भारत सरकार
विज्ञान और प्रौद्योगिकी मंत्रालय
जैव प्रौद्योगिकी विभाग
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY

सत्यमेव जयते

आज़ादी का अमृत महोत्सव

ब्लॉक-2, (6-8वाँ तल), एवं ब्लॉक-3, (4-5वाँ तल)
सी0जी0ओ0 कॉम्पलेक्स लोधी रोड, नई दिल्ली-110003
Block-2 (6-8th Floor) and Block-3 (4-5th Floor)
C.G.O. Complex, Lodhi Road, New Delhi-110003
Tele : 011-24365071 Fax : 011-24362884
Website : http://www.dbtindia.nic.in

File No. PID-15011(11)/2/2022-PPB-DBT (CN. 16893) **Dated 21.07.2025**

OFFICE MEMORANDUM

Subject: Guidelines on Genetically Engineered Plants Containing Stacked Events, 2025

In India, activities involving Genetically Engineered organisms (GE organisms) or cells, as well as hazardous microorganisms and their products, are governed by the 'Manufacture, Use/Import/Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells, Rules, 1989' (Rules, 1989), as notified by the Ministry of Environment, Forest and Climate Change (MoEF&CC), Government of India, under the Environment (Protection) Act, 1986.

2. To unlock the potential of genetically engineered (GE) plants containing stacked events while addressing biosafety concerns and ensuring the safety of humans and the environment, the Department of Biotechnology proactively initiated the preparation of a draft framework. Leveraging the powers conferred to the Review Committee on Genetic Manipulation (RCGM) under Sections 6, 8, and 25 of the Environment (Protection) Act, 1986, the draft framework has undergone public consultation, rigorous review and deliberation by the Working Group, Expert Committees, and RCGM.

3. The Review Committee on Genetic Manipulation (RCGM), as the Competent Authority under the Environment (Protection) Act, 1986 (Rules, 1989), approved and recommended notifying the 'Guidelines on Genetically Engineered Plants Containing Stacked Events' during its 313th meeting held on 25.06.2025

4. The Department of Biotechnology hereby notifies the "**Guidelines on Genetically Engineered Plants Containing Stacked Events, 2025**".

5. These guidelines provide a comprehensive roadmap for the development and sustainable utilization of GE plants containing stacked events, outlining specific biosafety considerations and delineating regulatory pathways to ensure safe and responsible innovation.

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6. These guidelines will supplement earlier guidelines and standard operating procedures notified by DBT and MoEF&CC pertaining to genetically engineered plants.

7. These guidelines shall be applicable to all public and private organizations involved in the research and development of the GE plants containing stacked events with this notification.

8. The "Guidelines on Genetically Engineered Plants Containing Stacked Events, 2025" can be accessed at **https://ibkp.dbtindia.gov.in/.**

(Dr. Nitin Kumar Jain)
Member Secretary, RCGM
Scientist- 'G' & Head, Regulation
Department of Biotechnology

To:

1. The Chairman, Member Secretaries and DBT Nominees of all IBSCs.

Copy to:
1. Sr. PPS/PPS, Secretary, DBT
2. IBKP Portal.

DEPARTMENT OF BIOTECHNOLOGY
Ministry of Science & Technology
Government of India

Guidelines on Genetically Engineered Plants Containing Stacked Events 2025

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सचिव
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ब्लॉक-2, 7वां तल, सी.जी.ओ कॉम्पलेक्स
लोधी रोड़, नई दिल्ली-110003

डॉ. राजेश सु. गोखले
Dr. RAJESH S. GOKHALE

SECRETARY
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
Block-2, 7th Floor, CGO Complex
Lodhi Road, New Delhi-110003

MESSAGE

India is pioneering the use of biotechnological innovations to tackle pressing challenges in agriculture, health, and sustainable development. A key advancement in this field is the development of genetically engineered (GE) plants with stacked events, which can simultaneously address multiple issues such as pest resistance, environmental stress, and crop yield improvement. To ensure the safety of human health and the environment, a robust and transparent regulatory framework is essential for overseeing these developments.

"The Department of Biotechnology is pleased to introduce the **'Guidelines on Genetically Engineered Plants Containing Stacked Events, 2025'**, a culmination of rigorous deliberations by the dedicated Working Group, Expert Committee, and Review Committee on Genetic Manipulation (RCGM). I extend my gratitude to the officers and experts involved in crafting this comprehensive guidance document, marking a significant milestone in our regulatory framework for GE plants.

These guidelines are designed to support researchers, technology developers, and regulators by establishing a robust regulatory framework for the development and biosafety assessment of stacked GE plants, ensuring the protection of human health and the environment. By doing so, they aim to enhance India's regulatory landscape while instilling confidence among the scientific community and the public, ultimately fostering a safer and more innovative environment for genetic engineering research and development.

We invite all stakeholders to familiarize themselves with these guidelines and incorporate them into their work, enabling India to maintain its leadership in biotechnology innovation and biosafety. By embracing these guidelines, we can collectively uphold a science-driven, transparent, and globally harmonized approach to regulating stacked GE plants, ultimately advancing the nation's scientific progress and environmental stewardship.

