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Retransformation of a male sterile *barnase* line with the *barstar* gene as an efficient alternative method to identify male sterile–restorer combinations for heterosis breeding

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Abstract We report in this study, an improved method for identifying male sterile–restorer combinations using the *barnase–barstar* system of pollination control for heterosis breeding in crop plants, as an alternative to the conventional line × tester cross method. In this strategy, a transgenic male sterile *barnase* line was retransformed with appropriate *barstar* constructs. Double transformants carrying both the *barnase* and *barstar* genes were identified and screened for their male fertility status. Using this strategy, 66–90% of fertile retransformants (restored events) were obtained in *Brassica juncea* using two different *barstar* constructs. Restored events were analysed for their pollen viability and copy number of the *barstar* gene. Around 90% of the restored events showed high pollen viability and ∼30% contained single copy integrations of the *barstar* gene. These observations were significantly different from those made in our earlier studies using line (*barnase*) × tester (*barstar*) crosses, wherein only two viable male sterile–restorer combinations were identified by screening 88 different cross-combinations. The retransformation strategy not only generated several independent restorers for a given male sterile line from a single transformation experiment but also identified potential restorers in the T_0 generation itself leading to significant savings in time, cost and labour. Single copy restored plants with high pollen viability were selfed to segregate male sterile (*barnase*) and restorer (*barstar*) lines in the T_1 progeny which could subsequently be diversified into appropriate combiners for heterosis breeding. This strategy will be particularly useful for crop plants where poor transformation frequencies and/or lengthy transformation protocols are a major limitation.

Keywords *Barnase–barstar* · *Brassica juncea* · Fertility restoration · Heterosis breeding · Retransformation

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Introduction

Hybrid vigor or heterosis is a well-known phenomenon in plant breeding and has been successfully used to break the yield barrier in various crop plants. Development of commercial hybrids in a crop requires a stable pollination-control mechanism and suitable combiners (parents) that would contribute to high heterosis. Hybrid production in agricultural crops requires introduction of male sterility into one of the combiners that would function as the female parent. In crop plants wherein seeds are the harvested product, the male parent must also contain a fertility restoration mechanism to ensure seed set in the F_1 hybrids.

The *barnase–barstar* system is one of the most extensively studied transgenic systems till date for the development of male sterile and restorer lines in crop plants. Induction of male sterility and its restoration by tissue (tapetum)-specific expression of a ribonuclease gene (*barnase*) and its corresponding inhibitor (*barstar*) from the bacterium *Bacillus amyloliquefaciens* was first reported by Mariani et al. (1990, 1992). We have reported significant improvements in the *barnase–barstar* strategy in our earlier work on development of male sterile and restorer lines in *B. juncea* (mustard),

a major oilseed crop of the Indian subcontinent (Jagannath et al. 2001; Bisht et al. 2004). Jagannath et al. (2001) described the use of a Spacer DNA fragment as an effective insulator to prevent deregulated expression of the *barnase* gene (driven by the tapetum specific promoter, TA29) to achieve high frequency generation of agronomically viable male-sterile lines in *B. juncea*. Significant improvements in restoration abilities were achieved by using improved *barstar* constructs based on the simultaneous use of a wild-type and a codon-modified sequence of the *barstar* gene under independent transcriptional control of two different tapetum-specific promoters (A9 and TA29) with overlapping expression profiles (Bisht et al. 2004). Using this strategy, the temporal expression pattern and the levels of barstar protein were altered favourably leading to the identification of efficient restorers for all the *barnase* lines that were tested in combination with the same.

However, development of agronomically suitable male sterile–restorer combinations using the above strategy required production of a large number of independent *barstar* transgenic lines, identification of single copy transformants, crosses between *barnase* and *barstar* lines in various possible combinations and screening a large population of F_1 progeny plants for their male sterility/fertility status, pollen viability and segregation of the *barnase* and *barstar* genes. For example, in our earlier work using TA29-*barstar* constructs (Jagannath et al. 2002; Bisht et al. 2004), 24 single copy *barstar* lines (identified by Southern analysis of 145 transgenic plants) were crossed with five single copy male sterile *barnase* lines in 88 different combinations. Following screening of about 6100 F_1 plants (approx. 70 progeny plants per cross) for their male fertility/sterility status, segregation of the *barnase* and *barstar* genes (by Tissue-PCR) and pollen viability, only two viable male sterile–restorer combinations could be identified. Using the improved two gene-two promoter strategy for enhanced expression of *barstar* gene (Bisht et al. 2004), 23 suitable male sterile–restorer combinations could be identified from 40 tested combinations. This required screening of around 2400 F_1 plants derived by crosses between ten single copy *barstar* lines (identified from 52 plants screened by Southern analysis) and four different *barnase* lines. This method for identification of agronomically viable male sterile–restorer combination(s) is therefore not only time-consuming, expensive and labour intensive, but also requires large tracts of transgenic containment area for screening the F_1 progeny plants. Moreover, using the line × tester cross method, a perfect male sterile–restorer combination can be identified only in the F_1 generation.

