ASSESSMENT OF FOOD AND ENVIRONMENTAL SAFETY (AFES) FOR

Environmental release of Genetically Engineered Mustard (*Brassica juncea*) hybrid DMH-11 and use of parental events (Varuna bn3.6 and EH2 modbs2.99) for development of new generation hybrids

Application submitted by

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CHAPTER 1
INTRODUCTION

The Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus, New Delhi has sought approval for environmental release of genetically engineered (GE) oilseed mustard (botanical name *Brassica juncea*) hybrid DMH-11 and use of parental events (Varuna bn 3.6 and EH-2 modbs 2.99) for development of new generation hybrids under Rules 1989 (The Manufacture, Use, Import, Export and Storage of Hazardous Micro Organisms/ Genetically Engineered Organisms or Cells) of Environment (Protection) Act, 1986.

CGMCP through extensive R&D work, financially supported by the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India and the National Dairy Development Board (NDDB), has developed male sterile and restorer lines using three transgenes- *barnase*, *barstar* and *bar* for hybrid seed production in *B. juncea*, a major oilseed crop of India. Research work on the development of the parental lines (Varuna bn 3.6 and EH-2 modbs 2.99) was published in 2001 and 2002 (Jagannath et al 2001, 2002). Studies on the biosafety of the parental lines and the resultant hybrid DMH-11 have been carried out since 2008 with the financial support extended by the Biotechnology Industry Research Assistance Council (BIRAC), a public sector enterprise set up by DBT.

The applicant in their research found that there are two diverse gene pools in mustard- the Indian gene pool and east European gene pool. Hybrids between lines of the two gene pools were found to be heterotic for yield (Pradhan et al 1993, Srivastava et al 2001). Heterosis breeding has potential for substantially increasing crop productivity. Since *B. juncea* is predominantly a self-pollinating crop (http://biosafety.icar.gov.in/wp-content/uploads/2015/11/Biology_of_Brassica_juncea_Ca.pdf), a pollination control mechanism is required to facilitate cross-pollination for production of hybrid seeds. To achieve this, one of the parents has to be male sterile. A novel way for developing male sterile (MS) lines through genetic engineering was developed by scientists in Belgium in early 1990s using two genes – *barnase* and *barstar* – isolated from a common soil bacterium *Bacillus amyloliquefaciens*. Both Barnase and Barstar protein encoding genes were expressed in specific cells (tapetum) of anthers that are the male part of the flowers using a tapetum specific promoter. Developing

pollen degenerates in the parental line expressing the *barnase* gene, thereby, providing a MS line. The other parent called restorer of fertility (RF) line, contains the *barstar* gene that also expresses in the tapetum cells. The MS line receives pollen from the RF line resulting in the production of hybrid seed. The hybrid seed thus produced has both the *barnase* and the *barstar* genes and the hybrid plants, cultivated by the farmer, are fully fertile. Thus, the MS/ RF system ensures that the MS line will only produce hybrid seeds by outcrossing with RF lines thereby providing an efficient system of pollination control for production of hybrid seed in mustard.

1.1 The application

The applicant has requested for approval of environmental release of the transgenic mustard (*Brassica juncea*) hybrid DMH-11 and use of parental events (Varuna bn 3.6 and EH-2 modbs 2.99) for development of new generation hybrids to the GEAC, MoEF&CC, Government of India. Summary of the application and information on the organizations that carried out the biosafety studies are provided in **Table 1.1.**

The applicant has conducted biosafety studies as per Rules, 1989 and biosafety guidelines and directives issued from time to time by the statutory bodies– Institutional Biosafety Committee (IBSC), Review Committee on Genetic Manipulation (RCGM) and Genetic Engineering Appraisal Committee (GEAC). The list of studies undertaken for the safety assessment of GE mustard parental lines and the hybrid is provided in **Table 1.2**.

The following lines were tested –

- Varuna bn 3.6 (Indian gene pool line Varuna containing the *barnase* gene);
- Non-GE Varuna parent;
- EH-2 modbs 2.99 (east European type breeding line containing the *barstar* gene);
- Non-GE EH-2 parent;
- GE mustard hybrid DMH-11 (Varuna bn 3.6 X EH2 modbs 2.99); and
- RL1359/ Maya (zonal checks)

Table 1.1: Information about the application

Project Title:	Environmental release of Genetically Engineered Mustard (Brassica juncea) hybrid DMH-11 and use of parental events (Varuna bn3.6 and EH2 modbs2.99) for development of new generation hybrids
Common name of the plant:	Indian mustard
Scientific name of the plant:	*Brassica juncea* (L.)
Introduced genes:	Male sterility, MS (barnase line), and restoration of fertility, RF (barstar line) Selection marker (bar), required only for hybrid seed production stage.
Field studies (BRL I and BRL II)	Conducted under the overall supervision of the Directorate of Rapeseed Mustard Research (DRMR), Indian Council of Agriculture Research (ICAR).
Biosafety Research Level I (BRL I) field trials **3 locations, for two years**	Conducted at 3 locations during 2010-11 and 2011-12. 1. Krishi Vigyan Kendra (KVK), Kumher, Bharatpur, Rajasthan. 2. Agricultural Research Station, Navgaon, Alwar, Rajasthan. 3. Agricultural Research Station, Sriganganagar, Rajasthan.
Biosafety Research Level II (BRL II) field trial **3 locations, for one year**	Conducted at 3 locations during 2014-15. i. Indian Agricultural Research Institute (IARI), New Delhi ii. Punjab Agricultural University (PAU), Ludhiana, Punjab iii. Regional Research Station (RRS), PAU, Bathinda, Punjab
Cloning, expression, purification and production of recombinant pure protein	Experiments carried out at M/s. Premas Biotech Pvt Ltd, Manesar. DSIR recognized, ISO 9001:2008 certified research and manufacturing facility located near New Delhi, India.
Compositional analysis	Conducted at Food and Drug Toxicology Research Centre (FDTRC) of the National Institute of Nutrition (NIN), Hyderabad. It is a Research Institute working under the aegis of Indian Council of Medical Research (ICMR), Ministry of Health and Family Welfare, Government of India.
ELISA kit development	Developed by M/s Amar Immunodiagnostics, Hyderabad. A research and development based Indian enterprise and a leading exporter of top quality GMO testing kits in India and across the world.
Allergenicity and Toxicity assessment	Conducted at FDTRC of NIN, Hyderabad, an ICMR institute
Soil microflora assessment	Conducted at CSIR-IMTECH, Chandigarh, a constituent establishment of the Council of Scientific & Industrial Research (CSIR), Ministry of Science and Technology, Government of India. The institute houses International Depository Authority (IDA) and Microbial Type Culture Collection and Gene Bank (MTCC), a depository for microbial cultures.
Environmental safety studies (weediness and crossability)	Conducted by CGMCP, University of Delhi, Delhi.

Table 1.2: List of studies undertaken for safety assessment of Varuna bn 3.6, EH-2 modbs 2.99 and hybrid DMH-11

Molecular characterization	• Gene sequences, constructs and molecular characterization • Expression studies of the three inserted genes – *bar, barnase* and *barstar* • Cloning, expression, purification and production of three expressed proteins
Food safety Studies	• Equivalence of the Bar, Barnase and Barstar recombinant proteins produced in bacteria with that expressed in GE plants • Bioinformatics analysis of the three proteins • 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	• Research & Development Phase including primary field trials 2004-2007. • BRL I trials for two growing seasons (2010-11, 2011-12) • BRL II trials for one growing season (2014-15) • Weediness potential and aggressiveness parameters • Impact on soil microflora during BRL I and BRL II trials • Crossability and pollen flow studies • Pollination behaviour, pollen morphology and physiology
Detection Protocols	• Protocol for testing at a level of detection (LOD) of 0.01% • Development of ELISA kits for Bar, Barnase and Barstar

1.2 Global status of hybrid seed production technology in *Brassica napus* using MS-RF system deploying the genes used for *B. juncea*

Between 1995 and 2003, the *barnase- barstar* based system for hybrid seed production has been approved for use in *Brassica napus* (rapeseed, commercially known as Canola) in Canada, USA and Australia. Rapeseed is a crop closely related to Indian mustard. While environmental release for large scale cultivation has been approved in Canada, USA and Australia, approval for food and feed use has been given in a large number of countries around the world including China, Japan, European Union, Mexico, and South Korea. List of regulatory status of GE rapeseed containing the *barnase* and *barstar* genes is provided in **Table 1.3**.

Table 1.3: Regulatory approval status of male sterile/ fertility restorer technology in *Brassica napus* in various countries

***B. napus* Event**	**Country**	**Approved for Environmental release**	**Approved for Food and Feed**	**Approved for Food**	**Approved for Feed**
ACS-BNØØ4-7 × ACS- BNØØ1-4 (MS1, RF1=>PGS1)	Australia	2003	2002		
	Canada	1995	1995		
	China		2004		
	European Union		2005		
	Japan	1996	1996		
	Korea			2005	2008
	South Africa		2001		
	United States	2002	1996		
ACS-BNØØ4-7 × ACS- BNØØ2-5 (MS1, RF2=>PGS2)	Australia	2003	2002		
	Canada	1995	1995		
	China		2004		
	European Union		2005		
	Japan	1997	1997		
	Korea			2005	2008
	South Africa		2001		
	United States	2002	1996		
ACS-BNØØ5-8 × ACS- BNØØ3-6 (MS8 ×RF3)	Australia	2003	2002		
	Canada	1996		1997	1996
	China		2004		
	European Union		2005		
	Japan	1998		1997	1998
	Korea		2005		
	Mexico		2004		
	South Africa		2001		
	United States	1999	1996		

Canada was the first country in 1996 to allow environmental release of lines containing the *barnase- barstar* and *bar* genes for commercial hybrid seed production (**Table 1.3**). Since the release of the technology, Canada has increased productivity of the crop and has emerged as the biggest exporter of rapeseed oil, seed and meal to Japan, China, Hong Kong and many other countries around the world including India (**Table 1.4**).

Table 1.4: Rapeseed ('Canola') oil exports (Historic) – Canada (000 Tonnes)

Country	Year												
	2002	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**
China	12	0	262	130	58	265	233	440	963	564	1003	855	501
Columbia	0	0	0	1	1	0	2	2	3	7	3	2	4
EU-27	5	22	0	49	193	56	0	0	28	183	31	10	7
Hong Kong	5	2	7	20	20	26	9	30	25	24	37	8	30
India	7	0	0	0	0	0	0	0	1	0	16	2	1
Iraq	0	0	0	0	0	0	0	0	16	0	0	0	0
Japan	3	0	10	24	4	10	12	8	6	23	18	10	5
Malaysia	0	2	5	20	26	15	27	5	17	26	24	3	12
S. Korea	28	2	8	19	20	18	39	30	38	66	56	30	76
Taiwan	15	3	17	37	24	22	4	18	12	12	15	18	9
U.S.A.	333	370	454	479	666	710	1005	971	1058	1492	1397	1227	1540
Others	4	9	220	51	31	32	30	7	9	91	65	66	144
Total	**412**	**409**	**982**	**829**	**1043**	**1153**	**1361**	**1509**	**2175**	**2487**	**2664**	**2261**	**2329**

CHAPTER 2
BIOLOGY OF INDIAN MUSTARD

In considering the environmental release of genetically engineered crops, it is essential to understand the biology, biodiversity, origin and domestication of that crop in the receiving environment. In the global regulatory system, biology documents are important resources for information and data in this regard (http://biosafety.icar.gov.in/wp-content/uploads/2015/11/Biology_of_Brassica_juncea_Ca.pdf).

2.1 Origin and domestication of *Brassica juncea*

Indian mustard (*B. juncea)* is a member of Brassicaceae family. Cultivated Brassica comprises of six closely related species, three diploids namely *B.campestris* or *B.rapa* (AA) *B. nigra* (BB) and *B. oleracea* (CC) and three allopolyploids namely *B.carinata* (BBCC), *B.napus* (AACC) and *B. juncea* (AABB). *B. juncea (2n=36)* is an amphidiploid, derived from natural inter-specific hybridization between *B. rapa* (AA genome, 2n=20) and *B. nigra* (BB genome, 2n=16) followed by endo-duplication (**Figure 2.1**). Vavilov (1949) reported that Afghanistan and its adjoining regions (Central Asia) constitute the primary centre of origin of *B. juncea*. The central and western China, eastern India and Asia Minor with Iran represent secondary centres of diversification. In India *B. juncea* is predominantly cultivated in the states of Punjab, Rajasthan, Uttar Pradesh, Assam, Gujarat, Haryana, Madhya Pradesh and West Bengal as a Rabi or winter crop. This wide acceptance of the crop is due to its adaptability to dry land agriculture where the crop is grown under limited moisture availability.

2.2 Brassica species present in India and their distribution

The Indian *Brassica* or rapeseed mustard group is constituted of species of two genera *Brassica* and *Eruca*. Cultivated Brassicas include three distinct species, viz., *B. campestris* (syn. *B. rapa*) - brown sarson, yellow sarson and toria, *B. juncea* and *B. nigra;* genus *Eruca* is represented by *Eruca sativa* (taramira or Duan).

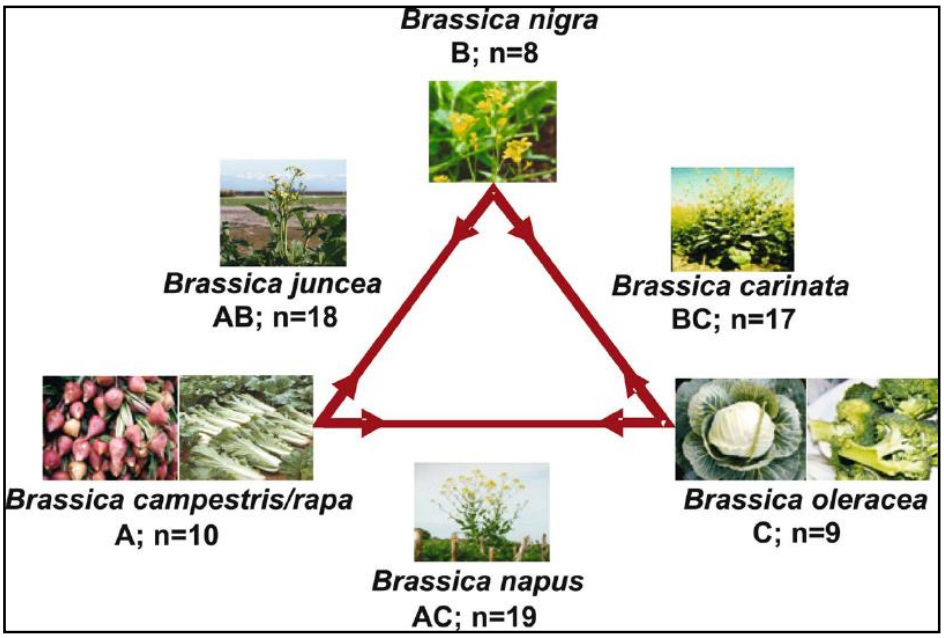


Figure 2.1.*Brassica* species relationships: The relationships among species were described in the classic work by Nagaharu (1935), in what is now termed the U-Triangle. At the tips of the triangle are the diploid species *B. nigra* (n=8), *B. oleracea* (n=9), and *B. rapa* (n=10) having two sets of chromosomes. Each of the diploid species has different basic chromosome number (N) found in either the pollen or egg. During the course of evolution, the diploid species spontaneously hybridized to produce the allotetraploids *B. carinata* (n=17), *B. juncea* (n=18), and *B. napus* (n=19), which have four sets of chromosomes, two sets from each parent species.

The differences observed among yellow sarson, brown sarson and toria are not enough to warrant separate species designation (Alam, 1945). Therefore, these subtypes have been placed together into one species *B. rapa (syn. campestris)*. A critical examination of the morphological traits of yellow sarson, brown sarson and toria reveals that these are morphologically different and have distinct mating systems. Toria is cross pollinated and self-incompatible, brown sarson *lotni* types are self-incompatible and highly cross-pollinated while yellow sarson types are self-compatible. These types are cultivated under distinct eco-geographical areas. Besides, there is another oilseed crop, taramira (*Eruca sativa*) belonging to the tribe *Brassiceae*. Initially it was placed in the genus *Brassica* and named as *Brassica eruca* L. Later, it was referred by Roxburgh (1832) as *Brassicaerucoides*. Subsequently, it was placed under a separate genus *Eruca* as *Eruca sativa* Lam.

The botanical names, genome, chromosome number and common names of commonly grown species of rapeseed-mustard in India are given in **Table 2.1**.

Table 2.1: Diversity in rapeseed-mustard and related species in India

Species	Common name	Genome	Chromo-some number (2n)	Mating system	Area of cultivation/Distribution
Brassica juncea (L.) Czern. & Coss	Indian mustard/Brown mustard	AABB	36	Self-compatible	Assam, Bihar, Haryana, Himachal Pradesh, Jammu and Kashmir, Madhya Pradesh, North East States, Orissa, Punjab, Rajasthan, Uttar Pradesh, West Bengal and Maharashtra (85%)
Brassica carinata A Br.	Abyssinian mustard/Ethiopian mustard	BBCC	34	Self-compatible	Uttrakhand, Himachal Pradesh, Punjab
Brassica nigra (L.) Koch	Black mustard	BB	16	Self-incompatible	Southern States
Brassica tournefortii Gouan	Sahara mustard	TT	20		Rajasthan, Haryana (Rain fed areas)
Brassica rapa L. ssp *toria* (syn B. campestris L. var. toria)	Indian rape/Rapeseed/Toria	AA	20	Self-incompatible	Assam, Orissa, West Bengal, Meghalaya, Tripura, Haryana, Himachal Pradesh, Jammu, Madhya Pradesh and Rajasthan; (8-6%)
Brassica rapa L. Brown sarson (syn B. Campestris L. var. brown sarson)	Brown sarson/rapeseed	AA	20	Self-compatible/ Self incompatible	Himachal Pradesh, Kashmir Valley
Brassica rapa L. yellow sarson (syn B. Campestris L. var. yellow sarson)	Colza yellow sarson/Rape seed	AA	20	Self-compatible	Assam, Bihar, Uttar Pradesh, West Bengal and North Eastern States particularly Meghalaya and Sikkim
Brassica napus (L)	Rape Rutabagas/Gobi sarson	AACC	38	Self-compatible	Himachal Pradesh, Haryana, Punjab and Rajasthan
Eruca sativa Mill.	Rocket/Salad	EE	22	Self-incompatible	Drought prone areas of Haryana, Himachal Pradesh, Punjab and Rajasthan
Brassica oleracea L. var. Botrytis	Cauliflower	CC	18	Self-incompatible	Common vegetable cultivated all over India
Brassica oleracea L. var. capitata		CC	18	Self-incompatible	Cultivated common vegetable

Source: Compiled from **Misra, 2005 (a and b).**

It is evident from Figure 2.1 and Table 2.1 that the genome of *Brassica juncea* is incompatible to cross with genomes of *B.napus, B.carinata*. Moreover, in rare cases even if interspecific crossing occurs, due to differences in ploidy levels, the resulting hybrid plants will have irregular meiosis, sterility and chromosomal imbalance. Thus the probability of persistence of progeny of such crosses in the environment are negligible.

2.3 Cultivation of *B. juncea:* soil and climate requirement

The general package of cultural practices, including pest, disease and weed situation is summarized below as guidance to understand cultivation of Indian mustard. Detailed information is available at Handbook of Agriculture, web link- http://www.icar.org.in/en/node/871.

Mustard thrives well in wide range of soil types ranging from light to heavy loam. Medium to deep soils with good drainage are well suited for its cultivation. Mustard is known for drought tolerance but does not withstand waterlogging conditions. Neutral soil pH (6-7.5) is ideal for optimal growth and development. In India, mustard is well suited for subtropical as well as temperate zones requiring cool and dry weather for optimal growth. Mustard is predominantly grown as a Rabi season crop. Temperatures between 18^{o}C to 25^{o}C and annual precipitation of 625-1000mm are considered to be ideal for satisfactory growth.

2.4 Standard agricultural practices for growing *B. juncea*

Seedbed is prepared with 1-2 ploughing followed by laddering. Mustard seeds are usually treated with fungicides before sowing for protection against soil and seed borne diseases. Sowing is performed by drilling or broadcasting method. Mustard is usually sown during September to October (Rabi). If sown as a pure crop, seed rate of 4-6 kg ha^{-1}, at a soil depth of 2-3cm and spacing of about 45 x 20cm is recommended. As a part of field preparation, 7-12t ha^{-1} of farm yard manure is recommended. Nutrient requirement depends on the soil type and organic matter content. Nitrogen is recommended at 80 kg ha^{-1} for rain fed conditions, and up to 120 kg ha^{-1} for irrigated conditions. The rate of phosphorus and potassium application varies between 30-50 kg ha^{-1} and 20-40 kg ha^{-1} depending upon the availability of soil moisture.

Split application of nitrogen has been recommended for optimal growth and development of the crop. Under irrigated conditions, half of nitrogen and full dose of phosphorus and potassium is recommended as basal dose at the time of sowing by placement method. The remaining half of nitrogen could be applied at the time of first irrigation. If the crop is rain fed, only half of the dose of recommended nutrients, except nitrogen, is used. In addition, mustard has a higher requirement of sulphur, therefore, nitrogen should preferably be

applied as ammonium sulphate, and phosphorous as single Superphosphate. Pre-soaking irrigation is preferred before seed sowing and three irrigations are recommended at 4 weeks interval after sowing, in particular, at the time of pre-bloom and pod filling stage.

Harvesting is done as soon as the pods turn yellow and seed becomes hard. Mustard crop matures in about 110 – 140 days. Harvested mustard plants are tied into bundles and exposed in sun for 5-6 days to dry before harvesting the seed.

2.5 Weeds, major pest and disease

Weeds in rape and mustard crop are reported to cause approximately 20-30 percent yield reduction. The most common troublesome weeds are *Chenopodium album*, *Lathyrus spp., Melilotus indica, Cirsium arvense, Fumaria parviflora and Cyperus rotundus.* Care should be taken to remove all weeds in the early stages of crop growth to avoid competition. 2-3 weeding and 2 hoeings are recommended at 2 weeks interval.

Mustard is highly susceptible to aphid attack, particularly, during cloudy weather in the month of December. At least two prophylactic sprays, once in the first week of November and the other in the first week of December using recommended insecticides has been recommended for the control of aphids. In addition, painted bug and pea leaf miner have also been reported to cause considerable damage. Mustard is affected by a number of diseases. *Alternaria* blight is considered to be the most important, disease, characterised by appearance of concentric black spots on leaves, stem and pods. Spraying with recommended fungicide as soon as the symptoms start appearing on the plants is usually practiced, White rust and stem rot are the other two important diseases of mustard in India. In the past few years, stem rot, has emerged as the most important disease of mustard in north-western India.

2.6 Zonalization of varietal testing

Indian Council of Agricultural Research (ICAR) started an All-India Coordinated Research Project on Oilseed crops in 1967 covering groundnut, rapeseed-mustard, sesame, linseed and castor. Activities of the coordinated research project were subsequently strengthened under the Technology Mission on Oilseeds. The All India Coordinated Research Project on Rapeseed-

Mustard is operational under the umbrella of National Research Centre on Rapeseed-Mustard (now DRMR) since 1993 with 21 research centres and 11 verification centres across the country. Various Brassica species are being grown in following six zones. Out of these six zones given below, ***B. juncea*** **is mainly grown in Zone II and Zone III.**

Zone I: Jammu & Kashmir, Himachal Pradesh

Zone II: Jammu & Kashmir, Punjab, Haryana, Rajasthan, Delhi

Zone III: Uttar Pradesh, Uttrakhand, Madhya Pradesh, Rajasthan

Zone IV: Rajasthan, Gujarat, Maharashtra

Zone V: Chhattisgarh, Bihar, Jharkhand, West Bengal, Orissa, Assam, NE Hill State

Zone VI: Andhra Pradesh, Tamil Nadu, Karnataka

CHAPTER 3
INDIAN BIOSAFETY REGULATORY FRAMEWORK

3.1 Introduction

Modern biotechnology, involving the use of recombinant DNA (rDNA) techniques, also known as genetic engineering, has emerged as a powerful tool with many potential applications in healthcare and agriculture. New plant varieties developed using rDNA techniques are commonly referred to as genetically engineered (GE), genetically modified (GM), or transgenic plants.

For the purposes of this document we use the terminology as: “Genetically engineered or transgenic plants”. GE Plants are being developed worldwide for a variety of purposes:

- enhancing agricultural productivity
- reducing dependence on the use of agricultural chemicals
- improving the agronomic qualities of plants
- enhancing the nutritional value of foods and feeds
- increasing tolerance to biotic and abiotic stresses
- providing cost effective and sustainable industrial products, including biofuels

Genetic modification of plants using selection and breeding has been carried out for centuries. The modern techniques of rDNA technology can obtain the same results by directly identifying the genes responsible for the desired character and transferring these into a variety of organisms. So, ideally, the risks associated with the introduction of GMOs/LMOs should be the same as those with conventionally modified hybrid crops and organisms. However, the main difference between the classical selection methods and improvement by rDNA technology is that the latter goes beyond the species barrier-a gene can be transferred across microorganisms, plants, and animals. Further, gene transfers are accomplished by manipulations outside the cells, which allow rearrangement and modification of genetic material before transfer, including the introduction of novel genes synthesized in the laboratory. Due to the novelty in the process of gene transfer all the products produced by genetic engineering are subjected worldwide to elaborate food/ feed and environmental risk assessment.

Several international organisations such as Organisation for Economic Cooperation and Development (OECD)http://www.oecd.org/biotrack; CODEX alimentaries (FAO-WHO food Code) (www.fao.org/fao-who-codexalimentarius/en);and Cartagena Protocol on Biosafety (CPB) (https://www.cbd.int/) under Convention on Biological Diversity (CBD and many functional regulatory systems such as USA Food and Drug Administration (FDA), the U.S. Department of Agriculture (USDA), and the Environmental Protection Agency (EPA) (http://www.usda.gov/, http://www.fda.gov/ , https://www3.epa.gov/);) European Union European Food Safety Authority (http://www.efsa.europa.eu/); Brazil; (http://ctnbio.mcti.gov.br/en/inicio), Japan (https://www.loc.gov/law/help/restrictions-on-gmos/japan.php; and Australia-Office of the Gene Technology Regulator (http://www.ogtr.gov.au/) have published several global consensus documents, protocols and data requirements for food/feed and environmental safety assessment of GE crops.

In India, the manufacture, import, use, research and release of GE organisms as well as products made thereof by the use of such organisms are governed by Rules 1989, of the Environment (Protection) Act 1986 (EPA) administered by the Ministry of Environment and Forests (MoEF); now the Ministry of Environment, Forests and Climate Change (MoEF&CC), Government of India. These rules and regulations commonly referred to as 'Rules 1989'available at the MoEF&CC website, (http://envfor.nic.in/legis/hsm/hsm3.html), cover the areas of research as well as large-scale applications of GE organisms and products made thereof. The regulatory agencies responsible for implementation of the Rules 1989 are MoEF&CC and DBT through the following six competent authorities:

- **Recombinant DNA Advisory Committee (RDAC):** The committee is serviced by DBT. It reviews developments in biotechnology at the national and international level and recommends suitable and appropriate safety regulations from time to time for India in r-DNA research, use and applications.

- **Genetic Engineering Appraisal Committee (GEAC) formerly known as the Genetic Engineering Approval Committee):**This is the apex body constituted in the Ministry of Environment, Forests and Climate Change (MoEF&CC)and is responsible for the approval

of proposals relating to the release of genetically engineered organisms and products into the environment including experimental field trials.

In relation to environmental release of genetically engineered plants, GEAC functions to provide approvals for the conduct of all types of confined field trials and final appraisal of the biosafety data submitted by the applicant for the release of a GE crop into the environment.

- **Review Committee on Genetic Manipulation (RCGM):** A statutory body of multidisciplinary experts – this committee functions independently and is serviced by the DBT. RCGM deals with specific procedures for regulatory and scientific risk assessment protocols, methods and processes of genetically engineered plants, animals and biopharmaceuticals and biologicals. RCGM is also involved in monitoring the biosafety related aspects in respect to all the on-going research projects and activities and brings out manuals and guidelines specifying procedures for the regulatory process with respect to activities involving genetically engineered organisms in research, use and applications by the industry as well as by the public funded institutions with a view to ensure environmental and food safety. RCGM provides research information and recommends the applications after thorough evaluation to the GEAC for environmental release of GE products including confined field trials.

- **Institutional Biosafety Committee (IBSC):** They serve as a nodal point for interfacing with RCGM regarding ongoing research activities within each institution and industry. IBSC is responsible for ensuring biosafety of the R&D work under laboratory and containment conditions.

- **State Biotechnology Coordination Committee (SBCC):** They serve as a nodal point at the State level for coordinating activities related to GMOs in the State with the Central Ministries including monitoring of the conditions stipulated by the RCGM/GEAC.

- **District Level Committee (DLC):** They serve as a nodal point at the district level for coordinating activities related to GMOs in the District with the SBCC and GEAC including monitoring of the conditions stipulated by the RCGM/GEAC.

The 'Rules 1989' are supported by a series of following guidelines (details available at http://dbtbiosafety.nic.in/files%5CCoverpage.pdfand http://dbtbiosafety.nic.in/field_trials_guidelines/combined_sops.pdf).

- Recombinant DNA Safety Guidelines, 1990
- Revised Guidelines for Safety in Biotechnology, 1994
- Revised Guidelines for Research in Transgenic Plants, 1998
- Guidelines and Standard Operating Procedures (SOPs) for Confined Field Trials of Regulated, Genetically Engineered (GE) Plants, 2008
- Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008
- Guidelines and Handbook for Institutional Biosafety Committees (IBSCs), 2nd Revised Ed. 2011

The *Guidelines for the Environmental Risk Assessment of Genetically Engineered Plants 2016* have been updated recently and is also considered in conjunction with the other documents cited above.

