Development of transgenic *barstar* lines and identification of a male sterile (*barnase*)/restorer (*barstar*) combination for heterosis breeding in Indian oilseed mustard (*Brassica juncea*)

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Transgenic lines containing the *barstar* gene (encoding for Barstar an intracellular inhibitor of the ribonuclease, Barnase both from *Bacillus amyloliquefaciens*) have been developed in Indian oilseed mustard, *Brassica juncea*, to develop a complete male sterility/restoration system for heterosis breeding in this crop. Transgenics were also raised using a modified sequence of the *barstar* gene based on parameters known to influence transgene expression in heterologous systems. The wild type and modified *barstar* lines were analysed for their restoration capabilities by crossing them with agronomically suitable male sterile *barnase* lines developed earlier in our laboratory. Of 30 different combinations of crosses tested between three male sterile *barnase* lines and several single-copy *barstar* lines, only one combination was found to restore male fertility among F1 progeny. Subsequent analysis of F2 progeny derived from such F1 restored events (containing both *barnase* and *barstar* genes) revealed stable inheritance of both genes in the segregating population thereby indicating proper functionality of the same. Further, pollen viability in restored events was found to be comparable to that observed in transgenic lines containing the *barstar* gene alone, indicating efficient restoration by the barstar protein in the presence of the ribonuclease. The male sterile line and its corresponding restorer identified in the present study constitute a complete, functional male sterility/restorer system in *B. juncea* and the traits can be diversified into appropriate combiners for heterosis breeding.

BRASSICA *juncea*, a major oilseed crop of the Indian subcontinent, is cultivated as a winter crop in about 6 million hectares of land in rain-fed areas of northern India covering Haryana, Rajasthan, Uttar Pradesh and northwestern parts of Madhya Pradesh. Despite ~ 28 million hectares of land under oilseed cultivation, India continues to remain one of the major importers of edible oil, with an annual import to the tune of 44,94,953 metric tonnes (valued at over 8000 crore rupees) in 1999–2000 (ref. 1).

Heterosis breeding could be successfully deployed for enhancing crop productivity in mustard. Studies on the analysis of heterotic potential of various *B. juncea* cultivars have reported significant heterosis under several test conditions[2–6]. Since *B. juncea* is a predominantly self-pollinating crop, production of hybrids would require introduction of male-sterility into one of the combiners that would function as the female parent. Availability of a suitable restorer system in the male parent is also required to achieve seed set since seeds of the F1 hybrids are the desired economic products. Although several cytoplasmic male sterility (CMS) systems (for example, *oxyrrhina*, *polima*, *tournefortii*, *moricandia*) have been extensively studied in oilseed *Brassicas*[7–11], these could not be successfully used for hybrid seed production due to various limitations, viz. breakdown of male sterility, chlorosis, abnormalities in petals, poor nectary function, lack of appropriate restorer lines, etc.[12–15]. Currently, no agronomically suitable CMS/restorer system is available in Indian mustard.

Several pollination-control mechanisms based on genetic engineering of nuclear male sterility and its restoration have also been described in literature and have emerged as tangible options for development of male sterile/restorer lines[14]. Mariani *et al.*[16] demonstrated induction of male sterility in transgenic tobacco and *Brassica napus* plants by expression of a ribonuclease gene (*barnase*) from *Bacillus amyloliquefaciens* in tapetal tissues of anthers using a tapetum-specific promoter, TA29 (ref. 17). However, several of these lines showed instability of the male sterile phenotype in subsequent generations[18]. In an earlier study on development of male sterile lines in *B. juncea*[19], we found that tissue-specific expression of the *barnase* gene was deregulated under the influence of a strong constitutive promoter (CaMV35S) used for expression of the marker gene (*bar*, confers resistance to the

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herbicide phosphinothricin, PPT). This not only reduced the recovery of transgenic shoots in transformation experiments but also affected several agronomically important traits (viz. vegetative morphology, female fertility, seed germination frequencies and inheritance of male sterility) of male sterile lines, rendering them unsuitable for agronomic applications. To circumvent this problem, we described a strategy of using a Spacer DNA fragment as an effective insulator to protect tissue-specific expression of the *barnase* gene which significantly enhanced recovery of agronomically viable male sterile lines in *B. juncea*[19].

