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

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Approved Type 1 Use Regulation

Name of the Type of Living Modified Organism	Maize resistant to Lepidoptera (Modified *vip3A*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR162, OECD UI: SYN-IR162-4)
Content of the Type 1 Use of Living Modified Organism	Provision as food, provision as feed, cultivation, processing, storage, transportation, disposal and acts incidental to them
Method of the Type 1 Use of Living Modified Organism	—

Outline of the Biological Diversity Risk Assessment Report

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

1. Information concerning preparation of living modified organisms

(1) Information concerning donor nucleic acid

1) Composition and origins of component elements

The composition of donor nucleic acid and the origins of component elements used for the development of maize resistant to Lepidoptera (modified *vip3A*, *Zea mays* subsp. *mays* (L.) Iltis) (MIR162, OECD UI: SYN-IR162-4) (hereinafter referred to as "this recombinant maize") are shown in Table 1.

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

Genetic element	Size (bp)	Origin and function
Insect pest-resistant gene cassette		
ZmUbiInt promoter	1,993	Promoter region from *Z. mays* polyubiquitin gene which contains the first intron (1,010bp). It provides constitutive expression of target gene in all the tissues of monocots (Reference 13).
Modified *vip3A* gene	2,370	A modified version of the native *vip3A* gene found in the *Bacillus thuringiensis* strain AB88, a gram-positive bacteria existing normally in soil (Reference 14), to accommodate the preferred codon usage in plants (Reference 15). The *vip3A* gene encodes the modified Vip3A protein which exhibits the insecticidal activity against Lepidopteran insect pests. In the modified Vip3A protein, the amino acid at position 284 in the amino acid sequence was substituted to glutamine from lysine. In addition, in the modified Vip3A protein expressed in this recombinant maize, methionine at position 129 was substituted by isoleucine during transformation.
iPEPC9	108	Intron #9 sequence from the phosphoenolpyruvate carboxylase gene from *Z. mays*. Used to enhance the expression of target gene (Reference 16).
35S terminator	70	Polyadenylation sequence derived from the cauliflower mosaic virus 35S RNA (Reference 17).
Selective marker gene cassette		
ZmUbiInt promoter	1,993	Same as described above
pmi gene	1,176	*manA* gene from the *Escherichia coli* strain K-12, encoding the phosphomannose isomerase (hereinafter referred to as "PMI protein"). Used as a selectable marker for gene-transferred transformant (Reference 18).
NOS terminator	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens*. Its function is to terminate transcription of mRNA by polyadenylation (Reference 19).

Other regions (hereinafter referred to as "backbone region")		
LB	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 20).
spec	789	Streptomycin adenylyltransferase gene (*aadA*) from *E. coli* transposon Tn7 (Reference 21). Used as a vector selectable marker to confer resistance to erythromycin, streptomycin, and spectinomycin.
cos	432	Cohesive end region of the linear DNA of lambda phage required for transferring of plasmid into *E. coli* and autonomous replication of plasmid in *E. coli* (Reference 22).
ColE1 ori	807	Origin of replication of plasmid in *E. coli* (Reference 23).
RB	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Reference 24).

2) Function of component elements

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

Functions of component elements of donor nucleic acid that was used for the production of this recombinant maize are shown in Table 1.

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

<u>Modified Vip3A protein</u>

The Cry protein derived from *Bacillus thuringiensis*, exhibiting insecticidal activity, is produced during sporulation of *B. thuringiensis* and inherent in the cells. In contrast, the Vip protein, to which the modified Vip3A protein expressed in this recombinant maize belongs, has been discovered as Vegetative Insecticidal Protein produced during vegetative growth of *B. thuringiensis* and secreted outside cells (Reference 14). As the Vip protein, Vip1, Vip2 and Vip3 proteins have been identified and they are classified into 3 ranks and 7 divisions by the *Bacillus Thuringiensis* Nomenclature Committee. The Vip1 and Vip2 proteins exhibit insecticidal activity against the insects of order Coleoptera, and the Vip3 protein exhibits its insecticidal activity against the insects of order Lepidoptera.

