Development of *barnase/barstar* transgenics for hybrid seed production in Indian oilseed mustard (*Brassica juncea* L. Czern & Coss) using a mutant acetolactate synthase gene conferring resistance to imidazolinone-based herbicide 'Pursuit'

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The use of herbicide-based field selection marker is an integral component in the development of hybrid seeds based on transgenic approach using *barnase* and *barstar* genes. It is an added advantage if the same marker can also be used efficiently as an *in vitro* selection marker. In the present study we report the development of male sterile transgenic lines and their restorers in *Brassica juncea* using the *barnase–barstar* system in conjunction with a selection marker gene ALS^{dm} conferring resistance to the imidazolinone, imazethapyr, the active ingredient of the herbicide 'Pursuit'. This herbicide is commercially available at low costs in India. It therefore provides a viable alternative to phosphinothricin/glufosinate herbicides used in the first-generation hybrids developed using the *barnase–barstar* system. Using constructs containing the ALS^{dm} gene we have developed stable male sterile *barnase* lines and homozygous *barstar* lines showing proper restoration, in appropriate combiners Varuna and EHII. We also successfully demonstrate the use of 'Pursuit' in field selections of male sterile lines in a segregating population.

Keywords: Acetolactate synthase, *barnase*, *barstar*, *Brassica juncea*, hybrid seed technology.

BRASSICA JUNCEA L. Czern & Coss (Indian mustard) is a major oilseed crop of the Indian subcontinent, being cultivated in around 6 million ha in the rainfed areas of northern India. Heterosis breeding has been successfully deployed for enhancing crop productivity in mustard, wherein hybrids between Indian and eastern European varieties lead to ~25–30% heterosis over the Indian check varieties[1]. As *B. juncea* is predominantly a self-pollinating crop, production of the hybrid seed is dependent on the development of a proper pollination control mechanism.

We have previously reported the development of a male sterility and restorer system in *B. juncea* based on transgenics with *barnase* and *barstar* genes respectively[2–4]. In this system male sterile lines were generated in one of the combiners by expressing the *barnase* gene from *Bacillus amyloliquefaciens* using a tapetum-specific TA29 promoter from tobacco. A major modification was made in the earlier *barnase* constructs to achieve more regulated expression of the tapetum-specific promoter[2]. Restoration of male fertility in the F_1 hybrids was brought about by an improved tapetum-specific expression[4] of the *barstar* gene, also from *B. amyloliquefaciens*, which was introduced into the other parental line.

Male sterile lines are maintained by backcrossing with an isogenic line without the *barnase* gene. The progeny thus obtained segregates for male sterility and fertility. In order to select for the male sterile lines in field, these lines are also made resistant to an herbicide by incorporating a gene conferring herbicide resistance in the *barnase* construct. Since for hybrid seed production both male sterile lines and *barstar* lines are grown together in the same plot and field selection of segregating male sterile population is concomitantly carried out by herbicide spray, the *barstar* transgenics are also made resistant to the same herbicide. Further, it is an advantage if the gene conferring resistance to herbicide can also be used for *in vitro* selection of initial transformants as it avoids the use of a second gene as a selection marker.

The commercial exploitation of this technology is dependent upon the availability and cost of the herbicide being used for field selections. The first generation *barnase* and *barstar* lines developed in our laboratory[2–4] used the *bar* gene (from *Streptomycin hygroscopicus*) for resistance against phosphinothricin/glufosinate, which is an active

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ingredient of commercially available herbicide like 'Basta' currently marketed in India for use in tea plantations (Bayer CropScience Ltd, pers. commun.). We looked for an alternate, less expensive herbicide being used in India. 'Pursuit', marketed by BASF India, is one such herbicide whose active molecule is an imidazolinone named Imazethapyr. This molecule inhibits the plant enzyme acetolactate synthase (ALS), a key enzyme needed for the synthesis of the branched-chain amino acids leucine, isoleucine and valine. In India, 'Pursuit' is used on soybean crop in Madhya Pradesh (BASF India, pers. commun.). Expression of a mutant acetolactate synthase gene, ALS^{dm} (wherein Pro at position 197 is changed to Ser, and Ser at position 653 to Asn) confers resistance to two groups of herbicides simultaneously, imidazolinones and sulfonylureas[5]. In a previous study, we developed this double-mutant version of the *ALS* gene from *Arabidopsis thaliana* and demonstrated its use in developing *B. juncea* transgenic lines resistant to the herbicides imidazolinones and sulfonylureas[6]. We further showed that the ALS^{dm} gene can be successfully used as an *in vitro* selection marker for developing *B. juncea* transgenics. The present study reports the successful development of male sterile transgenic lines and its restorers in *B. juncea* using the *barnase–barstar* system for heterosis breeding with this alternative herbicide selection marker.

