Frequently Asked Questions

GM MUSTARD
HYBRID TECHNOLOGY

BCIL
Biotech Consortium India Limited
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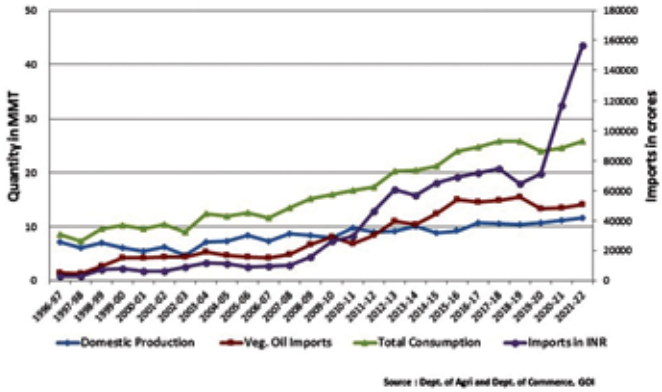
Introduction

Hybrid seed production technology using genetic engineering techniques in mustard (commonly referred to as **GM mustard**) was approved for environmental release in India by the Ministry of Environment, Forest and Climate Change (MoEFCC), Government of India on October 25, 2022.

In 2021-22 India imported 14.1 million tonnes of edible oils for Rs. 1,56,800 crores. The expenditure on edible oil imports in 2022-23 has further increased to Rs. 1,67,270 crores. Raising the yield of edible oil crops is therefore critical.

Domestic Production and Import of Vegetable Oils



Mustard is currently the number one oilseed crop in terms of its percentage contribution to total edible oil production in India. It is cultivated in 6-7 million hectare during Rabi winter season. The average mustard yield in the country is stagnant for almost two decades. The yield of mustard needs to be raised from the current national average of 1.2 tons/ hectare to at least 2 tons/ hectare in the next 10 years. This requires productive hybrids, yield stabilization through developing disease-resistant varieties and hybrids, and improvement in oil and meal quality for value addition.

Introduction of GM hybrids in rapeseed (a sister crop of mustard) has contributed significantly to yield enhancement globally since these were allowed first in Canada in 1996. Globally, GM canola is cultivated in more than 10 million hectares. The oil and seed meal produced from GM canola has been used for food and feed purposes all over the world for more than 25 years.

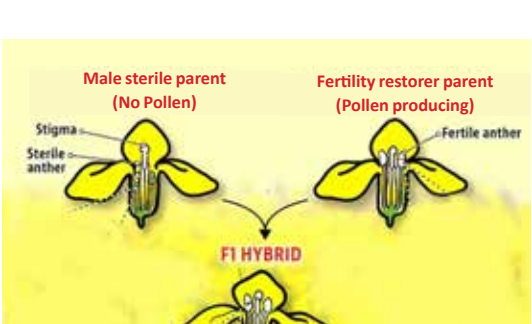
The release of GM mustard in India is expected to allow development of productive hybrids and contribute to enhancing mustard productivity in India, thereby improving farmers' incomes and reducing the import bill.

This brochure provides answers to some of the frequently asked questions on GM mustard hybrid technology in simple language with a view to facilitate easy understanding.

1 What is the GM mustard hybrid technology?

GM mustard hybrid technology refers to the use of genetic engineering to produce mustard hybrids using the *barnase-barstar* gene system.

Mustard is a predominantly self-pollinating crop. To facilitate cross-pollination male sterile (MS) lines containing the *barnase* gene are crossed with fertility restorer (RF) lines containing the *barstar* gene to produce hybrid seeds between two different parental lines. The barnase and the barstar lines also contain the *bar* gene which confers resistance to herbicide phosphinothricin. The *bar* gene is required for developing the *barnase* and *barstar* gene-containing lines and in the production of hybrid seeds.



2 What is the *barnase-barstar* gene system?

The *barnase-barstar* system is a novel way to develop male sterile (MS) lines and fertility restorer lines (RF) through genetic engineering. The system was developed by scientists in Belgium in the early 1990s using the *barnase* and *barstar* genes from a non-pathogenic soil bacterium *Bacillus amyloliquefaciens*. In nature, the bacterium excretes a defence protein called Barnase (a type of ribonuclease) which degrades the RNA of competing bacteria in its ecological niche. To protect itself from Barnase, the bacterium produces another protein called Barstar which tightly binds with Barnase and renders it ineffective.

