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Summary of analysis of dossier from Mahyco et al. in support of their claims of safety of fruit and shoot borer tolerant brinjal

Introduction

I am submitting to the Philippines Government this opinion on the testing of Bt brinjal conducted in India because I understand that the Philippines is currently evaluating a Bt brinjal event for commercial release and is relying in whole or in part on the results of Indian testing.

What follows is my opinion of the testing of the product known as Bt brinjal (EE-1 event) produced by a consortium of institutions including Mahyco of India. I have evaluated selected sections of the "Mahyco dossier"[1] released for review in India in 2008 and a response to criticisms (ECII)[2] of this data issued by the Genetic Engineering Approval Committee of India (in 2009). To my knowledge, there is no additional information that has been made available to the Philippines, and the dossier submitted for risk assessment contains the same data as that submitted to the Indian regulators; hence my comments are also relevant for the Philippines.

Importantly, only one country—India—has formally considered the risk assessment of Bt brinjal and they have decided that there is insufficient evidence of its safety. The Indian regulator, GEAC, failed in its evaluation of the assessment data to convince its minister, the Honourable Jairam Ramesh, that Bt brinjal was safe either as food or for open cultivation (e.g., Table 1). Overwhelming evidence from the independent scientific community submitted to the Minister and to GEAC justified a Ministerial moratorium on the product until proper and customised testing can be completed. To my knowledge, results of tests that address the flaws carefully examined by the independent scientific community have not been provided to India and therefore the Philippines are unlikely to have received any information that has not already been considered and dismissed by India.

[1] Mainly a document called Toxicology and allergenicity studies vol. 1.
[2] Report of the Expert Committee (EC-II) on Bt Brinjal Event EE-1 Developed by: M/s Maharashtra Hybrid Seeds Company Ltd. (Mahyco), Mumbai University of Agricultural Sciences (UAS), Dharwad and Tamil Nadu Agricultural University (TNAU), Coimbatore. Submitted to the Genetic Engineering Approval Committee of the Indian Ministry of Environment and Forests on October 8, 2009.

Who am I?

I am a professor of genetics and molecular biology in the School of Biological Sciences at the University of Canterbury, Christchurch, New Zealand and a senior adjunct professor of gene ecology at GenØk – Centre for Biosafety in Tromsø, Norway. I was previously a staff fellow at the US National Institutes of Health. My BSc with honors in biochemistry and molecular biology was from the University of Wisconsin – Madison and my PhD in molecular biology was conferred by the University of Oregon. I received the ICAAC Young Investigator Award from the American Society for Microbiology in 1993 and was the recipient of the New Zealand Association of Scientists Research Medal in 2002. I am listed by the United Nations as a biosafety expert and served on the 2009-2010 Ad Hoc Technical Expert Group on Risk Assessment and Risk Management established under the Cartagena Protocol on Biosafety. I have published broadly in the peer-reviewed scientific literature, authored invited works for the Food and Agricultural Organization of the United Nations (FAO) and the International Assessment of Agricultural Knowledge, Science and Technology for Development (IAASTD), advised various government agencies in several countries and have an active laboratory with four PhD students (with more on the way) and a postdoctoral scholar.

Purpose

Products of genetic engineering techniques, such as Bt brinjal, are by international consensus agreement subject to a risk assessment (Table 2). This consensus is reflected in the work of the Codex Alimentarius Commission (herein Codex) formed between the UN FAO and the World Health Organisation and which sets international standards for food safety, including for food derived from GMOs, and in the international agreement on the transboundary movement of living genetically modified organisms (LMOs/GMOs) called the Cartagena Protocol on Biosafety.

There are inconsistencies in how regulators in different countries prioritise the assessment and in which aspects of the assessment they prioritise. It is both proper and appropriate for different countries to come to different conclusions about the evidence available to assess risk or conclude safety. This is because: 1. different regulators may require different kinds of tests relevant to food safety in their country due to different levels of consumption, manner of preparation, and attention to scientific details; and 2. testing of the GMO in the food and/or environment of the country being asked to accept it is not always done and thus the dossiers submitted by companies are not addressing fundamental and specific risks in each country receiving the application. Customized, country-relevant, case-by-case risk assessment requires data pertinent to and developed in the Philippines.

Table 1: Selected standards allowed by Codex but enforced at discretion of GEAC.

