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Risks and side effects for humans and animals: What really goes wrong in the regulation of genetically engineered plants

– Risk assessment of genetically engineered plants used for food and feed and flaws in the work of the European Food Safety Authority EFSA –

Christoph Then & Andreas Bauer-Panskus 2016

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Risks and side effects for humans and animals:
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A Testbiotech Report by Christoph Then & Andreas Bauer-Panskus 2016

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Content

Summary

This report provides an overview of deficiencies in current European Food Safety Authority EFSA risk assessment in regard to genetically engineered plants for import and usage in food and feed.

The report is based on an analysis of EFSA opinions published within the last few years. It further takes in the outcomes of the EU research projects GRACE and MARLON as well as the EU Commission announcement that it will reconsider the Implementation Regulation (503/2013). This regulation sets the standards for the risk assessment of genetically engineered plants for use in food and feed. Amongst others, it includes a request that feeding studies with the plants are conducted in order to assess health risks. Industry is demanding that this request be abandoned.

As Testbiotech shows, the EU projects mentioned above only partially fulfilled their objectives. The GRACE project failed to develop sufficiently reliable and robust methods to replace the feeding studies. The MARLON project was unable to define suitable methods to monitor potential health effects after a market authorisation has been issued.

In the light of these findings and after a detailed analysis of EFSA opinion, Testbiotech recommends that the standards for risk assessment are substantially raised. Some of the relevant topics are:

- The requirements on the data to be provided for the first steps of risk assessment (which is the comparison between the genetically engineered events and mostly isogenic lines) need to be broadened to include more detailed methods and data on further substances (such as small RNAs) and on plant characteristics.
- The functional stability of the additional DNA and its interaction with the environment have to be given priority. The events should be exposed to a broad range of defined stress conditions. Data on metabolomics should be provided.
- Stacked events need to be examined just as carefully as the single plants. In field trials, the parental plants have to be included as comparators.
- Independently of the outcome of the first stage of risk assessment, further investigations must be performed to assess the impact on human and animal health from the consumption of whole food and feed. These investigations should also include potential impacts on the immune system and reproduction.
- Long-term effects have to be given priority. To assess these effects, feeding trials must take into account the whole lifetime of the animals, including their offspring.
- Residues from spraying with complementary herbicides must be assessed in detail.
- The toxicity of each of the Bt toxins produced in the plants must be assessed in detail. Whereby special attention needs to be paid to combinatorial effects with other substances (especially with stress factors) and the impact on the immune system.
- Independent control during the data generation is absolutely necessary during each step of risk assessment.
- Cut-off criteria such as a prohibition of market authorisation must be established for genetically engineered organisms able to spread into native populations.

- Specific guidance needs to be established for genetically engineered plants that are changed in their nutritional composition, in particular, taking into account the long-term effects of consumption.
- The precautionary principle, the limits of current knowledge and resulting uncertainties must be given much higher priority.

1. Introduction

All genetically engineered plants must undergo a process of authorisation, including risk assessment. Directive 2001/18 on the deliberate release of genetically engineered organisms into the environment and Regulation 1829/2003 on the use of genetically engineered plants in food and animal feed, provide the regulatory framework for this process. In addition, the European Food Safety Authority EFSA has issued further guidance relevant to risk assessment, including specific guidance for environmental risk assessment (EFSA, 2010) and for food and feed (EFSA, 2011).

Further, the EU Commission adopted a special Implementation Regulation (503/2013) for the risk assessment of genetically engineered plants for use in food and feed.[1] However, this Implementation Regulation is controversial: Unlike EFSA in their opinion, the EU Commission requests that genetically engineered plants used in food and feed undergo feeding trials. The Commission requests 90-day feeding trials with rats being fed the respective parts of the plants such as maize kernels. So-called stacked events, which are created by crossing several genetically engineered plants, are the only exemption to this rule.

Both industry and experts from EFSA[2] reject the need for feeding studies and point out that no feeding trials are requested in the US or Canada. For this reason, the Commission has announced that the Implementation Regulation might be revised again in 2016, after the results of the EU research project GRACE[3] are presented.

Testbiotech has followed the GRACE project closely and found evidence that experts with extensive ties to the biotech industry (Bauer-Panskus & Then, 2015) had a great deal of influence. The EU project GRACE did not succeed in finding sufficiently reliable and robust methods that could replace animal feeding studies given the current state of knowledge. Furthermore, the EU project MARLON[4], which was also conducted by experts with strong affiliations to industry, did not succeed in identifying methods for monitoring the effects on human and animal health due to the consumption of genetically engineered plants. (Bauer-Panskus & Then, 2015).

The purpose of this Testbiotech report is to analyse current EU standards for the risk assessment of genetically engineered plants for use in food and feed. Testbiotech has for several years looked closely at the way EFSA carries out risk assessment and commented numerous times on the individual opinions of EFSA[5]. Our experience and expertise in this respect form the basis and starting point for this report.

1 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:157:0001:0048:de:PDF
2 http://onlinelibrary.wiley.com/doi/10.15252/embr.201642739/abstract
3 http://www.grace-fp7.eu/
4 http://web.spi.pt/marlon/index.html
5 www.testbiotech.org/database

2. Overview of EU authorisations

In the European Union, up until June 2016, fifty-five events[6] have been authorised for use in food and feed.[7] Most plants are allowed for import only. Just one plant, maize MON810 produced by Monsanto, is also allowed for cultivation.

Out of the 55 events, 28 are for maize, 10 for cotton, 12 for soybeans, 4 for oilseed rape and 1 for sugar beet.

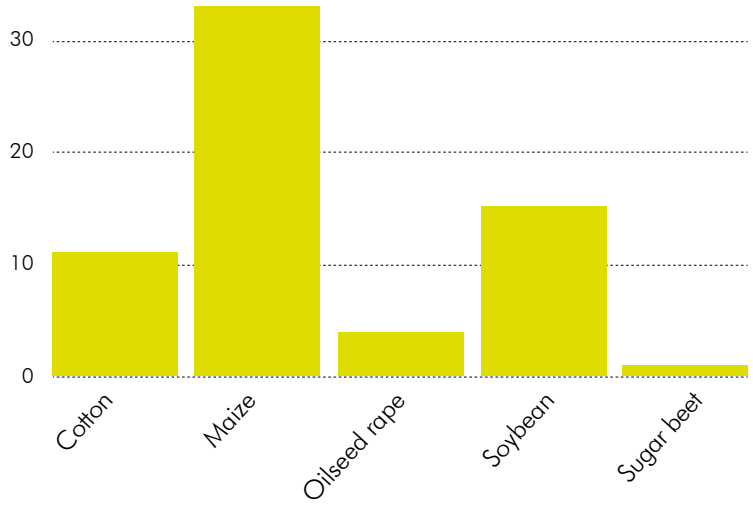

Figure 1: Genetically engineered plants (events) allowed for import into the EU and for usage in food and feed, list of species[8]

In respect to the traits (technical characteristics) the following grouping can be made:

- 7 produce insecticidal toxins
- 21 are resistant to herbicides
- 22 have a combination of insecticidal toxins and herbicide tolerance
- 1 soybean is changed in its nutritional compounds
- 2 soybeans are changed in their nutritional compounds and resistant to herbicides
- 1 maize is engineered to tolerate drought conditions
- 1 oil seed rape produces sterile pollen and is resistant to herbicides.

Furthermore, around mid-year 2016, there will be about 50 applications pending for other events in the EU, most of them for import.

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6 If the process for genetic engineering is successful, the resulting plant is called an event. The plant characteristics are called traits.

7 http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

8 http://www.testbiotech.org/en/gendatenbank_bilder

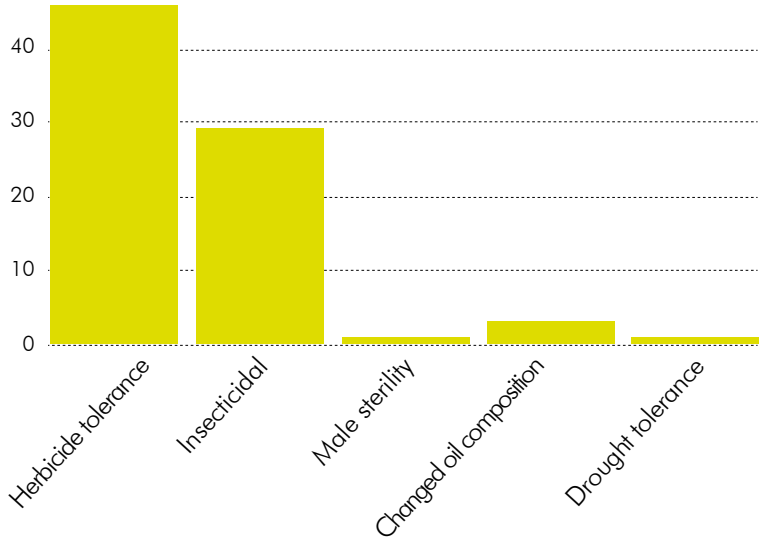

Figure 2: Genetically engineered plants (events) allowed for import into the EU and usage in food and feed, list of traits.[9]

9 http://www.testbiotech.org/en/gendatenbank_bilder

3. Overview of relevant risks

Genetically engineered plants for use in food and feed are a considerable challenge for risk assessment.