Material and methods

Transformation vectors

ALS^{dm} barstar construct: ALS^{dm} :: A9-bsmod :: TA29-bswt (ALS^{dm}barstar): The barstar construct (Figure 1 *a*) contains two copies of the *barstar* gene (wild type and codon modified)[4] under the control of two tapetum-specific promoters, TA29 from tobacco and A9 from *A. thaliana* respectively. It also contains a $35SALS^{dm}$ expression cassette cloned towards the left border of the binary vector pPZP200 (ref. 7).

ALS^{dm}barnase construct: ALS^{dm} :: DT :: T1T2 : TA29-bn (ALS^{dm}barnase): The TA29(870)–barnase–35SpA cassette[2] is cloned at the *Hin*dIII site of the binary vector pPZP200bsnp. This binary vector carries the native *barstar* gene with its own promoter outside the T-DNA borders of the binary vector pPZP200. Further, it carries a 35S–ALS^{dm}–35SpA expression cassette separated from the *barnase* expression cassette by a 4.6 kb spacer DNA fragment consisting of partial sequences of topoisomerase I (ref. 8) and diamine oxidase (ref. 9) genes. A transcription terminator T1T2 from the vector pKK232-8 was cloned[10] downstream to the spacer in the desired orientation (Figure 1 *b*).

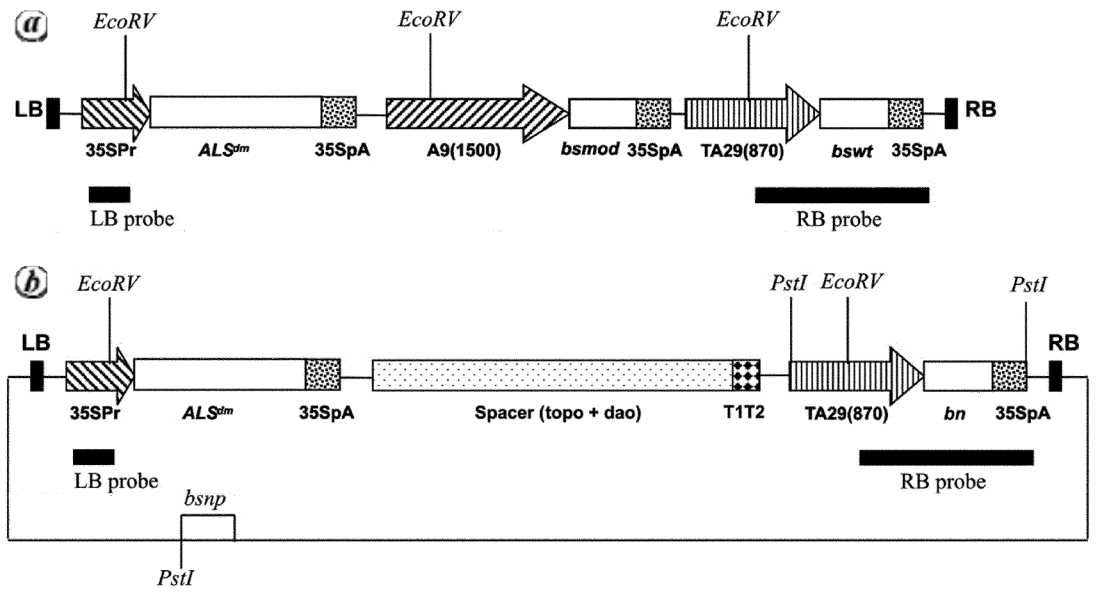

Figure 1. Map of T-DNA region of *barstar* and *barnase* gene constructs used for generation of transgenic lines in *Brassica juncea*. ***a***, ALS^{dm} *barstar* construct. ***b***, ALS^{dm} *barnase* construct. LB, Left border of T-DNA; RB, Right border of T-DNA; 35S Pr, CaMV 35S promoter; 35SpA, Poly A sequence of 35S transcript; ALS^{dm}, Acetolactate synthase gene with double mutations; A9(1500), 1500 bp of A9 promoter; TA29(870), 870 bp fragment of TA29 promoter; bsmod, Codon-modified sequence of *barstar* gene; bswt, Wild type *barstar* gene; Spacer (topo + dao), DNA fragment consisting of partial coding regions of *topoisomerase I* and *diamino oxidase* gene; bn, *barnase* gene; bsnp, *barstar* gene under its native promoter; T1T2, Transcription terminator sequences. Location of the *Eco*RV sites used for digestion of genomic DNA for Southern analysis and the probes used have also been indicated.