For review or revision or updating of protocols/ guidelines for safety assessment of GE crops, the approach followed is to critically examine and implement the best International practices along with other available peer reviewed research publications and documented experiences. The revised or updated documents are subjected to wide ranging consultations at multiple levels of stakeholders to arrive at consensus documents for wider adoption and harmonisation with practices in place at the global level.

Figure 3.1a explains R&D phase where several events and crosses are made for obtaining a stable and effective final event along with defined genetic background of initial genotype(s). In India, as in many countries, once the final event in a defined background is established, it requires that prior to the environmental release of GE plants, these undergo a case-by-case

risk assessment to evaluate any potential for adverse environmental impacts. Statutory committees (IBSC, RCGM and GEAC) examine each case through a step by step process from research to technology development (**Figure 3.1b**) to generate data on food and environmental safety.

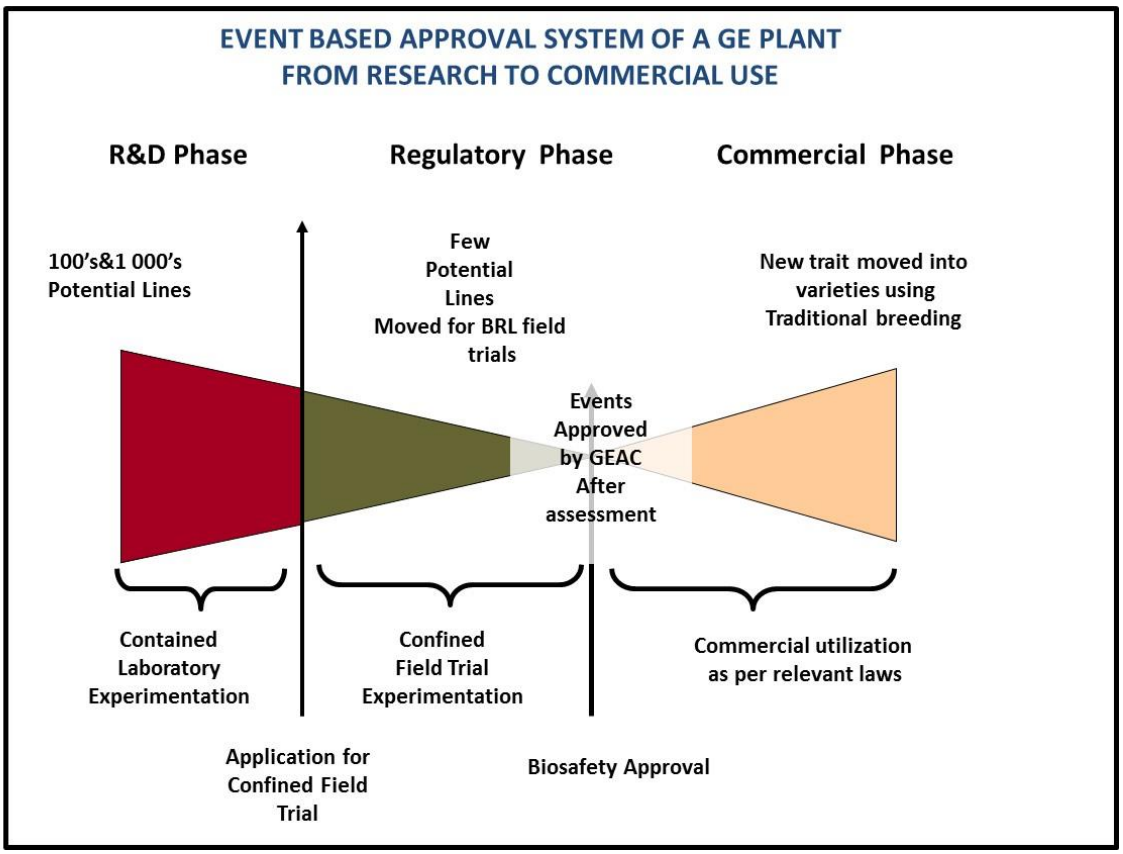


Figure 3.1a: Event based approval system of a GE plant from research to commercial use.

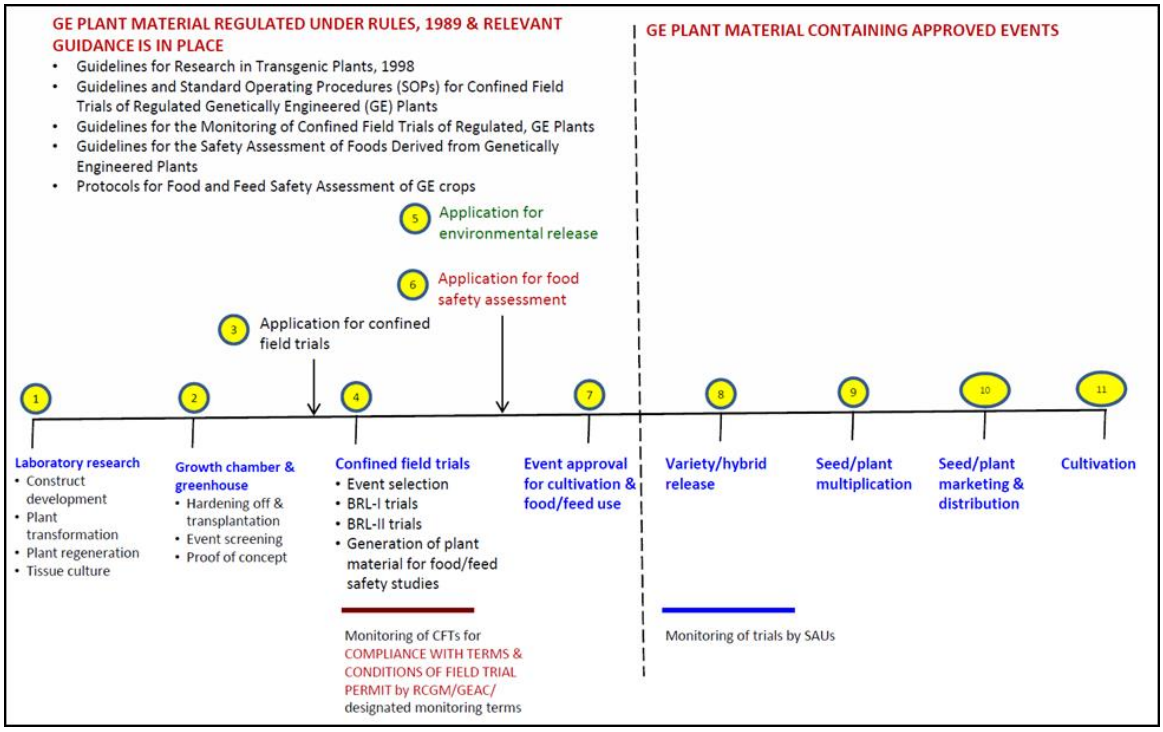


Figure 3.1b: Step by Step process followed by the applicant for regulatory approval

3.2 Step by step process to be followed by the applicant

In laboratory and contained conditions, the parameters to be examined include description of the host plant; centre of origin of the host plant; geographical distribution of the host plant in the country; source and sequence of transgene(s); cloning strategy; characteristics of expression vector(s); characteristics of inserted genes with detailed sequences; characteristics of promoters; cloning/transformation methods of target gene; genetic analysis including copy number of inserts, genetic stability, integration site characterization, level of expression of transgene(s), characterization of expressed gene product; mode of action of gene product; compositional analysis; rationale for the development of GE plants in terms of agronomic, nutritional and other benefits.

Experimental field trials, namely Biosafety Research Level I & II are conducted strictly under confined field testing conditions as a prerequisite for approval of a GE plant. These trials represent the first controlled introduction of a GE crop into the environment with experiments in confined facilities and large scale cultivation. A confined field trial is a field experiment of growing a regulated GE plant in the environment under specified terms and

conditions that are intended to mitigate the establishment and spread of the plant through the following parameters:

- Pollen- or seed-mediated dissemination of the experimental plant.
- Persistence of the GE plant or its progeny in the environment.
- Introduction of the GE plant or plant products into the human food or livestock feed chain.

On a case-by-case basis, specific methods of physical confinement are also advised to prevent herbivory or the destruction of plant material by foraging animals, or the unauthorized harvest or removal of plant material by humans.

A single confined field trial may be comprised of one or more events of a single plant species that are subject to the same terms and conditions of confinement which also include reproductive isolation, site monitoring, and post-harvest land use restrictions. Under Indian guidelines, these field trials are conducted at two stages namely Biosafety Research Level I and II.

In Biosafety Research Level I & II confined field testing, the data is generated to compare GE lines with their non-GE counterparts for various parameters including seed setting characteristics; germination rates; phenotypic characteristics; target gene efficacy ; possibility of transfer of gene to near relatives through out-crossing; implications of out crossing; invasiveness; susceptibility to diseases and pests and agronomic advantages etc. BRL studies also include assessment of food and feed safety parameters.

In addition, the statutory bodies can exempt or prescribe generation of additional information or data on biosafety depending on the nature and characteristics of the target gene and the GE crop history of safe use of products derived from same/similar GE crop as well as other legal provisions from time – to- time.

To ensure compliance during the confined field trials, Central Compliance Committees (CCC) with scientific experts nominated by GEAC, RCGM and ICAR, State Agriculture Universities and State Agriculture Departments are constituted and these committees visit the field trial

sites and inspect facilities to ensure compliance. Field trials are conducted in State Agriculture Universities or their Regional Stations with "No Objection Certificate (NOC)" from State Governments concerned.

On generation of above data in laboratory and confined field trials under authorized conditions of RCGM and GEAC as per the guidelines and protocols and in certified/ recognised laboratories/ institutions / universities, the applicant submits a detailed dossier to GEAC for environmental release. The evaluation of food/feed and environmental safety is carried out by the RCGM and re-assessed by GEAC using a detailed and thorough risk assessment procedure as described in the next section.

3.3 Step by step regulatory compliance and data generation in the case of GE mustard parental lines and hybrid DMH-11

All the relevant chronological approvals for this application, on GE mustard parental lines and hybrid DMH-11, by the Indian regulatory agencies are given in a tabular form in **Appendix 1**. A summary for the same is given below:

RCGM permitted CGMCP to conduct small scale confined field trials for R&D phase in 2003-2006 to finalize events and stablilize the genotypic background.

The BRL I first year field trials under confined conditions were conducted in Oct 2010 for environmental and food and feed safety assessments at three locations with approval from RCGM and GEAC. The trial sites were monitored by a Central Compliance Committees (CCC) in Rabi 2010-2011. The RCGM approved the protocols for environmental safety assessment studies submitted by the applicant in 2011. In Oct 2011, the applicant was permitted for BRL I 2nd year trial in Rabi 2011-2012 with monitoring from CCC team.

To generate toxicity data for food and feed safety assessment, the protocols were approved in Nov 2011. Based on the BRL I studies approval was granted by GEAC for BRL II field trials in Oct 2014and the trials were conducted in Rabi 2014-2015. The final dossier, incorporating comments of the GEAC and a sub-committee of experts, was submitted by the applicant to

GEAC in February, 2016. This final dossier forms the basis for evaluation of food/ feed and environmental safety assessment by the GEAC.

3.4 Assessment of Food/feed and Environmental Safety (AFES)-Risk Assessment Process

Indian law and the Cartagena Protocol on Biosafety, to which India is a signatory, require that a risk assessment be performed prior to the environmental release of a GE plant in India. The purpose of the risk assessment is to identify risks to the health and safety of people and the environment from the cultivation of the GE plant, when compared with the cultivation of the non-GE version of the plant, and to characterize the risks on the basis of severity and likelihood.

The *Risk Analysis Framework* describes the principles of risk analysis used by the Regulatory Agencies to protect human health and safety, and the environment, in accordance with the Environment (Protection) Act, 1986. Relevant excerpts, in the following section, have been taken from the guidelines for Environmental Risk Assessment that has recently been published by MoEF&CC, 2016. Incidently this Risk analysis approach is also in agreement with recently published report by National Academy of Science, USA (2016) and an article by Gregory et al. (2016).

Risk analysis integrates the assessment, management and communication of risks posed by GE plants. The risk context defines the parameters within which risk is assessed, managed and communicated.

Risk analysis approach used in India

The risk analysis method used for the environmental release of a GE plant is outlined in **Figure 3.2**. As illustrated, the process is not necessarily linear as there are steps where information flows in both directions, such as between risk assessment and risk management and between risk communication and stakeholders.

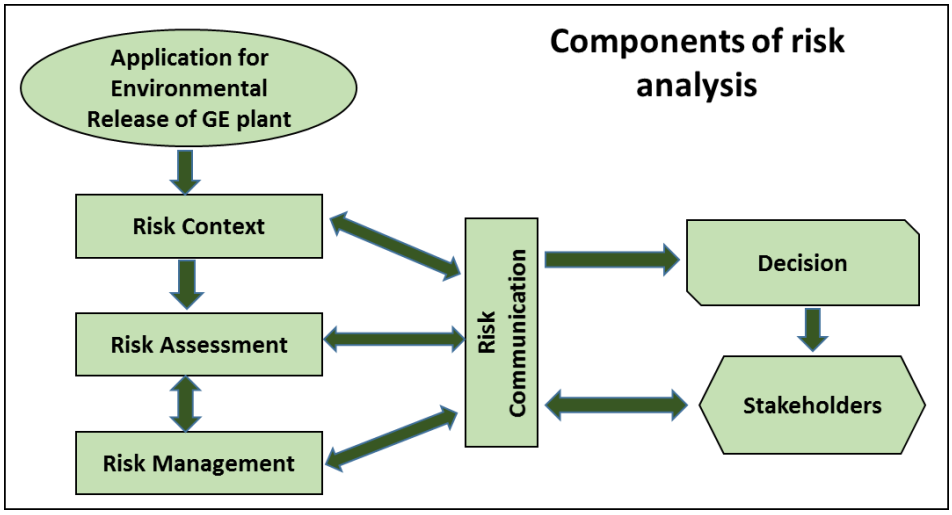


Figure 3.2: Risk analysis method for environmental release of a GE plant

Risk context: Establishing the risk context is the preparatory step that defines the scope and boundaries, sets the criteria against which risk will be evaluated, and describes the structures and processes for the analysis. This includes setting criteria for what is considered to be damage or injury to people or the environment.

Decisions on applications for the environmental release of a GE plant require case-by-case assessment, and details of the GE plant and the proposed activities, including any proposed controls, limits or containment measures, form the specific risk context. Details of the parent organism and the environment where activities with the GE plant will occur form the comparative baselines.

Risk assessment: Risk assessment is a structured, reasoned approach to consider the potential for harm from certain activities with a GE plant, based on scientific/technical evidence. Identifying and characterising risk relies on scientific/technical evidence, involving consultation with experts and other stakeholders. The aim is to identify, characterize, and evaluate risks to the health and safety of people or to the environment from GE plants. The risk assessment initially considers a wide range of potential pathways whereby harm might occur. Those pathways that identify substantive risks are considered in more detail by characterising how serious the harm could be (consequences) and how likely it is that harm could occur. The level of risk is then evaluated to determine whether the risk is acceptable or not.

Risk assessments are performed using the below mentioned fundamental principles:

- Risk assessments must be carried out in a scientifically sound manner.
- Risk assessments should be comparative. For example, according to the Cartagena Protocol on Biosafety, "**Risks associated with living modified organisms…should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment**."
- Risk assessments should be carried out on a case-by-case basis, taking into account the specific circumstances or context for each individual application.

The Risk Hypothesis is based on a comparison between the GE plant and the non-GE version of the plant, typically, the host variety or a near isogenic parental line, and so the data collection process must first collect sufficient information to fully characterize the biology of the non-GE version of the plant. This information establishes a background of long-standing familiarity with the crop and with the breeding of novel varieties using traditional methods. It is also important to collect data about aspects of the plant's biology that may alter the potential of the plant to cause harm. Relevant data should focus on characteristics that could likely have environmental implications, such as the plant's reproductive biology, whether the plant is known to have weedy or invasive properties, and whether the plant is known to produce toxic or allergenic substances. The goal is to identify specific ways, including both intentional changes and unintended ones, in which the GE plant is significantly different from the non-GE version and how those differences could impact the environmental resource in question. Useful data would be taken from a variety of sources: published scientific literature, applications submitted for confined field trial permits, past environmental risk assessments of GE plants with the same phenotype, including risk assessments from other countries, and professional experience of the risk assessors.

Risk Assessment Process: Risk assessment, including the assessment of risks from GE plants, can be described as a four-step process, the goal of which is to answer questions relating to
1. Risk identification ("What could go wrong?") Regulators consider a broad range of scenarios in which the release of a GE plant, for purposes of cultivation, could possibly cause harm to people or the environment. In each scenario there must be a causal link between the cultivation of the GE plant and the harm.

2. Risk characterization: consequence assessment ("How serious could the harm be?") Once a risk has been identified, regulators assess the severity of the potential harm.
3. Risk characterization: likelihood assessment ("How likely is the harm to occur?") Regulators examine the causal link between the cultivation of the GE plant and a particular harm and determine how likely it is that the harm will occur.
4. Risk evaluation ("What is the level of concern?") Once regulators have assessed the severity of the harm and the likelihood of its occurrence, they evaluate whether the risk is negligible, low, moderate, or high.
Problem formulation is a framework that provides the means to organize an environmental risk assessment so that the assessment is done in a logical and transparent way. It helps risk assessors decide what questions the assessment will address and what data are most relevant to those questions.

Risk Assessment in practice

The risk assessment process for GE plants described is based on a comprehensive, transparent, and science-based framework by which regulators can identify potential harms that might be caused by GE plants, collect relevant scientific data pertaining to the likelihood and severity of any harms, and consistently evaluate the level of risk posed by the use of GE plants. This framework uses a conventional approach to risk assessment similar to ones used in many other areas of risk assessment, and it incorporates a case-by-case approach that takes into account a variety of sources of information.
Using Problem Formulation, regulators will identify protection goals, formulate risk hypotheses that explore causal relationships between the cultivation of GE plants and the identified goals, and then determine which relevant data are needed to test the hypotheses. Using these data, regulators will assess the severity and likelihood of harms and ultimately evaluate the level of risk that would result from cultivating the GE plant. This process is performed for each risk hypothesis generated through Problem Formulation.
See **Table 3.1** for a matrix showing the relationship between the likelihood and the severity of a particular harm when evaluating the risk.

Table 3.1: Risk matrix used to estimate the level of risk posed by GE crop

LIKELIHOOD ASSESSMENT	LEVEL OF RISK			
Highly likely	Low	Moderate	High	High
Likely	Low	Low	Moderate	High
Unlikely	Negligible	Low	Moderate	Moderate
Highly unlikely	Negligible	Negligible	Low	Moderate
	Marginal	Minor	Intermediate	Major
	CONSEQUENCE ASSESSMENT			

Table 3.2: Scale of risk levels adopted

Level of risk	Risk level definition
Nil/Negligible	Risk is of no discernible concern and there is no present need to invoke actions for mitigation.
Low	Risk is of minimal concern, but may invoke actions for mitigation beyond standard practices.
Moderate	Risk is of marked concern and will necessitate actions for mitigation that need to be demonstrated as effective.
High	Risk is of considerable concern that is unacceptable unless actions for mitigation are highly feasible and effective.

The risk assessment process is frequently iterative in nature: regulators may analyse the data they have collected relative to a particular risk hypothesis and determine that they need to return to Problem Formulation to collect more data or to restate the risk hypothesis. This iteration is common in all fields of risk assessment and generally results in a better outcome from the assessment process. See **Figure 3.3** for a summary of this iterative process.

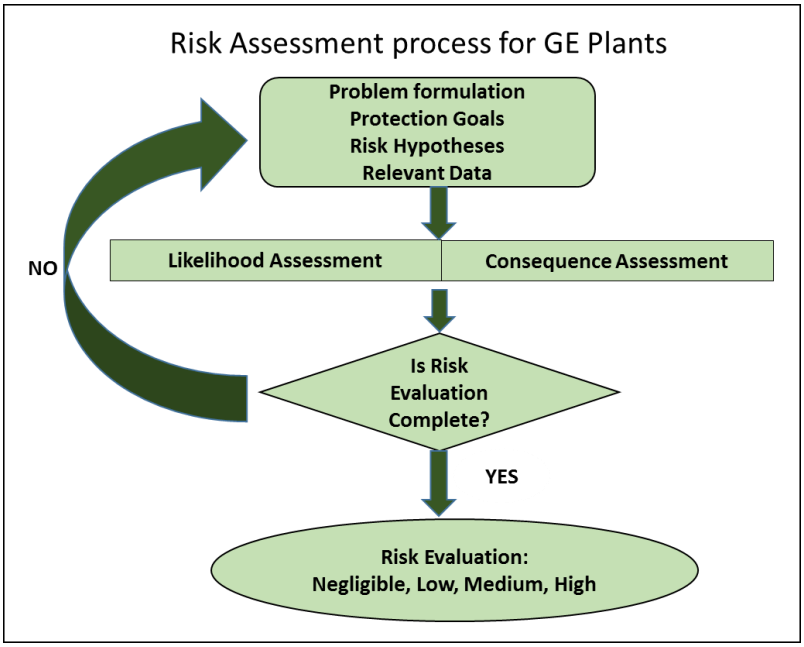


Figure 3.3: Risk assessment process for GE plant

After testing all the risk hypotheses that were identified during Problem Formulation, the risk assessors will make an overall risk evaluation to determine whether the GE plants likely to pose significantly different risks of adverse environmental impacts than a non-GE comparator. Once all the identified risks have been evaluated, the risk assessors will issue a risk assessment report.

Risk Assessment process for breeding stacked events: The Risk assessment process and the data requirement in case of stacked events primarily depends on the following two criteria:

1. The process used for generating the stacked event
2. Prior approval status of the individual event/s

Based on the following selection criteria, in case the application contains stacked events generated from breeding stack containing (all or some) events already approved in India, then the earlier data generated for characterization of approved event is acceptable and additionally data pertaining to environmental and food safety studies needs to be generated for stacked product only. However, in cases where none of the individual events are approved and to be used only for hybrid seed production, the applicant is required to submit a dossier containing detailed characterization report of the individual parent event (s), food and environmental safety studies data for hybrid as well as parental events.

Risk management: Risk management may be described as answering the following question: what can be done to mitigate any unacceptable risks identified during the risk assessment? Risk management measures are elaborated in a risk management plan that includes any conditions the regulators have imposed to control or reduce risk. Monitoring may be included to validate the original risk decisions and to adjust risk management measures to account for changes in circumstances or new information. The risk management plan helps the Regulatory Agencies decide whether to authorize an environmental release, and what conditions to impose, if any. If the Regulatory Agencies conclude that risks cannot be sufficiently mitigated to protect human health and safety and the environment, the environmental release of the GE plant should not be authorized.

Risk communication: Risk communication engages in dialogue about the risks to human health and the environment posed by GE plants. Risk communication is integral to the processes of risk assessment and risk management. It involves an interactive dialogue between the Regulatory Agencies and stakeholders to build trust in the Regulatory system by discussing issues and addressing concerns. The Regulatory Agencies undertake extensive consultation with a diverse range of expert groups and authorities and key stakeholders, including the public, before deciding whether to authorize the release of a GE plant into the environment. The *Risk Analysis Framework* is part of the Indian government's commitment to clarity, transparency and accountability for decision-making processes.

Following such due diligence through scientific appraisal process and consultations, the GEAC may determine that organism or product regulated under this Rules 1989 of Environmental (Protection) Act 1986 as safe for intended purpose or environmental release with post release risk management conditions, if any. Finally, the Ministry of Environment, Forests and Climate Change, Government of India through approval process provides authorization for intended use. However, such authorization shall also be subject to all other laws, rules and regulations made thereof in the Central and State Governments relevant at that time.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF GE MUSTARD HYBRID DMH-11 AND ITS PARENTAL LINES

4.1 Introduction

For ensuring the safety of human, animal and environment, it is imperative to undertake detailed molecular characterization of all the introduced genetic elements and the GE plant produced thereof. The molecular characterization of the introduced elements would include analysis of the introduced gene/s, the regulatory elements used for driving the expression of transgene(s) and any other elements in the vector, like the marker genes etc. These elements are assessed for any potential allergenicity/ toxicity/ pathogenicity through the analysis of the DNA and protein sequences and the source of the genes and promoter elements. A study of the GE plant biosafety would include method of transformation, characterization of the event through description of the transgene integration loci (no. of sites and flanking sequence analysis), transcript and protein expression (level, location and stability) and integrity and stability of the transgene in the GE plant. Another important aspect in the molecular characterization of the GE plant is to have a detection and identification protocol highly specific and sensitive to a particular GE event that is to be released. An event is a genotype produced from the transformation of a single plant species using a specific gene construct.

Molecular characterization of a GE event is the key step in the biosafety assessment. To sum up, the purpose of the molecular characterization is to provide a unique identification for each event as well as to understand the safety of all the introduced elements and the GE plant itself. In case the molecular data reveal any deviation, the phenotype of the resultant GE plant might be affected. This chapter deals with the molecular characterization of the parental lines Varuna bn3.6 and EH-2 modbs2.99 and the hybrid DMH-11 derived between these two parental lines.

4.2 The male sterility- fertility restorer technology

In the present case, a GE technology based hybrid seed production system has been developed by CGMCP. The system has the potential of hybrid seed production to bring about a substantial increase in crop productivity through utilizing genetic diversity available in *B. juncea* to produce hybrids that may show heterosis for yield – amount of yield increase depending upon the parental germplasm. The hybrid breeding system developed in *B. juncea* (Indian mustard) in this application consists of a *barnase* gene containing line that confers male sterility(MS) and a *barstar* gene containing line that restores fertility (RF). Heterosis breeding based on *barnase-bars*tar system has been successfully deployed for enhancing crop productivity in rapeseed (*B. napus*) in Canada, USA and Australia. *B*. *juncea* flowers contain both male and female organs and it is a predominantly self-pollinating crop, hence a pollination control mechanism is required to disallow self-pollination and facilitate cross-pollination for production of desired hybrid seeds. For this, one of the combiners (parental lines) of a hybrid has to be made male sterile that would function as the female parent so that it receives pollen from the other parent (male parent) to set seed. Since seeds of the F1 hybrids are the desired product, availability of a suitable restorer system in the male parent is also required to achieve seed set in the next generation. Genetic engineering of nuclear male sterility and its restoration have emerged as tangible options for the development of a robust male sterile/ restorer line system.

The GE *barnase-barstar* system for hybrid seed production has been reported to work efficiently in *B. napus* for more than 20 years. Initially, two *B. juncea* varieties, namely, RLM 198 for the *barnase* gene and Varuna for the *barstar* gene were used for genetic transformation experiments, along with *bar* gene as a selectable marker in both the constructs. *B. juncea* var. RLM 198 is a mustard variety developed through a combination of mutation and recombination breeding by Punjab Agricultural University. Varuna is extensively cultivated in northern India in the states of Haryana, Rajasthan, Uttar Pradesh and north-western parts of Madhya Pradesh. Later, the *barnase* elite event (bn 3.6) was transferred to Varuna genotype of *B. juncea* while the *barstar* gene construct event (modbs 2.99) was used to transfer the *barstar* gene to EH-2 genotype via backcross breeding. Most of the east European gene pool lines, however, are late in maturity under short-day conditions prevailing

in northern India during the Rabi season. The EH-2 genotype is developed from east European material by the University of Nagpur. This genotype has been found to be day-length neutral and has a maturity period similar to that of the mega variety Varuna belonging to the Indian gene pool. This combination of male-sterile (*barnase*) and restorer (*barstar*) lines in *B*. *juncea* constitutes a complete and functional male-sterility/restorer system which has been used to develop hybrid DMH-11. This can be diversified in future into better combiners and deployed for production of a series of hybrids in this crop.

The overall working of the system for hybrid seed production is shown in the **Figure 4.1**.

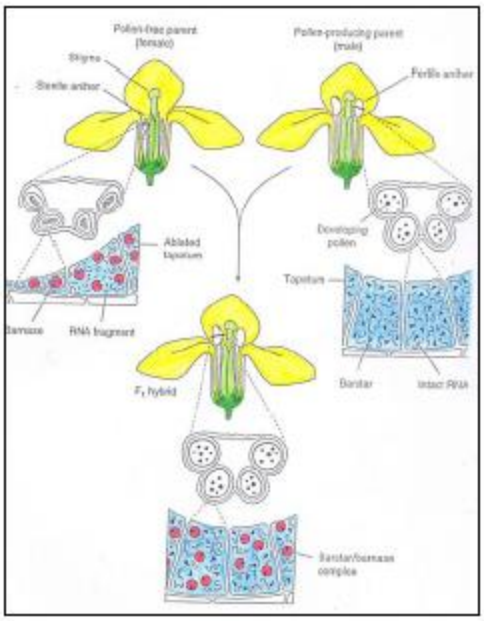


Figure 4.1: Mechanism of Barnase-Barstar system (adopted from Williams 1995).

4.3 Gene constructs

The sources of introduced genes and their products: The barnase and barstar genes are derived from *Bacillus amyloliquefaciens*, a commonly occurring soil bacterium, which colonizes plant roots and has been shown to have plant growth promoting activity by suppressing plant pathogens (Idriss et al. 2002). It is also frequently used as a source of industrial enzymes (Breccia et al. 1998). The bar gene introduced into both the MS and RF lines is derived from *Streptomyces hygroscopicus* (Thompson et al. 1987). Streptomyces species are saprophytic, soil borne microbes and are not reported to be pathogens of humans or animals.