Codex recommendation	Action	Next	Was the action requested?
Molecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects.	Profiling of the transcriptome and proteome (for novel RNA or protein molecules, or novel concentrations of normal RNA or protein molecules) for purposes of hazard identification.	If changes unique to the GM plant are found, these unintended changes can be further assessed if necessary.	No
The sensitivity of all analytical methods should be documented.	Proper reporting so that independent scientists could recreate the experiment and confirm the result.	If reporting is insufficient for independent confirmation, require the necessary details before advancing approval.	No
The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food does not cause harm when prepared, used and/or eaten according to its intended use.	Feeding and compositional studies using both whole food and food processed or prepared as humans would eat it.	If adverse effects are noted with the food in this form, then require further testing or reject on safety grounds.	No
[D]emonstrate whether deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its post-translational modification or affect sites critical for its structure or function	Isolate novel protein(s) from the plant and measure molecular mass using mass spectrometry.	Determine if any isoforms have been modified in any way and test for toxicity or allergenicity.	No
[D]emonstrate whether the newly expressed trait(s) are expressed as expected in the appropriate tissues in a manner and at levels that are consistent with the associated regulatory sequences driving the expression of the corresponding gene	Using targeted techniques for the isolation of in-planta produced novel protein, measure concentration in a tissue and time of development study across multiple test sites and years.	Evaluate implications of protein concentration for food (or environmental) risks.	No
[I]ndicate whether there is any evidence to suggest that one or several genes in the host plant has been affected by the transformation process	Profiling of the transcriptome and proteome (for novel RNA or protein molecules, or novel concentrations of normal RNA or protein molecules) and the metabolome for purposes of hazard identification.	If changes unique to the GM plant are found, these unexpected or unintended changes can be further assessed if necessary.	No

I have compiled a list of issues that may be of relevance for the risk assessment process in the Philippines and to the ultimate decision maker. The Philippines will have its own consumption patterns and local cooking/processing practices that may be relevant to the emergence of an unintended adverse effect caused by eating Bt brinjal. Codex recommends that local patterns be taken into consideration and that the GM food be tested after cooking: "The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered" (paragraph 47). The Philippines has its own unique environment and therefore non-target animals, from insects to nematodes through to mammals and birds, that will be exposed to cultivated plants. The Cartagena Protocol emphasises the case-by-case assessment and the need to generate information relevant to the receiving environment: "Risk assessment should be carried out on a case-by-case basis. The required information may vary in nature and level of detail from case to case, depending on the living modified organism concerned, its intended use and the likely potential receiving environment" (Annex III). Thus, the Philippines should be cautious in using environmental and food safety data from India in place of its own testing. The topics I have chosen to write about are within my area of expertise. This document was prepared with the purpose of assisting the regulatory process conducted by the Philippines.

Table 2: International consensus on the need for risk assessment

"Each Contracting Party shall, as far as possible and as appropriate: Establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health".	Article 8. Convention on Biological Diversity
The goal of each safety assessment is to provide assurance, in the light of the best available scientific knowledge, that the food will not cause harm when prepared or consumed according to its intended use, nor should the organism itself cause harm when viable organisms remain in the food.	Codex (paragraph 25)
In accordance with the precautionary approach contained in Principle 15 of the Rio Declaration on Environment and Development, the objective of this Protocol is to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements.	Cartagena Protocol on Biosafety

References

Codex, (2003). Principles for the risk assessment of foods derived from modern biotechnology. CAC/GL 44-2003.

Codex, (2003). Guidelines for the conduct of food safety assessment of foods derived from recombinant DNA plants. CAC/GL 45-2003.

1. Insertions

A proper safety assessment includes a molecular (genomic) level profile of the modified plant. The purpose of this stage of the assessment is to ensure that all intended and unintended effects of the process are detected and evaluated, including changes in expression of endogenous genes and new gene products (at the RNA and protein levels). This is consistent with Codex recommendations (paragraph 31):

"Information should be provided on the DNA insertions into the plant genome; this should include:

A) the characterization and description of the inserted genetic materials;

B) the number of insertion sites;

C) the organisation of the inserted genetic material at each insertion site including copy number and sequence data of the inserted material and of the surrounding region, sufficient to identify any substances expressed as a consequence of the inserted material, or, where more appropriate, other information such as analysis of transcripts or expression products to identify any new substances that may be present in the food; and

D) identification of any open reading frames within the inserted DNA or created by the insertions with contiguous plant genomic DNA including those that could result in fusion proteins."

However, for the Bt brinjal, Mahyco has not eliminated the possibility that there is more than one insertion of recombinant DNA and that all insertions are not free of vector "backbone" DNA. The following are reasons why one cannot conclude from Mahyco's data that there is a single insert and no additional inserts of unexpected size or sequence composition.

A. The Southern blot analysis is fundamentally flawed and incapable of finding unexpected inserts.
    a. The probe is described as "cry1Ac probe" or "Bt" probe (p. M34)[3], apparently meaning the cry1Ac transgene and the probe was presumably appropriately

[3] Page numbers beginning with "M" are from the document called Toxicology and allergenicity studies vol. 1.

sequence modified to reflect changes introduced by the developers[4]. ECII also refers to a single probe called "Bt" in section 3.1.4. Since the probe is specific to only this part of pMON10518 (the plasmid used in the construction of EE-1), the blot is inappropriate for establishing that there are no other inserts and no backbone DNA from pMON10518. This is the scientific equivalent of using a microscope to track asteroids.