Let us collaborate to unlock the transformative power of biotechnology, driving sustainable development and inclusive growth that benefits every segment of society.

(Dr. Rajesh S. Gokhale)

Phone : 24362950/24362881, Fax : 011-24360747, E-mail : secy.dbt@nic.in

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GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
Block-2, 7th Floor, C.G.O. Complex
Lodhi Road, New Delhi-110003

PREFACE

Breakthroughs in biotechnology have revolutionized the development of Genetically Engineered (GE) plants with multiple stacked events, enabling the expression of one or more target traits. These 'stacked GE plants' hold immense potential for agricultural innovation, but their complexity demands a rigorous and comprehensive biosafety assessment to safeguard human health and the environment.

In exercise of the powers conferred by the Environment (Protection) Act, 1986, and in accordance with the Rules for the Manufacture, Use/Import/Export, and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells, 1989, the Review Committee on Genetic Manipulation (RCGM) under the Department of Biotechnology (DBT), Government of India, is pleased to notify the **'Guidelines on Genetically Engineered Plants Containing Stacked Events, 2025'**. These guidelines provide a comprehensive framework and clear path for the biosafety assessment of stacked GE plants, ensuring a structured and transparent evaluation process.

These guidelines are designed to support stakeholders, including research institutions and technology developers, by detailing the data requirements and biosafety assessment processes necessary for the environmental release of stacked GE plants. They comprehensively cover a spectrum of topics, including molecular characterization, regulatory requirements, and biosafety assessments specific to stacked GE events. Furthermore, the guidelines are aligned with internationally recognized best practices, ensuring that India's regulatory framework remains robust, harmonized, and globally relevant.

We extend our sincere gratitude to the esteemed experts of the Working Group, Expert Committees, and the Review Committee on Genetic Manipulation (RCGM) for their invaluable contributions to the development of these comprehensive guidelines. We also acknowledge the tireless efforts of the Biosafety Support Unit (BSU); Regional Centre for Biotechnology, whose dedication and expertise played a pivotal role in shaping these guidelines.

These guidelines will be applicable across India to all public and private organizations engaged in developing stacked GE plants, specifically for conducting confined field trials preceding environmental release. We are confident that these guidelines will play a pivotal role in advancing the safe development and deployment of stacked GE plants, upholding a science-driven approach to biosafety and fostering innovation in India.

Prof. Y.K. Gupta
Chairman, RCGM
Former Director IITR, Lucknow &
President, AIIMS, Jammu

Dr. Nitin K. Jain
Scientist-'G', DBT &
Member Secretary, RCGM

Contents

1. INTRODUCTION

Genetically Engineered (GE) plants harbouring two or more GE events containing genes conferring one or more target trait(s) introduced sequentially or simultaneously to produce GE plants are generally referred as "stacked genetically modified plants", "stacked genetically engineered plants", "GE stacked (GES) events/lines", "stacked lines" or simply "stack". Stacked GE plants can be produced either through conventional breeding or genetic transformation or by combined approaches.

The *'Rules for the manufacture, use/import/export and storage of hazardous microorganisms/ genetically engineered organisms or cells, 1989'* (*Rules, 1989*) notified under the *Environment (Protection) Act, 1986* covers research as well as large-scale application of GE plants throughout India. Earlier, a compendium of the below-mentioned guidelines specific to GE plants has been notified:

- Guidelines and Standard Operating Procedures (SOPs) for Confined Field Trials of Regulated, Genetically Engineered (GE) Plants (2008)
- Protocols for Food and Feed Safety Assessment of GE Crops (2008)
- Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants (2008, Updated - 2012)
- Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants (2016)
- Regulations and Guidelines for Recombinant DNA Research and Biocontainment (2017)

This guidance document is a roadmap for biosafety assessment of stacked GE plants, including efficacy, stability and interaction among introduced genes. Biosafety assessment is a science-led approach comprising risk assessment, risk management and risk communication. This approach systematically evaluates safety of stacked GE plants within a framework of decision-making. A structured format analysing safety/risk is being adopted from the *Risk Analysis Framework, 2016* and *Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants, 2016*.

These recommendations align with the internationally accepted best practices for the assessment of stacked GE plants, and offer detailed data requirements in the context of plants containing GE stacked events to all stakeholders, including the regulatory agencies and technology developers.

2. SCOPE

The document outlines the data requirements for the biosafety assessment of stacked GE plants harbouring two or more events generated either through the conventional breeding method and/or through direct genetic transformation.

GE plants wherein multiple genes are deployed through genetic transformation using a single gene construct containing linked genes or multiple gene cassettes (that harbour two or more genes) are not considered equivalent to stacked GE plants harbouring stacked events developed through combining independent events by breeding or by sequential genetic transformation. Hence, such GE plants do not fall in the scope of this guidance document. Those plants will be considered as GE plants harbouring a single GE event.

Keeping in view of the rapid advancements in this field, RCGM shall review and update the document periodically.

