SAFETY ASSESSMENT OF GENETICALLY ENGINEERED PLANTS CONTAINING STACKED TRAITS

UNEP/GEF supported Phase II
Capacity Building Project on Biosafety

Ministry of Environment, Forest and Climate Change
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C O N T E N T S

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CONTAINING STACKED TRAITS

1. Introduction

Gene stacking refers to the process of using conventional plant breeding techniques to combine two or more genetically engineered (GE) traits in a single plant variety. This is analogous to the practice employed in conventional plant breeding whereby multiple desirable characteristics are combined in a single variety. GE plant varieties with multiple traits may also be produced *via* recombinant-DNA techniques, either by retransforming an existing GE plant to introduce one or more additional transgenes, or by transforming a non-GE plant with a vector containing more than one transgene.

There are several reasons why a crop variety may be developed through gene stacking:

- Farmers are under increasing pressure to manage multiple threats to crop production, such as diseases, insect pests and abiotic stresses, like drought or soil salinity. Conventionally bred crop varieties with combinations of several different useful traits have been common for decades. Similarly, varieties with multiple GE traits have also been developed to address the needs of farmers facing multiple challenges in successfully bringing a crop to market.

- Gene stacks have been developed to prevent or forestall the development of insect resistance to the insecticidal *Bt* proteins from *Bacillus thuringiensis*. Stacking multiple *Bt* genes so that the plant produced several different *Bt* proteins reduces the risk that insect pests will develop resistance, since it is unlikely that insects can develop resistance to two or more *Bt* proteins simultaneously (Prasifka *et al*. 2013).

- Genes for herbicide tolerance that have different modes of action may be stacked to enable farmers a choice of herbicides to use with the crop, while at the same time, reducing the chances that weeds will develop resistance to any of the herbicides, due to repeated use of the same herbicide (ISAAA 2015).

- Genes may be stacked to move a complete biosynthetic pathway into a plant. For example the production of provitamin A in rice has been accomplished by stacking each of the genes necessary for its production. Similarly, flower color has been altered by moving all the genes needed for pigment production into a plant (Naqvi *et al*. 2009).

The history of gene stacking is almost as long as the history of GE crops: the first GE crop received its regulatory approvals in 1992 and the first variety

containing stacked traits, an insect-resistant and herbicide-tolerant cotton, was first approved in 1995. Varieties with stacked insect resistance and herbicide tolerance GE traits have become increasingly popular: globally over 51 million hectares of stacked-trait GE crops were planted in 2014, by farmers in thirteen countries, ten of which are developing countries (James 2014). In line with these global trends, future generations of transgenic crops are expected to be developed with more diverse traits, such as compositional improvements, disease resistance and tolerance to abiotic stress.

However, stacking of two or more GE traits in one plant raises concerns that the traits may somehow interact with each other, or with the plant's genome, in unexpected ways, resulting in harm to the environment or to food safety.

The purpose of this document is to provide guidance, through citations to the published literature, documents developed by non-governmental organizations and regulatory decisions issued by governmental authorities, regarding the assessment of risks posed by GE stacks, when compared to GE plant varieties bearing the individual traits singly. This document follows India's established approach to environmental risk and food safety assessment as described in the *Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants* and *Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants*. These guidelines provide key considerations to be followed in complying with information requirements and procedures used by the Regulatory Agencies i.e., Review Committee on Genetic Manipulation (RCGM) and Genetic Engineering Appraisal Committee (GEAC).

2. Scope

This guidance document is intended to explain the risk assessment approach used in India with GE plants containing stacked GE traits proposed for commercial release into the environment under "Rules for the manufacture, use, import, export & storage of hazardous microorganisms, genetically engineered organisms or cells, 1989 (Rules, 1989)". The document is not intended to explicitly define data requirements for the assessment process or specific release conditions to be imposed on the GE plant.