***Barnase* construct:** The complete *35S-AMVL-bar-ocspA::*spacer*::TA29-barnase-35SpA* construct was cloned into the binary vector *pPZP200* to generate the final binary vector for plant transformation. The *barnase* gene (333bp) encodes an RNase, called Barnase that degrades ribonucleic acid (RNA), the biochemical intermediate between the gene (DNA) and the protein. RNases are ubiquitous in nature and serve many biological functions.

In order to express the *barnase* gene specifically in the tapetum cell layer that nurtures the developing pollen, a previously characterized tapetum-specific *TA29* promoter (870bp) from tobacco (*Nicotiana tabacum*) was used. Expression of *barnase* only in the tapetum cell layer of the pollen sac during anther development results in the degradation of the RNA only in those cells and prevents pollen formation thus resulting in male sterility (Mariani et al. 1990; De Block & De Bouwer 1993). The *polyA* signal, required for termination of gene expression in plants, was derived from the 35S RNA gene of *Cauliflower mosaic virus*. Complete male sterility is achieved in plants carrying the construct *TA29 –barnase-35SpA* (**Figure 4.2**).

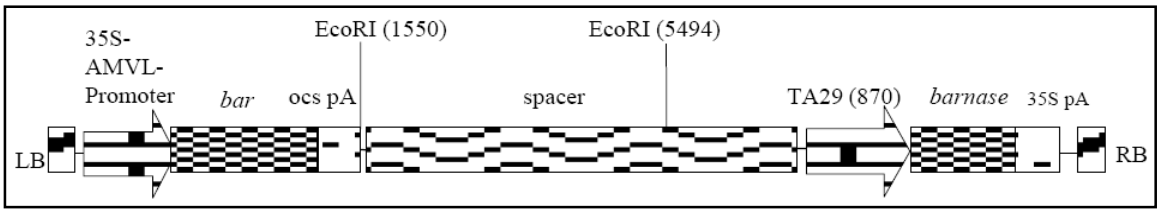


Figure 4.2: Map of the *barnase* gene construct.**LB**, left border of T-DNA; **RB**, right border of T-DNA; **ocspA**, polyA signal of octopine synthase gene; **35S**, CaMV 35S promoter with a single enhancer region; **AMVL**, leader sequence of the Alfaalfa Mosaic Virus; ***bar***, coding sequence of herbicide (Basta) resistance gene used as a selectable marker; **spacer**, partial sequence of pea *topoisomerase* gene and *Arabidopsis acetolactate synthase* gene together form the Spacer DNA; **TA29(870)**, 870 bp fragment of the tapetum-specific TA29 promoter; ***barnase***, coding sequence of the *barnase* gene, 35S gene polyadenylation signal.

In their earlier research, the applicant found that the tissue-specific expression of the *barnase* gene was influenced by the strong constitutive *35S* promoter when it is present next to the TA29-*barnase*gene. To circumvent this problem, a Spacer DNA fragment was incorporated between the *35S-AMVL-bar-ocspA* cassette and the *TA29 –barnase-35SpA* cassette as an effective insulator to protect the tissue-specific expression of the *barnase* gene in male-sterile lines of *B. juncea*, and thus prevent leaky expression of the *barnase* gene encoded RNase activity in tissues other than the tapetum cells. The spacer DNA comprised of a 5 kb sequence where truncated regions of the two genes: the *topoisomerase* gene (3 kb) from pea and the

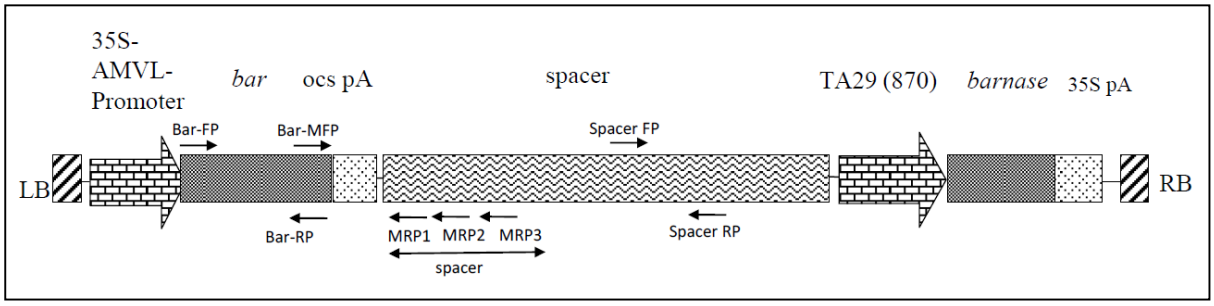
acetolactate synthase gene (2kb) from *Arabidopsis* were fused. These gene sequences are partial sequences with deletions at both the 5'- and 3'- ends of the genes.

Since the spacer comprises coding regions of two truncated genes, there was a possibility of formation of new open reading frames (ORFs). To identify the formation of any new ORFs in the spacer region, studies were conducted on the sequence to find out if it could encode for a protein in any of the probable six reading frames. This analysis showed that there are no potential ORFs created in the sequences to code for a new protein.

In order to rule out the possibility of any transcripts being generated from the spacer region due to the presence of CaMV35S promoter in the upstream region that is used to drive the expression of the *bar* gene, the applicant has performed RT-PCR experiments, specifically to identify the presence of transcripts corresponding to the spacer and the junction region of the two gene used to generate the spacer in the total RNA of event Varuna bn 3.6 and DMH-11 hybrid. For this purpose, a set of 4 primers were used covering the bar-spacer junction region and topoisomerase-acetolactate synthase (ALS) junction region (present within the spacer sequence, **Figure 4.3A**). Before preparing cDNA for RT-PCR, total RNA samples were checked for any genomic DNA contamination and were found to have no contamination.

The results obtained from the RT-PCR experiments are shown in **Figure 4.3B**. As expected DNA fragment corresponding to the *bar* gene transcript was observed in both Varuna bn 3.6 and DMH-11 hybrid. However most important, no DNA fragment was observed in any of the four primer sets designed to identify any transcript corresponding to the spacer region. Therefore, it can be concluded that there is no read through from the CaMV35S promoter of the *bar* gene into the spacer fragment. Also, for the lack of any PCR product in the RT-PCR experiment using Spacer FP and Spacer RP primers, it can be concluded that are no transcripts corresponding to the junction region within the spacer element.

A



B

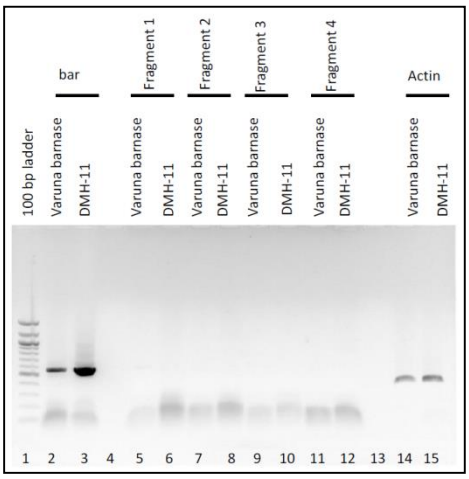


Figure 4.3:A. Schematic representation of T-DNA showing the primer binding sites with direction of PCR amplification. Spacer region constituted of partial coding region of two unrelated genes: *topoisomerase* gene from pea and *acetolactate synthase* gene from *Arabidopsis*. Four primer combinations were used in RT-PCR to identify the presence of any transcripts coming from the bar-spacer region. Expected size of PCR product with different combination of primers are: Bar-MFP and Spacer-MRP1(450bp, fragment 1), Bar-MFP and Spacer-MRP2 (697 bp, fragment 2), Bar-MFP and Spacer-MRP3 (994bp, fragment 3), Spacer FP and Spacer RP (679bp, fragment 4), Bar FP and Bar RP (550bp, positive control for transgene), Actin FP and Actin RP (500bp, internal control). **B**. Analysis of bar gene specific and read through transcripts in RNA isolated from the leaf tissue of Varuna barnase and DMH-11 hybrid lines (lanes 2 and 3). The *actin* gene was taken as an internal control (lanes 14 and 15). Note the lack of PCR amplification from the spacer region in any of the four primer combinations tested (lanes 5-12). No samples were loaded in the lanes 4 and 13.

***Barstar* construct:** The entire *35Sde-bar-ocspA::TA29-barstar (mod)-35SpA* construct was cloned into the binary vector *pPZP200* to generate the final binary vector for plant transformation. The *barstar* gene (273bp) encodes Barstar protein which selectively binds to Barnase by forming a one to one complex with the Barnase protein that results in suppression of the ribonuclease activity (Hartley 1988, 1989). The codons of bacterial *barstar* gene were optimized to maximize the expression of the barstar in the RF line. The amino acid sequence

of the protein encoded by the codon-modified barstar gene is exactly the same as that encoded by the wild type (native) bacterial gene. This codon modified sequence of the *barstar* gene was used for the development of restorer barstar lines so as to express the Barstar protein at a higher level for efficient fertility restoration in the barnase gene expressing male-sterile lines (**Figure 4.4**).

The *barstar* gene is under the control of the same regulatory sequences as the *barnase* gene i.e. the anther-specific *TA29* promoter from *N. tabacum* and polyadenylation signal sequence from the 3' non-translated region of the 35SpolyA from *Cauliflower mosaic virus*. The tapetum-specific TA29 promoter would lead to expression of Barstar protein in the tapetum cell layer during anther development. In the absence of a spacer region in the *barstar* construct, the expression of *barstar* is expected to be higher due to the influence of *35Sde* promoter (35S promoter with double enhancer element) driving *bar* gene in the same construct. The Barstar protein has a very high affinity for binding to the Barnase and thereby inactivates its RNase activity. As a result, the hybrid plants develop normal anthers and produce fertile pollen and full fertility is restored in the F1 (hybrid) seed derived plants.

In the *barstar* gene construct, the *bar* gene, used as a plant selectable marker, is controlled by *CaMV35S* double enhancer promoter (with duplication of the enhancer region from -90 to -343). This promoter confers a 10-fold increase in the expression levels over the corresponding single enhancer counterpart. The mRNA polyadenylation signal from the 3' non-translated region of *ocs poly(A)* has been used as transcription termination signal for *bar* gene in both the *barnase* and *barstar* gene constructs.

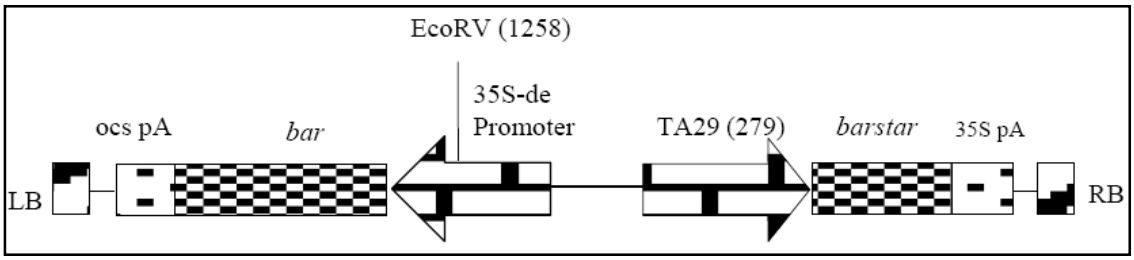


Figure 4.4: Map of the *barstar* gene construct.**LB**, left border of T-DNA; **RB**, right border of T-DNA; ***ocspA****, polyA* signal of *octopine synthase* gene; ***bar****,* coding sequence of the gene that confers resistance to herbicide Basta as selectable marker; ***35Sde***, *CaMV 35S* promoter with duplicated enhancer; *TA29* (279), 279bp fragment of the tapetum-specific *TA29* promoter; ***barstar* (mod)**, codon modified sequence of *barstar* gene; ***35SpA****,* 35S polyadenylation signal.

Components used in the different constructs are summarized in **Table 4.1**.

Table 4.1: Gene components introduced in the genetically engineered male-sterile as well as fertility restorer mustard events

Genetic components	Protein expressed/function	Source organism	Intended purpose
Genes			
***barnase* gene** (Gene Bank Accession no.: X12871)	Barnase ribonuclease for male-sterility	*Bacillus amyloliquefaciens*	Tapetum-specific *TA29* promoter driven expression destroys tapetum layer cells in the anther, preventing pollen formation, resulting in male sterility.
***barstar* gene**	Barstar functions as the Barnase ribonuclease inhibitor	*Bacillus amyloliquefaciens*	Barstar protein binds to the Barnase protein to inactivate its RNase activity. This restores fertility in the F1 hybrid plants to develop normal anthers and pollen and thus seeds
***bar* gene** (Gene Bank Accession no.: X17220)	Phosphinothricin acetyltransferase (PAT) protein for resistance to herbicide Glufosinate ammonium (Basta)	*Streptomyces hygroscopicus*	Used as a selectable marker to identify transformed plants during selection of parental lines.
Spacer sequence	A 3kb *topoisomerase* gene fragment	Pea	To regulate the function of CaMV 35S promoter, so as to avoid any leaky expression of the *barnase* gene.
	A 2 kb *acetolactate synthase* gene fragment DNA fragments representing truncated and non-functional genes were fused to generate the spacer DNA fragment	*Arabidopsis*	
Promoters			
TA29 (Gene Bank Accession no.: X52283)	To regulate expression of *barnase* and *barstar* genes	*Nicotiana tabacum*	Drive tissue specific expression of *barnase* and *barstar* genes in tapetum layer cells
CaMV 35S	To drive *bar* gene expression in the *barnase* gene construct	*Cauliflower mosaic virus*	Constitutive expression of *bar* gene in the *barnase* gene construct
AMV Leader sequence	To drive *bar* gene expression in the *barnase* gene construct	*Alfalfa mosaic virus*	Enhanced expression level of PAT (Bar) protein from the *barnase* gene construct
CaMV 35S with a duplicated enhancer (*35Sde*)	To drive *bar* gene expression in the *barstar* gene construct	*Cauliflower mosaic virus*	Enhanced constitutive expression of *bar* gene in the *barstar* gene construct
Terminators (Poly-A signal sequences)			
35S ***poly(A)*** signal [*35SpA*]	Polyadenylation signal sequence	*Cauliflower mosaic virus*	Transcription termination sequence for *barnase* gene and *barstar* gene
ocs poly(A) signal	Polyadenylation signal sequence	*Agrobacterium tumefaciens*	Transcription termination sequence for *bar* gene in both the gene constructs

4.4 Method of genetic transformation of *B. juncea*

GE Brassica lines were developed by *Agrobacterium*-mediated genetic transformation (**Figure 4.5**).MS and RF lines were developed based on the transformation method described by Mehra et al. (2000). GE plants containing the *barstar* gene were self-pollinated to produce seeds, whereas the plants transformed with the *barnase* gene were backcrossed with pollen from untransformed parent to obtain seeds. The next generation plants were evaluated for tolerance to herbicide Basta and screened for the presence of the transgenes.

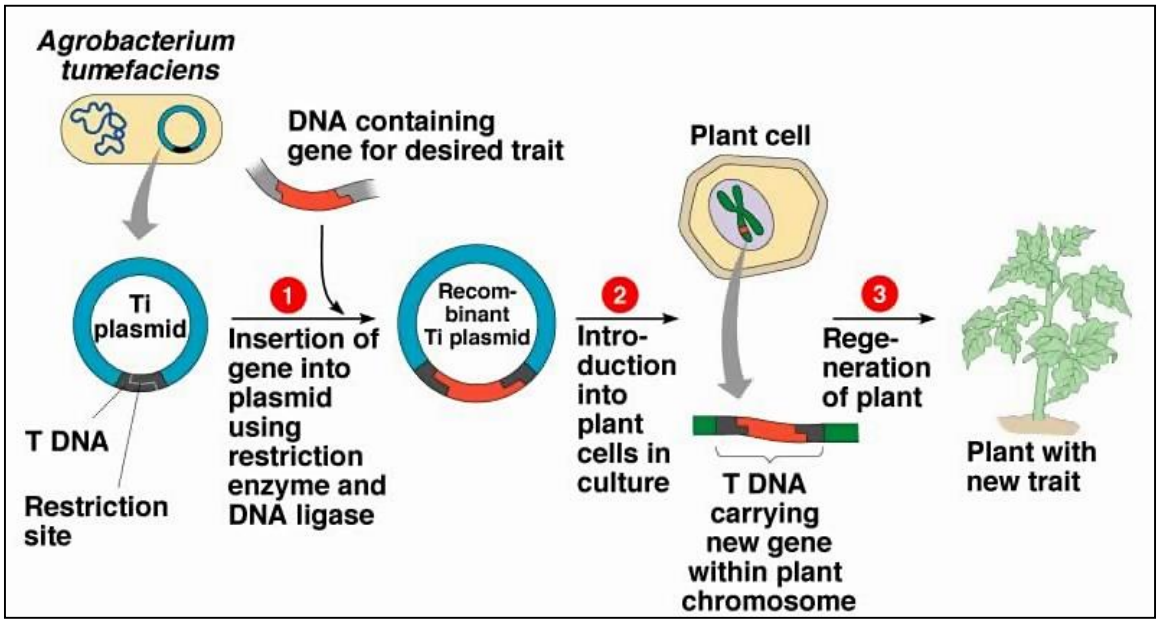


Figure 4.5: Generalized illustration of GE crop development through *Agrobacterium* mediated transformation. Source:www.whatisthebiotechnology.com

Out of several GE lines produced, the male sterile *barnase* event "bn 3.6"in RLM 198 genotype and the restorer *barstar* event "modbs 2.99" in Varuna genotype were selected for further assessment to develop hybrids. To develop a productive hybrid using the *barnase-barstar* system, the event bn 3.6 was backcrossed to variety Varuna and event modbs 2.99 was backcrossed to the line EH-2 (**Figure 4.6**). Event EH-2 modbs 2.99 was made homozygous by self-pollination and progeny testing. Subsequently this line has been maintained by self-pollination. Event Varuna bn 3.6 has been maintained by backcrossing to non-GE Varuna line (maintainer line).

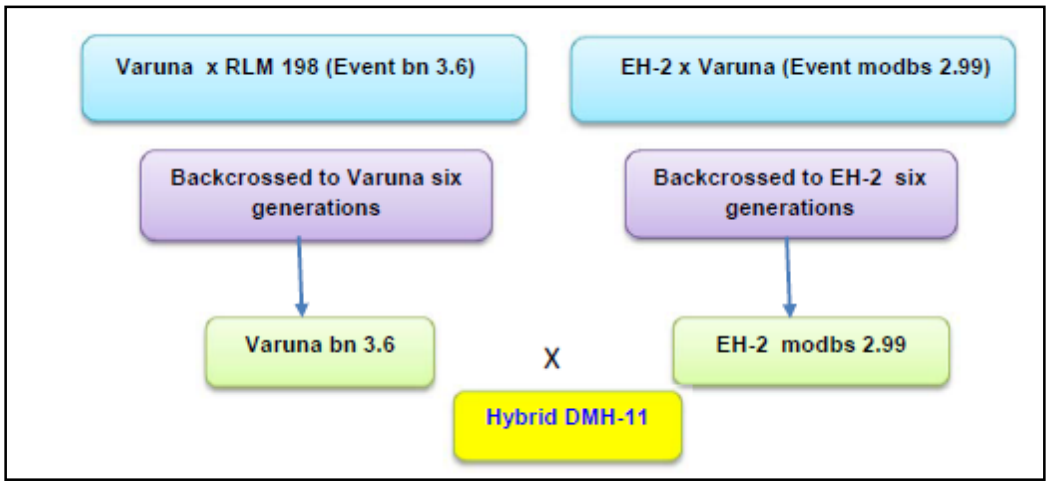


Figure 4.6: Scheme for backcrossing the initial GE events to productive lines for developing hybrid DMH-11

Varuna-bn 3.6 was found to stably maintain male sterility trait after going through several generations with no deleterious effect on the plant. EH-2 modbs2.99 was found to be a perfect fertility restorer of Varuna bn 3.6. Introgression of the two events in Varuna and EH-2 was used to develop F1 Dhara Mustard Hybrid 11 (DMH-11). Hybrid DMH-11, developed from crosses between Varuna bn3.6 and EH-2 modbs 2.99 was observed to be completely male fertile with no effects on female fertility and seed production in the F1 hybrid. The combining ability studies earlier had shown that hybrids between these two lines are significantly more productive than the parental varieties and the national and/ or regional checks.

4.5 Characterization of the inserted genetic material and stability of the genetic modification

Male sterile *barnase* line (event bn3.6): Several RLM198 GE lines transformed with the barnase construct were screened for their phenotype, extent of pollen sterility and stability of sterility trait. Event bn3.6 was selected as it was characterized to be a single copy insertion by Southern blot analysis and by genetic segregation analysis. MS event bn3.6 had normal morphology, was observed to be completely female fertile and had normal seed set when crossed to a maintainer line.

- *Southern blot and genetic stability analysis:* Genomic DNA, isolated from independently transformed events, was digested with *Eco*RV restriction enzyme, that cuts only once in the T-DNA region. Fragments were separated on agarose gels, blotted to a membrane and probed with the coding regions of the *bar* gene (present towards the Left Border) and with the *barnase* gene (present towards the Right Border) to determine the copy number of the integrated T-DNA in each of the putative transgenic events (**Figure 4.7**). Results of Southern hybridization confirmed the presence of a single copy integration (shown with arrow in **Figure 4.7a** and **4.7b**) (Jagannath et al. 2001, 2002), suggesting a single copy integration of the T-DNA in this transgenic event. This event was designated as "bn 3.6".

  Single copy integration for the *barnase* line 'bn3.6' has also been validated phenotypically by genetic analysis using backcrossed progeny of Varuna Barnase bn3.6 that segregated in a 1:1 ratio for resistance and sensitivity to the selection trait (when sprayed with Basta). The male-sterile *barnase* line has been analysed over ten generations under containment /field conditions and has been shown to stably inherit the male-sterile phenotype with no breakdown of sterility.

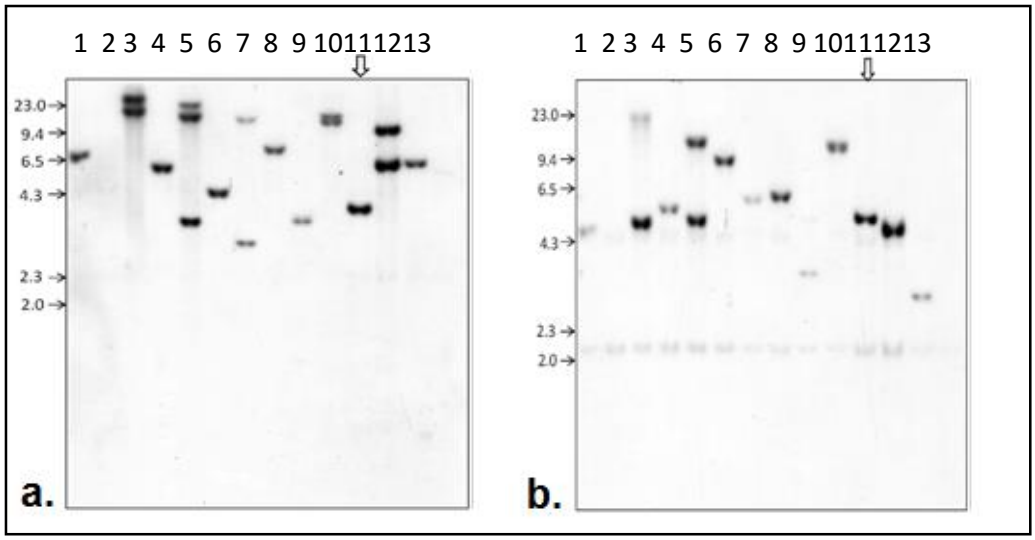


Figure 4.7: Southern blot to identify single copy insertion line for *barnase* construct. Event bn3.6 was picked as the best single-copy insertion event. Genomic DNA digested with EcoRI enzyme and (a) probed with bar gene coding sequence present towards the Left border of T-DNA and (b) probed with *barnase* gene coding sequence present towards the Right border. Lane 11 (marked with arrow) represents the selected Event bn 3.6.

- *Characterization of the integration site:*DNA sequence analyses was performed on genomic DNA of event barnase bn3.6 to determine the complete DNA sequence of the introduced T-DNA region and the flanking genomic DNA sequences by PCR based gene walking libraries. The sequence analysis confirmed the predicted organization of the genetic elements within the T-DNA and also confirmed that each genetic element in the genome-integrated T-DNA is intact and all sequence elements are identical to that of T-DNA present in the binary vector.

  The T-DNA was found to be integrated in A9 Linkage Group (LG A9) on the 'A' genome of *B. juncea* between 'Bra32488' and 'Bra32489' genes. The genomic sequences flanking the T-DNA insertion showed>95% sequence similarity with published sequence of *B. rapa* between the two genes. Translation of flanking DNA sequence in all the six reading frames showed that no new ORFs had been created due to T-DNA integration. The translation of flanking sequence (after joining both side sequences together) showed no disruption of any endogenous gene in the *barnase* event bn3.6.

  The original GE event bn 3.6 was in the genetic background of RML 198. It was later transferred to var. Varuna genetic background through six generations of backcrossing with Varuna. Insertion of any vector sequences other than the T-DNA into the plant genome would have been eliminated after six backcrossings.

Fertility restorer *barstar* line (event modbs2.99): Initially a large number of Varuna GE lines, developed with the barstar construct, were characterized for their phenotype and segregation of the bar-barstar cassette after selfing or back crossing. Copy number of the transgene was studied by Southern blotting and some of the selected lines were tested for their ability to restore male fertility of the MS line bn3.6. Finally event modbs2.99 was selected as the most optimal RF line for fertility restoration.

- *Southern blot and genetic stability analysis*: Genomic DNA, isolated from the events independently developed with the barstar construct, was digested with EcoRV restriction enzyme, that cuts only once in the T-DNA region. Fragments were separated on agarose

gels, blotted to membrane and probed with the coding regions of the *bar* gene (present towards the Left Border of T-DNA) and with the *barstar* gene (present towards the Right Border) to determine the copy number of the integrated T-DNA in each of the putative GE lines (**Figure 4.8**). Results of Southern hybridization showed the presence of a single band in some lines (shown with arrow in **Figure. 4.8**) (Jagannath et al. 2001, 2002)when hybridized with both the LB and RB probes, suggesting a single copy integration of the T-DNA in one of the GE events. This line was designated as "modbs2.99".

Single copy integration of the *barstar* event modbs2.99 was confirmed by genetic analysis using backcrossed progeny that segregated in 1:1 ratio for resistance and sensitivity to the selection trait when sprayed with Basta. The restoration capacity of the *barstar* event was also studied under field conditions for more than ten generations and the line has been shown to successfully restore male fertility of the *barnase* event bn3.6.

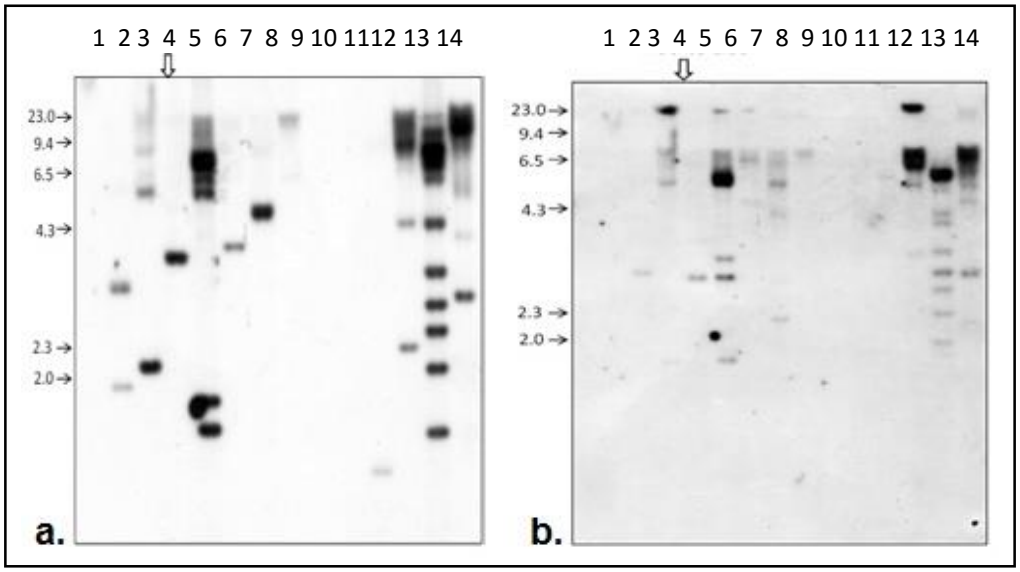


Figure 4.8: Southern blot analysis to identify single copy insertion line for the *barstar* construct (designated later as event modbs 2.99). Genomic DNA digested with EcoRV enzyme and (a) Probed with *bar* gene coding sequence present towards the Left border of T-DNA and (b) probed with *barstar* gene coding sequence present towards the Right border. Lane 4 (marked with arrow) represents the selected Event modbs 2.99.

- *Characterization of the integration site:* DNA sequence analyses was performed on genomic DNA of event modbs2.99 to determine the complete T-DNA sequence insertion and flanking genome sequences. The sequence analysis confirmed intactness and predicted organization of each genetic element in the T-DNA insert. The T-DNA sequence

determined for GE event modbs 2.99 is identical to that of T-DNA sequence present in the binary vector.

The flanking DNA sequences present on both the left (LB) and right (RB) border junctions were determined for the event modbs 2.99. The flanking DNA sequence analysis showed that the integration site is most probably in the 'B' genome of mustard as flanking sequences showed < 50% sequence similarity to the *B. rapa* sequence available in the BRAD database. The genomic flanking DNA sequence did not show any significant sequence similarity to any of the known genes in *B. rapa*, *Arabidopsis* and other crucifer species that have been sequenced. This analysis also showed that no new ORF was created and also no disruption of any known ORF was reported at the integration sites.