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3. COMMON METHODS OF STACKING EVENTS IN GE PLANTS

Stacking of events refers to a process where multiple GE events are introduced into the genome of a plant through genetic transformation and/or conventional crossing of GE plants with single or multiple transformation events. The following sections will illustrate the commonly followed strategies for developing stacked GE plants:

3.1 Stacking by Genetic Transformation

Event stacking by genetic transformation (also called as molecular stacking) brings together two or more GE events through genetic transformation process. Such molecular stacks developed by genetic transformation are considered as new GE events. The following sections describe the commonly used strategies for generating stacks through genetic transformation:

3.1.1 Re-transformation

In this process, a GE plant containing one or more GE events is re-transformed with a new gene construct. This strategy of combining multiple target genes by re-transformation allows sequential assessment of the function and/or efficacy of each introduced gene.

3.1.2 Co-transformation

Co-transformation is the process of simultaneous introduction of multiple gene constructs into a plant genome through a single genetic transformation experiment. As they are not linked together, this approach enables the removal of one or more introduced genes in the subsequent generations through segregation and selection, depending upon the traits required.

3.2 Stacking by Conventional Breeding

To develop stacked GE plants by breeding, a GE plant harbouring one or more events is crossed with another GE plant containing different event(s) that are to be combined. Such GE breeding stack plants can be created either by hybridising two genotypes, each containing distinct GE events, or by introgression of multiple events into a single genotype through repeated backcrossing.

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4. DATA REQUIREMENTS FOR MOLECULAR CHARACTERIZATION OF STACKED GE PLANT

Molecular characterization of inserted DNA sequences (*e.g.*, T-DNAs) is the key step in defining the genetic modifications that are introduced through stacking of multiple GE events. Different gene constructs in stacked GE plants are typically integrated at distinct loci in the same or different chromosomes, which may lead to various expression levels of introduced genes and their segregation in progeny plants. The basic molecular characterization data requirements for a stacked GE plant is similar to that of GE plants containing a single event (**Annexure I**) with some additional data requirements (**Annexure II**).

4.1 Description of Stacked GE Plant

i. Method of event stacking: By breeding or genetic transformation (re-transformation or co-transformation), or by a combination of these.
ii. Varietal name of the parent lines, basic phenotypic characteristics, introduced genes, gene-specific markers, and event-specific markers for each individual GE parent event.
iii. The number of generations studied, along with the pedigree and evidence demonstrating the genetic stability of stacked events/trait(s) in the stacked GE plant.

iv. The function of introduced genes and conferred traits.

4.2 Homology of Introduced DNA Sequences

The introduced genes in a stacked GE plant may influence the expression of other homologous introduced genes and/or may lead to co-suppression of homologous /orthologous endogenous genes in the host plant. Therefore, the following information is required:

i. Homology between introduced genes and regulatory sequences of all other introduced gene constructs in the stacked GE plant.
ii. Homology of the introduced genes and/or regulatory sequences with the host plant endogenous genes (where the whole genome sequence is available, or the homologous/orthologous genetic elements are already reported in the literature).

4.3 Gene Constructs Integration and Copy Number Analysis

The copy number of introduced genes has a significant impact on their expression levels in GE plants. There may be one or more copies of the gene construct at each insertion site and/or complex gene construct integration patterns. The site of gene construct integration in the host plant genome may influence the expression of introduced genes, depending on the presence of regulatory elements, upstream and/or downstream of the integration site. In addition, depending on the site of integration, there is a possibility of creating new Open Reading Frame(s) [ORF(s)]. Therefore, identification and analysis of flanking DNA regions on both sides of the integrated gene construct(s) is a critical requirement.

i. Confirmation of stable integration of introduced genes, as well as the integrity of gene cassettes within each gene construct (T-DNA) of the stacked GE plant is required.
ii. Copy number analysis of each introduced gene construct in the stacked GE plant using an appropriate method (e.g., Southern blot, NGS, etc.). *In the case of a GE breeding stack containing one or more approved events, the previously generated relevant copy number analysis of the approved events would be considered.*

4.4 Segregation and Zygosity Analysis

Segregation analysis is carried out (*wherever possible*) to determine the inheritance pattern, copy number and zygosity of integrated introduced genes. Stacked GE plants, where events differ in

zygosity status, may have different phenotypes due to gene dosage effect.

i. *For stacked events developed by genetic transformation*: Segregation analysis pertaining to the introduced genes of each individual event to be analyzed.
ii. *For a GE breeding stack*: Segregation analysis for each unapproved individual parent event to be analyzed. Zygosity status for each individual parent event in the stacked GE plant to be assessed.

4.5 Genetic Stability

The stability of a genetic modification reflects the integrity of the original structure of the integrated gene construct and function of the genetically engineered trait(s) over successive generations. Therefore, to ensure the stability and efficacy of introduced genes in a stacked GE plant, it is necessary to demonstrate the stable inheritance by confirming the intactness of the introduced gene cassette(s) (without alterations of the original gene cassette(s) present in the transformation vector), and consistent expression of the introduced genes over two generations.

Multiple introduced gene constructs with highly similar DNA sequences may be less stable and are more likely to undergo rearrangement(s) or recombination(s). Changes in the introduced gene construct may influence the detectability of stacked events in the GE plant.

i. The stability of stacked gene cassettes for a minimum of two successive generations in a particular genotype, which carries stacked events.
ii. Event-specific detection methods for each integrated gene construct in the stacked GE plants.

