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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.

Arun Jagannath[1,2,+], Panchali Bandyopadhyay[1,+], N. Arumugam[2], Vibha Gupta[2], Pradeep Kumar Burma[1] and Deepak Pental[1,2,*]

[1]*Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India;* [2]*Centre for Genetic Manipulation of Crop Plants (CGMCP), University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India;* [*]*Author for correspondence (fax: 91-11-6885270; e-mail: dpental@hotmail.com);* [+]*These authors contributed equally to this work*

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Abstract

Male-sterile lines were generated in oilseed mustard (*Brassica juncea*) with a cytotoxic gene (*barnase*) in conjunction with either of two tapetum-specific promoters, TA29 and A9. Several transformation vectors based on different promoter and marker gene combinations were developed and tested for their efficacy in generating agronomically viable male-sterile lines. Use of strong constitutive promoters (e.g. CaMV 35S or its double-enhancer variant) to express the marker gene (*bar*) in *barnase* constructs generated male-sterile plants at an extremely low frequency with most plants showing abnormalities in vegetative morphology, poor female fertility, low seed germination frequencies and/or distortion in segregation ratios of transgenes. Such abnormalities were considerably reduced on using weaker promoters (e.g. *nos*) to drive the marker gene (*nptII*) in *barnase* constructs and could therefore be attributed to leaky expression of the *barnase* gene under enhancing effects of strong constitutive promoters. We show that the use of a Spacer DNA fragment between the *barnase* gene (driven by a tapetum-specific promoter) and the CaMV 35S promoter-driven *bar* gene insulates tissue-specific expression of the *barnase* gene over all developmental stages of transgenic plants and significantly enhances recovery of agronomically viable male-sterile lines. All TA29-*barnase* male-sterile lines containing the Spacer DNA fragment exhibited normal morphology, growth and seed set on backcrossing as observed for wild-type plants. Around 75% of single-copy events tested further also showed proper segregation of the marker gene/male-sterile phenotype among backcross progeny. Constructs based on the use of Spacer DNA fragments as insulators could be successfully used to alleviate limitations associated with transformation of plant systems using cytotoxic genes for development of agronomically viable male-sterile lines in crop plants and for cell/tissue ablation studies in general.

Abbreviations: 35S – cauliflower mosaic virus (CaMV) 35S promoter with single enhancer, 35Sde – CaMV 35S promoter with double enhancer, AMVL – 5′ leader sequence of alfalfa mosaic virus RNA 4 gene, BAP – 6 benzylaminopurine, CTAB – cetyltriethyl ammonium bromide, IBA – indole-3-butyric acid, LB – left border of T-DNA of *Agrobacterium tumefaciens* Ti plasmid, MS medium – Murashige and Skoog basal salt medium, NAA – α-naphthalene acetic acid, OD_{600} – optical density at 600 nm; PPT – phosphinothricin, RB – right border of T-DNA of *A. tumefaciens* Ti plasmid.

Introduction

The amphidiploid *Brassica juncea* (mustard) is a major oilseed crop of the Indian subcontinent and is cultivated as a winter season crop in about 6 million hectares of land in rain-fed areas of northern India. It is also grown to a limited extent in Russia, China and Australia and is a potential crop for the western prairies of Canada. Earlier studies on hybrids in *B. juncea* have demonstrated the heterotic potential of crosses between genetically divergent lines and their role in enhancing crop productivity (Banga 1992; Pradhan et al. 1993). Since *B. juncea* is a predominantly self-pollinated crop, production of hybrids under field conditions would require introduction of male sterility into one of the combiners that would function as the female parent. Availability of a suitable restorer gene in the male parent would be essential in order to achieve seed set in F_1 hybrids.

Transgenic technology provides a powerful tool for genetic engineering of male sterility. Deployment of the *barnase* gene (from *Bacillus amyloliquefaciens*) or the *RnaseT1* gene (from *Aspergillus oryzae*) under transcriptional control of a tapetum-specific promoter (TA29) has been shown to induce male sterility in transgenic *Brassica napus* and tobacco plants (Mariani et al. 1990). However, in a later study on stability of the male-sterile phenotype and segregation of transformed genes among progeny of male-sterile *barnase* plants grown under controlled conditions, assays for pollen viability revealed the presence of viable pollen while a similar analysis of male-sterile *RnaseT1*-containing transgenics showed distortion in segregation ratios and reversion to fertility in as many as five of seven lines tested under different growth conditions (Denis et al. 1993). Further, the above strategies envisaged the use of two different marker genes, one primarily for *in vitro* selection (*nptII*) and another for field-level selection (*bar*) of transgenic plants. Although several other strategies were subsequently developed and tested for disruption of normal pollen development in transgenic plants (reviewed by Williams 1995), most of these were tested in model systems and their satisfactory performance in crop plants remains to be established. Moreover, most reports do not give a comprehensive analysis of other parameters, viz. transformation frequencies, frequency of male sterility and inheritance patterns of transgenes. Therefore, the ease of developing agronomically viable transgenic plants with conditional expression of lethal genes remains unclear.

The present study was initiated with the objective of developing methods that could be used for high-frequency generation of agronomically viable male-sterile lines in *B. juncea* (and other crop plants) to facilitate hybrid seed production. Several transformation vectors based on either the *bar* gene (conferring resistance to phosphinothricin (PPT), active component of the commercial herbicide preparation, Basta) or *nptII* gene (conferring resistance to kanamycin) as the selectable marker and either of two tapetum-specific promoters, TA29 (Koltunow et al. 1990) or A9 (Paul et al. 1992) to transcribe a cytotoxic gene (*barnase*) were developed and tested in this study. The efficacy of each construct was determined on the basis of its performance with reference to the following criteria that were chosen in order to represent different stages in the development and growth of transgenic (male-sterile) plants: (1) frequency of genetic transformation and regeneration, (2) frequency of obtaining male-sterile transgenic plants, (3) vegetative morphology and female fertility of male-sterile transgenic plants, (4) germination frequencies of backcross seeds, (5) segregation ratios of marker gene/male-sterile phenotype in the backcross progeny, and (6) stable inheritance of male sterility.

