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Testbiotech comment on EFSA's assessment of genetically engineered maize MIR604 for renewal authorisation under Regulation (EC) No 1829/2003 (application EFSA-GMO-RX-013) from Syngenta

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Introduction

The EFSA GMO panel assessed the renewal application for maize MIR604. The maize expresses genes producing a synthetic, artificial version of Cry3A (mCry3A), which is especially toxic for *Coleoptera* species (such as the Western corn rootworm larvae, *Diabrotica virgifera virgifera*). Further, the maize produces the PMI protein (phosphomannose isomerase) derived from Escherichia coli. Expression of PMI enables transformed maize cells to utilise mannose and, therefore, to survive on specific media used for selecting the maize plants after the process of genetic engineering (so-called marker gene). The integration of the additional DNA was performed by using *Agrobacterium tumefaciens* in two different lines of maize (EFSA, 2009a). The final event was derived from stacking (crossing).

Implementing Regulation 503/2003 was applied in the risk assessment performed by EFSA.

1. Molecular characterisation

Implementing Regulation 503/2003 requests that stacked events can only be assessed and authorised if the parental plants were previously assessed and authorised. However, there seems to be no data on the parental plants in regard to PMI and mCry3A in isolation.

In order to assess the sequences encoding the newly expressed proteins or any other open reading frames (ORFs) present within the insert and spanning the junction sites, it was assumed that the proteins that might emerge from these DNA sequences would raise no safety issues. Furthermore, other gene products, such as miRNA from additional open reading frames, were not assessed. Thus, uncertainties remain about other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs.

It is known that environmental stress can cause unexpected patterns of expression in the newly introduced DNA as well as the specific genomic background of the variety (see, for example, Trtikova et al., 2015). Indeed, the data presented in the original dossier assessed by EFSA (2009a) on kernels show a concentration of between 0.8 to 2 µg mCry3A protein/g tissue dry weight. However, data presented in EFSA (2016), EFSA (2010a) and EFSA (2010b) show a much lower concentration, while some data from the US (USEPA, 2010) show substantially higher values. These results seem to be, at least partially, caused by the genetic background of the specific varieties.

mCry3A levels on dry weight basis in event MIR604-derived maize plants (µg, dry weight)

	EFSA 2010a	EFSA 2010b	EFSA 2016	USEPA (2010) MIR604-B Hybrid	USEPA (2010) MIR604-C Hybrid	EFSA 2009 (as for renewal)
mCry3A	> 0.6	0.3-0.8	0.35-0.57	0.74-1.83	1.26-3.13	0.8-2.0

The applicant provided further data on gene expression from Spain and Romania in 2008; these were, however, were not summarised and presented by EFSA. Therefore, these data cannot be commented on within the given period for consultation.

Whatever the case, the available data indicate that gene expression is dependent on the varietal background and might also be influenced by site specific conditions. Therefore, EFSA should have requested more recent data on gene expression, taking into account a larger number of varieties and a broad range of environmental conditions.

From the data presented, it cannot be concluded to which extent specific environmental conditions, such as those caused by climate change, will influence the overall concentration of the enzymes (mCry3A and PMI) in the plants. Furthermore, the process of stacking might have been influenced by the concentration of the two newly produced enzymes. However, no data on the parental plants were made available.

Whatever the case, the data as presented do not represent the conditions of more extreme weather conditions which have been observed more frequently in recent years.

Furthermore, no data were presented from maize-producing regions such as Argentina and Brazil.

Already in 2009 (EFSA 2009b), experts from Member States complained:

> *"The analysis for the range of the expression of mCry3A and PMI relies solely on one field trial in one location. Since the expression can be affected by climatic conditions, soil fertility, agricultural practice or unknown gene x environment interactions, the data presented give only a crude estimate of the range and can not be regarded as sufficient for a market release."*

In regard to the expression of the additionally inserted genes, Implementing Regulation 503/2013 (which was not in place at the time of the original risk assessment) requests "*protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown*".

As shown, this requirement is not fulfilled by the data used and presented for the risk assessment.

We conclude that the available data indicate gene expression of the newly introduced genes is likely to depend on, or be influenced by, varietal background, and potentially by environmental conditions such as drought.

