Vår ref:2016/H_131
Deres ref: 2016/6108

Miljødirektoratet
Postboks 5672 Sluppen
7485 Trondheim
Dato: 26.08.16

Vedlagt er innspill fra GenØk – Senter for Biosikkerhet på høringen av søknad **EFSA/GMO/NL/2016/131**, genmodifisert mais **MON87427 x MON89034 x MIR162 x NK603**, fra Monsanto Company (Europe S.A) under EU forordning 1829/2003. Søknaden gjelder bruksområdene mat, fòr, import og prosessering.

Vennligst ta kontakt hvis det er noen spørsmål.

Med vennlig hilsen,

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Vår ref:2016/H_131
Deres ref: 2016/6108

Assessment of the technical dossier submitted under EFSA/GMO/NL/2016/131 for approval of MON87427 x MON89034 x MIR162 x NK603 maize

Sent to

Norwegian Environment Agency

by

GenØk- Centre for Biosafety
August 2016

ANBEFALING

GenØk–Senter for Biosikkerhet, viser til høring gjeldende for **MON87427 x MON89034 x MIR162 x NK603 mais** som omfatter bruksområdet import og prosessering og til bruk i fòr og mat eller inneholdende ingredienser produsert fra **MON87427 x MON89034 x MIR162 x NK603 mais**.

Søker har ikke inkludert den informasjonen som kreves for godkjenning av **MON87427 x MON89034 x MIR162 x NK603** iht til den norske genteknologiloven (NGTA), spesielt omkring samfunnsnytte og bærekraftighet av **MON87427 x MON89034 x MIR162 x NK603** (Appendix 4) for godkjenning i Norge. I denne sammenheng er det viktig å få dokumentert erfaringer med hensyn på effekter på miljø, helse og samfunnsaspekter. Denne type dokumentasjon er ikke tilstrekkelig i søknaden om omsetting av **MON87427 x MON89034 x MIR162 x NK603 mais** til import og prosessering og til bruk i fòr og mat eller inneholdende ingredienser produsert fra **MON87427 x MON89034 x MIR162 x NK603 mais.**

Vår anbefaling er at norske myndigheter ikke godkjenner bruk av **MON87427 x MON89034 x MIR162 x NK603 mais** til import og prosessering og til bruk i fòr og mat som det søkes om.

ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/NL/2016/131

GenØk, as a National Competence Center for Biosafety, aims at providing independent, holistic and useful analysis of technical and scientific information/reasoning in order to assist authorities in the safety evaluation of biotechnologies proposed for use in the public sphere.

The information in this assessment is respectfully submitted for consideration in the evaluation of product safety and corresponding impact assessment of event **MON87427 x MON89034 x MIR162 x NK603 maize**, setting out the risk of adverse effects on the environment and health, including other consequences of proposed release under the pertinent Norwegian regulations.

Specific recommendations

GenØk has previously assessed sub-combinations of the single events present in MON87427 x MON89304 x MIR162 x NK603. Below are recommendations from these, which we propose for the current application:

- The regulator is encouraged to ask the applicant to address the potential of non-target effects of Bt toxins, especially in the context of their combined use in a stacked event.
- The regulator is encouraged to ask the Applicant to consider the possibility of cross-resistance development to multiple Cry proteins due to the use of stacked events.
- The applicant should include a full evaluation of the co-technology intended to be used with MON87427 x MON89034 x MIR162 x NK603, namely glyphosate-based herbicide. Particular focus should be given to the level of accumulation of herbicides in the plants, particularly the parts used in food and feed production, and whether or not these levels of exposure could cause acute and/or chronic health issues. This needs to be tested in animal and feeding studies, separating the effects of the plant and the herbicide(s) by using both sprayed and unsprayed plant samples.
- The regulators are encouraged to ask the applicant to provide a full environmental risk assessment (ERA) of the life cycle of MON87427 x MON89034 x MIR162 x NK603 from being planted in the field and through the cultivation process, harvesting, transportation, processing, and as waste. Specifically, more information on risk management with regards to gene flow and herbicide regime should be included in the ERA (even when the application does not include cultivation).
- The regulator is encouraged to ask the applicant to demonstrate the lack of interactive effects between transgenic proteins through proper scientific testing and evidence gathering, rather than justify the lack of testing based on assumptions-based reasoning of no effects.
- It is unclear if the toxicity studies are performed on plant or bacterially derived transgenic proteins. We suggest that the Applicant perform toxicity studies with plant derived proteins from the stack the Applicant applies authorization for here.
- We encourage the Applicant to analyze proteins isolated from the stacked event to investigate proteins as they are expressed in the plant, and not base safety assessments on data from single events and stacks where proteins are expressed in another context.
- In order to meet the requirements for the NGTA, the regulator is encouraged to ask the Applicant to submit information relevant for the assessment of the social utility of MON87427 x MON89034 x MIR162 x NK603 and its contribution to sustainable development. The information provided by the Applicant must be relevant for the agricultural context in the producing country/countries. The information should include issues such as: Changes in pesticide use, emergence of herbicide resistant weeds, development of pest resistance in target populations, impacts on non-target organisms, potential for gene flow and possible impacts among poor and/or small-scale farmers in producing countries and share of the benefits among sectors of the society.

