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ACB'S OBJECTIONS TO SYNGENTA'S APPLICATION FOR COMMODITY IMPORT OF TRIPLE STACKED MAIZE (Bt11xMIR162xGA21)

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Description of Application

The application is made by Syngenta for authorsiation to the Registrar: Genetically Modified Organisms, to allow the importation into South Africa of a triple stacked GM maize bt11xMIR162xGA21 for use as food and animal feed.

The triple stacked maize contains transgenic cassettes from the following single events:-

Bt11 expressing a modified *Bt* insecticidal toxin (Cry1b) for control of certain Lepidopteran pests and the PAT gene for resistance to glufosinate herbicides;

MIR162 containing a modified version of the native vip3Aa1 gene from *Bacillus thuringiensis* that confers resistance to certain lepidopteran pest species and a phosphomannose isomerase (PMI) protein used as a selectable marker; and

GA21 maize expressing a modified maize 5-enolpyruvylshikimate-3-phosphate synthase enzyme (mEPSPS) that confers resistance to glyphosate herbicides.

Description of Data furnished to ACB

Upon our assessment of the data provided to us, we found a complete lack of data and were impeded in conducting an independent assessment of the application. We found all the Appendices containing data to have been omitted and we were unable to assess the validity of the statements made by the applicant.

It is universally agreed that GMOs must be assessed on a case-by-case basis. The triple stacked event represents a new GMO demanding assessment as such. Biosafety data relating to the single events respectively can be taken into account in so far as they are supportive in the biosafety assessments of the triple stacked GMO, but cannot replace the full molecular characterisation, compositional analysis, feeding studies and environmental risk assessments of the new GMO created by stacking three transgenic cassettes.

In essence, Syngenta is requesting authorization for a new GMO event, Bt11xMIR162xGA21, but relies on a large proportion of biosafety assessment from the individual GMO lines. It assumes that crossing 2 GMO maize events will not result in any polygenic or combinatorial effects. This assumption is severely flawed since the interaction between genetic elements are well known and widely studied in plant breeding and molecular biology (e.g. Xu 2003).

The description of the studies by the applicant refers to data, but in the absence of the data being furnished to us, this can only be considered conjecture and opinion.

In assessing the molecular characterization of the triple stacked GMO, Bt11xMIR162xGA21, the applicant states that Southern blots were carried out to determine the stacking and correct integrity transgenes, but supporting data was not provided to us since Appendix 1 was removed. The applicant also states that there are single insertion events in the genome, but evidence to back this up is not presented, nor is the site(s) of insertion into the genome provided. The integrity of the cassette and other unintended genetic effects has not been studied and it is therefore not known if there are any other genome changes.

Appropriate experiments would include quantitative Southern blots or quantitative PCR with several probes or primers (spanning the cassette and including flanking regions) on plants in field trials with the DNA sequencing of the amplified cassette. Furthermore it is assumed that no other genetic changes were introduced during the construction of Bt11xMIR162xGA21. In order to prove this assumption techniques such as repPCR, RAPD and comparative genome hybridization (CGH) are useful as these have been shown to be effective in establishing genome similarity(Bao *et al.* 1993, Pinkel and Albertson 2005) and will help establish if additional, unintended genetic changes were introduced.

Molecular characterisation: Unintended Genetic effects

The transgenic cassettes for the three individual events have been characterized as part of previous applications for authorizations and submissions and that data is available from previous Syngenta dossiers, accessible submissions to the EU/EFSA, referenced literature and so forth. The characterisation revealed mutations, deletions multiple insertions, and truncations in these transgenic cassettes.

Bt11: Bt11 expresses a synthetic truncated *crylAb* transgene from the soil bacterium *Bacillus thuringiensis kurstaki* that is effective against many Lepidopteran insects and a synthetic *pat* transgene from *Streptomyces viridochromogenes* for resistance to glufosinate herbicides. Each of these is driven by the 35S-CaMV promoter and terminated with the 3' untranslated region of the nopaline synthase (*nos*) sequence.

There are therefore in fact, two transgenic cassettes, each driven by a 35S- CaMV promoter. The company's dossier claimed a single copy insert with the structure: *35S-CaMV-Int II-pat-tnos-35S-CaMV Int VI-crylAb-tnos.* However, analyses by the Belgian Council for Biosafety revealed "primary insert with rearrangements, truncations and unexpected insertions", and "it is not certain if only one copy of the insert is present". Furthermore, 1.1kbp of the plasmid sequence was present at the 5' end of the insert, followed by plant DNA with homology to an 180bp knob specific repeat sequence.

