TESTBIOTECH Background 25 - 09 - 2016

Testbiotech comment on EFSA´s Scientific Opinion on an application by Syngenta (EFSA-GMO-DE-2011-99) for the placing on the market of maize Bt11 × 59122 × MIR604 × 1507 × GA21 and twenty subcombinations, which have not been authorised previously independently of their origin, for food and feed uses, import and processing under Regulation (EC) No 1829/2003

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Introduction

The transgenic maize Bt11 × 59122 × MIR604 × 1507 × GA21 produced by Syngenta was created through conventional crossing of five genetically engineered plants (EFSA, 2016). The resulting stack produces five insecticidal toxins (Cry1Ab, mCry3A, Cry34Ab, Cry35Ab, Cry1F). Moreover, the plants are resistant to the herbicides glyphosate and glufosinate – these herbicides leave residues in the plants. In addition, they produce a protein (phosphomannose isomerase, PMI) used as a marker for the selection of the plants. The application for authorisation further comprises 20 subcombinations of the maize stacked event, none of which have undergone EFSA risk assessment:

- Bt11 x MIR604 x 1507 x GA21
- Bt11 x 59122 x 1507 x GA21
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- Bt11 x 59122 x MIR604 x 1507
- 59122 x MIR604 x 1507 x GA21
- Bt11 x 59122 x MIR604
- Bt11 x 59122 x 1507
- Bt11 x 59122 x GA21
- Bt11 x MIR604 x 1507
- Bt11 x 1507 x GA21
- 59122 x MIR604 x 1507
- 59122 x MIR604 x GA21
- 59122 x 1507 x GA21
- MIR604 x 1507 x GA21
- Bt11 x 59122
- Bt11 x 1507
- 59122 x MIR604
- 59122 x GA21
- MIR604 x 1507
- 1507 x GA21

No specific data regarding any of the 20 subcombinations were submitted by the applicant.

Molecular characterisation
Many unintended changes and insertions are known to have occurred in the parental plants. For example, GA21 is known to have 3 full-length copies of the fragment. One copy has a base pair substitution in the NOS terminator region. Apart from these full-length copies, 3 other copies with specific individual deletions are present. Consequently, there is an identified disruption of a genomic maize gene; and several new putative open reading frames were created (EFSA 2015a). Research within the open reading frames shows similarities with known allergenic proteins. EFSA (2015b) assumed in an earlier opinion on maize GA21 that it is unlikely that these proteins are expressed in the plants. However, no empirical investigations (neither with the parental plants nor with the stacked events) were performed to prove that these proteins do not occur.

Unintended changes in the genome as a result of the genetic engineering process are also present in the other parental plants; many additional gene fragments and open reading frames have been identified in the single plants. These unintended changes and their gene products, such as RNA or proteins, are combined in the stacked event.

Furthermore, in the stacked event and its various sub-combinations, there is also an accumulation of intended gene constructs, such as promotors, stop codons and other elements that might interfere with each other or with parts of the plants genome. For example, six (intended) 35S genetic elements from Cauliflower mosaic virus and five (intended) nos – stop codons from Agrobacterium tumefaciens are present in the stacked event as assessed. These similar or identical genetic elements can, for example, interfere with each other in the expression rate – nevertheless, no investigations were performed to determine the interactivity of these elements in the stacked event and its various subcombinations.

These various genetic elements might also give rise to biologically active compounds, such as miRNA that interfere during the process of consumption by interacting with the microbiome of humans or animals; and might also be taken up from the intestine into the blood stream where they might interfere with cell regulation after consumption (see, for example, Zhang et al, 2012, Beatty et al., 2015; Yang et al., 2015; Liang et al., 2015; Hirschi et al, 2015; Baier et al., 2014; Lukasik & Zielenkiewicz, 2014, Zhou et al., 2015; Cavalieri et al., 2015) This can lead to long-term effects on health, particularly if the uptake is repeated over a longer period of time.