The original GE event (modbs2.99) was in the genetic background of Varuna. It was transferred to EH-2 background by six generations of backcrossing to EH-2. The possibility of the presence of any vector backbone sequences in the EH-2 genome after six backcrossing with EH-2 can be considered extremely low.

Efficacy of the MS/RF system: To test efficacy of the male sterility-restorer system, a hemizygous male-sterile barnase line was crossed with a hemizygous barstar line. All the F1 progeny containing bar gene marker showed segregation for male fertility and male sterility in a 2:1 ratio, respectively, indicating complete restoration of male sterility.

4.6 Expression of the introduced genes

Expression of each of the introduced genes in the two GE mustard lines Varuna bn3.6, EH-2 modbs2.99 and the F1 hybrid DMH-11 has been evaluated for expression of the transgenes at the transcriptional level and at the protein level.

Expression at the transcript level: Expression levels of the *barstar* and the *barnase* genes at various stages of plant development in the two events - Varuna bn3.6 and EH-2 modbs2.99 were determined by measuring transcript abundance of each gene using quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). In Varuna bn3.6 event, no barnase specific transcripts were observed in any tissue including anthers by RT-PCR. The lack of

detection in the anthers could be due to the fact that the expression of the barnase gene leads to degeneration of the tapetum tissue. This result proves that the expression of barnase gene under TA29 promoter is tightly regulated for temporal as well as spatial expression.

In the event EH-2 modbs2.99, highest expression of the *barstar gene* was observed in the anthers. Some level of expression was also observed in the leaf, stem and the root tissues. This could be due to the presence of a strong 35S double enhancer promoter, driving expression of the *bar* gene. The high expression of the Barstar protein would lead to complete inhibition of the Barnase enzyme.

In GE mustard hybrid DMH-11, expression of both the *barnase* and the *barstar* genes was observed in the anthers. *Barnase* and *barstar* are under the control of tissue specific promoter and are expressed only in the tapetum of anthers for a very short period of time i.e. at the anther development stage when tapetum is fully functional. Higher expression of the *barstar* gene in the anthers (tapetum) cells of DMH-11 hybrid protects the tapetum tissue from degeneration due to Barnase activity. That is the reason behind effective restoration of male sterility in DMH-11. In addition to anthers, low levels of *barstar* transcripts were also observed in other tissues of hybrid DMH-11.

Expression at protein level: To detect presence and levels of expression of the three proteins (Barnase, Barstar and Bar) in the GE events, quantitative enzyme linked immunosorbent assay (ELISA) kits were developed by M/s. Amar Immunodiagnostics, Hyderabad.

- *Detection limits*: These kits were checked for their detection sensitivity - the minimum level of detection for the Barstar protein is 0.150 ng/gm of total protein, for Barnase it is 0.190 ng/gm of total protein and for Bar it is 0.102 ng/gm of total protein.

As pure proteins are required for undertaking toxicity and allergenicity studies, recombinant Barnase, Barstar and Bar proteins were produced in *E. coli* using routine cloning methods. Activity assays were performed on the three proteins isolated from *E. coli* to confirm their functionality. The purified proteins besides being used for the development of ELISA based kits for detection of the three proteins in GE lines, were used for toxicity studies also.

Expression of the three proteins was studied first in the plants grown in a contained net house and later in the field grown plants during two years of the BRL I trials and one year of the BRL II trial. Data on protein expression in net house and field grown plants is summarized in **Table 4.2**.

Table 4.2: Expression level of Barnase, Barstar and Bar proteins in the GE mustard lines

	Leaf	Stem	Root	Whole bud	Seed	Seedling
Bar (µg / mg of the total protein) *						
Varuna barnase	0.006 ± 0.005	0.02 ± 0.02	0.03 ± 0.01	0.003 ± 0.002	0.01 ± 0.001	0.01 ± 0.004
EH-2 barstar	1.47 ± 0.68	0.97 ± 0.51	4.26 ± 1.81	1.24 ± 0.60	0.64 ± 0.125	1.32 ± 0.037
DMH-11	0.66 ± 0.32	0.46 ± 0.41	2.31 ± 1.12	0.31 ± 0.20	0.39 ± 0.06	0.83 ± 0.143
Barstar (µg / mg of the total protein)						
Varuna barnase	ND**	ND**	ND**	ND**	ND**	ND**
EH-2 barstar	0.00011 ± 0.00006	0.0001 ± 0.00007	0.00026 ± 0.00019	0.00164 ± 0.0006	ND**	0.00036 ± 0.00001
DMH-11	0.00006 ± 0.00003	0.00006 ± 0.00004	0.00013 ± 0.00009	0.00085 ± 0.00028	ND**	0.00022 ± 0.00001
Barnase (µg / mg of the total protein)						
Varuna barnase	ND**	ND**	ND**	ND**	ND**	ND**
EH-2 barstar	ND**	ND**	ND**	ND**	ND**	ND**
DMH-11	ND**	ND**	ND**	0.0017 ± 0.001	ND**	ND**

**bar* gene is driven by 35S CaMV promoter with double enhancer and is expected to express in all tissues.
**ND- Not detectable - below the level of detection

Barnase protein expression was detectable only in the buds of hybrid DMH-11 and that also at very low levels. The Barnase protein could not be detected in any other organs or tissues of the hybrid or parental lines.

Barstar protein expression was detected at a very low level in the buds of EH-2 modbs 2.99 and hybrid DMH-11. Negligible levels of Barstar protein were detected in other parts of EH-2 modbs2.99 and hybrid DMH-11.

Bar protein was found to be expressed in all the three lines, with low levels in leaves and barely detectable levels in the seeds. The expression level of Bar protein was observed to be significant in roots of the EH-2-barstar line and hybrid DMH-11.

The edible plant parts of Indian mustard are mainly leaves and seeds. While Barnase is not expressed in the leaves, very low expression of Barstar is detected in the hybrid plant leaves.

As expected, the Bar protein is present in all the edible plant parts in the two parental lines as well as in the hybrid.

Seeds of GE hybrid DMH-11, to be used for oil extraction and meal, contain only traces of the Bar protein. Seeds do not have any detectable levels of either the Barnase or the Barstar protein. Further, as edible oil produced from mustard seeds contains only fats the possibility of any of the three proteins being present in the oil to be consumed by human beings is remote.

4.7 Pleiotropic effect, if any, of the genetic modification

Pleiotropic effects, if any, arising from the integration of the T-DNA into plant genome were studied to identify any changes in the GE plants in terms of changes in their morphology, agronomical traits, compositional analysis, etc. The flanking DNA sequence analysis showed that no endogenous gene or open reading frame was disrupted. No pleiotropic effects were recorded for any of the traits studied in both GE parental lines as well as GE mustard DMH-11 hybrid, except that the pollen morphology in the GE Varuna bn 3.6 male sterile plant, as expected showed early degradation of the microspores.

4.8 Functionality of the introduced proteins for male sterility and restored fertility

Functionality of the *barnase* gene in the event bn 3.6 and its derivatives has been tested by morphological observations of anthers (i.e. absence of pollen production) and the functionality was confirmed by the absence of seed set upon self-pollination by bagging the inflorescence. The efficacy of the *barstar* gene in hybrids was undertaken by crossing hemizygous male sterile barnase line with a hemizygous barstar line. Pollen viability was found to be around 97%, tested by fluorescein di-acetate (FDA) test, in DMH-11 hybrid.

Flowers of the MS line Varuna bn3.6 are characterized by complete absence of viable pollen in the anthers, which remain rudimentary and flattened in comparison to the anthers in the normal fertile flowers. A moderate difference in size has been observed between the male sterile flowers and the normal fertile flowers, with the former being smaller. All other vegetative and floral organs of *barnase* containing plants are normal – similar to the

untransformed plants. Pollen production was studied by crushing the anthers in sucrose solution and by counting the microspores under a microscope. No viable pollen was observed in Varuna bn3.6, while non-GE Varuna line was found to have viable pollen grains. The restorer barstar event EH-2 modbs2.99 plants are indistinguishable in their morphological features from the non-GE plants of EH-2. Pollen production between EH-2 and its GE counterpart EH-2 modbs 2.99 showed no significant difference.

Similarly, pollen production between the GE DMH-11 hybrid (F1) plants and its non-GE counterpart VEH-2 (a hand-made F1 hybrid of non-GE Varuna x EH-2) showed no significant difference. Plants of the DMH-11 hybrid were indistinguishable in their morphological features from the non-GE plants of VEH-2 hybrid (**Figure 4.9**).

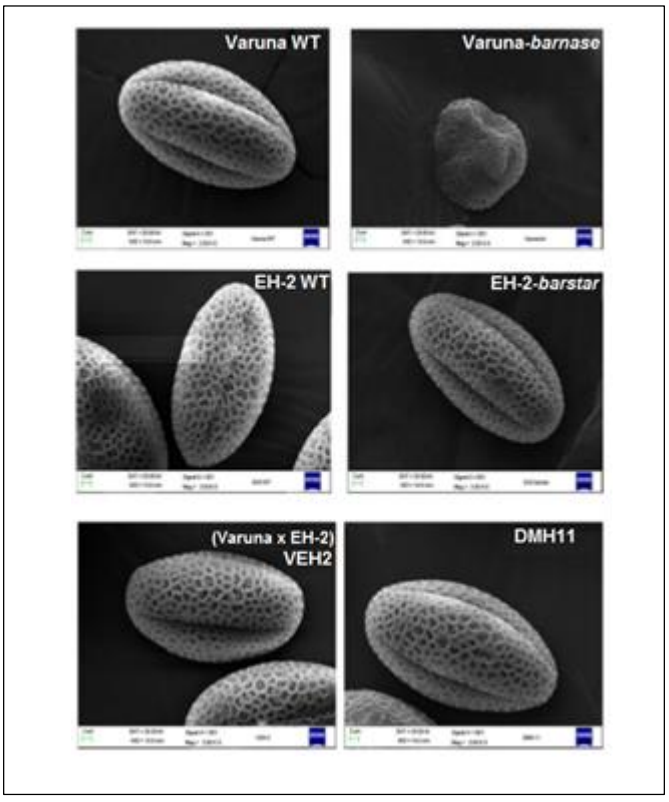


Figure 4.9: Scanning electronic microscopy (SEM) of pollen from *barnase, barstar* lines and their comparators and DMH-11. Only Varuna-*barnase* has shrivelled, non-viable, pollen.

4.9 Detection of GE seedlings in a population

As per the regulatory requirements, the applicant standardised a protocol, using selection agent 'Basta' spray followed by PCR analysis, for detecting the presence of a GE seedling

amongst non-GE seedlings at a frequency of 1 in1000. The protocol was checked with GE hybrid DMH-11, as it has all the three genes i.e. *barstar*, *barnase* and *bar*.

4.10 Cloning, purification and production of pure Barstar, Barnase and Bar proteins for biosafety studies

For undertaking toxicity as well as allergenicity studies, large amount of protein is required which cannot be isolated from GE plants. For the present study, Barstar, Barnase and Bar proteins were cloned and expressed in *E. coli* and purified and produced as per the protocols based on the Guidelines for Safety Assessment of Foods Derived from GE Plants, 2008 with prior approval of RCGM. In addition to the process followed for Bar and Barstar, two sequential clonings were included for the insertion of Barnase in pET30b_barstar expression construct. The strategy design was prepared this way because Barnase cannot be cloned as original sequence directly without presence of its inhibitor (Barstar). Interaction between Barnase and Barstar in the complex of two proteins was broken in presence of 6M urea in Diethylaminoethyl (DEAE) column during purification from periplasm. Removal of urea helped Barnase to refold, the protein was then eluted by Phosphate buffer saline (PBS). After purification process, purity of the barnase protein was achieved at more than 90%, purity of Barstar and Bar proteins was achieved at>98%.

It is important to show that the recombinant proteins isolated from a heterologous system (in this case *E.coli*) are similar to the ones expressed in plants. This was done by establishing:

- Confirmation of functionality of the three recombinant proteins by activity assays
- Equivalence of the Barstar, Barnase and Bar recombinant proteins expressed in *E.coli* versus GE plants

Confirmation of functionality of the three recombinant proteins by activity assays: For confirming functionality of the three proteins, activity assays were performed on the three proteins isolated from *E. coli*. The studies carried out were: Final buffer optimization activity assay; Visual Precipitation; Lyophilization study of Recombinant Barstar, Barnase and Bar proteins followed by activity analysis for the stability of the protein; Thermal stability study of recombinant protein sample at different temperatures ranging from 25°C to 95°C and

Spectrophotometric enzyme assay to establish the functions of respective recombinant proteins. The conclusions of activity assays of the three recombinant proteins are summarized in the **Table 4.3**.

Table 4.3: Functional properties of recombinantly expressed Barstar, Barnase and Bar proteins

	Barstar	Barnase	Bar
Final PBS buffer optimization activity assay	Significant activity.	Good activity	Good activity
Lyophilization study activity	Significant activity loss after lyophilisation	Significant activity loss after lyophilization	Significant activity loss after lyophilization
Thermal stability study	No decrease in activity at high temperatures	No decrease in activity	Decrease in protein activity after 55°C
Enzyme activity data	In a qualitative Barstar assay, its inhibitory action upon the ribonuclease activity of Barnase protein was studied. 2.5 µg of Barnase leads to complete degradation of yeast RNA, however increasing inhibition of RNase activity was observed upon increasing concentration of purified Barstar protein. At 160ug concentration of Barstar protein significant inhibition of Barnase was achieved.	• Spectrophotometric assay of the ribonuclease activity of the Barnase protein • 50 ng of Barnase was enough to completely degrade 20 µg of Yeast RNA in 1 hour. The specific activity was found to be $2.12X10^7$ Units/mg of protein	• Spectrophotometric assay of Bar N-acetyltransferase activity • Purified recombinant Bar protein has an activity of 11.47 units per mg of protein.

Equivalence of the Barstar, Barnase and Bar recombinant proteins expressed in *E.coli* versus proteins in the GE plants: For establishing equivalence, the applicant utilised Targeted Liquid Chromatography-Mass Spectrometry (LC-MS) to show that the three recombinant proteins Barstar, Barnase and Bar produced in *E. coli* were similar to the ones expressed in the GE mustard lines. Signature peptides for the three proteins – Barstar, Barnase and Bar were identified. Extracts of various tissues (leaf, stem, whole bud, seed and seedling) from Barnase, Barstar and DMH-11 GE lines were run on LC/MS to check for the presence of these peptides. The elution time and the MS/MS spectra for the selected peptides were the same for both

the recombinant protein as well as the proteins expressed in the GE lines (**Figure 4.10-4.13**). It is evident from the results that the expression of Barnase is very low in samples and is masked by a co-eluting highly-abundant peptide. The presence of the Barnase peptide was confirmed by matching the multiple fragment ions within mass accuracy of 5ppm as shown in the **Table** presented in **Figure 4.12**. The red colour indicates the presence of b and y-ion matching within the specified mass tolerance.

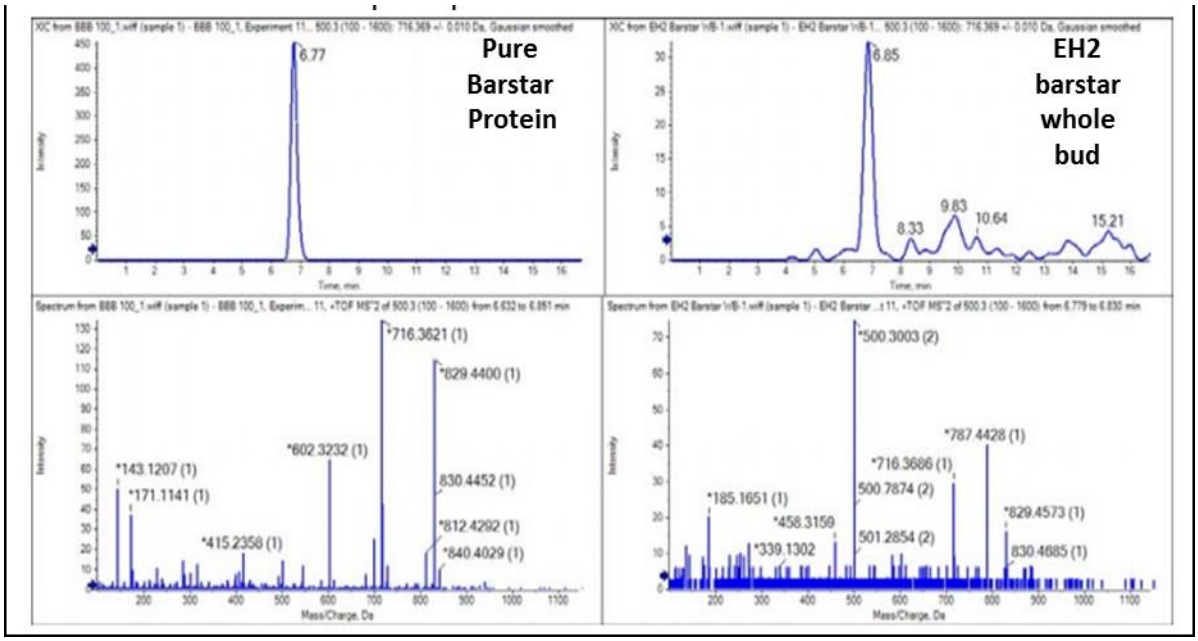


Figure 4.10: The extracted ion chromatogram for barstar peptide AVINGEQIR from the pure protein and EH2 barstar whole bud.

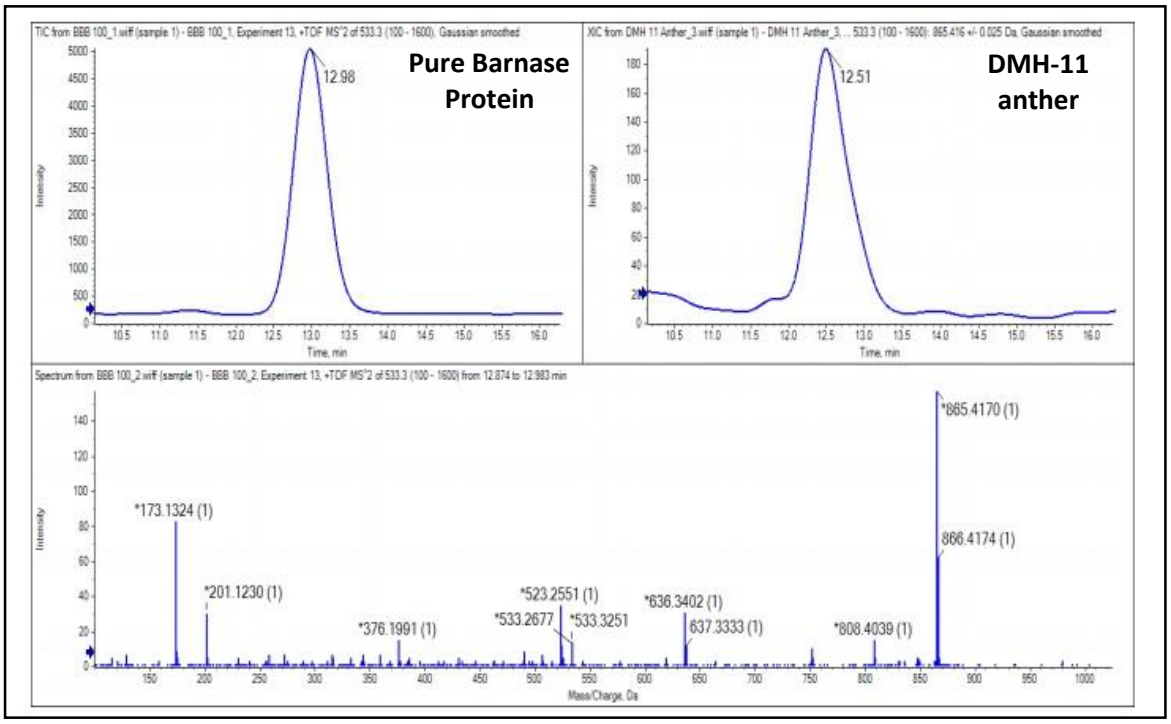


Figure 4.11: The extracted ion chromatogram for barnase peptide SIGGDIFSNR from the pure protein and DMH-11 anther. The MS/MS is shown for the pure protein.

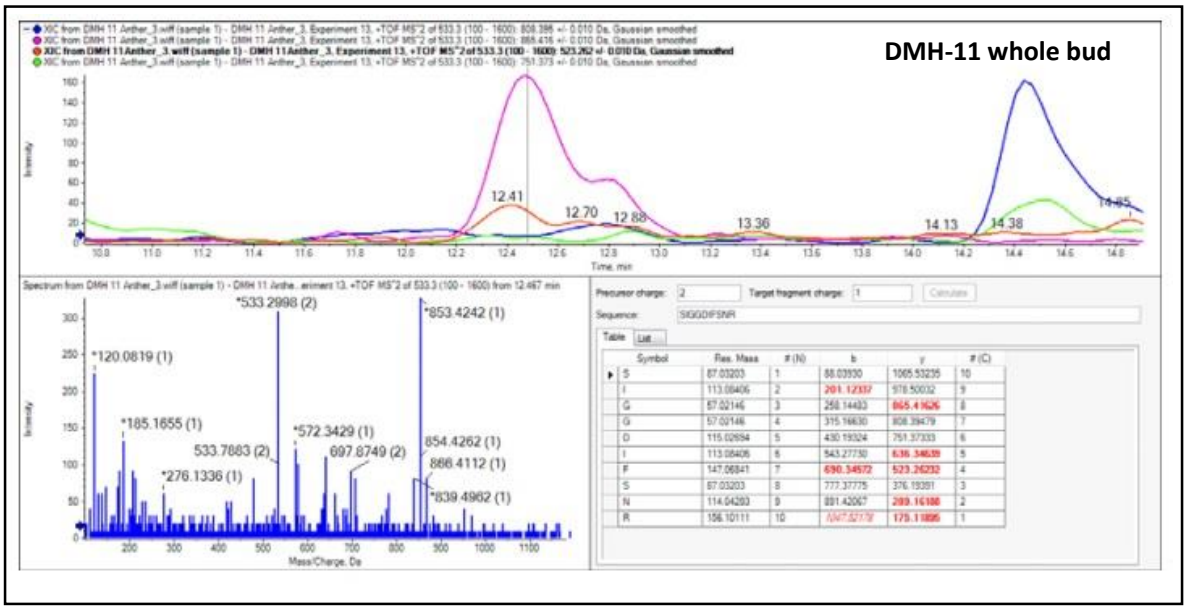


Figure 4.12: The extracted ion chromatogram for barnase peptide SIGGDIFSNR from DMH-11 whole bud

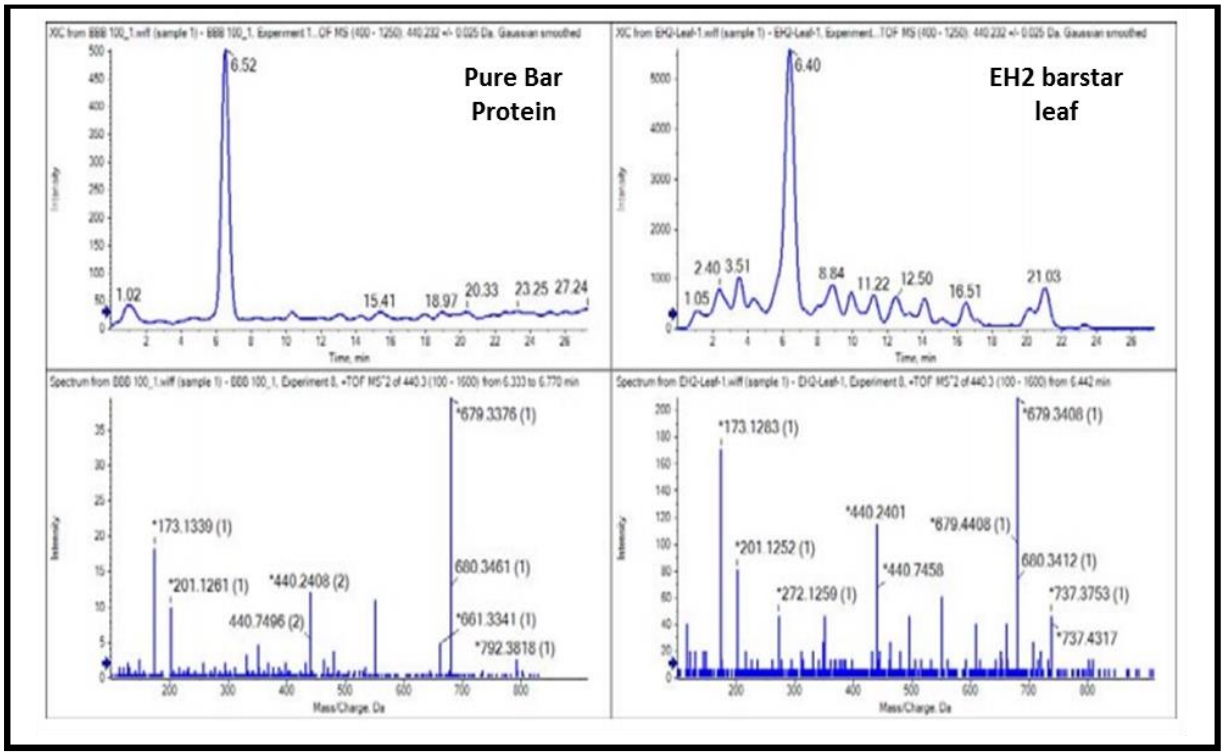


Figure 4.13: The extracted ion chromatogram for bar peptide SLEAQGFK from the pure protein and EH-2 barstar leaf

The study clearly confirmed that the Barstar, Barnase and Bar proteins expressed in GE plants were equivalent to the recombinant proteins isolated from *E. coli* in their MS/MS spectra for the selected peptides and all the three proteins expressed in *E. coli* and subsequently purified were functional.

4.11 Conclusions

The molecular characterization data reveals that the two parental events have a single copy of the transgene integrated in the genome. The insertion of these genes does not lead to disruption of any known endogenous gene coding for a protein. The transgenes are stably integrated and expressed and have been shown to be inherited across several generations. Hybrid DMH-11 was made by crossing the male sterile and fertility restorer lines. The MS line bn 3.6 has normal morphology, is completely female fertile and has normal seed set when crossed to a maintainer line. Flowers of the MS line Varuna bn 3.6 are characterized by complete absence of viable pollen in the anthers. In the MS line, the transgene is inherited stably with no breakdown in sterility over many generations. RF line has been shown to successfully restore male sterility of the MS line over many generations.

Molecular characterization of a GE event is the key step in the biosafety assessment. The extensive molecular analysis carried out on GE parental lines and hybrid DMH-11 clearly demonstrates that there are no unintended changes at the molecular level reflecting thereby that the phenotype is not expected to have any altered characters other than intended modification(s) as discussed in the following chapters.

CHAPTER 5
FOOD AND FEED SAFETY STUDIES

5.1 Introduction

As described in the Chapter 4, stable insertion and expression of introduced foreign gene(s) into the host plant genome has been demonstrated and the intended goal of developing stable MS and RF lines has been achieved. Compositional equivalency between GE crops and non-GE comparators is a fundamental component in the biosafety assessment of GE foods and feeds. It forms part of a weight-of-evidence approach in determining overall safety. The purpose of the compositional assessment is two-fold: first, to verify that the expected changes resulting from the genetic modification have not negatively affected the safety and nutritional quality of the food and, second, to verify that detrimental unintended changes to plant composition have not occurred as a result of the modification (i.e., as a check for "unintended effects"). Unintended changes that could be detrimental may include increased or decreased levels of nutrients, antinutrients, secondary metabolites, and/or natural plant toxins. Evaluation of compositional data involves comparison between the GE plant and its non-GE comparator. Any statistically significant difference is reviewed in comparison to values/ranges for conventional varieties available in literature or recognized databases. If the level of a particular component does appear to be outside of what is expected on the basis of the published ranges, further examination is warranted, to investigate possible unintended effects of the modification. Animal feeding studies may be needed to demonstrate the safety and nutritional quality of the food and feed derived from the GE plant.

An integral part of the food safety evaluation is an assessment of the potential toxicity and allergenicity of the introduced proteins being expressed in the GE *B. juncea* and its toxicity to human and other organisms. *B. juncea* being a food as well as a feed crop in India, the safety assessment included the following aspects: Whether the introduced proteins are expressed in the edible plant parts; whether the GE mustard produces any allergen due to introduced proteins; and whether the GE plant could potentially lead to toxicity after consumption by humans or animals.

5.2 Nutritional and compositional assessment studies

Keeping in view all the available information, biology of the crop and its use for diverse purposes, the regulatory authorities, RCGM and GEAC, had instructed the applicant to perform compositional analyses of key components in the seeds and leaves of GE mustard hybrid DMH-11, GE parents used in the hybrid seed production and the non-GE parents and local checks during the Biosafety research level (BRL) trials. The objectives of the study were:

- *Substantial equivalence of GE and non-GE mustard seeds including oil, seed meal for*-Oil, protein, carbohydrate, moisture, glucosinolates, erucic acid, fatty acids, allyl isothiocyanate, peroxide value, sodium, calcium, magnesium, potassium, and total mineral matter.

- *Substantial equivalence of GE and non-GE mustard leaf established for - p*rotein, carbohydrate, oil, ash, and moisture

Compositional analysis: The applicant has generated information on the composition of the key nutrients of genetically modified Varuna *barnase* event bn 3.6, EH-2-*barstar* event modbs 2.99 and their respective untransformed comparators, DMH-11 hybrid and zonal check.