The Vip3A protein has a total length of 88kDa, though the Vip3A protein, when fed by larvae of the target insects of order Lepidoptera, is partially digested in the digestive tracts and becomes a core protein having a length of 62kDa. It is known that the core protein binds to the specific receptors on the intestinal epithelium cells of target insects, causing disturbed ionic balance leading to destructed intestinal epithelium cells and resultantly inhibited digestive process, which contributes to insecticidal activity (Reference 12, Reference 25). This mechanism of action is also attained similarly in the Cry protein. In addition, Lee and his colleagues (Reference 12) have reported that the Vip3A protein and the Cry1Ab protein bind to the brush border membrane vesicles (BBMV) of mid-gut epithelium without any conflict with each other, and they also have revealed that the Vip3A protein does not bind to any aminopeptidase-like and cadherin-like molecules, which are known as the receptors of

Cry1Ab protein, in the BBMV of Tobacco Hornworm (*Manduca sexta*), a sensitive insect of the order Lepidoptera (Reference 12). Consequently, it is suggested that the Vip3A protein provides the similar mechanism of action as the Cry protein, though the Vip3A protein differs from the Cry1Ab protein regarding the receptors involved (Reference 12).

The Vip3A protein exhibits high insecticidal activity against Fall Armyworm (*Spodoptera frugiperda*), Corn Earworm (*Helicoverpa zea*), Black Cutworm (*Agrotis ipsilon*) and other order Lepidopteran insects which are the pest insects for cultivation of maize in US, though it is confirmed to exhibit no insecticidal activity against European Corn Borer (*Ostrinia nubilalis*) and Monarch butterfly (*Danaus plexippus*), the insects of order Lepidoptera against which the Cry1Ab protein exhibits its insecticidal activity (Reference 12).

Moreover, it has been also confirmed based on the homology search using the publicly available protein database (SWISS-PROT, FARRP, etc.) that the amino acid sequence in the modified Vip3A protein does not have any homology with known allergens and toxins examined.

PMI protein

The *pmi* gene is a gene derived from *E. coli*, which encodes the PMI protein (Phosphomannose isomerase), and the PMI protein has the capability of catalyzing the reversible isomerization of mannose 6-phosphate and fructose 6-phosphate, thereby allowing selection of transformed cells (Reference 18). Generally, maize and many other plants cannot utilize mannose as a carbon source, though the cells containing the *pmi* gene can use mannose for their growth. For this reason, with transferring the *pmi* gene into plant cells as a selective marker together with the target gene and subsequent incubation in the mannose-containing medium, it can be confirmed that the target gene is transferred into the cells together with the *pmi* gene (Reference 18). The PMI protein exists widely in nature including digestive system of human (Reference 26, Reference 27, Reference 28, Reference 29), and in fact, it is found present in soybean and other plants, though it has not been identified in maize (Reference 30, Reference 31, Reference 32).

The PMI protein encoded by the *pmi* gene in plants was identified equivalent to the PMI protein produced by microorganisms by the US Environmental Protection Agency (EPA) and then, the PMI protein encoded by the *pmi* gene, whether it is present in plants or microorganisms, was exempted from the requirement of a pesticide residue tolerance established by EPA (May 14, 2005).

In addition, it has been confirmed based on the homology search using the publicly available database (SWISS-PROT, FARRP, etc.) that the amino acid sequence in the PMI protein does not have any significant homology with known allergens and toxins examined.

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

The modified Vip3A protein expressed due to the modified *vip3A* gene is reported unlikely to possess any enzyme activity and thus, it is considered to function independently from the metabolic system of recipient organism. In addition, the PMI protein is a catalyst enzyme protein which has the capability of catalyzing the reversible isomerization of mannose 6-phosphate and fructose 6-phosphate, and its reaction is specific to mannose 6-phosphate and fructose 6-phosphate and the other

natural substrate of the PMI protein has not been reported (Reference 33).

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

(2) Information concerning vectors

1) Name and origin

For the development of this recombinant maize, the vectors pNOV1300 were used. This vector was constructed based on the vector pSB12 (Reference 34).

2) Properties

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

The total number of base pairs of the vector pNOV1300 is 14,405 bp, and the nucleotide sequences have been disclosed.

