show more than 53.8% homology with the sequence of the *DvSnf7* gene fragment. In addition, we also confirmed that there was no sequence that matched with the *DvSnf7* gene fragment with 21 base length in the same database (Table 6, p38; Table 5, p.9 of Appendix 11).

Therefore, the above fact suggested that the dsRNA of the *DvSnf7* gene fragment does not induce RNAi in these 7 eukaryotic organisms.

Fungi, which are eukaryotic organisms, have an RNAi mechanism (Dang et al., 2011). However, prokaryotic bacteria, etc. have a defense mechanism called a CRISPR / Cas system which is different from the RNAi mechanism (van der Oost and Brouns, 2009). This also suggests that there is very little

possibility that the dsRNA of the *DvSnf7* gene fragment has an influence on the bacteria and the like present in the soil.

2. Soil microbial test on soil cultivated the genetically modified maize

In order to investigate the effect of the genetically modified maize on soil microorganisms, we carried out a soil microbial phase test using this Genetically Modified Maize harvested at the isolation site of Kawachi Research Farm, Monsanto Co., Ltd. and on the cultivated soil of the non-recombinant control maize section in 2014 (1-2- (6) – ② - g, p.71; Appendix 12).

In this study, we measured the number of bacteria, actinomycetes and filamentous fungi present in the genetically modified maize collected at the time of harvest and the non-recombinant maize plot in the control.

As a result, no statistically significant difference was observed between the genetically modified maize and the non-recombinant control maize with respect to the number of bacteria, actinomycetes and filamentous fungi (1-2-(6) - ② - g, p.71; Appendix 12).

Therefore, there seems to be little possibility that the genetically modified maize has an effect on soil microorganisms.

As mentioned above (1 - 2 - (1) - B - 2 - iii, p.18 - 35), the insecticidal activity spectrum of the dsRNA of the *DvSnf7* gene fragment is extremely narrow and is thought to be limited to some insects belonging to Family Chrysomelidae among insects of Order Coleoptera. In order to induce RNAi by dsRNA ingested orally in insects belonging to the subfamily Galerucinae, Family Chrysomelidae, Coleoptera, it requires sequence homology of more than 21 consecutive bases. Systematically, the farther away from the subfamily Galerucinae, it has been clarified that insects with the *Snf7* gene are restricted to some insects belonging to Family Chrysomelidae as the lower homology with the *DvSnf7* gene of the *Snf7* gene and the sequence homology of 21 or more consecutive bases with the *DvSnf7* gene fragment. Therefore, it is considered there is little possibility that the dsRNA of the *DvSnf7* gene fragment showing specific activity to some insects belonging to Family Chrysomelidae have unintended effects on arthropods with respect to soil microorganisms which are phylogenetically distant was considered to be extremely low.

From the results of 1. Homology search comparing the sequences of the above 1. *DvSnf7* gene fragment and 7 typical eukaryotic living inhabiting in soil, and 2. Soil microflora test on soil cultivated the genetically modified maize, it was considered that there was little possibility where the dsRNA of the *DvSnf7* gene fragment has an unintentional effect on organisms living in the soil.

Table 6 Summary of homology search between the sequence of DvSnf7 gene fragment and seven eukaryotic gene sequences inhabiting soil[17]

Species name	Classification	Role in soil ecosystem	21 base length match[1]
Acanthamoeba castellanii	Amebazor	Bacterium feeding fungi	0
Blumeria graminis	Ascomycete (Pathogenic fungi)	Phytopathogenic fungus	0
Chlamydomonas reinhardtii	Green algae plant	Photosynthetic organisms	0
Laccaria bicolor	Basidiomycete	Exogenous mycorrhizae	0
Phanerochaete chrysosporium	Basidiomycete (Pathogenic fungi)	White rot dung Degradation of lignin	0
Rhodotorula gultinis	Basidiomycete (yeast)	Including iron Organic producer	0
Tetrahymena thermophila	Ciliata	Bacterium feeding fungi	0

[1] Number of sequences matching the DvSnf7 gene fragment sequence (240 bases) and 21 bases in the eukaryotic base sequence database as shown in Table 1

v. Effects of dsRNA of *DvSnf7* gene fragment on vertebrate

As mentioned above, the insecticidal activity spectrum of the dsRNA of the *DvSnf7* gene fragment expressed in the genetically modified maize is extremely narrow. Thus, we concluded that it is limited to some insects belonging to the family Chrysomelidae among insects of the order Coleoptera. However, for the sake of clarity, we examined that the dsRNA of the *DvSnf7* gene fragment does not show unintentional activity against vertebrates, including humans by discussing the following three points:

1. Various barriers preventing the induction of RNAi by orally ingested dsRNA,

2. History of safely eating crops containing RNA,

3. Expression amount of dsRNA of DvSnf7 gene fragment in the genetically modified maize

[17] Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

1. Various barriers preventing the induction of RNAi by orally ingested dsRNA

Since vertebrates have various barriers preventing the induction of RNAi by orally ingested dsRNA as shown below, it is considered that the dsRNA of the *DvSnf7* gene fragment does not affect the expression of the gene.

a. Nucleic acid degrading enzymes present in saliva, gastric juice, intestinal juice, pancreatic secretion fluid and blood vessels

Vertebrate animals including human have many degradation enzymes (such as saliva, gastric juice, and intestinal juice and serum degradation enzymes) that degrade orally ingested RNA. Generally, these degrading enzymes are thought to function on all nucleic acids entering the body regardless of the base sequence of the nucleic acid (Petrick et al., 2013). It is known that dsRNA orally ingested in mammals including humans is first degraded by ribonucleases present in saliva (Park et al., 2006) and then degraded under acidic conditions in the stomach (Loretz et al., 2006; Akhtar, 2009; O'Neill et al., 2011). Nucleic acid degrading enzymes and bile salts in the gastrointestinal lumen, digestive juice and pancreatic secretion fluid are also considered to be a barrier to decompose orally ingested RNA and impede the induction of RNAi (Loretz et al., 2006 ; O'Neill et al., 2011). Nucleic acid degrading enzymes are also present in blood vessels (Houck, 1958).

Regarding the structure and function of enzymes involved in digestion, it is common to all vertebrates, fish, amphibians, reptiles and birds also have nucleolytic enzymes that degrade nucleic acids like mammals (Stevens and Hume, 1995). Thus, barriers to orally ingested RNA present in mammals are also supposed to exist in vertebrates such as fish, amphibians, reptiles and birds.

b. Barriers preventing uptake of dsRNA into cells

Even if dsRNA is not degraded in saliva, gastric juice, intestinal juice and blood vessels, expression of the target gene cannot be suppressed unless it is taken into the cell. Generally, the molecular weight is large like RNA and the hydrophilic substance cannot permeate the cell membrane of intestinal epithelial organism, which is also a physical barrier to RNAi

induction by ingested RNA (Sioud, 2005; Akhtar, 2009; O'Neill et al., 2011). This is also a physical barrier to RNAi induction by ingested RNA (Sioud, 2005; Akhtar, 2009; O'Neill et al., 2011). In experiments in which siRNA is introduced into cultured cells at a high concentration of 250 nM, it has been reported that siRNA cannot be introduced into cultured cells without using a transfection reagent (Lingor et al., 2004). Even if it enters the cell, it is also known that foreign RNA is sequestered and decomposed by cell endocytosis and lysosomes (Gilmore et al., 2004; Sioud, 2005).

c. Numerous studies on the transport of RNA into cells

Regarding the uptake of RNA into cells, there has been an interest in the development of nucleic acid drugs that exert their effects through RNAi mechanisms such as RNA oligonucleotides due to their high specificity, and there have been numerous studies on the development of nucleic acid drugs. However, transporting RNA to systemic organisms still remains a challenge (Behlke, 2006; Nguyen et al., 2008; Vaishnaw et al., 2010; O'Neill et al., 2011). Transportation of RNA oligonucleotides to whole body organisms is difficult, which is one of the major factors impeding the development of nucleic acid drugs. Regarding treatment with oligonucleotides, when thorough measures are taken to keep it in a stable state by chemical modification, efficacy has been recognized only when formulated with special fat-soluble carriers or when both are used (Behlke, 2006). For example, it was reported that no suppression effect was observed even when 50 mg / kg of intravenous injection of siRNA stabilized with chemicals targeting apoprotein gene of mouse is administered (Soutschek et al., 2004). This amount of 50 mg / kg is a very large amount compared to the amount of dsRNA ingested by eating the genetically modified maize. In addition to this report, numerous studies have been made on the uptake of RNA into cells. So far it has not been reported that vertebrate cells efficiently take up RNA except under special conditions, but RNA uptake into cells is known to be a major barrier in RNAi mechanisms. Therefore, it is unlikely to, by eating the genetically modified maize, have an effect on animals other than insects as targets including human and other vertebrates.

2. History of safely eating crops containing dsRNA

Suppression of gene expression by RNAi is a universal phenomenon spontaneously occurring in eukaryotes including plants and animals consumed as food and feed, which relates to specific morphogenesis even for conventional crops, regulation of endogenous gene expression by RNA (Kusaba et al., 2003; Tuteja et al., 2004; Della Vedova et al., 2005). It is also known that food crops such as bean, pepper and barley have dsRNA caused by virus infection (Fukuhara et al., 2006). Furthermore, small RNAs are also present in rice seeds that are safely consumed by billions of people on a daily basis. It has been reported that a part of it has 100% homology with important gene sequences such as human cell cycle regulation, growth factors, signal transduction, metabolic enzymes and transcription factors (Ivashuta et al., 2009). These small RNAs were originally thought to have been produced from dsRNA by the RNAi mechanism, which supports the existence of dsRNA which became the origin of these small RNAs in rice plant. From these facts, it is not limited to RNA having a sequence that matches the important gene sequence of humans but also exists in other crops. Vertebrate animals including humans have a long history of safely eating dsRNA (Heisel et al., 2008; Ivashuta et al., 2009; Jensen et al., 2013).

L. Zhang et al. (2012a) reported the induction of RNAi by orally ingested RNA. After ingesting large amounts of uncooked rice seeds to mice (equivalent to human intake, about the same degree as when ingesting cooked rice grains to about 33 kg / day), multiple miRNAs from rice were detected in mouse serum and liver. The expression level of the protein (LDLRAP1) associated with the low-density lipoprotein (LDL) receptor encoded by the gene showing homology with miR168a, which is one of the detected miRNAs, decreases in the mouse body and the LDL Cholesterol increased (Zhang et al., 2012a). From these results, L. Zhang et al. (2012a) suggested that miRNAs in plants are involved in gene expression in animals based on sequence homology, suggesting that this phenomenon may be generated. Also, Heinemann et al. (2013) quoted a paper by L. Zhang et al (2012a) and claimed that the current method for safety review of genetically modified foods using RNAi is inadequate.

However, public institutions such as the Food Standards Australia New Zealand (FSANZ) claim that short dsRNA in genetically modified foods has a negative effect on humans than dsRNA already present in conventional foods (FSANZ, 2013) that there is no scientific basis to show that it has the risk of

exerting a harmful effect. As mentioned above, there is no concern about safety against oral ingestion of nucleic acid containing dsRNA in plants. It is considered that the existing method of screening genetically modified foods can be applied to genetically modified foods using RNAi such as the genetically modified maize. Below is a summary of the research and reports for the basis.