Based on an extensive analysis of transformants generated using constructs with various promoter and marker gene combinations, it was observed that use of a constitutive promoter with enhancing functions (to drive expression of the marker gene) deregulated temporal and spatial expression patterns of tapetum-specific promoters. This led to leaky expression of the *barnase* gene hindering successful production of male sterile plants. We describe in this report, a strategy of using a Spacer DNA fragment to effectively insulate tapetum-specific expression of the *barnase* gene from the influence of a neighboring constitutive promoter during various developmental stages of a transgenic plant thereby leading to high frequency generation of male-sterile transgenic lines in *B. juncea*. In addition to fulfilling various criteria outlined above, the proposed strategy would also allow use of a single selectable marker gene for *in vitro* as well as *in vivo* selection of transgenic plants thereby reducing the use of superfluous marker genes in transgenic lines.

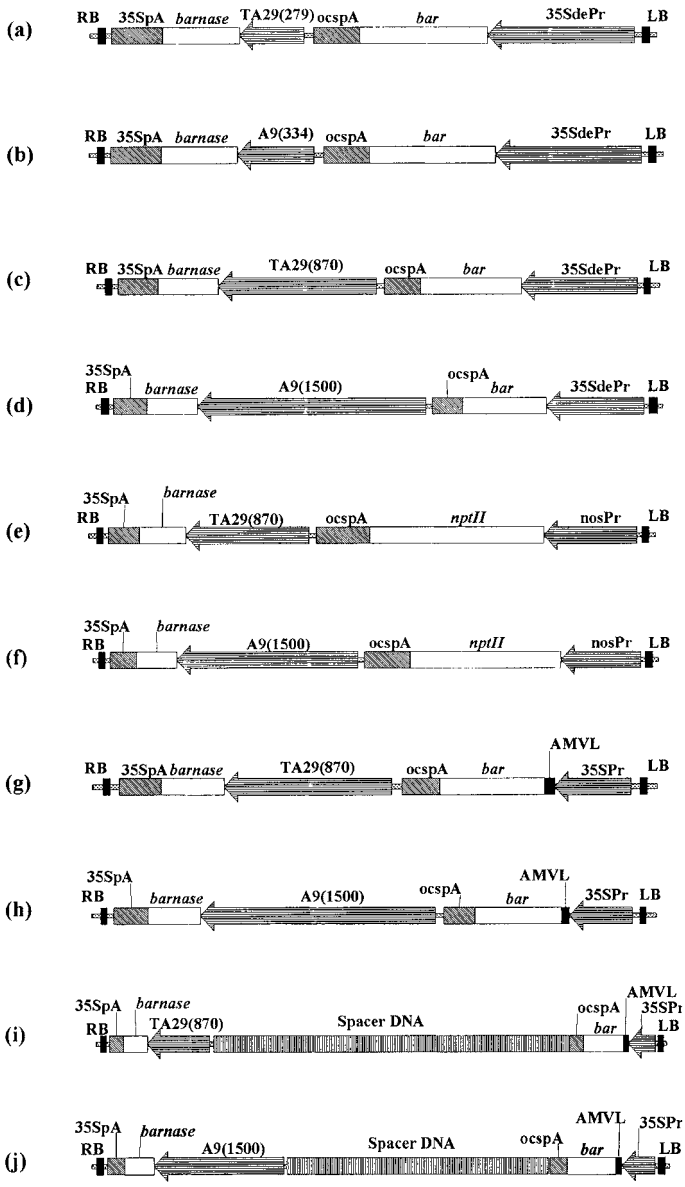

Figure 1. Map of T-DNA region of gene constructs tested for generation of male-sterile plants in *B. juncea* (a) de*bar*-TA29(279)*bn* (b) de*bar*-A9(334)*bn* (c) de*bar*-TA29(870)*bn* (d) de*bar*-A9(1500)*bn* (e) *nptII*-TA29(870)*bn* (f) *nptII*-A9(1500)*bn* (g) AMVL*bar*-TA29(870)*bn* (h) AMVL*bar*-A9(1500)*bn* (i) AMVL*bar*-Spacer-TA29(870)*bn* (j) AMVL*bar*-Spacer-A9(1500)*bn*. Abbreviations: Pr, promoter; *nos*, promoter of the nopaline synthase gene; *nptII*, neomycin phosphotransferase II gene; ocsp(A), polyA signal of the octopine synthase gene; TA29(279), 279 bp fragment of tapetum-specific TA29 promoter; TA29(870), 870 bp fragment of TA29 promoter including 50 bp downstream to the transcription start (+1) site; A9(334), 334 bp fragment of tapetum-specific A9 promoter; A9(1500), 1.5 kb fragment of tapetum-specific A9 promoter. The Spacer DNA used in construct i was constituted from coding regions of two dicot genes, *acetolactate synthase* from *Arabidopsis* and *topoisomerase* from pea. The Spacer DNA used in construct j comprised a linearized fragment of the plasmid pLitmus 38.