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

The vector pNOV1300 contains the *spec* gene which expresses the resistance to streptomycin, erythromycin, and spectinomycin as a selective marker for growth of vector in microorganisms, though the gene is not transferred in this recombinant maize.

(c) Presence or absence of infectious characteristics of vector and the information concerning the region of recipient organism if the infectivity of vector is found present

The vector pNOV1300 used for the production of this recombinant maize contains the cos, cohesive end region derived from the lambda phage which enables transfer of plasmid into *E. coli*, though there is no other known recipient organism reported than *E. coli* in the lambda phage. In addition, there is no sequence showing infectivity other than this.

(3) Method of preparing living modified organisms

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

Two gene expression cassettes (insect pest-resistant gene cassette and selective marker gene cassette) between RB and LB of T-DNA region of vector pNOV1300 used for the production of this recombinant maize were transferred in the recipient organism.

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

The T-DNA region of the vector pNOV1300 was transferred to the immature embryo of maize based on the *Agrobacterium* method.

3) Processes of rearing of living modified organisms

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

The vector pNOV1300 and the *Agrobacterium* containing a helper plasmid with the *vir* region acting to transfer the T-DNA region of the vector pNOV1300 into the recipient organism were co-cultivated and inoculated with the immature embryo of maize. Then, the immature embryo was incubated on the mannose-added medium for selecting the transformed cells.

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

After transferring of genes, the antibiotic Cefotaxime was added to the culture cell medium to remove any residual *Agrobacterium* used for transformation and then, regarding the regenerated individuals, the PCR analysis was conducted for the *spec* gene present in the backbone region of the vector pNOV1300. As a result, no *spec* gene was observed and thus, it was considered that there is no remaining *Agrobacterium*.

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

From the cells selected after transferring of genes, plant body was regenerated and conditioned then cultivated in a greenhouse. Then, plant body was analyzed based on the TaqMan PCR to identify presence or absence of the modified *vip3A* gene, and the T_0 plant body was selected. The T_0 plant body was crossed with an elite strain of dent type maize, and the progeny was used for collection of necessary information for assessment of Adverse Effect on Biological Diversity.

Regarding this recombinant maize, Type I Use (cultivation, storage, transportation, disposal and acts incidental to them in isolated fields) in accordance with the "Law concerning the conservation and sustainable use of biological diversity through regulations on the use of living modified organisms" was approved in July 2007 by the Ministry of Agriculture, Forestry and Fisheries and the Ministry of the Environment. In addition, in February 2008, application for approval of safety of use for food and application for approval of safety of use for feed were made to the Ministry of Health, Labour, and Welfare and the Ministry of Agriculture, Forestry and Fisheries, respectively.

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

1) Place where the replication product of transferred nucleic acid exists (on the chromosome, in the cell organelle, or in the protoplasm)

As a result of assessment of stability based on the genetic segregation ratio, it was found

that the modified *vip3A* gene and the *pmi* gene, the transferred genes in this recombinant maize, were both inherited across multiple generations in accordance with the law of Mendelian inheritance. Consequently, the modified *vip3A* gene and the *pmi* gene are considered to exist on the chromosome.

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

For determination of the number of copies of transferred genes in this recombinant maize, Southern blotting analysis was conducted using the genome DNA extracted from leaf tissues of this recombinant maize. As a result, it was found that one copy of each of the modified *vip3A* gene and the *pmi* gene is present on the genome of this recombinant maize at one site and that the backbone region of the vector pNOV1300 does not exist in this recombinant maize.

In addition, presence or absence of the modified *vip3A* gene and the *pmi* gene in individual generations of plant body was identified based on Southern blotting analysis using two different generations of this recombinant maize. As a result, the identical band was detected across the two different generations, this also suggesting that the modified *vip3A* gene and the *pmi* gene are both stably inherited in the posterity.

Based on the above results, it was confirmed that one copy of the transferred genes in this recombinant maize is present on the genome of chromosome and that the transferred genes are stably inherited in the posterity and the transferred genes in individual generations are identical to each other.