In addition, the following recommendations are proposed, based on our findings in the present application, summarized here and detailed in the critique below:

- The Applicant is encouraged to consider the complexity of a stacked transgenic plant in comparison to single events and evaluate potential safety issues based on that.
- We encourage the Applicant to specify more clearly, if the proteins characterized are from the stack or if the statements are based on previous analysis on single events.
- We strongly encourage the Applicant to analyze the proteins in the stack for homology to known toxins or anti – nutrients and not make assumptions based on data from analysis of previously assessed single events (constituting the stack in question).
- We encourage the Applicant to clearify if proteins analysed are from the maize stack or if the data are from the previous analysis of the single events only.
- We encourage the Applicant to clarify which pH levels stability analysis have been performed at. A broad pH range will better mimic the situation in the gastric system.
- We recommend the Applicant to perform 28 day oral toxicity analysis of the proteins isolated from the multi stack, as no analysis have been performed on the newly expressed proteins, only on proteins isolated from single events in parental lines, in previous analysis and also that the proteins in the stack has no history of safe use as they are expressed there, as this is a new combination of traits.
- We encourage the Applicant to perform a 90 day feeding study as the combined expression of traits in this multistack might be potentially distinct from each of the traits being expressed alone and also as no safety data is presented from the proteins isolated from the multistacks and the combined expression of these.
- We recommend the Applicant to consider performing the analysis for allergenicity on proteins as they are expressed in the multistack and not base assumptions on data from the single parental events alone.

Overall recommendation

In our assessment of maize event **MON87427 x MON89034 x MIR162 x NK603**, we find that the information provided in the technical dossier does not provide enough data to support claims of safe use, social utility and sustainable development.

A new application or reapplication should only be reconsidered with the delivery of the information requests recommended here, including any additional information deemed significant by the Norwegian authorities.

We therefore comment that the Applicant has not provided the information required under Norwegian law to warrant approval in Norway at this time.

Especially, the Applicant has not included information that is required to assess social utility and sustainability as required by the Norwegian Gene Technology Act (Appendix 4) for consideration of approval in Norway.

ASSESSMENT OF THE TECHNICAL DOSSIER RELATED TO EFSA/GMO/NL/2016/131

About the event

The **MON87427 x MON89034 x MIR162 x NK603** maize is a GM maize that is produced by crossing maize plants containing single events MON87427, MON89034, MIR162 and NK603 using traditional breeding. *Agrobacterium tumefaciens* mediated transformation methods were used for each of the single events, except NK603 that was developed through particle acceleration method.

This stacked maize event contains two CP4 EPSPS proteins (CP4 EPSPS and CP4 EPSPS L214P) that confers tolerance to glyphosate containing herbicides. It also contains proteins aiming at protecting the plants against damage caused by lepidopteran insects, namely the Cry1A.105, Cry2Ab2 and Vip3Aa20 proteins.

In addition, a phosphomannose isomerase (PMI) protein is used as a selection marker during transformation.

The Norwegian Scientific Committee for Food safety has previously concluded that the stack MON89034 x NK603 is compositionally, nutritionally, agronomically and phenotypically equivalent to its conventional counterpart (VKM report 2016:17). They also state that it is unlikely that the proteins expressed will increase the risk of allergenic reactions to food or feed based on this stack.

We have some assessment findings for the current application that we suggest must be considered for the further evaluation of the stack MON87427 x MON89034 x MIR162 x NK603.

ASSESSMENT FINDINGS

Stacked events

A stacked organism has to be regarded as a new event, even if no new modifications have been introduced. The gene-cassette combination is new and only minor conclusions could be drawn from the assessment of the parental lines, since unexpected effects (e.g. synergistic effects of the newly introduced proteins) cannot automatically be excluded. Stacked events are in general more complex, and it has been an increased interest in the possible combinatorial and/or synergistic effects that may produce unintended and undesirable changes in the plant – like the potential for up- and down regulation of the plants own genes. Interactions within stacked traits cannot be excluded and whether or not the expressed proteins in the plant can give specific immunological effects or adjuvant effects in mammals has been discussed previously (Halpin 2005, de Schrijver et al, 2006).

The stack MON87427 x MON89034 x MIR162 x NK603 maize combines several Bt proteins active against Lepidopteran insects pests. It is well known that synergistic and additive effects

both between Bt toxins and other compounds do occur (Then, 2009). Then (2009) reviews and discusses the evidence for changes in activity and specificity of Bt proteins dependent on synergistic interactions with extrinsic features. Such changes may critically influence the bioactivity and hence the potential for unintended effects and must be carefully considered in the development and risk assessments of stacked events. Robust data are necessary to identify whether the combined presence of transgenes influences expression levels.

Vegetative insecticidal proteins (Vip)
VIP is one of a number of extracellular compounds, in addition to crystal-associated toxin polypetides, that may contribute to the virulence of *B. thurungensis* (Liu et al 2007). These proteins have shown to have a broad insecticidal spectrum, which includes activity against a wide variety of lepidopteran as well as coleopteran pests and they may represent a new generation of insecticidal toxins that could be efficacious against insects that are resistant to Cry toxins (Asokan et al 2012, Mahon et al 2012). In that regard, one strategy involves the presentation of several toxins together, especially if a differing mode of action involving different receptors is available (Mesrati et al 2005).

In this stack, there are two Cry proteins and one VIP protein. The VIP and Cry proteins seem to have the same target species. Although the VIPs may have different mode of action dependent on the target (Li et al 2004). However, special concern or vigilance should be paid to GM stacks that combine events that have similar type of mode of action through their expressed transgenic proteins. Also, the Cry proteins can attach to the same receptor, changing their mode of action. In theory, the presence of two toxins can result in cross resistance and a changed effect on target and also non-target species (Schnepf et al 1998, Hua et al 2001, Estela et al 2004, Li et al 2004). Especially, an overall toxicity study of the GM stacked event should have been considered, but the applicant state that the 28 day toxicity study is not needed due to previous history on safe use of the of the proteins in the single events (Technical Dossier, p 53). For the VIP proteins, MIR 162 has previously been assessed expressing the Vip3Aa20 protein. Previous evaluations of this protein have especially noted the potential cross binding to receptors in the epithelial cells of the gut between Cry and VIP proteins. As this receptor has not been characterised, the similarity to human gut receptors cannot be clarified and should thus be further analysed. This is however not mentioned in this application as potential.