Materials and methods

Transformation vectors

The tapetum-specific TA29 and A9 promoter fragments were PCR amplified from *Nicotiana* and *Arabidopsis* genomic DNA, respectively. The *barnase* and *barstar* genes (the *barstar* gene encodes an intracellular inhibitor of the barnase protein) were PCR-amplified from the plasmid pMT416 (Hartley 1988). Amplification products were cloned and sequenced (Sequenase Version 2.0 system, Amersham Pharmacia Biotech) prior to their incorporation in cloning vectors. All DNA manipulations were performed using standard protocols (Sambrook et al. 1989). The *barstar* gene under transcriptional control of its native bacterial promoter was incorporated in all vectors (outside the T-DNA borders in case of binary vectors) used for cloning the *barnase* gene. This was necessary, as it was not possible to clone the *barnase* gene in the absence of background levels of barstar protein, presumably due to leaky expression of the *barnase* gene in bacterial systems. The marker gene cassettes based on the *bar* gene (driven by the 35S promoter or its variants) were derived from constructs described recently (Mehra et al. 2000) while the *nptII* gene-based cassettes (driven by the nos promoter) were derived from the binary vector pGSFR780A (De Block et al. 1989). Cloning steps involved in the development of various transformation vectors are not being described in the text but will be made available on request. Salient features of various transformation vectors are given below and a schematic representation of the same is given in Figure 1. Each vector is also assigned a code based on the particular promoter(s) and marker gene(s) used which would be used to refer to the same in subsequent sections of the text.

a. Construct 1. LB : 35Sde-*bar*-ocspA::TA29(279)-*barnase*-35SpA : RB (code: de*bar*-TA29(279)*bn*; Figure 1a)

The *bar* gene is driven by the 35Sde promoter and is fused at its 3′ end to a poly(A) signal of the *octopine synthase* gene (*ocspA*). The *barnase* gene is transcriptionally fused to a 279 bp fragment of the TA29 promoter (Koltunow et al. 1990) and contains at its 3′ end, the 35S polyA signal from pRT103 (Topfer et al. 1987).

b. Construct 2. LB : 35Sde-*bar*-ocspA::A9(334)-*barnase*-35SpA : RB (Code: de*bar*-A9(334)*bn*; Figure 1b).

The *barnase* gene is transcriptionally fused to a 334 bp fragment of the A9 promoter (Paul et al. 1992). All other features are similar to those described above for Construct 1.

c. Construct 3. LB : 35Sde-*bar*-ocspA::TA29(870)-*barnase*-35SpA : RB (Code: de*bar*-TA29(870)*bn*; Figure 1c).

An 870 bp fragment of the TA29 promoter, including sequences till the translation start site (ATG) of the TA29 gene, is translationally fused to the 5′ end of the *barnase* gene. All other features are similar to those described above for Construct 1.

d. Construct 4. LB: 35Sde-*bar*-ocspA::A9(1500)-*barnase*-35SpA : RB (Code: de*bar*-A9(1500)*bn*; Figure 1d).

A full-length 1.5 kb fragment of the A9 promoter is translationally fused to the 5′ end of the *barnase* gene. All other features are similar to those described above for Construct 2.

e. Construct 5. LB : nos-*nptII*-ocspA::TA29(870)-*barnase*-35SpA : RB (Code: *nptII*-TA29(870)*bn*; Figure 1e).

The *nptII* gene under transcriptional control of the nos promoter is used as a selectable marker along with the TA29-*barnase* cassette described in Construct 3.

f. Construct 6. LB : nos-*nptII*-ocspA::A9(1500)-*barnase*-35SpA : RB (Code: *nptII*-A9(1500)*bn*; Figure 1f).

The *nptII* gene under transcriptional control of the nos promoter is used as a selectable marker with the A9-*barnase* cassette described in Construct 4.

g. Construct 7. LB : 35S-AMVL-*bar*-ocspA::TA29 (870)-*barnase*-35SpA : RB (Code: AMVL*bar*-TA29(870)*bn*; Figure 1g).

A 56 bp leader sequence from the RNA 4 gene of alfalfa mosaic virus (Jobling and Gehrke 1987), was fused downstream to the 35S promoter and 5′ to the coding region of the *bar* gene. The *barnase* gene was translationally fused to the TA29 promoter as in Constructs 3 and 5.

h. Construct 8. LB : 35S-AMVL-*bar*-ocspA::A9 (1500)-*barnase*-35SpA : RB (Code: AMVL*bar*-A9(1500)*bn*; Figure 1h).

The AMV RNA 4 leader sequence was cloned downstream to the 35S promoter and 5′ to the coding region of the *bar* gene. The *barnase* gene was translationally fused to the A9 promoter as described above for Constructs 4 and 6.

i. Construct 9. LB:35S-AMVL-*bar*-ocspA::Spacer:: TA29(870)-*barnase*-35SpA:RB (Code: AMVL*bar*-Spacer-TA29(870)*bn*; Figure 1i).

The main feature of this construct is the pres-

ence of a 5 kb Spacer DNA fragment between the 35S-AMVL-*bar*-ocspA cassette and the TA29(870)-*barnase*-35SpA cassette of Construct 7 to insulate expression of the *barnase* gene from enhancing influences of the 35S promoter. Dicot gene sequences comprising a 3 kb *topoisomerase* gene fragment from pea (Reddy et al. 1998) and a 2 kb *acetolactate synthase* gene fragment from *Arabidopsis* (Li et al. 1992) were fused to generate the Spacer DNA fragment. A unique *Nco*I site within the topoisomerase gene was removed by polishing with *Pfu* DNA polymerase (Stratagene) prior to its use in cloning reactions.

j. Construct 10. LB:35S-AMVL-*bar*-ocspA::Spacer::A9(1500)-*barnase*-35SpA:RB (Code: AMVL*bar*-Spacer-A9(1500)*bn*; Figure 1j).