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

In 2006, in the fields in the US at 2 sites, this recombinant maize was cultivated, and individual tissue samples were taken at individual growth stages to determine the level of expression of the modified Vip3A protein and the PMI protein based on the ELISA method. As a result, it was confirmed that the modified Vip3A protein and the PMI protein are expressed in individual tissues of plant body throughout the growth period in two different generations.

In 2006, in a greenhouse at Syngenta Corporation in the US, multiple generations of this recombinant maize were cultivated to determine the level of expression of the modified Vip3A protein and the PMI protein based on the ELISA method. As a result, it was confirmed that the both proteins are stably expressed in plant body through multiple generations.

Based on the above understanding, it was considered that the modified Vip3A protein and the PMI protein in this recombinant maize are stably expressed across individuals and through generations.

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

The transferred nucleic acid does not contain any sequence allowing transmission.

Therefore, it is considered unlikely that the nucleic acid transferred to this recombinant maize could be transmitted to any other wild animals and wild plants.

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

Existence of the target genes in this recombinant maize can be confirmed based on the results of Southern blotting analysis using the modified *vip3A* gene as a probe after breaking the genome DNA by the restriction enzyme. In addition, a method for specific detection of this recombinant maize was developed based on the nucleotide sequence of transferred genes and the nucleotide sequence of the neighboring genome.

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

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

This recombinant maize is given the trait to be resistant to Lepidoptera due to the modified Vip3A protein that is expressed by the modified *vip3A* gene and the trait to be a selective marker due to the PMI protein that is expressed by the *pmi* gene. This recombinant maize, which expresses the modified Vip3A protein, exhibits resistance to Fall Armyworm (*Spodoptera frugiperda*), Corn Earworm (*Helicoverpa zea*), Black Cutworm (*Agrotis ipsilon*) and other order Lepidopteran insects which are the pest insects for cultivation of maize in the US.

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

In 2007, isolated field tests were carried out at the Kanza Site of Central Research Station, R&D Division, Syngenta Japan K.K. using this recombinant maize and the non-recombinant control maize.

(a) Morphological and growth characteristics

For the morphological and growth characteristics, comparison was made between this recombinant maize and the non-recombinant control maize regarding the progress of germination, germination rate, time of tassel exertion, time of silking, culm length, height of ear, plant type, maturation time, fresh weight at the time of harvesting, tiller number, number of ears, ear length, ear diameter, row number per ear, grain number per row, 100-kernel weight, grain color and grain shape. For the germination rate, culm length, height of ear, fresh weight at the time of harvesting, tiller number, number of ears, ear length, ear diameter, row number per ear, grain number per row and 100-kernel weight, statistical treatment was conducted. As a result, in all the items examined but culm length, no significant difference or difference was observed between this recombinant maize and the non-recombinant control maize. The culm length, in which a significant difference was observed, was found 191.5cm for this recombinant maize and 199.7cm for the non-recombinant

control maize.

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

For this recombinant maize and the non-recombinant control maize, cold-tolerance at the early stage of growth was evaluated. For the first leaf stage of plant body, evaluation was made on the growth under the low temperature conditions representing the winter season and as a result, this recombinant maize and the non-recombinant control maize both exhibited browning and/or withering leaves due to the low temperature stress and eventually they both died completely. Based on the findings, it was judged that there is no difference in cold-tolerance between this recombinant maize and the non-recombinant control maize.

(c) Wintering ability of the matured plant

Maize is a summer type annual plant, and after grain maturity it usually dies out. In fact, there is no report that, after maturity, maize has further propagated by vegetative parts or set seeds again and produced seeds. In addition, it was observed in the isolated field tests that this recombinant maize died after maturation similarly as the non-recombinant control maize.

(d) Fertility and size of the pollen

For this recombinant maize and the non-recombinant control maize, the shape, size and fertility of pollen were examined under a microscope to identify any difference between them. As a result of the observation with pollen stained with Acetocarmine solution, no significant difference was observed between this recombinant maize and the non-recombinant control maize regarding the diameter of pollen. In addition, no difference was observed in the shape of pollen between this recombinant maize and the non-recombinant control maize, and pollens were all found stained nearly 100% with Acetocarmine; therefore, the fertility was considered equivalent between the recombinant maize and the non-recombinant control maize. Consequently, no significant difference or difference was observed between this recombinant maize and the non-recombinant control maize in terms of fertility and size of pollen.