Therefore, the plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability, taking into account more extreme drought conditions. Further, they should have been tested in the

maize-producing countries in South America. In addition, EFSA should have requested data from several varieties, including those cultivated in South America. Furthermore, data from the parental plants need to be presented.

The material derived from the plants should have been assessed by using omics techniques to investigate changes in the gene activity of the transgene and the plant genome, as well as changes in metabolic pathways and the emergence of unintended biologically active gene products. Such in-depth investigations should not depend on any findings indicating potential adverse effects, they should always be necessary to come to sufficiently robust conclusions to inform the next steps in risk assessment.

2. Comparative analysis (for compositional analysis and agronomic traits and GM phenotype)
Implementing Regulation 503/2003 requests that stacked events can only be assessed and authorised if the parental plants were assessed and authorised previously. However, it appears there is no data on the parental plants, in regard to PMI and mCry3A in isolation.
Further, Implementing Regulation 503/2013 requests:

> *"The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly."*

However, the data presented do not represent expected agricultural practices or the different meteorological and agronomic conditions under which the crop is to be grown. The field trials were not conducted in all relevant regions where the maize will be cultivated, and no extreme weather conditions, such as those that have occurred more frequently in the last ten years, were taken into account.
As the significant findings from the field trials in 2002 and 2003 show, site by site and year by year effects have to be expected. It is also worrying that these data were interpreted not only in the light of the actual field trials, but other ranges of data found elsewhere in literature (EFSA, 2009a):

> *"These observed differences between maize MIR604 and its non-GM comparators all fell within the range of natural variability reported in literature, except for: i) campesterol in kernels of one GM line, which was slightly above the upper boundary of background values in one location; and ii) for values of oleic acid in kernels of two control lines and one GM line, which fell below the range of natural variability. With the exception of higher oleic acid levels, none of these differences were consistently observed over the seasons tested."*

Furthermore, there are no data from field trials with the parental plants. Therefore, effects caused by stacking cannot be excluded.

Taking into account the purpose of the genetic engineering in this case, it is not acceptable that EFSA (2019) failed to require further studies for renewal of the application even though

- No data from more recent field trials were made available, including varieties which are currently grown in the countries of cultivation.
- No data from Omics (proteomics, transcriptomics, metabolomics) were used to assist the compositional analysis and the assessment of the phenotypical changes.
- No data were generated representing more extreme environmental conditions, such as those caused by climate change resulting in more extreme droughts.
- No data were generated that represent the growing conditions in other relevant maize growing regions outside the US.

Based on the available data, no final conclusions can be drawn on the safety of the plants. The data do not fulfill the requirements of Implementing Regulation 503/2013.

Toxicology

Implementing Regulation 503/2003 requests that stacked events can only be assessed and authorised if the parental plants were assessed and authorised previously. There appears to be no data on the parental plants in regard to PMI and mCry3A in isolation.

Further, Implementing Regulation 503/2013 requests:

> *"Toxicological assessment shall be performed in order to:*
> *(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;*
> *(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;"*
>
> *"In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
> *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

The toxicological risk assessment originally performed by EFSA (2009a) has some substantial weaknesses: the Bt proteins used in the original risk assessment exhibited a different structure and biological activity compared to those produced in the plants.

Furthermore, a sub-chronic feeding study performed with MIR604 for the original risk assessment showed some significant findings as also mentioned in the comments from Member States (2009b):

> "*Noticeable is the partly significant lower food consumption of the male rats in both GMO-maize-fed-groups during the whole test, which leads to a significant lower increase in body weights in the group of 10% GMO-maize-fed male rats. Together with other results, especially the significant changes in the haemogram of male rats in the group which was fed with 10% GMO-maize thus can give a hint to possible adverse effects of MIR604 maize on the health of the test animals. As a consequence a subsequent feeding study should be requested to address the above uncertainties. The study should cover a longer exposure preferably over two generations to test for chronic effects.*"

Despite these findings, and in awareness of the lack of more specific data on the synthetic Bt protein, no further testing of the whole stacked plant (feeding study) was requested.