MON87427 x MON89034 x MIR 162 x NK603 maize combines two Bt proteins named Cry1A.105 and Cry2Ab2. These proteins, also called Bt-toxins are claimed to be safe, however the potential of non-target effects of Bt toxins, including alternative modes of action for Cry toxins have been addressed previously (Bøhn et al 2008, Gilliand et al 2002, Crickmore 2005, Hilbeck and Schmidt 2006).
Two meta-analyses of published studies on non-target effects of Bt-proteins in insects, (Lövei and Arpaia 2005) in relation to non-target and environmental effects, documented that 30% of studies on predators and 57% of studies on parasitoids display negative effects to Cry1Ab transgenic insecticidal proteins. Further, Cry toxins and proteinase inhibitors have often non-neutral effects on natural enemies, and more often negative than positive effects (Lövei et al 2009). A review by Hilbeck and Schmidt (2006) on Bt-plants, found 50% of the studies documenting negative effects on tested invertebrates.
Additionally, a recent review by van Frankenhuyzen (2013) indicated that several Cry proteins exhibit activity outside of their target orders. This study also found that many Cry proteins only

had been tested with a very limited number of organisms: thus, activity outside of the target organisms of many Cry proteins may be undocumented simply because testing has not included sensitive organisms (van Frankenhuyzen, 2013). As not every potentially sensitive species can be tested for sensitivity to Bt toxins, it cannot be excluded that sensitive species have been overlooked in testing until now. The issue is complicated further by the number of variables which can affect toxicity testing, which may include toxin preparation and purification, life stage of the specimens, differences in toxin expression hosts, as well as solubilization (or lack thereof) of the toxin, among other factors (van Frankenhuyzen 2009).

A quantitative review analysis based on 42 field experiments showed that unsprayed fields of Bt-transgenic maize plants have significantly higher abundance of terrestrial non-target invertebrates than sprayed conventional fields (Marvier et al. 2007). Thus, Bt-plants with a single Bt-gene inserted may represent an improvement for non-target organisms in the environment. However, an indication of some negative effects of the Cry1Ab toxin itself, or the Cry1Ab maize plant, on non-target abundance was shown in the same meta-analysis: when conventional (non-GM) fields were not sprayed, the non-target abundance was significantly higher than in the Bt-fields (Marvier et al. 2007).

Research on aquatic environments with emphasis on the impact of Bt-crops on aquatic invertebrates including *Daphnia magna* (Bøhn et al 2008) and caddisflies (Rosi-Marshall et al 2007) has also been performed. Given the potential load of Cry toxins (also in combination with herbicides) that may end up in aquatic environments, further studies are warranted. Douville et al. (2007) presented evidence of the persistence of the *cry1Ab* transgene in aquatic environments: more than 21 days in surface waters, and 40 days in sediments. A follow-up on this study in 2009 indicated possible horizontal gene transfer of transgenic DNA fragments to aquatic bacteria (Douville et al 2009). Impacts on soil microflora and fauna, including earthworms (Zwahlen et al. 2003), mychorizzal fungi (Castaldini et al. 2005) and microarthropods in response to Cry endotoxins have also been reported (Wandeler et al 2002, Griffiths et al 2006, Cortet et al 2007). The significance of tri-trophic effects of accumulation, particularly of insecticidal Cry toxins (Harwood et al. 2006, Obrist et al. 2006) is, however, yet to be firmly established. It has been demonstrated that sub-chronic dosages of Cry proteins may affect both foraging behavior and learning ability in non-target bees (Ramirez-Romero et al 2008), and may have indirect effects on recipient populations, and, given the key-stone role of bees as pollinators, on both primary production and on entire food-webs.

The use of multiple, related transgenes in a single (stacked) event may accelerate resistance development to both transgene products. This was the experience of Baxter et al (2005), who tested the effect of using broccoli plants containing Cry1Ac, Cry1C or both, on resistance development in a population of diamondback moths (*Plutella xylostella*). They found that the stacked use of similar Cry proteins in close proximity to single gene events led to accelerated resistance development to both traits (Baxter et al 2005). Bravo and Soberón (2008) commented on this effect, acknowledging that gene stacking is not a universal solution to resistance development towards Cry proteins. Studies such as these ask the question as to whether the stacked use of related Cry proteins, such as Cry1Ab and eCry3.1Ab, in the same event is advisable.

In relation to health impacts, a publication by (Dona and Arvanitoyannis 2009) reviews the potential health implications of GM foods for humans and animals, including incidences and effects of increased immunogenicity, amounts of anti-nutrients, possible pleiotropic and epigenetic effects, including possible reproductive and developmental toxicity. They conclude

that while there is strong evidence for health concerns on many fronts, exposure duration many have not been long enough to uncover important effects.
A recent study in mice showed that exposure to purified Cry1Ab resulted in specific anti-Cry1Ab IgG1 and IgE production, indicating inherent immunogenicity and allergenicity. Further, mice exposed to leaf extracts from both MON810 and unmodified maize demonstrated influx of lymphocytes and eosinophils in the broncho-alveolar lavage,and increased cytokine release in mediastinal lymph node cells (Andreassen et al 2015). Further studies should also include animals with immune-deficiencies and/or animals exposed to other stress agents simultaneously.