- Y. Zhang et al. (2012b) [18] reports that many small RNAs containing miR168a are registered as open-label databases as small animal-derived RNA despite being plant-derived. In particular, numerous miR168a registries are made in mammalian RNA databases. From these results, it is suggested that the miR168a found in mouse organisms in the test of L. Zhang et al. (2012a) may not be derived from rice, raising questions concerning the induction of RNAi in mice by orally ingested RNA (Zhang et al., 2012b).
- An examination to verify the report of L. Zhang et al (2012a) has also been conducted, but the results reported by L. Zhang et al. (2012a) were not reproduced (Dickinson et al., 2013). Dickinson et al. (2013) conducted a study where they gave mice miR 168a through the diet, and there was no change in the expression level of LDLRAP1 protein related to increase / decrease of LDL cholesterol by diet. LDL cholesterol level slightly changed in diets rich in rice fruit, but there was no change in nutritionally biased diet (Dickinson et al., 2013). Based on these findings, L. Zhang et al (2012a) attributed it due to variability in gene expression and protein expression and nutritional difference between diets, rather than caused by miR 168a (Dickinson Et al., 2013). The conclusion by Dickinson et al. (2013) is also consistent with previous studies reported in relation to the induction of RNAi by RNA from the oral intake described above (Loretz et al., 2006; O'Neill et al., 2011
- Snow et al. (2013) report that the amount of RNA absorbed by body organisms is limited, far below the amount of RNA that can induce RNAi despite the presence of substantial amounts of miRNA in human, mouse and honey diets.
- Similarly, Witwer et al. (2013) conducted that experiments were conducted to give mice rich in miRNA to primates and the presence or absence of miRNAs in the blood was examined, but there was no evidence that these miRNAs are present in the blood.

[18]Authors of Zhang et al. (2012a) and authors of Zhang et al. (2012b) are same names, but are totally different people.

- Tosar et al. (2014) suggested that the experimental results of L. Zhang et al (2012a) may have been caused by sample contamination and questioned the conclusion derived by L. Zhang et al. (2012a) (Tosar et al ., 2014).
- Other researchers, research institutes and regulatory bodies also support the application of the current safety assessment framework for genetically modified crops using RNAi technology (Parrott et al., 2010; ILSI- CERA, 2011; FSANZ, 2013).

3. Expression amount of RNA of *DvSnf7* gene fragment in the genetically modified maize

Nucleic acids such as RNA are universally present in plants as described above. For example, the total amount of RNA contained in the soybean seeds was 407.3 μg / g dry weight on average (range: 274.7-986.6 μg / g) and the average siRNA amount from 21 bp to 24 bp was 0.66 μg / g dry weight (Ivashuta et al., 2009). On the other hand, the average expression level of RNA of the *DvSnf7* gene fragment in the genetically modified maize kernel was 0.000104 μg / g dry weight (Table 10, p.62 to 63). The expression level of the dsRNA of the *DvSnf7* gene fragment in the genetically modified maize kernel is very low compared to the amount of siRNA present in the plant.

4. Conclusion

Vertebrates have various barriers that impede the induction of RNAi by orally ingested dsRNA. Since nucleic acids such as RNA are universally present in animals and plants, vertebrates have a long history of safely eating various RNAs. Also, the average expression level of RNA of the *DvSnf7* gene fragment in the genetically modified maize kernel was 0.000104 μg / g dry weight, which is extremely small compared to the amount of RNA generally contained in plant derived foods to be ingested. Therefore, even if the vertebrate ingests the dsRNA of the *DvSnf7* gene fragment from the genetically modified maize, the dsRNA is quickly degraded by various barriers. It is highly unlikely that the dsRNA of the *DvSnf7* gene fragment induces RNAi in the body of the ingested vertebrate.

vi. Homology with a protein that has been shown to have allergic properties

In general, it is highly unlikely that the dsRNA of the *DvSnf7* gene fragment expresses a new protein as dsRNA is structurally inhibited from ribosomal translation (Kozak, 1989). Therefore, it is considered that it does not have allergic property.

II. 【Modified Cry3Bb1 protein】

The modified Cry3Bb1 protein is the major Coleoptera pest (Coleoptera pest) of corn cultivation in the U.S., showing the insecticidal activity against CRW damaging the corn root (Table 7, p.44).

The insecticidal spectrum of the modified Cry3Bb1 protein is extremely narrow. And it shows insecticidal activity against CPB and CRW belonging to the subfamily Chrysomelinae and the subfamily Galerucinae among insect species of Coleoptera (Head et al., 2001; US EPA, 2010). There is no report showing insecticidal activity to other insects. It is shown that homologous closely related species with these two genera of insect species do not inhabit Japan (Nakane et al., 1963).

Also, strains that are genetically modified crops expressing the modified Cry3Bb1 protein and that are approved by the Type 1 Use regulation based on the Cartagena Protocol, include Coleoptera pest-resistant maize (MON 863, OECD UI: MON - ØØ 863-5) (approval date: June 1, 2004) (hereinafter referred to as "MON 863"), and herbicide, glyphosate-tolerant maize and Coleoptera pest-resistance maize (approval date: April 10, 2005) (hereinafter referred to as "MON 88017") of maize (MON 88017, OECD UI: MON - 88 Ø 17 - 3)). It is understood that neither strains have any possibility to affect biodiversity when used with contents of each Type 1 Use, etc.

Whether the modified Cry3Bb1 protein shares a similar amino acid sequence with the known allergen was compared using eight AD amino acids contiguous to the FASTA type algorithm using AD 2013[19], but similarity with known allergen was not recognized.

[19] Based on AD_2013: A database created on the basis of the sequence obtained from Food Allergy Research and Resource Program Database (FARRP) (http://www.allergenonline.com), as of January 2013, 1,630 amino acid sequences

Table 7 Insecticidal spectrum of modified Cry3Bb1 protein (susceptibility of various insects to modified Cry3Bb1 protein) *

Order	Family	Subfamily	Academic name	LC $_{50}$[a] or maximum concentration[b] (ng/mL diet)	Insecticidal activity*	Inhabitance in Japan
Hemiptera (Hemiptera))	Anthocoridae	Anthocorinae (Subfamily))	*Orius insidiosus*	930[b]	None	Congeneric species inhabits
Collembola	Isotomidae	Proisotominae	*Folsomia candida*	872.5[b]	None	Congeneric species inhabit
Neuroptera	Chrysopidae	Chrysopinae	*Chrysopa carnea*	8,000[b]	None	Congeneric species inhabit
Hymenoptera	Apidae	Apinae	*Apis mellifera*	360[b]	None	Congeneric species inhabit
	Pteromalidae	Pteromalinae	*Nasonia vitripennis*	400[b]	None	Inhabit
Lepidoptera	Crambidae	Pyraustinae	*Ostrinia nubilalis*	200[b]	None	Congeneric species inhabit
	Noctuidae	Heliothinae	*Helicoverpa zea*	200[b]	None	Congeneric species inhabit
Coleoptera	Curculionidae	Dryophthorinae	*Sitophilus oryzae*	200[b]	None	Inhabit
		Anthonominae	*Anthonomus eugenii*	200[b]	None	Congeneric species inhabit
			Anthonomus grandis	50[b]	None	Congeneric species inhabit
	Tenebrionidae	Tenebrioninae	*Tribolium castaneum*	200[b]	None	Inhabit
	Coccinellidae	Coccinellinae	*Hippodamia convergence* (A kind of ladybird)	8,000[b]	None	Congeneric species inhabit
			Coleomegilla maculate (A kind of ladybird)	MON 863 Pollen at 93 µg/g pollen	None	×
	Carabidae	Harpalinae	*Poecilus chalcites*	930[b]	None	Congeneric species inhabits
	Bruchidae	Bruchinae	*Callosobruchus maculatus*	200[b]	None	Inhabits
	Chrysomelidae	Chrysomelinae	**CPB** (*Leptinotarsa decemlineata*)	2.7[a]	Available	×
		Galerucinae	**CRW** (*Diabrotica virgifera*)	75[a]	Available	×

*It showed insecticidal activity to the species belonging to the two subfamilies of Chrysomelinae and Galerucinae of the family Chrysomelidae, Coleoptera.
[a] LC_{50}= Median lethal dose; [b] The maximum concentration administered
* Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

III.【DsRNA of DvSnf7 gene fragment + modified Cry3Bb1 protein】

Numerous studies have been conducted on the mechanism of action of Bt protein such as modified Cry3Bb1 protein (OECD, 2007). Cry protein is produced as a prototoxin (poison precursor) and converted from a crystal inclusion body to a core protein having activity by a proteolytic enzyme within a target insect body. It has been reported that by binding to specific receptors on the midgut epithelium of insects, cation selective pores are formed in the midgut epithelial cell membrane, resulting in inhibition of insect digestion process and exhibiting insecticidal activity (Vachon et al., 2012). On the other hand, since the dsRNA of the *DvSnf7* gene fragment acts at the nucleic acid level and exhibits insecticidal activity through the RNAi mechanism, it has a completely different action mechanism from the Bt protein. The difference in these mechanisms also influences the time during which the insecticidal effect of the dsRNA of the *DvSnf7* gene fragment and the modified Cry3Bb1 protein is observed. Therefore, it is necessary for a bioassay to investigate the interaction to design a test taking into consideration the time difference until the insecticidal effect between the dsRNA of the *DvSnf7* gene fragment and the modified Cry3Bb1 protein is recognized.

In Levine et al. (2015) reported that, Growth inhibition began to be observed after 3 days in either case when single stranded RNA (968 bases)[20] of the *DvSnf7* gene fragment and modified Cry3Bb1 protein were administered to the SCRW, respectively, and after 12 days, 80% Growth inhibition was observed in individuals (FIG. 7, p. 48) (Levine et al., (2015) (Appendix 4) in FIGS. 2-A, p 8). On the other hand, the insecticidal effect began to be observed after 6 days when the RNA was fed on day 2 after administering the modified Cry3Bb1 protein. As expected, it was confirmed that the case where the modified Cry3Bb1 protein was administered diet was about 4 days earlier than the case where the RNA was administered diet (FIG. 7, p48)) (Levine et al., 2015) (Attachment 4) Figure 2-B, p8). This means that, when the RNA and the modified Cry3Bb1 protein are simultaneously administered, the insecticidal effect of the modified Cry3Bb1 protein appears before the insecticidal effect of the RNA is observed, and the

[20] The RNA (968 bases) is an *in vitro* synthesized RNA transcribed from the *DvSnf 7* gene fragment expression cassette, which has the *DvSnf7* gene fragment in inverted repeat form. It also includes the *DvSnf7* gene fragment and intervening sequence, as well as subsequences of *hsp70* intron and E9 terminator. It is considered that the RNA (968 bases) forms double-stranded RNA (dsRNA) after synthesis. In fact, it has been confirmed that the insecticidal activity against WCRW and SCRW is equivalent to the dsRNA of the *DvSnf7* gene fragment (Attachement 7; Attachement 24).

interaction between the RNA and the modified Cry3Bb1 protein cannot be observed (Levine et al., (2015) (Attachment 4)).