- *Analysis of the key components:* Compositional analysis included whether any change(s) have occurred in the levels of key components like erucic acid and glucosinolates or are these within the normal range of crop varieties of the same species that are developed through conventional breeding methods and are currently being cultivated. Unlike Canola rapeseed (*B. napus*) mostly grown abroad, Indian mustard (*B. juncea)* seeds have high levels of erucic acid and glucosinolates and are considered less desirable for human consumption, especially in the western countries. One of the mustard crop improvement programs is to develop varieties with reduced content of glucosinolates (Chauhan et al, 2002; Sodhi et al, 2002; Augustine et al, 2013) and erucic acid (Agnihotri and Kaushik, 2003; Beniwal et al, 2015).The total glucosinolates (GSL) were measured by Near Infra-Red Spectroscopy (NIRS) and various components were analysed by High Performance Liquid Chromatography (HPLC). No significant difference was observed between the GE lines as compared to their non-GE parents for total glucosinolates in seeds collected from different entries in the BRL II trials at three locations: Ludhiana (PAU), Bhatinda (PAU) and Delhi

(IARI) (**Table 5.1**). Total GSL in hybrid DMH-11 was observed to be intermediate of the two parents Varuna (high GSL) and EH-2 (low GSL).

Table 5.1: Estimation of total glucosinolate among the GE lines along with their non-GE counterparts (growing season (2014-15)

Location	PAU Ludhiana	Bhatinda	IARI
Sample Name	Concentration (µmol/g seed)	Concentration (µmol/g seed)	Concentration (µmol/g seed)
Varuna	85.8	83.0	90.8
Varuna-Barnase	84.7	90.3	86.4
EH-2	17.8	11.8	10.9
EH-2 Barstar	16.4	10.9	11.6

Normal range of GSL in commercially cultivated varieties from Table 5.3- 10.9-96.1 µmol/g seed

Another important parameter analysed was the fatty acid composition. Levels of important fatty acids like Erucic acid (22:1), Omega-3 fatty acid (18:2), and Omega-6 fatty acid (18:3) were analysed. Erucic acid, Omega-3 fatty acid, Omega-6 fatty acid in Varuna barnase and EH-2 barstar are not significantly different from their respective non-GE comparators in leaves as well as in the seeds. The levels observed in the hybrid DMH-11 fall within the range of non-GE Varuna (**Table 5.2**) and zonal check RL-1359 (**Table 5.3**).Thus, the data submitted suggest that there are no significant differences in key fatty acids component levels in seeds or leaf of GE parental lines as well as in the hybrid DMH-11 as compared to their respective comparator.

Table 5.2: Estimation of key fatty acids (g/100g) in mustard seeds (dry).

Parameter	Varuna		EH-2	
	GE (Barnase)	Non GE	GE (Barstar)	Non GE
C22:1n9 Erucic)	18.59 ± 0.275	18.63 ±0.654	0.63 ±0.173	0.39 ± 0.170
C18 :3n3(α- Linolenic)	0.51 ± 0.425	0.46 ± 0.493	0.36 ± 0.624	1.44 ± 1.543
C18: 2n6c(Linoleic)	1.82 ± 1.028	3.18 ± 0.420	2.29 ± 2.939	5.99 ± 4.489

***significant at 5% level**
Values expressed Mean ± SD (g/100g)

Table 5.3: Glucosinolate and key fatty acids in hybrid DMH-11, Varuna and RL-1359

Parameter		Range of means from 3 locations for hybrid DMH-11	Normal range in commercially cultivated varieties (Varuna, Varuna barnase, EH-2,EH-2 barstar, RL-1359) from 3 locations
Glucosinolate	Seed (Dry)	60.5-63.5 µmol/g	10.9-96.1 µmol/g
Erucic Acid	Seed (Dry)	11.72-15.48 g/100g	0.23-19.19 g/100g
	Leaf (Dry)	2.19-2.74 g/100g	0.00-3.11 g/100g
α- Linolenic	Seed (Dry)	0.00-2.04 g/100g	0.00-4.26 g/100g
	Leaf (Dry)	0.53-1.30 g/100g	0.0-2.55 g/100g
Linoleic	Seed (Dry)	0.64-3.65 g/100g	0.45-8.97 g/100g
	Leaf (Dry)	1.02- 1.46 g/100g	0.63-1.98 g/100g

Other parameters like essential B-complex vitamins viz.Folic Acid, Riboflavin, Niacin, Pantothenic Acid, Thiamine, and Pyridoxine were found to be similar in the seeds as well as in the leaves of the GE parents as compared to their non-GE comparators. Similarly, there was no statistically significant difference in the levels of minerals like Calcium, Manganese, Copper, Iron, Selenium and Zinc in the leaf and seeds of GE line vis-à-vis their non-GE comparators across all the three locations. The values for these parameters in hybrid DMH-11 are comparable with the commercially cultivated Varuna and Zonal check RL-1359.

The compositional analysis also demonstrated that the GE parental lines were comparable in their nutrient composition, including values for proximates, fibres, amino acids and secondary metabolites to their non-GE comparators. The nutrient compositions of Hybrid DMH-11 are within the range of non-GE Varuna and the zonal check.

On the whole, the compositional studies of all key components involved in nutritional adequacy of leaves and seeds indicated that there is no evidence of any alteration in the nutrient or anti-nutrient quality with respect to parameters such as proximate, fibres, minerals, vitamins, amino acid, fatty acids and secondary metabolites in leaves and seeds of mustard due to the presence of the transgenes.

Conclusions: On the basis of data analysed, the compositional differences between GE line and their conventional comparators are within the range of natural variability encountered in mustard.

It is evident from the compositional analysis studies that GE parents are not significantly different from the non-GE comparators in terms of key parameters such as oil, proteins, carbohydrates, glucosinolates, erucic acid, fatty acids, allyl isothiocyanate, peroxide value, sodium, calcium, magnesium, potassium and minerals - when analysed in leaf and seeds. Also, hybrid DMH-11 is very similar, in its composition, to the commercially cultivated varieties in India which have a history of safe use.

These results collectively demonstrate that the introduction of *barnase, barstar* and bargenes into GE mustard does not show any unintended effects on the overall composition of GE plants – either the parental lines or the hybrid DMH-11.

5.3 Toxicity and allergenicity assessment studies

This section considers potential hazards that may be posed by the consumption of GE mustard as food or feed to human and animal health and safety. Studies were conducted at the National Institute of Nutrition, Hyderabad, an institute under the Indian Council of Medical Research, Ministry of Health & Family Welfare, Government of India.

As part of the risk assessment procedure the applicant was directed to conduct toxicity and allergenicity tests as per the requirements set out in the current guidelines of India (ICMR Guidelines for Safety Assessment of Foods Derived from Genetically Engineered Plants 2008, Protocols for Food and Feed Safety Assessment of GE crops 2008 based on Codex Alimentarius Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants, 2003). The proposed studies included:

Bioinformatics analysis of the three introduced proteins Bar, Barnase and Barstar for allergenicity;pepsin digestibility of the three proteins; thermal stability of these proteins; acute oral toxicity study of the purified recombinant proteins; and animal feeding studies by feeding leaves and seeds of GE and non-GE mustard lines to evaluate sub-chronic toxicity.

The assessment aims to evaluate the potential toxicity or allergenicity of GE mustard to humans and animals due to the expression of the introduced gene products or any unintended changes arising from the genetic modification at the site of T-DNA integration.

B. juncea is primarily cultivated as oilseed crop, and the leaves of young plant are used as vegetable, and are good source of vitamin A, vitamin C, calcium and iron (Swati and Das 2015). Both refined and unrefined (Kachi Ghani oil) oil is used in India in food. Further, mustard oil is commonly used for body and hair massage in certain parts of India. It is also used in soaps and as a counter irritant in folk medicine (Dhar et al., 2013).

The three genes used in the development of the *barnase-barstar* based male sterile hybrid system for mustard have been derived from a commonly occurring soil bacterium – *barstar and barnase* genes are from *Bacillus amyloliquefaciens* and *bar* gene from *Streptomyces hygroscopicus*. *B. amyloliquefaciens* is used in food industry and *S. hygroscopicus* is a known

non-pathogenic soil actinomycete. Carbohydrase and protease enzyme preparations derived from either *Bacillus subtilis* or *B*. *amyloliquefaciens* are Generally Recognized As Safe (GRAS) for use as direct food ingredients by US FDA (US FDA, 1999; 21 CFR Part 184). *Bacillus amyloliquefaciens* is approved as a source organism for food enzymes (Health Canada, 2015).

5.4 Expression levels of the Barnase, Barstar and Bar proteins in GE mustard parental lines and DMH-11 hybrid

- As mentioned in earlier chapters, the level of expression of the three proteins in the GE events was estimated by quantitative Enzyme Linked Immunosorbent Assay (ELISA) kits as part of the biosafety assessment.
- Data on the expression of the introduced genes in the two GE mustard parental lines (Varuna bn 3.6, EH-2 modbs 2.99) and hybrid DMH-11 was analysed in the plants and is presented in **Chapter 4.**
- The data showed that the Barnase expression levels in the Varuna bn3.6 are below the detection level and yet the expression level is sufficient to create male sterility trait, i.e the cells that express the protein are dead and so no protein expression can be detected.
- The data showed that Barstar is present in the buds and at very low levels in other parts such as leaves, stem, root and seedling in EH-2 modbs 2.99 and hybrid DMH-11. **No Barstar expression was detected in the seeds of EH-2 modbs 2.99 and hybrid DMH-11.**
- The data showed that the Bar protein is expressed in all the three lines, at low levels in leaves and at barely detectable levels in the seeds. The expression level of the Bar protein was observed to be significant in roots of the EH-2-barstar line and hybrid DMH-11 plant.
- **While Barstar, the inhibitor of Barnase, is present at low levels in the vegetative tissues, no Barnase protein was detected in any tissue other than the flower buds of the hybrid DMH-11. Importantly, seeds do not have any detectable levels of either the Barnase or the Barstar protein.**

As evident from the data, seeds of GE DMH-11 to be used for oil extraction and meal, contain only traces of the Bar protein. There is no expression of either Barnase or Barstar in the seeds. Further, edible oil extracted from the seed mainly consists of fats and the amount of any of the three proteins being present in the oil to be consumed by human beings is nil (Seed meal, however, will contain very low amount of the Bar protein).

5.5 Toxicity assessment of proteins encoded by the introduced genes – acute toxicity study

The potential for GE mustard to be toxic to humans and animals due to either expression of the three transgene encoded proteins or because of unintended effects of the genetic modification is assessed in this section.

Although, it has been reported in literature that Barnase, Barstar or Bar proteins have no sequence homology with any of the known toxins when compared with protein sequences present in various databases (Van den Bulcke, 1997, ANZFA; 2001), an acute toxicity study was conducted for all the three proteins, to rule out any possibility of any toxicity due to their presence in the GE mustard. The recombinant proteins expressed in *E. coli* and purified, as per the standard protocols approved by the RCGM, were assessed and shown to have equivalence to the proteins expressed in GE plants by LC-MS and functionality assays.

Acute toxicity studies were undertaken by administering the Barnase and Bar proteins orally at a single limit dose of 1000 mg/kg body weight and Barstar at 1700 mg/kg body weight to Swiss albino mice. These administered doses were several thousand-fold higher of the estimated dietary exposure of the Bar protein to humans (considering average daily intake of green vegetables and maximum expression level of Bar protein in leaves). The data generated showed that none of the three proteins cause mortality or any adverse effect in the test animals when administered orally.

Toxicity of the Pat[1] protein to humans and animals has been thoroughly addressed in several studies earlier. In all such studies, no adverse effects or mortality were observed at concentration as high as 2500 mg and 7792 mg/kg body weight. The highest concentration tested was 6 million times more than the Bar protein concentration in GE Canola (Merriman, 1996; Bremmer and Leist, 1996). It may be noted that this gene is used as a selectable marker in the experiments and does not imply that basta spray is required during cultivation of said hybrid.

5.6 Toxicity assessment of GE mustard for human consumption – subchronic toxicity study

Mustard oil from seeds of *B. juncea* is obtained either by mechanical crushing or solvent extraction, followed by further processes of refining and is extensively used as a cooking medium. Seeds are also used as condiments and in pickles in India as well as in China. The unrefined raw mustard oil is also consumed as Kachi ghani oil. In addition to the seeds, the leaves of young plant are used as vegetable. Therefore, the potential toxicity of the edible plant parts was assessed by a 90-day sub-chronic feeding toxicity study in Sprague Dawley rats. The study was carried out at the National Institute of Nutrition (NIN), Hyderabad as per ICMR Guidelines for Safety Assessment of Foods Derived from Genetically Engineered Plants 2008 and Protocols for Food and Feed Safety Assessment of GE crops 2008. The study was conducted by feeding leaves and seeds of the parental GE lines and their non-GE comparators and hybrid DMH-11 along with normal diet in rats for a period of 90 days. The recommended human daily dietary intake (DDI) of green leafy vegetables is 100g/day and for oilseed is 0.53 g/day for adult human, as per recommendations of the National Nutrition Monitoring Bureau (Dietary Guidelines for Indians, NIN, Hyderabad). GE *B. juncea* leaves were fed at a dose of 0.4 g/rat/day and seed at a dose of 20 mg/rat/day (equivalent to the recommended daily dietary intake for humans) to Sprague Dawley rats daily for 90 days.

[1]The *bar* (*S. hygroscopicus*) and *pat* (*S. viridochromogenes*) genes encode the same enzyme phosphinothricin acetyl transferase (PAT) which inactivates phosphinothricin (PPT), the active constituent of the non-selective herbicide glufosinate-ammonium. Both *pat* and *bar* genes are very similar, sharing 87% similarity at the nucleotide sequence level (Wohlleben *et al*., 1988). Further biochemical characterisation of the two enzymes found that they are so similar as to be functionally equivalent for the purpose of conferring tolerance to PPT (Wehrmann *et al*., 1996 cited in Safety assessment report of Canola; Food Standards Australia New Zealand, 2003).

The sub chronic toxicity studies with leaf and seed material showed that there was no significant difference in body weights, feed intake, cage side activities in the animal groups fed with GE and non-GE comparator material. Urine analysis, biochemical parameters and haematology was in the normal range and similar for the animals fed with normal and GE materials. No toxicologically significant adverse effects were observed in necropsy and histopathology studies of the vital organs of the test animals. Serum immunoglobulins were not altered, no allergenicity symptoms were observed.

Previously, a number of feeding studies have been conducted on chicken, rabbits and canaries for GE Canola (rapeseed) lines containing the same three proteins. No differences have been recorded and reported between the animals fed with GE seeds and those fed with conventional seeds for any of the measured parameters (Leeson 1999; Maertens 1994; Maertens *et al*. 1996; CFIA 1995). As such, it is noted that after extensive commercialization in Canada, for more than a decade of consumption, exports and trade there have been no reports of any observed ill effects. In addition, similar to the GE canola reports, the seeds of GE DMH-11, to be used for oil extraction and meal, also contained only traces of the Bar protein. There is no expression of either Barnase or Barstar in the seeds. The oil contains mainly fats, and no protein. Therefore, the chances of any of these three proteins being present in the oil, to be consumed by human beings or to be used for dermal application, are negligible ascertaining no concerns of risk associated with toxicological aspects of GE hybrid DMH-11 or its parental lines. Further, it has been assessed that the risk of allergenicity occurring from these proteins is nil, based on bioinformatic based analysis, pepsin digestibility and thermal stability of the introduced proteins.

5.7 Toxicity assessment of the Barnase, Barstar and Bar proteins for livestock and wildlife, including cattle, goats, and pigs

The oilseed meal cake generated as a by-product of processing of oilseeds, is used as an animal feed. The meal is rich in protein content but contains glucosinolates which are antifeedents for poultry and are not liked by cattle due to bitterness. In hybrid DMH-11, the expression of the Barnase and Barstar proteins is tightly controlled in the plant due to a promoter (TA29) used for tissue specific expression and both the proteins are not detectable in the seeds. Barstar is detected in traces in leaves and other edible parts of the plant. For this

reason, these proteins are not considered to contribute significantly to toxicity in livestock. Neither of these proteins show any sequence similarity with known allergens or toxins using data obtained from public genome and protein databases. No toxicity was observed in feeding of GE *B. juncea* leaves/seeds at a dose equivalent to recommended daily dietary intake for humans in the Sprague Dawley rats for 90 days. In addition, the *barnase-barstar* system in GE Canola was deregulated after bio-safety studies in Canada (1996), USA (1999), Japan (1996) and Australia (2003) for cultivation and in China (2004), European Union (2005), Korea (2005), Mexico (2004) and South Africa (2001) for food and feed. Seed and meal from GE Canola is being fed to animals in various countries and no adverse effects have been reported.

Further information available in literature supports rapid digestion of the three GE proteins in animals: In an *in vitro* digestibility study, the PAT protein was rapidly inactivated (within one minute) by acidic conditions in dog and pig gastric fluid and with bovine rennet-bag fluid (pH 1.3) (Schulz, 1993). In another *in vitro* study, the leaf material from GE Canola line Topas 19/2 (containing *pat* and *npt II*) were incubated in digestive stomach fluids extracted from pig, chicken and cow. The results showed that PAT protein was readily degraded after *in vitro* incubation in all the digestive fluids tested (Schneider, 1993; Safety assessment report of Canola, Food Standards Australia New Zealand, 2003).

Substantial equivalence has been established based on no difference between GE and their non-GE *B. juncea* lines through compositional analysis. Further, as per the national and internationally acceptable guidelines for the requirement of the animal feeding studies (ICMR Guidelines for Safety Assessment of Foods Derived from Genetically Engineered Plants 2008, Protocol for Livestock Feeding Study, DBT, 2008, Codex Alimentarius Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants, 2003, EFSA guidance 2008, etc.), the performance of livestock feeding studies is not recommended in cases where molecular, compositional, phenotypic, and other analyses have demonstrated equivalence between the GE plant derived food and feed and their conventional counterpart. Therefore, based on unambiguous database of history of safe use and adequate scientific justification (provided below), any need for conducting livestock feeding studies does not arise.

- Due to the tissue specific expression of *barnase* gene, the Barnase protein is expressed only in the anthers and not in any edible plant parts. Barstar predominantly expresses in the anthers. Very low levels are recorded in the leaves and some other tissues also. The Bar protein could be detected at a level of 0.06 – 26 μg/mg of total protein in different plant tissues.
- None of the three proteins is present in the extracted oil. Only Bar protein is present in the meal. Bar (PAT) has been studies extensively and has been found to be safe.
- Acute toxicity studies with the three proteins and sub-chronic toxicity studies with leaves and seeds of GE *B. juncea* did not show any adverse effects on mice and rats, respectively, which indicated that GE B. *juncea* is as safe as its non-GE comparators.
- All the three proteins get rapidly digested by pepsin and there is no sequence similarity of any of the three proteins to any allergenic protein.
- The *barnase-barstar* system in GE canola is deregulated after bio-safety studies in USA, Canada, Japan and Australia for cultivation; and China, European Union, Korea, Mexico and South Africa for food and feed (www.cera-gmc.org).
- Seed and meal from GE Canola, developed using the barnase-barstar technology, is being fed to animals in various countries and no adverse effects reported. As per statistics from Canadian International Merchandise Trade Database-Statistics, Canada (where more than 96% Canola crop is GE) has been a major exporter of seed and seed meal to various countries i.e. Bangladesh, China, Indonesia, Japan, Mexico, South Korea, Taiwan, Thailand, U.A.E., Vietnam, 28 countries of European Union etc. (www.statcan.gc.ca).
- No significant variations between the GE and their non-GE comparator lines has been observed for any of the parameters tested for compositional analysis of edible plant parts i.e. leaf and seed.

As per the ICMR Guidelines for 'Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008, adopted by RCGM and GEAC, the feeding studies are required only if the composition of the GE plant is not comparable to the conventional counterpart. The section 7.4 (page 19) is reproduced below:

"Some foods require additional testing. For example, animal feeding studies may be warranted for foods derived from GE plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also, foods designed for health benefits may require specific nutritional, toxicological or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a

thorough safety assessment, properly designed animal studies could be requested on the whole food".

In the Protocol for Livestock Feeding Study (section 1, page 28) published by DBT in 2008 it has been mentioned that

"The need for conducting livestock feeding trials should be carefully evaluated on a case-by-case basis. Generally, there are two situations in which livestock feeding trials may be of value: (1) if significant compositional differences are observed between the GE food and its comparator, then feeding trials may be used to investigate the biological significance of such differences; and (2) in the case of a GE food with enhanced nutritional characteristics, livestock feeding trials may be used to demonstrate that the expected nutritional benefit is achieved".

The requirement of animal studies as mentioned in the internationally accepted guidance by the Codex Alimentarius Commission i.e. "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants, CAC/GL 45-2003". The following was noted at Section 11:

"Animal studies cannot readily be applied to testing the risks associated with whole foods, which are complex mixture of compounds, often characterised by a wide variation in composition and nutritional value. Due to their bulk and effect on satiety, they can usually only be fed to animals at low multiples of the amounts that might be present in the human diet. In addition, a key factor to consider in conducting animal studies on foods is the nutritional value and balance of the diets used, in order to avoid the induction of adverse effects which are not related directly to the material itself. Detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can therefore be extremely difficult. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole foods. Another consideration in deciding the need for animal studies is whether it is appropriate to subject experimental animals to such a study if it is unlikely to give rise to meaningful information".

European Food Safety Authority (EFSA, 2008) has concluded, based on a detailed analysis on the role of animal feeding trials in safety and nutritional assessment of GE food/feed, that in cases where molecular, compositional, phenotypic, agronomic and other analyses have demonstrated equivalence between the GE plant derived food and feed and their conventional counterpart, except for the inserted trait(s), and results of these analyses do not indicate the occurrence of unintended effects, the performance of animal feeding trials with rodents or with target animal species adds little if anything to the overall safety assessment, and is not recommended. (It may be noted that in case of barnase, barstar lines and hybrid DMH-11 both acute and sub-chronic toxicity studies have been conducted).

Similarly in several other countries such as USA, Canada, Mexico, Brazil, Australia, Japan etc., such studies are not required unless there are some differences observed. The review of decision documents from various regulatory bodies available in public domain on GE Canola containing similar Barnase, Barstar combination indicates that no such studies were required for granting environmental release. It may be noted that GE Canola is being cultivated in several of these countries for more than 20 years.

Relevance of the whole food safety studies on GE crops has also been studied in two review articles published recently (Bartholomaeus *et al*., 2013;Van Eenennaam, 2013), wherein it has been concluded that comparative studies on compositional and agronomic considerations are robust and reliable and in such cases animal feed studies are not necessary and scientifically justified. These reviews are based on findings of hundreds of peer reviewed articles on animal feeding studies which have repeatedly shown that GE plants can be safely used for food and feed.

5.8 Assessment of cytotoxicity of Barnase

In the current technology, Barnase enzyme is used selectively to degrade RNA in tapetum layer of cells of developing anthers to create a male sterile line. However, RNases (Barnase comes under this category) are also being currently studied as non-mutagenic alternatives to the harmful DNA-damaging anticancer drugs commonly used in clinical practice. In view of this, the safety of cytotoxicity of the Barnase enzyme has been evaluated based on published data as mentioned below:

Barnase, a ribonuclease enzyme from *B. amyloliquefaciens*, is a cationic protein with isoelectric point of 9.2 (Ulyanova *et al*., 2011). Barnase showed selective toxicity to malignant fast dividing tumor cells which express tumor-specific proteins and markers (HER-2 antigen). Cancer cells expose more negatively charged phospholipids as well as more glycolipids and glycoproteins on the outer plasma membrane than do normal cells. This feature may be one of the reasons for the observed selectivity of cationic RNases that attract Barnase towards cancer cells (Smith *et al*., 1999; Edelweiss *et al*, 2008). Barnase enzyme is a protein molecule.

For anti-cancer treatment, protein drugs are given in injectable forms to avoid protein degradation in the gastrointestinal tract and to retain their pharmacological effects. For Barnase to act on a cell it has to be delivered at the target site in an active form. However, when barnase is sourced from GE mustard through the bud or anthers (only organs in GE mustard where barnase is synthesized) for oral consumption, it goes through the GI tract where it gets degraded due to acidic pH (as in dogs, pigs and cows), and loses its enzymatic activity, thus becoming non-functional. Therefore, with Barnase at below the detection level (selectively expressed in anthers) and with no possibility of Barnase to pass the GI tract, entering of the barnase protein into the intestinal epithelial cells is highly unlikely. Further, if the Barnase protein comes in contact with human blood by accidental exposure to the skin cuts of humans, the possibility of cytotoxicity potential is negligible because Barnase has more affinity towards fast dividing cancerous cells and has no particular affinity towards normal cells. In any case, there is no risk of the latter situation as no such possibility exists where pure barnase enzyme can directly come in contact with human beings during the cultivation process of GE DMH-11.

5.9 Toxicity assessment of GE Indian mustard in Ayurvedic uses

Mustard is used prominently in the Indian tradition and its medicinal properties have been systematically evaluated and documented in the classical Ayurvedic literature like *Caraka Saṃhitā, Suśruta Saṃhitā, Bhela Saṃhitā* and *Kāśyapa Saṃhitā* (Ram Manohar P, 2009). Mustard leaves are an ingredient of the decoction for steam fermentation (to ease pain), used to improve digestion, increases urine output and are useful as a laxative. Mustard seed is used externally as paste, fumigant, diaphoretic, massage powder, and scraping agent. Internally, mustard seeds are used for purging toxins from the human body. Mustard oil is indicated for external and internal uses in management of abdominal swelling, diabetes, skin diseases etc. (Ram Manohar P, 2009).

In the expression analysis, there is negligible to very low expression of the introduced proteins i.e. Barnase, Barstar and Bar in seed and leaf. Further, no adverse effects or mortality were observed in the acute toxicity study conducted with purified recombinant proteins in mice and in a 90-day subchronic feeding study of GE *B. juncea* leaves and seed to Sprague Dawley rats. Also, these proteins were rapidly digested by pepsin in Simulated Gastric Fluid (SGF).

Further, mustard oil contains mainly fatty acids and no protein. Therefore, the chances of presence of any of these three proteins in the oil, to be consumed by human beings or to be used for dermal application, would be negligible. Also, the risk of allergenicity occurring from these proteins is assessed to be negligible based on lack of allergenicity in the bioinformatics analysis, pepsin digestibility and thermal stability of the introduced proteins assays.

Conclusions: Based on the history of safe use of the host and donor organisms, transgene expression analysis, composition analysis, acute toxicity assays with purified proteins and sub-chronic studies with the whole leaf and seeds, it can be concluded that the use of leaves, seed and oil derived from transgenic mustard lines is not likely to pose any risk to humans and animals.

5.10 Potential allergenicity assessment of Barnase, Barstar and Bar proteins

As reported widely in literature (Kimber *et al*. 1999; Breiteneder *et al*. 2005) allergens usually share a number of characteristics:

- these are mostly proteins and are typically glycosylated
- have molecular weight ranging between 15-70 kD
- are stable in the mammalian digestive system
- are stable at high temperatures during cooking or processing

The Barnase, Barstar and Bar proteins have molecular weight of 12 kD, 10 kD and ~22kD, respectively. The molecular weight of the introduced proteins Bar (approx. 22kD) is within the molecular weight range of known allergens, while the Barnase and Barstar proteins are below this range (12kD and 10kD, respectively). However, all these proteins are expressed at extremely low levels in the two GE parental lines and in GE hybrid. Further, the Bar protein (~22 kD) that expresses in most of the tissues lacks glycosylation sites and many of the other characteristics which are common to allergens present in the plant derived food. **Therefore, none of these three proteins fall under allergen category as evident from comparison of molecular and biochemical properties with those of the known allergens**. A bioinformatic analysis was carried out to determine possible allergenicity of the Barnase, Barstar and Bar proteins by comparing their amino acid sequences to sequences of all known allergens using AllergenOnline.org (AOL) database versions 14 and 15 and NCBI Protein Database (BLASTp

version 2.2.30+). This analysis further confirmed lack of any significant sequence similarity of Barnase, Barstar or Bar proteins to any known allergenic protein. The bioinformatic analysis data for assessing potential risks of food allergy due to the presence of Bar, Barnase, and Barstar proteins in GE mustard have been published in a peer reviewed journal (Siruguri *et al*., 2015. Food and Chemical Toxicology 83: 93-102). **Nevertheless, in order to establish specifically the safety aspects with respect to consumption or contact of GE mustard DMH-11, allergenicity studies were carried out at the National Institute of Nutrition, Hyderabad**. In the pepsin digestibility assay, all the three test proteins (Barnase, Barstar and Bar proteins) were rapidly degraded by pepsin in a Simulated Gastric Fluid (SGF). Around 90% digestibility was achieved within half a minute. On the basis of the SDS–PAGE gel analysis of the pepsin-digested test proteins, it was concluded that the Barnase, Barstar and Bar proteins are rapidly digested by pepsin, and hence the risk of allergenicity from these proteins in food is extremely low. In another test, no protein could be detected after boiling the leaf samples even after two minutes. **Therefore, it can be concluded that cooked leaves, which are often used for human consumption as a popular delicacy in India i.e. 'Sarson ka Saag' is unlikely to have any of these proteins in an active form.**

In the thermal stability assay, the Bar protein showed a rapid decrease in acetyl transferase activity when subjected to temperatures from 55^0C to 95^0C. Barnase and Barstar proteins showed no specific change in ribonuclease enzyme activity and these proteins had high heat stability up to 95^0C. However, allergy risk is considered to be negligible since Barnase and Barstar proteins are mainly expressed in the anthers and are rapidly digested by pepsin in Simulated Gastric Fluid and do not share any similarity with any known allergens.