In the GM mustard, both the *barnase* and the *barstar* genes have been expressed under a tapetum-specific promoter. In the *barnase* gene-containing lines, the tapetum tissue ablates (dies) – consequently developing pollen degenerate, providing MS plants with no pollen. The other parental line is fully fertile, contains the *barstar* gene that also expresses in the tapetum cells and helps in the restoration of fertility in the hybrids. The MS line receives pollen from the RF line through wind pollination or bee pollination, resulting in the production of hybrid seed that has both the *barnase* and the *barstar* genes. The Barstar protein binds to the Barnase protein making it ineffective, thereby hybrids grown in the field by the farmers are fully fertile.

Thus, the *barnase-barstar* system ensures that the MS line will only produce hybrid seeds by outcrossing with RF lines and the hybrids are fully fertile thereby providing an efficient system of pollination control for the production of hybrid seed.

The *barnase-barstar* system for hybrid seed production has been used in rapeseed for hybrid seed production since 1996.

3 What are the benefits of GM mustard hybrid technology?

Crossing of genetically diverse parents results in hybrids with increased yield and wider adaptation, a phenomenon known as hybrid vigour or heterosis which has been widely exploited in crops like rice, maize, pearl millet, sunflower and many vegetable crops. Hybrids in general give 20-25% higher yield over the conventional varieties across the crops.

While India has several mustard varieties, it is a self pollinating plant and therefore a challenge for plant breeders to develop hybrids in the crop. A robust pollination control mechanism is required to disallow self-pollination and facilitate cross-pollination to produce hybrid seeds between two different parental lines. Hence, GM mustard hybrid technology can play a critical role in enhancing the productivity of mustard and thereby enhance the availability of oil for human consumption and seed meal for animals in the country.

4 How GM mustard hybrid technology is different from the already available hybrid production systems?

Non-GM hybrid seed production systems such as cytoplasmic male sterility (CMS) have been tested in mustard and some hybrids have also been released. However, these systems have limitations of breakdown of sterility under certain environmental conditions leading to lowering of seed purity in large-scale multisite hybrid seed production. As a consequence, the Ministry of Agriculture through Office Memorandum No. 15-13/2014-SD.IV reduced the usual purity standard of hybrid seeds of rapeseed and mustard from 95% to 85% under section 6(9) of the Seeds Act, 1966 in 2014. The current non-GE systems are not robust enough for large-scale hybrid seed production in comparison to the *barnase-barstar* based GM mustard hybrid technology.

The *barnase-barstar* system is extremely versatile and works in any genetic background to provide hybrid seeds with high purity.

5 Who has developed GM mustard hybrid technology in India?

The Centre for Genetic Manipulation of Crop Plants (CGMCP) at the University of Delhi South Campus, New Delhi has developed the two parental lines – Varuna bn 3.6 (MS line) and EH-2 modbs 2.99 (RF line), and their hybrid DMH-11 using the *barnase-barstar* technology. The two parental lines and hybrid DMH-11 have been developed by a team led by Prof. Deepak Pental, a well-known geneticist and former Vice Chancellor of the University of Delhi with financial support from the Department of Biotechnology (DBT), and the National Dairy Development Board (NDDB). Biosafety evaluation of the GM parental lines and the first hybrid DMH-11 was undertaken with support from the Biotechnology Industry Research Assistance Council (BIRAC). All the funding organisations belong to the Government of India.