The current study describes an efficient, cost-effective alternative to the conventional line × tester cross method for identifying agronomically viable male sterile–restorer combinations. In this strategy, a transgenic male sterile *barnase* line was retransformed with a suitable *barstar* construct and male fertile double transformants carrying both the *barnase* and *barstar* genes were identified among the T_0 plants. Fertile (restored) double transformants were analysed for their pollen viability and copy number status of the *barstar* gene to identify potential male sterile–restorer combinations. Selfing of single copy restored events with high pollen viability would segregate the *barnase* and *barstar* lines in the T_1 generation which could subsequently be diversified into appropriate combiners for heterosis breeding.

Materials and methods

Barstar gene constructs

Two different *barstar* constructs [developed using the basic binary vector pPZP200 (Hajdukiewicz et al. 1994)] were tested in the current study. The first construct (*hpt*::TA29-*bswt*) contains the wild-type *barstar* gene driven by an 870 bp fragment of the TA29 promoter (Koltunow et al. 1990) while the second construct (*hpt*::A9-*bsmod*::TA29-*bswt*; Bisht et al. 2004) harbours two different *barstar* expression cassettes – the modified sequence of the *barstar* gene driven by a 1.5 kb fragment of the *A. thaliana* A9 promoter (Paul et al. 1992) and the wild-type *barstar* gene driven by the TA29 promoter. Both constructs also contained a 35S-*hpt*-35SpA cassette [hygromycin phosphotransferase gene (*hpt*) driven by 35S promoter and with the 35S polyA signal at its 3′ end]. All DNA manipulations were performed using standard protocols (Sambrook et al. 1989). The constructs were mobilized into the *Agrobacterium tumefaciens* strain GV3101 by electroporation (Mattanovich et al. 1989) and used for genetic transformation of *B. juncea*.

Retransformation of a male-sterile *barnase* line (bn 3.6) with the *barstar* constructs

A male sterile *barnase* line of *B. juncea* (*bn* 3.6) developed earlier in the laboratory (Jagannath et al. 2001) was used for transformation with the *barstar* constructs. The *barnase* line contains the *barnase* gene under transcriptional control of tapetum-specific TA29 promoter and the *bar* gene [conferring phosphinothricin (PPT)-resistance] driven by the CaMV35S promoter (with the AMV leader sequence) as a plant selection marker. Genetic transformation of *B. juncea barnase* line (*bn* 3.6) was carried out following the protocols described earlier (Mehra et al. 2000; Jagannath et al. 2003). Selection of explants was performed on regeneration medium containing 20 mg l^{-1} of hygromycin. Regenerating explants were transferred after 25–30 days to regeneration

medium containing both hygromycin (20 mg l^{-1}) and phosphinothricin [10 mg l^{-1}; active component of the commercial herbicide formulation, Basta (Agrevo)]. Putative transformed shoots were transferred for rooting on MS medium supplemented with 2 mg l^{-1} IBA (indole-3-butyric acid), hygromycin (20 mg l^{-1}) and phosphinothricin (10 mg l^{-1}).

Transgenic plants were transferred to soil in the months of October–November and grown in a containment net-house in accordance with guidelines of the Department of Biotechnology, Government of India.

Characterization of transgenic plants

Male fertility/sterility status of transgenic plants was established based on morphological observations of anthers vis-à-vis pollen production and their ability to set seed on selfing. Two to three inflorescence axes (each containing ~ eight to ten unopened buds) of each plant were covered with pollination bags to test for the formation of selfed seeds. Presence/absence of selfed seeds was taken as a confirmation of male fertility/sterility respectively. Pollen viability assays were performed by fluorescein diacetate (FDA) staining following Heslop-Harrison et al. (1984). 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. Tissue-PCR for ascertaining the presence/absence of the *barnase* and *barstar* gene(s) was performed following Klimyuk et al. (1993). Transgenic plants were subjected to Southern analysis to identify plants with single copy insertions of the *barstar* expression cassette. Genomic DNA isolations, gel electrophoresis and hybridisations were performed according to protocols described earlier (Jagannath et al. 2001). Only single copy plants were selfed and analysed further.