The *barstar* gene, an intracellular inhibitor of *barnase*, was used to restore male fertility in *barnase*-containing lines of *B. napus* by Mariani *et al.*[20]. In this report, we describe the development of transgenic *B. juncea* lines containing the wild type or a modified sequence of the *barstar* gene (expressed using the tapetum-specific promoter, TA29) and present the results of our studies on the search for suitable restorers for three agronomically suitable male sterile lines (namely *bn*2.2, *bn*3.6 and *bn*3.4) that were reported by us earlier[19]. Of 30 cross-combinations tested between 3 male sterile *barnase* lines and 14 *barstar* lines (12 wild type *barstar* lines and 2 modified *barstar* lines), only one combination was found to restore complete male fertility in F1 progeny. This combination of male sterile (*barnase*) and restorer (*barstar*) lines constitutes a complete, functional male sterility/restorer system in *B. juncea* which could be diversified into appropriate combiners and deployed for hybrid seed production in this crop.

Materials and methods

Construction of transformation vectors

The tapetum-specific promoter, TA29, and the wild type *barstar* gene were PCR-amplified from tobacco genomic DNA and plasmid pMT416 (ref. 21), respectively. The amplified products were cloned in pPCR-Script Amp SK(+) (Stratagene) to generate pPCR-Script SK:TA29 and pPCR-Script SK:*barstar* and sequenced to verify the fidelity of amplification. The TA29 promoter fragment was subsequently sub-cloned as a *Hinc*II–*Nco*I fragment in pRT103 (ref. 22) to generate pRT103:TA29(279). All DNA manipulations were performed using standard protocols[23]. A modified sequence of the *barstar* gene was designed on the basis of various factors known to influence transgene expression levels in heterologous systems and was constructed using recursive PCR (unpublished data; sequence of the modified gene is available on request). The wild type (wt) and modified (mod) *barstar* genes were cloned in transformation vectors and expressed using a functional 279 bp fragment of the TA29 tapetum-specific promoter[17]. The selectable marker gene (*bar*) was placed under transcriptional control of CaMV35S double enhancer promoter (35Sde) and fused at its 3′ end with a polyA signal of the *octopine synthase* gene (ocspA) as described earlier[24]. Clones carrying the TA29 and 35Sde promoters adjacent to each other were selected for transformation experiments in order to benefit from enhancing effects of the latter (if any) on *barstar* gene expression. Cloning steps involved in the construction of transformation vectors are outlined below and a schematic representation of the vectors is given in Figure 1. Each vector is assigned a code, which would be used for referring to the same in subsequent sections of the text.

Construct 1: LB:ocspA–*bar*–35Sde::TA29(279)–*barstar* (wt)–pA:RB (*Code*:*wtbs*; Figure 1)

The 35Sde–*bar*–ocspA cassette was isolated from pMCS5: 35Sde–*bar*–ocspA[24] as an *Eco*RI–*Pst*I fragment and cloned into corresponding sites of the binary vector, pPZP100 (ref. 25), to generate pPZP100:35Sde–*bar*–ocspA**.** The wild type *barstar* gene was isolated from pPCR-Script SK:*barstar* as an *Xho*I fragment, end-filled with Klenow DNA polymerase and subsequently digested with *Xba*I to generate the insert fragment for the next sub-cloning. The vector plasmid, pRT103:TA29(279) was digested with *Nco*I, treated with Mung bean nuclease to generate blunt ends and subsequently digested with *Xba*I. Ligation of the vector and insert molecules generated the construct, pRT103:TA29 (279)–*barstar*(wt)–pA. The TA29(279)–*barstar*(wt)–pA cassette was isolated from the above clone as a *Hind*III fragment and cloned into pPZP100:35Sde–*bar*–ocspA to generate the final *wtbs* construct.