A 3 kb DNA sequence comprising linearized pLitmus38 plasmid (New England Biolabs) was used as a Spacer fragment between the 35S-AMVL-*bar*-ocspA cassette and the A9(1500)-*barnase*-35SpA cassette of Construct 8.

The above constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 using a gene pulser (BioRad) following the protocol described earlier (Mattanovich et al. 1989). The efficacy of each construct was studied by subjecting it to various selection criteria outlined in the Introduction. Plants derived from constructs that were found to falter in one or more of the selection criteria were not taken further for subsequent analysis.

Agrobacterium-mediated transformation of Brassica juncea

Transformation of *B. juncea* cultivars Varuna and RLM198 was performed according to the protocol described earlier (Bade and Damm 1995) with further modifications (Mehra et al. 2000). Hypocotyl explants were used for *Agrobacterium*-mediated genetic transformation. Following infection and co-cultivation in MSN1B1 medium (Pental et al. 1993), explants were washed with MSN1B1 containing 200 mg/l of the bacteriostatic agent Augmentin (Medreich Sterilab Limited, India) and plated on MSN1B1 medium containing appropriate selective agents (50 mgLl kanamycin or 10 mg/l, PPT (the active component in the commercially available herbicide Basta; Agrevo)). Only one regenerated shoot was taken from each explant and such shoots were transferred to MS medium supplemented with 2 mg/l IBA for rooting. Transgenic plants were maintained as nodal cultures until transplantation in the field.

Growth and maintenance of transgenic plants

Transgenic plants were grown in soil in a containment nethouse in accordance with guidelines laid down for growth of transgenic material by the Department of Biotechnology, Government of India. Plants with intact roots and a well-developed shoot system were transplanted directly to soil from *in vitro* growth conditions during the growing season. Transplantation was carried out during the months of October-November and plants were analyzed until the end of the growing season (March-April). T_0 transgenic plants were backcrossed with pollen from untransformed parent to obtain T_1 seeds, plants generated from which were used for subsequent analysis.

Analysis of male sterility/fertility status

Male-sterile plants were identified on the basis of morphological observations of anthers and absence of pollen production. Two to three inflorescence axes from each transgenic plant (each inflorescence bearing 10–15 unopened buds) were covered with a pollination bag to test for the formation of selfed seeds. Absence of selfed seeds was taken as a further confirmation of male sterility. In plants where pollen formation was observed, pollen viability was tested by fluorescein diacetate (FDA) staining (Heslop-Harrison et al. 1984). Transgenics with chimeric flowers (flowers with fertile as well as sterile anthers) or chimeric inflorescence (those which produced fertile as well as sterile flowers) were classified as semi-sterile plants.

Southern analysis

Genomic DNA was extracted from leaves of transgenic and untransformed (control) plants by the method described by Rogers and Bendich (1995). About 10 μg of each DNA sample was digested with an appropriate enzyme (which restricts the T-DNA between the marker gene, *bar* or *nptII*, and the *barnase* gene) for Southern analysis. Blots were sequentially probed with the *barnase* gene and the marker gene (*bar* gene or nos-*nptII*-pA cassette depending on the construct used) to determine copy number on the RB and LB flanks respectively of the T-DNA. Probes were labeled with α-[^{32}P]-dCTP by random priming using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech). After hybridization and washing in accordance with standard protocols (Sambrook et al. 1989), the membrane was subjected to autoradiography for 12–14 h at -80 °C.

Analysis of seed germination frequencies and segregation ratios

Backcrossed T_1 seeds from individual T_0 plants were surface-sterilized and germinated on non-selective media according to procedures described earlier (Mehra et al. 2000). Seed germination frequency for each progeny plant was calculated as a percentage of the ratio of number of seeds germinated over number of seeds inoculated. The apices of germinated seedlings were excised and placed on MS medium containing 2 mg/l IBA and the appropriate selective agent. Rooting and survival of plantlets was scored. Segregation ratios were calculated in terms of resistance (R)/sensitivity (S) to the selective agent and subsequently correlated with male sterility/fertility phenotype when T_1 plants came to flowering. Segregation data for each event was subjected to statistical analysis (χ^2 test at 95% confidence limit) to determine the goodness of fit.

Results and discussion

Development of male-sterile plants: transformation frequency, vegetative morphology, frequency of male sterility and female fertility

Initial *barnase* constructs developed in this study (constructs de*bar*-TA29(279)*bn* and de*bar*-A9(334)*bn*, Figure 1a, b) used truncated fragments of tapetum-specific promoters (reported to contain all the regulatory signals conferring tissue specificity) (Koltunow et al. 1990; Paul et al. 1992) to express the *barnase* gene. The 35Sde promoter was used to express the *bar* gene in the above constructs since earlier work in our laboratory had demonstrated higher transformation frequencies using the same as compared to the single enhancer 35S promoter (Mehra et al. 2000). However, transformation frequencies using the above *barnase* constructs were drastically reduced over those obtained from control experiments with the *bar* gene alone (Table 1). More significantly, an overwhelming majority of the few transgenic plants that were recovered on selection plates turned out to be fertile (Table 1). Only one male-sterile plant could be obtained from about 10 090 explants with the de*bar*-TA29(279)*bn* construct. No male-sterile plant could be generated with the de*bar*-A9(334)*bn* construct.