The donor organisms, from which these proteins are derived, are not known sources of allergens. The three genes used in the development of the barnase-barstar system for mustard have been derived from commonly occurring bacteria – barstar and barnase from *Bacillus amyloliquefaciens* and bar from *Streptomyces hygroscopicus*. *B. amyloliquefaciens* is used in food industry and *S. hygroscopicus* is a known non-pathogenic soil actinomycete.

Data on risk assessment based on hazard identification and exposure assessment shows that the GE mustard, the parental lines and hybrid DMH-11 do not raise any public health or safety concerns in human beings and animals with respect to overall nutritional characteristics. All the three introduced proteins i.e. Bar, Barnase and Barstar are expressed at low or negligible levels in the edible parts and have been derived from commonly occurring non-pathogenic bacteria. None of the three proteins has been shown to be toxic

or allergenic through bioinformatics and acute toxicity studies. All the three proteins are rapidly degraded in simulated gastric conditions of the mammalian digestive systems. Sub-chronic toxicity studies using edible plant parts i.e. leaves and seeds also did not show any adverse effects on the test animals. Oil extracted from seeds may contain negligible amounts of protein - if any. Therefore, the probability of oil extracted from DMH-11 or any other future hybrids to have any of the three proteins is negligible. Moreover, there is more than 20 years of history of safe use of proteins from the three genes – *bar, barnase* and *barstar* in rapeseed (*B. napus*), a sister crop of Indian oilseed mustard. Oil and meal extracted from GE rapeseed containing the same three proteins that have been expressed in mustard is being consumed in a large number of developed and developing countries and no toxicity or allergenicity have been reported till date. In conclusion, based on compositional analysis, GE mustard does not differ significantly from non-GE mustard and is as safe as commercially cultivated non- GE mustard varieties.

Conclusions: The risk assessment performed after examining and considering the existing information in literature and data provided in the dossier submitted by the developers, against the background of available knowledge in the subject areas, it is clear that GE mustard lines Varuna bn 3.6, EH-2 modbs 2.99 and the hybrid DMH-11 do not pose any risk of causing any adverse effects on human and animal health and safety.

CHAPTER 6
ENVIRONMENTAL SAFETY ASSESSMENT STUDIES

This chapter deals with environmental safety assessment examining aspects such as reproductive biology of mustard; interactions such as volunteers and weediness; wild relatives in India and potential of gene flow through naturally occurring intraspecific, interspecific and intergeneric crosses.

This chapter also deals with environmental risk assessment for understanding and analysing if the presence of GE plant would lead to any adverse or detrimental effects on the population and type of soil microflora. Another important aspect in risk assessment covered in this chapter is the effect of GE plant on the occurrence of pest, diseases and beneficial organisms.

6.1 Weediness Potential

Weeds become a problem to the community when their presence or abundance interferes with the intended use of the land. Weeds are of concern for environmental reasons whereby they outcompete with cultivated crop plants for resource and space and hence adversely affect agriculture productivity. In addition, the introduction of weeds in an environment may bring about ecological changes by altering the structure of food webs. Review Committee on Genetic Manipulation (RCGM) and Genetic Engineering Appraisal Committee (GEAC) recommended environmental safety studies on GE mustard hybrid DMH-11 and its GE parental lines for weediness potential and aggressiveness. The objectives of the study were:

- To generate data on biomass production, seed germination, seedling vigour test and speed of germination to assess the weediness potential.

The data provided by the applicant on these studies have been assessed for weediness potential and aggressiveness so as to protect the native plant communities and to maintain biodiversity in the receiving environment which is in turn important for maintaining ecological functions and agriculture productivity.

The goal of risk assessment is to assess any risk posed to the environment by GE mustard hybrid DMH-11 for the possibility of becoming an unmanageable weed. Weeds generally have

a number of characters that enable them to rapidly colonize and persist in an eco-system. These include germination and seed production under a wide range of environmental conditions, seed dormancy under adverse conditions, rapid seedling growth, rapid growth to early reproductive stage, long continuous seed production, mostly self-pollinating but not exclusively autogamous, good competitiveness etc. (Baker 1965; 1974). It is generally accepted that most crop plants that have undergone selective breeding and domestication have reduced competitiveness and grow optimally only under controlled agricultural conditions. Any GE plant would have weediness potential only if the introduced transgene when compared to its conventional comparator acquires characters associated with weediness.

The above traits can be analysed on the GE and normal mustard genotypes of DMH-11. Traits like germination pattern and seedling vigour can be measured using standard protocols. Data regarding seed germination percentage, speed of germination, seedling vigour and population establishment can also be undertaken in field conditions involving both GE mustard hybrid DMH-11 and parental lines along with their normal counterparts.

Although mustard is not prone to shattering, some seeds may remain in the soil until the following season when these germinate either before or following seeding of the successive crops. As a result, they could become weedy in subsequent crops. However, despite a long history of cultivation, mustard has not been reported to be a weed in India.

While conducting safety studies on GE plants, it is important to check whether the transgene has changed the germination and initial growth pattern in such a way that there is risk of the GE lines turning invasive in the form of a weed. Therefore, a comparison was carried out between the GE lines and their non-GE counterparts with respect to germination.

In part fulfilment of Indian regulatory obligations, applicant performed environmental safety study of GE mustard hybrid DMH-11 containing the *barnase*, *barstar* and *bar* genes in comparison with non GE counterparts and local checks i.e. GE Varuna bn3.6, Un-modified Varuna parent, GE EH-2 modbs 2.99, un-modified EH-2 parent, GE mustard hybrid DMH-11

and handmade non-GE hybrid (VEH2-F1). Non- GE hybrid VEH2 was a handmade hybrid and used as control only for assessing some of the issues related to environmental safety.

The likelihood of biodiversity loss due to DMH-11 cultivation is possible only if it is able to germinate in wide range of environmental conditions, and also establish in undisturbed habitats with shorter life span, with higher number of smaller seeds, with innate ability to shatter in summer in contrast to its counterpart non-GE hybrid.

All the estimates and observations that were concluded on weediness potential have been based on laboratory experiments and BRL I and BRL II field trial data. The data and results of the study are summarized below:

- **Seed germination**: The seed germination percentage was observed after 5 and 10 days under laboratory condition and up-to 15 days under field conditions. Seed germination percentage revealed that there was no difference between the GE and non- GE hybrid. Seed germination percentage of Varuna-barnase and Varuna, EH-2-barstar and EH-2, and GE mustard hybrid DMH-11 and its non-GE counterpart hybrid was found to be between 91-99%.
- **Speed of seed germination**: The number of seedlings emerged after 5, 10, and 15 days of sowing showed that speed of germination had stabilized by 10 days in both GE and non-GE hybrid.
- **Shoot and root weight**: Shoot and root weight were recorded as fresh and dry weight of the seedlings after 15 days of sowing. The data revealed significantly higher shoot and root weight in hand made non-GE hybrid (VEH2-F1) as compared to the GE hybrid DMH-11 under field conditions. Hence, the transgenes presence does not lead to any enhancement in the growth of DMH-11 as compared to the hand-made hybrid VEH2-F1.
- **Long continuous seed production**: The data on seed production shows no difference among GE hybrid DMH-11 and non- GE hybrid (VEH2-F1).
- **Seed size, Pod shattering**: The seed size of two parents of the hybrid DMH-11 i.e. Varuna and EH-2 are different. Small seed size is a characteristic of east European gene pool lines. Hybrid is intermediate in seed size and is non-shattering. Smaller seed size is an inherent

property of East-Europena gene pool lines and this germplasm has not been reported to be a weed anywhere in the world where it has been grown.

The data generated for each aspect of weediness potential of GE mustard hybrid DMH-11 showed no difference when compared with its conventional counterpart. Significantly higher shoot and root weight in hand made non-GE hybrid (VEH2-F1) than the GE hybrid DMH-11 under field conditions was observed, which indirectly reflects that hybrid DMH-11 is not showing growth rates higher than those observed in the handmade hybrid VEH2-F1. This is indicative that GE hybrid DMH-11 does not have any weediness or aggressiveness potential. The possibility of DMH-11 invading the natural ecosystems is highly unlikely. Therefore, it is concluded that GE hybrid DMH-11 poses negligible risk to biodiversity and agriculture productivity.

To determine the likelihood of potential of weediness and aggressiveness of GE hybrid DMH-11, the following parameters were also assessed –inherent weediness potential, if any, of *B. juncea*; weediness potential, if any, of GE hybrid DMH-11; selective advantage, if any, conferred by the introduced genes. The assessment is on the basis of data provided by the applicant and scientific evidence in published literature and reports of previous international biosafety assessments carried out for the release of GE *B. napus* (rapeseed) hybrids based on the *barnase-barstar* system.

Inherent weediness of *Brassica juncea:* The biology of *B. juncea* shows that seeds can escape harvest and remain in the soil until the following season when these will germinate either before or following seeding of the successive crop. As a result, *B. juncea* volunteers could grow and be present amongst subsequent crops. Normally, when no-tillage is used in the subsequent crop (other than mustard), or where land is kept fallow after mustard is harvested, the frequency of volunteer plants is observed to be relatively more. But that is neither specific to GE product nor non-GE product. Therefore, despite a long history of cultivation, *B. juncea* is not considered a weed in India.

Assessment of any potential weediness of GE hybrid DMH-11 and the parental lines: Laboratory studies with regard to seed germination, shoot length and root length indicated that there is no evidence of any change in weediness or aggressiveness with respect to the parameters like germination percentage, shoot and root length and shoot and root weight. Moreover, the significantly higher shoot and root weight in non-GE hybrid (VEH2-F1) than the

GE hybrid DMH-11 under field conditions is indicative of the fact that the GE lines have no potential for weediness. Similar results with respect to weediness potential have been earlier reported when MS/RF system based on the *bar*, *barnase*, *barstar* genes was used for production of hybrids in Canola rapeseed. The growth characteristics of GE rapeseed lines and hybrids in terms of phenology were found to be within the range for conventionally developed Canola (rapeseed) varieties. The regulatory authorities in various countries concluded that there was no evidence that the new traits introduced into GE rapeseed lines (including the hybrid system) would cause any of these to be weedier than conventional Canola. (CFIA 1995b, CFIA 1996b, ESCP 1998; USDA 1998, 1999, 2002 a, b, c, d,).

Regarding the weediness potential of GE mustard hybrids, it is important to note that hybrid vigour displayed in MS x RF hybrids of *B. juncea* is a result of hybridization between two genetically distinct parents. The three transgenes only provide a mechanism to allow controlled production of hybrid seeds which exhibit the natural phenomenon of hybrid vigour or heterosis. Hybrid vigour provides benefits of a healthier plant, less influenced by disease and environmental conditions and in agronomic terms - increased yield. Hybrid vigour manifested in the F1 generation declines in the subsequent generations (Falconer and Mackay et al. 1996). Therefore, although, F1 hybrid -DMH-11, will exhibit hybrid vigour, this will not result in increased weediness or invasiveness in F2 or in the subsequent generation seeds.

The data also shows that the GE hybrid DMH-11 is not prone to pod shattering. Other facts that also has to be considered here are - *B. juncea* is less prone to pod shattering than *B. napus* (Canola); *B. juncea* is a poor competitor and does not establish well in unmanaged areas; is not listed as a noxious weed or recorded as invasive in any natural ecosystem; there is no prior evidence of weediness characteristic.

Although GE mustard hybrid DMH-11 contains the *bar* gene, conferring resistance to herbicide Basta (Phosphinotricin), it is not exhibited as a functional trait till Basta is sprayed on the plants. Basta herbicide is required to be used only by seed producer for hybrid seed production employing the barnase-barstar system. In any case, farmers are not required to spray Basta in the hybrid GE DMH-11 field for weed control as Basta is not a recommended herbicide in the package of practices for mustard cultivation in India.

Even in countries like Canada where *B. napus* is being cultivated extensively with herbicide tolerance trait -either as Roundup Ready (Glyphosate resistance) or Liberty link (Basta resistance) technologies, there is no evidence that the GE herbicide tolerant crops are more invasive than their conventional counterparts (Crawley et al. 1993; Crawley et al. 2001b).
GE hybrid DMH-11 is as susceptible to other categories of herbicides as conventional varieties, so control could be achieved by use of herbicides other than Basta and/or by non-chemical techniques that are all part of best practices for weed management.

Study of the data submitted by the applicant showed that GE mustard hybrid DMH-11 is similar to its non-GE mustard hybrid in its aggressiveness or weediness potential. *B. juncea* is a poor competitor and does not establish well in undisturbed habitats, it is not listed as a noxious weed or recorded as invasive in any natural ecosystem. There is no evidence of weediness characteristics in *B. juncea*. No feral populations of *B. juncea* have been reported in India. Therefore, potential of GE hybrid DMH-11 becoming weedy and aggressive are highly unlikely.

Conclusions: The risk assessment of GE mustard hybrid DMH-11 establishing itself as a weed has been assessed and found to be negligible on the basis of:

- No differences observed in parameters tested for weediness potential and invasiveness for GE hybrid DMH-11 versus its conventional counterpart
- *B. juncea is* not reported to be a weed in India. Feral populations of *B. juncea* have not been reported.
- The genetic modifications are highly unlikely to enhance the weediness of the *B. juncea* hybrid DMH-11 lines compared to non-GE Indian mustard lines
- *B. juncea* DMH-11 hybrid is susceptible to other herbicides and can be controlled using alternative herbicides or non-chemical management methods.

A stepwise approach to risk assessment and conclusions is given below in **Figure 6.1**.

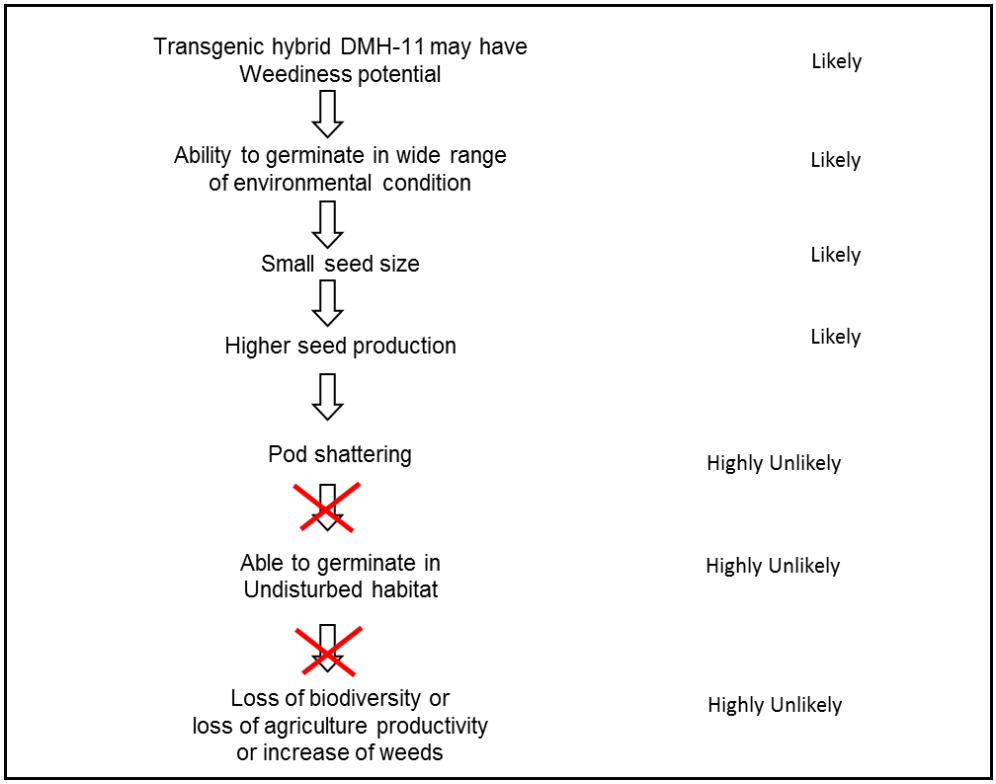


Figure 6.1: The causal pathway demonstrate that there are several steps involving multiple factors which contributes towards weediness potential of any GE plant. But it has to be also noted that mere presence of these factors does not ensure that the consequence would occur. In the case of hybrid DMH-11, several steps in the casual pathway are blocked and hence, it is concluded that the potential of DMH-11 to become a weed is highly unlikely. Therefore the risk of hybrid DMH-11 to become weed or invasive is assessed as negligible.

6.2 Crossability and gene flow studies

There are two major types of gene flow in agricultural crops, (1) GE crop to varieties of the same species and vice versa (intraspecific) and (2) GE crop to related species of the same genera and wild relatives (interspecific).

India is not a primary centre of diversity of Brassica species and presence of wild relatives growing in/near the mustard fields is low. Therefore, gene flow is not a concern particularly in the plains where the crop is mostly grown, as far as natural gene introgression between species is concerned. The wild relatives which are found in foothills or hilly regions in the north and north eastern hills, may have a probability of crossing with *B. juncea* and the wild relatives. This still does not indicate gene flow due to post-gene flow outcomes since the wild relatives are all monogenomic species and *B. juncea* (AABB) is an allotetraploid. Any hybrids formed between the diploid wild species and *B. juncea* will be a triploid and hence will remain sterile making the gene flow ineffective. The extremely low frequency of any survival of such a gene transfer shall have no risk in general since it will be similar to the natural process of evolution and in particular with the current trait of male sterility which is self-destructive with respect to the pollen carrying the *barnase* gene and non-existent with respect to *bar* gene as herbicide Basta is not a weedicide used in India, that too on wild vegetation.

To reduce gene flow from GE plant to the receiving agro-ecosystem and natural habitats, crossability studies are important to establish the distance to which pollen from the GE lines can travel and fertilize plants of the same and related species. Such a study is readily possible with the GE lines and hybrids that contain a selectable marker gene, in this case *bar* gene that confers resistance to herbicide 'Basta'. Seeds harvested from the recipient species or recipient mustard lines can be germinated at high density and sprayed with the herbicide to record frequency of cross-pollination.

This chapter considers whether there are chances of transfer of introduced gene from GE mustard hybrid DMH-11 to conventional varieties and hybrids and wild relatives RCGM, GEAC recommended conduct of crossability studies to study pollen flow from GE to non-GE material in the field. The objectives of the study were:

- Extent of cross-pollination between GE *B. juncea* hybrid DMH-11 (bn 3.6×modbs 2.99) and its related species
- Extent of cross-pollination between GE *B. juncea* hybrid DMH-11 (bn 3.6×modbs 2.99) and *B. juncea* var. Pusa bold as an adjoining crop.

Chances of escape of transgenes from GE mustard hybrid DMH-11 to related *Brassica* species and weeds may occur only if conventional crop, wild relatives and weeds are present in the receiving environment where hybrid DMH-11 is cultivated. The risk assessment was performed using scientific literature, background information on parental species including *Brassica* biology document and data on interspecific and intraspecific crossability studies as provided by the applicant.

Successful crossability requires that the populations of donors and recipients must overlap spatially and temporally and be sufficiently close biologically that the resulting hybrids are able to reproduce normally (den Nijs *et al*. 2004). For the flow of transgenes from GE plant to other plants, there are several fertilization and post fertilization barriers that need to be overcome.

In case of crossability, the following aspects need to be studied to determine the possibility of hybridization and introgression:

- The existence of same species of *B. juncea*/ close relatives/ weedy and wild relatives of Brassicas growing in a sympatric mode, with overlapping flowering times.
- Mating system allowing pollen flow
- The possibility of viable/ fertile plants produced in successive generations

To study the crossability of GE *B. juncea* hybrid DMH-11 with the various related species and also to study the extent of pollen flow from the GE hybrid to non-GE lines of *B. juncea*, an experiment was conducted at the Delhi University Research Farm in Bawana, New Delhi in the year 2010. Crossability studies were conducted as per the Guidelines and Standard Operating Procedures (SOPs) for Confined Field Trials of Regulated, Genetically Engineered (GE) Plants, 2008.

Effect of introduced DNA on pollination behaviour: Pollination behaviour of hybridDMH-11 was studied from the parameters such as anther morphology, pollen morphology, pollen production, pollen viability and pollen longevity. No significant difference either in anther morphology or pollen morphology were found between DMH-11 and its handmade non-GE hybrid counterpart VEH-2. The pollen viability of both the hybrids was found to be similar – in a range of 48-72 hours. Hence, the presence of transgenes does not have any unintended effect on the structural aspects of the pollen or its ability to pollinate. It is highly unlikely that genetic modification in GE DMH-11 will affect the outcrossing as compared to its non-GE counterpart.

Interspecies Crossability of DMH-11 with Brassica related species: For the interspecific hybridization possibility, data was generated by sowing the GE DMH-11 plants with the related species, alternatively, in the field**.** Ten different species related to *B. juncea* were sown along with DMH-11 in a ratio of 1:3 (one line of related species and 3 lines of DMH-11) to check for inter-species crossability with *B. rapa (*yellow sarson, brown sarson and Toria*) B. nigra, B. oleracea* (cauliflower), *B. napus* (rapeseed),*B. carinata. Sinapis alba, B. tournefortii, Eruca sativa and Raphanus sativus* (radish) (**Table 6.1**).

Table 6.1.Studies on the crossability of GE DMH-11 with other Brassica species

Entries	Genome and chromosome number	Days to 50% flowering	Days to physiological maturity	Any morphological abnormality in seed, pod, etc. (if any)
DMH-11 (bn 3.6×modbs 2.99)	AABB, n=18	58	144	None
*B. rapa (*Type Toria, var. Pant toria)	AA, n=10	40	110	None
B. rapa (Type Yellow Sarson var. Pusa Gold)	AA, n=10	62	130	None
B. rapa (Type brown Sarson var. BSH-1)	AA, n=10	52	125	None
B. nigra (var. IC-257)	BB, n=8	65	161	None
B. oleracea (Type Cauliflower var. Pusa Deepali)	CC, n=9	117	178	None
B. napus (var. IC-257)	AACC, n=19	58	122	None
B. carinata (var. BEC-184)	BBCC, n=17	112	161	None
Sinapus alba	SS, n=12	117	161	None
B. tournefortii	TT, n=10	70	130	None
Eruca sativa	EE, n=10	62	152	None
Raphanussativus	RR, n=9	112	171	None

It was observed that *B. oleracea*, *B. carinata, Sinapis alba* and *Raphanus sativus* take around 112 days to flower while GE mustard hybrid DMH-11 takes 58 days to flower. Hence, in the absence of synchronous flowering under growing season, the possibility of outcrossing with

these species is highly unlikely. However, for all the other species tested, the flowering time and flowering duration coincided with that of the hybrid DMH-11, providing ample opportunity for cross pollination. Bee activity was also observed to be normal during flowering ensuring pollination. Seeds were harvested from the related species, germinated and sprayed with herbicide Basta. The study showed no Basta resistant plant in F1 generation-hence it could be concluded that the likelihood of crossability of GE DMH-11 with other Brassica species under natural conditions is highly unlikely.

In an earlier study, Ghosh and Varma (1999) studied intercrossing between GE *B. juncea* (carrying barnase) and other related species such as *B. rapa*, *B. napus, B. carinata, B. nigra, B. tournefortii, Eruca sativa* and *B. oleracea* and it was observed that the number of seed set in the interspecies crossing were very few (a maximum of 6.38% of seed set was seen) and in most of cases, especially with the diploid species, the seed set was nil. Strong post-fertilization barriers were observed in most of the cases. Seeds of the progeny were mostly viable, but male sterility was observed in almost all cases. It shows that likelihood of interspecific cross to go to the next generation is highly unlikely.

Literature suggests that natural hybridization among *B. rapa*, *B. juncea* and *B. napus* do take place in northern hills at a low frequency. However, no such natural interspecific hybrids have been observed in the plains where mustard is predominantly grown. **However, as pointed out earlier in such rare cases even if interspecies crossing occurs, due to differences in ploidy levels, the resulting diploid plants will have irregular meiosis, chromosomal imbalance and sterility; its persistence in the environment will be negligible.** Most of the interspecific crosses made in the laboratories require embryo rescue and hybrid production has been reported to be even less than 0.1% under experimental conditions.

Intraspecies Crossability of DMH-11 with *B. juncea* varieties: Extent of cross-pollination between GE *B. juncea* hybrid DMH-11 (bn 3.6×modbs 2.99) and *B. juncea* var. Pusa bold as an adjoining crop was also studied. The study was to establish the distance travelled by GE hybrid DMH-11 pollen and its ability to pollinate and fertilize plants of the same species sown in its vicinity. In this test, non-GE *B. juncea* Pusa bold was sown surrounding an inner plot of DMH-11, up to a distance of 50 m starting from the outer boundary of the inner plot. To check for

the crossability, the progeny seeds harvested from Pusa bold plants were germinated and sprayed with Basta herbicide and checked for presence of any resistant plants. Seven plants were found to be resistant to herbicide Basta within 20 m distance from the inner plot. No seed harvested beyond 20m distance had any herbicide resistance. Therefore, DMH-11 pollen travelled only up to 20m, as no Basta resistant seedlings were observed in the progeny of Pusa bold plants beyond 20 meter distance from the boundary of the inner plot.

From the biology document of *B. juncea* it is very clear that it is mainly a self- pollinating crop and its pollen is relatively heavy and sticky and generally not carried to great distances by wind. Insects, particularly bees, are the primary cross-pollinators. The highest rate of cross-pollination occurs with plants in close proximity and more so in situations where there is a physical contact with the neighbouring plants. In *B. juncea* the rate of outcrossing up to 11 to 17.5%. (Labana *et al*. 1992, Singhal *et al.* 2005, Labana and Banga 1984, Chauhan *et al*. 1987, Dhillon and Labana 1988, Ram Bhajan *et al.* 1991, Abraham 1994) have been reported.

In farmer's field, one of the concerns is that the crossing could occur between intraspecific varieties growing in adjoining fields. In case of DMH-11, the crossing with neighbouring *B. juncea* would be similar with other non-GE hybrid/ varieties and no further effects are expected due to the presence of the transgenes. The introduced gene would not lead to any unintended increase in crossability of DMH-11 as compared to existing mustard varieties or conventional hybrids. Intraspecific crosses between DMH-11 and other varieties of *B. juncea* would not have any selective advantage in the absence of Basta herbicide spray.

It can be concluded that intraspecies gene flow could occur betweenDMH-11 and varieties of *B. juncea* grown in close proximity to GE hybrid DMH-11. However the cross between adjoining crops has been found to occur up to a distance of 20 m. Progeny of such crosses will not have any survival advantages unless sprayed with a specific herbicide Basta. In cases where *barnase* and *barstar* genes are transferred, the resultant plant would be normal due to co-expression of both the genes. In case, *barnase* is transferred alone, the result will be male sterility in the progeny and unless pollinated with normal pollen such plants cannot produce progeny and persist in the environment. The data generated for each aspect of crossability and pollen flow of GE mustard hybrid DMH-11 shows no interspecies crossability with related *Brassica species*. The pollen was found to be viable up-to 48 hrs and there is no alteration in pollination behaviour of GE hybrid DMH11 when compared to its conventional counterpart. In rare cases even if interspecies crossing occurs, due to differences in ploidy levels, the resulting triploid plants will have irregular meiosis, sterility and chromosomal imbalance thus persistence of progeny in the environment will be negligible.

Conclusions: The risk assessment of interspecies cross from DMH-11 to other *Brassicas* is assessed to be negligible. Further the risk for intraspecies cross between DMH-11 and *B. juncea* has been assessed as negligible to low (**Figure 6.2**). However, as a precautionary approach and sustainable use of this MS-RF technology of mustard an oversight post-release monitoring is suggested. Following are the observations:

- *B. juncea* is mainly a self-pollinating crop – and GE hybrid DMH-11 shows the typical behaviour of normal *B. juncea*.
- Cross pollination between *B. juncea* varieties occurs at very low frequencies and gets lower with increase in distance.
- Even if intraspecific crossing occurs, the progeny of such crosses will not have any survival advantages. Selective advantage will occur only if glufosinate is sprayed. Therefore, the chances of increase in the frequency of plants receiving transgene in subsequent generations are negligible in the absence of any selection pressure.
- Spontaneous outcrossing is low as flowering time of some of the crossable species is not synchronous with *B. juncea.*

The following figure (**Figure 6.2**) illustrates the stepwise analysis that has been carried out on gene flow and its consequences.