Construct 2: LB:ocspA–*bar*–35Sde::TA29(279)–*barstar* (mod)–pA:RB (*Code*:*modbs*; Figure 1)

The modified *barstar* gene, isolated as an *Xho*I–*Xba*I fragment from pCRScriptSK:*barstar*(mod), was used to replace the wild type *barstar* gene, removed as an *Xho*I–*Xba*I fragment from pRT103:TA29(279)–*barstar*(wt)–pA (generated above; *Xho*I site was recreated during ligation of blunt-ended *Nco*I and *Xho*I sites) to create pRT103:TA29(279)–*barstar*(mod)–pA. The TA29(279)–*barstar*(mod)-pA cassette was isolated from the above clone as a *Pst*I fragment

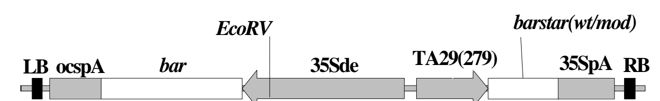

Figure 1. Map of T-DNA region of gene constructs (*wtbs* and *modbs*) used for generation of *barstar* lines in *B. juncea*. The two constructs differ only in the sequence of the *barstar* gene (wild type or modified) and have been represented in the same map. LB, left border of T-DNA; RB, right border of T-DNA; ocspA, polyA signal of *octopine synthase* gene; 35Sde, CaMV35S promoter with duplicated enhancer; TA29(279), 279 bp fragment of tapetum-specific TA29 promoter; *barstar*(*wt/mod*), wild type or modified sequence of *barstar* gene. Location of the *Eco*RV site used for digestion of genomic DNA for Southern analyses has also been shown.

and used to replace the TA29(279)–*barstar*(wt)–pA cassette (flanked by *Pst*I sites) in the *wtbs* construct to generate pPZP100:35Sde–*bar*–ocspA::TA29(279)–*barstar*(mod)–pA. The entire 35Sde–*bar*–ocspA::TA29(279)–*barstar* (mod)–pA cassette was isolated from the above clone as a *Hind*III fragment and cloned into the corresponding site of the binary vector pPZP200 (ref. 25) to generate the final *modbs* construct.

The constructs described above were mobilized into *Agrobacterium tumefaciens* strain GV3101 by electroporation using a Gene Pulser (BioRad) according to the protocol described earlier[26] and used for genetic transformation of *B. juncea*.

Development of transgenic plants in B. juncea

Transformation of *B. juncea* cv Varuna was performed using *A. tumefaciens* strain GV3101 following the protocol described earlier[24]. Transgenic plants were transferred to soil during October–November and grown in a containment net-house in accordance with guidelines provided by the Department of Biotechnology, Government of India. Morphological analysis and molecular characterization of transgenic plants was performed during the growing season (December–April). T0 transgenic plants were selfed for development of homozygous lines.

Southern analysis of transgenic plants

T0 transgenic plants were subjected to Southern analysis to identify plants containing a single-copy of the T-DNA insert. Genomic DNA from leaves of transgenic and untransformed (control) plants was extracted using the CTAB method[27]. About 10 μg of each DNA sample was digested with *Eco*RV (New England Biolabs), electrophoresed on a 0.8% agarose gel and blotted on nylon membranes (Hybond N^+, Amersham Pharmacia Biotech). Blots were sequentially probed with the coding sequences of the *barstar* (wt or mod) and *bar* genes to ascertain copy number both on the RB and LB flanks. Probes were labelled with α-[^{32}P]-dCTP using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech). Hybridization and washing conditions were based on standard procedures[21]. Prior to reprobing, blots were deprobed for 40 min in 0.4 N NaOH at 42°C followed by treatment with a neutralization solution (0.2 M Tris pH 8.0, 0.1X SSC, 0.5% SDS) for 40 min at 42°C.