Adjuvancy effects
The potential adjuvancy of Cry proteins has previously been addressed by the GMO Panel of the Norwegian Scientific Committee for Food Safety (VKM, 2012). Also, scientific studies have shown that the Cry1Ac protein is a potent systemic and mucosal adjuvant (Moreno-Fierros et al, 2003). In the evaluation of another GM maize, MIR604 x GA21, the panel found that it was difficult to evaluate if kernels from this stack would cause more allergenic reactions than kernels from unmodified maize. The Panel continues with: "*As the different Cry proteins are closely related, and in view of the experimental studies in mice, the GMO Panel finds that the likelihood of an increase in allergenic activity due to Cry1Ab and mCry3A proteins in food and feed from maize Bt11 x MIR604 x GA21 cannot be excluded. Thus, the Panel's view is that as long as the putative adjuvant effect of Cry1Ab and mCry3A with reasonable certainty cannot be excluded, the applicant must comment upon the mouse studies showing humoral antibody response of Cry1A proteins and relate this to a possible adjuvant effect of the Cry1Ab and mCry3A proteins expressed. Furthermore, although Cry1Ab and mCry3A proteins are rapidly degraded in gastric fluid after oral uptake, there is also the possibility that the protein can enter the respiratory tract after exposure to e.g. mill dust. Finally, rapid degradation is no absolute guarantee against allergenicity or adjuvanticity" (Norwegian Scientific comitee for Food Safety (2013), Evaluation of EFSA/GMO/UK/2007/48).*

We also agree with these concerns and highlight them for the present stack of maize and that this potentially also might be the case for the proteins expressed in MON87427 x MON89034 x MIR162 x NK603 maize.

Recommendation:
- The regulator is encouraged to ask the applicant to address the potential of non-target effects of Bt toxins, especially in the context of their combined use in a stacked event.
- The regulator is encouraged to ask the Applicant to consider the possibility of cross-resistance development to multiple Cry proteins due to the use of stacked events.

Herbicides
The stacked maize event MON87427 x MON89034 x MIR132 x NK603 contains several CP4 EPSPS genes providing herbicide tolerance.

Herbicides as co-products
Herbicide tolerant (HT) plants are specifically designed to be used in combination with herbicides, and will always be sprayed with the intended herbicide. Without spraying, the

introduction of HT plants would be useless. Surprisingly, these herbicides are often not tested as part of the assessment and risk evaluation of HT plants. In feeding studies with HT GM plants for quality assessment the herbicide is systematically overlooked, which represents a serious flaw in the testing and risk evaluation. Viljoen et al. (2013) found that in 13 out of 16 published feeding studies with HT GM crops the plant material used had not been sprayed with the intended co-technology herbicide. There is also a gap in knowledge regarding herbicide accumulation and residues, including metabolic pathways and metabolites thereof. Bøhn et al. (2014) documented high levels of glyphosate residues in HT GM soybeans grown in the USA, and the same research group have published papers showing that such residues negatively affect the feed quality of HT GM soybeans (Cuhra et al., 2015). Moreover, safety testing (in relation to health and environmental issues) has focused on the active ingredient in the co-technology herbicides, and not the commercial formulations actually used, providing unrealistic and possibly misleading results (Mesnage et al., 2014). Stacked HT GM plants are tolerant to one or more agrochemicals, allowing for combinatory and alternating use of several herbicides. Tolerance to multiple herbicides is also often combined with multiple other proteins (like Cry toxins) that could have additive or even synergistic effects on non-target species and the environment.

In the toxicology assessment of a gene modified plant used for food or feed the focus is mostly on the resulting protein from the inserted gene, and the potential of herbicide exposure through consumption of herbicide treated maize is not considered. A recent study found that glyphosate and AMPA, constituents of the herbicide Roundup accumulated in soybeans (Bøhn et al., 2014), highlighting the importance of including the herbicides in the comparative and toxicological assessment of GM crops with herbicidal co-technology.

Glyphosate tolerance

The *cp4 epsps* and *cp4 epsps L214P* genes present in MON87427 x MON89034 x MIR162 x NK603 maize confers tolerance to herbicide products containing glyphosate.

Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvoyl-shikimate-3-phosphate synthase (EPSPS), necessary for production of important amino acids. Some microorganisms have a version of EPSPS that is resistant to glyphosate inhibition.

Glyphosate has been announced as an ideal herbicide with low toxicity for operators, consumers and the environment surrounding agriculture fields (Duke and Powles 2008, Giesy et al 2000). However, it has received more risk-related attention due to its potential for negative effects on both aquatic and terrestrial ecosystems (Blackburn and Boutin 2003,Solomon and Thompson 2003), as well as in studies in animals and cell cultures that have indicated possible negative health effects in rodents, fish and humans (Axelrad et al 2003, Dallegrave et al 2003, Benachour et al 2007).

Recent studies indicate that agriculture of GM plants is associated with greater overall usage of pesticides than the conventional agriculture (Benbrook 2009). Large proportions of the GM agricultural system is crops that is tolerant to glyphosate (GT-cultivars) (James 2010).

A restricted number of recent publications indicate unwanted effects of glyphosate on health (Dallegrave et al 2003, Malatesta et al 2002), aquatic (Solomon and Thompson 2003) and terrestric (Ono et al 2002, Blackburn and Boutin 2003) organisms and ecosystems.
A study of Roundup effects on the first cell divisions of sea urchins (Marc et al 2002) is of particular interest to human health. The experiments demonstrated cell division dysfunctions at the level of CDK1/Cyclin B activation. Considering the universality among species of the CDK1/Cyclin B cell regulator, these results question the safety of glyphosate and Roundup on human health. In another study (Axelrad et al 2003) it was demonstrated a negative effect of glyphosate, as well as a number of other organophosphate pesticides, on nerve-cell differentiation. Surprisingly, in human placental cells, Roundup is always more toxic than its active ingredient. The effects of glyphosate and Roundup were tested at lower non-toxic concentrations on aromatase, the enzyme responsible for estrogen synthesis (Richard et al, 2005). The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation. The authors conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. They suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

Additionally, the International Agency for Research on Cancer (IARC) recently released a report concluding that glyphosate is “probably carcinogenic to humans” (Fritschi et al., 2015).