It should be acknowledged that, in regard to toxicology or potential combinatorial effects, the negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action and are altered in their biological quality; therefore, they are not identical to their natural templates (Hilbeck & Otto, 2015). It should not be overlooked that the mode of action of mCry3A was changed to become more effective in pest insects, thus data from the naturally occurring Bt toxins are not sufficient.

In general, it is known that not all modes of action of the insecticidal proteins produced in the plants depend on the specific mechanisms occurring only in the target insect species. Only very few Bt toxins (especially Cry1Ab, for overview see, Then, 2010) were investigated in more detail in regard to their exact mode of action, and there is no data on the Bt toxins produced in the maize. On the other hand, several publications exist showing the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestine of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2013) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. Two recent publications (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hema toxicity of several Cry toxins, including those being used in genetically engineered plants such as Cry 1Ab and Cry1Ac. These effects seem to occur after high concentrations and tend to become stronger after several days. Such observations call for the study of effects after long-term exposure to various dosages.

Studies to determine effects after long-term exposure to various dosages are also relevant in regard to potential immune toxicity. This is underlined by EFSA's original risk assessment (EFSA 2009):

> *"The EFSA GMO Panel also considered possible immunogenicity and adjuvanticity of Cry proteins. After intraperitoneal (i.p.), intranasal (i.n.) or intragastric administration of Cry1Ac and i.p. and i.n. administration of Cry3A to mice at relatively high dosage, IgG, IgM and mucosal IgA response were induced, but no IgE response was reported (Guerrero et al., 2004; Vazquez-Padron et al., 1999; 2000). (...)"*

It is not clear why EFSA (2009a and 2019) did not request data regarding the dose-response relationship to test this assumption on immunogenicity and adjuvanticity. Further, in regard to potential immune reactions, which often coincide with inflammation, data on chronic exposure would be highly relevant, but no such data were made available.

For the assessment of the renewal application (EFSA, 2019), Syngenta provided data on sequence analogies with other known toxins, allergens and immunogenic gluten-related epitopes. However, such data do not address the specific questions raised above.

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

In this regard it also has to be considered that the concentration of the insecticidal proteins will be enriched in processed products such as gluten meal, and that they can reach much higher concentrations compared to the kernels.

EU legal provisions such as Regulation 1829/2003 (as well as Implementing Regulation 503/2013) state that "*any risks which they present for human and animal health and, as the case may be, for the environment*" have to be avoided. We conclude that the health risk assessment performed by EFSA is not sufficient to fulfill this requirement.

Allergenicity

Implementing Regulation 503/2013 requests:

> *"In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity."*
>
> *"In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
> *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

However, EFSA did not request the applicant to provide data to verify whether the source of the transgene is allergenic, even though there were some indications: according to Santos-Vigil et al. (2018), the Bt toxin Cry1Ac can act as an allergen if ingested. However, no specific experimental data on the allergenic or immunogenic potential of the mCry3A were requested.

Furthermore, as mentioned, there are several studies indicating that immune responses such as adjuvanticity in mammals are triggered by Bt toxins and have to be considered in this context.

In this regard, it further has to be considered that the concentration of the insecticidal proteins will be enriched in processed products such as gluten meal, and that it can reach a much higher concentrations compared to the kernels.

In its risk assessment, EFSA did not consider that under real conditions and, contrary to what is suggested by the findings of in-vitro studies, Bt toxins will not be degraded quickly in the gut but are likely to occur in substantial concentrations in the large intestine and faeces (Chowdhury et al., 2003; Walsh et al., 2011). In addition, in regard to the degradation of the Bt toxins during ingestion, there is specific cause for concern that the maize or gluten is likely to be fed together with soybeans that naturally produce enzymes, which can substantially delay the degradation of Bt toxins in the gut (Pardo-López et al., 2009). In addition, soybeans are known to produce many food allergens. Therefore, the immune system responses caused by the allergens in the soybeans might be enhanced by the adjuvant effects of the Bt toxins.

In this context, it also should be remembered that the PMI enzyme was also suspected of being potentially allergenic (EFSA, 2009a and 2009b). Although no conclusive evidence is shown that PMI is allergenic, the combination of the two enzymes deserves specific attention.