Characterization of barstar lines for fertility restoration

Heterozygous T0 plants of single-copy *barstar* lines were used as pollen donors and crossed with single-copy male sterile *barnase* lines (identified earlier) to obtain F1 progeny. Suitable fertile F1 plants containing both *barnase* and *barstar* genes were selfed to raise F2 progeny.

At the molecular level, presence of *barnase* and *barstar* genes among segregating F1 and F2 progeny derived from crosses between *barnase* and *barstar* lines was analysed by tissue PCR. Leaf samples (used as source of DNA template) were subjected to alkali treatment following Klimyuk *et al.*[28]. A small piece of the treated tissue was added to the PCR reaction mix and subjected to 40 cycles of PCR using *barnase* or *barstar* gene-specific primers under appropriate cycling conditions.

At the morphological level, male fertility/sterility in plants among segregating F1 and F2 progeny was primarily established based on morphological features of anthers *vis-à-vis* pollen production and their ability to set seed on selfing. Two to three inflorescence axes (each containing ~ 8–10 unopened buds) of each plant were covered with pollination bags to test for the formation of selfed seeds. In F2 events showing pollen formation, viability of pollen grains was tested by fluorescein diacetate (FDA) staining[29]. For each sample, pollen from freshly-opened flowers was tested and a minimum of three independent observations was taken to determine the percentage of viable pollen in the same.

Results

Development of barstar lines in B. juncea cv Varuna: Genetic transformation and Southern analysis

Hypocotyl explants of *B. juncea* cv Varuna were transformed with the *wtbs* and *modbs* constructs (Figure 1) using *A. tumefaciens* strain GV3101. Transformants were selected on any of three concentrations (5, 10 or 20 mg/l) of PPT with transformation frequencies ranging from 5% to 35% in independent experiments. Transgenic plants were transferred to field during the growing season and subjected to Southern analysis to identify single-copy transformants for further studies. Fourteen single-copy, wild type *barstar* plants and 11 single-copy modified *barstar* plants were identified from a tested population of 39 and 47 transgenic plants, respectively. A representative Southern blot of *wtbs* plants is shown in Figure 2. Single-copy wt *barstar* and mod *barstar* lines were crossed with male sterile *barnase* lines as described in the subsequent section in order to identify suitable male sterile/restorer combinations in *B. juncea*.

Identification of restorer lines: Crosses between barnase and barstar lines and analysis of F1 progeny

In order to analyse restoration capabilities of single-copy heterozygous *barstar* lines generated in the present study, several wt *barstar* lines were crossed with 3 agro-

nomically suitable male sterile *barnase* lines, namely *bn*2.2, *bn*3.6 and *bn*3.4 (ref. 19). Two mod *barstar* lines (*modbs*2.99 and *modbs*2.78) were also crossed with one of the male sterile *barnase* lines (*bn*3.6). F1 seeds derived from the above crosses were germinated *in vitro* and shoot apices were transferred to appropriate selective media[24] containing 10 mg/l PPT to check for resistance to the herbicide (presence of the marker gene). The expected segregation profiles of herbicide resistance *vis-à-vis* the *barnase* and/or *barstar* gene(s) was analysed among F1 progeny derived from the above crosses. Since *barnase* as well as *barstar* lines contain the *bar* gene as a selectable marker, segregation of resistance : sensitivity to the selective agent (PPT) was observed at a ratio of 3 : 1 (data not shown). Progeny plants carrying the marker gene (*bar*) were transferred to field conditions in the growing season (1999–2000) to study segregation of male fertility/male sterility among the same. In cases where restoration of male fertility has occurred, segregation of male fertile and male sterile plants is expected in a 2 : 1 ratio, whereas in situations wherein restoration of male fertility does not occur (due to *barstar* being ineffective), the ratio of male fertile: male sterile plants would be 1 : 2 in the segregating population. A summary of crosses between *barnase* and *barstar* lines and results obtained are outlined in Table 1.