Firstly, the risk assessment of the composition of compounds is much more complex than for isolated and chemically well-defined substances. Plant constituents encompass thousands of compounds such as carbohydrates, amino acids and fatty acids, in addition to compounds of the so-called secondary metabolism such as those for natural defence mechanisms. All these compounds, including their interactions, have to be considered in the assessment. Further, there are biologically active compounds to be considered such as miRNA, which can interfere with gene regulation and be transferred at the stage of consumption without losing biological activity. The concentration of these components and their composition are dependent on environmental conditions and the stages of vegetation. The risk assessor needs to look at genetically engineered plants as organisms undergoing steady changes due to various impacts rather than as static entities that always have the same characteristics.

Secondly, the potentially negative impacts of using genetically engineered plants in agriculture and food production, are not restricted to specific applications as is the case with many pharmaceuticals or pesticides. In the case of genetically engineered plants, a much broader range of interactions has to be considered. No matter where the plants are cultivated and for which food or feed purposes they are used, independently which combinatorial effects will emerge, it must be ensured that the plants do not cause any harm to health or the environment even after long-term exposure.

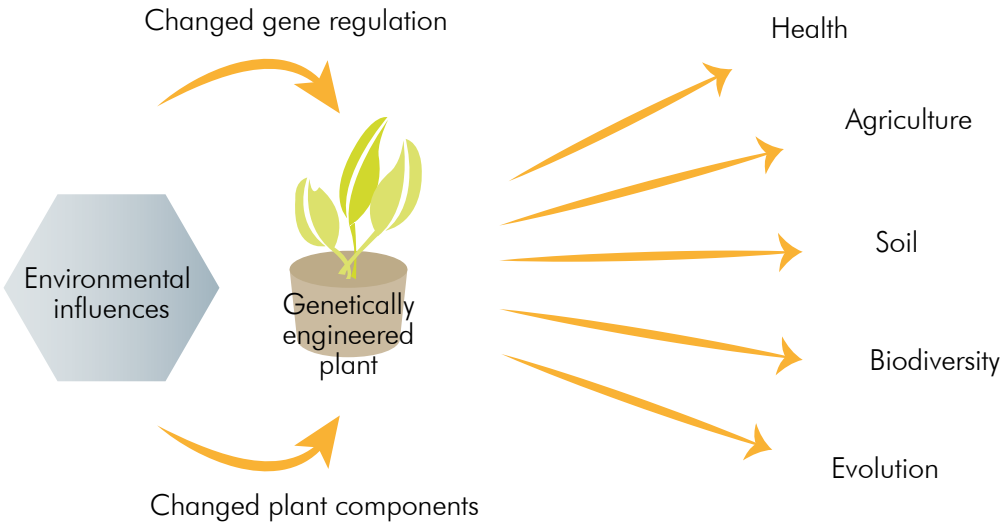

Figure 3: Overview of some risk categories for genetically engineered plants, taking into account interactions with the environment

One has to assume that these challenges cannot easily be met by science, companies, authorities or political decision makers. Indeed, faced with this huge challenge, companies and risk assessment authorities have reacted with an overly simplistic approach: For example, the newly produced compounds produced in the plants are taken out of context and assessed in isolated form. Experimental field trials are only conducted over a short period of time and only on very few field sites. If this very limited testing does not provide evidence for potential hazards, no further detailed investigations are required.

Moreover, there is a substantial discrepancy between the range of risks and the technical means available to perform risk assessment. It would be a mistake to believe that our current knowledge and scientific methods are adequate to identify the real risks or for efficient control.

In fact, it seems to be more or less impossible to assess the multiplex risks of genetically engineered plants used in food and feed in a way that safety can be guaranteed. Safety cannot be concluded from a retrospective assessment. As yet, there is no sufficiently reliable system for monitoring the negative impacts of genetically engineered plants that are granted market authorisation.

In conclusion, the real risks from the cultivation of genetically engineered plants and their use in food and feed are much more complex than the technical means available to predict and control those risks, or to protect health and the environment in the long-term.

Added to this, there appears to be a substantial amount of non-knowledge that is seemingly intentional. In many cases, crucial investigations that would not pose major technical challenges, are neither carried out nor requested. The difference between inevitable non-knowledge and intentional ignorance is made more explicit in Table 1, which is based on the work of Boeschen et al (2006).

Tabelle 1: Dimensionen des Nichtwissens (Nach: Boeschen et al., 2006).

1st dimension	knowledge (or awareness) of non-knowledge fully recognised ↔ completely unrecognised
2nd dimension	intentionality of non-knowledge unintended ↔ consciously refused
3rd dimension	temporal stability (or reducibility) of non-knowledge not yet known ↔ entirely unknowable

In general, it is important to be aware of the limits of current knowledge. Where reliable knowledge or evidence is not available, precaution and prevention have to take priority. This is the reason why the regulatory system in the EU is based on the precautionary principle in regard to genetically engineered plants. If there are reasons to doubt safety, they can and should be prevented from being marketed.

For further consideration, it is useful to identify larger groups of risk. Systemic risks due to the processes used for genetic engineering, and more particular risks such as the specific traits that are introduced into the plants and the products derived thereof.

3.1 Systemic risks

Gene activity in the plant cells is normally controlled by the plants´ own gene regulation. It enables the plants to keep some kind of steady, dynamic balance when exposed to various environmental conditions. If biotic or abiotic stressors impact the plants, they will have various reactions such as the production of specific compounds to defend themselves. If adaptability is insufficient, the plants will show signs of distress and might decay.

Similarly, there is a balance between the environment and the various species: After millions of years of co-evolution, the genetic conditions and plant characteristics have reached a stage of optimal adaptation to each other, and will continue to evolve further in steady interaction with the ecosystems.

The genetic plasticity of the plants (which are also called epigenetic mechanisms) enabling them to adapt to new environmental conditions is sometimes astonishing. For example, there are cases where weeds reacted to the cultivation of herbicide resistant genetically engineered plants by multiplying some DNA sequences within their genome. This enabled them to adapt to the use of glyphosate (Sammons & Gaines, 2014; Gaines et al., 2009) very much more rapidly than expected.

The dynamic balance between plants and the environment is also known as homoeostasis. This expression is used to refer to the systemic capability of cells and organisms to safeguard their stability, encompassing the totality of their regulatory processes. [10]

Very generally spoken, homoeostasis in genetically engineered plants is changed in comparison to their conventional counterparts. However, these changes are neither developed by interaction with ecosystems nor on the basis of the genetic plasticity of the plants. Genetic engineering is not concerned with seeing the plants and their cells as a self-regulating system. Instead, genetic engineering intervenes directly at the level of the genome in order to create a desired genetic condition.

This might be seen as similar to mechanisms that are known from viruses, which can enforce genetic conditions in plants and even be detrimental to the cells affected. However, the techniques used in genetic engineering are very different from other methods that can cause a change in the genetic conditions of plants such as:

- Random mutations: These evolutionary mechanisms do not generally put the plants' own regulatory capacity into question. In many cases, the cell will be able to silence a mutation.
- Methods of traditional breeding by sexual crossing: The genetic conditions of the individual plants being used are recombined in the next generation, but the biological mechanisms involved are based on the system driven by evolution. In some cases, plants may display undesired characteristics. However, this problem is not caused by the methods used for breeding. On the contrary, it is related to specific aims of the breeders, which, for example, can result in non-sustainable forms of agriculture.

To conclude, from a regulatory perspective there is no scientific justification for considering genetically engineered plants to be equivalent to those derived from traditional breeding as is the case in the US and Canada. In this regard, the scientific approach taken by the EU is much better suited to the purpose because here the technical process is decisive for the regulatory requirements.

10 See for example www.spektrum.de/lexikon/biologie-kompakt/homoeostase/5621

The systemic risks are relevant for risk assessment for food and feed as well as, for example, the long-term consequences of introducing the DNA from genetically engineered plants into native populations. The insertion of the additional genetic material can change the activity of the plants´ own gene activity (see for example Batista et al., 2008; Jiao et al., 2010). In addition, there are several publications showing that genetically engineered plants do not react to environmental stress in the same way as plants derived from conventional breeding (see Meyer et al., 1992; Gertz et al., 1999; Matthews et al., 2005; Zeller et al., 2010). The impacts on the plants can be various: The plants might show a higher susceptibility to pests or lower yield or lower resistance to conditions such as climate change. But it can also render higher fitness (such as a higher pollen count or seeds) or an increased content of unhealthy compounds. These unintended changes might only emerge under specific environmental conditions or after several generations. In general, there is a greater urgency around these issues due to ongoing climate change.

In conclusion, there is a need for detailed investigations of the interactions between newly introduced DNA, the plant genome and the environment. The investigations should cover several stages of vegetation, last more than one generation and involve a broad range of environmental conditions. Otherwise, we will not know if the homoeostasis of the plants has been disturbed by the process of genetic engineering in a way that could cause harm to health and the environment. However, as yet, no systematic investigation of the interactions of genetically engineered plants with their environment has ever been requested by the EFSA.