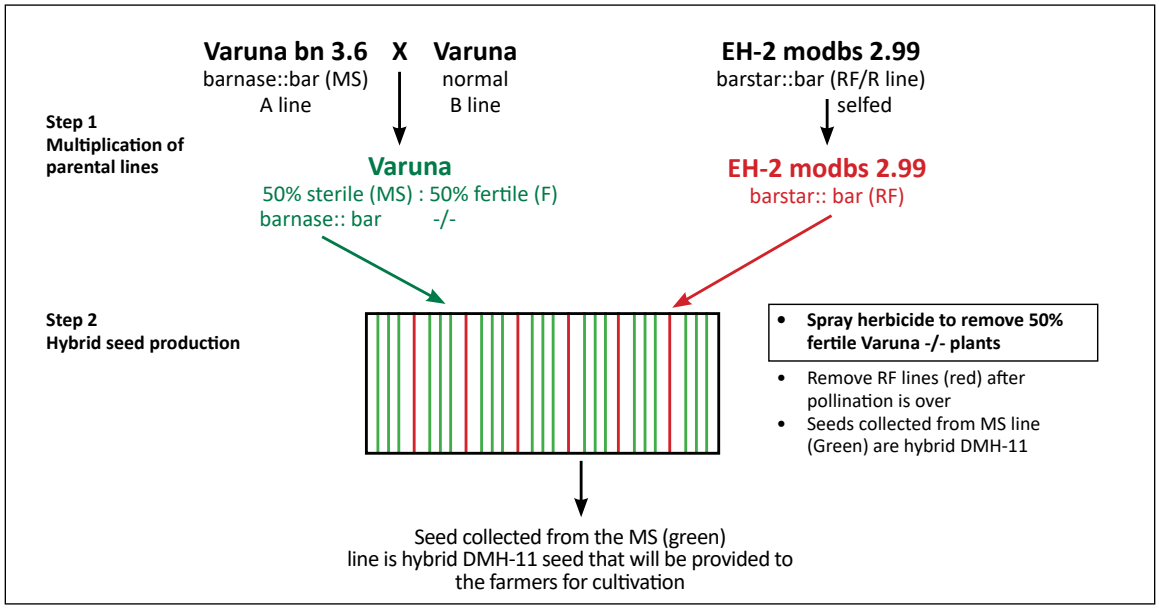
6 How are hybrid seeds produced with the *barnase-barstar* systzem?

This is illustrated with the example of DMH-11, the first GM mustard hybrid developed by the University of Delhi.

Male sterile (MS) lines cannot self-pollinate to reproduce and are maintained (multiplied) by crossing with a normal line. For example, Varuna bn 3.6 (MS line) is crossed with Varuna. The progeny of this cross segregates 1:1 – MS (contain *bar::barnase* transgenes in a linked manner) : fertile (no transgenes) plants. The restorer line EH-2 modbs 2.99 containing the *bar::barstar* transgenes in a linked manner is multiplied by self-pollination since it is fully fertile.

For the production of the hybrid seed, the production plot is sown with three to five rows of seeds obtained from the MS x normal line cross which would segregate 1:1 male sterile (*bar::barnase*): male fertile (no transgenes) plants alternating with a row of the fertility restorer (RF) line (*bar::barstar*). After germination herbicide phosphinothricin will be sprayed on the hybrid seed production plot to eliminate the fertile plants (that contain no transgenes) from the MS x normal cross progeny; the remaining plants will be MS plants since the *bar* gene confers tolerance to herbicide phosphinothricin in the male sterile progeny. These MS plants will receive pollen from the RF parent to provide hybrid seeds with very high purity. Hybrid seeds will give completely fertile plants in the farmers' fields.

The use of herbicide phosphinothricin to eliminate the fertile plants with no transgenes in the hybrid seed production plots is an essential step for hybrid seed production with the *barnase-barstar* system. However, there is no need for the use of phosphinothricin in the farmers' fields.



7 Is GM mustard released as an herbicide-tolerant (HT) crop?

No. The GM mustard hybrid DMH-11 has not been released as an herbicide-tolerant (HT) crop even though the hybrid contains the *bar* gene that confers resistance to phosphinothricin. The use of herbicide phosphinothricin is neither needed nor has been allowed in the farmers' fields. The use of phosphinothricin is approved only in hybrid seed production plots where its use is essential to produce hybrid seed. The government of India has not permitted the use of phosphinothricin in the farmers' fields.

Numerous studies have shown that phosphinothricin degrades very quickly (half-life is around 2 – 3 days in an open field) and does not impact soil health. The amount of phosphinothricin to be used in the hybrid seed production is well within the safety limits.