Recommendation:

- Maize event MON87427 x MON89034 x MIR162 x NK603 should be checked for level of accumulation (level of heribicide residues) of glyphosate prior to use in food and feed.

INFORMATION RELEVANT FOR THE GENETIC MODIFICATION

1.2. Molecular characterization

1.2.1 .Information relating to the genetic modification.

The technical dossier does not provide enough data to support claims of safe use of the maize stack MON87427 x MON89034 x MIR162 x NK603, but is based on the previous assumptions that the single parental events are safe. However the combination of the transgenes from the single events might potentially behave completely different in the stacked maize plant on a molecular level in real life, than the theoretical addition of the characteristics (see p.8 for further elaboration on this).
For example it is mentioned that the open reading frames that are created as a result of the genetic modification either at the junction sites with genomic DNA or due to internal rearrangements, are previously evaluated to be safe for MON 87427, MON 89034, MIR162 and NK603. Could it be possible that the combination of all of them by conventional breading technics might lead to deletions and possible frameshift mutation? Conventional breeding in plant production occasionally generates foods with undesirable traits, and some of these could

potentially be hazardous to human health (Safety of Genetically Engineered Foods, Chapter 5), the likelihood of this happening when crossing four already genetically modified plants should therefore be seriously taken into account, and all molecular characterizations should be done on the specific stacked event.

Other points to consider from the present application:

- The applicant states that sequences of the inserts are commercially sensitive information, but the applicant also claims that "The DNA sequence of the MON87427, MON89034, MIR162 and NK603 inserts and flanking sequences in MON87427 x MON89034 x MIR162 x NK603 are 100% identical to DNA sequence determined for the respective single events…". This information as well as plasmids and or vectors used during the experiment should be provided.
- The Applicant was responsible for the development of the transgenic maize event MON89034. This was done by transformation of two T-DNAs. The one expressed Bt proteins Cry1A.105 and Cry2Ab2 and the other T-DNA contains the *npt II* gene encoding neomycin phosphotransferase II. There is a cause for concern for the possible potential of horizontal gene transfer of inserted fragments, if present.
- The Applicant states "…the probability of foreign DNA entering and recombining with the host DNA in humans and animals is negligible…", because there are various barriers that limit any exogenous DNA. There are indeed DNA degrading enzymes that are released by the pancreas in intestinal cells throughout the gastro intestinal tract (GIT), but it has also been shown that DNA can persist in the GIT (Jonas et al 2001, Kleter et al 2005). This indicates that free transgenic DNA could be available for uptake by intestinal bacteria. A study done by Netherwood et al (2004), demonstrated how the *cp4 epsps* gene survived in the small intestine of humans consuming a GM soy product. According to Kleter et al (2005) the most likely site for bacterial transformation is the colon, since DNA is less rapidly degraded and the colon contains the biggest fraction of bacteria within the GIT. Even though only a fraction of the consumed product will reach the colon, the possibility that the transgenic DNA could transform should be considered.
- The applicant states "…horizontal gene transfer from genetically enhanced plants to environmental micro-organisms is unlikely due to the overall complexity and probability of the process". According to Pontiroli et al (2009) the DNA from degrading plants can persist and remain biologically active for periods of time. Nutrients released during this process provide a copiotrophic[1] environment that encourages bacterial growth (Pontiroli et al 2009). According to Pontiroli et al (2009) transformation frequency may be less in nature than in situ laboratory conditions, but a single transformation event, depending on the transgene, could lead to a chain reaction of destructive outcomes. The likelihood of these events occurring in natural systems cannot be written off as negligible.
- The application in its whole is characterized by data from the single parental events previously applied to EFSA. Stacked events are considered as new GMOs and should be tested "where the combined expression of the newly introduced genes has unexpected effects on biochemical pathways. This assessment will clearly require a case-by-case approach" (EFSA, 2007). This means that one use methods for analyzes that provide detailed description of the genome of the new stack and expression of its genes.

[1] Copiotropic environment: rich in nutrients/organic matter

- In the present application, sequencing and bioinformatics analyzes are performed on the stack itself by the Applicant. Such analyzes should also be performed by independent laboratories to strengthen the data.
- The single event MIR162 has been sequenced by another laboratory (Syngenta). The origin of the material is however a bit unclear from the data presented in the Application. What generation(s) have been analysed? This is important to look at the stability of the insert over time.
- The raw data (electropherograms) from the sequencings performed should have been available to be able to say something about the quality of the analysis. In one of the references for analysis of MON87427 x MON89034 x NK603 it is written that " some of the data is rejected due to poor quality and irrelevance (Vest et al 2015). It would have been nice to see at what level these data have been rejected.
- The primer sequences should have been made available for MON87427 x MON89034 x NK603 (Vest et al 2015) as these have been made available for the single parental event MIR162.
- It is not clear from the Application if sequencing have been performed on more than one generation of the stacked event. As small rearrangements and deletions have been shown after multiple generations (de Shrijver et al 2007) these should be analysed further for analysis of stability of the insert.
- Southern blots have been used to verify presence and copy number of inserted genes in the parental single events. Southern blot is a rather "rough" and non-sophisticated method and newer methods should have been used in addition, especially since some of the single parental events have been assessed years ago. The inserts have been sequenced in the stack, but here southern blots have not been used in addition to verify further.
- We suggest a map of the genome showing where the different genes and their flanking sequence are present. Whole genome sequencing could be used here.
- In the Application, inserted genes and flanking sequences have been sequenced. It would have strengthened the analysis and assessment if data from larger areas of the genome also had been sequenced in order to discover potential insertions, deletions, point-mutations and rearrangements (Schnable et al 2009 and Zastrow-Hayes et al 2015).
- The use of more "up-to-date" techniques may help put to rest some of the residual uncertainties regarding the stability and placement of transgene within the crop genome.
- The applications have many assumption based statements, not necessarily based on scientific evidence; like
    - "*there is low likelihood of molecular interactions between the inserts….and therefore, low likelihood of any changes in the molecular characteristics of the inherited inserts…. (p.22).* It is unclear what this means, it refers to sequencing data, and is not discussed further. If it means that there is a low probability of interactions and unforeseen changes to occur but difficult to foresee the consequences, this is not mentioned in the assessment.
    - On page 33: "*There are no scientific basis to support the notion that these sequences would be intrinsically more unstable when combined together by..*" and "*There is no known mechanism by which two inserts at different locations on…*"…We comment that we do not know about the unstable areas and potentials for genetic rearrangements and this must be considered as knowledge gaps.