This kind of 'non-knowledge' is intentional. It would not require a huge effort to collect more relevant data, for example, by using climate chambers. Further, the so-called omics (metabolomics, transcriptomics, proteomics) can be quite useful in this context since they allow lots of data on changes in gene regulation and metabolism in the plants to be generated. These data might not allow any direct conclusions to be drawn on the risks, but they can provide important information to facilitate more targeted down-stream investigations.

In general, issuing any authorisation for the use of genetically engineered plants in food and feed without first requesting comprehensive data on the reactions of the plants to a broad range of defined environmental conditions, is an act of negligence.

3.2 Specific risks

Specific risks associated with genetically engineered plants are, for example, intentionally introduced traits such as insecticidal toxins, herbicide resistance or changes in nutritional quality.

In addition to combinatorial and accumulated effects, risk assessment has to take into account the new proteins, metabolites and biologically active compounds such as miRNA. As yet, only a few of the relevant factors are taken into account during EFSA risk assessment. Some of the risks are discussed in the following chapters.

3.3 Assessment of long term effects

Deliberate release and cultivation of genetically engineered organisms exposes the environment and the chain of food production to new, large-scale biological functions and compounds, which were not evolved and adapted to in evolutionary processes. It is a matter of serious concern that we are already seeing the uncontrolled spread of transgenic plants into native populations in several regions (see Bauer-Panskus et al., 2013).

The lack of balance between our capability to perform risk assessment and the real risks is especially relevant in these cases. No one can predicted how these plants will evolve in further evolutionary processes, which will among other things be influenced by ongoing climate change.

This problem is also relevant to future food production since it can result in continuous and non-controllable contamination of seeds and / or harvests. All the uncertainties around the long-term impacts on health and the environment will be continued into future generations. Moreover, in many cases, future generations will have no way of removing these plants from the environment.

Therefore, from the perspective of the intention of the precautionary principle, any release of genetically engineered organisms that might persist in the environment should be prohibited. So far, however, there are no clear "cut-off" criteria to reject applications where there is no effective spatio-temporal control in place for the genetically engineered organisms.

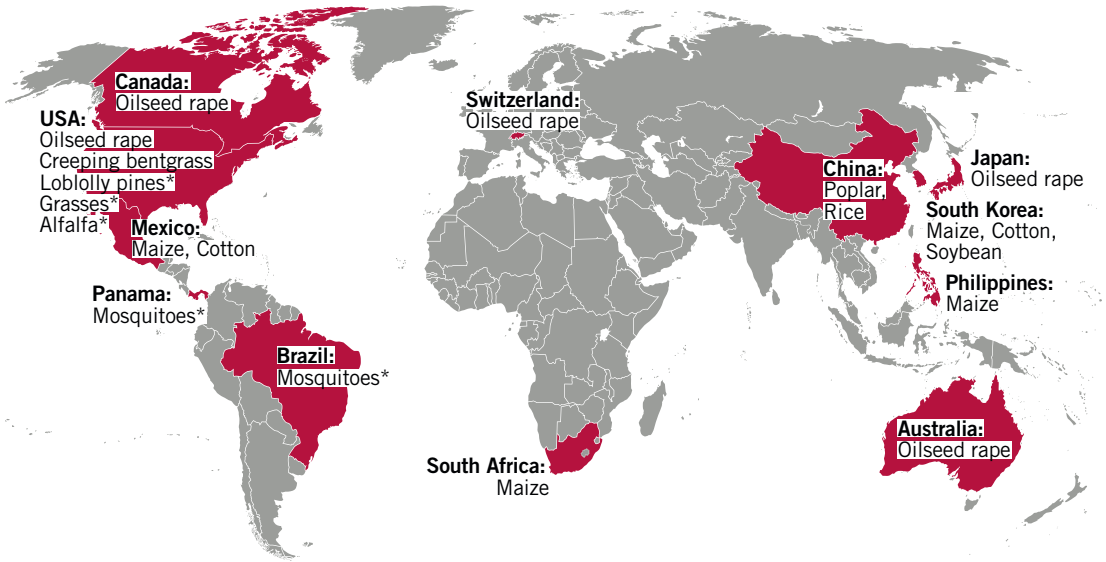

Fig. 4: Overview of case studies of uncontrolled spread of genetically engineered organisms (* = lack of regulatory oversight or missing scientific data)

Regardless of whether the genetically engineered plants can persist in the environment, there must be an assessment of their long-term effects on health and the environment, including their combinatorial and accumulative effects. Some of these issues will be discussed in the following chapters.

4. Comparative risk assessment and substantial equivalence

Existing EU regulations such as Regulations 178/2002 and 1829/2003 as well as Directive 2001/18 foresee a high level of protection for consumers and the environment. For example, in Recital 9 of Regulation 1829/2003 it says:

> *"Thus, genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of the European Food Safety Authority (Authority), of any risks which they present for human and animal health and, as the case may be, for the environment."*

According to Regulation 1829/2003 (Article 4), it must be excluded that food and feed derived from genetically engineered plants has adverse effects on human health and the environment:

> *"(1) Food (…) must not:*
> *(a) have adverse effects on human health, animal health or the environment;*
>
> *(3) No GMO for food use or food referred to in Article 3(1) shall be authorised unless the applicant for such authorisation has adequately and sufficiently demonstrated that it satisfies the requirements of paragraph 1 of this Article."*

These regulations pose a huge challenge for the risk assessor. As already mentioned, the use of food plants is not restricted to specific purposes as it is with pharmaceuticals or pesticides. Rather, there are all kinds of uses and possible impacts on health and the environment that have to be considered together with agriculture and food production. Regardless of where these plants are grown or in which food and feed they might be used, the plants must be proven to have no adverse effects on health or the environment.

For each relevant area of concern, specific and robust methods need to be developed together with assessment criteria. The following diagram gives an overview of some of the relevant topics.

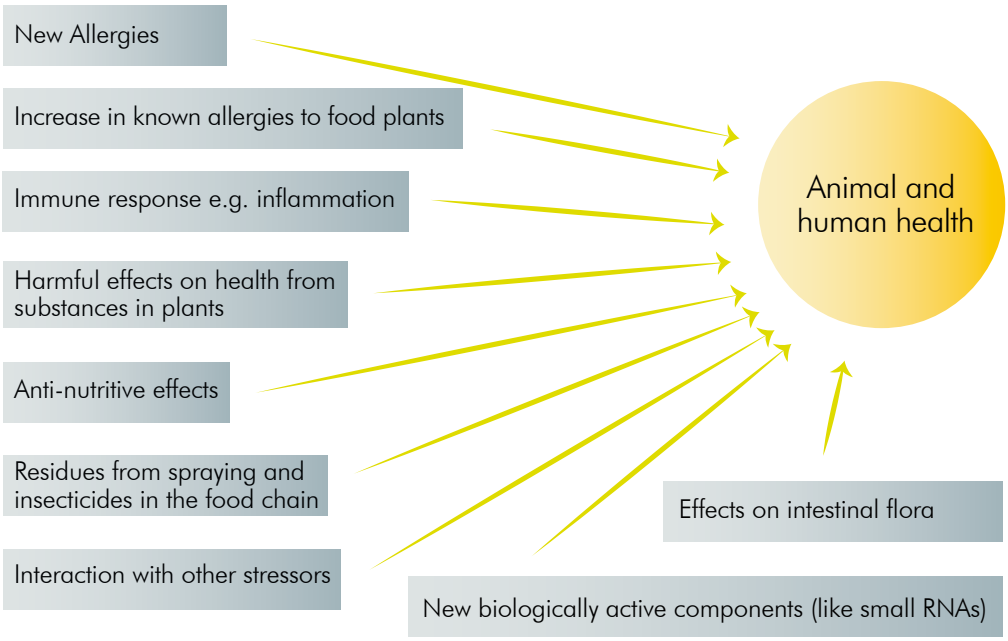

Figure 5: Schematic overview onf some health risks ofposed by genetically engineered plants

In the toxicological assessment of single isolated compounds, it is very often possible to develop a solid hypotheses for targeted investigations, but this is much more difficult in the context of genetically engineered plants. In the risk assessment of genetically engineered organisms we are not dealing with clearly defined compounds, but with thousands of components that can show large variations in their composition.

Consequently, the risk assessment of genetically engineered plants has to start with the uncertainties, and cannot at this point be narrowed down to well-defined potential hazards or be reduced to very specific risks. From a scientific point of view, this is a much bigger challenge than the assessment of chemically well-defined single substances.