The presence of plasmid sequence is of particular concern since this may contain genetic origins of replication (Col1E1) and the marker gene *pat* that confers resistance to the antibiotic from *Streptomyce viridochromogenes* (phosphinothricin is an antibiotic naturally produced by *Streptomyces viridochromogenes).*

There is additional uncertainty as to the molecular characterisation of Bt11 because Bt11 was contaminated with an unapproved GMO, Bt10. From 2001- 2004, 165 000 tons of maize was sold on international markets (including to South Africa who approved Bt11 for commercial release in 2003. (http://cat.inist.fr/?aModele=afficheN&cpsidt=18553781; and http://www.i-sis.org.uk/BT10DMA.php; and http://gmo-crl.jrc.it/doc/Bt10_Executive%20summary.pdf).

Therefore, many batches of Bt11 seed may contain the unapproved and significantly different Bt10 GMO. Importantly, Bt10 contains the ampicillin resistant gene that could be transferred to bacteria, thereby compromising the ability to treat diseases. The ampicillin resistant gene should not be present in GMOs since it will spread antibiotic resistance to pathogens, thereby compromising our ability to treat present and future disease. This statement is supported by every organisation, including the WHO and EFSA and it is for this reason that the EU decided not to approve GMOs with ampicilin resistance genes in 2004.(http://www.gmo-compass.org/pdf/documents/efsa_marker.pdf).
In light of this fact, there is even greater need to fully characterize

Bt11xMIR162xGA21.

MIR162
MIR162 expresses the modified vip3Aa1 insecticidal toxin from the soil bacteria *Bacillus thuringiensis* that is effective against lepidopteran pests. This cassette uses the PMI gene as a selectable marker in contrast to the antibiotic resistance markers commonly used in any other GMO food crops on the market. The PMI (phosphomannose isomerase) gene is driven by the Ubiquity promoter (ZmUbiIntr) from *Zea mays* and enables transformants to grow on mannose as the sole carbon source. The applicant states that sequencing and Southern blot data have demonstrated that MIR162 maize contains a single DNA insertion with one copy of both the vip3Aa1 and the PMI genes, however, this data was not provided (Appendix 1 and others absent).

GA21 Maize
The mutated EPSPS gene, (3-enoyl pyruvyl shikimate 5-phosphate synthase) which confers resistance to glyphosate herbicides is from the bacteria *Agrobacterium* spp. The expression of this gene is driven by a promoter consisting of the actin 1 gene promoter as well as the first exon of the actin 1 gene from *Oryza sativa* (rice). mEPSPS gene was fused to an optimized chloroplast transit peptide to allow targeting to the chloroplast (the site of the shikimate pathway and mode of action of the glyphosate herbicides). The chloroplast transit peptide is a synthetic peptide derived from peptide sequences from maize and sunflower RuBisCo genes. Sequence analysis of the GA21 event revealed multiple (six) insertions of the transgenic cassette! Additionally, many of these copies contain deletions or truncations. Copy 1 has a 5' deletion of 696 bp; copies 2, 3 and 4 are intact; copy 5 contains a truncated mEPSP gene (only 288 bp of the mepsps gene) while copy 6 is contains only the rice actin promoter and a truncated actin first exon; with all other genetic elements truncated.

The use of the viral 35S-CamV promoter also presents new biosafety risks. These risks are due to increased rearrangements and deletions that affect genome integrity and stability since there is evidence from the laboratory (Koholi et al. 1998 and 2003, Vaden and Melcher 1990) and field studies (Quist and Chapela 2001, Collonier et al. 2000, Ho et al. 2000) that the 35S-CaMV is a recombination hotspot. The increased recombination with other genomes may have many effects. For example the recombination with viral elements can result in the creation of new viruses (Falk et al 1994; Wintermantel et al. 1996, Greene et al. 1994; Ho and Cummins 2000a and b).

The complete biosafety risks of these unintended genetic changes are unknown and uncertain, but may include the production of novel allergenic or toxic proteins, changes in cellular gene expression and metabolism as well as increased recombination and horizontal gene transfer (HGT).

Risks to human and animal health and the environment

The commodity import of maize may include maize kernels (seed), flour and oil to be used in animal feed and human food. Since maize and maize meal are a staple diet of the majority of South Africans, the diet of South Africans is very different from the Europeans and Americans (where maize makes up a smaller percentage of the diet and is usually in a highly processed form such as corn/maize oil or high fructose syrup). Therefore, the assumptions used for the approval of this GMO maize in other countries (low exposure, absence or protein from highly processed maize products) does not apply. Equally maize (kernels, cobs, leaves, stalks) are a popular animal feed in South African husbandry and the increased exposure raises increased risks of possible toxic or allergenic effects to animals used in agriculture (predominantly chickens and cattle).