Results

Retransformation of the *barnase* line *bn* 3.6 with *barstar* constructs and analysis of T_0 double transformants

The *B. juncea* male sterile *barnase* line, *bn* 3.6 (Jagannath et al. 2001) used for retransformation by two *barstar* constructs contained a single copy of the *barnase* gene and the *bar* gene as a selectable marker in hemizygous condition. The design of the retransformation experiment has been schematically represented in Fig. 1. A total of 95 and 114 double transformants were obtained using the *hpt*::TA29-*bswt* and *hpt*::A9-*bsmod*::TA29-*bswt* constructs, respectively in a single retransformation experiment (Table 1).

A representative population of T_0 double transformants obtained was analysed for their ability to form pollen. The plants were classified as complete male fertile (successful restoration wherein the barstar protein completely inhibits the activity of barnase in tapetum), complete male sterile (unsuccessful restoration or presence of *barnase* gene alone) and semi-fertile (bearing fertile as well as sterile anthers and/or flowers indicating chimeras).

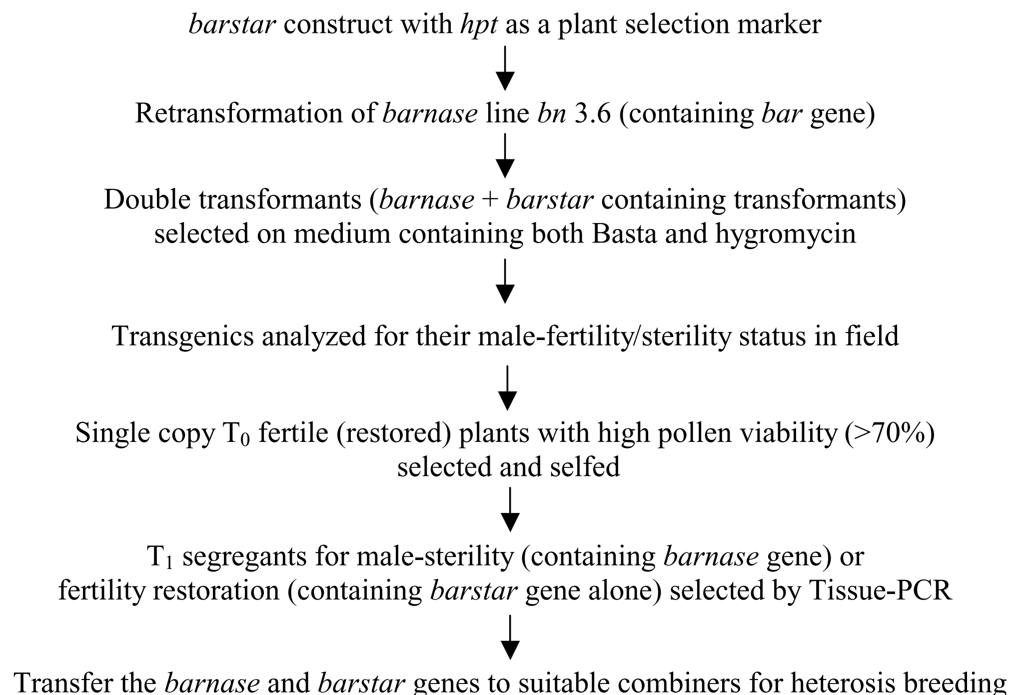

Fig. 1 Schematic representation of various steps performed in retransformation of *barnase* line, *bn* 3.6 for obtaining male sterile/restorer combinations

Table 1 Retransformation of a male sterile *barnase* line (*bn* 3.6) with two different *barstar* constructs: fertility restoration studies

	Construct	
	hpt::TA29-*bswt*	*hpt*::A9-*bsmod*::TA29-*bswt*
No. of explants inoculated	920	1276
No. of $Hyg^R + Bas^R$ plants	95	114
No. of plants analysed in field	64	88
Fertility status based on pollen formation		
F	42	79
S/SF	22	9
Frequency of fertile (restored) plants	65.6	89.8
Pollen viability of fertile events		
Class I (> 70%)	38	73
Class II (< 70%)	4	6
No. of plants (class I) analysed for copy number	20	44
No. of single copy plants obtained	6	14

TA29—tapetum specific promoter TA29, A9—tapetum specific promoter A9, *hpt*—hygromycin resistance conferring gene, *bswt*—wild-type sequence of *barstar* gene, *bsmod*—modified sequence of *barstar* gene, *bn*—*barnase* gene, Hyg^R - hygromycin resistant plants; Bas^R—Basta resistant plants, F—fertile, S—sterile, SF—semi-fertile.