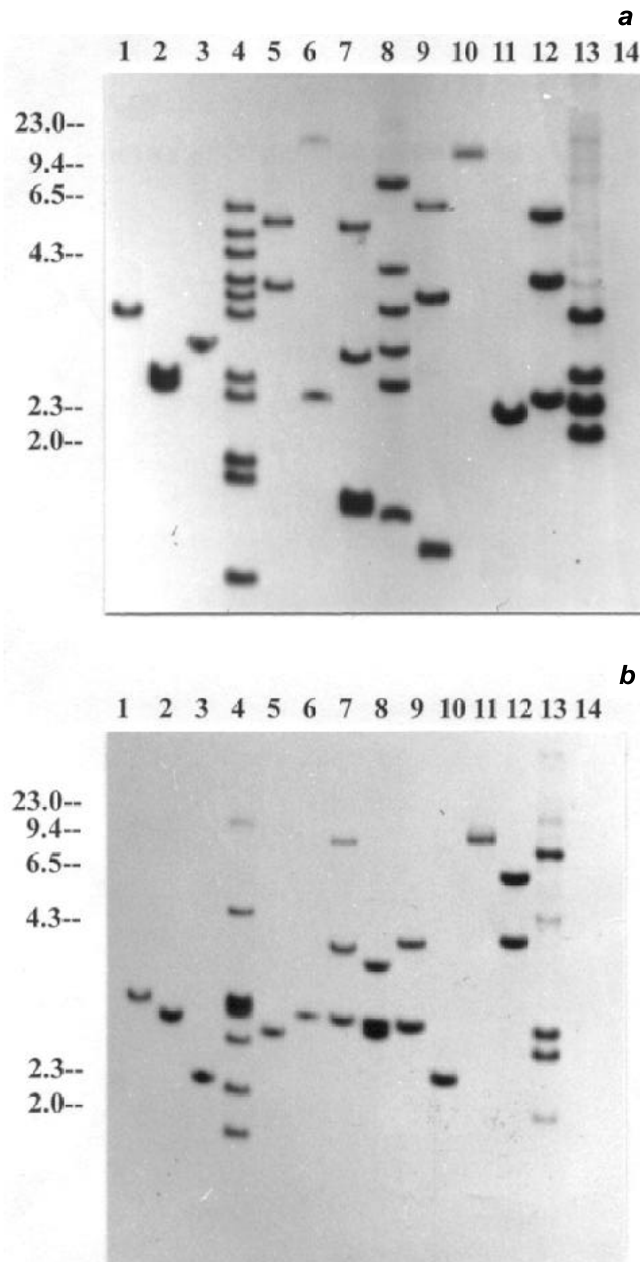

Figure 2. Southern analysis of a representative population of *wtbs* plants. ***a***, Copy number on the LB flank was determined by probing *Eco*RV digests of genomic DNA with the *bar* gene sequence. Loading profiles of samples are as follows: Lanes 1–13, Transgenics 13.17.1, 3.8.2, 13.19.1, 13.18.2, 13.18.1, 13.21.1, 4.7.1, 13.35.1, 13.75.3, 13.69.2, 3.6.1, 13.103.1 and 13.55.1 in that order; lane 14, control (untransformed) plant. ***b***, Copy number on the RB flank was determined by reprobing the blot in (***a***) with the wild type *barstar* gene sequence. Lanes 1, 3, 10 and 11 represent hybridization profiles of transgenics highlighting a single-copy integration event on both flanks of the T-DNA.