- On page 36: "*there is no evidence for interaction between the multiple copies of the cp4 epspe gene. The effect on protein expression levels from the presence of multiple gene copies is not completely understood.....* We comment that it should be the responsibility of the Applicant to analyze potential combinatorial and synergistic effects of the distinct inserted genes. Combinatorial effects in stacked plants can not be excluded, and the Applicant has not tried to show the opposite.
- We have previously written that there is a lack of precise methods to perform characterisations of the gene modified plants, as well as making them (GenØk report 2015/03). To use (and develop) techniques that are more in depth and more precise is necessary to get more information about:
  - The novelty of the introduced genes and their products within the context of the recipient organisms, and the potential effects (e.g. pleiotropic effects, interactions with endogenous proteins etc.) that may occur as a result of their introduction.
  - Knowledge regarding unintended changes (e.g. recombination, positional effects etc.) introduced into the genomes of recipient organisms due to genetic modification is sparse, and exacerbated by lack of access to test materials and sequence information.
  - The imprecision of both the methods used to perform genetic modification, as well the techniques used to characterize the resultant GM organisms.

Recommendation

- The Applicant is encouraged to consider the complexity of a stacked transgenic plant in comparison to single events and evaluate potential safety issues based on that.

1.2.2. Information relating to the genetically modified plant

1.2.2.3 Information on the expression of the inserted/modified sequences (p.51)

Expression analysis of CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI proteins was conducted on forage and grain samples collected from MON87427 x MON89034 x MIR162 x NK603 maize using ELISA (Enzyme-linked immune-sorbent assay) on various tissues (forage and grain). Plants were treated with glyphosate prior to protein isolation and analysis of expression.
The technical dossier does not state if the measured protein levels are as expected or not (one has to go into the reference to find that).
Also, there is no mentioning of analysis of interactive effects between transgenic proteins.

Recommendation:

- The Applicant should demonstrate the lack of interactive effects between transgenic proteins in this **stacked event** through proper scientific testing and evidence gathering, rather than justify the lack of testing based on assumptions-based reasoning of no effects.

1.4. Toxicological assessment (p.51)
Accordingly, the Codex (2009) assessment is utilized for evaluation of toxicity. Here the "weight of evidence" approach is used. These evidences include:

- Characterization of the newly expressed protein
- Bioinformatics sequence analysis to search for similarities to toxins, anti-nutrients, allergens, etc.
- Stability of protein *in vitro*
- Toxicity studies (acute oral in this case)

1.4.1 Testing of newly expressed proteins

Proteins CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI were assessed for their potential toxicity. In this assessment, the Applicant refers to previous assessments of the single events of the stack MON87427 x MON89034 x MIR162 x NK603 for the evaluation and the description of the safety aspects.

1.4.1.1 Molecular and biochemical characterization of the newly expressed proteins
The Applicant refers to analysis of the proteins CP4 EPSPS, Cry1A.105, Cry2Abs, Vip3Aa20 and PMI in their single parental events.
In the text the Applicant write that "This results in the combined expression of….". From the text in the current application it is not clear if the molecular and biochemical analysis of the newly expressed proteins from the stack is analysed or if the data are from the previously assessed single events. We assume the latter as the rest of the Application has data from that.

No data are provided for analysis of the newly expressed protein from the stack MON87427 x MON89034 x MIR162 x NK603 in the current application.

Recommendation

- We encourage the Applicant to specify more clearly, if the proteins characterized are from the stack or if the statements are based on previous analysis on single events.

1.4.1.2 Molecular and biochemical characterization of the newly expressed proteins
Bioinformatic analysis of amino acid sequence of the newly expressed proteins for analysis of sequence homology to known toxins of anti-nutritional proteins was performed on the expressed proteins. No biologically relevant sequences were observed according to the Applicant. However, the Applicant refers to observations from the assessments performed for each of the single, parental events constituting the stack in the current application, and not observations from proteins isolated from the stack themselves.
Seemingly, no analysis have been performed on the proteins isolated from the stack MON87427 x MON89034 x MIR162 x NK603.

Recommendation

- We strongly encourage the Applicant to analyze the proteins in the stack for homology to known toxins or anti – nutrients and not make assumptions based on data from analysis of previously assessed single events (constituting the stack in question).

1.4.1.3 Stability of the newly expressed proteins under relevant processing and storage conditions and the expected treatment of the food and feed.
Each of the proteins expressed in the maize stack MON87427 x MON89037 x MIR162 x NK603 has previously been demonstrated to:

- Loose functional actitivy due to heat treatment, indicating that these proteins are degraded upon treatment.
- Remain intact after pH treatment at different pH conditions.