When the male sterile line, *bn* 3.6 was transformed with the *hpt*::TA29-*bswt* construct, around 66% (42/64) of the double transformants were completely male fertile (Table 1). These restored plants were analysed for their pollen viability by FDA staining to determine the extent of restoration and were classified into two classes based on their pollen viability: < 70% and > 70%. Our earlier observation for pollen viability and seed set indicate that pollen viability of ~70% or above is sufficient to achieve good seed set (unpublished data). Approximately 90% of the T_0 fertile plants showed > 70% pollen viability (Table 1) and proper seed set on selfing. In case of retransformation with the two gene-two promoter construct (*hpt*::A9-*bsmod*::TA29-*bswt*), the frequency of fertile plants among the double transformants increased significantly to around 90% as compared to the *hpt*::TA29-*bswt* construct. A majority of these fertile plants (92%) showed > 70% pollen viability reflecting enhanced expression of the *barstar* gene in tapetal tissues.

A representative population of T_0 fertile (restored) plants with > 70% pollen viability were subjected to Southern analysis for identifying single copy events (Table 1). Genomic DNA of transgenic plants was digested with *Hinc*II and blots were probed with the wild-type *barstar* gene for copy number analysis on the RB flank. Single copy events thus identified were subsequently digested with *Xba*I and probed with a partial fragment of the 35S-*hpt*-pA cassette for analysis of copy number on the LB flank (Fig. 2a). Plants showing single copy integration on both flanks of the T-DNA were considered as true single copy events. A representative example of a Southern blot for the (re)transformants developed using the *hpt*::A9-*bsmod*::TA29-*bswt* construct and probed with the *barstar* gene for RB integration is given in Fig. 2b. A total of 6 and 14 single copy *barstar* double transgenics were obtained out of 20 and 44 plants analysed for the *hpt*::TA29-*bswt* and *hpt*::A9-*bsmod*::TA29-*bswt* constructs respectively indicating that single copy plants were obtained at approximately similar frequencies (~30%) with each construct.

Characterization of T_1 progeny: segregation of *barnase* (*bar*::*bn*) and *barstar* (*hpt*::TA29-*bswt* or *hpt*::A9-*bsmod*::TA29-*bswt*) plants

Six single copy T_0 restored events obtained using the *hpt*::TA29-*bswt* construct (L2-1, L2-7, L2-25, L2-26, L2-48 and L2-64) and four events for the *hpt*::A9-*bsmod*::TA29-*bswt* construct (K2-25, K2-31, K2-40 and K2-47) were taken for further analysis. Ten T_1 plants (selfed progeny of T_0 plants) for each event were grown in the field and analysed for the presence of the *barnase* and *barstar* genes by tissue PCR. Of a total of 97 T_1 plants (derived from ten independent restored events, Table 2) analysed by tissue-PCR, 48 plants contained both *barnase* and *barstar* genes, 21 plants contained only *barnase* gene, 23 plants contained only *barstar* gene and 5 plants were free from both *barnase* and *barstar* genes. This segregation profile was observed to fit to the expected digenic segregation ratio of 9:3:3:1. The segregation profiles for these genes were correlated with the male sterility–fertility status and pollen viability of each T_1 plant (Table 2). All sterile plants contained only the *barnase* gene (*bar*::*bn*) while the fertile plants carried three types of gene combinations: *barstar* gene alone (*hpt*::*bs*), both *barnase* and *barstar* genes (*bar*::*bn*-*hpt*::*bs*) and absence of both *barnase* and *barstar* genes. However, it was not possible to ascertain the genotype (homozygous or heterozygous) of male sterile *barnase* and male fertile *barstar* segregants in this generation. All the fertile T_1 progeny containing both *barnase* and *barstar* genes as well as *barstar* gene alone were also found to have a pollen viability > 70% comparable to that of the parent T_0 event reflecting stable inheritance of the restoration ability in subsequent generations.