The presence of interaction between the RNA and the modified Cry3Bb1 protein was examined by a bioassay, as an index, a concentration that causes growth inhibition with a small time difference, not by a time-dependent lethal concentration until an effect is recognized between the RNA and the modified Cry3Bb1 protein, (Levine et al., (2015) (Appendix 4)).

In this study, the RNA and the modified Cry3Bb1 protein were administered to the SCRW instead of WCRW. This is because SCRW belongs to the same subfamily Galerucinae as WCRW, it is relatively easy to rear in a laboratory (Marrone et al., 1985) and suitable for observing growth inhibition because of the fast growth. In addition, we also established three groups in total as follows (low reaction, medium reaction and high reaction, respectively); (i) the RNA and the modified Cry3Bb1 protein mixed with artificial diet at their respective GI_{35} values (Growth Inhibition; a concentration that suppresses the average body weight of SCRW by 35% as compared with the control) (ii) with GI_{50} values, and (iii) with GI_{65} values. Under these three concentration conditions, we examined the number of surviving SCRW and its total weight after administering the modified Cry3Bb1 protein, the RNA of interest, and both of these to SCRW (3 replicates) for 12 days in order to evaluate the extent of growth inhibition (Figure 8, p.48) (Levine et al., (2015) (Attachment 4) Figure 3, p10). Furthermore, we constructed an additive reaction model (Faust et al., 2000) from the GI_{35} value, GI_{50} value and GI_{65} value of the modified Cry3Bb1 protein and the RNA, and predicted the GI value (GI expected value) when the RNA and modified Cry3Bb1 protein additively act under each concentration condition 8, p. 48) (Levine et al., (2015) (Attachment 4) Figure 3, p10). We compared statistically the calculated GI expectation value with the GI value (GI observation) observed when the RNA and the modified Cry3Bb1 protein were actually fed together.

As a result, there was no statistically significant difference between the GI observation value and the GI expectation value under three concentration conditions ($p > 0.05$; Figure 8, p48) (Levine et al., (2015) Figure 3, p.10 of the attached document 4). It was confirmed that the GI observation value obtained by administering the RNA and the modified Cry3Bb1 protein together is indicative of an additive change as compared with the RNA and the modified Cry3Bb1 protein.

From the above fact, it was demonstrated that the dsRNA of the *DvSnf7* gene fragment and the modified Cry3Bb1 protein do not show interaction.

We conducted additional analysis in order to confirm the evaluation of Levine et

al., 2015 which investigated the possibility of interaction between RNA of *DvSnf7* gene fragment and modified Cry3Bb1 protein. In this analysis method, we set two models of regression analysis, A + B (additive effect model) and A + B + A * B (interaction model), and compatibility with observations is calculated by AIC (Akaike's Information Criterion), and compared the two models. AIC is a relative evaluation where a model with a smaller AIC is interpreted as having a high degree of conformity with data.

As a result, the AIC of the additive effect model was -64.44, and the AIC of the interaction model was -63.88. Also, the additive action model AICc was -62.62, and the interaction model AICc was -61.02. The difference between AIC and AICc in the additive model and the interaction model was slight, and in each case there was no significant difference to be discussed with regard to the fitness of the model.

Based on the principle of economy (Occam's razor) that multiple models should show equivalent equality, it should not be necessary to choose an interaction model based on the savings principle that should be done with the least parameter model (Attachment Material (Appendix) 13 Table 1, p 6). This result supported the results of Levine et al., 2015 which showed that the RNA of the *DvSnf7* gene fragment and the modified Cry3Bb1 protein acted additively.

Also, as described in the 1- 2- (1) - B - ② (p. 9 - 44), it is considered that the dsRNA and modified Cry3Bb1 protein of the *DvSnf7* gene fragment expressed in this recombinant maize (genetically modified maize) act specifically on the target insect, showing independent insecticidal effect. Furthermore, because the functions that they have are different, the insecticidal effect possessed by the dsRNA of the *DvSnf7* gene fragment and the modified Cry3Bb1 protein may be additionally increased; however, the possibility of exhibiting functional interaction may be low. Therefore, it is concluded that the dsRNA of the *DvSnf7* gene fragment expressed in the genetically modified maize and the insecticidal spectrum by the modified Cry3Bb1 protein are restricted to Coleoptera insects and are not expanded elsewhere

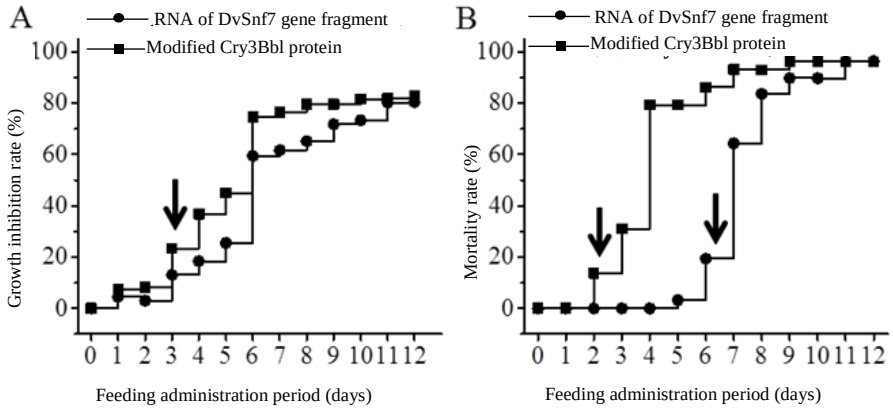

Figure 7 Comparison[21] between growth inhibition (A) and lethal effect (B) for RNA (968 bases) (●) of the *DvSnf 7* gene fragment and modified Cry3Bb1 protein (■) in bioassay by SCRW for 12 days diet administration

(A) Arrows in the figure indicate the points at which significant growth inhibition began to appear. Regarding the growth inhibition (A) effect, we provided diet with the RNA and the modified Cry3Bb1 protein respectively with GI80 values (0.012 ug / ml diet: the relevant RNA, 25 ug / ml diet of the modified Cry3Bb1 protein).

(B) The arrows in the figure indicate the points at which significant lethality began to appear. Regarding the effect of lethality (B), we provided diet to each of the RNA and modified Cry3Bb1 protein with LC95 values (0.050 ug / ml diet: the relevant RNA, 250 ug / ml diet of modified Cry3Bb1 protein).

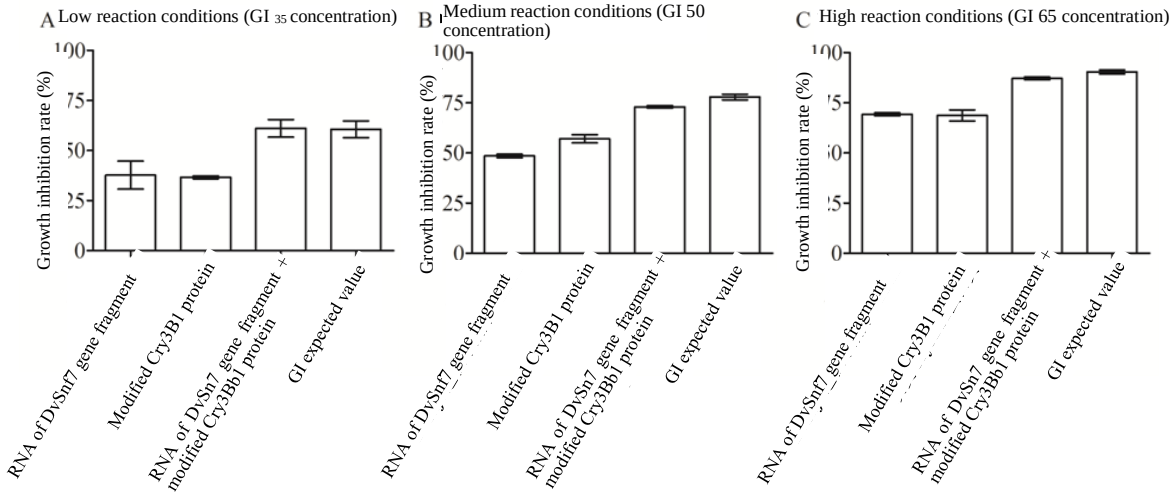

FIG.8 Comparison between GI observation value and expected GI value for RNA (968 bases) (●) of the *DvSnf 7* gene fragment and modified Cry3Bb1 protein (■) in bioassay by SCRW for 12 days diet administration

We presented the GI observations of the modified Cry3Bb1 protein, the RNA of interest and both, and their standard errors under three concentration conditions of GI 35 value (low response), GI 50 value (medium response) and GI 65 value (high response). Also, we predicted the GI value (GI expected value) when the RNA and the modified Cry3Bb1 protein additively act under each concentration condition.

[21] Responsibility for the rights and contents pertaining to the information described in this figure belongs to Monsanto Japan Limited.

IV.【Modified CP4 EPSPS protein】

The modified *cp4 epsps* gene expressed in the genetically modified maize is isolated from the *Agrobacterium* CP4 strain and encodes 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), expressing a modified CP4 EPSPS protein with high tolerance to herbicide glyphosate. When plants treat the glyphosate, 5-enolpyruvylshikimate-3-phosphate synthase (enzyme number: EC 2.5.1.19, hereinafter referred to as "EPSPS protein") is inhibited, whereby an aromatics essential for protein cannot synthesize amino acids and withers. However, the activity of modified CP4 EPSPS protein which is produced by the modified *cp4 epsps* gene is not prohibited even in the presence of glyphosate. As a result, shikimate synthesis can function normally and grow in recombinant plants expressing this protein.

At present, there are 6 crop plants and 15 lines (3 lines in maize) that are genetically modified crops expressing the CP4 EPSPS protein and approved by the Type 1 Use regulations based on the Cartagena Protocol (excluding stack strains). It is considered that there is no fear that biodiversity will be affected if any strains are used according to the method such as the Use of each Type 1 species.

We compared whether the modified CP4 EPSPS protein shares an amino acid sequence similar to the known allergen, using AD 2013, with 8 amino acids contiguous with the FASTA type algorithm. However, similarity with known allergens was not observed.

③ When changing the host's metabolic system, its details.

【DsRNA of DvSnf7 gene fragment】

The *DvSnf7* gene fragment is not an endogenous gene of maize but is a highly conserved gene sequence among insect species belonging to the subfamily Galerucinae (family Coleoptera, Coleoptera). Therefore, it is not considered that it will suppress the expression of the host gene.

In fact, we performed a homology search to see if there is a nucleotide sequence that is consistent with 21 base length between the sequence of the

transcript in maize and the sequence of the *DvSnf7* gene fragment using the transcript product database of corn. As a result, we confirmed that in transcripts derived from maize, there is no sequence that matches 21 base length with the sequence of the *DvSnf 7* gene fragment (Appendix 11).

Also, since dsRNA is structurally inhibited from ribosomal translation (Kozak, 1989), the possibility of the dsRNA of the *DvSnf7* gene fragment expressing a new protein is extremely low. From these facts, it is considered that the metabolic system of the host is not changed.

【Modified Cry3Bb1 protein】

The modified Cry3Bb1 protein is one of the insecticidal proteins (Bt protein) of crystal derived from *Bacillus thuringiensis.* A number of studies have been made on the mechanism by which Bt protein exerts insecticidal activity (OECD, 2007). However, so far there is no report that the Bt protein has other functions. Therefore, it is unlikely that these Bt proteins have enzymatic activity, and it is considered that they do not change the metabolic system of the host.