Seemingly, the proteins are stable over pH but degraded upon processing (temperature treatment).
From the Application, it seem as none of these analysis have been performed on the proteins isolated from the maize stack itself. No data are presented that indicate that proteins have been isolated from the stack and analysed for pH or temperature stability.
It is also unclear from this section what pH values that have been used for the analysis.
The pH in the human digestive tract varies greatly. It ranges from 1.5 to 8.5 depending on how long time it was since food was eaten, disease state, where in the stomach the measure is made and several other issues. This can indicate that a proteolytic degradation assay should be performed over a pH range to look at stability of proteins over pH range, and also over time.

It is however possible that processing, and also the matrix used for analysis, might have an impact on the digestibility of the proteins analysed by altering the "susceptibility to gastrointestinal enzymes (Takagi et al 2003). In Verhoeckx et al (2015) it is therefore suggested that a "combination of processing and digestions" should be performed in the assessment (allergenicity assessment specially mentioned) to look at impacts resulting from protein and peptide fragments in functional assays. The solubility of the proteins after these treatments is also an issue that must be considered as it might impact the results of the assays.

Recommendation
- We encourage the Applicant to clearify if proteins analysed are from the maize stack or if the data are from the previous analysis of the single events only.
- We encourage the Applicant to clarify which pH levels stability analysis have been performed at. A broad pH range will better mimic the situation in the gastric system.

1.4.1.4 Resistance of the newly expressed proteins to proteolytic enzymes
The Applicant claims "evidence suggests that proteins are more rapidly cleaved by digestive proteases in their denatured form than in their native form". They expect their proteins to behave the same way based on the demonstrations of digestion of the proteins analysed in the single events.

No new data is presented for analysis of resistance to digestion of prteins isolated form the maize stack.

See section 1.4.1.3 for comments.

1.4.1.5 Repeated dose 28 day oral toxicity study with newly expressed proteins in rodents
A repeated dose 28 day study was not performed as there is "no testable hypothesis to justify the use of experimental animals to conduct a 28 day oral toxicity study".
The Applicant also state that "such testing would not further inform the robust and well established history of safety of these proteins".

We comment that no analysis have been performed on the "newly expressed proteins", but that the assumptions are based on the data provided from the studies of the single parental lines.

According to the Applicant, CP4 EPSPS, Cry1A.105, Cry2Ab2, Vip3Aa20 and PMI proteins "have no synergistic or antagonistic effects to each other".
However, the Applicant do not present any new data on proteins isolated from the stack MON87427 x MON89034 x MIR162 x NK603 showing that this is the case. The Applicant refers to previous analysis performed on the single proteins for their safety and specificity.

The Applicant refers to the history of safe use of each of the single proteins and also that these proteins are expressed in very low levels in grain and forage, loose activity upon heating and digested in gastric juice model system.
There is however no history of safe use for the combined use of the four distinct proteins in combination as this multi-stack is new with a new combined expression of the four proteins.

Recommendation

- We recommend the Applicant to perform 28 day oral toxicity analysis of the proteins isolated from the multi stack to verify these data as no analysis have been performed on the newly expressed proteins, only on proteins isolated from single events in parental lines, in previous analysis and also that the proteins in the stack has no history of safe use as they are expressed there, as this is a new combination of traits.

1.4.4 Testing of the whole genetically modified food or feed
No 90 day feeding study was performed in rodents as the Applicant refer to EFSA OECD guidelines stating:
"*whole food and feeding studies are unnecessary om GM crops that have already been demonstrated not to be biologically different from their conventional counterparts by molecular, compositional, phenotypic and agronomic analysis*".
Seemingly, this is not required.

And"*as there are no evidence that there is any adverse effects of any of the four proteins expressed on human or animal health*".

However, as no feeding trials have been performed with material from the maize stack with the combination of proteins, this has not been analysed fully yet. Only the single proteins expressed in each single parental line have been assessed.

Recommendation

- We encourage the Applicant to perform a 90 day feeding study as the combined expression of traits in this multistack might be potentially distinct from each of the traits being expressed alone and also as no safety data is presented from the proteins isolated from the multistacks and the combined expression of these.

1.5 Allergenicity assessment

The Applicant refers to Codex guidelines (2009) for assessment of the allergenic potential where one compare characteristics of the proteins of interest with those from known allergens. As the Applicant refers to the allergenic assessment performed for each of the single parental lines and the proteins expressed there, they state that there is no reason to expect that it will be different in the current multistack expressing these proteins in combination.

We refer to section on stacked events and data presented there and strongly encourage the Applicant to consider the potential for change to proteins and their behavior due to that.

Recommendation:

- We recommend the Applicant to consider to perform the analysis for allergenicity on proteins as they are expressed in the multistack and not base assumptions on data

Social utility and sustainability aspects

In addition to the EU regulatory framework for GMO assessment, an impact assessment in Norway follows the Norwegian Gene Technology Act (NGTA). In accordance with the aim of the NGTA, production and use of the GMO shall take place in an ethically and socially justifiable way, under the principle of sustainable development. This is further elaborated in section 10 of the Act (approval), where it is stated that: *"significant emphasis shall also be placed on whether the deliberate release represent a benefit to the community and a contribution to sustainable development"*. These issues are further elaborated in the regulations relating to impact assessment pursuant to the NGTA, section 17 and its annex 4. In the following we identify areas that are relevant to consider in order to assess social utility and sustainability aspects, and highlight information that is missing from the Applicant.