No restorer line could be identified among the wild type *barstar* transgenics tested in the present study in crosses with any of the three *barnase* lines (Table 1). However, one of the modified *barstar* lines (*modbs*2.99) restored fertility of the *barnase* line, *bn*3.6 (Table 1). Tissue PCR analysis of a representative population of fertile F1 plants (derived from the cross *bn*3.6 × *modbs*2.99) to test for the presence of *barnase* and *barstar* genes revealed the presence of both genes in the expected 50% of male fertile, PPT-resistant progeny (data not shown). Two such restored plants (designated P1 and P2), derived from the cross between *barnase* line *bn*3.6 and the *barstar* line *modbs*2.99, were selfed to obtain F2 progeny, which were subjected to further analysis as described below.

Analysis of F2 progeny: Segregation of male fertility/sterility, tissue PCR and pollen viability assays

F2 seeds obtained by selfing restored plants P1 and P2 (containing both *barnase* and *barstar* genes) were screened for resistance to the selective agent (PPT). PPT-resistant progeny were transferred to field conditions in the 2000–2001 growing season and analysed for their male fertility/sterility status. In both cases, male fertile and male sterile plants were found to segregate among F2 progeny in the expected 12 : 3 ratio following selection on the herbicide (Table 2). Additionally, the occurrence of male sterile plants in F2 progeny as a result of independent assortment of *barnase* and *barstar* genes also served to indicate stability in expression of both genes.

Table 1. Analysis of F1 progeny derived from crosses between *barnase* and *barstar* lines

Cross *barnase* line × *barstar* line	Segregation of male fertility and male sterility in PPT-resistant F1 progeny[a] No. of PPT^R plants analysed	Fertile	Sterile	Segregation ratio c^2 value[b]
*bn*2.2 × *wtbs*3.6.1	40	12	28	24.2
*bn*2.2 × *wtbs*13.17.1	33	12	21	13.6
*bn*2.2 × *wtbs*13.25.4	33	11	22	16.5
*bn*2.2 × *wtbs*13.33.1	36	11	25	21.12
*bn*2.2 × *wtbs*13.69.2	28	5	23	30.01
*bn*2.2 × *wtbs*13.83.2	28	11	17	9.44
*bn*2.2 × *wtbs*13.96.1	32	14	18	7.57
*bn*2.2 × *wtbs*14.97.1	31	11	20	13.56
*bn*2.2 × *wtbs*14.108.2	34	15	19	7.78
*bn*2.2 × *wtbs*14.130.1	36	14	22	12.49
*bn*3.6 × *wtbs*3.6.1	29	7	22	23.62
*bn*3.6 × *wtbs*13.17.1	27	8	19	16.66
*bn*3.6 × *wtbs*13.69.2	51	16	35	28.59
*bn*3.6 × *wtbs*13.83.2	32	16	16	4.0
*bn*3.6 × *wtbs*14.97.1	29	10	19	13.5
*bn*3.6 × *wtbs*14.108.2	30	6	24	29.4
*bn*3.6 × *modbs*2.78	58	15	43	43.46
*bn*3.6 × *modbs*2.99	61	42	19	0.13
*bn*3.4 × *wtbs*3.6.1	30	7	23	25.35
*bn*3.4 × *wtbs*13.17.1	30	9	21	18.15
*bn*3.4 × *wtbs*13.25.2	37	8	29	33.78
*bn*3.4 × *wtbs*13.25.4	31	12	19	10.9
*bn*3.4 × *wtbs*13.33.1	34	16	18	5.88
*bn*3.4 × *wtbs*13.40.2	31	9	22	19.76
*bn*3.4 × *wtbs*13.69.2	27	6	21	24.0
*bn*3.4 × *wtbs*13.83.2	33	10	23	19.63
*bn*3.4 × *wtbs*13.96.1	24	11	13	4.68
*bn*3.4 × *wtbs*14.97.1	36	15	21	10.12
*bn*3.4 × *wtbs*14.108.2	33	8	25	26.72
*bn*3.4 × *wtbs*14.130.1	30	10	20	15.0

[a], F1 progeny derived from crosses between *barnase* and *barstar* lines were screened initially for resistance to the selective agent, PPT (active component of the herbicide, Basta). Since both *barnase* and *barstar* lines carry the *bar* gene as a selectable marker, F1 progeny would segregate in a ratio of 3 : 1 for resistance : sensitivity to the herbicide (data not shown). In cases where successful restoration occurs, male fertile and male sterile progeny would be obtained in a 2 : 1 ratio among the PPT^R lines.
[b], c^2 tests were performed at 95% confidence limit ($P < 0.05$) to determine the goodness of fit.