Development and maintenance of transgenic lines

The *barnase* and *barstar* constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101 by electroporation using a Gene Pulser (BioRad), according to the protocol described earlier[11], and used for genetic transformation of *B. juncea* cv. Varuna and cv. EHII.

Cultivar Varuna was transformed with the *ALS*^{dm} *barnase* construct following the protocol published earlier[12]. The EHII line was transformed with the *ALS*^{dm} *barstar* construct. A modified regeneration medium was used for transformation of the EHII line. The regeneration medium containing MS salts and vitamins was supplemented with 1.5 mg/l BAP, 0.075 mg/l 2,4-D and 20 μM silver nitrate and the medium was solidified with 0.6% agarose (Sigma). Transformants were selected on the herbicide 'Pursuit'. A concentration of 0.2 μM (60 μg/l) imazethapyr was used for *in vitro* selections.

Transgenic plants were transferred to soil during winter season and grown in containment net-houses in accordance with the guidelines given by the Department of Biotechnology, Government of India. T_0 transgenic plants with single copy of the transgene were backcrossed to non-transgenic *B. juncea* cv. to yield the BC_1 seeds in the first year. In the second year the BC_1 seeds were germinated *in vitro* and the seedlings were screened for resistance to 'Pursuit'. The resistant plants were transferred to the net-house and observed for their vegetative and reproductive morphology.

Southern analysis of transgenic plants

Genomic DNA from leaves of transgenic and untransformed (control) plants was extracted using the CTAB method[13]. About 10 μg of genomic DNA was digested with *Eco*RV, electrophoresed on a 0.8% agarose gel and transferred onto nylon membrane (Hybond N+; Amersham). Junction sequences towards the left and right borders were analysed using appropriate probes as shown in Figure 1. DNA was labelled with α-[^{32}P]-dCTP using the Megaprime DNA Labeling System (Amersham). Hybridization and washing conditions were based on standard procedures. 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, 0.1× SSC, 0.5% SDS for 40 min at 42°C). The membrane was subjected to autoradiography for 36–48 h at –80°C.

Male sterility status of barnase transgenic lines

Vegetative and reproductive morphology of barnase plants was monitored for two growing seasons. Male sterile plants were identified on the basis of anther morphology and absence of pollen production. Two to three inflorescences with 10–15 unopened buds from each transgenic plant were covered with a pollination bag to test for selfed pod set. Absence of selfed pods was taken as a further confirmation of male sterility.

Seed germination percentages and segregation for resistance to herbicide

BC_1 seeds from individual T_0 plants of both *barnase* and *barstar* plants were surface-sterilized and germinated on non-selective media. Germination percentages of BC_1 seeds for each single-copy T_0 *barnase* line was estimated. The apices of the germinated seedlings were excised and placed on MS medium containing 2 mg/l IBA and selection agent 0.2 μM of imazethapyr. Seedlings which successfully formed roots in this medium and survived were scored as resistant. Segregation ratio were tested using the χ^2 test at 95% confidence limit to determine the goodness-of-fit.

Analysis of barstar lines for effective restoration

Heterozygous T_0 plants of single-copy *barstar* lines were used as pollen donors and crossed with single-copy male sterile *barnase* lines. The F_1 seedlings were screened *in vitro* for resistance to 'Pursuit'. Resistant F_1 progeny from individual crosses were transferred to the net-house and scored for their pollen fertility/sterility status. Pollen viability was analysed by staining the pollens with fluorescein diacetate[14]. Pollen from these freshly opened flowers was tested. Restored events were confirmed by PCR amplification of *barnase* and *barstar* genes in the F_1 lines using gene-specific primers on 100 ng of genomic DNA.

Results and discussion

Development of barnase construct

The *barnase* construct was developed in the binary vector pPZP200 which carried the wild type *barstar* gene with its native promoter outside the T-DNA borders. The *Als*^{dm} expression cassette was cloned towards the left border followed by the spacer fragment. In initial experiments the *barnase* gene was being cloned downstream the spacer fragment that did not carry the T1T2 terminator. Most of the *Agrobacterium* clones obtained after electroporation with this construct carried deletion of the 1.4 kb *barnase* gene cassette as observed on digestion with *Pst*I (Figure 2). Further, sequencing of the *barnase* gene in all clones which did not show deletion revealed the presence of nonsense mutations in the gene, thereby encoding a truncated non-functional protein. This showed that *Agrobacterium* cells expressed functional barnase protein, making such cells non-viable. Further, this barnase activity could not be blocked by the barstar protein being

Table 1. Transformation of *Brassica juncea* cultivars with the *ALS^{dm} barstar* and *ALS^{dm} barnase* constructs

Constructs used	*B. juncea* cultivars used	Total number of explants in transformation experiments*	Total number of of independent events obtained	Number of events analysed by Southern hybridization	Number of single-copy events obtained
ALS^{dm} barstar	EHII	3772	3	3	2
ALS^{dm} barnase	Varuna	6098	153	143	72

*Indicates total number of explants used in several experiments.