3. Determination of the Risk Assessment Process for GE Plants with Stacked Traits

As discussed above, there are three different methods for combining multiple GE traits in a single plant:

i)   Conventional cross of two GE plants

ii) Re-transformation of a GE plant with one or more new traits

iii) Transforming a non-GE plant with a vector bearing multiple traits

The method used to combine different GE traits and the regulatory history of each of the individual GE traits determine the appropriate risk assessment process. The following flowchart (Figure 1) summarizes how this determination is made.
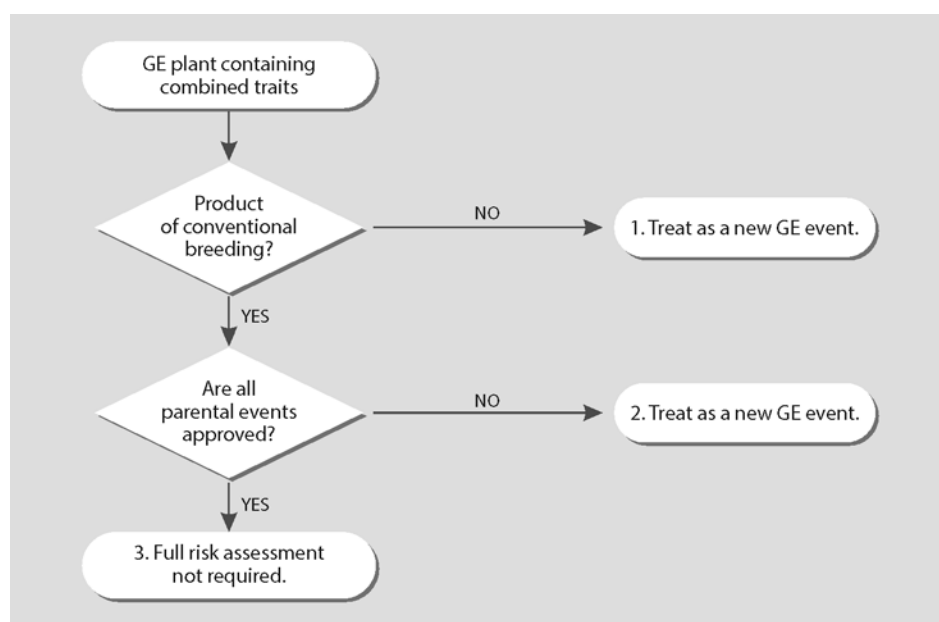

Figure 1: Determination of risk assessment process for GE plants with stacked traits

3.1 Stacked Traits Combined *via* Genetic Engineering

Outcome "1" from the flowchart represents varieties created via the re-transformation of an existing GE plant and varieties resulting from the transformation of a non-GE plant using a vector bearing multiple traits. Such plants will be treated as new transformations. That means that these GE plants must undergo the same full risk assessment process required for any new transformation event. Approvals for traits combined *via* genetic engineering will be for the new event and its progeny.

3.2 Stacked Traits Combined *via* Traditional Breeding

Outcomes "2" and "3" from the flowchart represent varieties created *via* conventional plant breeding, the most common way of stacking traits in GE crops. The approach for risk assessment will depend on whether each of the traits in the parental varieties has been previously approved for cultivation in India.

Outcome "2": In cases where one or more of the traits has not received prior approval for cultivation, the stacked variety will require a full safety assessment consistent with the requirements for a completely new event. This includes a complete molecular characterization; assessment of the potential toxicity and potential allergenicity of all expressed gene products and their patterns of expression in relevant plant tissues; agronomic and phenotypic characterizations; and compositional analysis of key nutrients, anti-nutrients and secondary metabolites. If any of the expressed gene products, i.e., novel proteins, have been subject to a risk assessment as part of a prior review, data relevant to assessing potential allergenicity and potential toxicity of the novel protein need not be re-submitted, but may be referenced in the application.

Data will be required from at least three growing seasons in different locations representative of the cultivation conditions for the crop in India. It is recommended that all the parental varieties be included as comparators.

Consistent with the *Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants*, 2008, the need for sub-chronic toxicity testing in animals and/or livestock feeding studies will be evaluated on a case-by-case basis. Normally, such studies may only be required when there are biologically significant compositional differences between the stacked event product and its conventional counterpart.