4.6 Expression of Introduced Genes: Pre-Stacking vs. Post-Stacking

Various factors might influence the level of expression of the introduced genes at both transcript (mRNA) and protein levels, leading to altered trait efficacy in the stacked GE plant. In either case, it may result in an unintended effect on trait performance. To address this, the following is required:

i. Expression analysis of introduced genes of each event in the stacked GE plant at multiple locations/agro-climatic zones where the stacked GE plant is intended to be cultivated.
ii. Individual parent events, of stacked GE plants either developed by breeding or by re-transformation, should be included in each expression analysis as comparator(s).

4.7 Comparators

Comparators are used to establish the extent to which the expected trait utility is achieved or the extent of unintended changes that occur due to stacking. They are also used in compositional analysis to establish equivalence, to address biosafety concerns that might arise due to a combination of events in stacked GE plants. Therefore, the selection of appropriate comparators is important.

Data interpretation for biosafety assessment of GE plants primarily relies on the non-GE isogenic line as the primary comparator. In cases where a non-GE isogenic genotype is not available, use of the closest available non-GE genotype may be used as a comparator. In the case of GE breeding stack hybrids (F1 hybrids); an ideal comparator would be the isogenic non-GE hybrid.

However, to check for potential cumulative (synergistic, additive or antagonistic) and/or combinatorial trait effects (towards assessing trait efficacy); one of the comparator entries could be the GE parental line(s) having one or more individual GE parent events, wherever possible.

The GE parental line(s) harbouring introduced events that must be included as comparators, wherever applicable, are:

i. In case of GE breeding stack, all the individual parent events.
ii. For stacked GE plant, parent lines having stacked individual events expressing the same gene.
iii. For re-transformation molecular stack, the base event wherein transformation was made.
iv. For co-transformation molecular stack, the comparator would be the isogenic non-GE line.

The comparators shall also include one or more of the following non-GE counterpart(s), however, the list is not exhaustive, and the use of comparator(s) may vary case-to-case:

v. The non-GE isogenic genotype.
vi. A non-GE line derived from the same breeding scheme that was used to develop the stacked GE plant.
vii. A non-GE line with phenotypic properties closest available to the stacked GE plant.
viii. Commercial local /national check variety(ies) according to the receiving environment(s) of the stacked GE plant.

4.8 Detection of Events in the Stacked GE Plant

Detection of events in GE plants primarily rely on DNA-based techniques (*e.g.*, PCR) to identify a specific gene construct integrated at defined locus in the host plant genome. The detection and identification method, however, must be specific and sensitive

enough to detect each individual event stacked in the GE plant at the Limit of Detection of 0.01% (LoD 0.01%). To ensure analytical efficiency, wherever feasible, multiplex or consolidated assays may be conducted to enable the simultaneous event-specific detection of all stacked events within a single experimental setup. Furthermore, to ensure the reliability and reproducibility of the event-specific detection method at LoD 0.01%, the method should be independently validated by a third party using the appropriate combination of GE and non-GE tissue samples.

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5. BIOSAFETY ASSESSMENT

The *Risk Analysis Framework (2016),* published by the Ministry of Environment, Forest and Climate Change (MoEF&CC), describes the principles of biosafety analysis used by Regulatory Agencies of the country to protect human health and the environment, in accordance with the *Environment (Protection) Act, 1986*. While the biosafety assessment of food derived from GE organisms is based on the principles prescribed in the "*Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008*", the environmental safety assessment is established through "*Environmental Risk Assessment of Genetically Engineered Plants, 2016*".

In the case of stacked GE plants, the following two parameters are critical in evaluating the risks: i) the method of gene stacking, and ii) potential interaction among the genes in the events, if any. Based on these two parameters, the data requirements for safety assessment are elaborated, particularly for events stacked by genetic transformation, and **Annexure III** summarizes these data requirements.

5.1 Biosafety Assessment of Stacked GE Plant Developed by Genetic Transformation

Stacked GE plants developed by genetic transformation will be treated as "new transformation event" and are required to undergo comprehensive characterization,

evaluation and biosafety assessment. Data requirements, based on the strategy used for developing a stacked GE plant have been illustrated at **Annexure III.A.**

In the case of a re-transformation led molecular stacked plant, wherein the individual parent events are not intended for environmental release, a complete biosafety assessment shall be required for the stacked GE plants only, but is not required for the unapproved individual parent events (as summarized in **Annexure III.A**). However, the individual parent events should be used as comparators for evaluating trait efficacy.

5.2 Biosafety Assessment of GE Breeding Stack

The biosafety assessment of a stacked GE plant developed by breeding shall depend on the approval status of each of the individual parent events in India. The following sections elaborate on the data requirements for various possible combinations of individual parent events used in stacking:

5.2.1 Approach to be followed for unapproved individual GE parent events in India used for stacking

In cases where the individual GE parent events of the stacked GE plant have no prior approval for environmental release in India, but may also be intended for environmental release along with the stacked GE plant, each individual GE parent event and stacked GE plant shall require thorough evaluation and biosafety assessment consistent with the requirements for a new transformation event. This includes detailed molecular characterization, trait efficacy assessment, compositional equivalence, environmental safety assessment and, food and feed safety assessment of the individual parent event as well as the stacked GE plant.