【Modified CP4 EPSPS protein】

EPSPS protein, which is functionally identical to the modified CP4 EPSPS protein, is an enzyme protein that catalyzes the shikimate pathway for biosynthesizing aromatic amino acids. However, it is not the rate-limiting enzyme in this pathway, even though the activity of the EPSPS protein increases, it is considered that the concentration of the aromatic amino acid which is the final product of this pathway will not increase. It is also known that EPSPS protein specifically reacts with phosphoenolpyruvate (PEP) which is a substrate and shikimate-3-phosphate (hereinafter referred to as "S3P") (Gruys et al., 1992). Besides these, shikimic acid, the analog of S3P, is known to react only with the EPSPS protein. However, when comparing the EPSPS protein with shikimic acid and S3P with the specificity constant k_{cat} / K_m showing the likelihood of reaction, the reaction specificity of EPSPS protein with shikimic acid is only about two millionth of the specificity of EPSPS protein with S3P (Gruys et al., 1992). This shows a very low possibility where shikimic acid will react as a substrate for EPSPS protein. Therefore, it is considered that the modified CP4 EPSPS protein does not change the metabolic system of the host.

【DsRNA of *DvSnf7* gene fragment + modified Cry3Bb1 protein + modified CP4 EPSPS protein】

As described above, the dsRNA, the modified Cry3Bb1 protein and the modified CP4 EPSPS protein of the *DvSnf7* gene fragment expressed in this recombinant maize (genetically modified maize) have different action mechanisms, they are supposed to work independently. In addition, since the dsRNA sequence of the *DvSnf7* gene fragment does not have compatibility / incompatibility with the sequence of the modified *cry3Bb1* gene, the modified *cp4 epsps* gene and the corn endogenous gene by 21 bases, it is unlikely that RNAi will suppress the expression of these genes. In fact, they have confirmed the expression of the modified Cry3Bb1 protein and the modified CP4 EPSPS protein in the genetically modified maize (Tables 11 to 12, p64 to 67; Appendix (Appendix) 20 Table 1 to 2, p 18 to 21). Also, we considered that these expression products do not produce a protein, not have enzymatic activity, or have high substrate specificity, so they do not affect the plant metabolic pathway. Therefore, it is considered that there is no possibility that the dsRNA of the *DvSnf7* gene fragment, the modified Cry3Bb1 protein and the modified CP4 EPSPS protein mutually affect in the plant.

(2) Information on vectors

A Name and origin

PV-ZMIR10871 used for the creation of the genetically modified maize was constructed based on the vector pBR 322 (Sutcliffe, 1979) derived from *Escherichia coli* and the like.

B Characteristic

① Number of bases and base sequence of vector

The number of bases of PV-ZMIR10871 was 16,497 bp, which was used for the production of the genetically modified maize. The nucleotide sequence of PV-ZMIR10871 is listed in Appendix 14.

② If there is a base sequence having a specific function, its function.

The *aadA* gene is derived from the *E. coli* transposon Tn7 which confers resistance to spectinomycin and streptomycin as a selection marker gene of the construction vector in *E. coli* and is present outside the T-DNA region

③ The presence or absence of infectivity of the vector and information on its host range

The infectivity of this vector is unknown.

(3) Method for preparing living modified organisms

A Constitution of the entire nucleic acid transferred into the host

Components of this plasmid vector transferred into the host are described in (p. 5 - 8). The position of the constituent elements of donor nucleic acids in the vector is shown in FIG. 1 (p. 4).

B Method for transferring nucleic acid transferred into host

Using the Agrobacterium method, we introduced the T-DNA region in PV-ZMIR10871 into immature germ cells of conventional corn variety LH 244, which is classified as a dent type.

C The course of development of living modified organisms

① Method of selecting cells into which nucleic acid has been transferred

We co-cultured immature embryos of conventional maize variety LH244 with *Agrobacterium tumefaciens* ABI strain containing PV-ZMIR10871, then transferred the immature embryos to organism culture medium supplemented with glyphosate and carbenicillin. We used herbicide glyphosate to select transformed individuals.

② If the method of transferring nucleic acid is the Agrobacterium method, the presence or absence of remaining Agrobacterium cells

Agrobacterium cells used for transformation have been removed by organism culture medium supplemented with carbenicillin. Also, we performed Taqman PCR analysis targeting the outer skeletal region of PV-ZMIR10871 used for transformation in the seed of R4F 1 generation[22] of the genetically modified maize. As a result, there was no outer framework region of PV-ZMIR10871 in the genetically modified maize (Appendix 15, Table 1, p. 12). From this, it was confirmed that Agrobacterium cells used for transformation do not remain in the genetically modified maize.

③ The progress of development for strains in which the state of existence of the copy of the transferred nucleic acid is confirmed from the cell into which the nucleic acid has been transferred, as well as those used for isolated field tests or others used to collect information necessary for Biological Diversity Risk Assessment

We transplanted redifferentiated individuals (R0) obtained by redifferentiation from dividing cells on the medium into the soil. Thereafter, the transgene was homogenized by self-propagation five times in total, and the progeny of the selected individuals was analyzed and subjected to the morphological character investigation. As a result, the genetically modified

[22]We bulked harvested seeds, randomly picked about 20 grains, extracted DNA and used for PCR analysis.

maize was ultimately selected as a commercialized line.

Analysis of the transgene in this recombinant maize (genetically modified maize), the stability of expression of the transgene, and the generation to be tested in isolated field test in Japan are described in the breeding diagram of FIG. 9 (p 54). The subject of this application is all crossbreed progeny derived from LH244 R4 generation and LH244 R4 generation.

【Non-disclosed due to confidentiality】

Figure 9 Training diagram of the genetically modified maize

(4) Existence of the nucleic acid transferred into the cell and stability of the expression of the trait by the nucleic acid

① Place where replicate of transferred nucleic acid exists

In order to investigate whether the T-DNA region of this recombinant maize (genetically modified maize) is present on the chromosome, we analyzed the separation ratio of T-DNA region by Chi-square test in TI: BC2F1 generation, TI: BC2F2 generation and TI: BC3F1 generation of this recombinant maize (genetically modified maize) (Appendix 16).

In order to produce the TI: BC2F1 generation (FIG. 9, p 54) to be tested, we first selfed the transformed regenerated individual (R0). Then, using Real-Time TaqMan PCR method, In the R1 generation, the individuals with homozygous T-DNA region were selected and selfed, thereby creating the R2 generation. After that, we self-propelled two more times to create R4 generation. We used R4 generations of the genetically modified maize homozygous for the T-DNA region as a repetitive parent (F1266Z) without either of the DvSnf7 gene fragment, the modified cry3Bb1 gene or the modified cp4 epsps gene and the conventional breeding method, then created the R4F1 (F1266Z x LH244) generation heterozygous for the T-DNA region. We backcrossed with the recurrent parent (F 1266Z) against the resulting R4F1 generation and created the TI: BC1F1 generation. We further selected the individuals heterozygous for the T-DNA region in the TI: BC1F1 generation obtained by End-Point TaqMan PCR method and backcrossed again to create the TI: BC2F1 generation. We also created the BC2F2 generation by selfing the individual with heterozygous T-DNA region in TI: BC2F1 generation, and created the TI: BC3F1 generation by backcrossing individuals with heterozygous T-DNA region in the TI: BC2F1 generation with recurrent parents.

We confirmed presence or absence of T-DNA region by End-Point TaqMan PCR method or Real-Time TaqMan PCR method in these TI: BC2F1 generation, TI: BC2F2 generation and TI: BC3F1 generation and calculated the separation ratio and chi-squared test using the separation ratio.

As a result, no statistically significant difference was observed between the measured value and the expected value by the chi-square test. Thus, it was confirmed that the transgene was inherited without contradiction to Mendel's separation law (Table 8, p.56; Table 9, p.56). The T-DNA region of the genetically modified maize was considered to be present on the chromosome.

Table 8 Separation ratio of T-DNA region in BC2F1 and BC3F1 generation of the genetically modified maize [23]

				1:1 Separation			
Generation	Number of specimens	Observed value Transgene Positive / hetero population	Observed value Transgene Negative population	Expected value Transgene Positive / hetero population	Expected value Transgene Negative population	χ^2	p- value[2]
TI:BC2F1[1]	351	172	179	175.50	175.50	0.14	0.709
TI:BC3F1[1]	223	104	119	111.50	111.50	1.01	0.315

[1] The measured value was confirmed by presence or absence of T-DNA region by End-Point TaqMan PCR method.

[2] Separation ratios obtained from the above 2 generations were analyzed by chi-square test ($p < 0.05$).

Table 9 Separation ratio of T-DNA in the BC 2 F 2 generation of the genetically modified maize [23]

					1:2:1 Separation				
Generation	Number of specimens	Observed value Transgene Positive / homozygous population	Observed value Transgene Positive / hetero population	Observed value Transgene Negative population	Expected value Transgene Positive / homozygous population	Expected value Transgene Positive / hetero population	Expected value Transgene Negative population	χ^2	p-value[2]
TI:BC2F2[1]	623	152	314	157	155.75	311.50	155.75	0.12	0.942

[1] The presence or absence of T-DNA region was confirmed by Real-Time TaqMan PCR.

[2] Separation ratios obtained from the BC 2 F 2 generation were analyzed by chi-square test ($p < 0.05$).

[23] Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

② Copy number of transferred nucleic acid and stability of transmission of multiple copies of transferred nucleic acid in multiple generations

In order to investigate the number of insertion sites of the transgene introduced into the genetically modified maize, the copy number, the presence or absence of the transgene and the stability of transmission of the transgene in plural generations. We analyzed the junction region including the transgene by Next Generation Sequencing Technology [24] and bioinformatics (Next Generation Sequencing/Junction Sequence Analysis: NGS/JSA)[25]and PCR and sequence analysis of the transgene region (Appendix 17). In the following, we describe the method of this analysis and the results of the analysis carried out using the genetically modified maize.

NGS analyzes the nucleotide sequence of the plant genome with redundancy[26] of 75 or more by fragmenting the plant genome into a DNA fragment of about 100 bp and analyzing the nucleotide sequence of this DNA fragment with the next generation sequencer (Illumina) (Figure 10-1, p.59). Next, the nucleotide sequence of all DNA fragments is compared[27] with the nucleotide sequence of the plasmid for introduction (Figure 10-2, p. 59). In this result, we selected a DNA fragment homologous to the plasmid for introduction and confirmed the presence or absence of sequence homologous to the outside skeleton region in this selected DNA fragment (Figure 10-2, p.59). We further selected DNA fragments having both homologous sequences and homologous sequences in the JSA as a junction sequence between the

[24]Next Generation Sequence Technology (NGS) is a generic term for technologies that can simultaneously analyze enormous nucleotide sequences. This analysis is a method using Illumina out of NGS, where the nucleotide sequence of the entire genomic region can be deciphered by randomly cutting the genome to prepare a large number of fragments, amplifying each fragment, and then analyzing the nucleotide sequence.