Compositional analysis, allergenicity and toxicity

There are several aspects to consider when determining whether a novel protein is likely to be an allergen:- sequence similarity to known allergens, ability to survive simulated digestion using gastric fluid, as well as monitoring the immune response in feeding studies. There is limited sequence similarity between the PMI protein and two allergens, Hev b 13, from the latex of the rubber tree (*Havea brasiliensis*) and alpha-parvalbumin from the frog (*Rana* spp). Similarly, vip3Aa gene product (vip3Aa20) shows similarity to the Allergen V5/Tpx-1 family protein precursor. Both these warrant further *in vivo* studies to determine allergenicity and biosafety. The studies of the allergenic potential have been limited to establishing that the protein is destroyed by heat (95^{o}C for 30min) and was degraded by acid and/or enzymatic hydrolysis when exposed to simulated gastric or intestinal fluids in the laboratory. The allergenic potential has not been studied in animal models nor have antibody levels been determined in controlled feeding studies in order to monitor the immune response.

The compositional analysis of Bt11xMIR162xGA21 maize is stated as being substantially equivalent to conventional maize, but the data was not presented or available to support this statement- it is *assumed* that the triple stacked Bt11xMIR162xGA21 maize is a composite of the individual events and there are no other differences. Interestingly, although the data is omitted (Appendix 13 absent), a paragraph in the application reveals that there are substantial differences between Bt11xMIR162xGA21 and the non-GMO parental line. It states: “Of the 56 analytes measure in grain of Bt11xMIR162xGA21, statistically significant differences were noted for B1, B3, B6, 14 different amino acids, stearic acid, oleic acid and phytic acid and so can clearly not be considered substantially equivalent! The use of non- comparators (i.e. vastly different maize varieties or transgenic lines) in an attempt to nullify any differences is clearly inappropriate and unjustified.

Similarly, although the individual events have been tested in some

feeding studies, the triple stacked maize has not. There is reference to stacked maize (quadruple stacked Bt11xMIR162xMIR604xGA21) which is inappropriate since it is not the same as the GMO in this application. At the very least bovine and chicken feeding studies should be carried out with observations not only for toxicity, but also other physiological and behavioral changes so that any changes in morbidity and mortality can be detected. These experiments are required to provide the required evidence to establish compositional equivalence, but have not carried out.

Herbicide resistance, horizontal gene transfer (HGT) and gene escape

There are risks associated with the consumption of maize and maize products by animals and humans as well as threats to biodiversity. In South Africa many of the maize derived staple food products are not highly processed (maize cobs, samp and maize-meal) and both the transgenic proteins and the DNA will be present. It is now well known that DNA can persist in soil, and in many processed food products. Furthermore, evidence shows that horizontal gene transfer (HGT) to bacteria does occur and is significant and occurs at a high frequency when sequence homology is present (de Vries and Wackernagel 1998). The company states that "No changes in the ability of the Bt11 x MIR162 x GA21, Bt11, MIR162 or GA21 maize to transfer genetic material to other organism are expected compared to conventional maize since no sequences have been introduced to allow this to occur." This is incorrect since the EPSPS, vip3Aa1 and PMI genes all have gene homologs in soil bacteria indicating an increased risk for recombination and horizontal gene transfer (HGT) compared to the non-GM parental maize line. Furthermore, a study carried out to determine if transgenic DNA transferred to bacteria of the human gut by HGT, found that this did indeed occur (Netherwood 2004).

There is also a more obvious manner in which GM transgenes may escape into the environment. It seems likely that Bt11xMIR162xGA21 maize kernels that are approved for food or feed will inevitably be planted by farmers and thus be released into the environment. The distinction between seed and feed is unclear in the eyes of many farmers where part of the maize harvest is always saved as seed. A poor harvest may necessitate the purchase of seed for consumption (food or feed), but naturally some will be saved for planting in the hope of a better crop the next year. Since the Bt11xMIR162xGA21 will be indistinguishable in the field from many other varieties, this type of contamination, or co-existence and co-mingling, will be unnoticed and may spread following years of seed saving and planting.