If only the stacked GE plant, and not the individual parent events, are intended for environmental release, then, a complete biosafety assessment of the resultant stacked GE plant shall be carried out. A complete biosafety assessment of unapproved individual parent events shall not be required (as summarized in **Annexure III.B.**). However, individual parent events should be used as comparators for trait efficacy and compositional equivalence studies. In the case of GE stacked hybrids (F1 hybrids), relevant GE individual parent events should also require a complete biosafety assessment.

5.2.2 Approach to be followed for a combination of approved and unapproved individual GE parent events in India used for stacking

For the individual GE parent events already approved in India, previously approved data on molecular characterization and assessment of potential allergenicity and toxicity of the expressed gene product (*i.e.,* novel protein) shall be acceptable.

However, for all unapproved individual GE parent events, used in the stack, these data must be generated. The data requirements for the evaluation of stacked GE plants in this category will include an assessment of the genetic stability and expression analysis of all introduced genes as described in Section 4 and **Annexure II**; a complete biosafety assessment to be carried out is summarized in **Annexure III**.

Expression data of introduced genes that confer trait(s) of interest in relevant tissues of the stacked GE plant compared with the parental lines harbouring individual events shall be required.

The potential for interaction between the expressed gene products that could likely result in adverse environmental or health impacts must be assessed. For this purpose, a scientific rationale addressing i) the likelihood of creating a new allergen or toxin as a consequence of any interaction between gene products, ii) substantial equivalence, and iii) the potential for any changes to the reproductive biology of the plant as a consequence of stacking of GE events shall be provided. If one or more of the gene product(s) confer insect or disease resistance, the potential for any antagonistic or synergistic effects that could affect the efficacy of pest or disease control; and any anticipated changes to cultural or crop management practices relevant to the product with stacked events need to be assessed.

5.2.3 Approach to be followed, where all individual GE parent events used in stacking are previously approved in India

Stacked GE plants, in which all individual parent events have been previously approved for environmental release in India, do not require to undergo the entire biosafety assessment. In such cases, data only on food and feed safety assessment with plant parts, the effect on non-target organisms and compositional equivalence would be required.

In cases of subset (lower order) GE breeding stacks, where the higher order GE breeding stack and corresponding individual parent events are deregulated, a complete biosafety assessment of such subset GE breeding stacks is not required. However, data on the trait efficacy of such subset GE breeding stack shall be required to be considered for deregulation.

5.2.4 Data portability for stacked GE plants expressing some or all events approved outside India

Confined Field Trials (CFTs) are conducted to generate samples for food and feed safety studies, for environmental safety assessment, and to evaluate phenotypic equivalence and trait efficacy of the regulated GE plant under realistic environmental conditions. Trial end-points vary depending on the risk hypothesis being tested, but most CFTs aim at identifying any significant differences

between the regulated GE plant and its non-GE/GE comparator resulting from intended or unintended consequences of the genetic modification across a range of agro-ecosystems. Therefore, the field studies used to evaluate the potential environmental risks associated with a stacked GE plant should be conducted in the relevant receiving environment of the country, even if relevant and potentially sufficient data are already available from studies conducted elsewhere (outside India). However, the data generated in CFTs conducted "outside the country" can be submitted for reference.

The biosafety assessment requirements of a stacked GE plant containing one or more events that have been deregulated in other country(ies) shall be similar to those of stacked GE plants containing unapproved events. However, laboratory data on the molecular characterization and food and feed safety studies with pure protein of such events generated in other country(ies) is acceptable, and a complete data set as submitted to the regulatory agency of the approving country(ies) shall be required to be submitted.

Data on compositional analysis from other countries is not acceptable as the background germplasm of GE breeding stack and the environmental conditions and their (G x E) interaction in the country would be different. Therefore, compositional analysis involving biochemical parameters to establish substantial equivalence of the proposed GE breeding stack must be carried out with the samples from confined field trials conducted in India.

GLOSSARY

Acute Oral Toxicity — Adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.

Agrobacterium tumefaciens — A naturally occurring soil bacterium (also known as *Rhizobium radiobacter*) of plants that can transfer a part of its DNA present in T-DNA region of plasmid/vector into plant cells.

Allergen — An allergen is a usually harmless substance capable of triggering a response that starts in the immune system and results in an allergic reaction.

Anti-nutrient — Substance that interferes with the absorption or utilization of one or more nutrients by the body (e.g., oxalate and phytate, which prevent calcium absorption).

Backcross — A cross between a hybrid and one of its parents. Subsequent backcrosses of offspring to same (recurrent) parent produce offspring of increasing similarity to that parent.

Biosafety — The maintenance of safe conditions in biological research to prevent harm to workers, non-laboratory organisms and the environment.

Biotechnology — The application of scientific and engineering principles to the processing of materials by biological agents to produce goods and services.

Breeding Stack — Stacked GE plants developed by conventional breeding wherein a GE plant harbouring one or more events is crossed with another GE plant containing other event(s).

Cartagena Protocol — The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international agreement that aims to ensure the safe handling, transport and use of living modified organisms (LMOs) resulting from modern biotechnology, which may have adverse effects on biological diversity, also taking into account risks to human health. It was adopted on 29 January 2000 by India and entered into force on 11 September 2003.