[25] NGS / JSA provides molecular biological evaluation of the same extent as conventional Southern blot analysis by using next-generation type and conventional type sequence analysis and bioinformatics. NGS/JSA. In NGS / JSA, NGS first amplifies the sequence corresponding to the whole region of the genome of this recombinant maize as a fragment of about 100 bp. Then, by specifying the junction region between the T-DNA region and the host's endogenous sequence by JSA, these fragments are used to determine the number of introduction sites and copy number of the T-DNA region (Kovalic et al

[26] Redundancy: A measure of how many nucleotide sequences are analyzed against a specific DNA (genomic DNA or gene).

[27] In the BLAST search, sequences having an E-score of 1×10^{-5} or less and homology of 96.7% or more in the region of 30 bp or more were selected.

transgene and the plant genome. Then, by aligning overlapping regions in this junction sequence, we identified the junction region between the transgene and plant genome (Figure 10-3, p. 59). If one copy transgene is present in one place in the genome, two junction regions are identified (Kovalic et al., 2012). Furthermore, by examining the nucleotide sequence of the introduced gene existing between the junction regions by PCR and nucleotide sequence analysis, it is possible to determine the actual DNA sequence of the transgene.

As a result of test of genome extracted from the genetically modified maize and control non-recombinant maize to NGS / JSA, we were able to analyze the nucleotide sequence of 334.9 Gb (average redundancy 125) in the R4 generation of this recombinant maize (genetically modified maize) and 346.9 Gb (average redundancy 126) in the control non-recombinant maize (Appendix Table 3, p. 45 of Appendix 17). In this recombinant maize (genetically modified maize), two junction regions were identified (FIG. 11, p60), which were sequences containing the 5 'end and the 3' end of the transgene, respectively (Appendix 17 of Figure 4 Panel C, pp. 57-58). In the non-recombinant control maize, junction areas were not identified (Appendix 17, p. 29). Furthermore, as a result of examining the homology of all the DNA fragments obtained from the genetically modified maize with the sequence of the introducing plasmid PV-ZMIR10871, it was shown that the outer skeleton region was not included (Supplementary material of Appendix 17, Figure 3, p 5). From the above, it was confirmed that one copy of the T-DNA region was incorporated in one nuclear genome of the genetically modified maize, and the outer framework region was not inserted.

Also, as a result of amplifying the junction region and transgene-containing region detected in the genetically modified maize by PCR and analyzing the sequence, it was confirmed that the target T-DNA region was introduced (Appendix 17 Appendix Figure 5, p 59, Appendix Figure 6, p 60 to 64 and Appendix Figure 7, pp 65 to 79).

From the above, it was confirmed that one copy of the T-DNA region was incorporated in one nuclear genome of the genetically modified maize. Also, the junction region detected in NGS / JSA was only the junction region resulting from the transgene (FIG. 11, p 60), and no junction sequence having homology with the outer skeleton region of PV-ZMIR10871 was observed (Figure 3 of additional material of Appendix 17). Therefore, it was confirmed that the outer

skeleton region was not inserted. In addition, by PCR analysis and nucleotide sequence analysis of the transgene region, we confirmed that the nucleotide sequence of the transgene was identical to the T-DNA region of the transduction plasmid PV-ZMIR10871.

Also, in NGS / JSA targeting the genetically modified maize of multiple generations (R4, R4F1, R5, R5F1 and R6 generations), it was confirmed that the transgene was stable and inherited in the progeny (Appendix 17 Appendix 17 p 32 - 34). A schematic diagram of the transgene in the genetically modified maize is shown in Figure 11 (p60).

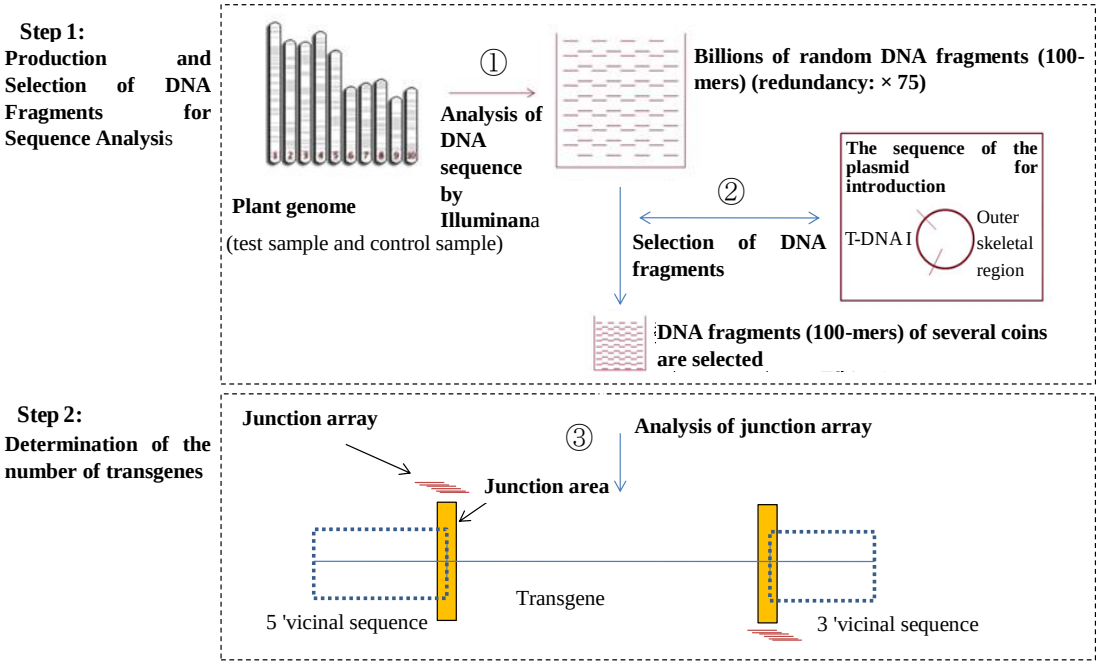

Figure 10 Conceptual diagram of analysis method of NGS / JSA (Kovalic et al., 2012) [28]

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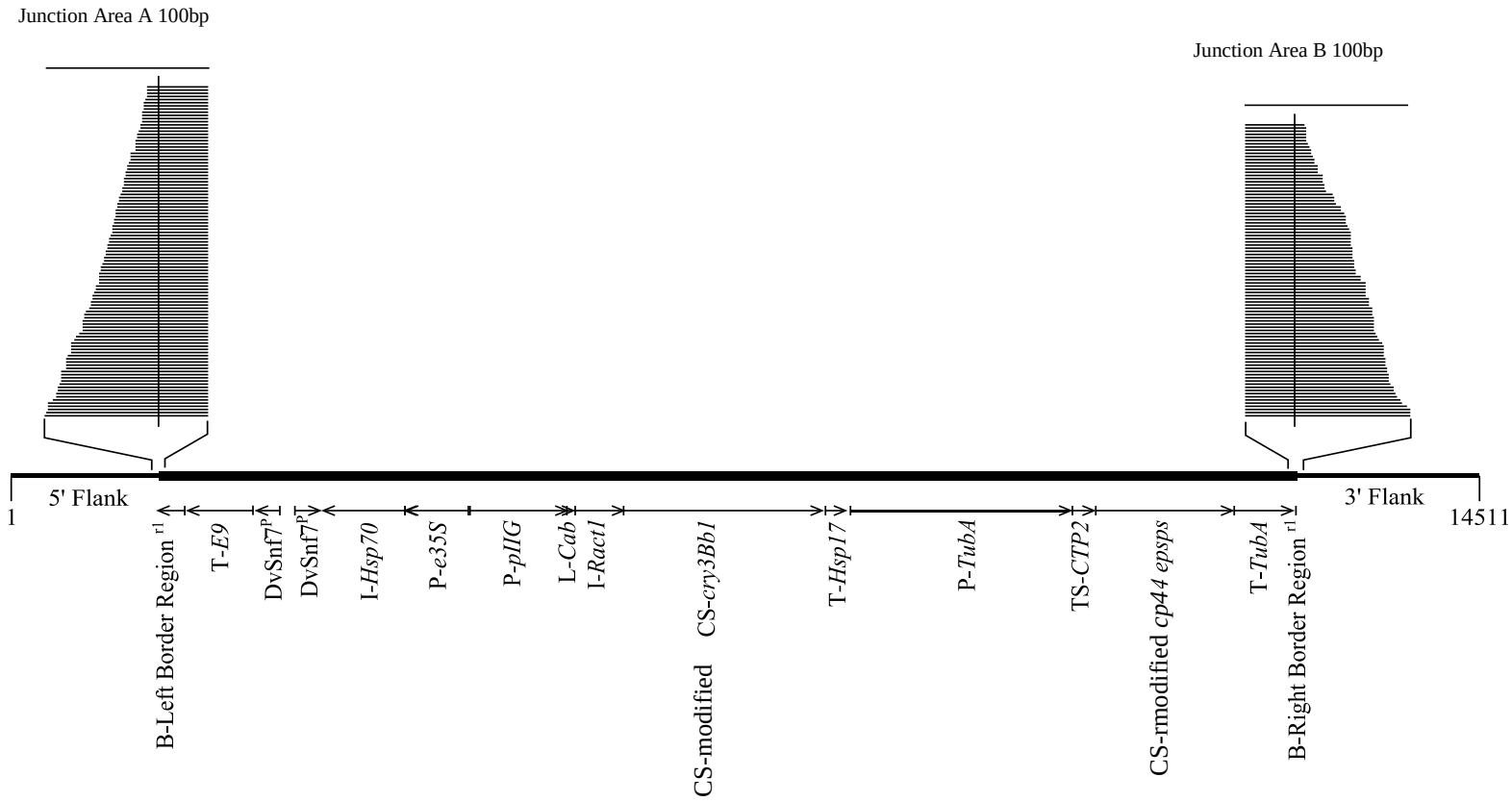

Figure 11 Transgenic map of the genetically modified maize [29]

The figure is a schematic diagram of the transgene and neighboring sequences in the genetically modified maize. Arrows in the transgene (→) indicate the direction of the sequence of the components in the genetically modified maize. In the figure, “rl” means that the B-Right Border Region and the B-Left Border Region are shorter than this PV-ZMIR10871 in the genetically modified maize. Also, p means partial sequence. We showed the schematic diagram of the junction areas A and B detected in the NGS / JSA at the top of the figure.

[29] Responsibility for the rights and contents pertaining to the information described in this figure belongs to Monsanto Japan Limited.

③ If there are multiple copies on the chromosome, whether they are adjacent or separate

Since it is a copy, it does not apply (Appendix 17 of p. 29).

④. Stability of expression among individuals and between generations under natural conditions with respect to the characteristics specifically shown in (6)-①

Using western blot analysis, we found that the modified Cry3Bb1 protein and the modified CP4 EPSPS protein were stably expressed in multiple generations (R4, R4F1, R5, R5F1 and R6 generation) of the genetically modified maize (Appendix 18 in Figures 2 to 3, p.17 to 18 in Appendix 18).

We also collected over-season leaf (OSL 1 to 4), roots (over-season root; OSR 1 to 4), aerial parts (over-season whole plant; OSWP 1 to 4), stems of maturity, root of maturity, root of yellowish maturity, aerial part of ground part, pollen and silk of the genetically modified maize grown in 4 repetitions in 5 locations in Buenos Aires, Argentina from 2011 to 2012. Then, we analyzed the expression level of RNA of DvSnf7 gene fragment, modified Cry3Bb1 protein and modified CP4 EPSPS protein by QuantiGene® Plex 2.0 Assay[30] or ELISA (Appendix 19, Appendix 20). As a result, expression of the RNA, the modified Cry3Bb1 protein and the modified CP4 EPSPS protein was confirmed in all the organisms of the genetically modified maize (Tables 10 to 12, pp 62 to 67; Appendix Appendix) 19 Table 1, p18 to 21; Appendix 20 Table 1 to 2, p 18 to 21).