For the management of weeds in mustard crops, besides manual weeding, farmers are already using two pre-emergence herbicides Pendimethalin and Fluchloralin which have been approved by the Central Insecticides Board & Registration Committee for use on mustard. Therefore the option of using herbicides is already available to the farmers growing mustard.

8 What is the performance of GM mustard hybrid vis-à-vis pure line varieties?

In over three years of field studies (BRL-I and BRL-II trials carried out under the biosafety analysis) at multiple locations undertaken by the Indian Council of Agricultural Research-Directorate of Rapeseed Mustard Research (ICAR-DRMR), hybrid DMH-11 demonstrated 28% more yield than the national check variety Varuna which also happens to be one of the parents of the hybrid DMH-11. The heterotic potential of the first GM mustard hybrid has been established.

The *barnase-barstar* system developed by the University of Delhi for mustard is a complete and functional male-sterility/restorer system that could be diversified into better combiners and used to produce new hybrids with higher yields than DMH-11 in the years to come.




9 Is GM mustard safe? Has it been tested for biosafety and environmental safety?

Yes. Extensive biosafety studies were carried out on the two parental lines Varuna bn 3.6, EH-2 modbs 2.99, their comparators – normal Varuna and EH-2, and hybrid DMH-11 developed by University of Delhi, despite very involved biosafety analysis of the rapeseed lines containing the three transgenes – *bar, barnase*, and *barstar* in Canada, the US, and Australia before the environmental release of GM hybrids. Biosafety studies were undertaken following the guidelines of the government of India. Protocols and methodology for all the biosafety studies were approved by the Review Committee on Genetic Manipulation (RCGM) and Genetic Engineering Appraisal Committee (GEAC). Most of the studies were carried out in the leading publicly funded institutions of the country under the guidance of subject-specific experts.

Key findings were:

- None of the three proteins – Bar, Barnase and Barstar have been shown to be toxic or allergenic through bioinformatics and acute toxicity studies. All the three proteins are rapidly degraded in the mammalian digestive systems. Sub-chronic toxicity studies using edible plant parts i.e. leaves and seeds also did not show any adverse effects.
- The composition of the GM parental lines does not vary as compared to the non-GM comparators. The glucosinolate content also does not vary between the transgenic and non-transgenic lines.
- All the three proteins – Bar, Barnase, and Barstar express at low or very low levels in the target plant tissues and there are no post-translational modifications of the three proteins in the plant cells.
- Extensive field studies demonstrated that agronomic, phenotypic, and other characteristics are similar in GM mustard lines and their non-GM comparators.
- Intra-specific and inter-specific crossability studies demonstrated that the potential of outcrossing in the transgenic lines is no different from that of their non-transgenic comparator. No inter-specific hybrid seed was obtained under natural field conditions.

Studies undertaken for safety assessment of GM mustard

Parental lines (Varuna bn 3.6, EH-2 modbs 2.99) and Hybrid (DMH-11)

Category	List of Studies	Conducted/Undertaken by
Molecular characterization	➢ Gene sequences, constructs, and molecular characterization of integration sites in the mustard genome.	➢ CGMCP[1], University of Delhi
	➢ Expression studies of the three inserted genes – *bar, barnase*, and *barstar*	
Food safety studies	➢ Cloning, expression, purification, and production of the three proteins in *E. coli*	➢ Premas Biotech Pvt. Ltd., Manesar
	➢ Equivalence of the Bar, Barnase, and Barstar recombinant proteins produced in E. coli with the proteins expressed in the transgenic plants	➢ Premas Biotech Pvt. Ltd. Manesar ➢ CGMCP, University of Delhi
	➢ Bioinformatics analysis of the three proteins for allergenicity	➢ ICMR[2]- National Institute of Nutrition (NIN), Hyderabad
	➢ Pepsin digestibility of the three proteins	
	➢ Heat stability of the three proteins	
	➢ Acute oral toxicity of the three proteins in mice	
	➢ Sub-chronic toxicity of leaves and seeds containing the three proteins in rats	
	➢ Compositional analysis	
Environmental safety studies	➢ Field trials from 2004 – 2007	➢ CGMCP, University of Delhi & ICAR
	➢ BRL-I field trials for two growing seasons (2010-11, 2011-12) ➢ BRL-II field trial for one growing season (2014-15)	➢ ICAR[3]- Directorate of Rapeseed & Mustard Research (DRMR), Bharatpur
	➢ Weediness potential and aggressiveness parameters	➢ CGMCP, University of Delhi
	➢ Impact on soil microflora during BRL-I and BRL-II trials	➢ CSIR[4]- Institute of Microbial Technology, Chandigarh
	➢ Crossability and pollen flow studies	➢ CGMCP, University of Delhi
	➢ Pollination behavior, pollen morphology and physiology	➢ CGMCP, University of Delhi & ICAR- Directorate of Rapeseed & Mustard Research (DRMR), Bharatpur
Detection protocols for the three proteins – Bar, Barnase, and Barstar	➢ Protocol for testing at a level of detection (LOD) of 0.01%	➢ Developed by CGMCP, University of Delhi ➢ Validated by ICAR – National Institute of Plant Biotechnology (NIPB), New Delhi
	➢ Development of ELISA kits for Bar, Barnase and Barstar	➢ Amar Immunodiagnostics Pvt. Ltd., Hyderabad