For all events except two (L2-7 and K2-47), the T_1 progeny segregated for the *barnase* and *barstar* genes in accordance with their male fertility–sterility status (Table 2). In case of the above events, some semi-fertile plants were also observed in the T_1 progeny. Tissue-PCR analysis of such plants showed the presence of both *barnase* and *barstar* genes indicating the breakdown of fertility restoration in the T_1 generation for these events. Since the retransformation strategy provides a backup of several independent restorers for a given *barnase* line, problems arising due to the

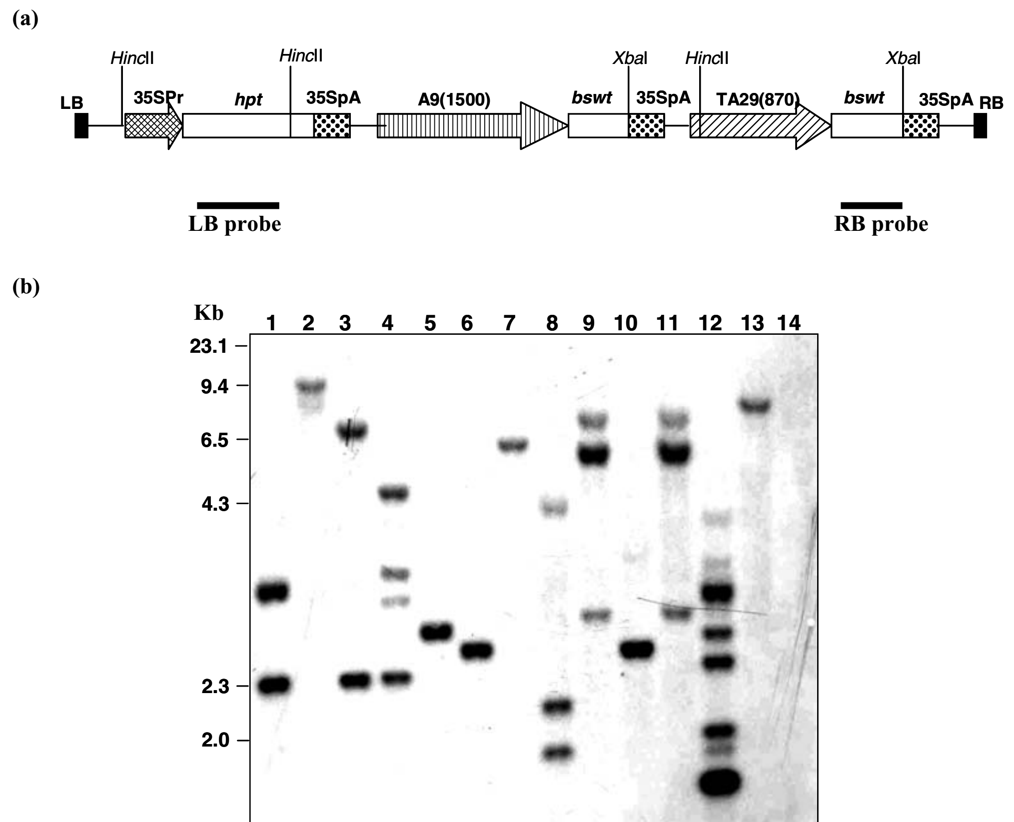

Fig. 2 (**a**) T-DNA map of *hpt*::A9-*bsmod*::TA29-*bswt* construct. (**b**) Southern analysis of a representative population of T_0 restored (fertile) plants obtained from a retransformation experiment using the above construct. Genomic DNA of T_0 restored events was digested with *Hinc*II and probed with *bswt* fragment for analysis of copy number at the RB flank. The single copy plants thus obtained were digested with *Xba*I and probed with a partial fragment of *hpt* gene for analysis of copy number at the LB flank (data not shown). Positions (in kb) of Lambda DNA/*Hind*III size marker are indicated. Lane no. 14 indicates untransformed wild type plant (negative control). Abbreviations: 35SPr – cauliflower mosaic virus (CaMV) 35S promoter, 35SpA – polyA sequence of 35S transcript, LB and RB – left and right border of T-DNA of *A. tumefaciens* Ti plasmid respectively

breakdown of restorer lines in subsequent generations can readily be addressed.