Maize plants may also escape into the environment accidentally through the spillage of seeds on transport routes so that feral plants are established. This has been clearly observed in other countries where

people have looked for feral GMOs in the environment. For example, the occurrence of genetically modified maize at a grain receiving port and along transportation routes in the Republic of Korea (Kee Woong Park *et al.* 2009). These effects will be exacerbated if the Bt11xMIR162xGA21 has increased seed dormancy and germination, but Syngenta has failed to produce data to support the contention that this is not the case. These problems with feral Bt11xMIR162xGA21 will only be detected as part of a monitoring program or when maize is shipped to other countries. In terms of South Africa's obligations under the Cartagena Protocol on Biosafety, a transboundary movement requires monitoring and prior notification to the affected Party. The current commodity clearance application for Bt11xMIR162xGA21 should therefore specifically exclude whole maize (kernels or seed) and only permit cracked maize and more processed maize Bt11xMIR162xGA21 products (e.g. maize flour).

There may be several consequences of gene escape and hybridisation with other maize varieties or landraces. Maize has undergone many generations of breeding and natural selection to create numerous varieties suited to South Africa (adapted for increased resistance to soils, drought, pests etc.)- this forms part of the indigenous knowledge systems and unique seed banks of maize varieties and landraces.

Since GMO maize will freely cross-pollinate with non-GMO maize, there are risks of contamination of South Africa's landraces and loss of South Africa's unique maize seed diversity. A lack of co-existence of GMO with non-GMO maize can result in rejection of maize from importing countries that have not approved this transgenic as well as the spread of herbicide resistance, and non-target effects on other plants animals (Cui and Xia 1999, Hillbeck 1999) and soil microorganisms (Benbrook 1999 and Kowalchuk 2003, Koskella and Stotzky 1999, Tapp and Stotzky, 1998).

After almost three decades of world-wide use, confirmed resistance to glyphosate exists in *Lolium rigidum* (annual ryegrass) in Australia, South Africa, and California; *Lolium multiflorum* (Italian ryegrass) in Chile, *Eleusine ind*ica (goosegrass) in Malaysia; and *Conyza canadensis* (marestail) in certain states of North America (http://www.cropscience.org.au/icsc2004/symposia/2/5/2166_killmer.htm) .

Furthermore, if unintentional field release were to occur, the farmer may still incur financial liability since the GMO is patented. Despite these facts, there is no measure to ensure that farmers purchasing Bt11xMIR162xGA21 are informed that the seed cannot be planted, nor is there a monitoring system in place.

Lack of monitoring and compliance with legislation

The detection methods developed for the single events could be used for the stacked event. However, a specific and sensitive method is required so that Bt11xMIR162xGA21 can be distinguished from the single events Bt11, MIR162 and GA21. PCR with primers flanking or over-lapping the insertion site would easily enable the events to be distinguished, but this has not been carried out.

There is no proposal to monitor any of possible unintended effects (mentioned above) which contravenes South Africa's obligations under the Biosafety Protocol on Biosafety, National Environmental Management Act (1998) as amended and its GMO Act, in failing to monitor changes in biodiversity as well as transboundary movements of GMOs. There is no proposed method for the specific and sensitive detection of Bt11xMIR162xGA21 so that transboundary movements as well as contamination or mingling of with other maize in the food and feed chain can be monitored. Additionally, it is unclear if the shipments will be recorded at the Biosafety clearing house and whether the sale of maize as food or feed (maize seed or kernels) will be labeled as GMO with information that it must not be planted/cultivated or financial liability will be incurred as a result of Syngenta's patent rights.

Conclusion

In summary, there is a total lack of data to support any of the evidence that bt11xMIR162xGA21 is substantially equivalent to the non-GM counterpart. Most of the data supporting the application is absent (Appendices absent). In particular, the results from the molecular characterisation and compositional analysis (mainly Appendix 1 and 13) are absent: this is required to determine the validity of the applicants' statements and cannot be considered confidential business information.

On these grounds alone, Bt11xMIR162xGA21 should not be approved. The importation of Bt11xMIR162xGA21 for human food and animal feed carries additional biosafety risks in South Africa (compared to the EU or North America) since unprocessed maize is a staple for many Africans and may also provide the bulk of animal feed, resulting in greater exposure to transgenic proteins and DNA.

The importation of maize kernels also carries unacceptable risks of gene escape since maize bought for feed is saved for seed by farmers and planted. Furthermore, evidence from other countries shows that these seeds will be spilled along transportation routes or on farms (where the seed has been purchased for seed) and feral plants will be established. This will impact food security and sovereignty of South Africa's landraces and may jeopardize maize exports (that are contaminated with Bt11xMIR162xGA21). In order to comply with local and international legislation, a monitoring system with a detection system specific for the triple stacked Bt11xMIR162xGA21 must be put on place so that changes in biodiversity, gene escape and transboundary movements can be detected.

In light of these facts, and the significant biosafety risks and uncertainties of the individual events, it is recommended that Bt11xMIR162xGA21 not be approved for commodity import.

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