[30] Quantigene® Plex 2.0 Assay is a technique to capture target RNA using magnetic beads and sequence-specific probes and measures the expression level by detecting fluorescence emission. In this analysis, the *DvSnf7* gene fragment (240 bases) is used as a probe. Also, RNA extracted from each tissue of corn contains dsRNA. However, we analyzed the expression level of all dsRNAs after denaturing them into single strands in this analysis.

Table 10 Expression level of RNA of *DvSnf7* gene fragment in each organism of the genetically modified maize (2011-2012, Argentina)[31]

Organism[1]	Growth stage[2]	Average value (standard deviation) Range [μg / g fresh weight][3]	Average value (standard deviation) Range [μg / g dry weight][4]	Detection limit value / Quantitation limit value [Mg / g fresh weight]
Leaf (OSL1)	3 - 4 leaf stage	12.6×10^{-3} (2.11×10^{-3}) 8.53×10^{-3} - 16.6×10^{-3}	73.9×10^{-3} (14.5×10^{-3}) 43.3×10^{-3} - 103×10^{-3}	0.129×10^{-3}/ 0.566×10^{-3}
Leaf (OSL2)	6 - 8 leaf stage	13.2×10^{-3} (4.08×10^{-3}) 7.77×10^{-3} - 20.1×10^{-3}	67.3×10^{-3} (19.4×10^{-3}) 37.1×10^{-3}- 98.9×10^{-3}	0.114×10^{-3}/ 0.502×10^{-3}
Leaf (OSL3)	10 - 13 leaf stage	10.3×10^{-3} (1.89×10^{-3}) 6.19×10^{-3} - 12.8×10^{-3}	44.6×10^{-3} (8.51×10^{-3}) 27.5×10^{-3} - 58.8×10^{-3}	0.106×10^{-3}/ 0.468×10^{-3}
Leaf (OSL4)	14 leaf stage - Silking stage	14.4×10^{-3} (6.71×10^{-3}) 5.40×10^{-3} - 33.8×10^{-3}	56.9×10^{-3} (28.5×10^{-3}) 22.1×10^{-3} - 153×10^{-3}	0.110×10^{-3}/ 0.482×10^{-3}
Root (OSR1)	3 - 4 leaf stage	3.15×10^{-3} (1.79×10^{-3}) 1.74×10^{-3} - 8.00×10^{-3}	23.9×10^{-3} (15.1×10^{-3}) 12.5×10^{-3} - 67.0×10^{-3}	0.029×10^{-3}/ 0.128×10^{-3}
Root (OSR2)	6 - 8 leaf stage	2.32×10^{-3} (0.758×10^{-3}) 0.928×10^{-3} - 3.76×10^{-3}	16.3×10^{-3} (4.84×10^{-3}) 6.62×10^{-3} - 25.7×10^{-3}	0.021×10^{-3}/ 0.093×10^{-3}
Root (OSR3)	10 - 13 leaf stage	1.81×10^{-3} (0.749×10^{-3}) 0.942×10^{-3} - 4.00×10^{-3}	10.2×10^{-3} (4.77×10^{-3}) 5.13×10^{-3} - 24.3×10^{-3}	0.020×10^{-3}/ 0.088×10^{-3}
Root (OSR4)	14 leaf stage - Silking stage	1.28×10^{-3} (0.471×10^{-3}) 0.530×10^{-3} - 2.40×10^{-3}	6.84×10^{-3} (2.67×10^{-3}) 2.66×10^{-3} - 13.0×10^{-3}	0.015×10^{-3}/ 0.067×10^{-3}
Aerial part (OSWP1)	3 - 4leaf stage	10.5×10^{-3} (4.25×10^{-3}) 6.78×10^{-3} - 23.1×10^{-3}	84.8×10^{-3} (43.8×10^{-3}) 51.1×10^{-3} - 213×10^{-3}	0.078×10^{-3}/ 0.345×10^{-3}
Aerial part (OSWP2)	6 - 8leaf stage	8.54×10^{-3} (3.54×10^{-3}) 5.01×10^{-3} - 16.0×10^{-3}	55.1×10^{-3} (23.1×10^{-3}) 33.0×10^{-3} - 106×10^{-3}	0.054×10^{-3}/ 0.239×10^{-3}
Aerial part (OSWP3)	10 - 13leaf stage	3.53×10^{-3} (1.17 × 10-3) 2.03×10^{-3} - 5.89×10^{-3}	25.5×10^{-3} (9.53×10^{-3}) 13.0×10^{-3} - 45.9×10^{-3}	0.027×10^{-3}/ 0.119×10^{-3}
Aerial part (OSWP4)	14leaf stage - Silking stage	3.16×10^{-3} (1.03×10^{-3}) 1.89×10^{-3} - 5.37×10^{-3}	18.5×10^{-3} (6.27×10^{-3}) 10.3×10^{-3} - 32.2×10^{-3}	0.028×10^{-3}/ 0.123×10^{-3}

[31]The responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

Table 10 Expression level of RNA of *DvSnf7* gene fragment in each organism of the genetically modified maize (2011-2012, Argentina) (continued)

Organism[1]	Growth stage[2]	Average value (standard deviation) Range [μg / g fresh weight][3]	Average value (standard deviation) Range [μg / g dry weight][4]	Detection limit value / Quantitation limit value [Mg / g fresh weight]
Root	Maturity stage	0.536×10^{-3} (0.295×10^{-3}) 0.086×10^{-3} - 1.07×10^{-3}	2.37×10^{-3} (1.29×10^{-3}) 0.425×10^{-3} - 4.61×10^{-3}	0.013×10^{-3}/ 0.059×10^{-3}
Aerial part	Yellowing-ripe stage	1.28×10^{-3} (0.361×10^{-3}) 0.601×10^{-3} - 2.31×10^{-3}	4.26×10^{-3} (1.26×10^{-3}) 2.00×10^{-3} - 7.72×10^{-3}	0.036×10^{-3}/ 0.157×10^{-3}
Root	Ripening stage	0.353×10^{-3} (0.203×10^{-3}) 0.127×10^{-3} - 0.947×10^{-3}	1.39×10^{-3} (0.815×10^{-3}) 0.478×10^{-3} - 3.68×10^{-3}	0.015×10^{-3}/ 0.065×10^{-3}
Stem and leaf	Ripening stage	0.310×10^{-3} (0.077×10^{-3}) 0.190×10^{-3} - 0.449×10^{-3}	0.677×10^{-3} (0.201×10^{-3}) 0.401×10^{-3} - 1.04×10^{-3}	0.047×10^{-3}/ 0.207×10^{-3}
Pollen	Detasseling stage - Silking stage	0.103×10^{-3} (0.069×10^{-3}) 0.056×10^{-3} - 0.224×10^{-3}	0.134×10^{-3} (0.090×10^{-3}) 0.073×10^{-3} - 0.292×10^{-3}	0.013×10^{-3}/ 0.057×10^{-3}
Silk	Silking stage	0.530×10^{-3} (0.190×10^{-3}) 0.215×10^{-3} - 0.893×10^{-3}	5.42×10^{-3} (2.05×10^{-3}) 1.99×10^{-3} - 9.03×10^{-3}	0.004×10^{-3}/ 0.019×10^{-3}
Kernel	Ripening stage	0.091×10^{-3} (0.028×10^{-3}) 0.049×10^{-3} - 0.153×10^{-3}	0.104×10^{-3} (0.033×10^{-3}) 0.056×10^{-3} - 0.175×10^{-3}	0.008×10^{-3}/ 0.036×10^{-3}

[1] OSL= over-season leaf; OSR= over-season root; OSWP= over-season whole plant

[2] Growth stage of each organism collected

[3] The expression level of RNA of the *DvSnf7* gene fragment is calculated as μg per gram of fresh weight of the organism. The mean value, standard deviation and range (minimum value - maximum value) are calculated for each organism taken at all five fields (roots (ripening stage), stems, pollen and kernels Except for n = 20 for all organisms, n = 19 for roots (ripening stage), n = 16 for stems and leaves, n = 5 for pollen and n = 18 for kernels.) The expression level in two pollen samples was less than the detection limit value (LOD). Samples of other roots (ripening stage), foliage, pollen and kernel were below the quantification limit (LOQ).

[4] The expression amount of RNA of the *DvSnf7* gene fragment is calculated as μg per 1 g of dry weight of the organism. The mean value, standard deviation and range (minimum value - maximum value) are calculated for each organism taken at all 5 fields (n = 20 in all organisms, except for roots (ripening stage), foliage, pollen and kernels; roots (ripening stage) n = 19, stems and leaves n = 16, pollens n = 5 and kernels n = 18).)

The expression level in two pollen samples was less than the detection limit value (LOD). Samples of other roots (ripening stage), foliage, pollen and kernel were below the quantification limit (LOQ). Dry weight was obtained by dividing fresh weight by dry weight coefficient obtained from moisture analysis data.

Table 11 Expression level of modified Cry3Bb1 protein (2011-2012, Argentina) in each organism of the genetically modified maize[32]

Organism[1]	Growth stage[2]	Number of days after sowing	Average value (standard deviation) Range [µg / g fresh weight][3]	Average value (standard deviation) Range [µg / g dry weight][4]	Quantitation limit value/ Detection limit value [Mg / g fresh weight]
Leaf (OSL1)	3 - 4 leaf stage	21-22	45 (9.0) 31 – 64	270 (65) 160 - 390	0.035/0.006
Leaf (OSL2)	6 - 8 leaf stage	35-44	40 (7.8) 26 – 56	210 (40) 120 – 270	0.035/0.006
Leaf (OSL3)	10 - 13 leaf stage	50-55	40 (7.9) 21 – 52	170 (35) 92 – 220	0.035/0.006
Leaf (OSL4)	14 leaf stage - Silking stage	59-78	56 (19) 31 – 89	220 (63) 130 – 340	0.035/0.006
Root (OSR1)	3 - 4 leaf stage	21-22	25 (4.6) 16 – 32	180 (43) 130 – 280	0.035/0.028
Root (OSR2)	6 - 8 leaf stage	35-44	16 (4.0) 9.4 – 25	120 (24) 67 – 170	0.035/0.028
Root (OSR3)	10 - 13 leaf stage	50-55	15 (4.0) 9.6 – 24	84 (21) 54 – 130	0.035/0.028
Root (OSR4)	14 leaf stage - Silking stage	59-78	14 (3.3) 9.0 – 21	75 (19) 43 – 120	0.035/0.028
Aerial part (OSWP1)	3 - 4 leaf stage	21-22	44 (4.9) 33 – 53	340 (49) 250 – 460	0.035/0.008
Aerial part (OSWP2)	6 - 8 leaf stage	35-44	30 (5.3) 21 – 40	190 (30) 130 – 270	0.035/0.008
Aerial part (OSWP3)	10 - 13 leaf stage	50-55	20 (6.8) 9.2 – 33	140 (39) 59 – 210	0.035/0.008
Aerial part (OSWP4)	14 leaf stage - Silking stage	59-78	20 (4.8) 12 – 29	120 (28) 71 – 170	0.035/0.008
Leaf and stem	Ripening stage	136-155	10 (6.2) 1.9 – 19	21 (13) 4.7 – 44	0.035/0.008
Root	Ripening stage	136-155	4.8 (3.1) 0.76 – 12	19 (13) 3.0 – 50	0.035/0.028
Root	Yellowing-ripe stage	101-111	7.9 (3.5) 2.6 – 15	36 (16) 13 – 66	0.035/0.028
Aerial part	Yellowing-ripe stage	101-111	12 (4.9) 5.5 – 23	39 (17) 18 – 75	0.035/0.008