Case-by-case basis — An individualistic review of a proposal against defined assessment criteria, which are relevant to the particular proposal.

Confined Field Trial — A field experiment of a regulated GE plant under terms and conditions that are intended to mitigate the establishment and spread of the plant.

Conventional non-GE counterpart — The related non-genetically engineered plant genotype, its components and/or products for which there is experience of established biosafety.

Detection — Determining the existence of a change in the genetic

	material of an organism (for instance at the level of DNA through the presence of a novel DNA sequence)
Event	A genotype developed from transformation of a single cell of a plant species with a specific gene construct integrated at a particular locus of the plant genome. This is also referred as an independent GE event. Different GE events of a particular genotype differ in integration site of the same gene construct at different chromosomal locations.
Gene	The physical and functional unit of heredity of which is transmitted to progeny cells and is held to determine characteristics of the offspring.
GE Plant	Plant harbouring exogenous genetic information into cells from non-sexually compatible or related species that leads to the transmission of the input genetic information (introduced genes) to successive generations.
Gene Construct	An engineered DNA fragment containing, but not limited to, the DNA sequences to be integrated into the genome of the target plant.
Genetic Engineering	Techniques by which heritable material, which does not usually occur or will not occur naturally in the organism or cell concerned, generated outside the organism or the cell is inserted into said cell or organism. It shall also mean the formation of new combinations of genetic material by incorporation of a cell into a host cell, where they occur naturally (self-cloning) as well as modification of an organism or in a cell by deletion and removal of parts of the heritable material.
Genetic Modification	Modifying organism genetic makeup by the introduction of a gene or set of genes into its cells in a way that follows the transfer of the genes to successive generation.
Germplasm	Collection of genetic stocks (genotypes) of an organism.
Genotype	Plant genotype refers to the specific genetic constitution of a plant, characterized by its unique set of alleles and gene sequences across the genome.
Hazard	Any source that has the potential to cause an adverse effect on human health, animal health or the environment.
Health Hazard	A factor or exposure that may adversely affect the health of a human population.
Introduced Gene	An exogenous DNA fragment, comprising one or more gene cassettes, that is stably integrated into the genome of a host organism and is heritable across successive generations
Identification	Identifying a specific genetic engineering that has been intentionally introduced.

Locus	The specific position of a gene or a DNA sequence on a chromosome.
Molecular stack	GE plants that contain two or more events combined by genetic transformation.
Mutations	Any detectable and heritable change in the genetic material, not caused by segregation or genetic recombination, that is transmitted to daughter cells and even to succeeding generations giving rise to mutant cells or mutant individuals provided it does not act as a dominant lethal factor.
Phenotype	The observable structural and functional properties of an organism, produced by the interaction between the organism's genetic potential (genotype) and the environment in which it finds itself.
Promoter	A DNA segment to which RNA polymerase binds to allow the initiation of the transcription of downstream (3') gene.
Protocol	The step-by-step experiments proposed to describe or solve a scientific problem, or the defined steps of a specific procedure.
Recombination	The process of intermolecular exchange of DNA or chromosomes combining genetic information from different sources. Site specific, homologous, transpositional and non-homologous (illegitimate) types of recombination are known.
Risk	A situation in which there is a probability that the use of, or exposure to an organism or contaminated product will cause adverse health consequences or death.
Risk Assessment	The qualitative or quantitative estimation of the likelihood of adverse effects that may result from exposure to specified health hazards.
Rules, 1989	The rules for the manufacture, use/import/export and storage of hazardous microorganisms/ genetically engineered organisms or cells, 1989 notified under the Environment (Protection) Act, 1986.
Segregation	The separation of allele pairs from one another and their distribution to different cells, usually at meiosis and sometimes at mitosis.
Source Organism	The organism from which a DNA fragment/genetic element is obtained (isolated or served as a template to design a new DNA fragment/genetic element) to introduce into the recipient/host organism.
Trial Location	The geographic location of a confined trial site, *e.g.*, village, address and plot number.

Annexure I: Data requirements for introduced individual GE events in a stacked GE plant

Data requirements for individual GE events are part of confined field trial applications that are submitted at the IBKP portal. These data requirements are essential and must be accompanied by the specific data needs of a stacked GE Plant **(Annexure II).**

1. Information on the host /recipient plant:

Name and basic phenotypic characteristics of the plant genotype, information on whether the host plant species is a source of allergens, toxins or anti-nutrients.

2. Information on the transformation method:

Transformation method followed; Selection agent(s) and method(s) used to select transformed cell/plant.

a. For *Agrobacterium* mediated transformation, species and strain of *Agrobacterium* used, data ascertaining removal of *Agrobacterium* cells from plant tissues.

b. For direct transformation, the method of transformation (electroporation, particle bombardment, etc.), the nature of carrier DNA used (if any), the method of preparation and the purity of the DNA.

3. Information on the GE event/ line:

Unique Identifier of the events/line; purpose of the modification (introduced traits); phenotypic description of the new trait; capacity to reproduce or transfer genes to other organisms; pleiotropic effects on the recipient plant; etc.

