# Import and processing of alpha-amylase encoding maize 3272

## COGEM advice CGM/070905-01


### Summary

*The present application by Syngenta Seeds S.A.S. of file EFSA/GMO/UK/2006/34, 'alpha-amylase encoding maize 3272', concerns the import and processing for use in feed and food of a genetically modified maize line. Cultivation is not part of this application.*

*Maize line 3272 is genetically modified by insertion of the amy797E gene, which encodes a thermostable alpha-amylase. Alpha-amylase is used in the production of ethanol from maize. In addition, maize line 3272 contains the pmi gene, which enables the plant to use mannose as a sole carbon source and functions as a selection marker.*

*During the long domestication process, maize has lost its ability to survive in the wild. In the Netherlands, the appearance of maize volunteers is very rare and establishment of volunteers in the wild has never been reported. There are no reasons to assume that expression of the amy797E and pmi genes increases the potential of maize to establish feral populations. The introduced genes cannot spread to closely related species as wild relatives of maize are not present in Europe. Therefore, COGEM is of the opinion that incidental spillage of maize will probably not pose a risk to human health and the environment.*

*However, COGEM points out that the molecular analysis of maize line 3272 is incomplete. Therefore, it cannot be excluded that new open reading frames were created due to the insertion. If new open reading frames were created, these could theoretically give rise to potential toxic or allergenic products. In view of the deficiencies in the molecular analyses, COGEM cannot advice positively on the application for import and processing of 3272.*


### Introduction

The present application by Syngenta Seeds S.A.S., file EFSA/GMO/UK/2006/34, concerns the import and processing of maize line 3272 for use in feed and food. This line contains a thermostable alpha-amylase (*amy797E*) gene, which is expressed in the endosperm. Alpha-amylase catalyzes the digestion of starch components amylose and amylopectin, into dextrins, maltose and glucose. In addition, a constitutively expressed phosphomannose isomerase (*pmi*) gene was inserted into maize line 3272. The *pmi* gene serves as a selectable marker because it enables the plant to use mannose as a sole carbon source. Maize line 3272 has not been cultivated in other countries and consequently it has

no history of safe use. According to the applicant 3272 has been notified for use in the United States of America and in China.

**Previous COGEM advices**

COGEM has never advised on an organism genetically modified to express an alpha-amylase. In 2005, COGEM advised positively on import and processing of maize line MIR604, which contains amongst others the *pmi* gene (1).

**Aspects of the crop**

Maize (*Zea mays*) is a member of the *Poaceae* family (grasses). Maize was domesticated in Central America and is nowadays cultivated throughout the world (2). Although maize is tolerant to a wide range of temperatures, it is typically grown in temperate regions due to the moisture level and the number of frost-free days required to reach maturity (2). Depending on cultivar and climate the period from planting to harvesting ranges from 70 to 200 days (3). The minimum temperature for germination is 10°C. Usually, the stem emerges from the soil 4 to 6 days after planting and flower initiation occurs 20 to 30 days after germination. The tassel of a 4-month cultivar emerges 50 to 60 days after planting and the silk appears about a week later (3). Fertilization occurs through cross-pollination, and maize pollen is usually distributed by the wind (2). Hybridization with other species cannot occur as wild relatives of maize are not present in Europe (2).

During the long process of domestication, maize has lost the ability to survive in the wild and it needs human intervention to disseminate its seed (2). Maize kernels exhibit no dormancy and only survive under a narrow range of climatic conditions. Furthermore, maize is very sensitive to weed competition during the first 4 to 6 weeks after emergence (3) and it cannot persist as a weed (2). In the Netherlands, the appearance of volunteers is very rare and establishment of maize plants in the wild has never been observed.

**Molecular characterization**

*Origin and function of the introduced genetic elements*

Maize line 3272 was genetically modified by *Agrobacterium*-mediated transformation. The introduced sequences are:

- GZein promoter,
  promoter providing endosperm-specific expression, which has been derived from the *zein* gene of *Zea mays*, which encodes a seed storage protein;
- AMY797E coding sequence,
  chimeric thermostable alpha-amylase gene, which codon usage has been optimized for maize. The gene is composed of alpha-amylase genes from

three hyperthermophilic microorganisms of the archaeal order *Thermococcales*. The gene is fused to the maize gamma-zein signal sequence which targets the protein to the endoplasmic reticulum and to the SEKDEL retention signal which retains the protein in the endoplasmic reticulum;

- PEPC9 intron,
  intron from the phosphoenolpyruvate carboxylase (*pepc*) gene from *Zea mays*;
- 35S terminator,
  terminator sequence derived from the *Cauliflower mosaic virus* 35S RNA, which provides a polyadenylation sequence;
- ZmUbiIntron promoter,
  promoter providing constitutive expression derived from the polyubiquitin gene of *Zea mays*;
- PMI coding sequence,
  *manA* gene from *Escherichia coli*, which encodes phosphomannose isomerase;
- NOS terminator,
  terminator sequence of the nopaline synthase gene of *Agrobacterium tumefaciens*, which provides a polyadenylation sequence.

*Properties of the introduced genes*

Maize line 3272 was genetically modified by the insertion of the *amy797E* and the *pmi* genes. *Amy797E* is a chimeric alpha-amylase gene and the expression of this transgene in the endosperm is under control of the Gzein promoter. It encodes a thermostable alpha-amylase, which has been designed for use in ethanol production. Alpha-amylase is an enzyme that catalyzes the digestion of starch. Alpha-amylase cleaves the internal α-1,4-glucosidic bonds of starch components amylose and amylopectin, which generates dextrins, maltose and glucose (4). Normally, during ethanol production alpha-amylase produced by microorganisms is added to the ground maize kernels. The mixture is then heated to cleave and rupture the starch molecules (5). The thermostable alpha-amylase produced by 3272 was designed to be used in ethanol production and could replace the external addition of alpha-amylase.