Subsequently GEAC asserted that Mahyco had used "the entire pMON10518 plasmid as a probe, as well as the nptII gene and 7S terminator-right border regions as probes. No additional bands were detected using these probes, indicating that there are no additional fragments from the construct at other locations in the genome." *However, I could not find these data either in the materials released in 2008 or in late 2009. In addition, the GEAC summary does not address the important point that there are no sensitivity parameters upon which to build confidence that any unintended insertions would be detected (see below). The conclusions of GEAC on this point are based on the strength of a negative result (non-detection), which could have multiple causes besides the absence of additional inserts.*

b. As recommended by Codex, Mahyco should disclose all details that are necessary to establish the sensitivity of their analyses. Therefore, the size of the probe and the stringency of the wash procedure should be reported. All probes should be shown on the plasmid map. Partial fragments of transgenes or genomic DNA interspersed into transgenes have been detected as fragments as small as 300 bp[5]. Mahyco would have to show that all its probes would have detected such small insertions at the sensitivity of 0.5 copies per diploid genome in order to establish with reasonable certainty that the negative result was meaningful.

   It is impossible to determine from the documents provided the sensitivity of the methods used by Mahyco to attempt to detect unintended additional inserts.

[4] "Both regions of the cry1Ac gene were genetically modified for increased plant expression using a strategy comparable to that described by Perlak…" (p. 33).

[5] https://bat.genok.org/bat/?sp=html/practical_assessment/ch2_DNA_to_insert/molecular_characterization/southern_blots.html

Note that Codex (paragraph 20) makes specific recommendations on molecular characterization:
"The sensitivity of all analytical methods should be documented."

c. The sensitivity of genomic profiling methods (e.g. Southern blotting) for surveying insertions of partial transgenes should be at least to the standard of published studies[6]. These other published studies found that even to their much higher standard, they failed to detect all insertions initially. They found for example that "[t]ransgenic oat line 3830…was previously characterized with FISH, fiber-FISH, and Southern analyses and shown to have a single major transgene locus estimated to be ca. 15 kb in length. However, when T1 progeny of line 3830 were analyzed by Southern blot hybridizations with longer exposure times and more genomic DNA per lane compared to these previous analyses, two additional minor transgene loci were detected" (p. 424-425 Makarevitch et al. 2003). Their work emphasizes how vulnerable analyses are to arbitrary exposure times, probe sizes and wash stringency.

d. The PCR data does not substitute for the required Southern data because small fragments cannot be expected to insert in the correct order or proximity to primers for easy amplification[7].

e. The Southern blot provided as evidence (p. M46) is below acceptable standard for other reasons as well. A light band of the same size can be seen in control lanes and in the lanes with DNA taken from transgenic plants. This result can arise from sloppy handling and loading of samples. It can also result from contamination of control lines, and thus the use of controls that are also GM plants (see section 4 on comparators, below). Since we do not know the sensitivity of the probes, the possibility that the control lines carry a single simple insert cannot be ruled out with this data. In addition, larger bands are seen to hybridize and these could have secondary inserts to which the probe binds. The only way to resolve these possibilities is to clone and sequence all visible bands.

f. Mahyco should meet the dual standard of demonstrating comprehensive coverage in their search for pMON10518 DNA (which the list of probes does not) and demonstrating appropriate sensitivity to small inserts (which the data do not).

[6] https://bat.genok.org/bat/?sp=html/practical_assessment/ch2_DNA_to_insert/example.html

References
Codex, (2003). Guidelines for the conduct of food safety assessment of foods derived from recombinant DNA plants. CAC/GL 45-2003.
Makarevitch, I., Svitashev, S.K. & Somers, D.A. (2003). Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. Plant Mol. Biol. 52, 421-432.

B. Mahyco previously argued incorrectly that there could be no vector sequences transferred and did not to my knowledge provide any evidence of having verified their assumption. GEAC never addressed this criticism.

The claim by Mahyco was that during Ti plasmid-mediated DNA transfer from *Agrobacterium tumefaciens* to plants, only the T-DNA is transferred. These claims begin on page M32 (section 3.3). This view is clearly wrong. First, the expectation is inconsistent with the biochemical process (Waters and Guiney, 1993). Second, at least since the mid-1990s it has been known that the "long transfer – the collinear transfer of DNA past the traditional left border – is a common phenomenon" (p. 914 Wenck et al. 1997).