[32]Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

Table 11 Expression level of modified Cry3Bb1 protein in each organism of this recombinant maize (genetically modified maize) (2011-2012, Argentina) (continued)

Organism[1]	Growth stage[2]	Number of days after sowing	Average value (standard deviation) Range [µg / g fresh weight] [3]	Average value (standard deviation) Range [µg / g dry weight] [4]	Quantitation limit value/Detection limit value [Mg / g fresh weight]
Kernel	Ripening stage	139-154	3.5 (0.45) 2.7 – 4.4	4.0 (0.56) 3.1 – 5.1	0.035/0.007
Pollen	Detasseling stage - Silking stage	65-80	29 (3.0) 23 – 34	36 (4.0) 30 – 42	0.035/0.018
Silk	Silking stage	65-81	16 (3.8) 8.5 – 23	160 (37) 89 – 220	0.035/0.010

[1] OSL= over-season leaf; OSR= over-season root; OSWP= over-season whole plant

[2] Growth stage of each organism collected

[3] The expression level of protein is represented by arithmetic average value and standard deviation (shown in parentheses). The protein weight is expressed in µg per gram of fresh weight of the organism. The mean value, standard deviation and range (minimum value - maximum value) are calculated for each organism taken at all fields (n = 20 in all organisms).

[4] The expression level of protein is represented by arithmetic average value and standard deviation (shown in parentheses). The weight of the protein is expressed in µg per 1 g of dry weight of the organism. Dry weight was obtained by dividing fresh weight by dry weight conversion coefficient obtained from moisture analysis data.

Table 12 Expression amount of modified CP4 EPSPS protein in each organism of the genetically modified maize (2011 - 2012, Argentina)[33]

Organism[1]	Growth stage[2]	Number of days after sowing	Average value (standard deviation) Range [μg / g fresh weight][3]	Average value (standard deviation) Range [μg / g dry weight][4]	Quantitation limit value/ Detection limit value [Mg / g fresh weight]
Leaf (OSL1)	3 - 4 leaf stage	21-22	7.1 (0.83) 5.8 – 9.0	42 (5.9) 33 – 55	0.137/0.071
Leaf (OSL2)	6 - 8 leaf stage	35-44	7.0 (0.64) 6.0 – 7.9	36 (3.1) 29 – 39	0.137/0.071
Leaf (OSL3)	10 - 13 leaf stage	50-55	7.4 (0.78) 6.4 – 8.9	32 (3.8) 27 – 42	0.137/0.071
Leaf (OSL4)	14 leaf stage - Silking stage	59-78	7.8 (0.85) 6.6 – 9.5	31 (3.5) 24 – 37	0.137/0.071
Root (OSR1)	3 - 4 leaf stage	21-22	6.5 (0.86) 4.4 – 8.0	48 (6.6) 38 – 63	0.068/0.033
Root (OSR2)	6 - 8 leaf stage	35-44	5.2 (1.0) 3.8 – 7.1	37 (7.0) 23 – 48	0.068/0.033
Root (OSR3)	10 - 13 leaf stage	50-55	5.6 (0.84) 4.0 – 7.1	31 (4.7) 24 – 37	0.068/0.033
Root (OSR4)	14 leaf stage - Silking stage	59-78	5.7 (0.80) 4.2 – 7.1	30 (4.8) 20 – 38	0.068/0.033
Aerial part (OSWP1)	3 - 4 leaf stage	21-22	8.1 (0.90) 6.6 – 9.8	63 (6.7) 54 – 76	0.137/0.070
Aerial part (OSWP2)	6 - 8 leaf stage	35-44	5.6 (0.94) 3.4 – 7.4	36 (5.8) 21 – 46	0.137/0.070
Aerial part (OSWP3)	10 - 13 leaf stage	50-55	4.6 (1.1) 2.3 – 6.6	33 (6.2) 21 – 45	0.137/0.070
Aerial part (OSWP4)	14 leaf stage - Silking stage	59-78	4.3 (0.87) 2.9 – 5.5	25 (5.0) 17 – 32	0.137/0.070
Stem and leaf	Ripening stage	136-155	1.0 (0.60) 0.30 – 2.1	2.2 (1.2) 0.59 – 4.9	0.137/0.070
Root	Ripening stage	136-155	1.4 (0.69) 0.49 – 2.6	5.4 (2.9) 1.8 – 11	0.068/0.033

[33]Responsibility for the rights and contents pertaining to the information described in this table belongs to Monsanto Japan Limited.

エラー! 参照元が見つかりません。Table 12 Expression level of modified CP4 EPSPS protein in each organism of this recombinant maize (genetically modified maize) (2011-2012, Argentina) (continued)

Organism[1]	Growth stage[2]	Number of days after sowing	Average value (standard deviation) Range [μg / g fresh weight] [3]	Average value (standard deviation) Range [μg / g dry weight]][4]	Quantitation limit value/Detection limit value [Mg / g fresh weight]
Root	Yellowing-ripe stage	101-111	2.2 (0.81) 1.1 – 4.1	10 (3.7) 5.1 – 19	0.068/0.033
Aerial part	Yellowing-ripe stage	101-111	2.4 (0.71) 1.5 – 3.8	8.0 (2.3) 5.2 – 13	0.137/0.070
Kernel	Ripening stage	139-154	1.7 (0.27) 1.4 – 2.7	1.9 (0.31) 1.6 – 3.1	0.228/0.152
Pollen	Detasseling stage - Silking stage	65-80	15 (1.9) 12 – 19	19 (2.8) 16 – 24	0.137/0.099
Silk	Silking stage	65-81	4.0 (0.69) 3.1 – 5.1	40 (5.0) 32 – 49	0.137/0.121

[1] OSL= over-season leaf; OSR= over-season root; OSWP= over-season whole plant

[2] Growth stage of each organism collected

[3] The expression level of protein is represented by arithmetic average value and standard deviation (shown in parentheses). The protein weight is expressed in μg per gram of fresh weight of the organism. The mean value, standard deviation and range (minimum value - maximum value) are calculated for each organism taken at all fields (N = 20 in all organisms except stem and leaves, n = 19 in stems). The expression level in one sample of stem and leaf was less than the quantification limit value (LOQ).

[4] The expression level of protein is represented by arithmetic average value and standard deviation (shown in parentheses). The weight of the protein is expressed in μg per 1 g of dry weight of the organism. Dry weight was obtained by dividing fresh weight by dry weight conversion coefficient obtained from moisture analysis data.

⑤ If the transferred nucleic acid via virus infection or other route is likely to be transmitted to wild animals and plants etc, the presence or absence of the degree and degree

There is no possibility that the sequence of transferred nucleic acid is transmitted to wild animals and plants etc. via virus infection and other routes because there is no function to enable transmission.

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

Using primers specific for the genetically modified maize, it is possible to detect and identify by Real-Time TaqMan PCR method (Appendix 21).

The detection limit value of this PCR method is 0.04% in genomic DNA quantity ratio (Appendix 22, p.11).

The reliability of this PCR method has been verified and confirmed in Monsanto Company, USA and BioDiagnostics Inc. in USA (Appendix 22, p.13 to 15).

(6) Differences from taxonomic species to which the host or host belongs

① Specific contents of physiological or ecological characteristics provided by expression of replicates of transferred nucleic acid

The *DvSnf7* gene fragment introduced into the genetically modified maize expresses the dsRNA of the *DvSnf7* gene fragment showing insecticidal activity against CRW, providing resistance to CRW. Also, by expressing the modified Cry3Bb1 protein and the modified CP4 EPSPS protein, respectively, the modified *cry3Bb1* gene and the modified *cp4 epsps* gene provide resistance to Coleoptera pest and herbicide glyphosate tolerance.

② Regarding the physiological or ecological characteristics listed below, if there is a difference or existence between genetically modified agricultural crops and taxonomic species to which the recipient organism belongs, the extent thereof[34]

Maize has been used for a long time since introduction in Japan in 1579, but no case has been reported where corn has naturally grown under natural conditions.

In 2014, we conducted an isolated field test of the genetically modified maize in an isolated field of Monsanto Co., Ltd. Kawachi research farm (hereinafter referred to as the "Isolated Field"). We used R4F1 (HCL645 x LH244) generation of the genetically modified maize for isolated field test (Fig. 9, p.61). As a control non-recombinant maize we used HCL645 x LH244 with a genetic background similar to the genetically modified maize. Also, we conducted a cold tolerance test in the early stage of growth (1- 2- (6) - ② - b, p. 69) in 2012 in the artificial weather room in the United States.

a Morphology and growth characteristics

In order to evaluate the morphology and growth characteristics, we investigated the following 9 items: Detasseling stage (month), silking stage (month), main stem length (cm) height of ear in corn plant (cm), number of tillers, ripening stage (month), weight of the aerial part at the harvesting period (kg), grain shape, grain color).

We performed statistical treatment on main stem length (cm), height of ear in corn plant (cm), tiller number and aerial weight (kg) at harvesting stage. However, no statistical processing was performed with respect to the detasseling stage (moon day), silking stage (month), ripening stage (month), grain type, grain color since they have no quantitative variation.

As a result, no statistically significant difference or difference was observed between the genetically modified maize and the non-genetically modified maize plants in any of the above items (Appendix 12, Table 3, p.10).

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b Low Temperature Resistance in Early Growth

The cold tolerance test in the early stage of growth was carried out in 2012 in the Monsanto Company's artificial weather room in the United States. The seedlings of the genetically modified maize, the control non-recombinant maize and the conventional commercial variety four varieties on the 14th day (about 3 leaf stage) after seeding were cultivated at low temperature conditions of 12 ° C during the day / 5 ° C at night. We studied the growth stage and plant length on the 10th day before the start of the low temperature treatment and at the start of the same treatment. Then, on the 19th day after the initiation of the low temperature treatment, the growth stage, plant height, fresh weight and dry weight were investigated (1 individual / repetition, 10 iterations). Statistical treatment was performed on plant height (cm), fresh weight (g) and dry weight (g). Regarding the growth stage, statistical processing was not carried out because there was no quantitative variation.

