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Double recoverable block of function – a molecular control of transgene flow with enhanced reliability

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Despite all the achieved benefits and potential promises from recombinant DNA technology of plants, the potential of transgene spread to wild relatives and to non-transgenic crops is still of a wide-spread concern. We continue to develop recoverable block of function (RBF) technology for gene flow control in transgenic plants. RBF consists of two elements: blocking construct (BC) and recovering construct (RC). Natural expression of the BC (*barnase*) in embryos and sprouts blocks a physiological function essential for survival or reproduction of the transgenic plant (mRNA synthesis and germination). Artificially induced (heat shock treatment) RC (*barstar*) recovers the blocked function enabling transgenic plant to reproduce. In natural conditions without artificial induction of RC the transgenic plant can not reproduce itself. However, a single RBF may still fail because of the potential for mutations and gene silencing of the inserted constructs. To minimize the frequency of such an inactivation, we developed a double RBF, in which a single insert comprising two BC, flanking a transgene of interest, was constructed and transferred into tobacco (*Nicotiana tabacum* (*L.*)). We used a *barstar* gene driven by a heat shock or 35S promoter as a RC, and two different promoters were used for *barnase* genes in the BC. One BC contained a seed germination specific cysteine endopeptidase promoter (BC_1) and the other contained the cruciferin promoter (BC_2), which is active during fruit development and embryogenesis. Three alternative constructs of double RBF are described, and a segregating two-insert as well as a one-insert cassettes, were compared. One-insert system comprising two BC with different nucleotide sequences but degenerate codons that expressed the same Barnase protein appeared to be the most reliable choice. The biological and molecular data obtained suggest that double RBF is a potent transgene containment technique that can safely be applied in agriculture.

Key words: *barnase* / *barstar* / control of gene flow / gene containment / GMO risk management / tobacco (*Nicotiana tabacum* (*L.*)) / recoverable block of function

Abbreviations: RBF: recoverable block of function; **BC:** blocking construct; **RC:** recovering construct; **TGI:** transgene of interest; **UTR:** untranslated region; **HSp:** heat shock promoter; **CRUp:** cruciferin promoter; **SH-EPp:** cysteine endopeptidase promoter

INTRODUCTION

The potential for transgene flow has received a wide attention from the scientific community. Gene flow has been reported for squash, carrot, maize, sorghum, sunflower, strawberries, sugar beet and Brassica species (Gray, 2000; Kling, 1996; Lazzeri and Shewry, 1993). Several research groups have been working with gene flow mitigation concepts and have developed systems based on the action of negative selection and rescuing factors (Bright et al., 1994; Fabijanski et al., 2004; Kuvshinov et al., 2002; 2005; Shernthaner et al., 2003).

We reported earlier a recoverable block of function (RBF) with transgenic tobacco (*Nicotiana tabacum* (*L.*)) as a model plant (Kuvshinov et al., 2001; 2004). This system consists of a blocking construct (BC) linked to a transgene of interest (TGI) and a recovering construct (RC). The gene product of the BC, regulated by a developmental or tissue-specific promoter, blocks a physiological function vital for the reproduction of the host plant. To recover the blocked function of the host plant, the expression of the RC is deliberately induced by

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an external stimulus, such as heat shock. The blocking construct used in our previous study (Kuvshinov et al., 2001; 2004) consisted of a *barnase* gene, regulated by the seed germination specific cysteine endopeptidase promoter (Akasofu et al., 1990; Yamauchi et al., 1996), which is specifically active during embryogenesis and seed germination (Kuvshinov et al., 2001). We have also used a cruciferin promoter originating from *Brassica napus*, which is active during embryogenesis (Rodin et al., 1992). The RC consisted of a *barstar* gene driven by the soybean heat shock promoter (Czarnecka et al., 1989). In natural conditions, the transgenic plants and their hybrids would produce seeds incapable of germination. This situation would be reversed upon the activation of the recovering construct through heat treatment of the mother plant during flowering and seed setting. All of the proposals mentioned above use a single blocking gene to stem transgene flow however a single RBF is subject to failure due to spontaneous mutations or gene silencing.

To minimize the frequency of such events, we developed a double RBF strategy, in which two BC are placed in an insert flanking the TGI. This strategy greatly minimizes the possibility of gene silencing and failure due to spontaneous mutations. Herein we describe three functional constructs of double RBF consisting of a segregating two-insert and one-insert system both of which use a single RC. In addition we discuss different possible constructs of double RBF.