Analysis of F_1 progeny obtained from crosses between *barnase* (*bar*::*bn*) and *barstar* (*hpt*::TA29-*bswt* or *hpt*::A9-*bsmod*::TA29-*bswt*) T_1 segregants

The efficacy of the retransformation strategy in identifying suitable restorers was further corroborated by analyzing the F_1 progeny derived from a cross involving randomly selected male sterile *barnase* (*bar*::*bn*) and male fertile *barstar* (*hpt*::*bs*) segregants from T_1 generation, for each *barstar* construct. Approximately, 70 F_1 plants were grown in the field and sprayed with Basta (at a final concentration of 200 mg l^{-1} phosphinothricin). The F_1 progeny segregated in a 1:1 ratio for Basta resistance/sensitivity, thereby indicating that the T_1 *barnase* segregants taken in the two crosses were heterozygous in nature (Table 3). Further the presence of both male sterile and fertile plants in the Basta resistant F_1 progeny of the two crosses indicated that the *barstar* gene in the T_1 plant was also in the heterozygous condition (Table 3). While the Basta resistant F_1 progeny derived from L2-25 line segregated in the 1:1 ratio for male sterility/fertility, the progeny derived from K2-25 line showed distortion in segregation which could probably be due to the small number of individuals analysed in this cross.

Discussion

The strategy outlined in this paper is a more efficient alternative for obtaining suitable restorers for a given male sterile *barnase* line over the line × tester cross method which is both time-consuming and labour intensive. In the current study, two different *barstar* constructs were used to transform a male sterile *barnase* line, *bn* 3.6, and the double transformants carrying both *barnase* and *barstar* genes were selected and analysed for their male fertility/sterility status and pollen viability. Using the basic *hpt*::TA29-*bswt* construct, about 66% of the double transformants were fertile (restored) while the two gene-two promoter construct (*hpt*::A9-*bsmod*::TA29-*bswt*) provided ∼90% of fertile (restored) double transformants. In both the cases, about 90% of the restored plants showed pollen viability > 70%, which is necessary for normal self seed formation. Thus, there was a major improvement in obtaining restorer lines by retransformation as compared to the conventional methodology of developing independent, single copy *barnase* and *barstar* lines and identifying restorers by crossing *barnase* and *barstar* lines in line × tester crosses. For example, in our previous studies using the TA29-*barstar* construct, when a total of 24 single copy *barstar* lines were crossed with five different *barnase* lines, only two *barstar* lines were identified as potential restorers for only one of the *barnase* lines, following analysis

Table 2 Analysis of a representative population of T_1 plants (Progeny #1–10) derived by selfing single copy T_0 restored events: segregation of male-fertile/sterile plants and pollen viability vis-à-vis segregation of the *barnase* and *barstar* genes

Restored event	Construct code									
	hpt::TA29-*bswt*						*hpt*::A9-*bsmod*::TA29-*bswt*			
	L2-1	L2-7	L2-25[$]	L2-26	L2-48	L2-64	K2-25[$]	K2-31	K2-40	K2-47
PV(%) in T_0	97	98	98	95	90	87	93	91	92	87
Prg #1	F(-/bs) [86]	F(bn/bs) [80]	S(bn/-)	F(-/bs)	S(bn/-)	F(bn/bs)	F(-/bs)	F(-/bs) [94]	F(bn/bs) [87]	F(bn/bs) [88]
Prg #2	S (bn/-)	F(bn/bs)	F(bn/bs) [80]	S(bn/-)	F(bn/bs) [87]	F(-/bs)	F(-/bs)	S(bn/-)	F(-/-)	S(bn/-)
Prg #3	–	F(bn/bs) [90]	F(bn/bs) [75]	F(-/bs)	S(bn/-)	F(-/bs)	F(-/-) [93]	F(bn/bs)	F(bn/bs)	SF(bn/bs)
Prg #4	F (bn/bs)	S(bn/-)	F(bn/bs)	S(bn/-)	F(bn/bs) [85]	F(bn/bs) [84]	F(-/bs) [93]	F(-/-)	F(bn/bs) [85]	nd(-/bs)
Prg #5	F(bn/bs) [93]	SF(bn/bs)	F(-/bs)	nd(bn/bs)	F(-/bs)	S(bn/-)	F(-/bs)	F(-/bs)	S(bn/-)	F(-/-)
Prg #6	F (-/bs)	S(bn/-)	S(bn/-)	S(bn/-)	F(-/bs)	F(bn/bs)	S(bn/-)	S(bn/-)	F(bn/bs)	F(bn/bs)
Prg #7	F(bn/bs) [88]	F(bn/bs)	F(bn/bs)	F(-/bs)	S(bn/-)	F(bn/bs) [83]	F(bn/bs) [71]	F(-/-) [91]	S(bn/-)	F(bn/bs)
Prg #8	F (bn/bs)	SF(bn/bs)	F(bn/bs) [80]	F(bn/bs)	F(-/bs)	F(-/bs)	F(bn/bs) [74]	F(bn/bs) [88]	F(bn/bs)	F(bn/bs) [84]
Prg #9	F (bn/bs)	F(bn/bs)	S(bn/-)	S(bn/-)	F(bn/bs)	F(bn/bs)	S(bn/-)	F(bn/bs) [88]	F(-/bs)	
Prg #10	F (bn/bs)	F(-/bs)	F(-/bs)	F(bn/bs)	F(bn/bs)	F(bn/bs)	F(-/bs)	F(bn/bs)	F(bn/bs)	