4. Introduced DNA

a. ***Details of each transformation vector:***

Size, source organism, vector map with base pair positions and important restriction sites; Nucleic acid sequence; GenBank accession number of the vector backbone; Selection agent; description of changes introduced in the vector.

b. ***Details of each gene construct:***

Gene construct map with base pair positions of each genetic element and important unique restriction sites; size, annotated complete nucleotide sequence (from right border to left border); GenBank accession number (if known), Open Reading Frame (ORF) analysis on all six reading frames; bioinformatics analysis to assess homology of products of each ORF with known allergens, toxins and anti-nutrients.

c. *Details of each genetic element:*

Feature Type (Antisense, Coding, Promoter, Intron etc.); GenBank accession number (if known); start and end positions (bp), size, source organism; modification(s) made in native DNA sequence (*e.g.*, codon optimization, truncation, etc.), function and mode of action; details if the genetic element(s) gives rise to any infectious agent.

d. *Details of each expressed ORF:*

Start and end positions of the coding region (bp) in the gene construct; size (number of aa) and molecular weight (kDa); amino acid sequence; GenBank accession number (if known); type of expression (*e.g.*, constitutive, inducible, etc.); known post-translational modification in source organism; any known human allergenicity or toxicity to humans or non-target organisms; history of safe use of the expressed genetic element.

5. Characterization of the introduced DNA:

Confirmation of the presence of each introduced gene; copy number of each detectable gene construct insert; complete nucleotide sequence of the insert (from RB to LB); integrity of inserted gene construct in the event {complete or/and partial copy(ies)}; fidelity of the inserted gene construct if significant rearrangement(s) observed in integrated gene construct(s) and/or at the insertion locus of the plant genome; data to confirm absence of vector backbone DNA sequence(s).

6. Characterization of site of integration in the recipient genome:

Nucleotide sequences of flanking regions bordering the site of gene construct insertion; nucleotide sequence of the pre-insertion locus in the non-GE host plant genome; Genomic location (Chromosome locus) of the site of integration; information on endogenous genes of the host plant in the flanking region; homology of the flanking region nucleotide sequences to the pre-insertion locus nucleotide sequences of the recipient non-GE host plant; analysis of the junction site for rearrangements and deletions in comparison to the pre-insertion locus; identification of any new ORF (on all six reading frames) within the inserted DNA and created by the insertion with contiguous plant genomic DNA; ORF analysis (on all six reading frames) of the insertion locus after joining RB and LB flanking sequences and comparative analysis with ORFs present at the pre-insertion locus; bioinformatics analysis for assessing homology of each new ORF with known allergens, toxins and anti-nutrients; and expression profile of potential new ORFs identified at junction sites or within the integrated gene construct.

7. Detection of the GE event:

Validated event-specific identification protocol at Limit of Detection 0.01% (LoD 0.01%), and oligonucleotide sequence of event-specific primers.

8. Studies with introduced protein(s) purified after overexpression in non-plant expression systems:

Thermal stability; Pepsin digestibility; Immunological assays for assessment of possible allergenicity (for proteins that have sequence homology with a known allergen or originate from a source known to be allergenic); Acute oral toxicity study; Functionality of the purified proteins used for biosafety studies (functional and biochemical equivalence of the non-plant expressed protein with the plant expressed protein).

Annexure II: Molecular characterization data requirements for stacked GE plants

1. Description of stacked line

a. Method of the stacking: Event stacking by breeding, event stacking by genetic transformation (re-transformation or co-transformation), or a combination of these.

b. Name/Unique Identifier of the stacked GE plants; Name and basic phenotypic characteristics of the background genotype (variety/hybrid).

c. Purpose of the modification, stacking (introduced trait(s)) and phenotypic description of the new trait(s).

d. Basic phenotypic characteristics, DNA fingerprint of the stacked line as well as that of individual parent events (in case of event stacking by breeding or re-transformation).

e. Markers for introduced genes along with event-specific markers for all individual GE parent events.

f. Number of generation cycles and pedigree of the final stacked line (starting from primary transformation of each event).

2. Homology of introduced DNA sequences

a. Homology among the introduced genes and regulatory sequences.

b. Homology of any introduced gene or regulatory sequences of gene constructs with endogenous genes/ regulatory sequences of the host plant used for genetic engineering, if genome sequence of the host plant is available:

i. Name of homologous/ orthologous genetic elements in the host plant.

ii. GenBank Accession Numbers of homologous/ orthologous genetic elements (wherever available) and nucleic acid sequence in an appropriate format (*e.g.*, .txt, .fasta, .fsa, .doc, .docx).

iii. Extent (%) of similarity between the nucleic acid sequences.

3. Gene construct integration and copy number analysis

a. Confirmation of transformation as well as the presence of every major genetic element of each gene construct.

b. Copy number analysis of each DNA insert (gene construct) in the stacked line by an appropriate method (*e.g.*, Southern blot). In case of GE breeding stack containing approved events, the previously generated data on copy number analysis of that approved event may be considered.

4. **Segregation and zygosity analysis**

a. For stack developed by genetic transformation, segregation data pertaining to introduced genes of each gene construct in T_1 progeny. For GE breeding stack, segregation analysis of each unapproved introduced event.

b. Zygosity status of each introduced event in the stacked GE plant proposed for confined field trial.