Table 2. Segregation of male sterility/male fertility among F2 progeny of the cross *bn*3.6 (male sterile line) × *modbs*2.99 (restorer line)

Restored event	Segregation of male fertility/sterility among F2 progeny No. of PPT^R plants analysed	Fertile	Sterile	Segregation ratio c^2 value[a]
P1	46	38	8	0.19
P2	51	39	12	0.39

[a], c^2 tests were performed at 95% confidence limit ($P < 0.05$) to determine the goodness of fit.

In order to correlate segregation pattern of both genes with the male sterile/male fertile phenotype of F2 progeny, a representative population of PPT-resistant plants [derived from selfed seeds of one of the restored F1 plants (P1)] was subjected to tissue PCR analysis. All eight male sterile plants analysed were found to contain only the *barnase* gene. Of 33 fertile plants tested, 27 were found to contain both the *barnase* and *barstar* genes while six were found to contain only the *barstar* gene, which is in consonance with their expected segregation ratios [3 (*barnase* and *barstar*) : 1 (*barstar*)].

Representative samples of plants containing both *barnase* and *barstar* genes (restored events) and plants containing the *barstar* gene alone were subjected to FDA staining assays along with an untransformed control to determine pollen viability in restored events. Results of this analysis are summarized in Table 3. Significantly, plants containing both the *barnase* and *barstar* genes showed a high percentage of viable pollen unlike the corresponding male sterile segregants, wherein no pollen formation could be detected. More importantly, the range of pollen viability obtained in restored plants was comparable to that observed in plants containing the *barstar* gene alone thereby indicating efficient restoration of male fertility by the line *modbs*2.99.

Discussion

In an earlier study on restoration of male fertility in *B. napus*, as many as six of nine crosses performed between single-copy *barnase* and *barstar* lines were found to restore male fertility in F1 progeny with at least one restorer being identified for three of the four male sterile lines tested[20]. However, in our study, majority of crosses made between three single-copy *barnase* lines and several single-copy *barstar* lines failed to restore fertility in the three stable male sterile lines tested (Table 1). Only one modified *barstar* line (*modbs*2.99) was found to function as an efficient restorer for a male sterile line (*bn*3.6) that was tested in combination with the same (Table 1). Although a greater number of restorers could be identified for male sterile *B. napus* lines[20], it is important to note that these *barnase* lines were also shown to be unstable, leading to a breakdown of male sterility at higher temperatures[18]. In contrast, male sterile *B. juncea* lines tested in the present study were identified using stringent selection criteria under field conditions and have been shown to retain stable expression levels of the *barnase* gene[19]. Inability to identify more restorers for male sterile *B. juncea* lines could therefore be attributed to higher levels of *barnase* expression (conferring greater stability to the male sterile phenotype) in the same, requiring enhanced levels of barstar protein to successfully inhibit barnase activity.

An important observation with respect to the male-sterile/restorer combination identified in the present study, is the frequency of viable pollen observed among F2 progeny containing both the *barnase* and *barstar* genes (Table 3). This is particularly relevant in case of oilseed crops such as *Brassicas* wherein seed set is of paramount importance in achieving full yield potential of the crop. While earlier reports on restoration of male fertility recorded the inheritance of male sterility/fertility[20], data on pollen viability were lacking. In the present study, F2 plants containing both *barnase* and *barstar* genes showed high levels of viable pollen comparable to that observed in plants containing the *barstar* gene alone (Table 3). None of the male sterile plants obtained among the segregating progeny showed pollen formation. This also implied that formation of viable pollen in plants with both genes was not due to breakdown of male sterile phenotype, but due to efficient inhibition of the ribonuclease (barnase) by the barstar protein.

