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Testbiotech comment on EFSA GMO Panel Scientific Opinion on the assessment of genetically modified maize 4114 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2014-123) of company Pioneer (DowDupont)

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Introduction

Maize 4114 produces three insecticidal proteins classified as Cry1F, Cry34Ab1 and Cry35Ab1, as well as the PAT protein that confers tolerance to the active herbicide ingredient glufosinate-ammonium. It was genetically engineered using *Agrobacterium tumefaciens*.

Regulation (EU) No 503/2013, which foresees 90-day animal feeding studies, an extended literature review, specific monitoring requirements and specific statistical analysis was applied in the risk assessment of maize 4114.

1. Molecular characterisation

Maize 4114 was genetically engineered with the help of *Agrobacterium tumefaciens*. It expresses truncated versions of the Cry toxins Cry1F, Cry34Ab1 and Cry35Ab1, as well as the PAT protein that confers tolerance to the active herbicide ingredient glufosinate-ammonium. The DNA sequences used for the expression of these proteins have previously been used for maize 1507 (Cry1F and PAT) and maize 59122 (Cry34Ab1, Cry35Ab1 and PAT).

Insertion of this construct creates several new open reading frames. In addition, a gene with similarities to a hypothetical glutaredoxin-like (GRX-like) protein sequence was identified in the flanking downstream position. GRX proteins are involved in many cellular functions, including DNA synthesis, signal transduction and defence against oxidative stress.

EFSA (2018a) did not assess unintended gene products, such as miRNA, that can emerge from the insertion of the transgenes. Some comparisons were made, but only between the intended proteins produced in the plants with known allergens and toxins. No detailed consideration was undertaken regarding the extent to which the truncation of the Bt proteins will change its biological characteristics.

EFSA only assessed a few of the relevant issues. Therefore, in order to enable further independent risk assessment, the full DNA sequence inserted into the plants should be made available, including all open reading frames.

In addition, EFSA (2018a) did not request any detailed analysis based on so-called -omics (transcriptomics, metabolomics, proteomics) to investigate changes in the overall metabolism in the plants. EFSA assumed that the data from phenotypic characteristics and compositional analysis

would not indicate any need for further investigations. However, these data did show many significant changes (see below). In general, data on phenotypic characteristics and compositional analysis can be used as complementary data, but these are not as sensitive as omics data and cannot replace them.

Expression data were provided on the new intended proteins. These data show rather wide ranges for the Bt toxins that require further investigation. It is known that the Bt content in the plants depends on environmental impact. For example, environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). Therefore, the plants should have been subjected to a much broader range of defined environmental conditions and stressors in order to gather reliable data on gene expression and functional genetic stability.

Further, the method used to determine the amount of Bt toxins (ELISA) is known to be dependent on the specific protocols used. The data are not sufficiently reliable without further evaluation by independent labs. For example, Shu et al. (2018) highlight difficulties in measuring the correct concentration of Bt toxins produced by the genetically engineered plants (see also Székács et al., 2011).

Without fully evaluated test methods to measure the expression and the concentration of the Bt toxins, risk assessment will suffer from substantial methodological gaps. This is also a problem for food safety. Despite EFSA mostly ignoring the data, there are indications that Bt toxins can impact human and animal health (see below). Furthermore, based on such poor and inconclusive data, the dietary exposure to Bt toxins within the food chain cannot be determined as required by Regulation (EU) No 503/2013.

Consequently, the risk assessment of molecular characteristics is not conclusive and is not sufficient to show food and feed safety.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Field trials were only performed in the US and Canada and not in other maize producing regions.

Interestingly, two varieties were introgressed with the event. Data from more than one genetically engineered variety can help to investigate potential interactions of the gene constructs with the genetic background of the plants. However, as explained by EFSA (2018a and b), the data from these two varieties were pooled for statistical analysis. Therefore, the additional data might not be of additional value and only increase what is known as data noise.

A large part of the parameters measured for plant composition and phenotypic characteristics were significantly different. For example, in plant composition, more than one third of the parameters were different, with an increasing tendency for the plants sprayed with glufosinate: 71 parameters were evaluated showing 27 significant differences for the untreated maize, and 33 significant differences for maize 4114 treated with glufosinate. Taken as isolated data these differences might not directly raise safety concerns, nevertheless, the large number of effects should have led to further investigations.