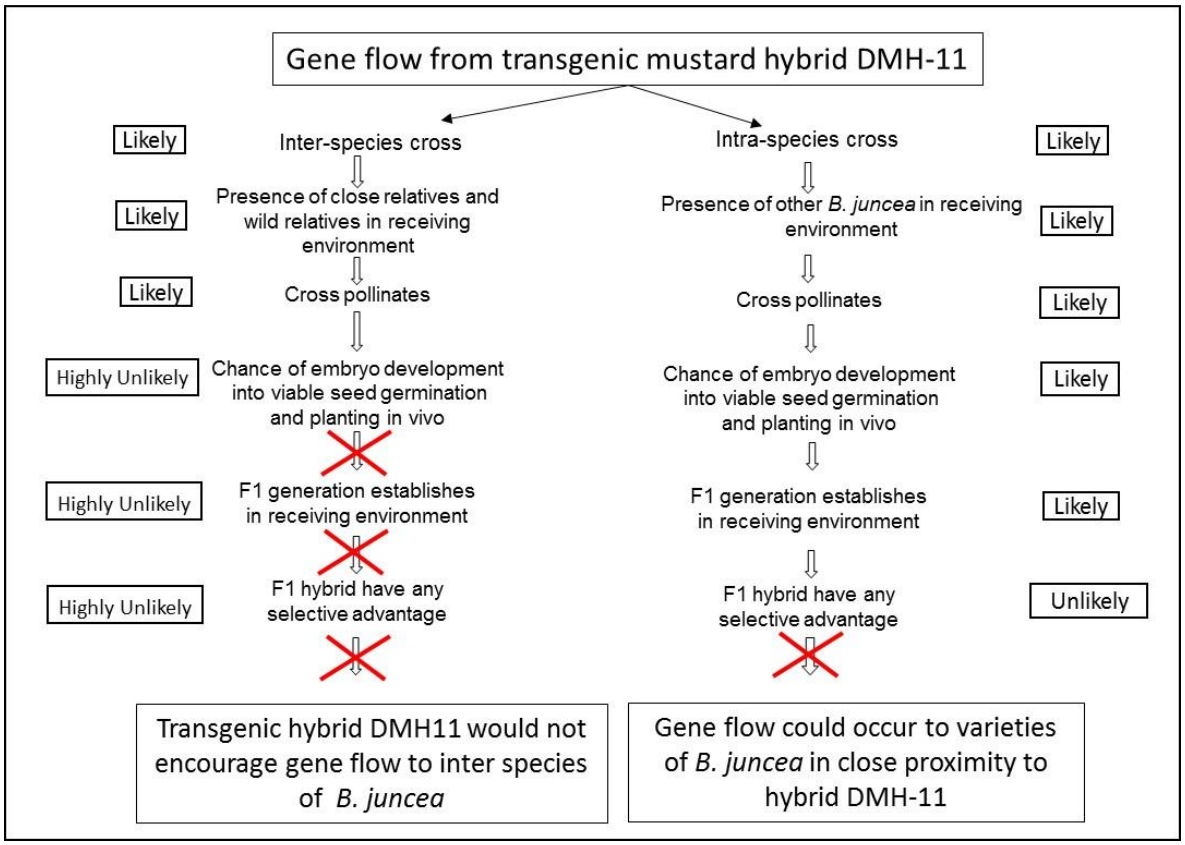


Figure 6.2: The causal pathway demonstrate that there are several steps involving multiple factors which contribute to crossability (inter and intra-species) due to pollen flow from GE plant. But it has to be also noted that mere presence of these factors does not ensure that the consequences would occur. In case of inter-species crossability due to GE hybrid DMH 11 several steps in causal pathway are blocked hence the risk of interspecies outcrossing is assessed to be negligible. However, in case of intra-species crossability the pathways continues till end with likely probability of cross between DMH-11 and *B. juncea.* Even in case of occurrence of intra-species cross, the progeny will not have survival advantage in the absence of selection pressure. Hence the risk due to intraspecies crossability have been assessed to be negligible to low.

6.3 Studies on soil microbial community

Soil microbiota are considered valuable ecological entities that have significant role in determining the soil health, functionality and agricultural productivity. Competent regulatory authorities acknowledge that soil microflora especially those associated with rhizospheric region, may get affected if exposed to the product/compound arising from the introduced genetic modification that are not present in the non-GE comparators. Hence, regulatory authority carefully considered all possible risks associated with the potential hazard[2] produced by the GE plant and its exposure[3] to compare and assess the effect on rhizospheric microbial population of GE crop vs. conventional non-GE comparator

Current Indian regulatory risk assessment is based on culturable approach where residential rhizospheric microbial population abundance is assessed from the experimental fields where both GE and non-GE comparator have been grown. If there is any compromise in microbial abundance due to the cultivation of GE crop then microbial diversity is assessed to ensure that microbial shift is not affecting beneficial microbes. Such a process reduces complexity and generates sufficient and necessary background information on microbial abundance in the receiving environment as well as identifying "normal" functions within a soil for developing or discussing possible management options, if there is any harm from the cultivation of a GE crop. For such study, the protocol after discussion is approved by the competent authority where measurement endpoint is focused on identifying rhizospheric microbial abundance and predominant species along with data regarding source and function of the introduced genes, their products and possibility of their adversely affecting the soil microbes.

The regulatory requirements were set by the two regulatory agencies operative in India i.e. RCGM and GEAC to(a) to determine culturable microbial population abundance in rhizospheric zones and (b) identification of predominant bacteria present in that location.

[2]Potential hazard, if: Transgenic product has known toxicity/ pathogenicity/ antimicrobial properties towards native soil microflora.

[3]Possible exposure, if:

- Expressed in high amount in root tissues.
- Transgenic product present in root exudate.
- Dislodging and decay of plant material releasing transgenic product in soil.

After soil sampling all microbiological tests were performed in an internationally accredited microbiological laboratory at IMTECH, Chandigarh. Population abundance of bacteria, fungi and actinomycetes were determined in cfu/gm of soil at 0 d (day) (before sowing), 30, 60, 90 and 120 d (post-harvest) at three different rhizospheric depths i.e. 30, 60 and 90 cm. Further, predominant bacterial strains were identified following morphological, biochemical and 16S rRNA gene sequence analysis. The quality of data generated was sufficiently detailed and satisfied the criteria for successful risk assessment.

Assessment of the effect of Barnase on soil microflora: One of the major concern was on the deposition and decay of sufficient quantity of whole buds of DMH-11 in soil resulting in exposure of soil microflora to the Barnase-Barstar complex, further, dissociation of Barstar may lead to restoration of Barnase activity in the soil and may affect susceptible microbes present in the rhizosphere either by killing and allowing rise of opportunists that are tolerant to the Barnase. The association between Barnase and its inhibitor Barstar is fast (with a rate constant of 6×10^8 M^{-1} s^{-1}) and with high affinity ($k_d \sim 10^{-14}$M) corresponding to a binding free energy of about 80 kJ/mol whereas the dissociation is extremely slow (rate constant = 8×10^{-6} s^{-1}) (Schreiber and Fersht 1993). It is considered among the strongest known interactions between proteins, hence spontaneous dissociation of complex is thermodynamically unfavourable (Hartley 1993; Schreiber and Fersht 1993). Moreover, the binding is dependent on pH (Wang et al. 2004). For the functional folding of Barnase, Barstar and binding of the two proteins requires pH-optima of 5.0, 7.0, and 8.0 respectively, whereas Barstar unfolds at high pH (beyond pH 10) (Mitra et al. 2011).**The dissociation of complex after decay of buds of DMH-11 is not possible under normal soil pH suitable for agricultural production.**

Even if functional Barnase enters in soil, it will not affect microbial community abundance, diversity and functionality as the Barnase concentration released in soil from the buds of DMH-11, after the decay of buds will be insufficient to affect the rhizospheric populations. Moreover, half-life of Barnase in harsh physico-chemical conditions of soil will be far less than 102 min(Prior et al. 1996).This duration of exposure will not be sufficient to completely eliminate susceptible microbial population present in the soil to a level that requires further monitoring and critical assessment. In addition, soils harbour diverse populations of

proteolytic strains that secrete non-specific proteases sufficient enough to cleave the traces of protein before it shows activity.

Summarizing the information on risk assessment based on hazard identification and exposure assessment, it can be concluded that inserted genes in GE mustard lines have been derived from highly abundant non-pathogenic soil microorganisms. Therefore, the proteins expressed by the GE mustard are expected to be already widely present in nature and their presence in the GE mustard is not expected to present any new toxicity risks to soil microorganisms in these environments. In addition, the introduced proteins are expressed at very low levels as intracellular proteins and not found in the root exudates of GE mustard. Even Barnase, the protein that may show activity against microbes, has not been found in any part of the GE lines except present at a very low level, as expected, in the buds of DMH-11 where Barstar, the inhibitor of Barnase is also co-expressed. Overall, not a single physical or chemical stressor was introduced in soil by GE mustard. Hence, considering the remote possibility of exposure of soil micro-organisms to GE hybrid products, if it occurs under any circumstances, will not generate any harm to microbes or will not disturb microbial gene pool (in terms of microbial abundance and diversity). Therefore, GE mustard is expected to exert an equivalent effect on rhizospheric microbial population similar to non-GE conventional parents and local checks.

All estimates and observations that were concluded in earlier sections support BRL I and BRL II field trial data on microbial abundance as tested at IMTECH, Chandigarh.

Compared to its non-GM counterpart, all GE line(s) tested in this study (both BRL I and BRL II trials) exhibited similarities in:

- Microbial count was relatively high at top soil (0-30 cm) and declined as depth increased. This observation is in agreement with standard microbiological concept i.e. availability of proper physiologic conditions like nutrients, oxygen etc. for microbial growth become limited as depth increases. Further microbial activity in the top soil is reported to be more influenced with plant developmental stage due to inherent metabolic activity of the plant in GE as well as non-GE comparator.
- In fulfilment of a second objective i.e. determination of predominant bacteria present in the rhizosphere, -taxonomic characterization based on physiological, biochemical and molecular analysis was performed. In the report submitted by the applicant, for all lines in this study (including GE and non GE) at every depth of sampling and at every time point of analysis, species belonging to genera *Bacillus* were found to be most abundant. In addition

to *Bacillus* sp., species representing the genera *Serratia, Microbacterium, Paenibacillus, Pseudomonas, Kocuria, Burkholderia, Streptomyces,Cupriavidus, Rhodococcus, Pseudoxanthomonas, Azospirillum, Agromyces, Mesorhizobium, Methylobacterium, Cellulosimicrobium etc* were also present. All the predominant genera reported in this study are common in soil flora and are responsible for maintaining important ecosystem functions and have no role in adversely influencing plant growth. Further, uniformity in their abundance in rhizosphere of all GE lines and non GE-counterpart eliminates any possibility of adverse effect induced by GE mustard.

Minor variations were recorded in bacterial count taken from rhizosphere of DMH-11 at two locations (out of 6 locations tested) in BRL I trial season. Based on the weight of evidence approach in combination with earlier observations, the regulatory agency has concluded that these differences are not significant.

In summary, based on the data provided by the applicant, it can be concluded that the effect of GE mustard lines on rhizospheric soil microflora is as same as its non-GE counterpart indicating nil/ negligible risk. However, as a precautionary approach and sustainable use of this MS-RF technology of mustard an oversight post-release monitoring is suggested.

In general, the shifts that occurred in both GE and non-GE plants are similar and can be correlated with the developmental stage of the plants such as flowering and senescence. Further, prevalence of predominant bacterial genera remained same in both GE and non GE mustard indicating an undisturbed diversity associated with rhizosphere. Hence, irrespective of the year, trial site and locations, and comparing the pattern of changes in microbial population between GE and non-GE mustard, it can be concluded that the GE lines exert an equivalent effect on soil microflora as its non-GE counterparts and hence confirms that GE lines will have similar effect on microbial abundance in rhizospheric soil as non-GE lines.The risk on soil microflora due to cultivation of GE mustard is negligible or nil for which no further risk management is required.

The **Figure7.1** below summarises step by step risk assessment carried out on the soil microflora and other non-target organisms.

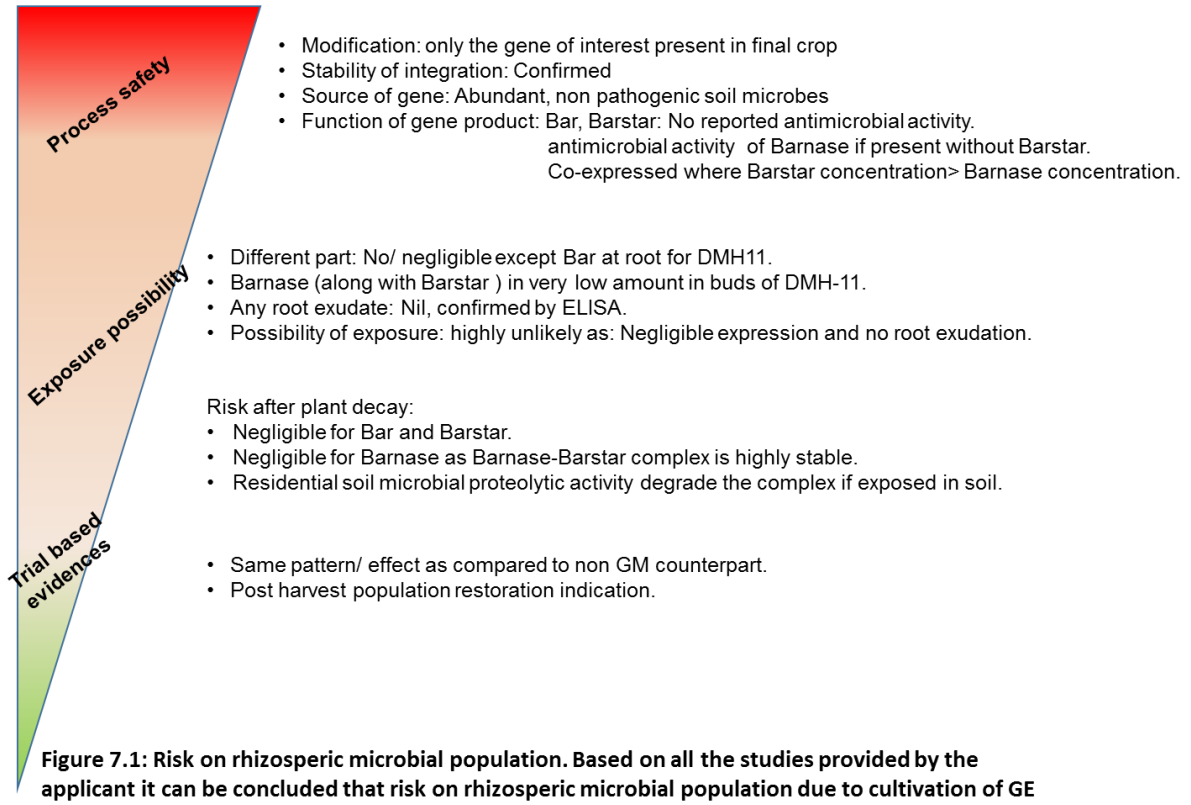


Figure 7.1: Risk on rhizosperic microbial population. Based on all the studies provided by the applicant it can be concluded that risk on rhizosperic microbial population due to cultivation of GE mustard is negligible as it will exert an equivalent effect on rhizospheric soil microflora as its non-GM counterpart for which no management is sought.

6.4 Studies on pests, diseases and beneficial organisms

Environmental safety assessment was performed (refer **Figure 7.1**) to identify and evaluate even the remotest possibilities of changes that can cause unintended effects on the environment after the release of GE hybrid DMH-11 and its parental lines Varuna bn3.6 and EH-2 modbs 2.99. Transgenic proteins encoded by the foreign genes may change the plant such that it may become more attractive to pests and diseases and conversely unattractive for the predators and pollinators. Therefore, on the basis of the experimental data presented in the dossier submitted by the applicant and a review of the past breeding studies - safety assessment was carried out to identify and evaluate the likelihood of any harmful or unintended effects of the transgenic material on the occurrence of pests, predators, diseases and pollinators.

Major insect pests of mustard: 1. Aphids (*Lipaphis erysimi*), 2. Painted bug (*Bagrada hilaris*), 3.Leaf minor (*Chromatomyia horticola*), 4.Cabbage butterfly (*Pieris brassicae*), 5. Mustard saw fly (*Athalia lugens proxima*) and 6. Bihar Hairy Caterpillar (*Diacrisia obliqua*).

Major diseases of mustard: 1. Alternaria blight (*Alternaria brassicae*), 2. White rust (*Albugo candida*), 3.Downy mildew complex (*Hyaloperonospora parasitica*), 4. Sclerotinia stem rot (*Sclerotinia sclerotiorum*) and 5. Powdery mildew (*Erysiphe cruciferarum*) are of great economic importance. Among different species of the U's triangle, *B. juncea* and *B. rapa* are more susceptible to diseases than *B. carinata* and *B. napus*.

Beneficial organisms: Pollinators like **honeybees** are the most important beneficial organisms that help in pollination and as a result contribute to the yield of the crop. Though *B. juncea* is self-compatible crop it is also cross pollinated by insects. Honey bees are one of the most important pollinators whose foraging behaviour is favourable to increase the crop productivity.

Major predators of mustard pests are *Coccinella septempunctata* (commonly called lady bird beetles, feeding stages are larvae and beetles), larvae of syrphid flies, *Syrphus confrater* and *Syrphus balteatus* and *Chrysoperla carnea*. Abundance of predators and parasitoids in GE

mustard field maintains the environmental biodiversity and also plays a significant role in agricultural productivity by keeping a check on pests and diseases spread.

Orobanche is a major devastating parasitic weed of mustard.

Earlier studies on the foraging behaviour of bees on GE *B. napus* Canola lines vs. non-GE comparators and MS1xRF1 hybrids found no significant differences in bee behaviour. Similarly, no negative effects on the foraging or brood behaviour of bees was observed in the lines T45 (USDA-APHIS 1998b), Topas 19/2 (Canadian Food Inspection Agency 1995a; European Scientific Committee on Plants 1998a) or RF3 and MS8 of Canola (European Scientific Committee on Plants 1998b; USDA-APHIS 1999b). Similar observations have been noted in several other studies where no negative impact has been reported on bees foraging on Basta (glufosinate ammonium) tolerant Canola (rapeseed) plants (Malone 2002).

USDA-APHIS in its reports for the application having Barnase-Barstar protein concluded that knowledge of the mode of action, and the lack of known toxicity of the newly expressed proteins in the GE canola suggest no potential for deleterious effects on beneficial organisms such as earthworms (USDA-APHIS 1999b).

The regulatory agencies have set the objectives for the biosafety assessment of Varuna barnase, EH-2 barstar and DMH-11 such that these GE parents are equivalent to the non-GE parents and the GE hybrid is comparable to the checks used in the study in terms of its reaction to the pests, predators, diseases and receptibility to pollinators. The assessment is targeted for the following points:

- Assess any change in the incidence of pests and diseases for GE mustard in comparison to non-GE comparators i.e. whether there is an increase or a decrease in the pest and disease prevalence.
- Assess effects on organisms that contribute to the biological control of pests and pathogens eg. predators and parasitoids of mustard pests.
- Assess any negative effect or stress on beneficial organisms - e.g. pollinators like honeybees.

Biosafety Assessment on pests: The applicant took observations on occurrence of the following insect pests viz. Mustard aphids (*Lipaphis erysimi*), Painted bug (*Bagrada hilaris*), Leaf minor (*Chromatomyia horticola*), Cabbage butterfly caterpillar (*Diacrisia oblique*), Mustard saw fly (*Athalia lugens proxima*) and termites on all the entries and in all the three year trials**.** Aphids were found to be the most prevalent pest. Pest attack incidences were found to be similar for GE and non-GE comparators. No aphid attack was reported by the applicant in Bhatinda and Ludhiana in the BRL II trial (2014-15) and Sriganganagar in BRL I second season trial (2011-12). In the BRL II trial at Bhatinda (2014-15) no occurrence of any pest was reported. In this site, insecticide was sprayed to control the infestation of termites and this could be one of the possible reason for the above mentioned observation.

Biosafety assessment of GE mustard on disease causing organisms: The applicant scored for the following diseases during the BRL I trials (2010-11, 2011-12) and BRL II trial (2014-15); *Alternaria* leaf blight, white rust and powdery mildew. Incidence of the disease was similar between the GE parents and their non-GE comparators for *Alternaria*. Hybrid DMH-11 was recorded to be having lesser incidence of *Alternaria* leaf blight as compared to the zonal check varieties, over all the locations in three years of BRL trials. Varuna *barnase* and Varuna had similar observations for white rust – both were susceptible. No white rust was observed on EH-2 barstar, EH-2 and DMH-11.

Powdery mildew was observed in Kumher and Navgaon in BRL I trial during both the seasons. Similar level of powdery mildew infestation was recorded in the GE parents and non-GE comparators. *Orobanche,* a parasitic weed, was reported only from Navagaon in the BRL I trials. *Orobanche* incidence at Navgaon was similar for all the entries.

Biosafety Assessment GE mustard on predators and pollinators: Honey bees are the major pollinators of mustard. No significant differences were observed for honey bee foraging on the GE and non-GE lines (**Table 6.2**). Expression of the introduced proteins, encoded by the *barnase* and *barstar* genes, is controlled by a tapetum specific promoter and no expression could be detected in the pollen. As a consequence, the exposure of the pollinating insects to these proteins is likely to be negligible. In addition, no adverse effects of these genes has been observed on pollination in GE canola for the last 20 years.

Table 6.2: Comparative data (mean values) for honey bee foraging in BRL I and BRL II trials

		Honey bees						
	Trial Sites	Varuna Barnase	Varuna	EH-2 barstar	EH-2	DMH-11	Local Check	CD
BRL I, 1st year (2010-11)								
	Kumher	24	25	25	24	26	21	4.05
	Navgoan	21	22	25	27	23	21	5.1
	Sriganganagar	8	9	13	12	8	8	3.0
BRL I, 2nd year (2011-12)								
	Kumher	31.25	30	30.75	30.5	30.75	28	1.1
	Navgoan	26.94	29	27.63	31.94	36.63	23.13	4.34
	Sriganganagar	13	13.25	16	15.5	13.5	12.5	1.418
BRL II (2014-15)								
	IARI, Delhi	13.56	13.58	13.44	13.04	14.08	14.06	0.9
	Bathinda	2.25	2.83	4.33	4.25	2.41	2.5	0.5
	Ludhiana	4.1	3.6	5.85	5.9	4.25	5.85	1.18

In most of the sites predators were not observed. Only coccinelids were found in Navagaon (BRL I, 2nd year trial) and IARI, Delhi (BRL II trial). There was no report of Chrysoperla larvae and Syrphid fly larvae from any of the trial sites.

In summary, it was observed that natural occurrence of all the major pests and diseases of mustard were similar in GE hybrid DMH-11 as well as non GE conventional local check variety. Honey bee foraging was also observed to be similar on GE mustard as it was on conventionally grown mustard variety. The transgenes have been found to have no unintended effect on the pest and disease occurrence and on non-target organisms. However, as a precautionary approach and sustainable use of this MS-RF technology of mustard an oversight post-release monitoring is suggested.

CHAPTER 7
EVALUATION OF AGRONOMIC PARAMETERS FOR GE HYBRID DMH-11 AND THE PARENTAL LINES

It is clearly evident from the earlier sections that the integration of the transgenes in GE mustard hybrid DMH-11 and the two parental lines Varuna bn3.6 and EH-2 modbs2.99 do not reveal any undesirable changes. In the evaluation studies of the GE plant, it is imperative to evaluate and understand the effect of the presence of transgenes on the agronomic and phenotypic parameters. To evaluate these agronomic parameters, based on the recommendation of regulatory agencies, the applicant has compared the key vegetative, reproductive and survival biology characteristics between GE and non-GE parental lines. Also, the bio-efficacy of the GE lines and hybrid DMH-11 in terms of the efficacy of the male sterility-fertility restorer technology and extent of hybrid vigour were assessed.

The GE *B. juncea* hybrid DMH-11containing the three transgenes viz. *bar, barnase* and *barstar* was field tested to

- Assess reproductive and survival biology (e.g. crop growth, plant height, dry matter yield) of GE lines in comparison with non-GE comparators.
- Collect data regarding yield performance of GE hybrid DMH-11 in comparison to national and zonal checks.

BRL I trials (1st and 2nd Rabi seasons, i.e. (Oct 2010 - Mar 2011, and Oct 2011 - Mar 2012) were conducted at three different locations (viz. Kumher, Navgaon, and Sriganganagar), all in the state of Rajasthan. Field trial at Sriganganagar was discontinued prematurely (two weeks before harvest) in the BRL I, 2nd season trial.

Based on the outcomes of biosafety and efficacy assessment of BRL I trials (1st and 2nd season), RCGM recommended the applicant's request for conducting BRL II trials to GEAC as a prerequisite for environmental release of the GE lines and hybrid DMH-11 in India.
BRL II trials were conducted in Rabi season (Oct 2014-Mar 2015) at three different locations viz. New Delhi, Ludhiana, Bhatinda. These trials were supervised by DRMR, Bharatpur an institute of the Indian Council of Agricultural Research. Members of the Central Compliance

Committee (CCC), authorised by GEAC inspected/monitored the trial sites. The applicant followed standard cultivation practices for mustard under irrigated conditions.

As recommended by RCGM/GEAC, all the trials were conducted in a randomised block design with 6 entries replicated in 5 blocks. The entries included: Varuna *barnase* (event bn 3.6); EH-2 *barstar* (event modbs 2.99); Varuna (non-GE line); EH-2 (non-GE line); GE hybrid DMH-11(bn 3.6 X modbs 2.99); Maya (zonal check at Kumher)/ RL1359 (zonal check at Navgaon and Sriganganagar). Size of each plot was 9 x 5m. Row to row spacing was maintained at 45cm and plant to plant spacing within a row was 20cm. In addition, an isolation distance was maintained on all the sides of the trial plot to avoid any unintended pollen flow.

Among the five blocks, block 1 to 4 were used for recording comparative observations of yield and yield associated parameters (agronomic – phenotypic characterisation) that include plant population at 30 days; plant population at maturity; days to 50% flowering; leaf shape; days to maturity; plant height; number of primary branches/plant; number of secondary branches/plant; number of pods per plant; shape of pods; average pod length (cm); average number of seeds per pod; seed colour; pod shattering; seed yield and test weight. The 5^{th} block was used for assessing dry matter yields and plant height at 30, 60 DAS and at maturity.

To analyse the data one way statistical analysis was used across the 6 entries, with the assumption that the residual variance is constant across the experiment. The means with statistical parameters such as Critical Difference (CD to explain the difference between the performance of different entries) at 5% from the ANOVA [The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means of three or more independent (unrelated) groups] was reported for validation.
A GE crop is compared with an appropriate conventional counterpart or control as a part of comparative risk assessment mainly to identify whether the GE crop is different from its non-GE counterpart and/or equivalent to commercial varieties apart from the inserted trait(s). Assumption for this approach is that conventionally grown crops have gained a history of safe use for consumers and animals, and familiarity for the environment. Thus the agronomic and phenotypic characterisation aims to identify and measure agronomic and phenotypic differences between GE plant and its conventional counterpart and to confirm that good

agricultural practices were followed for the acquisition of the data and to identify possible factors that might bias the outcomes of the comparative analysis

Based on each year trial data across each location (BRL I trials for two years and BRL II trial for one year), the comparison of vegetative, reproductive and survival biology parameters (e.g. plant height, days to flowering, primary and secondary branches, seeds per pod, and pod length) between GE lines viz. Varuna bn3.6, EH-2 modbs 2.99 and their non-GE comparators were very similar (**Table 7.1**). This indicates that the genetic modification did not have any unintended effects on the morphology and reproductive parameters, as compared with their non-GE comparators, apart from the inserted trait(s).

Table 7.1: Comparison of parameters for key vegetative, reproductive and survival biology characteristics between GE and non-GE parental lines

S. No.	Parameter	Female parent		Male parent	
		Varuna bn 3.6	Varuna	EH-2 barstar	EH-2
1	Days to 50% Flowering	63.44±9.9	63.00±9.82	59.66±9.78	59.83±10.02
2	Plant Height (cms)	194.38±23.55	197.73±21.66	221.61±16.89	220.82±18.25
3	Leaf Length (cms)	39.77±13.94	39.63±14.01	34.85±11.02	34.38±11.77
4	Leaf Width (cm)	15.87±3.77	16.83±3.46	18.19±4.97	18.57±4.79
5	Leaf color	Green	Green	Light green	Light green
6	Number of Primary Branches/ Plant	6.87±1.92	7.59±1.89	7.42±2.01	7.3±2.59
7	Number Secondary Branches/ Plant	19.30±5.85	20.21±5.69	20.03±4.74	21.09±5.25
8	Average Number of Pods/ Plant	747.66±259.02	779.0±264.12	887.89±286.89	855.32±277.53
9	Average Number of Seeds/ Pod	12.65±2.48	13.05±2.99	15.42±2.80	15.15±3.47
10	Pod Length (cms)	4.52±0.35	4.54±0.40	2.92±0.21	2.93±0.40
11	Seed Coat Color	Brown	Brown	Yellow	Yellow
12	1000 Seed Weight (gms)	4.82±0.47	4.71±0.52	2.49±0.38	2.48±0.46
13	Days to Maturity	139.11±6.44	138.94±5.98	136.17±7.0	135.29±7.05
14	Oil Percent**	40.19±1.54	40.59±1.05	38.76±2.12	38.61±2.52

GE hybrid DMH-11 showed yield advantage in each year trial across each location (BRL I trials for two years and BRL II trial) in comparison with the national and zonal checks (**Table 7.2, 7.3** and **7.4**).

Table 7.2 Seed yield (kg/ha) under BRL I trial, 1styear (2010-11 growing season)

S. No.	Entry	ICAR Centre			Total	Mean
		Kumher	Navgaon	Sriganganagar		
1	Varuna(barnase)	1986	1789	2513	6287	2096
2	EH-2 (barstar)	1730	1842	2455	6026	2009
3	Varuna	1866	1741	2670	6278	2093
4	EH-2	1793	1716	2182	5691	1897
5	DMH-11	2285	2515	3000	7801	2600
6	Maya/RL-1359	2057	1767	2287	6112	2037

Table 7.3 Seed yield (kg/ha) under BRL I trial, 2nd year (2011-12 growing season)

S. No.	Entry	ICAR Centre		Total	Mean
		Kumher	Navgaon		
1	Varuna(barnase)	2484	2098	4582	2291
2	EH-2 (barstar)	1640	1581	3221	1611
3	Varuna	2375	2169	4544	2272
4	EH-2	1873	1608	3481	1741
5	DMH-11	2892	3157	6049	3025
6	Maya/RL-1359	2195	1836	4031	2016

Table 7.4 Seed yield (kg/ha) under BRL II trial (2014-15 growing season)

S. No.	Entry	Punjab Agricultural University			Mean
		Ludhiana	Bhatinda	IARI	
1	Varuna	2006	1911	1746	1887
S 2	Varuna Barnase	1938	1947	1700	1861
3	EH-2	1740	1443	953	1378
4	EH-2 Barstar	2001	1563	1110	1558
5	RL 1359	1965	1792	1571	1776
6	DMH-11	2543	2734	1879	2386

Conclusions: Agronomic and phenotypic data for comparative assessment is complementary to other data for the assessment of any GE plant. Based on the assessment of the agronomic and phenotypic parameters, it can be concluded that presence of transgenes in the hybrid does not lead to any unintended effect on the agronomic parameters. The efficacy evaluation has proven the presence of hybrid vigour in the hybrid. The hybrid DMH-11 which is the result of cross between varuna bn 3.6 and EH-2 mod bs 2.99 is superior as compared to the parents proving proof-of-concept of the technology and showing heterosis and hybrid vigour.