The EU risk assessment tries to overcome this problem with a so-called "comparative approach": Genetically engineered plants are grown side by side in experimental field trials. A comparison is made of some plant characteristics (such as height, yield and flowering) and the composition of several plant components. Although at first sight this may appear to be a reasonable approach, current practice does not allow sufficiently reliable conclusions. Some of the deficiencies are:

- The very limited number of field sites (mostly below ten) and the short duration of the field trials (very often only one season) that do not allow reliable conclusions to be drawn on the risks of large-scale cultivation. In fact, these trials are nothing but a "snapshot" and certainly cannot reflect the whole picture. Once these plants have been granted authorisation for commercial cultivation, they will be grown under very different environmental conditions, where the opportunities for them to interact with the environment are very much increased. Moreover, their traits will be introgressed into other varieties that have a different genetic backgrounds. This can cause further unpredictable interaction between the genomes of the plants and the newly introduced genes. What is missing are comprehensive data on the interactions with the environment and biotic or abiotic stressors, including those varieties that are actually cultivated in the field.

- The components used for comparison such as carbohydrates, amino acids and fatty acids and minerals, represent only a small percentage of relevant plant ingredients and their metabolic processes. For example, omics-data are not requested and neither is data on biologically active substances such as chemical transmitters or biological messengers such as miRNA. The problem arising from selecting of data that is too narrow is also relevant for the plant characteristics. For example, secondary seed dormancy, resistance to (non-target) pest organisms or reactions to more extreme climate conditions are not generated in a systematic manner. Furthermore, in many cases, it is not the whole plant that is investigated but only specific parts of it such as the kernels. Most data on plant components only concern the harvest, there are not many data on the different stages of vegetation. Consequently, relevant changes in plant characteristics and components can easily be missed.
- Nearly all data show significant unintended changes in comparison to the plants´ composition. However, the biological relevance of these differences very often remains a matter of uncertainty. In most cases, these differences are not investigated further but are assumed to be irrelevant at the early stage of the investigations. Without generating further more specific data on the real range of variations under defined environmental conditions, such assumptions are not sufficiently reliable.
- In parallel to the conventional plants that were used to create the genetically engineered plants (which are its 'comparators'), there are further varieties used in the field trials as further 'references'. These additional plants can show a large range of compositional differences, which are not relevant for the risk assessment. Data stemming from these reference plants can hide relevant differences between the genetically engineered plants and their true comparators. In fact, data from these reference plants are used by EFSA to justify not carrying out further investigations.
- Some crucial data are not requested at all. For example, in trials with so-called stacked events (which are derived from crossings of genetically engineered plants), the parental plants are not requested to be grown in parallel. While EFSA, in its first guidance, previously requested such data (EFSA, 2007) these requirements were abandoned without sufficient justification in the following years.
- All data from field trials are generated by industry without any independent control.

In recent years, some progress has been made as, for instance, a more detailed statistical analysis is now requested. Furthermore, data in the so-called ILSI database (see Then & Bauer-Panskus, 2010) can no longer be used for the purpose of specific comparisons. But nevertheless, this has not solved the general problems with comparative risk assessment.

The deficiencies as described have a huge impact: Nearly all further steps in risk assessment carried out by EFSA are dependent on the outcome of the first comparison. If no evidence is found for potential hazards in this first step, then more specific investigations such as in-vitro tests or feeding studies with the plant or any long term investigations are not requested.

Also in the opinion of EFSA, there is a substantial difference between the "comparative risk assessment" and a "comprehensive risk assessment" (EFSA 2011). Indeed, the comparative risk assessment is not much more than a cursory check and certainly nothing like a comprehensive, robust and reliable risk assessment.

Industry had considerable influence in setting the standards for comparative risk assessment. Especially relevant in this context is the International Life Sciences Institute (ILSI), an institution funded by food and agrochemical companies (see Then & Bauer-Panskus, 2010). Consequently, current EFSA practice is not in line with the requirements of the EU regulations: According to EU Regulation 1829/2003, the risk assessment has to be substantially different from an approach known as "substantial equivalence" that is applied in the US. This concept assumes that plants derived from conventional breeding can, in general, be regarded as substantially equivalent in comparison to genetically engineered plants, and only specific traits have to be taken into account for risk assessment.

Until 2003, the concept of substantial equivalence, which is not sufficiently based on science, was also applied in the EU (Regulation 258/97), but was abandoned with the adoption of Regulation 1829/2003. As stated in the Regulation 1829/2003, Recital 6:

> *"Regulation (EC) No 258/97 also provides for a notification procedure for novel foods which are substantially equivalent to existing foods. Whilst substantial equivalence is a key step in the procedure for assessment of the safety of genetically modified foods, it is not a safety assessment in itself. (…) this notification procedure should be abandoned in respect of genetically modified foods."*

But according to the experts who developed the comparative approach for EFSA, the concept of substantial equivalence remained unchanged (Kok & Kuiper, 2003):

> *„Although the Principle of Substantial Equivalence has received comments from all types of stakeholders (producers, regulators, consumers, evaluators, etc.), the basic idea behind the principle remains untouched. When evaluating a new or GM crop variety, comparison with available data on the nearest comparator, as well as with similar varieties on the market, should form the initial part of the assessment procedure."*

Indeed, the comparison of data as described above, is not only the starting point of risk assessment as performed by EFSA, but is in most cases also the end point. It is mostly based on insufficient data, but still the authority fails to request any further more specific toxicological investigations. As a consequence, EFSA is applying the 'comparative approach' as if the concept of 'substantial equivalence' were still be in place. In doing so, the authority is in conflict with the legal requirements of the EU.

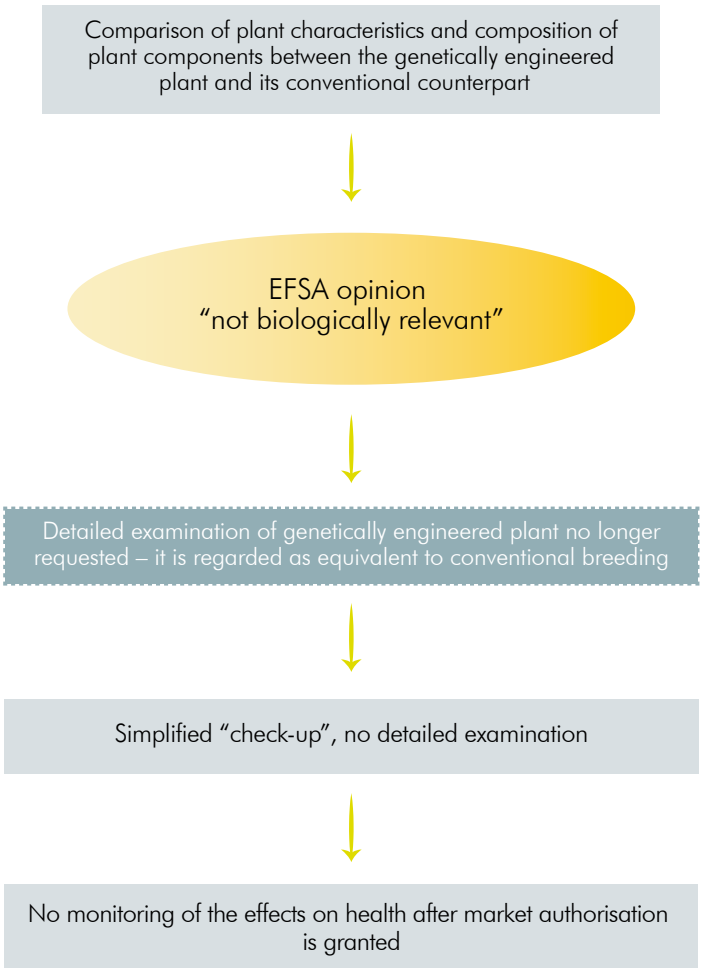

Figure 6: Schematic overview of EFSA health risk assessment of genetically engineered plants, applying the so-called comparative approach.

In contrast, the EU Commission with its Implementation Regulation (503/2013) has substantially widened the remit of EFSA: For the first time, the request for 90-day feeding trials has become mandatory. However, the adequacy of such sub-chronic feeding trials that were developed for the testing of defined chemicals compounds is open to dispute. Furthermore, there can be no justification (as given in the Implementation Regulation) for exempting so-called stacked events, which can inherit very complex interactions emerging from the crossing of genetically engineered plants. But in regard to the overall authorisation process, these feeding trials as requested by the Commission do make a significant difference: It makes clear that – independently of the outcome of the first comparative step of risk assessment - further investigations are necessary.

Necessity of feeding trials and the GRACE-Project

The EU research project GRACE was conducted from 2012 up until 2015. One of the goals of the project was to provide more clarity as to whether 90-day feeding trials are needed for the risk assessment of genetically engineered plants. The EU Commission will be making further decisions on standards of risk assessment according to the outcome of the project.

Testbiotech followed the GRACE project very closely and found evidence that the project was heavily influenced by experts with close ties to the biotech industry Bauer-Panskus & Then, 2015). In fact, the GRACE project concluded that the mandatory feeding trials as requested for applications from the beginning of 2014, were not necessary. If the opinion of GRACE were to prevail, it would substantially lower the standards of risk assessment and the requirements for data to be provided by companies. Specific toxicological investigations of the genetically engineered plants would not be requested in most cases.

GRACE experts state that feeding trials are in general not suitable for investigating the health risks of genetically engineered plants. In this context, concerns were raised that it would be problematic if significant findings emerged with no clear biological relevance.