The drastic reduction in transformation frequencies using the above constructs is, in all probability, due to leaky expression of the *barnase* gene in transformed cells preventing survival/further development of the same. Similar observations were reported earlier in studies on targeted expression of the *barnase* gene with another tapetum-specific promoter (TA56), which failed to produce any *barnase* plants in tobacco (Beals and Goldberg 1997). Biochemical analysis of transgenic calli generated using TA56-*gus* constructs revealed intense blue colour indicating that failure to obtain TA56-*barnase* plants was due to leaky expression of the *barnase* gene at the callus stage. Two factors were therefore analyzed in this study for their role in influencing leaky expression of the *barnase* gene: (1) absence of stringent regulatory controls over truncated fragment(s) of tapetum-specific promoter(s) and (2) enhancing effects of the double enhancer 35S promoter (Kay et al. 1987) leading to deregulated expression of the *barnase* gene.

To resolve the first question, constructs were developed with longer version(s) of tapetum-specific promoter(s) to transcribe the *barnase* gene (constructs de*bar*-TA29(870)*bn* and de*bar*-A9(1500)*bn*, Figure 1c, d). Use of an 870 bp fragment of the TA29 promoter to express the *barnase* gene failed to generate any male-sterile plant with transformation frequencies remaining extremely low (Table 1). On using a 1.5 kb fragment of the A9 promoter with the *barnase* gene, although the number of shoots obtained initially was higher, a large number of them failed to develop normally. Only two male-sterile plants could be obtained among 17 transgenic events tested (Table 1). To test whether enhancing effects of the 35Sde promoter were responsible for low transformation frequencies, a weaker promoter (nos) was used for expression of the selectable marker gene (*nptII*) in *barnase* constructs. Use of a nos-*nptII*-pA cassette as the selectable marker (constructs *nptII*-TA29(870)*bn* and *nptII*-A9(1500)*bn*, Figure 1e, f) registered a substantial increase in frequency of male-sterile plants over those obtained with constructs containing the 35Sde-*bar*-ocspA cassette: 94% of TA29-*barnase* transgenics and 87% of A9-*barnase* transgenics were male-sterile (Table 1). Most of the male-sterile plants obtained were normal in their vegetative growth (Table 1) and set proper seed on backcrossing.

The observations described above conclusively established the role of a strong constitutive promoter (35Sde) in influencing leaky expression of the *barnase* gene leading to low transformation frequencies and a corresponding decrease in the frequency of male-sterile transgenics. Although it was possible to

Table 1. Transformation frequency, frequency of male sterility and vegetative morphology of male-sterile transgenics generated using various *barnase* constructs.

No.	Construct	Number of explants used	Number of shoots obtained	Transformation frequency[1] (%)	Analysis of male sterility/fertility status of T_0 transgenic plants				Frequency of male-sterile plants (%)	Vegetative morphology of T_0 male-sterile plants[2]	
					No. of plants analyzed	Sterile	Semi-sterile	Fertile		Normal	Abnormal
1.	de*bar*-TA29(279)*bn*	10 090	50	0–2	50	1	2	47	2	1	–
2.	de*bar*-A9(334)*bn*	10 996	38	0–0.8	12	0	0	12	0	–	–
3.	de*bar*-TA29(870)*bn*	2 670	24	0–1.5	20	0	0	20	0	–	–
4.	de*bar*-A9(1500)*bn*	2 734	116	0.8–6.8	17	2	2	13	12	2	–
5.	*nptII*-TA29(870)*bn*	5 454	788	8–26	70	66	2	2	94	64	2
6.	*nptII*-A9(1500)*bn*	2 561	292	4.4–14	30	26	0	4	87	26	–
7.	AMVL*bar*-TA29(870)*bn*	7 855	151	0–3.5	46	27	7	12	59	0	27
8.	AMVL*bar*-A9(1500)*bn*	9 975	42	0–0.8	4	0	0	4	0	–	–
9.	AMVL*bar*-Spacer-TA29(870)*bn*	5 997	402	2–6	97	71	6	20	73	71	0
10.	AMVL*bar*-Spacer-A9(1500)*bn*	4 921	191	0.6–6.4	40	23	1	16	58	0	23
	Control : 35Sde-*bar*-ocspA	831	250	12–17	All plants were fertile						
	Control : 35S-AMVL-*bar*-ocspA	5 645	710	3–10	All plants were fertile						

[1] Calculated on the basis of the number of PPT- or kanamycin-resistant shoots obtained over number of explants used. Numbers represent the range of transformation frequencies obtained in independent transformation experiments.
[2] As compared to features of untransformed (control) plants.

obtain a large number of male-sterile plants using nos-*nptII*-ocspA cassette as a marker in *barnase* constructs, it was important to achieve better transformation frequencies with a combination of *bar* (or any other herbicide resistance-conferring gene) and *barnase* genes to enable use of a single marker (gene) for *in vitro* as well as field-level selection of transgenic lines. This, in turn, required the use of an adequately strong constitutive promoter, viz. 35S, to transcribe the marker gene (*bar*) but with reduced enhancing activities than the 35Sde promoter. Earlier reports in literature have described the role of leader sequences in enhancing gene expression without influencing promoter strength (Gallie et al. 1987; Jobling and Gehrke 1987; Day et al. 1993). Fusion of the AMV RNA 4 leader sequence at the 5′ end of the bar gene (35S-AMVL-*bar*-ocspA) has also been shown to increase transformation frequencies and gene expression levels over those obtained with the 35S promoter alone (Mehra et al. 2000).