RESULTS

Two-insert double RBF (segregating)

Tobacco plants were twice-transformed with two constructs shown in Figure 1A and 1B. Positive shoots containing GUS and *barstar* were rooted and then transferred to the greenhouse. Plants having abnormal morphology (25–30% of all transgenics), presumably caused by unspecific excessive expression of *barnase*, were removed from further experiments. Plants of each transgenic line, which were included in subsequent experiments, all presumably expressed moderate levels of *barnase* mRNA, flowered and set fruits in ambient and heat shock conditions. Pollen grains from transgenic plants were used to pollinate non-transgenic plants. GUS- and *barstar*-positive transgenic lines exhibiting normal phenotype were divided into three groups:

(1) those that flowered and produced fruits with seeds that germinated without heat shock treatment, indicating insufficient *barnase* activity;

(2) those that formed normal flowers in ambient temperature but the flowers dried before forming fruits, indicating *barnase* activity and heat shock treatment enabled these plants to form fruits and to produce viable seeds;

(3) those that were able to form fruits and produce seeds but germination capacity of the seeds depended on whether heat shock treatment was applied to their parent plant or not. Distribution of transgenics between these three groups was even deviating from 25 to 40%. The same distribution was in the regenerants carrying one-insert RBF. Finally, properly acting RBF were found from 10 to 25% of transgenic plants depending on particular construct.

Germination tests, conducted on transgenic seeds originating from self-pollinated or plants that were back-crossed with non-transgenic parental lines, demonstrated a clear Mendelian segregation of the blocking and recovering trait. As an example, germination of seeds from the line HSp-*barstar*-10/5 demonstrated independent segregation of blocking construct containing the transgene of interest, and the recovering construct (Tab. 1). More intensive expression of *barnase*, as was the case in line HSp-*barstar*-2/1, was associated with the death of all self-pollinated seeds in the absence of heat shock treatment.

Harboring 35Sp-*barstar* RC and BC (Fig. 1A and 1B), twice-transformed tobacco plants did not show the adverse symptoms of *barnase* expression. *Barstar*-mRNA and GUS positive lines grew vigorously and produced seeds. Several lines, such as 35Sp *barstar*-5/1 and 35Sp *barstar*-6/3 demonstrated segregation of RC from BC in the germination tests (Tab. 1). Proportions of germinated and ungerminated seeds are in agreement with Mendelian segregation of two genetically unlinked RCs and one BC-TGI-BC insert.

The lines harboring the segregating RBF, with 35Sp-*barstar* as the recovering construct (Fig. 1A and 1B), expressed *barstar* RNA constitutively at a level of 0.3–0.5 pg.μg^{-1} of total RNA. According to Northern blot analysis, *barnase1* RNA was expressed in embryos at the level of 0.02–0.05 pg.μg^{-1} of total RNA. The segregating RBF with HSp-*barstar* transgenic plants were also tested for *barnase* expression. Because BC in segregating RBF encoded the same coding sequences of *barnase*, it was obvious that mRNA transcripts of the two *barnase1* genes were too homologous to separate in Northern hybridization. Therefore, expression of the two versions of BC encoding for *barnase1* was identified separately using RT-PCR with primers designed to anneal to the sequences in the 5' and 3' UTRs, unique to each of the two *barnase1* genes (not shown). Amplified products were