Table 2. Analysis of morphological traits (reproductive and vegetative) of T_0 *ALS^{dm} barnase* transgenic lines

Number of single-copy T_0 events transferred to net-house	63
Male sterility status[a]	Sterile: 58; Fertile: 1; Semi-fertile[c]: 3
Plant type	Normal: 61; Abnormal: 2
Female fertility[b] of male sterile plants	Fertile: 56; Sterile: 1
Number of male sterile lines available	56

[a]Based on visual observation of pollen as well as absence of seed set on selfing.
[b]Based on seed set in backcrossing with parental lines.
[c]Some plants showed presence of both male fertile and male sterile flowers. These are semi-fertile plants.

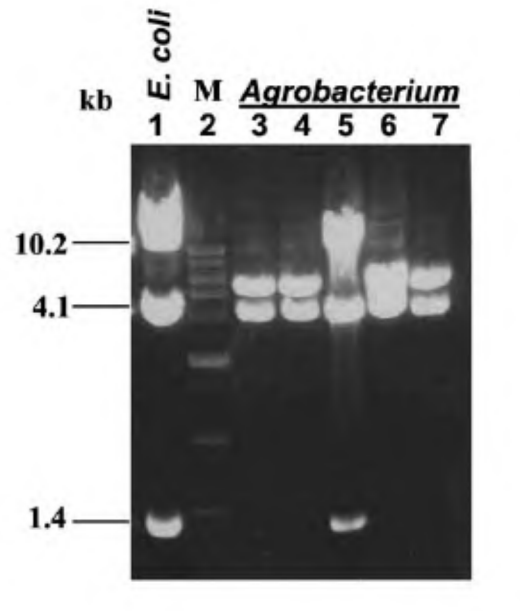

Figure 2. Restriction profile of *ALS^{dm} barnase* construct without the T1T2 terminator isolated from *Escherichia coli* (lane 1) and different *Agrobacterium* colonies (lanes 3–7) on digestion with *Pst*I. While plasmid isolated from *E. coli* showed the presence of 1.4 kb *barnase* cassettes, plasmid isolated from most of the *Agrobacterium* colonies showed a deletion of the same (lanes 3, 4, 6 and 7). In clones (e.g. lane 5) showing the presence of the *barnase* gene cassette, sequencing the fragment revealed the presence of nonsense mutations. The changes were found to occur at positions 170 and 212 of the *barnase* coding region converting a TCA codon to TAA and TGG to TAG respectively. M, 1 kb ladder (New England Biolabs, USA).

encoded for by the *barstar* gene incorporated in the backbone of the vector. It was felt that the *barnase* gene was being transcribed as a read-through transcript probably from the upstream CaMV 35S promoter known to be functional in *Agrobacterium* cells. This problem was circumvented by cloning the T1T2 terminators upstream the TA29 promoter::*barnase* gene cassette. The T1T2 are rho-independent transcription terminators of the rRNA operon of *E. coli*[10] and are used to block transcription in vectors like pKK232-8.

Genetic transformation with barstar and barnase constructs and analysis of T_0 lines

Line EHII and cv. Varuna were transformed with the *ALS^{dm}*barstar and *ALS^{dm}*barnase constructs respectively. Line EHII failed to regenerate using the regeneration/transformation protocol developed earlier for Indian cultivars[12]. However, few transgenics (Table 1) could be obtained in one of the experiments when BAP (1.5 mg/l) and 2,4-D (0.075 mg/l) were used instead of BAP (1 mg/l) and NAA (1 mg/l) in the regeneration medium and agarose instead of agar was used as the solidifying agent in this case. A total of 153 events in Varuna were generated using the *ALS^{dm}*barnase construct.