As a result, no statistically significant difference or difference was observed between the genetically modified maize and the non-genetically modified maize plants in any item (Appendix 23 Table 2, p.6).

c Overwintering of adult plants

Maize is a summer-type annual plant, and normally it dies naturally in winter after fruiting. They do not reproduce and propagate vegetatively or produce seeds. In fact, we continued to grow the genetically modified maize and control non-genetically modified maize plants grown in this isolated field even after the ripening stage. And we observed the growth situation in our winter season, observing the growth situation in our winter season. As a result of observing individuals cultivated in the overwintering test area on November 19, 2014, both the genetically modified maize and the non- genetically modified maize plants were dead (Appendix 12, FIG. 5, p.12).

d Fertility and size of pollen

Pollen collected from the genetically modified maize grown at this isolated field and controlled non- genetically modified maize grown plants was stained with Alexander solution and pollen fertility (degree of fulfillment) and size were

measured. As a result of statistical processing on these items, statistical analysis was conducted between the genetically modified maize and the control non-recombinant maize in both items of pollen fertility (degree of fulfillment) and size, there was no significant difference (Appendix 12, Figure 6, p.13 and Table 4, p.13).

e Seed production, shedding, dormancy and germination rate

Seed production:

We investigated the following six items concerning seed production for the genetically modified maize plants grown at this isolated field and non-recombinant control maize: effective ear number, ear length (cm), ear diameter (cm), number of grain rows, number of grains, 100 grain weight (g).

As a result of statistical processing on these items, a statistically significant difference was observed between the genetically modified maize and the genetically modified maize plants only at the ear diameter. (Appendix 12, Table 5, p14). The average value of the ear diameter was 4.6 cm for the genetically modified maize plants, and 4.5 cm for the non- genetically modified maize plants. The value of the genetically modified maize was higher (Appendix 12, Table 5, p. 14).

Shedding:

Regarding the shedding property, we observed the genetically modified maize and the control non-genetically modified maize plants, regarding whether or not they are wrapped in bracts by visual inspection, presence or absence of after removal of the bract and the degree.

As a result, in each of the genetically modified maize and the control non-genetically modified maize plants, the ears at the time of harvest were covered with bracts, and no shedding under natural conditions was confirmed. The ears after removal of the husk were also difficult to shed. There was no difference in seed shedding between the genetically modified maize and the controlled-genetically modified maize plants (Appendix 12, Table 5, p 14).

Dormancy and germination rate:

Regarding the dormancy and germination rate, we counted the number of germinated individuals in a thermostat set at 25 ° C on the 16th day after harvesting the seeds on a petri dish.

As a result, the germination rates of the genetically modified maize and the controlled-genetically modified maize plants were 100% and 99.5%, respectively. There was no statistically significant difference (Appendix 12, Table 5, p. 14).

f Crossability

Since there are no closely related wild species that can be crossed in Japan, we did not test the crossing rate.

g Productivity of harmful substances

In order to confirm that no substances affecting soil microorganisms or other plants have been produced from the genetically modified maize plants, we conducted soil microbial test, plow-in test and post-production test.

As a result, there was no statistically significant difference in the number of soil microorganisms, the germination rate of radish, and the dry weight between the genetically modified maize and the controlled non-genetically modified maize plants (Appendix 12, Tables 6 to 8, p.17).

Ⅱ Results of examination at the Study Group on Biological Diversity Risk Assessment

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

1 On the results of Biological Diversity Risk Assessment

The genetically modified maize has been produced by introducing the T-DNA region of PV-ZMIR10871 constructed on the basis of plasmid pBR322 derived from E. coli by the Agrobacterium method.

In the genetically modified maize, one copy of the expression cassette containing the following is integrated on the chromosome:

① *DvSnf7* gene fragment derived from Western corn rootworm (*Diabrotica virgifera virgifera*) * designed to produce double-stranded RNA (dsRNA) that produces RNA interference effect *

② A modified *cry3Bb1* gene encoding a modified Cry3Bb1 protein derived from *Bacillus thuringiensis*

③ A modified *cp4 epsps* gene encoding a modified CP4 EPSPS protein derived from *Agrobacterium* CP4 strain

It has been confirmed by gene isolation mode and bioinformatics analysis that it is stably transmitted over multiple generations.

In addition, Western blot analysis confirmed that the target gene is stably expressed over multiple generations.

(1) Advantage in competition

Maize has a long history of being cultivated in Japan, but there have been no reports that it has grown up in the natural environment so far.

We cultivated this recombinant maize (genetically modified maize) and control non-recombinant maize in the isolation field of our country in 2014. We then compared the traits associated with competitive advantage (morphology and growth characteristics, overwintering of adults, fertility and size of pollen, seed production, shedding, dormancy and germination rate). However, it is

unlikely that this significant difference will enhance the superiority of the competitiveness of genetically modified maize plants.

In addition, resistance to Coleoptera pest is imparted to the genetically modified maize by the dsRNA produced from the *DvSnf7* gene fragment and the modified Cry3Bb1 protein. However, only damage caused by Coleoptera pest cannot be a factor to regulate the growth of maize in the natural environment of this country. It is unlikely that the genetically modified maize grows naturally in the natural environment, further enhancing the competitive advantage.

Also, the genetically modified maize has herbicide glyphosate tolerance (herbicide, glyphosate-tolerant maize) due to the production of the modified CP4 EPSPS protein. Under the natural environment that glyphosate is not expected to be sprayed, it is unlikely that being resistant to glyphosate will enhance the competitive advantage.

Based on the above, we concluded that the applicant's conclusion that the genetically modified maize is not likely to cause biodiversity effects due to competitive advantage.

(2) Productivity of harmful substances

Maize has a long history of being grown in our country, but there have been no reports that corn has produced toxic substances so far.

The *DvSnf7* gene fragment has low homology with the mRNA of the endogenous gene of maize, and high homology is found among insect species belonging to the subfamily Galerucinae, the family Coleoptera subsp., Coleopter. Therefore, we do not believe that the dsRNA produced in the genetically modified maize suppresses the expression of genes internal to corn by the RNAi mechanism. Moreover, it is hard to imagine that it is difficult to produce a new protein, so it is difficult to think that it acts on the metabolic system of the host to produce harmful substances. In addition, dsRNA produced in the genetically modified maize has been introduced with the purpose of conferring insecticidal activity against Western corn rootworm. However, its insecticidal spectrum is extremely narrow, and it is limited to some insects belonging to family Chrysomelidae among insects of the order Coleoptera.

In addition, the insecticidal spectrum of the modified Cry3Bb1 protein produced in the genetically modified maize is extremely narrow. It shows

insecticidal activity only in the Colorado potato beetle and corn rootworm belonging to the subfamily Chrysomelinae of the family Chrysomelidae and the subfamily Galerucinae among insect species (Coleoptera), respectively, and no toxicity to other wild animals and plants has been recognized.

Since dsRNA, modified Cry3Bb1 protein and modified CP4 EPSPS protein produced by the genetically modified maize each have different action mechanisms, it seems that there is no possibility of interacting to synergistically increase insecticidal activity against specific insects or expand insecticidal spectrum.

In addition, the EPSPS protein, which is functionally identical to the modified CP4 EPSPS protein, is an enzyme protein that catalyzes the shikimate pathway for biosynthesizing aromatic amino acids. However, it is unlikely that the concentration of aromatic amino acid, which is the final product of this pathway, will increase even if the activity of EPSPS protein is increased instead of the rate-limiting enzyme in this pathway.

Also, it has been confirmed that the modified Cry3Bb1 protein and the modified CP4 EPSPS protein do not have an amino acid sequence similar to the known allergen.

As wild animals and plants etc. that cannot be denied the possibility of being affected by eating pollen or plants of the genetically modified maize, four species of Coleoptera insects designated as endangered or semi-threatened species inhabiting Japan were identified.

However, the amount of pollen accumulating around the corn cultivation field is extremely low when it is 10 m away from the field, and it can be considered that it is in a state where it can be almost ignored if it is separated by 50 m or more. Thus, it is unlikely that the Coleoptera insect species live locally in this area, and the possibility of being affected is considered to be extremely low.

In addition, when we conducted a plow-in test and a post-production test at the isolation field of our country in 2014, there was no statistically significant difference between the genetically modified maize and the non-genetically modified maize plants with respect to radish germination rate and dry weight. In addition, a statistically significant difference was not found between the genetically modified maize and the non- genetically modified maize plants

with regard to the number of bacteria, actinomycetes and filamentous fungi as a result of soil microbial test.

Based on the above, we concluded that the conclusion by the applicant that the genetically modified maize is not likely to cause biodiversity effects due to the productivity of harmful substances is appropriate.

(3) Crossability

Corn can be crossed with teosint, a closely related wild type, but in Japan there is no report of nutrition of teosint. For this reason, no wild animals or plants were identified that may be affected by biodiversity due to the crossability of the genetically modified maize.

Based on the above, we concluded that the applicant's conclusion that the genetically modified maize is unlikely to cause biodiversity effects due to crossability is appropriate.

2 Conclusion based on Biological Diversity Risk Assessment

From the above, when the genetically modified maize is used according to the First Type (Type 1 Use) regulation, the conclusion of the Biological Diversity Risk Assessment Report states that there is no fear of affecting biodiversity in Japan, which we judged that it was valid.

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Appendix List

Appendix 11 Bioinformatic Comparison of the 240 Nucleotide DvSnf7 Sequence with Eukaryotic Soil Microorganism and *Zea mays* Sequence Databases (RAR-2013-0260) (Confidential)

Appendix 12 Biological Diversity Risk Assessment Test Result Report (Confidential) in an isolated field of Coleoptera pest-resistant and herbicide glyphosate tolerant maize (*DvSnf 7, modified cry 3 Bb 1, modified cp 4 epsps, Zea mays subsp. Mays (L.) Iltis*) (MON 87411, OECD UI: MON-87411-9)

Appendix 13 Additional Analysis Using Akaike's Information Criterion to Support the Conclusion of No Toxicological Interaction Between the Cry3Bb1 Protein and DvSnf7RNA (MSL0027233) (Confidential)

Appendix 14 Sequence of Genetic Elements in PV-ZMIR10871 (Confidential)

Appendix 15 PCR analysis to confirm the absence of *Agrobacterium tumefaciens* used to produce MON 87411 (MSL0025553) (Confidential)

Appendix 16 Segregation of the T-DNA Insert in MON 87411 Across Three Generations (MSL0024728) (Confidential)

Appendix 17 Amended Report for MSL0025314: Molecular Characterization of MON 87411 (MSL0025845) (Confidential)

Appendix 18 Demonstration of the Presence of Cry3Bb1 and CP4 EPSPS Proteins in Maize Leaf Samples across Multiple Generations of MON 87411 (MSL0024801) (Confidential)

Appendix 19 Assessment of DvSnf7 RNA Levels in Maize Organisms Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012 (MSL0024697) (Confidential)

Appendix 20 Assessment of Cry3Bb1 and CP4 EPSPS Protein Levels in Corn Organisms Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012 (MSL0024586) (Confidential)

Appendix 21 A Recommended Procedure for Real-Time Quantitative TaqMan PCR for MON 87411 (Confidential)

Appendix 22 In-House Validation Summary for “A Recommended Procedure for Real- Time Quantitative TaqMan PCR for MON 87411 using 109 bp Event- Specific product at the 3’ End” (Confidential)

Appendix 23 Assessment of the Effect of Cold Stress on Maize MON 87411 under Controlled Environment Conditions in 2012 (MSL0024455) (Confidential)

Appendix 24 Characterization of DvSnf7 RNA Extracted from MON 87411 and Comparison of the Molecular and Functional Properties of Plant-Produced and *in vitro*-Produced DvSnf7 RNA (MSL0025263) (Confidential)