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

Regarding seed production, no significant difference was observed between this recombinant maize and the non-recombinant control maize in the number of ears, row number per ear, grain number per row, and 100-kernel weight.

Regarding shedding habit of the seed, the seeds of maize never shed spontaneously, since they adhere to ears and the ears are covered with husk (Reference 2). Also in this recombinant maize, similarly as the non-recombinant control maize, the ears were found covered with husk at harvest time and thus, it was judged that the shedding habit was not observed in the both plants, this recombinant maize and the non-recombinant control maize, under the natural condition.

Regarding the germination rate, no significant difference was observed in the seeds

of this recombinant maize and non-recombinant control maize.

Dormancy has not been examined, though the possibility is considered low that the dormancy of this recombinant maize is significantly different from that of the non-recombinant control maize, since no significant difference was observed in the germination rate of sowing seeds and harvested seeds between this recombinant maize and the non-recombinant control maize.

(f) Crossability

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

(g) Productivity of harmful substances

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

Succeeding crop test：
Soils in the root zone of maize were collected from individual experimental plots and mixed with each other, to which the seeds of radish were sown to determine the germination rate and dry weight. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize in the germination rate and dry weight examined.

Plow-in test:
Leaves and stems at harvest time were dried and powdered then mixed with granular clay, to which the seeds of radish were sown to determine the germination rate and dry weight. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize in the germination rate and dry weight examined.

Soil microflora test：
At the time of harvesting of this recombinant maize and the non-recombinant control maize, soil was sampled from the cultivation field to measure the number of colonies of filamentous fungi, bacteria and Actinomyces for the microorganisms in soil based on the dilution plate technique. As a result, no significant difference was observed between this recombinant maize and the non-recombinant control maize.

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

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

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

(1) Competitiveness

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

This recombinant maize is given the trait to be resistant to Lepidoptera due to the transferred modified *vip3A* gene. In addition, as a result of isolated field tests in Japan, regarding the culm length among the characteristics relating to competitiveness, a significant difference was observed between this recombinant maize and the non-recombinant control maize. However, it is considered that the insect damage by Lepidopteran insects is not the major cause making the maize difficult to grow in the natural environment in Japan. Moreover, it is considered that the difference observed in culm length would not increase the competitiveness of this recombinant maize.

In addition, due to the transferred *pmi* gene, the PMI protein is expressed and then, mannose can be a carbon source, though it is considered unlikely that this trait enhances the competitiveness of this recombinant maize.

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

(2) Productivity of harmful substances

There has been no report that maize, the species to which the recipient organism belongs, produces any harmful substances that could affect wild animals and wild plants.

This recombinant maize produces the modified Vip3A protein which possesses insecticidal activity against the insects of order Lepidoptera and the PMI protein which is encoded by the *pmi* gene transferred as a selective marker, though it has been confirmed that neither of the proteins has significant homology with any known allergen and toxin.

The modified Vip3A protein is considered unlikely to have any enzyme activity, and the PMI protein is specific to mannose 6-phosphate and fructose 6-phosphate and there is

no other known natural substrate of the PMI protein. Therefore, it is not considered that these proteins would affect the metabolic pathway of recipient organism and produce any harmful substances. There is a concern about possible impacts of pollens of this recombinant maize on the non-target species of insects of the order Lepidoptera, though the non-target species of insects of order Lepidoptera are considered unlikely to locally inhabit around the fields where this recombinant maize is cultivated. Consequently, it is very unlikely that the non-target species of insects of order Lepidoptera could be affected at the level of individual population by the pollens of this recombinant maize.

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

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

(3) Crossability

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

2. Conclusion based on the Biological Diversity Risk Assessment Report

Based on the above understanding, the Biological Diversity Risk Assessment Report concluded that there is no risk that the use of this recombinant maize in accordance with Type 1 Use Regulation causes Adverse Effect on Biological Diversity. It was judged that the conclusion above made by the applicant is reasonable.

Bibliography

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