5. **Genetic stability**

The stability of stacked gene constructs for at least two additional generations to determine if there is any DNA rearrangements in introduced gene constructs and/or at the insertion locus of the plant genome.

6. **Expression of introduced genes: pre-stacking vs. post-stacking**

a. Protein or transcript expression of all introduced genes in the stacked GE plant at multiple receiving environments where the stacked GE plant is intended to be cultivated.

b. Individual parent events of stacked GE plant developed by breeding and/or by re-transformation should be included as comparator for expression analysis.

c. ***Transcript expression analysis of each transcript*** *(essential for untranslated RNA and intractable proteins):*

Type of expression (*e.g.*, constitutive, inducible, tissue specific, developmental stage); Quantitative analysis (range and mean ± error) of expression level of transcript in each major relevant tissue/organ at key developmental stages; details of induction stimuli/conditions, tissues (for tissue specific expression) etc. of introduced genes used for the analysis, method used for the expression analysis.

d. ***Protein expression analysis of each translation product*** *(Expression data in multiple environmental conditions, and/or generations, seasons, years. For vegetatively propagated species and for species with long reproductive cycles, evaluation expression in multiple environments or over multiple years):*

Type of expression (*e.g.*, constitutive, inducible, tissue specific, developmental stage); Quantitative analysis (w/w, range and mean ± error) of expression level in each major relevant tissue(s)/organ(s) at key developmental stages; Maximum expression level in edible parts; Details of induction stimuli/conditions, particular tissue(s), and/or the particular developmental stage for inducible and/or development stage specific expression of introduced genes. Details of the protein detection method used for the expression analysis and the limit (ng) of the detection method followed.

e. Analysis of partial or complete gene silencing (if applicable to the introduced trait(s)), in case of RNAi and antisense transgenics.

f. Analysis of silencing of the introduced genes where expression of the introduced gene is significantly lower in the stacked GE plant in comparison to the individual parent events (GE breeding stacks and/or re-transformation molecular stacks), if required.

g. Characterization of Post-translational modification(s), if required.

Annexure III: Biosafety assessment studies of stacked GE plants

III.A. Data to be generated for stacked GE plants developed by genetic transformation

Stacking method	Approval status of individual events		Molecular characterization	Trait Efficacy	Food and feed safety studies		Environmental safety studies		Phenotypic equivalence
	Approved events	Individual event / Stacked GE plant			Studies with pure protein	Studies with plant part(s)	Non-target organisms	Pollen Flow & Crossability	
Event stacking by Re-transformation	None	Stacked GE plant	R	R	R	R	R	R	R
	Some (*One or More*) (*)	Approved events	A	NR	A	NR	NR	NR	NR
		Stacked GE plant	R (#)	R	R (▲)	R	R	R	R
Event stacking by Co-transformation	None	Stacked GE plant	R	R	R	R	R	R	R

R - Required
NR - Not required
A - Earlier data used for deregulation of the event is acceptable (To be resubmitted for records)
(#) - Genetic stability and expression analysis for all introduced genes through individual events in stacked GE plant will be done.
(▲) - Study required for new protein only
(*) - One or more events used for re-transformation are approved earlier.

Note: Refer section 4.7 for comparator(s) to use in biosafety assessment of stacked GE plant.

III.B. Data to be generated for stacked GE plants developed by breeding

Stacking method	Approval status of individual events		Molecular characterization	Trait Efficacy	Food and feed safety studies		Environmental safety studies		Phenotypic equivalence
	Approved events	Individual event / Stacked GE plants			Studies with pure protein	Studies with plant part(s)	Non-target organisms	Pollen Flow & Crossability	
Event stacking by Breeding	None	Unapproved events	NR	NR	NR	NR	NR	NR	NR
		Stacked GE plant (*)	**R**	**R**	**R**	**R**	**R**	**R**	**R**
	Some	Approved events	**A**	NR	**A**	NR	NR	NR	NR
		Unapproved events	**R**	NR	**R**	NR	NR	NR	NR
		Stacked GE plant (*)	**(#)**	**R**	NR	**R**	**R**	**R**	**R**
	All	Approved events	**A**	NR	**A**	NR	NR	**A**	NR
		Stacked GE plant	**(#)**	**R**	NR	**R**	**R**	NR	**R**

R - Required (Data to be generated)
NR - Not required
A - Earlier data used for obtaining deregulation is acceptable (To be resubmitted for records)
(#) - Genetic stability and expression analysis for all introduced genes through individual events in stacked GE plant will be done.
(*) - If subset (lower order) GE breeding stack are also intended to be deregulated, individual parental events will require complete environmental, food and feed safety studies.

Note: Refer section 4.7 for comparator(s) to use in the biosafety assessment of the stacked GE plant.

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- ✓ **Working Group** under the Chairmanship of Prof. Deepak Pental, Former Vice Chancellor, University of Delhi, New Delhi

- ✓ **Review Committee of Genetic Manipulation** (RCGM)

- ✓ **Biosafety Support Unit, New Delhi** (Regional Centre for Biotechnology, Faridabad)

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