(L2-1, L2-7, L2-25, L2-26, L2-48, L2-64) and (K2-25, K2-31, K2-40 and K2-47) are single copy T_0 restored events obtained by retransforming *barnase* line *bn* 3.6 with *hpt*::TA29-*bswt* and *hpt*::A9-*bsmod*::TA29-*bswt* constructs, respectively. Each cell represents the fertility status (F—fertile, S—sterile; SF—semi-fertile) followed by presence of *barnase* (bn) and/or *barstar* (bs) gene in the T_1 progeny. The pollen viability (in %) observed in some cases is represented in square brackets [ ]. nd—not determined. [$]The T_1 *barnase* (*bar*::*bn*) containing progeny of this event was crossed to a corresponding *barstar* plant (*hpt*::*bs*) and the F_1 progeny thus obtained were analysed in the subsequent generation.

Table 3 F_1 progeny profile of crosses between T_1 male-sterile (*bar*::*bn*) and T_1 restorer (*hpt*::*bs*) plants. Values in parentheses represent the chi-square value for the respective 1:1 ratios

Fertile (restored) event (T_0)	L2-25	K2-25
Female × male progeny (T_1)	#1 × #5	#9 × #5
No. of seeds transplanted	70	70
Basta resistance status	30 res : 38 sens (0.94)	26 res : 23 sens (0.18)
Male-fertility status	16F : 12S (0.57)	4F: 13S (4.76)

res—resistant, sens—sensitive, F—male-fertile, S—male-sterile.

of F_1 progeny. The retransformation strategy reported here also helped in the identification of potential restorers in the T_0 generation itself, thereby saving cost and labor in developing and analyzing several hundred F_1 progeny obtained from each *barnase* × *barstar* cross.

The frequency of single-copy plants in the restored population obtained using both the constructs was around 30% which clearly indicated that the higher frequency of fertile (restored) plants using the two gene-two promoter construct (*hpt*::A9-*bsmod*::TA29-*bswt*) was a reflection of construct efficacy alone and not due to any bias towards multiple copy integration in the restored plants. The two gene-two promoter construct, therefore, provides very high restoration capabilities. This observation is in consensus with our earlier observation wherein we have shown the use of the two gene-two promoter strategy for effective restoration of different male sterile *barnase* lines using the line × tester cross method (Bisht et al. 2004).

Using the strategy mentioned in this study, a large number of effective single-copy restorers could be identified in the T_0 generation, following stringent selection based on pollen viability, self seed formation and Southern analysis. The *barnase* and *barstar* genes could be segregated subsequently in the T_1 generation to obtain effective restorer lines. Since every T_0 restored plant is an independent transformation event, T_1 plants (derived from each restored event) containing the *barstar* gene alone would function as an independent restorer line for the same male sterile *barnase* line and could be maintained by selfing or could be transferred to a desired combiner by backcrossing. The retransformation strategy confers an additional advantage by providing a substantial backup of many independent restorers for a given *barnase* line which would be most useful if some restorer lines falter over subsequent generations. Further, the combined use of the retransformation strategy and the two

gene-two promoter construct mentioned in this study would be particularly useful for crop plants like cotton and grain-legumes, wherein transformation protocols are difficult and lengthy, as a large number of restored events can be easily obtained in the T_0 generation.

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