Deployment of the 35S-AMVL-*bar*-ocspA cassette as a selectable marker with the TA29-*barnase*-35SpA cassette (construct AMVL*bar*-TA29(870)*bn*, Figure 1g) did not lead to any significant improvement in transformation frequencies over earlier *barnase* constructs based on the *bar* gene (Table 1). Nevertheless, a substantial increase was observed in the frequency of male-sterile transgenic plants with 27 of 46 plants (ca. 59%) being male-sterile (Table 1). However, most of these male-sterile plants exhibited vegetative abnormalities such as chlorosis and wrinkled leaves (Table 1) and poor seed set on backcrossing. These abnormalities could be attributed to leaky expression of the *barnase* gene under enhancing effects of the single enhancer 35S promoter (Kay et al. 1987). No male sterile plant could be generated using the AMVL*bar*-A9(1500)*bn* construct (Table 1).

To test whether enhancing influences of the 35S promoter on temporal and spatial expression patterns of tapetum specific promoters could be minimized, the 35S-AMVL-*bar*-ocspA and TA29(870)-*barnase*-35SpA cassettes were separated by a 5 kb Spacer DNA fragment to insulate expression of the *barnase* gene (construct AMVL*bar*-Spacer-TA29(870)*bn*, Figure 1i). Although this construct registered only a modest increase in transformation frequencies over those obtained with the AMVL*bar*-TA29(870)*bn* construct, a majority of transgenic plants grown in the field (71 of 97 plants) were male-sterile (Table 1). More significantly, no vegetative abnormalities were observed among these male-sterile plants and they appeared similar in their vegetative morphology to untransformed (control) plants. All the male-sterile *barnase* plants that were backcrossed set seed and were therefore female-fertile. Although male-sterile plants could be generated at a reasonable frequency (ca. 58%) with a corresponding A9 construct containing a 3 kb Spacer DNA fragment (AMVL*bar*-Spacer-A9(1500)*bn*, Figure 1j, Table 1), seed set in most of these plants was poor and in some cases, backcrossed seeds obtained were shriveled. The above results indicate that use of a 3 kb Spacer fragment with the A9 promoter does not confer sufficient protection against deregulated expression of the *barnase* gene while use of a 5 kb Spacer fragment adequately protects tissue- specific expression pattern of the TA29 promoter.

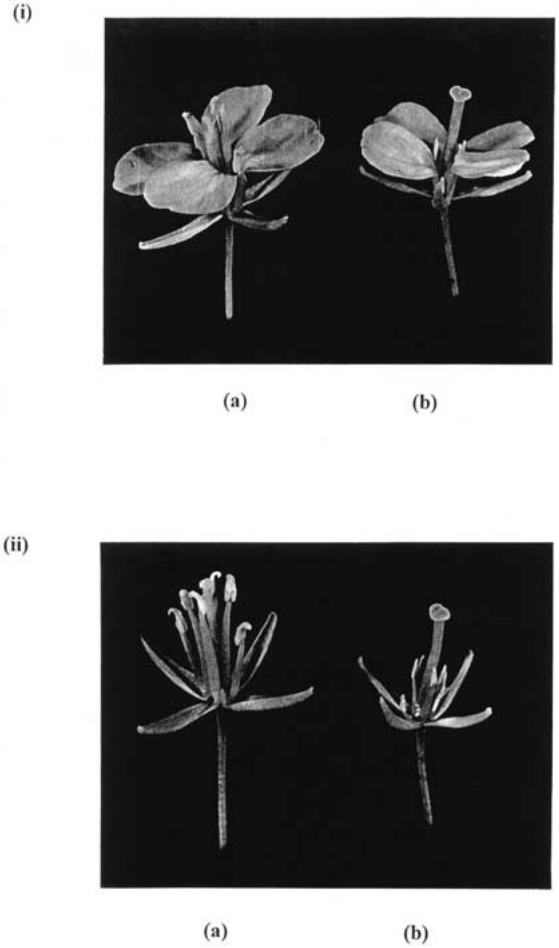

Figure 2. i Individual flowers of (a) untransformed plants and (b) male-sterile *barnase* lines of *B. juncea*. ii Individual flowers of untransformed and male-sterile lines. Petals have been removed for greater clarity of anther phenotypes.

Close monitoring of male sterility in the T_0 transgenics was done over the entire growing season to study stability of the male-sterile phenotype under field conditions. Male-sterile flowers (Figure 2) were characterized by the complete absence of pollen production in anthers, which were rudimentary and flattened in comparison to anthers of control fertile flowers. A moderate difference in size was also seen between male-sterile flowers and control fertile flowers with the former being smaller. None of the male-sterile plants obtained using the above constructs either showed reversion to fertility or set seed on selfing. In addition to male-sterile plants, semi-sterile and fertile plants were also obtained at varying frequencies with different *barnase* constructs (Table 1) with most semi-sterile plants producing flowers with fertile as well as sterile anthers. Some semi-sterile plants eventually showed a breakdown of male sterility and reverted to producing male-fertile flowers.

Southern analysis of transgenic plants

Male-sterile plants generated using constructs *nptII*-TA29(870)*bn*, AMVL*bar*-TA29(870)*bn*, AMVL*bar*-Spacer-TA29(870)*bn* and AMVL*bar*-Spacer-A9(1500)*bn* (Figure 1) were subjected to Southern analysis to identify single-copy plants for further analysis. Representative examples of Southern blots of AMVL*bar*-Spacer-TA29(870)*bn* plants with probes used to determine copy number on the left-border and right-border flanks of T-DNA are shown in Figure 3. Interestingly, single-copy insertions were obtained at a much higher frequency amongst the *barnase*-containing transgenics analyzed (Table 2) in contrast to earlier observations on transformants containing the marker gene (*bar*) alone wherein the reported frequency of single-copy insertions varied between 15% and 20% (Mehra et al. 2000). This might be largely due to preferential selection and survival of single copy plants over those with higher copy numbers wherein probability of deregulated expression of one or more copies of the *barnase* gene under the influence of flanking genomic sequences (position effects) is higher.