At the same time, it was claimed that GRACE could provide more precise alternative methods.[11] However, it became very clear from the final presentations made by several GRACE experts that the alternative methods referred to have not yet been developed to the extent that they could replace animal feeding studies. They only make sense if performed in addition. This is especially true for the so-called Omics methods, which can be used to measure gene activity and metabolism in the plants.

Moreover, in-vitro methods using cell cultures for the assessment of genetically engineered plants are not yet fit for purpose. The relevant GRACE experts commented that a period of ten years would be needed to develop sufficiently advanced methods and protocols. The methods currently available might be quite helpful if they are used additionally, but they cannot replace animal feeding trials at the present time (Bauer-Panskus & Then, 2015).

In general, most potential health effects due to genetically engineered plants are much more difficult to investigate than those due to plants composed of defined chemical substances. The composition of these plants is not as clearly defined as specific chemical compounds, and the mechanisms that can cause negative health effects can be various, such as altered plant composition, effects of intended additional proteins or any unintended gene products. The negative effects might only be detected after some period of time and in combination with other compounds present in food and feed. Consequently, risk assessment should build on methods that allow the assessment of combinatorial effects present in whole food and feed.

11 http://www.grace-fp7.eu/sites/default/files/GRACE_Conclusions%20&Recommendations.pdf

90-day feeding trials are so far the only method frequently used to assess the health risk of whole food and feed derived from genetically engineered plants. Further, feeding trials with whole feed are carried out with poultry; normally lasting for a period of 42 days. However, these trials are only meant to provide information on the nutritional quality of the feed, and cannot provide reliable information on health effects. This fact is also acknowledged by EFSA.

Currently, more than 50 genetically engineered events have been assessed and authorised for import

into the EU. Many of those were never tested in a 90-day feeding trial. One example is the genetically engineered maize known as SmartStax, which produces six insecticides and is engineered to be resistant to two herbicides. The EU Commission issued market authorisation for this stacked event without requesting any feeding trials with whole food and feed to assess potential health effects. It should further be noted that the combinatorial effects of genetically engineered plants mixed into food and feed have likewise never been assessed.

There are further levels of complexity that will add to these problems in the near future: Market applications for so-called stacked events such as SmartStax are increasing. In addition, several applications have been filed for plants that are changed in their nutritional quality. The risk assessment of these plants might prove to be much more complicated than for plants that were only made resistant to one herbicide.

It has to assumed, that in general, 90-day feeding trials will in most cases not be sufficient to assess the health risks of genetically engineered plants. Long-term, combinatorial and accumulative effects can only be assessed in feeding trials over the lifetime of the animals, including their offspring.

GRACE experts as well as industry reject the trials for several reasons. Amongst others, they allege the proponents of feeding trials are responsible for thousands of animals being used in the studies. However, this allegation is not substantiated: Industry and EU political decision-makers have to ask themselves if the supposed positive effects of genetically engineered plants are sufficient to justify animal experiments. In the light of constant consumer rejection in the EU, the answer to this question is likely to be a resounding no. But if this question is answered with yes, they have to be prepared to adequately protect human and animal health as well as the environment. In this regard, there can be no compromise.

5. Residues from spraying with complementary herbicides

In 2015/ 2016, the EU Commission, experts from Members States and the EU Parliament held discussions on the issue of market authorisation for genetically engineered soybeans produced by Bayer and Monsanto. These soybeans can be sprayed with glyphosate in combination with dicamba or isoxaflutole (MON87708 x MON89788 und FG72). Isoxaflutole is classified as probably carcinogenic (ref), whilst glyphosate is suspected of being carcinogenic (IARC, 2015).

Residues from spraying with these complementary herbicides (i.e. the herbicides that the genetically engineered plants were made resistant to) are present in the harvest. Hence, the harvested soybeans that are imported into the EU will contain residues from herbicide formulations allowed in countries such as Argentina, Brazil or the US. They are applied in very high dosages in some regions, especially where there are problems with herbicide resistant weeds.[12] The EU has though, as yet, failed to assess the specific formulations applied in these countries or any health risks associated with them. At the same time, it is well-known that the formulations can be much more toxic in comparison to an active ingredient in its isolated form. Moreover, the combination of the formulations can be much more toxic than each of them separately. Despite these well-known facts, the EU has still not requested an assessment of the residues from spraying.

Indeed, there are major gaps in the current risk assessment of herbicide resistant plants imported into the EU. In 2015, EFSA published its opinion on the risks of the herbicide glyphosate, which it considers to be only low risk for human health (EFSA 2015a). However, the commercial formulations such as Roundup with glyphosate as the active ingredient, were considered to have a much higher degree of toxicity (EFSA 2015b). At the same time, according to EFSA, it is not possible to assess the health impact of the residues from these commercial formulations because no data are available.

There is a similar problem with isoxaflutole: According to EFSA (2016), there are metabolites from this herbicide that have been found in the genetically engineered soybeans, none of which have ever been investigated in regard to health risks.

These gaps in risk assessment prompted the EU Commission to take action. In a letter the Commission sent to the EFSA that was published in 2016,[13] these deficiencies in current risk assessment were acknowledged for the very first time. And now the assessment of health risks due to residues in imported feed from spraying with herbicide formulations such as Roundup is requested.

Over and above this circumstance, the risk assessment of genetically engineered plants needs to be completely revised in regard to the residues from spraying with the complementary herbicides. For example, it should also be taken into account that a permanent exposure to these residues can impact human and animal health through changes in the intestinal microbiome: The residues might lead to a change in the composition of the microorganisms, and thereby increase the probability of diseases. It is known that glyphosate can change the composition of soil microorganisms (see for example, EFSA, 2012). In addition, glyphosate acts as an antibiotic on some bacteria such as E. coli (Forlani et al., 1997; Carlisle & Trevors, 1988). Thus, it is not at all unlikely that the intestinal microbiome can be affected.

12 www.testbiotech.org/node/926
13 www.testbiotech.org/node/1636

6. Insecticidal Bt toxins

Generally, the genetically engineered plants authorised for import into the EU already contain around a dozen different Bt toxins. These toxins originate from the soil bacteria *Bacillus thuringiensis*, which naturally produce around 200 insecticidal substances. The Bt toxins produced in the transgenic plants are, however, changed in their structure to enhance their toxicity. In addition, genetically engineered plants can be crossed to produce so-called stacked events. The stacked events that are then marketed not only contain a combination of toxins but have an overall higher concentration of the toxins (see below).

The Bt toxins produced in the plants are considered to be mostly specific for targeted pest insects, and therefore 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, risks for human and animal health cannot be excluded a priori but need to be investigated empirically. In this context, there are some open questions that need to be considered:

- The detailed mode of action for most of the Bt toxins produced in genetically engineered plants is not known, and is different for each of the toxins. Further, there is a lack of relevant data and the existing data are partially contradictory (see Then, 2010; Hilbeck & Otto, 2016). As a 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 produced in the plants is substantially changed. The Bt toxins produced in genetically engineered plants are not the same as natural Bt toxins and some do not even have a natural template. Consequently, there are substantial uncertainties regarding the safety assumed for health and the environment.
- There are some indications that Bt toxins can have negative effects in humans or, more generally, in mammals (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 be substantially enhanced by interaction with other stressors such as residues from spraying with herbicides (Then, 2010). Combinatorial effects have already been described in some model organisms (Kramarz et al., 2007, Bohn et al., 2016). However, such interactions are not investigated in the context of EFSA risk assessment.
- The toxicity of Bt toxins can vary. Even small changes in their structure can render a higher toxicity (Pardo-López et al., 2009). However, even if the structure is deemed to be identical, toxicity can vary in dependency on the source as shown by Saeglitz et al. (2008). More detailed investigations are missing so far.
- There are further open questions about the true Bt content in the various parts of the plants, which can vary substantially in response to environmental conditions (Then & Lorch, 2008). But, as yet, evaluated methods to reliably determine the Bt content in the plants are largely missing (Székács et al., 2011). As investigations under defined stress conditions show, the Bt content in the plants can change unpredictably (Trtikova et al, 2015).
- It is known that at least some of the Bt toxins produced by transgenic plants can impact the immune system in mammals (see Rubio-Infante & Moreno-Fierros, 2015). In this context, it is a matter of special concern that Bt toxins are produced in some genetically engineered soybeans. Soybeans naturally produce a broad range of allergenic substances. Combinatorial effects may lead to an enhanced immune response to these allergens or cause new allergies (overview: Testbiotech 2012). To some extent these issues are also relevant to maize, since some allergenic compounds

have been described for maize plants. Furthermore, this adjuvant effect may also be relevant to other compounds that are mixed in food and feed along with the Bt producing plants. Contrary to claims made previously, after ingestion the Bt toxins are not rapidly degraded but 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 to trigger or enhance immune responses.