Where sufficient data were provided in the application to also complete a risk assessment of the parental lines, the approval should include the stacked event product, its progeny, any intermediate stacked events and the relevant parental lines.

Outcome 3: In cases where the GE parental lines used to create the stack have all been previously approved for cultivation and determined to be as safe as other commercials varieties, there is no need to undertake a full risk assessment of the stacked event. Instead, the evaluation of the stacked event will focus on the assessment of the potential for interactions between the expressed gene products and for the potential for the combination of multiple GE traits in a single plant to cause genetic instability. This assessment is discussed in detail in section 3.

As with Outcome 2, the authorization for stacked event varieties developed through conventional breeding methods will include the stacked event product, its progeny and any intermediate stacked events.

4. Assessment of Inadvertent Interactions of Stacked GE Traits

There are two possible undesirable outcomes that might result when GE traits are combined through conventional breeding. The first is that the combination

of genes could reduce the genetic stability of the plant. The second is that there could be unexpected metabolic or biochemical interactions between the GE traits. Either of these outcomes could result in adverse impacts on the environment or human health, so the risk assessment for stacked events must evaluate the possibility of these outcomes.

4.1 Background

Traditional plant breeding relies on crosses to combine useful genes, for characteristics such as disease resistance and abiotic stress tolerance, in a single plant. Frequently, crosses are performed between different species, ones that would not ordinarily cross pollinate under natural conditions and this type of gene stacking has been used extensively for crop improvement. However, plant breeding is an imprecise process and each cross results not only in the transfer of genes that the breeder wants to incorporate into the crop plant but also in the movement of many genes that are inadvertently transferred to the crop plant. In spite of the fact that the genetic and biochemical mechanisms underlying desired traits, as well as traits moved inadvertently, are largely unknown, gene stacking accomplished through conventional crosses of non-GE plants has a long history of environmental and human safety.

In addition, over 2,500 crop varieties have been developed through mutagenesis to create novel, beneficial crop characteristics. However, this process introduces genetic changes in hundreds of genes besides the ones which result in the desired phenotype and the molecular and biochemical impacts of these numerous changes are typically unknown. Yet again, crops improved through mutagenesis have a long history of environmental and human safety and are used in all types of agriculture, including organic production (Trewavas 2008).

As plant breeders incorporate new genetic variation into a crop species through wide crosses and mutagenesis to generate new, desirable phenotypes, they also use a screening process to eliminate gene alterations with undesirable phenotypes, including lethality. Initial screening is typically followed by a backcrossing or selfing process to stabilize the desired genetic changes they have made. When a commercial variety is finally derived, the breeders are reasonably certain that their breeding efforts have not contributed either genetic instability or undesirable phenotypes to the crop.

4.2 Genetic Instability in GE Plants

Just as traditional plant breeders use selection and back crossing to identify and stabilize novel desirable traits, developers of new GE plants also use these methods to eliminate plants with undesirable phenotypes and evidence of genetic instability. Because the same methods are used, the GE plants and the

novel traits they possess are as stable as varieties resulting from a traditional breeding program. Significant genetic changes, such as recombination between two transgenes or chromosomal rearrangements, are likely to affect either the expression of the desired GE traits or the plant's viability and fertility. These aberrations would be eliminated during the initial selection process and subsequent confined agronomic trials. Harmful genetic instability is therefore no more likely to appear in a GE plant with stacked traits than in a traditionally bred plant bearing traits from a number of parental lines (Steiner *et al*. 2013).

The applicant should supply data demonstrating that the genetic material inserted into the genome of the GE plant has been stably incorporated, that the sequences of all inserted genetic material are as expected and that the sequences do not change over subsequent generations of the GE plant. The application should also provide agronomic and phenotypic data from confined field trials demonstrating that, except for the intentionally added GE traits, the GE plant performs in other respects comparably to its non-GE counterpart. Data requirements addressing genetic stability of the GE plant are provided in Chapter 9 of the *Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants*, *2016*, specifically in sections 9.1 and 9.5, as well as in Chapter 10.