Southern analysis was carried out to identify single-copy integration events. Two out of the three barstar events and 72 of the barnase events were observed to have single copy of the respective transgene (Figure 3). The T_0 lines were grown in containment net-house. The *barnase* lines were analysed for morphological characters, male sterility and female fertility. Out of the 63 plants transferred to the net-house, 56 were male sterile, female fertile and with normal morphology (Table 2). The *barnase* and *barstar* lines were maintained by backcrossing to non-transgenic lines.

Analysis of BC_1 barnase progeny: Germination percentages, segregation analysis and inheritance of male sterility

The BC_1 progenies from 24 *barnase* lines were analysed for (i) seed germination percentage, (ii) segregation ratio for

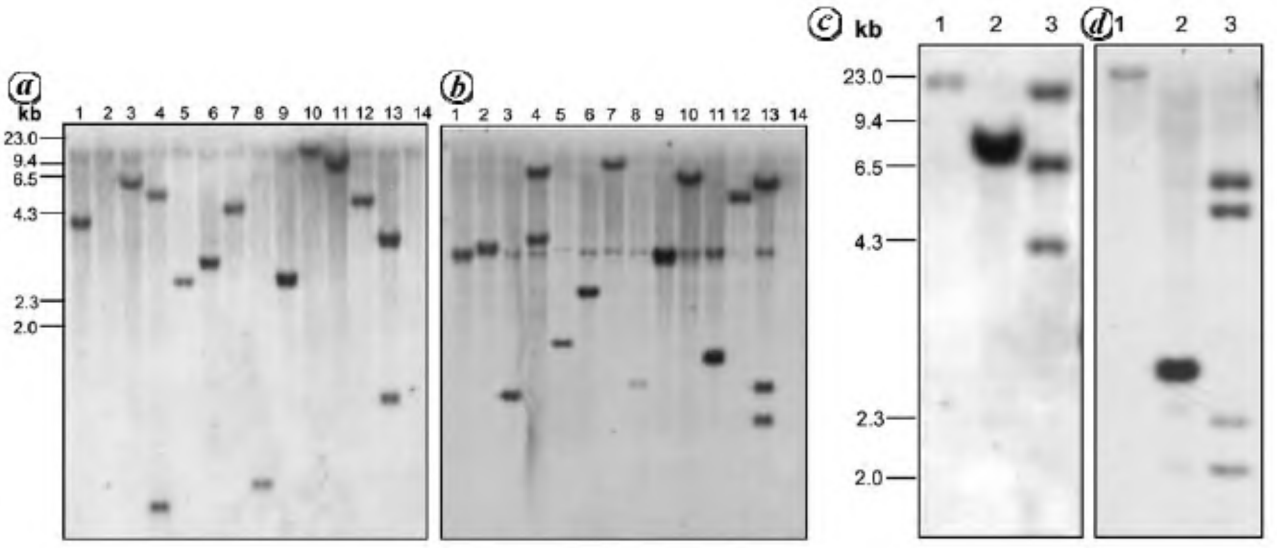

Figure 3. ***a***, ***b***. Southern analysis of a representative population of T_0 *ALS*^{dm} *barnase* plants. Junction fragments towards the left and right borders of the T-DNA cassette are presented in (***a***) and (***b***) respectively. Lanes 1–13, Transgenic lines and lane 14, Control (untransformed) plant. ***c***, ***d***. Southern analysis of three T_0 *ALS*^{dm} *barstar* plants analysed for their junction fragments towards the LB (***c***) and RB (***d***) flanks. Lanes 1 and 2 represent transgenic lines showing integration of a single T-DNA cassette.

Table 3. Analysis of BC_1 progeny obtained from maintainer crosses of single-copy *barnase* lines for germination percentage and segregation of resistance to 'Pursuit'

Transgenic events	Germination (%)	Segregation ratio for BC_1 *barnase* lines on 'Pursuit' selection		
		Resistant	Sensitive	χ^2
bnALS 3.04	100	13	13	0*
bnALS 1.42	87	36	40	0.2*
bnALS 3.03	100	17	20	0.24*
bnALS 3.44	100	18	28	2.16*
bnALS 3.52	100	17	18	0.028*
bnALS 3.61	100	33	34	0.014*
bnALS 4.4	100	27	26	0.018*
bnALS 1.3	100	26	22	0.33*
bnALS 3.06	91	11	22	3.6*
bnALS 1.52	80	15	18	0.27*
bnALS 3.11	100	18	20	0.1*
bnALS 3.53	96	25	21	0.34*
bnALS 3.33	93	41	21	6.4
bnALS 4.3	95	14	30	5.8
bnALS 5.11	100	0	60	30
bnALS 3.41	95	6	49	33.6
bnALS 3.6	100	0	47	23.5
bnALS 1.2	89	23	22	0.02*
bnALS 4.04	91	23	24	0.02*
bnALS 1.32	100	20	24	0.36*
bnALS 3.09	100	46	26	5.5
bnALS 3.13	100	27	31	0.28*
bnALS 1.44	97	27	22	0.5*
bnALS 3.47	84	15	14	0.034*