[1] *Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus*
[2] *Indian Council of Medical Research (ICMR)*
[3] *Indian Council of Agricultural Research (ICAR)*
[4] *Council of Scientific & Industrial Research (CSIR)*

10 Are products such as “Sarson da saag” derived from GM and non-GM mustard nutritionally equivalent?

Yes, GM mustard is nutritionally equivalent to non-GM mustard. Nutritional and compositional analysis of seeds and leaves of the GM lines Varuna bn 3.6, and EH-2 modbs 2.99 have been compared with their non-transgenic counterparts Varuna and EH-2. Based on the results of these comparisons, it can be concluded that there will be no difference in the products derived from GM and non-GM mustard including “Sarson da saag”.

11 How oil and seed meal derived from the GM mustard are different from the products of the currently grown mustard varieties?

Mustard oil does not contain any protein. Therefore, oil extracted from DMH-11, or any other future hybrids will not have either of the three proteins - Bar, Barstar, or Barnase. The meal from hybrid DMH-11 will not contain any Barnase or Barstar proteins, only Bar protein at low levels will be present.

Oil and seed meal extracted from GM rapeseed containing the same three proteins that have been expressed in mustard has been consumed in many developed and developing countries, including India for more than two decades and no adverse effects have ever been reported.

12 Is India a centre of origin or diversity of mustard? What would be the impact of growing GM mustard on biodiversity in India?

No, India is not the centre of origin of mustard. Mustard (*Brassica juncea*) most likely originated in the middle- east.

A pangenome sequencing study of 408 mustard accessions from all over the world has shown five sub-gene pools in mustard. The Indian gene pool is one of them and has been clearly shown to be very narrow in diversity.

Studies on the crossability of GM mustard hybrid with the related cultivated and wild species present in India conducted as a part of the biosafety studies have demonstrated that the possibility of hybridization in the field and viable seed set is virtually nil.

13 Will GM mustard cross-pollinate and contaminate non-GM mustard?

The varieties and hybrids are multiplied in isolation of 100-200 meters to maintain seed purity following the well-established ICAR guidelines. All the earlier and new varieties are maintained in the breeding stations by multiplying the seeds in isolation. Seeds of the varieties are deposited in the National Bureau of Plant Genetic Resources (NBPGR).

The spread of transgenes follows well-established models of population genetics. Any concern about contamination is only valid before the biosafety studies are completed on the effect of the transgenes. In the case of GM mustard, the biosafety regulatory authorities, after a detailed evaluation of the biosafety studies have concluded that these lines are as safe as their non-GM counterparts for human and livestock consumption and will have no ill effects on the environment. The three proteins – Bar, Barnase, and Barstar have been shown to be completely innocuous in the biosafety tests and contamination should not be a matter of concern.