Therefore, EFSA should have requested data on the emergence of new variations, combinations and concentrations of small, biologically active RNA in the parental plants as well as in the stacked event and all subcombinations.

Furthermore, the expression of the additional gene constructs and their various elements should have been tested under a wide range of defined environmental conditions, taking into account stressful conditions that, for example, emerge under ongoing climate change. It is known that under stress conditions, genetically engineered plants can show reactions that are not obvious under normal agricultural conditions, and these can be very different from those of conventionally bred plants. Environmental stress can also cause unexpected patterns of expression of the newly introduced DNA (see for example Trtikova et al., 2015).

However, no data concerning the stacked event and its various subcombinations were assessed by EFSA.

Comparative analysis (for compositional analysis and agronomic traits and the phenotype)
Field trials with maize Bt11 × 59122 × MIR604 × 1507 × GA21 (and isogenic line) were conducted solely in the USA and only for one year.

Significant differences were found for several compounds and agronomic characteristics. For example, for grain components, 16 significant differences with respect to the conventional counterpart were found for untreated maize Bt11 x 59122 x MIR604 x 1507 x GA21 and 15 statistically significant differences for maize Bt11 x 59122 x MIR604 x 1507 x GA21 treated with target herbicides.

Significant differences were also found in the agronomic and phenotypic analysis: the five-event stacked maize had a lower early and final stand count.

These differences should have been investigated over more than one year and under a broad range of environmental conditions, including defined biotic and abiotic stressors.

However, EFSA did not request any further investigation regarding maize Bt11 x 59122 x MIR604 x 1507 x GA21 as, for example, testing the whole food and feed.

Furthermore, no specific data were provided for any of the 20 subcombinations. However, according to a minority opinion (EFSA 2016) of the EFSA GMO Panel member Jean-Michel Wal,

> *"compositional data and actual concentrations of the NEPs are crucial to detect possible unintended effect and achieve a grounded safety assessment."*

Toxicology
Eight proteins are newly expressed in the five-event stacked maize. Five of them are Bt toxins. Following "a weight of evidence" approach, EFSA claims that (EFSA 2016):

> *"On the basis of the known biological function of the individual newly expressed proteins (Table 4), there is currently no expectation for possible interactions relevant to the food and feed safety assessment of the five-event stack maize Bt11 x 59122 x MIR604 x 1507 x GA21."*

No toxicological studies with the whole plants were conducted to underpin this claim. Further, no data were provided for any of the 20 subcombinations. This issue is also raised by the minority opinion of EFSA GMO Panel member Jean-Michel Wal (EFSA 2016):

> *"In its opinion the GMO Panel states that it expects no adverse effect on human health; this expectation or assumption is based on so called "weight of evidence approach" and extrapolation of the data available for the singles, the five-event stack and some subcombinations, i.e. those already assessed in previous applications that are out of the scope of AP 99. However what kind of extrapolation is being made is not precisely defined. The criteria, procedure and the level of confidence that should be required for this extrapolation are not given and there is no critical appraisal of its limitations. No evaluation of the resulting uncertainty has been performed, e.g. using a probabilistic analysis, as recommended by the Draft Guidance on Uncertainty in EFSA Scientific Assessment (Revised for Internal Testing) of the EFSA Scientific Committee."*

Existing evidence – largely ignored in EFSA´s opinion - shows that more investigations would indeed be needed to conclude risk assessment on this stacked genetically engineered plant. The Bt toxins as produced in the plants are considered to be mostly specific for targeted pest insects and

therefore being safe for human and mammalian health in general. However there is evidence that for several Bt toxins, the range of susceptible organisms is broader than assumed. Therefore also risks for human and animal health can not be excluded *a priori* but have to investigated empirically. In this context there are some open questions that have to be taken into account:

- For most of the Bt toxins being produced in genetically engineered plants, their detailed mode of action which is different for each of the toxins, is not known. Not only relevant data are missing, but also the existing data are in partial contradicting with each other (see Then, 2010; Hilbeck & Otto, 2016). In result, the specificity of the toxins remains a matter of uncertainty. Also relevant in this context is – as mentioned – that the structure of the toxins as produced in the plants is substantially changed (for example by truncation). In result there are substantial uncertainties regarding the safety assumed for health and the environment.
- There are some indications that Bt toxins also can act in humans or, more general, in mammalians (Thomas and Ellar, 1983; Shimada et al., 2003; Huffmann et al. 2004; Ito et al. 2004; Mesnage et al., 2012; Bondzio et al., 2013). These effects might substantially be enhanced by interaction with other stressors such as residues from spraying with herbicides (Then, 2010). Combinatorial effects were already described in some model organisms (Kramarz et al., 2007, Bohn et al., 2016). However such interactions are not subjected to examination in context of EFSA´s risk assessment.
- The toxicity of Bt toxins can vary. Already small changes in their structure can render a higher toxicity (Pardo-López et al., 2009). However, even if the structure is supposed to be identical, its toxicity can vary in dependency of the source (for the company which is providing the toxin) as shown by Saeglitz et al. (2008). More detailed investigations are missing so far.
- There are further open questions concerning the true Bt content in the various parts of the plants which can vary substantially in reaction to environmental conditions (Then & Lorch, 2008). But so far, evaluated methods to reliably determine the content in the plants, are largely missing (Székács et al., 2011). As investigations under defined stress conditions show, the Bt content in the plant can change unpredictably (Trtikova et al, 2015).
- In is known that at least some of the Bt toxins produced by transgenic plants can impact the immune system of mammalian (see Rubio-Infante & Moreno-Fierros, 2015). These effects which are mostly described as enhancing immune reactions (adjuvant mode of action), are are not dependent on specific modes of action but on dosage effects. This is relevant in this context since the stacked plants show a higher overall concentration of Bt toxins than the parental plants. Thus for example, the reaction to allergens might become enhanced or even new allergies might emerge. To some extent these questions also concern maize, since also in these plants, some allergenic compounds are described. However, there also might be other, non-allergic immune reactions. Further, this adjuvant effect also can concern other compounds that get mixed with the Bt producing plants in food & feed. Contrary to assumptions made previously, the Bt toxins after ingestion do not get degraded rapidly but can persist throughout the intestine in relatively large quantities (Chowdhury et al., 2003; Walsh et al. 2011). In consequence, there is sufficient time for the Bt toxins to interact with all kind of compounds from the food plants to trigger or enhance immune reactions.
- The investigations should not only cover direct effects on health but also indirect effects via changes in the microbiological composition in the gut (see, for example, Shehata, et al., 2012).

Therefore, potential combinatorial health effects must be assessed in detail before any conclusion can be drawn on food safety. No conclusion on potential health effects can be drawn from the nutritional study performed with poultry – this study does not even fulfill requirements for Good Laboratory Practice (GLP).

Also relevant in this context, but omitted in the risk assessment of the GMO Panel, is the potential toxicity caused by the residues from spraying with the complementary herbicides. Due to the specific agricultural practices that go along with the cultivation of these herbicide resistant plants, there are, for example, specific patterns of applications, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention. For example, commercial large-scale cultivation of these plants results in a strong selective pressure on weeds to develop resistance to these herbicides (Sammons & Gaines, 2014), this can lead to increasing amounts of sprayed herbicides and subsequently of residues in the harvest. Further, herbicide-tolerant plants are meant to survive the application of the complementary herbicide while most other plants will die after short time. Thus, for example, residues of glyphosate, its metabolites and additives to the formulated product might accumulate and interact in the plants. As a publication by Kleter et al. (2011) shows, using herbicides to spray genetically engineered herbicide-resistant plants does indeed lead to patterns of residues and exposure that are not taken into account in regular pesticide registration:

> *"1. GM herbicide-resistant crops can change the way that herbicides can be used on these crops, for example: (a) post-emergent over-the-top applications (i.e. on the crop itself) instead of directed sprays, avoiding herbicide contact with the crop; or (b) pre-emergent and pre-harvest applications made to the conventional crop and not, or in different quantities, to the GM crop. 2. The residue profile of the applied pesticide may have been altered on the basis of the nature of the modification. 3. The overall pattern of pesticides applied to the particular crop may have been altered, leading to different exposure to pesticide residues overall."*

According to a reasoned legal opinion drawn up by Kraemer (2012), residues from spraying with complementary herbicides have to be taken into account in the risk assessment of genetically engineered plants from a regulatory point of view:

> *"It is the objective of Directive 2001/18 to avoid any adverse effect of the genetically modified plant on human health. The provisions of the Directive on the environmental risk assessment are very broad and try to cover - in the abstract, it is true – all possible cases, where direct or indirect, immediate, delayed or unforeseen adverse effects might occur. Then, it is only logical that, when genetically modified plants which are tolerant to certain herbicides, are exposed to pesticide or herbicide treatment, the effects of such treatment on the plant – and later on human or animal health – must be examined during the environmental risk assessment."*

Following on from this, that the applicants have to provide a comprehensive environmental risk assessment of the genetically engineered plants, which includes all and potential adverse effects on the environment as well as on human and animal health. This requirement includes long-term potential and accumulative effects and also all other harmful effects on human or animal health which are, in one way or another, related to the genetically modified plant, such as residues from spraying with complementary herbicides.

This is also in accordance with pesticide regulation, which requires specific risk assessment of imported plants if the usage of pesticides is different in the exporting countries compared to the one in the EU: Recital 26 of Regulation 396/2005 requires Maximum Residues Levels (MRLs) are set for food and feed produced outside the Community if produced by different agricultural practices as regards the use of plant protection products. Article 14 of Regulation 396/2005 requires that the presence of pesticide residues arising from sources other than current plant protection uses and their known cumulative and synergistic effects are determined. Further, Article 29 of Regulation

1107/2009 states that active substances and synergists have to be approved, and the maximum residue levels for each specific agricultural product have to be determined.

In any case, both the EU pesticide regulation and the GMO regulation require a high level of protection for health and the environment. Thus, in regard to herbicide-resistant plants, specific assessment of residues from spraying with complementary herbicides must be considered to be a prerequisite for granting authorisation. In addition, cumulative effects have to be investigated if a plants contains or produces other compounds of potential toxicity.

A basic prerequisite for risk assessment in this context is the availability of valid and reliable data on residue loads from spraying with herbicides. This is especially relevant in the case of glyphosate: A study published in 2015 (IARC) found that glyphosate is probably carcinogenic. While carcinogenicity of the active ingredient remains a matter of debate (EFSA 2015c), there is a scientific consensus that additives and their mixtures used in commercial formulations for spraying glyphosate can show a much higher toxicity than the active ingredient alone (Mesnage et al., 2014).

The amount of these residues depends on the specific agronomic management used in the cultivation of the herbicide resistant plants. Data from some publications (Bøhn et al., 2014, Cuhra, 2015) show a considerable amount of residues from spraying can be expected in genetically engineered soybeans resistant to glyphosate formulations. In general, the level of residues is likely to increase due to increasing problems with herbicide resistant weeds (Benbrook, 2016). However, as the EFSA Pesticide Panel stated (EFSA 2015c), safety of residues from spraying glyphosate formulations could not be concluded on the data provided so far. Thus, EFSA was unable to deliver a conclusive risk assessment on the actual risks of residues from spraying with glyphosate and the various glyphosate formulations. In regard to glufosinate, similar gaps in risk assessment exist. Glufosinate will be phased out in Europe in 2017 because of its reproductive toxicity. Thus combintorial effects have to be assessed.

Furthermore, the residues from spraying might interact with the Bt toxin and might act as a potent co-stressor. Thus, the combinatorial effects between the effects of glyphosate and the Bt toxins also need to be assessed in more detail. As, for example, Kramarz et al. (2007) show, interaction with co-stressors can render toxicity of Bt proteins to organisms that are not susceptible to Bt toxins alone. Bøhn et al. (2016) show there are signs that the toxicity of Bt toxins can indeed be enhanced by the presence of glyphosate. This issue seems to be of specific relevance if several Bt toxins are combined as they are in this case. However, these aspects were completely omitted during EU risk assessment.