Impacts in producer countries

The NGTA, with its clauses on societal utility and sustainable development, also comes into play with a view to health, environmental and socio-economic effects in other countries, such as where the GMOs are grown or in this case where the maize MON87427 x MON89034 x MIR162 x NK603 is cultivated. Especially with regard to the ethical justifiability, it is not sufficient to only state that MON87427 x MON89034 x MIR162 x NK603 will not be cultivated in the EU. Indeed, to accept a double standard of safety criteria for Norway on one hand, and other safety criteria for countries from which Norway may import its food and feed on the other hand is not adhering to the principle of sustainable development and is unacceptable. Currently, no information is provided that demonstrate reflection on how the monitoring, assessment or

evaluation of the GM crop in countries where the crop will potentially be cultivated is assessed. It is important to explain the process of evaluation of the environmental and socio-economic consequences for other countries as well, as this will provide the required information to assess the application on the criteria in the NGTA.

Social impact relevant for sustainability
Published reviews on sustainability-relevant aspects of social impacts from cultivating GM crops (e.g. impacts among poor and/or small-scale farmers in developing countries, share of the benefits among sectors of the society) indicate that these effects have been very complex, mixed and dependent on the agronomic, socio-economic and institutional settings where the technology has been introduced (Glover, 2010). Fisher et al. (2015) performed a literature review on empirical studies concerning social implications from cultivating GM crops, and found that from 2004 – 2015 there has only been 15 studies corning social implications of cultivating Bt-maize. They show that published literature is dominated by studies of economic impact and conclude that very few studies take comprehensive view of social impacts associated with GM crops in agriculture. Importantly, it is difficult to extrapolate on hazards or risks taken from data generated under different ecological, biological, genetic and socio-economic contexts as regional growing environments, scales of farm fields, crop management practices, genetic background, interactions between cultivated crops, and surrounding biodiversity are all likely to affect the outcomes. Hence, it cannot be expected that the same effects will apply between different environments and across continents. In order to meet the requirements in the NGTA, further investigations of social implications (e.g. economic, distribution of benefits, access to seeds and wellbeing) in countries where maize MON87427 x MON89034 x MIR162 x NK603 is intended for cultivation is needed.

Impacts of the co-technology: glyphosate
The evaluation of the co-technology, that is, secondary products that are intended to be used in conjunction with the GMO, is also considered important in the risk assessment of a GMO (Dolezel et al 2009). Therefore, considerations of the co-products also warrant an evaluation of safe use and data required for such an assessment is, not provided by the Applicant.

The MON87427 x MON89034 x MIR162 x NK603 maize confers tolerance to glyphosate and offers protection against insect pests. Recent studies have shown negative effects from glyphosate, both on species present in terrestrial and aquatic ecosystems and on animals and cell cultures (for further elaboration and references on this issue see p.12). Consequently, glyphosate is now increasingly recognized as more toxic to the environment and human health than what it was initially considered to be. This is particularly a concern as the introduction of glyphosate tolerant GM plants has led to an increase in the use of glyphosate (Dill et al 2010). As the event MON87427 x MON89034 x MIR162 x NK603 is genetically modified to possess two *cp4 epsps* genes (providing glyphosate tolerance), it is likely to assume that this GM maize is tolerant to higher doses of glyphosate. This could further increase the use of glyphosate. Moreover, studies have shown increased levels of herbicide residues in herbicide tolerant GM crops (Bøhn et al. 2014), which could have health impacts on humans and animals consuming food/feed based on ingredients from this type of GM plants. Finally, weed resistance to glycines

in maize cultivation has been vastly documented[2]. The Applicant has not provided information on the contribution of the MON87427xMON89034xMIR162xNK603 maize to the emergence of glyphosate resistance in weeds, nor if there are already cases of this in the areas intended for cultivation of the variety.

Impacts of the Bt-toxin on target and non-target organisms
MON87427 x MON89034 x MIR162 x NK603 maize does also confer resistance to certain lepidopteran and coleopteran pests. Evaluation of resistance development within the target pest population and strategies suggested to halt this development, as well as impacts on non-target organisms is crucial in a sustainability assessment.

Co-existence management and assessing alternatives
The applicant highlights that the appearance of "volunteer" maize in rotational fields following the maize crop from the previous year is rare under European conditions. Still, an evaluation of the occurrence of volunteer plants in the producing countries and suggested control strategies is important for a sustainability assessment. Information about the occurrence of volunteers and which herbicides that will potentially be used for killing volunteers is required to evaluate potential health and environmental impacts of these.
Moreover, the cultivation of GM plants in general is causing problems with regard to co-existence. For instance, Binimelis (2008) has investigated consequences on co-existence of Bt maize in Spain among small-scale farmer and has found that co-existence is very difficult and that farmers in some areas have given up growing non-GM maize. Even though the cultivation of MON87427 x MON89034 x MIR162 x NK603 is not planned in Europe/Norway, it is important to obtain information about the strategies adopted to ensure co-existence with conventional and organic maize production and information about consequences on co-existence in the countries intended for production of maize MON87427 x MON89034 x MIR162 x NK603 is required for a coherent analysis for the criteria in the NGTA.

Recommendations

- In order to meet the requirements for the NGTA, the regulator is encouraged to ask the Applicant to submit information relevant for the assessment of the social utility of MON87427 x MON89034 x MIR162 x NK603 and its contribution to sustainable development. The information provided by the Applicant must be relevant for the agricultural context in the producing country/countries. The information should include issues such as: Changes in pesticide use, emergence of herbicide resistant weeds, development of pest resistance in target populations, impacts on non-target organisms, potential for gene flow and possible impacts among poor and/or small-scale farmers in producing countries and share of the benefits among sectors of the society

Conclusion
The applicant does not attempt to identify socio-economic implications, nor demonstrate a benefit to the community and a contribution to sustainable development from the use of the MON87427 x MON89034 x MIR162 x NK603 maize and does therefore not provide sufficient information as required by the NGTA.

[2] http://www.weedscience.org/Summary/Crop.aspx?SituationID=8

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