CHAPTER 8
CONCLUSIONS AND SUMMARY OF RISK ASSESSMENT

8.1 Level of risk associated with various biosafety studies

Assessment for biosafety	Comparison between GE and Non-GE counterpart	Risk assessed
Assessment for toxicity and allergenicity to humans and toxicity to animals		
Composition Analysis	Alteration in nutrient composition of leaf and seed	Nil/Negligible
	Alteration in toxin/anti-nutrient composition in leaf and seed	Nil/ Negligible
Toxicity potential	Expression levels of the introduced proteins i.e. Bar, Barnase and Barstar proteins in the edible plant parts (leaf and seed)	Bar is expressed in leaves but risk is Nil/ Negligible
	Acute oral toxicity of the purified proteins	Nil/ Negligible
	Sub-chronic toxicity with the edible plant parts (seed and leaf)	Nil/ Negligible
Allergenicity potential	Bioinformatics analysis of the Bar, Barnase and Barstar proteins	Nil/ Negligible
	Pepsin digestibility	Nil/ Negligible
	Thermal stability	Nil/ Negligible
Environmental risk assessment		
Weediness potential	Seed germination	Nil/ Negligible
	Speed of Seed germination	Nil/ Negligible
	Seedling vigour	Nil/ Negligible
	Small seed size	Nil/ Negligible
	Long continuous seed production	Nil/ Negligible
	Pod shattering	Nil/ Negligible
Crossability and gene flow	Extent of cross pollination between GE *B. juncea* hybrid DMH-11 and its related species	Negligible

	Extent of cross pollination between GE *B. juncea* hybrid DMH-11 and *B. juncea* variety Pusa bold as an adjoining crop (Pollen flow)	Crosses could occur but unlikely to provide selective advantage for transgene hence risk is Negligible to low
	Alteration in pollen production	Nil/ Negligible
Effect on soil microflora	Alteration in abundance (cfu/gm) if any of bacteria, fungi and actinomycetes in rhizosphere	Nil/ Negligible
	Alteration in predominant bacterial species in rhizosphere	Nil/ Negligible
Effect on pests, diseases and beneficial insect	Change in the susceptibility for insects and diseases	Nil/ Negligible
	Change in the predators abundance	Nil/ Negligible
	Change in the receptibility towards honeybee and any toxicity to honeybee feeding on pollen and nectar	Nil/ Negligible

8.2 Summary

The Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus, New Delhi sought approval from GEAC, MoEF&CC, Government of India for environmental release of GE mustard (*B. juncea*) hybrid DMH-11 and use of parental events (Varuna bn 3.6 and EH-2 modbs 2.99) for development of new generation hybrids. CGMCP through extensive R&D supported bythe Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India and also with financial support from the National Dairy Development Board (NDDB), has developed the technology. Further studies on biosafety including field performance have been carried out with financial support extended by the Biotechnology Industry Research Assistance Council (BIRAC), a public sector enterprise. The biosafety studies were carried out in various national institutes like National Institute of Nutrition (NIN) Hyderabad an ICMR institute; Institute of Microbial Technology (IMTECH) Chandigarh, a CSIR institute; Directorate of Rapeseed Mustard Research (DRMR) Bharatpur, an ICAR institute.

Statutory committees (IBSC, RCGM and GEAC) examined the case through a step by step process from research to technology development and generation of data on food and environmental safety. The conclusions have been drawn on the basis of mandated risk assessment carried out based on thorough analysis of all the submitted data and information from literature. The goal for the assessment is that any substantial differences between the GE lines when compared with the non-GE comparators and the national and zonal check varieties, if identified could be further evaluated.

GEAC started the evaluation of the final dossier submitted along with detailed biosafety data for environmental release and constituted a sub-committee of scientific experts to thoroughly investigate each aspect of the biosafety data. The sub-committee members examined the dossier on the following aspects of food/feed safety and environmental safety and compliance aspects.

The sub-committee made the following observations:

- The applicant has followed all the regulatory Compliances under Rules, 1989.

- The technology is safe for food/feed and environment in view of the following analysis that have emerged after the assessment.

A transgenic technology based hybrid seed production system has been developed. *B*. *juncea* is a predominantly self-pollinating crop, hence a pollination control mechanism is required to disallow self-pollination and facilitate cross-pollination for production of desired hybrid seeds. Therefore, a transgenic technology based hybrid seed production system has been developed. The genetic engineering based hybrid developed consists of the *barnase* gene for male sterility (MS) and *barstar* gene for restorationof male fertility (RF). The hybrid was made by crossing the MS and RF lines. The MS line Varuna bn 3.6 had normal morphology, was completely female fertile and had normal seed set when crossed to a maintainer line. Flowers of the MS line Varuna bn 3.6 are characterized by complete absence of viable pollen in the anthers. In the MS line, the transgene was inherited stably with no breakdown in sterility. RF line has been shown to successfully restore male sterility of the MS line over many generations. This combination of male-sterile (*barnase*) and restorer (*barstar*) lines in *B*. *juncea* constitutes a complete and functional male-sterility/restorer system, which could be diversified into appropriate combiners and deployed for production of new hybrids in this crop.

The molecular characterization data reveals that the two GE parental events each have a single copy of the transgene integrated in the genome. The insertion of these genes does not lead to disruption of any known endogeneous genes. These genes are stably integrated and their stable expression and inheritance has been shown across several generations.

The Food and Feed Safety Studies have led to the following conclusions: from the compositional analysis studies it is clearly evident that GE parents are substantially equivalent to their non-GEcomparators in terms of key parameters such as oil, protein, carbohydrate, glucosinolate, erucic acid, fatty acids, allylisothiocyanate, peroxide value, sodium, calcium, magnesium, potassium and minerals when analysed in leaf and seeds. Also, hybrid DMH-11 is very similar, in its composition, to the commercially cultivated varieties in India which have a history of safe use.

From the toxicity and allerginicity studies, it was concluded that the GE mustard, the parental lines and hybrid DMH-11, does not raise any public health or safety concerns for human beings and animals with respect to overall nutritional characteristics. The introduced proteins i.e. Barnase and Barstar are expressed at negligible to non detectable levels in the edible parts and have been derived from commonly occurring non-pathogenic bacteria. None of the three proteins has been shown to be toxic or allergenic through bioinformatics and acute toxicity studies in experimental animals. All the three proteins are rapidly degraded in simulated gastric conditions of the mammalian digestive systems. Sub-chronic toxicity studies in experimental animals using edible plant parts i.e. leaves and seeds also did not show any adverse effects. Mustard oil does not contain any proteins. Therefore, the probability of oil extracted from DMH-11 or any other future hybrids to have any of the three proteins is nil/ negligible. There is more than 20 years of history of safe use of proteins from the three genes – *bar, barnase* and *barstar* in rapeseed (*B. napus*), as oil and meal extracted from GE rapeseed is being consumed in a large number of developed and developing countries and so far no toxicity or allergenicity have been reported. In conclusion, based on the history of safe use of the host and the donor organisms, transgene expression analysis, composition analysis, acute and sub-chronic toxicity assays of purified proteins and whole leaf and seeds, respectively, it can be concluded that the use of leaves, seed and oil derived from GE mustard lines is not likely to pose any risk to humans and animals.

From the environmental risk assessment studies, following conclusions were drawn–

a) The weediness Potential of GE mustard hybrid DMH-11 is similar to that of the varieties commonly grown in India. There is no risk of any aggressiveness or any weediness potential in the hybrid DMH-11. Therefore, potential of GE hybrid DMH-11 becoming a weed in mustard fields or in natural ecosystem is highly unlikely.

b) Crossability and geneflow study lead to the conclusion that intraspecies gene flow could occur between DMH-11 and varieties of *B. juncea* grown in close proximity to GE hybrid DMH-11. However, the cross between adjoining crops has been found to occur upto a distance of 20 m. Moreover, the progeny of such crosses will not have any survival advantages in the absence of selection pressure. The data on interspecies crossability of

GE mustard hybrid DMH-11 shows no crossability with related *Brassica species*. Thus the probability of persistence of progeny of such crosses in the environment are negligible.

c) As for the effect on Soil microbial community-it can be concluded that the transgenes present in GE mustard lines have been cloned from highly abundant non-pathogenic soil microorganisms. In addition, the introduced proteins are expressed at very low levels as intracellular proteins and not found in the root exudates of GE mustard. Hence, considering the remote possibility of exposure of soil micro-organisms to barnase/barstar or bar proteins, such an exposure will not create any harm to microbes or will not disturb microbial gene pool in terms of microbial abundance and diversity in the soil. Further prevalence of predominant bacterial genera remained same in both GE and non GE mustard. Therefore, GE mustard is expected to exert an effect on rhizospheric microbial population that is similar to the effect of non-GE conventional parents and local checks.

d) Effect on pests, diseases and non-target organisms- it was observed that natural occurrence of all the major pests and diseases and predators of mustard were similar in GE hybrid DMH-11 to their non-GE comparators and conventional zonal check variety. Honey bees foraging were also observed to be similar on GE mustard as it was on conventionally grown mustard varieties. The transgenes have been found to have no unintended effect on the pest and disease occurrence and on non-target organisms.

Agronomic and phenotypic data demonstrates that presence of transgenes in the hybrid does not lead to any unintended effect on the agronomic parameters. The efficacy evaluation has proven the presence of hybrid vigour in the hybrid. The hybrid DMH-11 which is the result of cross between varuna bn 3.6 and EH-2 mod bs 2.99 is superior as compared to the parents proving proof-of-concept of the technology and showing heterosis and hybrid vigour.

These results collectively reveal that the introduction of the *barnase, barstar* and *bar* genes to mustard did not lead to any unintended effects on the food/feed and environmental safety of the GE plants – either the parental lines or the hybrid DMH-11.

Sub- committee prepared this document on “Assessment of food and environmental safety (AFES)” and critically examined the data requirement particularly as the resultant hybrid is based on stacked events. The evaluation of such staked events depends primarily on the two criteria: The process used for generating the stacked event and prior approval status of the individual event/s. Since in this case none of the individual events are approved and are to be used only for hybrid seed production, the applicant was required to submit a dossier containing detailed characterization report of the individual parent event (s), food and environmental safety studies data for hybrid as well as parental events. It was noted that the data generated and submitted in final dossier by the applicant to GEAC is comprehensive and in compliance with all the existing guidelines published, protocols and measurable end points prescribed by RCGM and GEAC as well as the international best practices.

The AFES report presented by the sub-committee herein contains thorough assessment of biosafety data generated by the applicant, its comparison with other such international assessment by well known regulatory agencies such as EFSA, OGTR and Canadian regulatory authorities and exisiting scientific literarure on the subject in peer reviewed journals yet addressing the specific uses of mustard in Indian context. Therefore, sub-committee is of the opinion that both the genetically engineered parents (Varuna bn3.6 and EH-2 modbs 2.99) and the hybrid DMH-11 are substantially equivalent to non-GE parents and conventional mustard, and its consumption is safe for human and animal health. With regard to the environment, the sub-committee concluded that environmental release of parental lines for hybrid production DMH-11 may not pose any risk to biodiversity and the agro-ecosystem as the GE material under review have been demonstrated to have no/ negligible effect on non-target organisms.

As mandated by the Environment (Protection) Act, 1986, it is essential to ensure protection of the health and safety of people and the environment by identifying risks posed by, or as a result of, modern biotechnology and managing those risks. Rules, 1989 of this act regulates the authorization of genetically engineered products in India through statutory committees and series of biosafety guidelines for determining food and environmental safety assessment in Indian context. The provisions also include authorization, supervision and monitoring of multi-location biosafety research confined field trials.

Accordingly Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi has submitted final dossier with biosafety data generated as per the Rules 1989 and directions of RCGM and GEAC from time to time. Based on the biosafety data in the final dossier, peer reviewed scientific literature on the subject, evaluation and opinion of sub-committee of GEAC constituted for the purpose and Risk Assessment Unit of RCGM, this document on "Assessment of Food And Environmental Safety (AFES) has been prepared on the proposal requesting Environmental release of genetically engineered mustard (*Brassica juncea*) hybrid DMH -11 and use of parental events (varuna bn3.6 and EH-2 modbs2.99) for development of new generation hybrids.

Although, food and environmental biosafety assessment elaborated in this document did not reveal any measurable risk, for sustained use of technology in breeding for newer hybrids some post-release monitoring/stewardship is suggested as a precautionary measure. These measures include: monitoring honey bee behavior particularly with respect to presence of target proteins in honey; impact on non-target organisms and intra and inter -specific interactions. Additional measures should be taken not to include any chemicals for weed control in the package of practices.

To facilitate informed decision making, the GEAC invites written comments before final decision by the GEAC.

Appendix-I

Chronology of approvals for this application by the regulatory authority, Government of India.

Date	Letter No.	Activities
Research and development phase		
17.09.2003	BT/BS/17/30/97-PID	Small Scale open field trial of hybrid DMH-11 at Delhi location was conducted during 2003-04 under the supervision of RCGM.
17.09.2004	BT/BS/17/30/97-PID	Application seeking permission to carry out contained limited experimental field trial and hybrid seed production of transgenic mustard (*Brassica juncea*) expressing *barnase*, *barstar* and *bar* genes at Jaunti Village, Delhi during Rabi 2004.
28.10.2004	BT/BS/17/30/97-PID	RCGM permitted Centre for Genetics Manipulation of Crop Plants (CGMCP) to conduct contained limited experimental field trial and hybrid seed production of transgenic mustard (*Brassica juncea*) expressing barnase& barstar genes at Field Research Station, village Jaunti, Delhi during Rabi -2004 for research purpose
10.10.2005	BT/BS/17/30/97-PID	RCGM permitted CGMCP to conduct multi-location field trials on transgenic B. juncea hybrid DMH-11 containing barnase/barstar gene during Rabi 2005-06
08.02.2006	BT/BS/17/78/2002-PID	Central Monitoring Team for inspection of the field trials of transgenic mustard constituted by the RCGM
25.02.2006		The MEC (Monitoring and evaluation committee) visited trial location and made recommendations to the DBT. The MEC recommended conducting the Multi location trial for one more year in 2006-07 by All India Coordinated Research Project on rapeseed-mustard under the supervision of NRCRM, Bharatpur.
01.08.2006		IBSC forwarded the proposal to RCGM for conducting multi-location field trial based on barnase/barstar transgenic mustard for generating biosafety data and to produce hybrid seeds
30.10.2006	BT/BS/17/30/97-PID	RCGM recommended conduct of multi-location field trials of transgenic mustard hybrid DMH-11 and recommend GEAC to accord approval for the same.
06.02.2007	BT/BS/17/30/97-PID	Monitoring Team for inspection of the field trials of transgenic mustard constituted by the RCGM

Date	Letter No.	Activities
Biosafety data generation as per the guildeines		
10.1.2008	BT/BS/17 /30/97-PID	RCGM permitted to carry out toxicity, allergenecity and other biosafety tests
2008		Adoption of new guideline; introduction of BRL I and BRL II trials
20.08.2010	VC/DU/2010	Application submitted through IBSC to conduct BRL I 1st year study under confined conditions at three locations namely Agricultural Research station experimental Farm, Navgaon, Agricultural Research Station Sriganganagar and KVK, Kumher during Oct, 2010
15.10.2010 and 30.10.2010	BT/BS/17/30/97-PID (13/7/2007-CSIII)GEAC	RCGM and GEAC approved conduct of BRL I 1st year study under confined conditions For environmental and food and feed safety assessments at three locations namely Agricultural Research station experimental Farm, Navgaon, Agricultural Research Station Sriganganagar and KVK, Kumher during Oct, 2010. For experimental seed production under confined condition at Jaunti Village, Delhi and Environmental safety studies (Crossability studies) at Bawana, Delhi during Oct, 2010.
07.02.2011	BT/BS/17/30/97-PID	RCGM approved the following protocols submitted by Centre for Genetic Manipulation of Crop Plants, University of Delhi South Campus, New Delhi for conducting studies for environmental safety assessment of transgenic *Brassica juncea* containing *barnase*, *barstar* and *bar* genes: Expression studies for Bar, Barnase and Barstar in various plant tissues of transgenic *B. juncea* Comparative assessment of potential for weediness and aggressiveness Parameters affecting pollen flow. Impact on soil microflora 0.01% level of detection of barnase-barstar contamination
3.05.2011	IR/PA/M-333	Central Compliance Committee (CCC) submitted report after inspection on 9 April 2011 of Biosafety Research Level-I confined field trial at University of Delhi South Campus, New Delhi and Agricultural Research Station, Sriganganagar (RAU, Bikaner).

Date	Letter No.	Activities
27.06.2011	BT/BS/17/30/97-PID	RCGM recommended submission of expression data of the two proteins i.e. Barnase and Barstar in various tissues of mustard.
26.07.2011		RCGM in its 103rd meeting noted submission of UDSC conducting BRL I 2nd year study during Rabi 2011 and recommended the application.
		Final report submitted to RCGM for BRL I 1st year trial and assessment
17.10.2011	BT/BS/17/30/97-PID	RCGM permitted to conduct BRL I 2nd year study under confined conditions at three locations namely Agricultural Research station experimental Farm, Navgaon, Agricultural Research Station, Sriganganagar and KVK, Kumher during Oct, 2010 for experimental seed production.
17.10.2011	13/7/2007-CSIII	GEAC permitted to conduct BRL I 2nd year study under confined conditions at three locations
20.12.2011	BT/BS(MM)/17/360/2009-PID	Constitution of CCC for monitoring the Biosafety Research Level-I 2nd year trials
12.01.2012	BT/BS/17/30/97-PID	RCGM approved protocol for food and feed safety analysis of transgenic mustard.
08.01.2012		Visit of the CCC team to trial site at Agriculture Research station Sriganganagar for monitoring the BRL I 2nd year trial of transgenic *Brassica juncea.*
28.01.2012		Visit of the CCC team to trial site at Agriculture Research Station Experimental Farm, Navgaon and KVK, Kumher, Bharatpur for monitoring the BRL I 2nd year trial of transgenic *Brassica juncea*.
12.03.2012	BT/BS/17/30/97-PID	Withdrawal of NOC by Department of Agriculture Govt. of Rajasthan for conducting BRL 1 2nd year trial on transgenic mustard at three locations namely Agricultural Research Station Experimental Farm, Navgaon, Agricultural Research Station, Sri Ganganagar and KVK, Kumher during Rabi 2011-12
		Data submitted to RCGM for BRL I 2nd year trial and assessment
22.04.2014	BT/BS/17/48/2001-PID	In 133rd meeting, RCGM recommended to GEAC for BRL II trials during Rabi 2014-15.

Date	Letter No.	Activities
16.10.2014	1499/JDA(HYVP)	NOC issued by State government of Punjab to conduct BRL II trial at Ludhiana and Bhatinda
28. 10. 2014	12013/35/2012-CS-111	GEAC permitted BRL II field trial in Rabi 2014-15 at two different locations viz. Ludhiana and Bhatinda
05.11.2014	3/1/P&P/JDA/2014-15/4075	NOC issued by State Government of Delhi to conduct BRL II trial
7.11.2014	12013/35/2010-CS-III	GEAC permitted BRL II field trials in Rabi 2014-15 at IARI, New Delhi
9.03.2015		Visit of the CCC team to trial site at Punjab Agriculture University (PAU) Ludhiana for monitoring the BRL II trial of transgenic Brassica juncea.
10.03.2015		Visit of the CCC team to trial site at Regional Research Station, Bhatinda (PAU) Ludhiana for monitoring the BRL II trial of transgenic *Brassica juncea*.
11.03.2015		Visit of the CCC team to trial site at IARI, New Delhi for monitoring the BRL II trial of transgenic Brassica juncea.
15.09.2015		Biosafety dossier submitted to GEAC for environmental release of hybrid DMH-11
2015-2016		Preparation of Risk Assessment and Risk Management document

List of Abbreviations

^{0}C	degree Celsius
35S	CaMV 35S promoter with single enhancer
35SpA	35S polyadenylation signal
AMV	*Alfalfa mosaic virus*
AMVL	Alfaalfa Mosaic Virus Leader sequence
ANZFA	Australia New Zealand Food Authority
AOAC	Association of Official Analytical Chemists
AOL	AllergenOnline.org
APHIS	Animal and Plant Health Inspection Service
B. juncea	*Brassica juncea*
bar	coding sequence of Basta herbicide resistance gene obtained from *Streptomyces hygroscopicus*
Barnase	ribonuclease
barstar (Mod)	Codon optimized *barstar* gene;
Barstar	Barnase ribonuclease inhibitor
BIRAC	Biotechnology industry research assistance council
BRL	Biosafety Research Level
BRL1	Biosafety Research Level 1
BRL 2	Biosafety Research Level 2
BW	Body Weight
CaMV	*Cauliflower mosaic virus*
CD	Critical Difference
cfu	Colony forming unit
CCC	Central Compliance Committee
CGMCP	Centre for Genetic Manipulation of Crop Plants
cm	centimetre
CMS	cytoplasmic male sterility
DBT	Department of Biotechnology
DDI	Daily Dietary intake
DMH-11	Dhara Mustard Hybrid 11, *B. juncea* hybrid produced using parent lines containing barnase (Event bn 3.6) and barstar (modbs 2.99)
DNA	Deoxyribonucleic acid
DRMR	Directorate of Rapeseed Mustard Research

EFSA	European Food Safety Authority
EH-2	An EMS induced early mutant of the line 'Heera', a '00' line developed by the University of Nagpur
ELISA	enzyme-linked immunosorbent assay
EPA	Environment Protection Act, 1986
EU	European Union
FYM	Farm Yard Manure
g/kg	gram per kilogram
GE	Genetically Engineered
GEAC	Genetic Engineering Appraisal Committee
GIT	Gastrointestinal Tract
Ha	hectare
HER 2	human epidermal growth factor receptor 2
HL-60	Human promyelocytic leukemia cells
IARI	Indian Agricultural Research Institute
ICAR	Indian Council of Agricultural Research
ICMR	Indian Council of Medical Research
K	potassium
Kg	Kilogram
Kg/ha	kilogram per hectare
KVK	Krishi Vigyan Kendra
LB	Left border
LC-MS	Liquid chromatography-mass spectrometry
LGA9	A9 Linkage Group
mg	milligram
mg/Kg	milligram per kilogram
min	minute
ml	millilitre
mm	millimetre
MS	Male-sterile
MS1, MS8	Male sterile lines
NDDB	National Dairy Development Board
NIN	National Institute of Nutrition, Hyderabad, India
NOAEL	No Observed Adverse Effect Level

NPK	Nitrogen Phosphorus and Potassium
ocspA	*polyA* signal of *octopine synthase* gene
ORFs	Open Reading Frames
PAU	Punjab Agricultural University
PCR	Polymerase Chain Reaction
polyA	polyadenylation signal
PPT	DL-Phosphinothricin
RARM	Risk Assessment and Risk Management
RB	Right border
RCGM	Review Committee on Genetic Manipulation
RF	Restorer of fertility
RF1, RF3	fertility restorer lines
RFLP	Restriction fragment Length Polymorphism
RNA	Ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SOPs	standard operating procedures
SDS–PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SGF	Simulated Gastric Fluid
Spacer	*topoisomerase* gene component and *acetolactate synthase* component as the Spacer DNA
t ha^{-1}	tonnes per hectare
TA29	Tapetum-specific TA29 promoter
USA	United States of America
USDA	United States Department of Agriculture
UDSC	University of Delhi South Campus

Glossary:

***bar* gene**: The *bar* gene encodes an enzyme, phosphinothricin acetyl transferase (PAT) that detoxifies the active compound DL-Phosphinothricin (PPT) in the commercial preparation named –'Basta'.

Barnase* gene:* In nature, the bacterium excretes a defense protein called Barnase (a type of ribonuclease) which degrades the RNA of competing bacteria in an ecological niche

Barstar* gene:* To protect itself from Barnase, the bacterium produces another protein called Barstar which tightly binds with Barnase and renders it ineffective.

Bioagents – Bioagents include parasitoids, predators and microbial agents and materials derived from animals, plants, fungi, bacteria and viruses used for the management of insect pests and diseases.

BRL I field trials: Biosafety Research Level-I Trials, Limited in size to no more than 1 acre (0.4 ha) per trial site location and a maximum cumulative total of 20 acres (8.1 ha) for all locations for each plant species/ construct combination, per applicant, per crop season.

BRL II field trials: Biosafety Research Level-II Trials, Limited in size to no more than 2.5 acre (1 ha) per trial site location and number of locations to be decided on a case by case basis for each plant species/ construct combination, per applicant, per crop season.

Case-by-case: Is defined as the approach by which the required information may vary depending on the GE plant, their intended use and potential receiving environment, taking into account i.e. GE plant already in the environment

cfu:A colony-forming unit (CFU) is used to estimate the number of living bacteria or fungal cells in a sample.

Chrysoperla - Chrysoperla is a genus of green lacewings in the neuropteran family Chrysopidae which is a widely used beneficial insect to naturally control many different pests.

Coccinelids - Coccinellids also called Ladybird beetles, coccinellid beetles, ladybugs or lady beetles belong to the family Coccinellidae (order: Coleoptera) are general predators of other insects. Both the larvae and the adults are predators, feeding on aphids, mites, whiteflies, small insects, insect eggs, etc.

ELISA: Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive and specific test that uses antibodies and color change to identify and quantifya protein of interest.

Event: A genotype produced from the transformation of a single plant species using a specific gene construct.

Feral population: Those crop derived plants that exist in the field margins of receiving environment, outside cultivated parts of field and such are distinct from volunteer crops.

GE crop: A genetically engineered (GE) crop is that in which the basic genetic material i.e. DNA has been altered or modified using genetic engineering techniques to improve the attributes or make it perform a new function. GE crops are also referred as genetically modified (GM) crops, transgenic crops or biotech crops. The two terminologies have been used interchangeably in the document although GE or transgenic are more appropriate terms.

GEAC: Genetic Engineering Appraisal Committee (GEAC) established under Ministry of Environment and Forest and Climate Change (MoEF&CC) is the apex body, notified under Rules 1989, of the Environment Protection Act 1986 for approval of activities involving large scale use of hazardous microorganisms and recombinants in research and industrial production from the environmental safety angle. The GEAC is also responsible for approval of proposals relating to release of genetically engineered organisms and products into the environment including experimental field trials (Biosafety Research Level trials-I and II also known as BRL I and BRL II).

Gene flow: Newly introduced gene could potentially disperse into nearby population of the crop species or wild species, bringing about the new phenotypic trait

IMTECH: The Institute of Microbial Technology (IMTECH), based in Chandigarh, India, is one of the constituent establishments of the Council of Scientific & Industrial Research. It houses Microbial Type Culture Collection and Gene Bank (MTCC), a modern facility that act as a depository to supply authentic microbial cultures and provide related services to the scientists working in research institutions, universities and industries. It is an affiliate member of the World Federation for Culture Collections (WFCC) and is registered with the World Data Centre for Microorganisms (WDCM, registration number 773).

Likelihood (exposure): is defined as causal link between the cultivation of the GE plant and a particular harm and to determine how likely it is that the harm will occur.

Parasitoids - A parasitoid is an organism that spends a significant portion of its life history attached to or within a single host organism in a relationship that is in essence parasitic; unlike a true parasite, however, it ultimately sterilises or kills, and sometimes consumes, the host. Most beneficial insect parasitoids are wasps or flies.

Pollinators - A pollinator is an animal that causes plants to make fruit or seeds. They do this by moving pollen from one part of the flower of a plant to another part. This pollen then fertilizes the plant.

Predators – The predator is defined as an animal which feeds upon other animals (prey) that are usually smaller and weaker than itself, frequently devouring them completely and rapidly. *Predatory insects* such as mites, ladybugs, green lacewings, dragonflies and spiders eat harmful *insect*s.

RCGM: Review Committee on Genetic Manipulation (RCGM) established by the Department of Biotechnology, Ministry of Science and Technology is to monitors the safety related aspects in respect of on-going research projects and activities (including small scale field trials) and bring out manuals and guidelines specifying procedure for regulatory process with respect to activities involving genetically engineered organisms in research, use and applications including industry with a view to ensure environmental biosafety.

Receiving environment: is defined as the environment into which the GE plant will be released.

Risk assessment: A case- by- case, science- based process consisting of the following steps: 1) risk identification; 2) risk characterization: consequence assessment; 3) risk characterization: likelihood assessment and 4) risk evaluation.Consequence (hazard): is defined as the potential of an organism to cause harm to or adverse effects on human health and/or the environment

Risk management: The risk management plan provides an answer to the question: "How any risks posed by an environmental release of GE plant might be managed in such a way as to protect the health and safety of human, animal and the environment?"

Risk: In relation to any GE plant, the probability that some valued environmental resource (including human and animal health) will be adversely affected by exposure to a hazard caused by the plant. Risk is commonly expressed as an equation: Risk = ∫ (Hazard • Exposure).

Selective advantage: The characteristic of GE plant that enables it to survive and reproduce better than other organisms in a population in a given environment

Stressor: The GE plant itself, the transgene(s) in this organismal context and its products that was absent in non GE crop and can harm the protectable entities

Syrphid: Syrphid flies also called Hoverflies or Flower flies, make up the insect family Syrphidae which are seen hovering or nectaring at flowers. Larvae of many species of this family are insectivores and prey on aphids, thrips, and other plant-sucking insects.

Weediness potential: Potential for these GE plant and parental events to establish as problematic weeds after environmental release

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