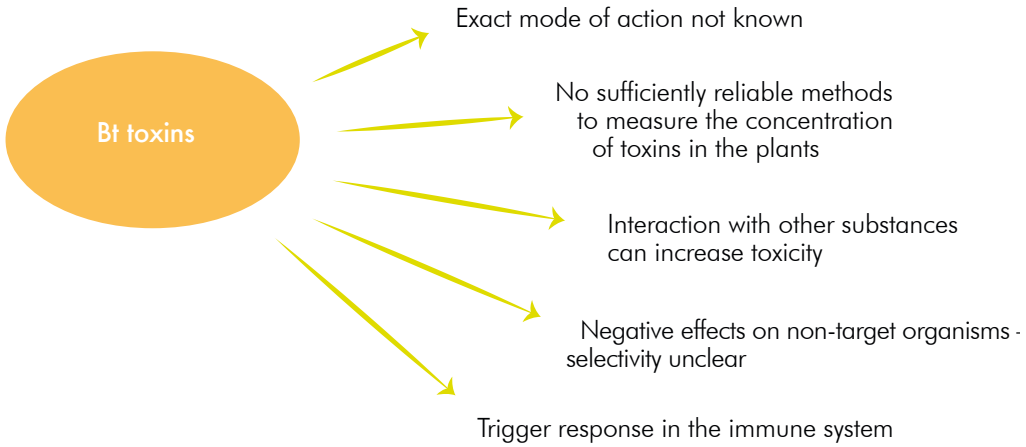

Figure 7: Overview of some problems in regard to the risk assessment of Bt toxins

7. Change in nutritional quality

Already several genetically engineered soybeans that have been changed in their nutritional quality have been authorised for import into the EU. For example, the composition of oil is altered in genetically engineered soybeans produced by Monsanto and DuPont/Pioneer.

The mechanisms established in the plants are various:

- Additional DNA is inserted with the intention of producing new fatty acids that do not naturally occur in soybeans.
- Gene activity is reduced in a way that the level of some fatty acids is substantially reduced. These plants are altered by using the mechanisms of RNA interference (RNAi, see below).

Genetically engineered soybeans are engineered to enhance the level of Omega-3-fatty acids in food and feed. However, it is controversial as to whether the higher levels of these fatty acids have a health value. They are actually suspected of promoting some specific forms of cancer (GeneWatch UK & TestBiotech, 2015). But despite all these concerns, EFSA has not requested any investigation into the real health impact of these soybeans.

In some cases feeding trials were performed, but the rats were fed with defatted soybeans. This meant that the data were inconclusive for the assessment of the effects on health of soybeans with altered oil composition (see table). Feeding studies to assess carcinogenicity were not performed.

In short, EFSA has not as yet issued any specific guidance to assess the health risks of plants that are changed in their nutritional quality, despite its own dossiers making this recommendation (EFSA, 2011).

Table 2: Overview of feeding trials performed with genetically engineered soybeans that are changed in their oil quality, as assessed by EFSA

Event	Year	Company	Species	Traits	Feeding trials for 90 days	Comment
MON87705 x MON89788	2015	Monsanto	Soybean	Resistance to glyphosate / changed oil composition	(Yes)	The soybeans were defatted – the changed oil composition was not part of the trials
MON 87769	2014	Monsanto	Soybean	Changed oil composition	(Yes)	The soybeans were defatted, the changed oil was tested separately.
MON87705	2012	Monsanto	Soybean	Changed oil composition	(Yes)	The soybeans were defatted – the changed oil composition was not part of the trials
DP305423	2014	DuPont/ Pioneer	Soybean	Resistance to ALS-inhibitors / changed oil composition	(Yes)	Plants were not treated with herbicides.

8. Biologically active compounds such as miRNA

Biologically active compounds collectively known as small RNAs (miRNA, siRNA) are of increasing interest for researchers. Small RNAs are decisive for gene regulation in plants, animals, humans and microorganisms. Some of the known mechanisms are based on so-called gene silencing by adding blocking transcription of DNA (by methylation) or by the degrading of messenger substances (mRNA) needed for the production of proteins. These and other mechanisms are also collectively known as RNA interference (RNAi).

It is known that plants in particular have a large variation of small RNAs enabling them to react to changing environmental conditions (Borges & Martienssen, 2015). In addition, funghi, bacteria and other microorganisms such as those which are part of the intestinal microbiome produce a great diversity of small RNAs (Beatty et al., 2014).

Many small RNAs are stable in a way that they can persist outside the cells, for example, in the bloodstream. Many experts assume that there are specific mechanisms that support the uptake of small RNAs from the environment (or the intestine). For example, they can be packed in vesicles and thereby transferred to other cells. This might be one reason why miRNA produced by intestinal microorganisms can be found in the blood and organs of humans and animals (Wang, et al. 2012; Beatty 2014).

Small RNAs also play a role in the context of genetically engineered plants: For example, RNAI is used to reduce the production of specific fatty acids in soybeans and thereby to change their oil composition (see above). Furthermore, the plants are genetically engineered to produce small RNAs that act like an insecticidal toxin: If pest insects feed on the plant, they will take up additional miRNA, this will then interfere with the gene regulation of the insects in a way that will kill them (see for example Zotti & Smagghe, 2015). As yet, these plants are not allowed in the EU.

In 2012, it was reported for the first time that miRNA produced by plants can enter the blood of mammals (including humans) at the stage of consumption (Zhang et al, 2012). These findings were called into question by several experts.[14] However, looking at more recent publications, one has to assume that plant miRNA can indeed enter the blood, organs and urine of mammals after ingestion (Yang et al., 2015; Liang et al., 2015; Hirschi et al, 2015). At the same time, the amount being taken up and the factors influencing biological impact need further research. This uptake of small RNAs via ingestion, but also via the lungs is relevant for risk assessment because many small RNAs have a structure that can universally interact with the gene regulation of plants, animals, humans and microorganisms.

There is evidence that small RNAs taken up from the intestine do indeed interfere with gene regulation in humans and animals. For example, it was found that miRNA transferred via milk shows biological activity (Baier et al., 2014). Small RNAs produced by plants are able to interfere with the immune system in humans and animals (Zhou et al., 2015; Cavalieri et al., 2015).

Further, one has to assume small RNAs from the plants can also interfere with the microbiome during ingestion, and can thereby change the composition of the intestinal flora. In consequence this would change also the composition of the small RNAs that are taken up from the intestine and entering the blood stream and organs. Very generally, it has to be assumed that the genetic engineering of plants always causes a change in the composition of their small RNAs. And the real impact on human and animal health remains unknown. The biological relevance of these changes might be different from case to case. But, so far, EFSA has not requested any data in this context.

14 See for example FIFRA Expertenpanel des US EPA, 2014: https://www.epa.gov/sites/production/files/2015-06/documents/012814minutes.pdf

9. Combinatorial, accumulative and long-term effects

Combinatorial and accumulative effects can lead to substantial risks for humans, animals and the environment. Such effects can be antagonistic, additive or synergistic. As to the latter, the combinatorial effect is higher than the combined single effects. Combinatorial effects can result on occasion, some are predictable resulting from the:

1. Combination of traits in stacked events
2. Mixtures of genetically engineered plants in food and feed
3. Interaction with other components within the food chain such as allergens or toxins.

While the overall impact of all combinatorial effects within the food chain is hard to examine, stacked events with a specific combination of traits such as insecticidal toxins and herbicide resistance are well-suited to detailed examinations. Mixtures of genetically engineered plants authorised for food and feed can also be assessed. However, current EFSA practice does take into account not request such investigations, despite EU Directive 2001/18 requesting the assessment of accumulative effects.

Several flaws in current regulation have been observed in the case of the genetically engineered maize known as SmartStax. It was authorised for import into the EU for usage in food and feed without any examination of health risks in a single feeding study (see also Testbiotech, 2014):

- SmartStax is a joint Monsanto and Dow AgroSciences product; it is a genetically engineered maize currently grown in the US, that produces six insecticidal proteins (Bt toxins) from different subspecies of Bacillus thuringiensis and has been engineered to be resistant to two herbicides (glyphosate and glufosinate). One of the insecticidal proteins (Cry1A.105) is derived from synthetic DNA that does not have a natural variant. In addition, the combination of these toxins does not occur naturally. Some uncertainties in regard to effects on health did emerge in feeding studies performed with the individual parental plants. Nevertheless, EFSA did not request a feeding study with the stacked maize to investigate health risks resulting from combinatorial effects. SmartStax has a much higher Bt content than any other genetically engineered plant to date, precipitating a higher likelihood of there being an impact on the immune systems in humans and animals. These health risks were left aside by EFSA.
- In addition, SmartStax has been engineered to be resistant to the herbicides glyphosate and glufosinate. Consequently, we have to assume there will be a mixture of insecticidal toxins in combination with residues from spraying. None of these were investigated.