It is in fact surprising that in 2008 anyone would claim by argument alone that Ti-mediated DNA transfer was precisely and routinely limited to the material between the left and right borders, given the extensive studies over 10 years prior showing the opposite. For example: *"In this study, we report the surprising result that approximately 75% of the transgenic plants that we generated using Agrobacterium-mediated T-DNA transfer contained binary vector 'backbone' sequences integrated into the plant genome. We obtained these data using both DNA blot and PCR analyses of the DNA of these transgenic plants"* (p. 951 Kononov et al. 1997, emphasis added).

Outdated views such as those expressed by Mahyco derive from previous experiments that were not designed to detect backbone transfers. *"Usually, transfer of only the non-T-DNA sequences to the plant would remain undetected because: (1) there is no selection for the transfer of such sequences; and (2) scientists generally have not looked for the transfer of these sequences"* (p. 954 Kononov et al. 1997, emphasis added). Mahyco continues this tradition. The amount of DNA that can transfer can be many times the length of the T-DNA region: *"extremely long regions of DNA (greater than 200 kbp) can transfer to and*

[7] https://bat.genok.org/bat/?sp=html/practical_assessment/ch2_DNA_to_insert/example.html

integrate into the genome of plants" (p. 954 Kononov et al. 1997, emphasis added). More troubling, short backbone sequences can transfer and be difficult to detect. *"In many instances, vector 'backbone' regions of a binary vector are smaller than what is conventionally termed the 'T-DNA' region"* (p. 954 Kononov et al. 1997, emphasis added).

These scientific facts invalidate additional claims made by Mahyco, notably that "[t]he border sequence itself is not entirely transferred during the T-DNA insertion in the plant genome. This means that the inserted DNA is no longer functional T-DNA, i.e., once integrated into the plant genomic DNA, it can not be remobilized into the genome of another plant even if acted upon by vir genes again" (p. M32). Note that Mahyco has rested its understanding of the T-DNA transfer process on highly selective references that are 1992 or older, and has reached erroneous conclusions.

Mahyco also makes the claim that the disarmed Ti plasmid "does not transfer to the plant cells but remains in the Agrobacterium" (p. M32). The Ti plasmid is a conjugative plasmid. It has been known since the 1980s that conjugative plasmids transfer to eukaryotes including plants. At some frequency, the Ti plasmid itself may transfer to the plants (Buchanan-Wollaston et al., 1987, Ferguson and Heinemann, 2002, Heinemann, 1991). It is Mahyco's burden to demonstrate with proper molecular analyses that this did not happen. No data are presented by Mahyco to establish the absence of Ti DNA, *and this was never denied by GEAC*.

Finally, there is no indication of a left border on the pMON10518 map (p. M40) or on the list of genetic elements (Table 3.2, p. M48). If the left border is indeed missing, then the entire plasmid will transfer.

The Philippines should be aware of these facts and should, in my view, require the company to properly demonstrate that no unintended additional inserts exist.

In its review of the molecular characterization (ECII section III), GEAC failed to indicate that it was aware of the deficiencies in Mahyco's experiments, and committed its own errors of logic. For example, GEAC asserted that: "*Agrobacterium*-mediated transformation has been used for the development of numerous biotech crops grown around the world for the past two decades, and has a proven track (*sic*) from a biosafety standpoint."

GEAC was wrong to associate its views on the safety of some commercialized products with the conclusion that the method of development has a history of safe use. Furthermore, such sweeping generalizations violate international consensus advice that assessments should be "case-by-case". While these products are considered safe by competent authorities in *some* countries based on both a pre-market assessment and their use in structured agricultural systems, *other* countries have reached different conclusions, at least for environmental assessments. There are a limited number of commercialized products derived from *Agrobacterium*-mediated transformation. The number of products being used is still very small and does not warrant an endorsement of the safety of a particular procedure. For example, considering the number and the kinds of products based on nuclear fission (from bombs to power plants) it could be asserted that in the context in which products of nuclear fission are deployed these products have a safe track record. This does not mean that every nuclear fission reaction is inherently safe and by extension every bomb or powerplant in every context is inherently safe just because it is based on nuclear fission.

References

Buchanan-Wollaston, V., Passiatore, J. E. and Cannon, F. (1987). The mob and oriT mobilization functions of a bacterial plasmid promote its transfer to plants. Nature 328, 172-175.

Ferguson, G. C. and Heinemann, J. A. (2002). Recent history of trans-kingdom conjugation. In Horizontal Gene Transfer, M. Syvanen, and C. I. Kado, eds. (London and San Diego, Academic Press), pp. 3-17.

Heinemann, J. A. (1991). Genetics of gene transfer between species. Trends Genet. 7, 181-185.