Another interesting observation made during Southern analysis of *barstar* lines in the present study was that the frequency of single-copy integrations among *barstar* lines (23–36%) was substantially lower than that observed for *barnase* transgenic lines (45–64%)[19]. In *barnase* lines, it was envisaged that selection and survival of transformed events containing multiple copies of the transgene (*barnase*) may be impaired because of increased probability of leaky expression of one or more copies of the *barnase* gene under the influence of flanking genomic sequences[19]. Such a preferential selection mechanism for single-copy integration events is however not necessitated for *barstar* transformants thereby leading to a higher frequency of transgenics with multiple copy integrations.

Table 3. Analysis of pollen viability in segregating F2 progeny derived from selfed seeds of the restored event P1 and untransformed control

Plant type (no. of plants analysed)	Percentage pollen viability[a]
Plants with both *barnase* and *barstar* genes (14)	84–96
Plants with *barstar* gene (4)	71–96
Untransformed control (6)	100

[a], Minimum of 100 pollen grains per sample were analysed. Three independent observations per sample were taken from freshly-opened flowers. Numbers in parenthesis indicate the number of plants of each type analysed for their pollen viability.

The difficulties encountered in identification of efficient restorer lines as evidenced in the present study clearly demonstrate the need for improved molecular strategies to achieve better temporal and spatial expression patterns of restorer gene(s). The modified sequence of the *barstar* gene was primarily designed for enhanced and stable expression in dicot systems. Investigations are currently in progress to determine variation in expression levels (if any) between the wild type and modified *barstar* gene sequences. Other possible combinations between single-copy wild type/modified *barstar* lines generated in the present study against all available *barnase* lines are also being analysed to identify more male-sterile/restorer combinations.

The male sterile *barnase* line (*bn*3.6) and its corresponding restorer (*modbs*2.99) identified in the present study constitute a complete male sterility/restorer system in *B. juncea* and have been developed following stringent selection criteria over two successive generations under field conditions. These lines have not shown any instability in the functionality of either trait and can therefore be deployed in appropriate combiners and used for hybrid seed production in Indian mustard using strategies described earlier[14].

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Occurrence of pockmarks and gas seepages along the central western continental margin of India

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High-resolution seismic reflection profiling has revealed the occurrence of acoustic masking, pockmarks, gas seepages and plumes on the inner shelf, middle shelf and upper continental slope of western India, between 20 and 50 m, 60 and 75 m, and 170 and 260 m water depths, respectively. A typical 40-km long seismic section trending in a NW-SE direction – from the upper slope is characterized by anticline–syncline structure and culminates toward the NW. In some places fluid or gas escape features and plumes appear 2–12 m above the seafloor. Active and relict-type pockmarks are noticed. Out of thirty well-recognized pockmarks along the section, six are buried. In general, the pockmarks are 80–130 m in diameter and 0.75–2.5 m deep. Biogenic gas due to organic-rich sediments in the slope or thermogenic gas emanating from deep-seated faults, fractures and lineaments in the region may have given rise to these features.

POCKMARKS are increasingly being recognized as widespread morphological phenomena on the seabed and are often indicative of the venting of natural gas, mainly methane[1]. Pockmarks found at the bottom of lakes and oceans around the world[2] are somewhat v-shaped depressions that form from the escape of natural gas and interstitial water from unconsolidated muddy sediments[3]. They range from a few metres to several hundreds of metres in diameter, with a maximum relief of 30 m. Pockmarks have been considered as indicators of subsurface petroleum deposits[1,4], offshore geologic hazards[5], contributors to global warming through greenhouse gas (methane) release[6,7], index of enhanced biological pro-

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