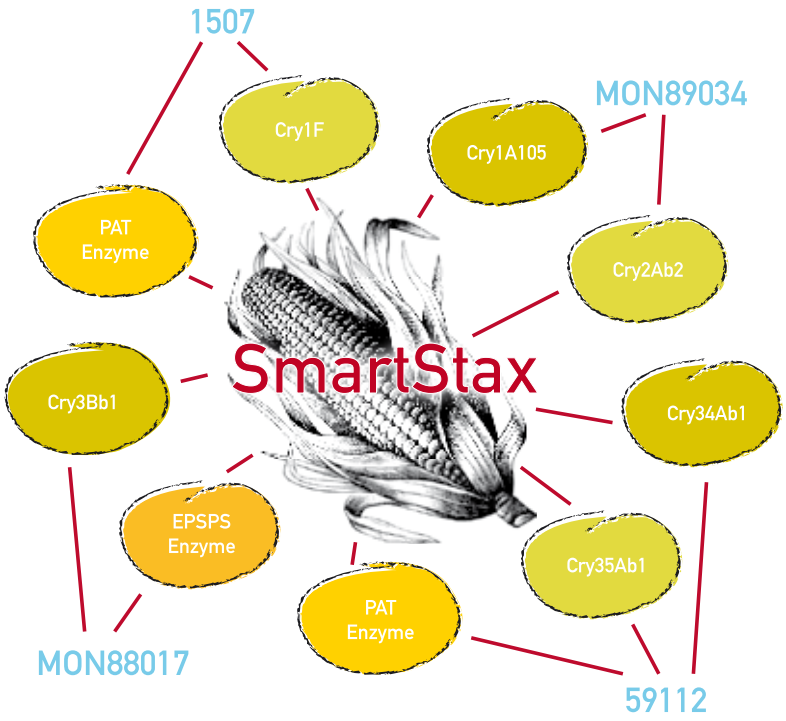

Figure 8: SmartStax, produced by Monsanto and Dow AgroSciences. This maize is a combination of four genetically engineered events (MON88017, MON89034, 59122, 1507). It produces six insecticidal toxins (Cry toxin is derived from several strains of Bacillus thuringiensis, one of which, Cry1A105, is synthetically manufactured) and is tolerant to two herbicides (glufosinate through the PAT enzyme and glyphosate through the EPSPS enzyme).

More generally, EFSA is failing to examine the long-term effects of the consumption of genetically engineered plants and the combinatorial effects of mixtures of genetically engineered plants:

- No feeding trials are requested over the lifetime of the animals, including their offspring.
- There have been absolutely no investigations into the accumulated effects resulting from mixtures of several genetically engineered plants being used in food and feed.

10. Conclusions and recommendations

Within the bounds of the current state of knowledge, the EU project GRACE was unable to find sufficiently reliable and robust methods that could replace animal feeding studies. Furthermore, the EU project MARLON did not succeed in identifying methods for monitoring the effects on human and animal health from the consumption of genetically engineered plants (ref). In the light of these findings, and in awareness of substantial deficiencies in current EFSA risk assessment, there is an urgent need to raise the standards of risk assessment for genetically engineered plants before they are granted market authorisation. We recommend the following measures:

First steps in risk assessment:

- The requirements for data that has to be provided for the first steps of risk assessment (which is the comparison between the genetically engineered events and mostly isogenic lines) must be broadened to include more detailed methods and data on further substances (such as small RNAs) and plant characteristics.
- Priority must be given to the functional stability of the additional DNA and its interaction with the environment. All events should be exposed to a broad range of defined stress conditions. Data on metabolomics should also be provided.
- Data must be provided for all parts of the plants and represent several stages of vegetation.
- Stacked events must be examined at least as carefully as the single plants. In field trials, the parental plants must be included as comparators.
- The genetically engineered varieties, which are actually used for cultivation also need to be assessed. Data stemming from these varieties could also be part of post-market monitoring.
- Where there is some doubt, only data stemming from the comparison of the comparators (isogenic lines and parental plants) can be relied on for making a decision on market authorisation.
- There must be a requirement to provide reliable methods allowing independent scientists to determine the expression of all newly produced substances in the plants such as enzymes or Bt toxins. The variations in the expression of the substances must be examined under defined stress conditions.
- If no new substances are produced in the plants, but the plants natural composition is changed by, for example, RNAi, the changes in the concentration also have to be examined under defined stress conditions.

Second stage of risk assessment:

- Independently of the outcome of the first stage of risk assessment, further investigations must be performed to assess the impact of the consumption of the whole food and feed on human and animal health. These investigations should also include potential impacts on the immune system and reproduction.
- Long-term effects have to be given priority. To assess these effects, feeding trials must take into account the whole lifetime of the animals, including their offspring.
- Data generated from feeding trials should include metabolomic data of the animals.

- The potential impacts on the microbiome of humans and animals must be added in at this stage.
- Accumulated effects, which, for example, can result from mixing genetically engineered plants in food and feed have to be assessed in detail.
- Residues from spraying with complementary herbicides have to be assessed in detail. Not only the active ingredients, but also the formulations of the herbicides used for their cultivation have to be taken into account, in addition to the combinatorial effects if more than one complementary herbicide can be applied.
- The toxicity of each of the Bt toxins produced in the plants must be assessed in detail, focussing, in particular, on the combinatorial effects with other substances (especially with stress factors) and the impact on the immune system. Simple assumptions derived from the mode of action of naturally occurring Bt toxins are not sufficient.
- Special attention has to be given to the empirical investigation of combinatorial effects emerging in stacked events.

Further recommendations :

- For each step of the risk assessment, independent control is absolutely essential during data generation.
- Cut-off criteria such as a prohibition of market authorisation for genetically engineered organisms able to spread into native populations, have to be established.
- If new relevant methods for data generation or data assessment become available or new risk-related questions do emerge, these must be included within a short period of time. In this case, the events that have already been granted authorisation will have be to be re-assessed without delay.
- Specific guidance needs to be established for genetically engineered plants that are changed in their nutritional composition, in particular, taking into account the long-term effects from consumption.
- Any decision on market authorisation must prioritise the precautionary principle, and reflect the limits of current knowledge and resulting uncertainties.

Literature

Batista, R., Saibo, N., Lourenco, T., Oliveira, M. (2008) Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. PNAS 105 (9): 3640-3645.

Bauer-Panskus, A., Breckling, B., Hamberger, S., Then, C. (2013) Cultivation-independent establishment of genetically engineered plants in natural populations: current evidence and implications for EU regulation. Environmental Sciences Europe 2013, 25: 34. http://www.enveurope.com/content/25/1/34

Bauer-Panskus, A. & Then, C. (2015) The impact of industry on publicly-funded risk research projects on genetically engineered plants, www.testbiotech.org/node/1129

Baier, S.R., Nguyen, C., Xie, F., Wood, J.R. Zempleni, J. (2014) MicroRNAs Are Absorbed in Biologically Meaningful Amounts from Nutritionally Relevant Doses of Cow Milk and Affect Gene Expression in Peripheral Blood Mononuclear Cells, HEK-293 Kidney Cell Cultures, and Mouse Livers. The Journal of nutrition, 144(10): 1495-1500.

Beatty, M., Guduric-Fuchs, J., Brown, E., Bridgett, S., Chakravarthy, U., Hogg, R.E., et al. (2014) Small RNAs from plants, bacteria and fungi within the order hypocreales are ubiquitous in human. Plasma, 15: 1–12. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4230795/

Boeschen, S., Kastenhofer, K., Marschall, L., Rust,I., Soentgen, J., Wehling, P., (2006) Scientific Cultures of Non-Knowledge in the Controversy over Genetically Modified Organisms (GMO) The Cases of Molecular Biology and Ecology, GAIA 15/4: 294 – 301.

Bøhn, T., Rover, C.M., Semenchuk, P.R. (2016) Daphnia magna negatively affected by chronic exposure to purified Cry-toxins, Food and Chemical Toxicology, 91: 130-140.

Bondzio, A., Lodemann, U., Weise, C., Einspanier, R. (2013) Cry1Ab treatment has no effects on viability of cultured porcine intestinal cells, but triggers hsp70 expression. Plos One, 8(7): e67079.

Carlisle, S.M. & Trevors, J.T. (1988) Glyphosate in the environment. Water, Air, and Soil Pollution, 39(3-4): 409-420.

Cavalieri, D., Rizzetto, L., Tocci, N., Rivero, D., Asquini, E., Si-Ammour, A., Bonechi, E., Ballerini, C., Viola, R. (2016) Plant microRNAs as novel immunomodulatory agents. Scientific Reports, 6: 25761. www.nature.com/scientificreports/

Chen, X., Zen, K., Zhang, C.Y. (2013) Reply to Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nature Biotechnology, 31(11): 967-969. www.nature.com/nbt/journal/v31/n11/full/nbt.2741.html

Chowdhury, E. H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K. S., Saito, M., Nakajima, Y. (2003) Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. J. Anim. Sci., 81: 2546-2551.

EFSA (2007), Guidance Document of the Scientific Panel on Genetically Modified Organisms for the risk assessment of genetically modified plants containing stacked transformation events. The EFSA Journal 512, 1-5.

EFSA (2010) Guidance on the environmental risk assessment of GM plants. EFSA Journal 2010; 8(11): 1879.

EFSA (2011) Guidance for risk assessment of food and feed from GM plants. EFSA Journal 2011; 9(5): 2150.

EFSA (2012) Scientific Opinion on an application (EFSA-GMO-NL-2005-24) for the placing on the market of the herbicide tolerant genetically modified soybean 40-3-2 for cultivation under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 2012; 10(6): 2753.

EFSA (2015a) Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA Journal 2015; 13 (11): 4302.