Kononov, M. E., Bassuner, B. and Gelvin, S. B. (1997). Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Pl. J. 11, 945-957.

Waters, V. L. and Guiney, D. G. (1993). Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. Mol. Microbiol. 9, 1123-1130.

Wenck, A., Czakó, M., Kanevski, I. and Marton, L. (1997). Frequent collinear long transfer of DNA inclusive of the whole binary vector during Agrobacterium-mediated transformation. Pl. Mol. Biol. 34, 913-922.

2. Novel cry gene has no natural analogue or history of safe use

The GEAC claimed in its ECII report (p. 11) that: "The cry1Ac protein produced in Bt brinjal is similar in structure and activity to that found in nature and in commercial B.t.k. microbial formulations." This is inaccurate (see below).

The cry1Ac gene was in fact a chimeric formed by fusing selected parts of two cry genes found in nature (p. M32). Moreover, these parts are not identical to the parts found in nature, with the potentially significant substitution of a serine for a natural leucine at position 766 and other unspecified changes to increase "insecticidal efficacy" (p. M33). Serine is an amino acid that is frequently modified through linkages to sugar residues in-planta after protein production, thus making inexcusable the absence of analyses on post-translational modifications (see section 3, below).

Mahyco go on to claim that the part of the protein with the newly introduced serine will be of no consequence because this portion of the protein is removed when the pro-toxin is activated through protease cleavage in the insect gut ("Since the Cry1Ac protein produced in the Bt brinjal yields an insecticidally active trypsin resistant core product of approximately 600 amino acids in size, the amino acid at position 766 will be lost in the insecticidally inactive fragment upon exposure to trypsin (or the proteases within the insect gut…" p. M33). This claim is misleading. The entire pro-toxin is produced in the plant. People and wildlife will be exposed to the pro-toxin and all its subsequent modifications when they eat the plant. Its cleavage in the insect gut has no relevance to food safety or for assessing non-target effects.

There are no direct scientific data that support the Indian regulator's claim that the gene used in Bt brinjal is structurally or functionally equivalent to that found in commercial B.t.k. microbial formulations.

3. Expression: Novel RNA and Proteins

Mayhco did not provide information on potential novel RNAs and proteins produced in the six possible open reading frames created by the EE-I event or by undetected secondary insertions. In fact, Mahyco has provided no information whatsoever on novel RNAs. This is a significant omission. Moreover, since Mahyco uses the nos3′ terminator in its construct it has an added obligation to look for novel RNAs. The nos3′ is not an efficient terminator in eukaryotes, leading to read-through, longer mRNA molecules and potential fusion proteins.

According to Codex (paragraph 32):
"Information should be provided on any expressed substances in the recombinant-DNA plant; this should include: the gene product(s) (e.g. a protein or an untranslated RNA); the gene product(s)' function…".

GEAC has commented that "the insertion sites from Bt brinjal event EE-1 was isolated and sequenced. The 3′ end of the *cry1Ac* gene was examined and found to have the expected stop

codon, followed by the 7S terminator and right border. Genomic flanking sequence examined on either side of the insertion showed no significant matches [to] any sequence in GenBank, and does not encode any open reading frames in all six frames. This suggests that no novel RNAs or proteins were generated as a result of the EE-1 insertion."

A. The GEAC response is based on assumption, not evidence. Moreover, the assumptions are not reliable.

    a. These sequences at the end of a gene do not stop transcription initiation 5′ of themselves. Reference to a reading frame is not relevant, since Codex advises to find *untranslated* RNA too.

    b. The presence of stop codons and terminator sequences are not sufficient to prove the absence of unintended RNA and protein products.

    For example, Mahyco has used the nos3′ terminator adjacent to the nptII gene. The nos3′ is not an efficient terminator in eukaryotes, leading to read-through, longer mRNA molecules and potential fusion proteins (Rang et al., 2005).

    Researchers first reported this in a transgenic soybean called 40-3-2. Interestingly, 40-3-2 is a Monsanto product and Monsanto is presumably the source of pMON10581. Even more troubling, "the read-through transcript [in 40-3-2] was processed in four different RNA-variants" (p. 440 Rang et al., 2005). The variants might arise from splicing pathways or other pathways that are not predicted from Genbank DNA sequences.

    A significant concern raised by the authors about the variant RNA molecules in 40-3-2, if they arise through a splicing mechanism, was that the nos sequence itself harbors a splice site. "The cis regulatory regions that initiate and mediate splicing are located within the removed region of spliced transcripts. If this is also true for the mechanisms mediating posttranscriptional processing of the described variants, it seems reasonable to assume that the transcribed nos terminator region might be responsible for processing the RNA. *Since the nos terminator was and still is commonly used as regulatory region in the production of genetically modified crops, read-through products and RNA variants could also be expressed in these plants*" (p. 442 Rang et al., 2005, emphasis added). Since nos is also used in Bt binjal, these established

research findings require that it be evaluated for variant RNA species arising from the inserted DNA.