In general, it has to be taken into account that so far only very few Bt toxins produced in genetically engineered plants have been investigated in regard to their potential impact on the immune system. As yet, only two Bt toxins (Cry1Ac and Cry1Ab) have been tested in more detail for their possible effects on the immune system. This is especially relevant for mCry3A which has so far not been subjected to more detailed analysis regarding potential immunological effects.

Given the fact that potential effects of Bt toxins on the immune system have meanwhile been discussed for many years (for overview see, for example, Then & Bauer-Panskus, 2017), and already around 40 GE crop events producing Bt toxins have been approved for the EU market, any further delay in resolving these crucial questions cannot be accepted.

In accordance with EU Regulation 1829/2003, safety of whole food and feed has to be demonstrated before approval for import can be issued. Since this is not the case with the stacked maize, the risk assessment is not conclusive and no market authorisation can be granted.

Others

Overall process

Syngenta presented new data, e.g. on gene expression and monitoring for the renewal application. However, these data were not presented and summarised in the EFSA opinion. Given the tight timeframe of one month for public consultation, these data cannot be accessed and assessed by the public. Therefore, in order to turn the process for public consultation into a useful exercise, it needs to be reorganised to allow an accurate understanding of all relevant data and respective findings during the consultation period.

Monitoring:

If approval for import is given, the applicant has to ensure that post-market monitoring (PMM) is developed to collect reliable information on the detection of indications showing whether any (adverse) effects on health may be related to GM food or feed consumption. Thus, the monitoring report should, at very least, contain detailed information on: i) actual volumes of the GE products imported into the EU; ii) the ports and silos where shipments of the GE products were unloaded; iii) the processing plants where the GE products was transferred to; iv) the amount of the GE products used on farms for feed; and v) transport routes of the GE products. Environmental monitoring should be run in regions where viable material of the GE products, such as kernels, are transported, stored, packaged, processed or used for food/feed. In case of losses and spread of viable material (such as kernels) all receiving environments need to be monitored. Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing GE products during or after the production process, and during or after human or animal consumption, should be part of the monitoring procedure.

The applicant made available some data on monitoring which could not be assessed during the consultation period. However, in awareness of current general practice, it has to be assumed that the above requirements were not fulfilled.

Reliability of data:

As existing evidence shows (Székács et al., 2011; Shu et al., 2018), the methods need to be carefully evaluated to ensure that the results are reliable, comparable and reproducible. Therefore, fully evaluated methods have to be published that allow the Bt concentration in the maize to be measured by independent scientists, as is the case for other plant protection compounds used in food and feed production. This is necessary to make sure that the environment as well as human and animals coming into contact with the material (for example, via dust, consumption or manure) are not exposed to higher quantities of Bt toxins than described in the application.

Literature research:

In regard to the literature review, the way in which it was carried out is unacceptable. Out of nearly 3000 publications, the applicant only selected 16 publications which were considered to be relevant (!). 11 of these publications were produced with the involvement of the company and the experts who had applied for a patent on the specific Bt toxin (!). Two of the other publications have been published in the Russian language only and not in a peer reviewed magazine. In essence, this shows that the data on literature provided by Syngenta are not reliable, and that EFSA should not have accepted this kind of literature review.

Environmental risk assessment
The appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al., 2017) has to be considered in the context of the renewal application process. As Pascher et al, (2016) show, the volunteer potential of maize is higher than currently assumed.

The hypothesis that hybrid offspring from maize MIR604 and teosinte will show a higher fitness compared to conventional maize, is plausible since the Bt toxins may be present in the offspring and teosinte shows higher survival rates compared to maize.

EFSA should have requested data from the applicant to show that no adverse effects can occur through gene flow from the maize to teosinte and / or from teosinte to the maize volunteers. In the absence of such data, the risk assessment and the authorisation have to be regarded as not valid.

Without detailed consideration of the hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize, no conclusion can be drawn on the environmental risks of spillage from the stacked maize.

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

Conclusions and recommendations
The EFSA risk assessment cannot be accepted.

Beyond that, since MIR604 has to be considered to be a stacked event derived from crossing two distinct lines of maize, data have to be requested on the parental plants. In the absence of such data, the market authorisation and the renewal application for the maize does not fulfill the standards required by EU law. This is a problem for all further stacked events with MIR604.

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