Allergenicity

It is known that at least some of the Bt toxins produced by transgenic plants can impact the immune system of mammals (see Rubio-Infante & Moreno-Fierros, 2015). These effects which are mostly described as enhancing immune reactions (adjuvant mode of action), are are not dependent on specific modes of action but on dosage effects. This is relevant in this context since the stacked plants show a higher overall concentration of Bt toxins than the parental plants. Thus for example, the reaction to allergens might become enhanced or even new allergies might emerge. To some extent these questions also concern maize, since also in these plants, some allergenic compounds are described. However, there also might be other, non-allergic immune reactions. Further, this adjuvant effect also can concern other compounds that get mixed with the Bt producing plants in food & feed.

Since these effects are likely to be dose dependent, it is important to take into account that the stacked event and several of its subcombinations produce a much higher concentration of Bt toxins than the single plants. Furthermore, the concentration of Bt toxins in the stacked event and its subcombinations can vary substantially, due to genetic background (Adamczyk et al., 2004) and environmental conditions (Then & Lorch, 2008).

Contrary to previously made assumptions, the Bt toxins are not degraded rapidly after ingestion, they can persist throughout the intestine in relatively large quantities (Chowdhury et al., 2003; Walsh et al. 2011). Consequently, there is sufficient time for the Bt toxins to interact with all kinds of compounds from the food plants and to trigger or enhance immune system reactions, that are not solely to do with allergenicity.

Certainly, there is substantiated concern that the stacked event and its subcombinations can indeed trigger immune system responses. These need to be assessed in detail.

These concerns were also reflected in the dissenting opinion of EFSA GMO Panel member Jean-Michel Wal (EFSA 2016), who states in regard to the subcombinations which were not assessed:

> *"Indeed it has been shown that the genetic background of the recipient plant has a major effect on Cry1Ac expression in GM cotton and therefore it may cause an important variability in Bt protein concentrations which might impact on the safety. The risk of increased expression of the newly expressed Bt proteins in some of the "future" sub-combinations and of a possible cumulative effect of their combination on the immune system (e.g. resulting in an adjuvant activity) cannot be ruled out although it is difficult to evaluate in the absence of actual experimental data."*

Other

EFSA did not make the comments of experts from Member States publically available. These can be decisive for the comments made by third parties. This failure should be reconsidered by the Commission and the period for filing comments should be prolonged.

Environmental risk assessment

EFSA did not take into account that teosinte plants have been spreading in EU Member States, such as Spain and France, for several years. This means that environmental risk assessment has to be completed before any decision is taken on the application.

Monitoring

The JRC report (JRC, 2014) confirmed that event-specific PCR-based methods validated for single events can also be applied to DNA extracted from stacked maize Bt11 x 59122 x MIR604 x 1507 x GA21. However, no validated method was made available to distinguish the single event from any of the stacked events named in the application. Thus, no targeted monitoring or general surveillance can be performed. Therefore, legal requirements for case specific identification and monitoring have not been met and market authorisation cannot be issued.

As a legal dossier compiled by Professor Ludwig Kraemer (Kraemer, 2012) shows, EU regulations require the monitoring of effects on health at the stage of consumption in cases where there are uncertainties. Thus, for example, there must be a requirement for the monitoring of health effects that takes residues from spraying with herbicides into account.

In this case, case specific monitoring is needed to investigate negative health impacts from residues of spraying as well as effects stemming from the intended and unintended changes in the plants´ composition. Further, any spillage of the kernels has to be closely monitored, since teosinte plants allow genetic exchanges from plant to plant.

Conclusions and recommendations

Based on the data presented and assessed, the risk assessment cannot be concluded. Consequently, the application should be rejected.

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