4.3 Metabolic or Physiological Changes

With thousands of genes present in most crop plants, there is the potential for countless interactions between these genes when different varieties are crossed. Yet, in spite of this enormous potential for interactions, the incidence of unexpected metabolic or physiological changes occurring as a result of traditional plant breeding, including wide crosses, is extremely rare (Weber *et al*. 2012). When such rare interactions occur, they are typically self-limiting, by causing impaired viability or fertility or they are culled during the selection process. The situation is no different for transgenes combined through genetic engineering and it is unlikely that such unexpected interactions would occur in a GE plant that has been properly selected and adequately tested in confined field trials.

There are three potential interactions which could theoretically cause harm to the environment or human health:

i)   The gene product of one transgene affects the expression of the other.

ii) The gene products interact directly with each other.

iii) The gene products encode enzymes that share substrates, either with each other or with existing metabolic pathways in the plant

The approach to assessing potential environmental or human health risks from these unexpected metabolic or physiological interactions due to gene stacking is no different from the approach used for GE plants bearing a single

transgene. This approach is laid out in the *Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants*, *2016* and as described in the Guidelines, it is essential to formulate a plausible risk hypothesis to test, using data supplied by the applicant. For example, if the stacked GE traits result in the production of two or more different *Bt* toxins for insect resistance, the hypothesis would propose that the toxin molecules may interact with each other in a way that results in harm to the environment or human health.

Then, as with a risk assessment for a single-trait GE plant, the applicant should provide data relevant to the hypothetical question, i.e., whether there is evidence that different *Bt* toxin proteins do associate with each other in ways that have the potential to cause harm to the environment or to human health. The risk assessors would then use this data to test the risk hypothesis and then characterize any identified risks. Any risk hypothesis formulated to investigate potential inadvertent interactions between stacked traits can be addressed in this manner.

5. References

ISAAA. 2015. "Pocket K No. 42: Stacked Traits in Biotech Crops." http://www.isaaa.org/resources/publications/pocketk/42/.

James, Clive. 2014. "Global Status of Commercialized Biotech/GM Crops: 2014." Ithaca, NY.

Naqvi, Shaista, Changfu Zhu, Gemma Farre, Koreen Ramessar, Ludovic Bassie, Jürgen Breitenbach, Dario Perez Conesa, *et al*. 2009. "Transgenic Multivitamin Corn through Biofortification of Endosperm with Three Vitamins Representing Three Distinct Metabolic Pathways." *Proceedings of the National Academy of Sciences* 106 (19): 7762–67. doi:10.1073/pnas.0901412106.

Prasifka, P. L, D. M. Rule, N. P. Storer, S. P. Nolting and W. H. Hendrix. 2013. "Evaluation of Corn Hybrids Expressing *Cry*34Ab1/*Cry*35Ab1 and *Cry*3Bb1 against the Western Corn Rootworm (Coleoptera: *Chrysomelidae*)." *Journal of Economic Entomology* 106 (2): 823–29. doi:10.1603/EC12392.

Steiner, Henry-York, Claire Halpin, Joseph M Jez, John Kough, Wayne Parrott, Lynne Underhill, Natalie Weber and L Curtis Hannah. 2013. "Evaluating the Potential for Adverse Interactions within Genetically Engineered Breeding Stacks." *Plant Physiology* 161 (April): 1587–94. doi:10.1104/pp.112.209817.

Trewavas T (2008) Redefining "Natural" in Agriculture. PLoS Biol 6(8): e199. doi:10.1371/journal.pbio.0060199

Weber, N., C. Halpin, L. C. Hannah, J. M. Jez, J. Kough and W. Parrott. 2012. "Crop Genome Plasticity and Its Relevance to Food and Feed Safety of Genetically Engineered Breeding Stacks." *Plant Physiology* 160 (December): 1842–53. doi:10.1104/pp.112.204271.