The *pmi* gene encodes phosphomannose isomerase and is constitutively expressed. The expression of this transgene is controlled by the ZmUbiIntron promoter. Phosphomannose isomerase is an enzyme that catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate (6). Most plants are unable to use mannose-6-phosphate, but can use fructose-6-phosphate as a carbon source. Expression of the *pmi* gene in 3272 thus enables the maize plant to use mannose and functions as a selection marker.

*Molecular analysis*

The applicant demonstrated by hybridization analysis that the *amy797E* and *pmi* genes are present in a single copy and that 3272 contains a single copy of the T-DNA. Furthermore, hybridization analyses carried out on four generations of 3272 showed that the T-DNA stably integrated.

Hybridization analysis was performed using the complete backbone of the plasmid that has been used for transformation as a probe. The results demonstrated that the complete backbone of the plasmid is absent in 3272. In addition, sequence analysis showed that no backbone fragments were inserted in the T-DNA flanking regions.

According to the applicant 1000 base pairs were sequenced on both sides of the T-DNA. However, the method that has been used to generate the sequences has not been described. Therefore, it is uncertain whether the sequences that were generated correspond to the flanking regions.

BLAST analysis performed on the regions flanking the 3' end of the T-DNA showed that these regions were homologous to maize transcription regulation elements. The regions that display homology to regulatory elements are short and positioned downstream of the inserted genes. In COGEM's view it is unlikely that these regions are functional regulatory elements which influence the expression of the inserted genes.

BLAST analysis of the region flanking the 5' end of the T-DNA did not indicate any homology to maize sequences. In the opinion of COGEM the applicant did not convincingly demonstrate that the region flanking the 5' end of the insert is maize DNA. Hybridization analyses on 3272 and a non-transgenic maize line with probes derived from the regions flanking the T-DNA could indicate whether these regions are indeed maize genomic DNA. Another possibility to demonstrate that the flanking regions are maize genomic DNA is to perform a PCR on 3272 and a non-transgenic maize line using primers developed on the region that flanks the T-DNA.

The junction between the T-DNA and its flanking regions was examined for the presence of potential novel open reading frames (ORFs). The applicant defined an ORF as a region of at least fifty amino acids in length that initiates with an ATG codon and ends with any of the three stop codons TAA, TAG or TGA. No novel ORFs were identified. Although most ORFs initiate with an ATG codon, translation may initiate with other codons. In addition, the junction between the T-DNA and its flanking regions may be part of a very long ATG-initiated ORF. Therefore, the COGEM is of the opinion that all ORFs in the junction between the T-DNA and its flanking regions should have been examined. In addition, the applicant did not prove that the flanking regions are maize DNA. Therefore, it cannot be concluded that all novel ORFs were identified.

COGEM is of the opinion that the environmental risks of the import of maize line 3272 are probably negligible. However, this opinion cannot be sufficiently substantiated because of the missing molecular data.

**General surveillance plan**

A general surveillance plan is supplied by the applicant. Several organizations that import or use viable maize, e.g. COCERAL, UNISTOCK and FEDIOL, are mentioned in the surveillance plan. According to the applicant these organizations are well-placed to detect effects on human health or the environment. However, information about their expertise in human health or the environment is not given. In addition, it is unclear whether these organizations have agreed to cooperate in the general surveillance of 3272. COGEM would prefer independent organizations which have expertise on human health and the environment to cooperate in general surveillance.

According to the applicant indirect effects will be reported at the stage of re-evaluation or at the end of a given consent. As stated before, in COGEM's opinion any indirect effects should be reported annually.

**Advice**

COGEM has been asked to advice on import and processing for use in feed and food of maize line 3272.

Maize is a crop that has lost the ability to survive in the wild. In the Netherlands, volunteers are rarely found and establishment of maize plants in the wild has never been observed. In addition, maize needs human intervention to disseminate its seed. There is no reason to assume that the expression of the *amy797E* and *pmi* genes in 3272 increases the potential of maize to run wild. In addition, introgression of the introduced genes into closely related species cannot occur, as wild relatives of maize are not present in Europe. In view of the above, COGEM is of the opinion that incidental spillage of maize will not pose a risk to man and the environment.

In COGEM's view, it is sufficiently proven that only one copy of the T-DNA is present in 3272. In addition, the applicant showed that the complete backbone of the plasmid was absent. However, the applicant did not describe which method has been used to obtain the sequences of the regions that flank the T-DNA insert. Moreover, it was not convincingly proven that the region flanking the 5' end of the T-DNA is maize genomic DNA. In COGEM's opinion the applicant did not prove that the complete site of integration was characterized. Therefore, it cannot be excluded that novel ORFs were created due to the insertion. In addition, only the presence of novel ORFs that initiate with an ATG codon has been examined. However, translation may initiate with other codons. In addition, the junction between the T-DNA and its flanking regions may be part

of a very long ATG-initiated ORF. COGEM is of the opinion that all ORFs in the junction between the T-DNA and its flanking regions should have been examined. If novel ORFs were created, these could give rise to potential detrimental products.

COGEM is of the opinion that import of 3272 most likely poses negligible risks to man and the environment. However, in view of the missing data concerning the molecular characterization, this opinion cannot be sufficiently substantiated. Until the missing data have been provided COGEM cannot advice positively on import and processing of maize line 3272.

**References**


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