χ^2 was calculated for the ratio 1 : 1 and subjected to statistical analysis at 95% confidence limit ($P > 0.05$) to determine the goodness-of-fit. A 1 : 1 ratio was observed in each of the lines marked with an asterix.

herbicide resistance, (iii) vegetative morphology and (iv) male sterility. While the first two were carried out *in vitro*, vegetative morphology and male sterility were studied in the net-house.

BC_1 seeds (30–60) derived from single-copy male sterile *barnase* events were germinated on MS medium without selective agent. All the lines showed germination percentage comparable to that of untransformed control plants (Table 3). The seedlings were then tested for their resistance to 'Pursuit'. Out of the 24 T_0 transgenic plants whose BC_1 progeny was analysed, 18 events showed the expected 1 : 1 segregation ratio of resistance : sensitivity (Table 3). These were then transferred to the net-houses to check for plant morphology and male sterility.

All the lines except two (bnALS 3.53 and bnALS 4.04) were male sterile. These two lines though initially male sterile, showed pollen formation at the end of the growing season when the day temperatures were around 35–37°C. Earlier studies have also reported similar temperature-dependent breakdown in male sterility[15]. The rest satisfied all the criteria of a potential male sterile line as outlined earlier[2], and could be used as a female parent in hybrid seed production. These lines were crossed with the *barstar* lines for identification of an appropriate restorer.

Identification of restorer lines: Analysis of F_1 progeny from crosses between barnase and barstar lines

Twenty-seven different crosses between the two-single copy heterozygous EHII barstar transgenics (as pollen

Table 4. Analysis of 'Pursuit'-resistant F_1 progeny derived from crosses between male sterile *ALS*^{dm} *barnase* lines in Varuna and *ALS*^{dm} *barstar* lines in EHII

Crosses between *barnase* line and *barstar* line	No. of 'Pursuit'-resistant plants analysed	F	SF	S	χ^2
bnALS1.42 and EHII(2)bsALS	87	31		56	25.56
bnALS1.42 and EHII(3)bsALS	65	35		30	4.88
bnALS1.44 and EHII(2)bsALS	47	17		30	19.8
bnALS1.44 and EHII(3)bsALS	21	7		14	10.5
bnALS1.32 and EHII(2)bsALS	65	30		35	12.41
bnALS1.32 and EHII(3)bsALS	53	25		28	8.19
bnALS3.04 and EHII(2)bsALS	84	36		48	21.7
bnALS3.04 and EHII(3)bsALS	101	34		67	49.65
bnALS3.13 and EHII(2)bsALS	42	14		28	21
bnALS3.13 and EHII(3)bsALS	59	17		42	38.2
bnALS1.2 and EHII (2)bs ALS	**45**	**32**		**13**	**0.19**
bnALS1.2 and EHII (3)bsALS	**36**	**27**		**9**	**1.13**
bnALS3.52 and EHII(2)bsALS	20	5	3	12	
bnALS3.52 and EHII(3)bsALS	32	16	3	14	
bnALS1.3 and EHII(2)bsALS	43	13		30	25.51
bnALS1.53 and EHII(2)bsALS	27	23		4	4.17
bnALS 3.61 and EHII(3)bsALS	32	9	1	22	
bnALS3.03 and EHII(2)bsALS	31	4		27	40.13
bnALS3.03 and EHII(3)bsALS	33	8	1	24	
bnALS3.06 and EHII(3)bsALS	26	12	2	12	
bnALS3.44 and EHII(3)bsALS	65	30		35	12.41
bnALS3.47 and EHII(2)bsALS	27	9		18	13.5
bnALS3.47 and EHII(3)bsALS	24	11		13	4.69
bnALS1.52 and EHII(2)bsALS	26	8		18	15.24

Identified male sterile–restorer combinations are shown in bold. F, Fertile; SF, Semi-fertile; S, Sterile.

donors) with the 15 male sterile *barnase* transgenic lines of cv. Varuna were made. The F_1 progeny was analysed for restoration of the *barnase* lines based on: (i) segregation for resistance: sensitivity to herbicide in 3 : 1 ratio, (ii) segregation for male fertility : sterility among the herbicide-resistant progeny in a 2 : 1 ratio, (iii) absence of *barstar* gene in all male sterile progeny (as analysed by PCR) and (iv) segregation of male fertile F_1 progeny in 1 : 1 ratio for plants carrying *barstar*, and both *barnase* and *barstar* genes. F_1 progenies from all the 27 different crosses except one [bnALS 1.3 crossed to EHII(3)bsALS line] showed the expected ratio for segregation for resistance : sensitivity to herbicide.