EFSA (2015 b) Statement of EFSA on the request for the evaluation of the toxicological assessment of the co-formulant POE-tallowamine. EFSA Journal 2015; 13(11): 4303.

EFSA (2016) Conclusion on the peer review of the pesticide risk assessment of the active substance isoxaflutole. EFSA Journal 2016; 14(2): 4416.

Forlani, G., Kafarski P., Lejczak, B., Wieczorek, P. (1997) Mode of Action of Herbicidal Derivatives of Aminomethylenebisphosphonic Acid. Part II. Reversal of Herbicidal Action by Aromatic Amino Acids J Plant Growth Regul 16: 147–152.

Gaines, T.A., Zhang, W., Wang, D., Bukun, B., Chisholm, S.T., Shaner, D.L., Nissen, S.J., Patzoldt,W.L., Tranel, P.J., Culpepper, A.S., Grey, T.L., Webster, T.M., Vencill, W.K., Sammons, R.D., Jiang, J., Preston, C., Leach, J.E., Westra. P. (2009) Gene amplification confers glyphosate resistance in Amaranthus palmeri. PNAS, 107: 1029–1034.

GeneWatch UK & TestBiotech (2015) Request for a review of the authorisations for GM crops with altered oil content, www.testbiotech.org/node/1284

Gertz, J.M., Vencill, W.K., Hill, N.S. (1999) Tolerance of Transgenic Soybean (Glycine max) to Heat Stress. British Crop Protection Conference – Weeds, 15-19 Nov 1999,. Brighton: 835-840.

Hilbeck, A. & Otto, M. (2015) Specificity and Combinatorial Effects of *Bacillus thuringiensis* Cry Toxins in the Context of GMO Environmental Risk Assessment. Frontiers in Environmental Science, 3, 71.

Hirschi, K. D., Pruss, G. J. & Vance, V. (2015) Dietary delivery: a new avenue for microRNA therapeutics?. Trends Biotechnol. 33: 431–432.

Huffmann, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, G., Aroian, R.V. (2004) Mitogenactivated protein kinase pathways defend against bacterial pore-forming toxins: PNAS, 101: 10995-11000.

IARC (2015) Glyphosate Monograph, http://monographs.iarc.fr/ENG/Monographs/vol112/mono112-02.pdf

Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., Ohba, M. (2004) *Bacillus thuringiensis* crystal protein with selective cytocidal action on human cells. J Biol. Chem, 279: 21282-21286.

Jiao, Z., Si X.X., Li, G.K., Zhang, Z.M., Xu X.P. (2010) Unintended Compositional Changes in Transgenic Rice Seeds (Oryza sativa L.) Studied by Spectral and Chromatographic Analysis Coupled with Chemometrics Methods. J. Agric. Food Chem., 58: 1746–1754.

Kramarz, P.E., Vaufleury, A., Zygmunt, P.M.S, Verdun, C. (2007) Increased response to cadmium and *Bacillus thuringiensis* maize toxicity in the snail Helix aspersa infected by the nematode Phasmarhabditis hermaphrodita. Environ. Toxicol. Chem., 26(1):73–79.

Kok, E.J., Kuiper, H.A. (2003) Comparative safety assessment for biotech crops, Trends in Biotechnology, 21: 439–444.

Liang, H., Zhang, S., Fu, Z., Wang, Y., Wang, N., Liu, Y., ... & Chen, X. (2015) Effective detection and quantification of dietetically absorbed plant microRNAs in human plasma. The Journal of nutritional biochemistry, 26(5): 505-512. www.sciencedirect.com/science/article/pii/S0955286315000169

Matthews, D., Jones, H., Gans, P., Coates, St. & Smith, LMJ (2005) Toxic secondary metabolite production in genetically modified potatoes in response to stress. Journal of Agricultural and Food Chemistry, 53(20): 7766-7776.

Meyer, P., Linn, F., Heidann, I., Meyer, H., Niedenhof, I., Saedler, H. (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. Mol. Gen. Genet., 231: 345-352.

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.-E. (2012a) Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide, Journal of Applied Toxicology, 33(7): 695-699. http://onlinelibrary.wiley.com/doi/10.1002/jat.2712/abstract

Pardo-López, L., Muñoz-Garay, C., Porta, H., Rodríguez-Almazán, C., Soberón, M., Bravo, A (2009) Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*. Peptides, 30(3): 589–595.

Pigott, C.R. & Ellar, D.J. (2007) Role of Receptors in *Bacillus thuringiensis* Crystal Toxin Activity. Microbiol Mol Biol Rev, 71 (2): 255–281.

Rubio-Infante, N. & Moreno-Fierros, L. (2015) An overview of the safety and biological effects of *Bacillus thuringiensis* Cry toxins in mammals, J. Appl. Toxicol., 36(5): 630–648.

Saeglitz, C., Bartsch, D., Eber, A., Gathmann, K., Priesnitz, K.U., Schuphan, I. (2006) Monitoring the Cry1Ab Susceptibility of European Corn Borer in Germany, J. Econ. Entomol., 99(5): 1768-1773.

Sammons, R.D., & Gaines, T.A. (2014) Glyphosate resistance: State of knowledge. Pest Management Science, 70, (9): 1367–1377.

Shimada, N., Kim, Y.S., Miyamoto, K., Yoshioka, M., Murata, H. (2003) Effects of *Bacillus thuringiensis* Cry1Ab toxin on mammalian cells. J Vet Med Sci, 65: 187-191.

Székács, A., Weiss G., Quist, D., Takács, E., Darvas, B., Meier, M., Swain, T., Hilbeck, A. (2011) Inter-laboratory comparison of Cry1Ab toxin quantification in MON 810 maize by ezyme-immunoassay. Food and Agricultural Immunology, 23(2): 99-121.

Testbiotech (2012) Technical background for a complaint under Article 10 of Regulation (EC) No. 1367/2006 against the decision of the EU Commission to give market authorisation to stacked soy MON87701 x MON89788, www.testbiotech.de/node/691

Testbiotech (2014) Technical background for a complaint under Article 10 of Regulation (EC) No. 1367/2006 against the decision of the EU Commission to give market authorisation to stacked maize MON89034 × 1507 × MON88017 × 59122 (SmartStax), www.testbiotech.org/sites/default/files/Testbiotech_Complaint_SmartStax_a.pdf

Then, C. (2010) Risk assessment of toxins derived from *Bacillus thuringiensis* - synergism, efficacy, and selectivity. Environ Sci Pollut Res Int, 17(3): 791-797.

Then, C. & Bauer-Panskus A (2010) European Food Safety Authority: A playing field for the biotech industry, www.testbiotech.de/en/node/431

Then, C. & Lorch, A. (2008) A simple question in a complex environment: How much Bt toxin do genetically engineered MON810 maize plants actually produce? In: Breckling, B., Reuter, H. &Verhoeven, R. (eds), 2008, Implications of GM-Crop Cultivation at Large Spatial Scales, Theorie in der Ökologie 14. Frankfurt, Peter Lang, http://www.mapserver.uni-vechta.de/generisk/gmls2008/index.php?proceedings=ja&call=ja

Thomas, W.E. & Ellar, D.J. (1983) *Bacillus thuringiensis* var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. Journal of Cell Science, 60(1): 181–197.

Trtikova, M., Wikmark, O.G., Zemp, N., Widmer, A., Hilbeck, A. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions, PLoS ONE 10(4): e0123011. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0123011

Walsh, M.C., Buzoianu, S.G., Gardiner G.E., Rea M.C., Gelencser, E., Janosi, A., Epstein, M.M., Ross, R.P., Lawlor, P.G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS One 6(11): e27177.

Wang K., Li H., Yuan Y., Etheridge A., Zhou Y., Huang D., et al. . (2012) The complex exogenous RNA spectra in human plasma: an interface with human gut biota? PloS one, 7(12): e51009.

Yang, J., Farmer, L. M., Agyekum, A.A.A. Hirschi, K.D. (2015) Detection of dietary plant-based small RNAs in animals. Cell Res. 25, 517–520.

Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J., Bian, Z., Liang, X., Cai, X., Yin, Y., Wang, C., Zhang, T., Zhu, D., Zhang, D., Xu, J., Chen, Qu., Ba, Y., Liu, J., Wang, Q., Chen, J., Wang, J., Wang, M., Zhang, Q., Zhang, J., Zen, K., Zhang, C.Y. (2012) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Research, 22(1): 107-126.

Zeller, S.L., Kalininal, O., Brunner, S., Keller, B., Schmid, B. (2010) Transgene x Environment Interactions in Genetically Modified Wheat. PLoS One, 5(7): e11405. http://www.plosone.org/article/info:doi/10.1371/journal.pone.0011405

Zhou, Z., Li, X., Liu, J., Dong, L., Chen, Q., Liu, J., ... & Zhang, L. (2015) Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. Cell research, 25: 39–49.

Zotti, M.J. & Smagghe, G. (2015) RNAi Technology for Insect Management and Protection of Beneficial Insects from Diseases: Lessons, Challenges and Risk Assessments. Neotropical Entomology, 44(3): 197-213.