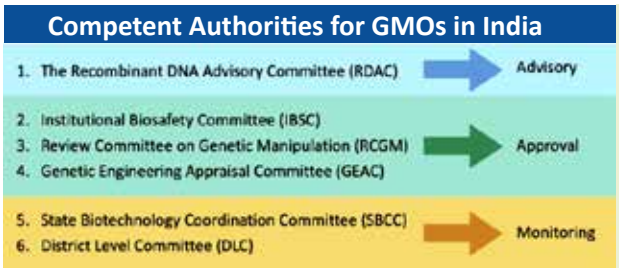
14 What would be the impact of GM mustard on honeybees and honey production?

Mustard flowers attract honeybees and provide pollen and nectar as sources of food for honeybees. In the BRL-I and BRL-II trials, the male sterile lines have seed set equivalent to normal comparator lines indicating that bees visit male sterile and restorer transgenic lines without any preference or discrimination. Bee foraging studies indicated no adverse impact of GM mustard.

In Canada, GM rapeseed is grown in around 8.64 million hectares every year. No adverse effects of the transgenic rapeseed on honeybees have been reported from Canada or the other countries growing GM rapeseed and they continue to maintain healthy production and export of honey.

15 Who has reviewed and approved GM mustard in India?

India has a systematic and structured regulatory framework for biosafety evaluation of genetically modified organisms (GMOs) and products thereof as per "Rules for manufacture, use/import/ export & storage of hazardous microorganisms/ genetically engineered organisms or cells, 1989" notified under the Environment (Protection) Act, 1986. India's biosafety regulatory system is fully aligned with provisions of the Cartagena Protocol on Biosafety.



Six Competent Authorities, their composition, and roles have been notified in the Rules. While the Recombinant DNA Advisory Committee (RDAC) is advisory in function, the Institutional Biosafety Committee (IBSC), Review Committee on Genetic Manipulation (RCGM) and Genetic Engineering Appraisal Committee (GEAC) are responsible for regulatory function. State Biotechnology Coordination Committee (SBCC) and District Level Committee (DLC) are for monitoring purposes.

Rules, 1989 are implemented by MoEFCC jointly with the Department of Biotechnology (DBT), Ministry of Science & Technology and the state governments.

Every proposal is subjected to in-depth review at each stage of the development viz. initiation of research in laboratory, greenhouse, field assessment of safety, impact on food, feed, and environment by appropriate committees mentioned in the figure above. The responsibility of recommending the final environmental release is with GEAC.

Development of GM mustard was undertaken with due approval from the IBSC of University of Delhi and other institutions. All the stipulated biosafety studies were designed and carried out after approvals from the RCGM and GEAC. A 3251-page document containing the observations and results of all the biosafety studies (listed on page 9) was submitted to GEAC. An expert committee was set up by GEAC to undertake a detailed assessment of the dossier. A report on Assessment of Food and Environmental Safety (AFES) was posted on the GEAC website for public consultations.

GEAC recommended the environmental release of the GM parental lines Varuna bn 3.6, EH-2 modbs 2.99, and the first GM mustard hybrid DMH-11 in its 147th meeting held on 18.10.2022. The parental lines containing events bn 3.6 (*barnase* :: *bar* genes) and modbs 2.99 (*barstar* :: *bar* genes) could be used for developing new sets of MS and RF lines that would provide next generation of hybrids with higher yield, quality traits, and resistance to pests. GEAC allowed seed production of hybrid DMH-11 as per the ICAR guidelines and asked for mutisite trials of DMH-11 along with the checks by ICAR-DRMR in All India Coordinated Research Project on Rapeseed-Mustard (AICRP-RM) for two growing seasons for the eventual commercial release of the hybrid. In addition, studies with respect to the effect of GE mustard on honey bees and

other pollinators are to be conducted post-environmental release, within two years, under the supervision of ICAR.

In the Rabi season 2022-23, GM mustard hybrid DMH-11 was tested at six locations as part of AICRP-RM trials conducted by ICAR-DRMR. Seed multiplication was undertaken by CGMCP. A post release Monitoring Committee visited all the locations.

16 What are the guidelines and protocols used by regulatory authorities for assessing safety of GM plants like GM mustard?

Regulatory authorities in India have well defined guidelines and protocols for the safety assessment of GM crops, documents describing these are mentioned in the box below. The guidelines and protocols are in consonance with the internationally accepted principles and methodologies.