F_1 progenies resistant to 'Pursuit' from each of the crosses were transferred to the net-house to study segregation for male fertility/sterility. Out of the 15 male sterile *barnase* lines tested for restoration, only the line bnALS1.2 could be restored. This line was restored by both the *barstar* lines EHII(2)bsALS and EHII(3)bsALS (Table 4).

The F_1 progenies derived from the two restored combinations were analysed for the presence of the *barnase* and *barstar* genes by PCR. All male sterile progenies showed the presence of *barnase* gene. The fertile F_1 progeny segregated in an expected 1 : 1 ratio for only the *barstar* gene and those that carry both the *barnase* and *barstar* genes (restored events) are summarized in Table 5. Further the pollen viability was similar to that observed in lines containing only the *barstar* gene. This established proper restoration of the 1.2bnALS line by both the *barstar* lines. Homozygous *barstar* lines have been developed which would be used for production of hybrid seeds in large amounts.

The present study strengthens our earlier observations that the modifications made in the *barnase* and *barstar* constructs[2,4] lead to efficient generation of male sterile and restorer lines. The use of an insulator fragment[2] in the present *barnase* construct with the *ALS*^{dm} marker gene, as an alternate to the *bar* gene helped in generating male sterile lines at a high frequency. Similarly, use of the 'two promoter–two gene' strategy for *barstar* constructs[4] for high and overlapping *barstar* expression, allowed identification of restorers from only two *barstar* lines.

We have thus successfully developed male sterile and restorer lines in appropriate combiners of *B. juncea*, viz. cultivars Varuna and EHII. Hybrids derived from these combiners developed earlier gave ~25–30% heterosis over Indian check varieties[1]. The first generation *barnase*/*barstar*-based hybrid seed technology developed by this laboratory used the herbicide 'Basta' for field selection. The present transgenics utilize an imadizolinone-based low-cost herbicide 'Pursuit', available in India. 'Pursuit' can be used both as a contact (foliar spray) as well as systemic herbicide. In small (25 sq. m) plot trials two foliar sprays with a gap of 2–3 days of 'Pursuit' (1.2 ml of Pursuit in 15 l of water) on one-month-old plants were enough to distinguish between the resistant and sensitive lines (Figure 4). Thus, under field conditions male sterile lines can be easily selected from a segregating population of sterile

Table 5. PCR analysis and pollen viability of fertile progenies obtained from *barnase* and *barstar* crosses

Male sterile/restorer combination	bnALS1.2X EHII(2)bsALS	bnALS1.2 X EHII(3)bsALS
No. of fertile plants	32	27
Presence of *barstar* gene	21	12
Presence of both the *barnase* and *barstar* genes	11	15
χ^2 value for an expected 1 : 1 ratio of progenies with *barstar*: progenies with both *barnase* and *barstar* genes	3.13	0.33
Pollen viability in plants having only the *barstar* genes	81.5–100%	92–100%
Pollen viability in plants having both *barnase* and *barstar* genes (restored events)	90–100%	93.5–100%

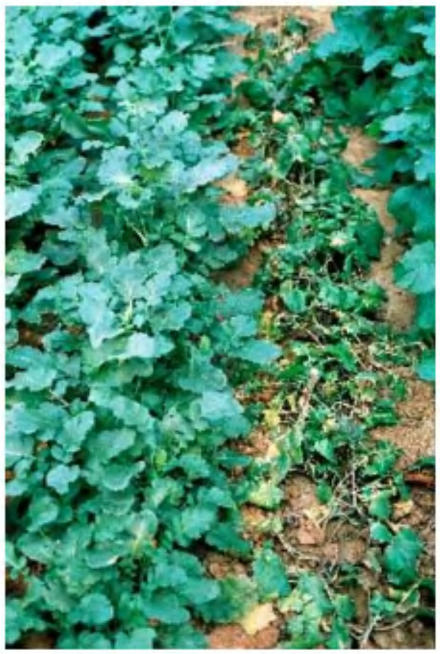

Figure 4. Homozygous *ALS*^{dm} *barstar* lines and untransformed control lines in *B. juncea* sprayed with herbicide 'Pursuit'. After 10 days of initial spray the transgenic plants grew normally, while the untransformed control lines were killed.

and fertile plants by spraying 480 ml of 'Pursuit' per hectare. The use of 'Pursuit' as a contact herbicide also reduces its residual persistence in the soil. Further, ALS the target enzyme for imazethapyr is absent in animals, which gives an added advantage. Also, as the *Als*^{dm} gene can be used as an *in vitro* selection marker, an additional transgene in plants for initial selection of transformants is not needed. These advantages make the present hybrid seed technology suitable for commercial use. The constructs and strategy developed and confirmed in the present study can be extended to other crops like cotton and rice for hybrid seed production.