This research has been extended to other genetically engineered plants (Box 1).

Box 1. General problems with nos3′ termination.
The experiments showed the synthesis, due to the known loss of the NOS terminator, of fusion RNA variants coding for putative CRY fusion protein showing 2 or 18 putative additional amino acids, composed of the 3′ end of truncated cry1A(b) gene and the putative HECT 3′ sequences transcribed in antisense orientation. Different transcripts showed a series of deletions some of which in common to all observed RNAs…In silico translation of putative fusion RNAs did not show significant identities with known protein domains…Taken together, our data, while suggesting the insertion of the transgenic sequence in a putative HECT gene, *show the transcription of new fusion RNAs, a result similar to that obtained by Rang and et al. (2005) in Roundup Ready Soybean 40-3-2* (p. 280 Rosati, A. et al., 2008, emphasis added).

Note that these novel variants (Rang et al. and Box 1) *were not predicted from Genbank analyses of open reading frames.* This is emerging as a general theme in molecular biology and cannot be ignored by the industry or the regulator. In addition to transcriptome changes, there are unpredicted changes in the proteome. For example, see Box 2.

Box 2. Testing to detect unintended changes in the proteome.
A comparison of a commercial maize variety carrying the MON810 event [from Monsanto] and its isogenic relatives (Zolla, L. et al., 2008) indicates both that unanticipated changes occur as a result of the engineering process and that these can be more carefully characterized using profiling techniques that are not common in the scientific dossiers provided to regulators (Heinemann, J.A., 2007).

The commercial line (33P67) was confirmed by Southern blotting to have a single insert (Zolla, L. et al., 2008). The subsequent analysis was on the proteome, comparing seeds of the commercial line and the seeds of its immediate F1 generation (33P67F1) with the seeds of the isogenic comparator (33P66) and the seeds of its immediate F1 generation (33P66F1). These comparisons allowed the researchers to

measure variability in the proteome that was caused by the environment (33P66 vs. 33P66F1 and 33P67 vs. 33P67F1), effects caused only by the engineering process (33P66F1 vs. 33P67F1), and the combined effects of environment and engineering (33P66 vs. 33P67).

Approximately 100 of the identifiable proteins varied either quantitatively (increases or decreases but protein in both) or qualitatively (protein present only in one) in the comparison of the seeds from the conventional line and the seeds it produced (33P66F1). Similar numbers of statistically significant changes were observed in the seeds of the transgenic 33P67 and its F1 progeny (78 changes). This is the effect of breeding and the environment on proteome changes because the tested seeds (33P66 and 33P67) came from plants grown in different environments. Comparisons between 33P66 and 33P67 reveal the combination of differences in environment and effects of the genetic engineering process. There were 27 statistically significant proteome differences between the conventional and transgenic seeds.

This illustrates that only thorough scientific testing, and not assumption-based reasoning of the kind used in the ECII, can properly be used for risk assessment.

References[8]

Heinemann, J.A. (2007). Letter to the Editor. Environ Plann Law J 24, 157-160.

Zolla, L., Rinalducci, S., Antonioli, P. & Righetti, P. G. (2008). Proteomics as a complementary tool for identifying unintended side effects occurring in transgenic maize seeds as a result of genetic modifications. J Proteome Res 7, 1850-61.

Regardless of whether the variant RNAs arise from a cryptic splice site within nos or through other processing pathways, all novel RNA species in Bt binjal must be reported for a proper safety assessment. I encourage the Philippines to adhere to the full extend of Codex guidelines, and avoid picking and choosing which standards to enforce and which to excuse.

References

Codex, (2003). Guidelines for the conduct of food safety assessment of foods derived from recombinant DNA plants. CAC/GL 45-2003.

[8] https://bat.genok.org/bat/?sp=html/practical_assessment/ch2_DNA_to_insert/breeding_trees_equivalence.html

Rang, A., Linke, B. & Jansen, B. (2005). Detection of RNA variants transcribedfrom the transgene in Roundup Ready soybean. Eur. Food Res. Technol. 220, 438–443.
Rosati, A., Bogani, P., Santarlasci, A. & Buiatti, M. (2008). Characterisation of 3' transgene insertion site and derived mRNAs in MON810 YieldGard maize. Plant Mol. Biol. 67, 271-81.

c. The failure to adequately assess the kinds of novel RNAs produced at the site of insertion is compounded by the very real possibility that there exist additional uncharacterized unintended insertions, each of which could be responsible for generating unknown and unintended novel RNA and proteins. Two kinds of experiments would put this question to rest:
    i. proper and full profiling of the genome by a combination of techniques (done properly, as discussed above); and
    ii. transcriptome[9], proteome and metabolome[10] profiling:
    "Non-targeted, analytical approaches at the gene, transcript, protein and metabolite levels are the methods-of-choice for investigating the physiology of the GM plants as comprehensively as possible, thus increasing the chances of detecting unintended effects" (p. 103 Rischer et al. 2006).