Guidelines for safety assessment of foods derived from genetically engineered (GE) plants were prepared by the Indian Council of Medical Research (ICMR) in 2008 and were accepted by GEAC in its 85th meeting held on 28/05/2008. These guidelines are based on the Principles and Guidance published in 2003 by the Codex Alimentarius Commission, an FAO and WHO joint body. Guidelines for environmental risk assessment of GE plants, 2016 are based on the processes and protocols provided in the Cartagena Protocol on Biosafety. Copies of the guidelines can be accessed at www.geacindia.gov.in.

Government of India is following a case-by-case safety assessment of the GE plants. The information requirements and analysis may vary depending on the crop specific trait and intended use.

Safety evaluation of GM mustard comprised of food and feed safety assessment and the environmental risk assessment coupled with information on the molecular characterization of the events and characterization of the expressed, transgenic proteins. All the stipulated guidelines and protocols contained in the documents mentioned below were followed.

Biosafety Guidelines for GE Plants in India	
Contained use (DBT)	• Regulations and Guidelines on Biosafety of Recombinant DNA Research & Biocontainment, 2017 • Revised Guidelines for Research in Transgenic Plants, 1998
Confined Field Trials (MoEFCC and DBT)	• Guidelines for Conduct of Confined Field Trials (CFTs) of Regulated, Genetically Engineered Plants (GE) Plants, 2008 • Standard Operating Procedures (SOPs) for CFTs of Regulated, GE Plants, 2008 • Guidelines for Monitoring of CFTs of Regulated, GE Plants, 2008
Food Safety Assessment (DBT and ICMR)	• Guidelines for the Safety Assessment of Foods Derived from GE Plants, 2008 (Updated in 2012) • Protocols for Food and Feed Safety Assessment of GE Crops, 2008
Environmental Safety Assessment (MoEFCC and DBT)	• Guidelines for Environmental Risk Assessment (ERA) of GE Plants, 2016 • Risk Analysis Framework, 2016 • ERA of GE Plants: A Guide for Stakeholders, 2016

17 What is the source of the *barnase-barstar* technology deployed in mustard for hybrid seed production? Is it IPR protected?

CGMCP scientists have made their own constructs with the *bar*, *barnase*, and *barstar* genes with substantial modifications to the original system and received both national and international patents. The herbicide phosphinothricin (Basta) required in the hybrid seed production plots is currently out of patent protection globally. A few agrochemical companies in India manufacture the herbicide, mainly for export purposes.

18 Do farmers have to pay royalties?

No, the technology has been developed with public funding and hence will be transferred to seed producers as per the government of India norms. The farmers will not have to bear the cost of technology development. Hybrid seed costs, therefore, will remain only a very small percentage of the total cost of cultivation.

19 Is GM mustard approved and cultivated in other countries?

Yes, *barnase-barstar*-based GM rapeseed was approved for hybrid seed production in Canada in 1996, in the USA in 2002, and in Australia in 2003.

Currently, more than 90% percent of Canadian rapeseed (also called Canola) is hybrid. The first productive hybrid was released in Canada in 1998. This hybrid showed a yield increase of only 13% over the Canadian rapeseed mega variety Westar. Since then, many hybrids have been released with higher yields. In 2008, a hybrid was released with a 46 % yield advantage over Westar.

Canada is a big exporter of rapeseed oil and seed meal. Oil and seed meal from Canola rapeseed have been consumed as food by humans and seed meal by livestock and poultry all over the world with no ill effects reported either in the growing country or in the consuming countries.

20 How will GM mustard benefit farmers in India?

Seed replacement (farmer buying fresh seed) rates in mustard are around 79 % and the area under irrigation has increased to 80 % of the total area under mustard. Despite all these investments, the yields of mustard are stagnating.

The release of GM mustard parental lines would allow public and private organizations to produce productive hybrids of their own. DMH-11 is the first hybrid developed using the *barnase-barstar* system, more will follow. A good pollination control mechanism is a one-time development. Developing productive hybrids is a continuous activity that will contribute to enhancing mustard productivity in India.

If productive hybrids are available, farmers will adopt these readily to increase their income. India will reduce its import bill. Farmers of the country will earn the amount which is currently going to the farmers of other countries.

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