Most semi-sterile and fertile plants showed hybridization with the marker gene indicating that they were not selection escapees from transformation experiments. The *barnase* gene was however found to be lacking in a majority of them (data not shown) indicating rearrangements and truncations in the T-DNA. Such rearrangements have been reported earlier in literature and are known to occur at a low frequency during T-DNA transfer and its integration into the plant genome (Spielmann and Simpson 1986; Bhattacharya et al. 1994; Krizkova and Hrouda 1998).

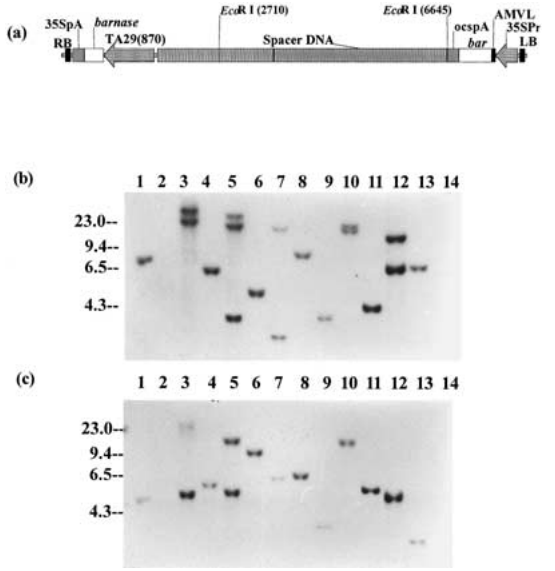

Figure 3. Southern analysis of a representative population of male-sterile plants obtained using the AMVL*bar*-Spacer-TA29(870)*bn* construct. (a) Schematic representation of T-DNA region of the above construct showing location of the *Eco*RI sites used for genomic DNA digestions. (b) Copy number on the left border flank was determined by probing Southern blots with coding sequence of the *bar* gene. (c) Copy number on the right-border flank was determined by reprobing the blot in (b) with coding sequence of the *barnase* gene. Lanes 1, semi-sterile line; 2, fertile line; 3–13, male-sterile lines; 14, untransformed plant. Lanes 6, 8, 11 and 13 represent hybridization profiles of transgenics highlighting a single-copy integration event of the T-DNA.

Single-copy *barnase*-containing plants thus identified were backcrossed to the wild-type parent and T_1 seeds obtained were subjected to further analysis as described below.

Analysis of T_1 progeny: germination frequencies, segregation analysis and inheritance of male sterility

Backcrossed T_1 seeds of single-copy male-sterile transgenic plants generated with the constructs AMVL*bar*-Spacer-TA29(870)*bn* (Figure 1i) and AM VL*bar*-TA29(870)*bn* (minus Spacer DNA, used as a control; Figure 1g) were germinated on MS medium (without selective agent) to study germination frequencies. Most AMVL*bar*-TA29(870)*bn* plants were characterized by poor germination frequencies (ranging from 4% to 69%, Figure 4), with no transformant showing germination frequencies comparable to those

Table 2. Copy number of transgenes in male-sterile plants as determined by Southern hybridization.

No.	Construct	Number of male-sterile plants analyzed	Number of single--copy transgenics	Frequency of single-copy events (%)
1.	*nptII*-TA29(870)*bn*	22	11	50
2.	AMVL*bar*-TA29(870)*bn*	22	14	64
3.	AMVL*bar*-Spacer-TA29(870)*bn*	22	10	45
4.	AMVL*bar*-Spacer-A9(1500)*bn*	22	14	64

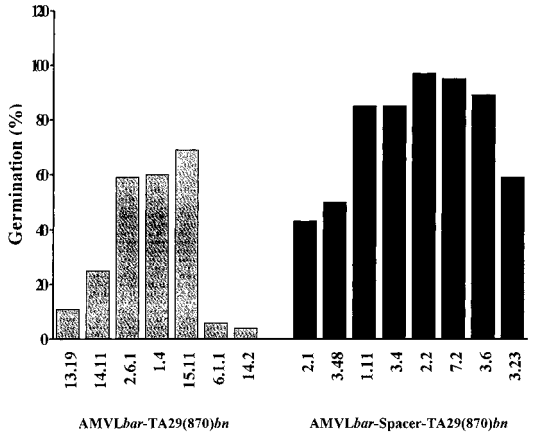

Figure 4. Germination frequencies of T_1 seeds derived from single-copy male-sterile transgenic plants generated with the AMVL*bar*-TA29(870)*bn* and AMVL*bar*-Spacer-TA29(870)*bn* constructs. Numbers on the X-axis represent independent transgenic events.

observed for untransformed (control) plants (ca. 85% and above). On the other hand, transgenic plants generated using the AMVL*bar*-Spacer-TA29(870)*bn* construct showed a substantial increase in germination frequencies (ranging from 43% to 97%, Figure 4) with around 60% of transgenic events showing germination frequencies of >85%, comparable to that observed for untransformed (control) plants.