1. Pradhan, A. K., Sodhi, Y. S., Mukhopadhay, A. and Pental, D., Heterosis breeding in Indian mustard (*Brassica juncea* L. Czern and Coss): Analysis of component characters contributing to heterosis for yield. *Euphytica*, 1993, **69**, 219–229.
2. Jagannath, A., Bandyopadhyay, P., Arumugam, N., Gupta, V., Burma, P. K. and Pental, D., The use of a spacer DNA fragment insulates the tissue-specific expression of a cytotoxic gene (*barnase*) and allows high frequency generation of transgenic male sterile lines in *Brassica juncea* L. *Mol. Breed.*, 2001, **8**, 11–23.
3. Jagannath, A., Arumugam, N., Gupta, V., Pradhan, A. K., Burma, P. K. and Pental, D., Development of transgenic *barstar* lines and identification of a male sterile (*barnase*)/restorer (*barstar*) combination for heterosis breeding in Indian oilseed mustard (*Brassica juncea*). *Curr. Sci.*, 2002, **82**, 46–52.
4. Bisht, N. C., Jagannath, A., Gupta, V., Burma, P. K. and Pental, D., A two gene–two promoter system for enhanced expression of a restorer gene (*Barstar*) and development of improved fertility restorer lines for hybrid seed production in crop plants. *Mol. Breed.*, 2004, **14**, 129–144.
5. Mourad, G., Haughn, G. and King, J., Intragenic recombination in the CSR1 locus of *Arabidopsis*. *Mol. Gen. Genet.*, 1994, **243**, 178–184.
6. Ray, K., Jagannath, A., Gangwani, S. A., Burma, P. K. and Pental, D., Mutant *acetolactate synthase* gene is an efficient *in vitro* selectable marker for the genetic transformation of *Brassica juncea* (oilseed mustard). *J. Plant Physiol.*, 2004, **161**, 1079–1083.
7. Hajdukiewicz, P., Svab, Z. and Maliga, P., The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.*, 1994, **25**, 989–994.
8. Reddy, M. K., Nair, S. and Tewari, K. K., Cloning, expression and characterization of a gene which encodes a *topoisomerase I* with positive supercoiling activity in pea. *Plant Mol. Biol.*, 1998, **37**, 773–784.
9. Rossi, A. and Petruzzelli, A. F., c-DNA derived amino acid sequence of lentil seedlings' amine oxidase. *FEBS Lett.*, 1992, **301**, 253–257.
10. Brosius, J., Ullrich, A., Raker, M. A., Gray, A., Dull, T. J., Gutell, R. G. and Noller, H. F., Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *E. coli*. *Plasmid*, 1981, **6**, 112–118.
11. Mattanovich, D. *et al.*, Efficient transformation of *Agrobacterium* spp. by electroporation. *Nucleic Acids Res.*, 1989, **17**, 6747.
12. Mehra, S., Pareek, A., Bandyopadhyay, P., Sharma, P., Burma, P. K. and Pental, D., Development of transgenics in Indian oilseed mustard (*Brassica juncea*) resistant to herbicide phosphinothricin. *Curr. Sci.*, 2000, **78**, 1358–1364.
13. Rogers, S. O. and Benedich, A. J., In *Plant Molecular Biology Manual* (eds Gelvin, S. B. and Schilperoort, R. A.), Kluwer, Dordrecht, 1995, vol. D1, pp. 1–8.
14. Heslop-Harrison, J., Heslop-Harrison, Y. and Shivanna, K. R., The evaluation of pollen quality and a further appraisal of the fluorochromatic (FCR) test procedure. *Theor. Appl. Genet.*, 1984, **67**, 367–375.
15. Denis, M., Delourme, R., Gourret, J. P., Mariani, C. and Renard, M., Expression of engineered nuclear male sterility in *Brassica napus*: Genetics, morphology, cytology and sensitivity to temperature. *Plant Physiol.*, 1993, **101**, 1295–1304.

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