Therefore, EFSA should have requested further studies e.g.

- data from omics (proteomics, transcriptomics, metabolomics),
- data representing more extreme environmental conditions such as those caused by climate change,

- data representing more areas of commercial maize cultivation,
- more data on stress reactions under controlled conditions,
- and impact of the dosage of the complementary herbicide sprayed onto the plants.

Instead, EFSA (2018a) has relied solely on the newly introduced statistical method known as the "test of equivalence". This method can be helpful to make some assumptions on the relevance of the significant findings. However, it cannot replace a detailed assessment of the high number of significant differences.

Based on the available data, no final conclusions can be drawn on the safety of the plants.

Toxicology

Pioneer conducted a 90-day feeding trial with maize 4114. In this feeding trial only one dosage of maize (33 %) was included as part of the diet, instead of several dosages as requested by existing guidance. Nevertheless, EFSA (2018a) still accepted the data.

The feeding trial findings were alarming: two male animals fed with maize 4114 (unsprayed) were diagnosed with renal tubule adenomas and/or carcinoma associated with tubule hyperplasia. These findings led to a further 90 day-feeding study to assess the original findings. In this second study, the findings could not be confirmed.

There may be several reasons why findings from a study may, or may not, be repeated, and so uncertainties remain. The feeding studies with maize 1507 (see Dona & Arvanitoyannis, 2009) and maize 59122 (see EFSA, 2007) also led to significant findings. Therefore, we conclude the need for more detailed investigation. Further, more detailed (e.g. using several dosages) and long-term feeding studies would be necessary to assess potential health impacts. These studies should include omics data from animals, as well as detailed assessment of the impact of higher dosages of glufosinate sprayed on the plants (as can be expected under practical conditions). Besides the renal system, the immune system should also have been investigated in more detail (see below).

The need for more detailed assessment is underlined by publications showing that Bt toxins raise further questions in regard to feed and food safety:

(1) There are several partially diverging theories about the exact mode of action of the Bt toxins at the molecular level (see Then, 2010; Hilbeck & Otto, 2015). Thus, it cannot be excluded a priori that the toxins are inert in regard to human and animal health as considered under risk assessment for food and feed.

(2) There are further uncertainties regarding the specificity of Bt toxins (Venter and Bøhn, 2016). Changes in specificity may emerge from structural modifications performed to render higher efficacy. For example, the proteins are truncated to become activated (see Hilbeck and Schmidt, 2006).

(3) In addition, there are findings in mammalian species showing that Bt toxicity is a relevant topic for detailed health risk assessment: some Cry toxins are known to bind to epithelial cells in the intestines of mice (Vázquez-Padrón et al., 1999).

(4) As far as potential effects on health are concerned, several publications (Thomas and Ellar 1983; Shimada et al., 2003; Mesnage et al., 2013; Huffman et al., 2004; Bondzio et al., 2013) show that

Cry proteins may indeed have an impact on the health of mammals. For example, de Souza Freire et al., (2014) confirm haematoxicity of several Cry toxins. Some of these effects seem to occur where there are high concentrations and tend to become stronger over longer periods of time.

(5) Further, the toxicity of Bt toxins can be enhanced through interaction with other compounds, such as plant enzymes (Zhang et al., 2000, Zhu et al., 2007; Pardo-López et al., 2009); other Bt toxins (Sharma et al., 2004; Tabashnik et al., 2013; Bøhn et al. 2016, Bøhn 2018); gut bacteria (Broderick et al., 2009); residues from spraying with herbicides (Bøhn et al. 2016, Bøhn 2018) and other (Kramarz et al., 2007; Kramarz et al., 2009; Khalique and Ahmed, 2005; Singh et al., 2007; Zhu et al., 2005; Mason et al., 2011; Reardon et al., 2004).

In this context, it is relevant that Bt toxins can survive digestion to a much higher degree than has been assumed by EFSA. Chowdhury et al., (2003) and Walsh et al. (2011) have found that when pigs were fed with Bt maize, Cry1A proteins could frequently and successfully still be found in the colon of pigs at the end of the digestion process. This means that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed; and that there is enough time for interaction between various food compounds.