Further, only two AMVL*bar*-TA29(870)*bn* plants (2.6.1 and 15.11) of seven single-copy events tested, showed segregation profiles that fit exactly (χ^2 tests at $P < 0.05$) to the expected 1:1 ratio for resistance/sensitivity to the selective agent (PPT) upon transfer to selection medium (Table 3). Similar distortions in segregation ratios were also observed in about 90% of AMVL*bar*-Spacer-A9(1500)*bn* plants and, quite surprisingly, in around 40% of *nptII*-TA29(870)*bn* events (data not shown). Distortions in segregation ratios among *nptII*-TA29(870)*bn* plants were unexpected because the nos promoter is much weaker than 35S promoter (Sanders et al. 1987) and is therefore not expected to possess any strong enhancing functions that could induce deregulated expression of the *barnase* gene. Observations made in this study however suggest that deregulated expression of a cytotoxic gene could also occur in the presence of weaker promoters (e.g. nos).

In contrast to the above observations, it was seen that six of eight (75%) single-copy AMVL*bar*-Spacer-TA29(870)*bn* transgenics tested (2.2, 2.1, 3.6, 3.23, 3.4 and 3.48) showed segregation profiles corresponding to the expected ratios for resistance/sensitivity to the selective agent, PPT (as determined by χ^2 test at $P < 0.05$; Table 3). All herbicide-resistant progeny of the six single-copy AMVL*bar*-Spacer-TA29(870)*bn* transgenics listed above were completely male-sterile when grown under field conditions thereby indicating stable inheritance of the male-sterile phenotype. These lines satisfy all the selection criteria outlined in the Introduction and are therefore potential male-sterile lines for use in hybrid seed production.

Observations made during the present study clearly indicate that a crucial step in the development of agronomically viable male-sterile transgenic lines in crop plants by using tissue-specific promoters to transcribe cytotoxic genes is to prevent leaky expression of the gene over all developmental stages of transgenic plants. Results presented in this study demonstrate the efficacy of using a Spacer DNA sequence to insulate tissue-specific expression of a cytotoxic gene (*barnase*) from deregulation by strong enhancers. While there are no limitations to the sequence(s) per se that can be used as Spacers, their selection in the present study was primarily based on the absence of regulatory or enhancer elements in the same in order to prevent deregulating effects on expression of neighboring genes. The Spacer DNA used in this study has a GC-content of ca. 42% which is in the range of GC content of most dicot genes (Salinas et al. 1988) and in consonance with that of transcriptionally active regions of

Table 3. Segregation profiles among T_1 progeny of single-copy AMVL*bar*-TA29(870)*bn* and AMVL*bar*-Spacer-TA29(870)*bn* transgenic plants.

Construct AMVL*bar*-TA29(870)*bn*					Construct AMVL*bar*-Spacer-TA29(870)*bn*				
Transgenic line	Number of seedlings on selection	Number of seedlings[a]		χ^2 value	Transgenic line	Number of seedlings on selection	Number of seedlings[a]		χ^2 value
		PPT^R	PPT^S				PPT^R	PPT^S	
13.19	NT[b]	–	–	–	2.1*	24	12	12	0
14.11	38	11	27	6.7	3.48*	32	12	10	3.2
2.6.1*	71	33	38	0.36	1.11	92	36	56	4.3
1.4	72	1	71	68	3.4*	28	14	14	0
15.11*	52	21	31	1.9	2.2*	96	42	54	1.5
6.1.1	NT	–	–	–	7.2	96	31	65	12.0
14.2	NT	–	–	–	3.6*	96	48	48	0
–	–	–	–	–	3.23*	42	16	26	2.4

[a]R, resistant; S, sensitive.
[b]NT, not tested; these plants were not subjected to segregation analysis since percent germination of backcrossed seeds derived from these events was very low (Figure 4).
Plants marked by an asterisk (*) show the expected 1:1 ratio of segregation of transgenes as determined by χ^2 test at $P < 0.05$.

the host genome. The length of the Spacer used to insulate expression of the cytotoxic gene would vary depending on the nature and strength of the constitutive promoter used for expression of the marker gene. The optimum length of the Spacer is governed by the primary objective of generating agronomically viable male-sterile transgenic plants in large numbers. Additionally, *barnase* constructs based on Spacer DNA sequences (as described in this study) allow use of a single selectable marker gene for *in vitro* as well as field-level selection of transgenic plants thereby reducing the use of superfluous marker genes in transgenic crops.

Several naturally occurring Insulator elements have been well described particularly in animal systems (e.g. scaffold/matrix attachment regions, SARs/MARs; locus control regions, LCRs; specialized chromatin sequences, scs/scs′; suppressor of Hairy wing (su[Hw])-binding region) and their deployment in constructs has been shown to protect transgenes from position effects by their ability to establish independent functional domains within a chromosome (reviewed by Geyer 1997). Although a few such elements (e.g. SARs/MARs) have been shown to function as insulators of transgene expression in plant systems (Breyne et al. 1992; Mlynarova et al. 1994), they are also known to contain enhancing activities (Allen et al. 1993, 1996; Vain et al. 1999) and are therefore unsuitable for use in context of the present study. The scs/scs′ and su[Hw]-binding region insulators (identified from the *Drosophila* genome) lack any significant enhancer activity and have been shown to contain multiple components (*cis* and *trans*-acting factors) which function cooperatively to block enhancer-promoter communication when placed between the enhancer and the promoter (Geyer 1997). However, to the best of our knowledge, such insulators lacking enhancer function have not been tested in plant systems. In this study, the efficacy of using a simple Spacer DNA sequence as an insulating element has been established using one of the strongest constitutive promoters (35S) and a highly potent cytotoxic gene (*barnase*). It follows therefore, that the proposed strategy would be applicable to any other sequence combination with similar properties and can be used to circumvent problems associated with transformation of other crop plants with cytotoxic genes for development of male-sterile lines or for cell/tissue ablation studies.

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