    Disappointingly, GEAC (ECII) dismissed Dr. P.M. Bhargava's previous recommendation for profiling for the following reasons:
    - the techniques are expensive and have "little value"; and
    - they are not validated.

    These assertions are not based on evidence and are not in my opinion—and that of other biosafety experts—correct[11]. In fact, even Mahyco's collaborator, Monsanto, has demonstrated its ability to perform such profiling (Box 3).

[9] https://bat.genok.org/bat/?sp=html/topic_guides/ch1_basics/profiling_hazards/molecular_methods/transcriptome_techniques/main.html
[10] https://bat.genok.org/bat/?sp=html/topic_guides/ch1_basics/profiling_hazards/molecular_methods/proteome_techniques/main.html
[11] see quote above by Rischer et al. and https://bat.genok.org/bat/?sp=html/topic_guides/ch1_basics/profiling_hazards/main.html

Box 3. Industry use of profiling.

Transcriptome

Monsanto researchers conducted a survey of small RNAs found in soybean seeds, corn kernels, and rice grains (Ivashuta, S.I. et al., 2008). Although this survey was incomplete, it represents the largest such survey that I am aware of to date. These RNAs were ≤30 nucleotides long. The RNA molecules isolated from rice were sequenced using high throughput 454 procedures, for a total of 285,864 unique and sequenced RNA molecules.

The researchers were able to quantify the amount of RNA in size range of ≤30 nucleotides for mature soybean and corn seeds. Soybean had approximately 0.70 g of small RNA/gram of tissue, and corn and rice reportedly had similar amounts.

This study demonstrates that it is within the capacity of developers to perform profiling experiments for the purpose of hazard identification. The number of small RNAs reported in this study would likely exceed the number of anticipated and unintended small RNAs generated as a result of the engineering process and insertions, and thus the exercise would be even simpler when applied to the GMO.

References: Monsanto study published under Ivashuta, S.I., Petrick, J.S., Heisel, S.E., Zhang, Y., Guo, L., Reynolds, T. L., Rice, J.F., Allen, E. & Roberts, J.K. (2009). Endogenous small RNAs in grain: semi-quantification and sequence homology to human and animal genes. Food Chem Toxicol 47:353-360.

Proteome

This study demonstrated that 2DE [2D gel electrophoresis] can be utilized to reliably analyze the seed proteome of transgenic A. thaliana (p. 2176).

Quote from a Monsanto publication under Ruebelt, M. C., Lipp, M., Reynolds, T. L., Schmuke, J. J., Astwood, J. D., DellaPenna, D., Engel, K. H. & Jany, K. D. (2006). Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of gentically

modified crops. 3. Assessing unintended effects. J. Agric. Food Chem. 54, 2169-2177.

"[T]his method [2D gel electrophoresis] could be used to interrogate proteome alterations such as a novel protein, fusion protein, or any other change that affects molecular mass, isoelectric point, and/or quantity of a protein" (p. 2154).
Quote from a Monsanto study published under Ruebelt, M. C., Leimgruber, N. K., Lipp, M., Reynolds, T. L., Nemeth, M. A., Astwood, J. D., Engel, K. H. & Jany, K. D. (2006). Application of two-dimensional gel electrophoresis to interrogate alterations in the proteome of genetically modified crops. 1. Assessing analytical validation. J. Agric. Food Chem. 54, 2154-2161.

GEAC and all other regulators do accept profiling evidence. Southern blotting (done properly) to determine if unintended additional insertions were made during transformation is a profiling of the genome. This procedure has not been "validated", but is ubiquitously accepted (Heinemann, 2007).

References

Heinemann, J.A. (2007). Letter to the Editor. Environ Plann Law J 24, 157-160.
Rischer, H. & Oksman-Caldentey, K.-M. (2006). Unintended effects in genetically modified crops: revealed by metabolomics? Trends Biotechnol. 24, 102-104.

4. Comparator[12]

All scientific studies that form part of a safety evaluation must involve a comparator. The comparator must be appropriate and used consistently. The purpose of the comparator is to provide the standard baseline for all measurements, and be the single common element in all experiments using material grown in multiple locations and years. It is impossible to determine if either of these rules were followed in the dossier for Bt brinjal.