Further, as far as the exposure of the food chain with Bt toxins is concerned, EFSA should have requested data on the overall combined exposure to Bt toxins caused by the introduction of Bt plants in the EU. Currently, there are already 30 events that produce Bt toxins authorised for import. The exposure stemming from these imports should have been added to that of maize 4114.

Consequently, the toxicological assessment carried out by EFSA is not sufficient to show food and feed safety.

Allergenicity

Bt toxins are known to be immunogenic. They seem to act as allergens and adjuvant effects are likely to occur. In regard to immunogenicity (non-IgE-mediated immune adverse reactions), it is generally acknowledged that Bt toxins are immunogenic (Rubio-Infante & Moreno-Fierros, 2016; Adel-Patient et.al., 2011; Andreassen et.al., 2015a,b; Andreassen et.al., 2016; see also Then & Bauer-Panskus, 2017). Thus, there are some substantial reasons for concern that reactions to allergens can be enhanced. This is relevant since in food/feed the Bt toxins can be mixed with allergens from soybeans, amongst others. Mixing with soybeans can also substantially prolong the degradation of the Bt toxins in the gastric system (Pardo-López et al., 2009).

New findings (Santos-Vigil et al., 2018) now indicate the allergenic potential of Cry toxins after intra-gastric administration in a murine model. Thus, the EFSA assumption that a detailed assessment of the allergenic potential of Cry toxins is not necessary is simply wrong.

Consequently, the assessment on allergenicity cannot be regarded as conclusive.

Others

(1) In its risk assessment, EFSA (2018a) refers several times to previous experience with the Bt proteins (as expressed in maize 1507 and maize 59122) to explain why further data are not needed. However, EFSA rejected the request from experts of Member States (EFSA, 2018b) to also take into account the data from these other events, such as that on the expression rate of the Bt proteins. Further, EFSA (2018a) did not request a literature review, including those other events. Instead,

EFSA concluded, that a systematic literature review would not be justified because of the low number of specific publications on the event maize 4114:

> "A systematic literature review as referred to in Regulation EU No 503/2013 has not been provided in support of the risk assessment of application EFSA-GMO-NL-2014-123, because of the limited number of relevant publications available on maize 4114."

This clearly is a violation of the requirements of Regulation (EU) No 503/2013 and cherry-picking of information in regard to experience with similar events already on the market.

(2) According to Regulation (EU) No 503/2013, the applicant has to ensure that post-market monitoring is developed to collect reliable information with respect to the detection of indications of whether any (adverse) effects on health may be related to genetically modified food or feed consumption. Some experts from Member States (EFSA 2018b) have made appropriate demands regarding the implementation this obligation. Accordingly, the monitoring report should deliver detailed information on
i) actual volumes of maize 4114 imported into the EU,
ii) the ports and silos where shipments of maize 4114 were unloaded,
iii) the processing plants where maize 4114 was transferred to,
iv) the amount of maize 4114 used on farms for feed, and
v) transport routes of maize 4114.

The applicant is further requested to explain how the PMM of maize 4114 in mixed GMO commodities imported, processed or used for food/feed is put into practice. Since traders may co-mingle maize 4114 with other imported commercial GM maize that is processed or used for food/feed, the applicant is requested to explain how the monitoring will be designed to distinguish between potential adverse effects caused by maize 4114 and those caused by other GM maize.

The monitoring should be run in regions where viable maize 4114 will be transported, stored, packaged, processed or used for food/feed. In case of substantial losses and spread of maize 4114, all receiving environments need to be monitored.

Environmental risk assessment
EFSA (2018a) acknowledges that potential gene transfer between maize and weedy Zea species, such as teosintes and/or maize-teosinte hybrids can occur (Trtikova et al., 2017).

However, EFSA only vaguely considers the consequences of potential hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize. Much more detailed investigation would be needed to assess the introgression of wild teosinte populations with gene constructs inserted in maize 4114 and its effects on fitness of any progenies.

Further, as shown by Pascher (2016), EFSA is also underestimating the risks posed by the occurrence of volunteers from maize plants.

Consequently, environmental risk assessment carried out by EFSA is not acceptable.

Conclusions and recommendations
The EFSA risk assessment should be rejected.

When making his decision the risk manager should also take into account issues that are related to pesticide regulation. In this case, glufosinate-ammonium is about to be prohibited in the European Union.

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