The Philippines should be aware that failure to adhere to these simple scientific rules has resulted in withdrawal of other commercial products. Monsanto's dossier for LY038 and LY038 x MON810, two GM corn varieties, also failed to follow these rules. Despite LY038 being

[12] https://bat.genok.org/bat/?sp=html/topic_guides/ch2_dna_to_insert/breeding_trees.html

approved by several jurisdictions, both Food Standards Australia New Zealand (FSANZ) and the European Food Safety Authority (EFSA) required Monsanto to redo crucial experiments because Monsanto did not use the proper comparator. While some revised experiments were submitted to FSANZ and LY038 was ultimately approved by them, in early 2009 EFSA required other experiments to be redone. Despite an estimated US$1 billion investment in LY038 and its derivatives' marketing, processing and development, Monsanto instead chose to withdraw the product from further evaluation by EFSA and discontinued all plans for commercial production. This story illustrates the critical importance of the comparator to all the scientific findings. Getting this wrong can kill the product in other jurisdictions and for good reason: using different and/or inappropriate comparators in the many different experiments takes away the ability to identify true hazards and assess them.

The comparator is defined by Codex (footnote 5) as the conventional counterpart derived from the non-GM parent, and cannot be a product of modern biotechnology:
"[It] is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts."

EFSA (2008 p. S9) requires that:
"The appropriate comparators have all traits in common except for the newly introduced ones."

And EFSA (2006, p. 22-23):
"In the case of vegetatively propagated crops, comparative analyses should include the non-genetically modified isogenic variety used to generate the transgenic lines. In the case of crops that reproduce sexually, comparators would include appropriate information required in applications for GM plants and/or derived food and feed non-GM lines of comparable genetic background."

While Codex and EFSA do not preclude the use of control lines in addition to the proper comparator, these additional lines should not substitute for the comparator.

What was the comparator for Bt brinjal? Was it the non-GM parent, closely related (e.g. ≥50%)? Was a single comparator consistently used in all molecular, toxicological, immunological, feeding and compositional studies? I could find little or no information on the comparator in any experiment that I reviewed. The GEAC report (ECII) mentions the comparator zero times. GEAC does mention the "conventional counterpart", but does not indicate that this was a single closely related parent used consistently in all experiments, nor does GEAC provide any details on the genotype or history of this conventional counterpart. Hence, I have low confidence that the

simple, but critical, scientific practice of using the same and the appropriate comparator has been followed.

References

Codex, (2003). Codex Work on Foods Derived from Biotechnology. CAC/GL 45-2003.

EFSA, (2006). Guidance Document of the Scientific Panel on Genetically Modified Organisms for the Risk Assessment of Genetically Modified Plants and Derived Food and Feed. EFSA J. 99, 1-100.

EFSA (2008). Safety and nutritional assessment of GM plants and derived food and feed: The role of animal feeding trials. Food Chem. Toxicol. 46, S2-S70.

Conclusion/Summary

In my opinion, the dossier and the subsequent GEAC analysis (ECII) fail to meet fundamental and even routine hazard assessment standards for molecular characterization.

- I advise against any substitution of Philippine-generated data with that provided by Mahyco because the data used by India:
    - is not customized to the consumption patterns and local food of the Philippines and the biodiversity of the Philippines; and
    - was fundamentally flawed (in the ways described above).
- The molecular characterization of event EE-1 was incapable of detecting additional inserts which have a high likelihood of existing based on the methods used.
- There were no satisfactory descriptions of changes to the genome, and hence I have no confidence in the conclusion that unintended additional gene products (e.g. novel RNA and protein) are not made. All unintended gene products should be described and evaluated as potential hazards in human food or to non-target wildlife and microorganisms.
- There were no satisfactory descriptions of changes to the proteome, including isoforms of the intended protein.
- Critical scientific details are systematically lacking, including those necessary to evaluate experimental design (e.g., controls, particularly the isogenic comparator) and method sensitivity.

Since molecular characterization is the starting point of all risk assessments of genetically modified organisms, the downstream effects of a poor molecular characterization on the overall assessment of risk can be significant. If indeed Bt brinjal is safe for human consumption, safe for

environmental release, and the right technology for the Philippines, then the certainty of this can be demonstrated using existing, affordable and effective scientific analyses.

I urge the Philippines to insist on the proper standards being met to ensure that the product matches the claims. The Philippines should also insist upon its own in-country testing of any similar Bt brinjal product, and culture-specific testing of cooked/processed foods that include a brinjal ingredient. A checklist of tests is provided by the Biosafety Assessment Tool (https://bat.genok.org/bat/) which is freely available to all. This checklist allows a careful regulator to confirm that the science has been done to high and appropriate standards and that the guidelines set by Codex and other competent food and environment safety agencies have been fully addressed by the data.

Respectfully submitted,

Prof. Jack Heinemann
1 September 2010