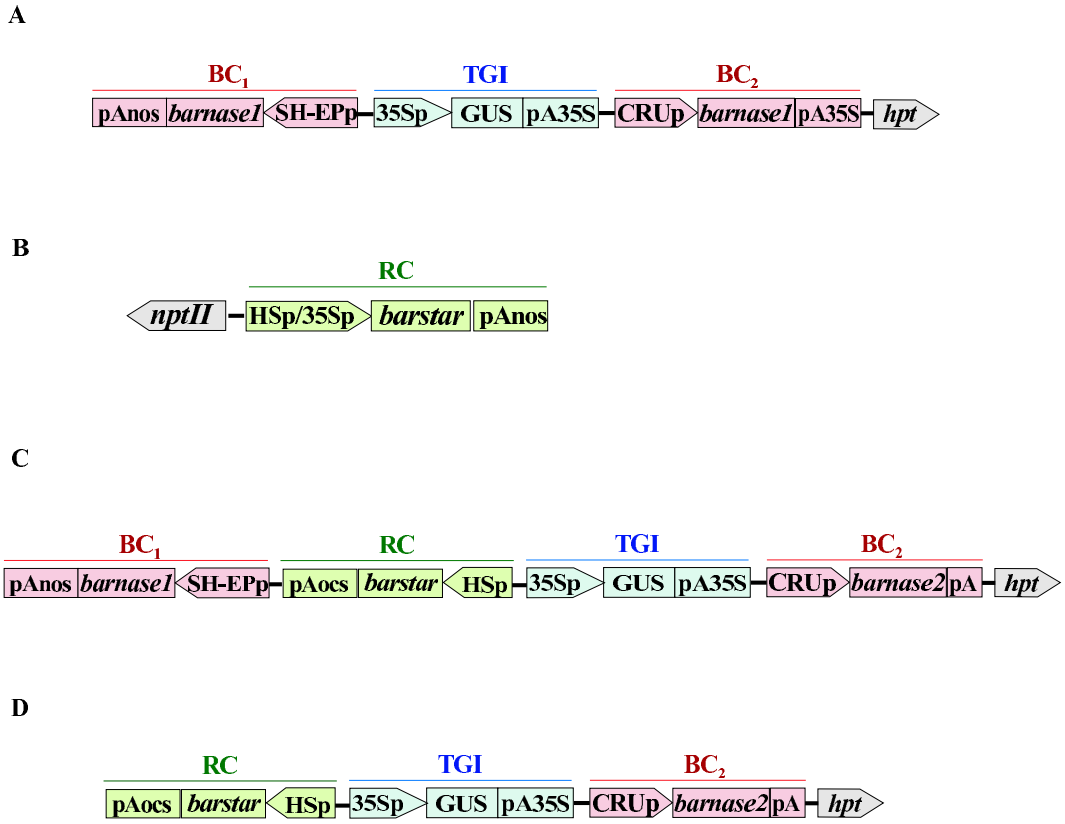

Figure 1. DNA constructs.
A. Two-insert segregating double RBF containing two blocking constructs encoding two identical *barnase1* mRNA and driven by different promoters: cysteine endopeptidase (SH-EPp) from *Vigna mungo* (Akasofu et al., 1990) and cruciferin promoter (CRUp) from *Brassica napus* (Rodin et al., 1992). The GUS gene models the transgene of interest.
B. Recovering construct comprises barstar driven by a heat shock promoter (HSp) from soybean (Czarnecka et al., 1989) or 35Sp.
C. One-insert double RBF (pVK34) with an enhanced GC-enriched *barnase1* driven by cysteine endopeptidase promoter (SH-EPp) and an enhanced AT-enriched *barnase2* driven by cruciferin promoter (CRUp).
D. Simple one-insert RBF (pVK35) comprises *barnase2* driven by CRU promoter.
pA: -polyadenylation signal; P: promoter; *npt*II: neomycin phosphotransferase; *hpt*: hygromycin phosphotransferase; nos: nopalin synthase; ocs: octopine synthase.

sequenced. The sequence of either *barnase* cDNA coincided with its respective physical map in the plant transformation vector. This result supports the expectation that *barnase* mRNA was expressed from both blocking constructs.

One-insert double RBF

The construct as shown in Figure 1C, was developed to demonstrate the efficacy of our double RBF as a single transgenic insert. The two versions of *barnase* genes used in the BC differ in DNA sequences (Fig. 2). Because the functionality of BC_1 was described earlier (Kuvshinov et al., 2001), a vector carrying only a single BC_2, as in Figure 1D, was developed to show that the second BC, used in the study, is also functional. Single insert tobacco lines, which were positive in both GUS and Southern blot analyses, were grown in ambient and in heat shock conditions. Excessively high expression of *barnase*, possibly because of multiple insertions, was associated with an abnormally dwarf phenotype and with poorly developed inflorescences. Lines that expressed insufficient levels of *barnase* showed normal morphology and produced fruits with viable seeds that were able to germinate. Lines expressing a moderate level of *barnase* were selected for further studies. Expression of *barnase2*

Table 1. Germination assays and genetic analysis of transgenic tobacco seeds carrying the recovering constructs HSp-*barstar* or 35Sp-*barstar* (first transformation) and the blocking construct SH-EPp-*barnase1* and CRUp-*barnase1* and 35Sp-GUS as the gene of interest (second transformation). Experimental samples ranged from 100 to 1200 seeds. Lines 2, 5, 6 and 10, which were successfully transformed with the recovering construct, were re-transformed with the blocking construct and the recovered transgene lines were given the serial numbers 1, 2, 3, and 5.

Transgenic line		Pollination	Heat treatment °C	% Germinated
1st transformation	2nd transformation			
HSp-*barstar* 2	1	self-pollinated	42	100
HSp-*barstar* 2	1	self-pollinated	ambient	0
HSp-*barstar* 2	1	crossed with NTS	42	97.5
HSp-*barstar* 2	1	crossed with NTS	ambient	0.8
HSp-*barstar* 10	5	self-pollinated	42	95
HSp-*barstar* 10	5	self-pollinated	ambient	0
HSp-*barstar* 10	5	crossed with NTS	42	72
HSp-*barstar* 10	5	crossed with NTS	ambient	49
35Sp-*barstar* 5	1	self-pollinated	ambient	96
35Sp-*barstar* 5	1	crossed with NTS	ambient	91
35Sp-*barstar* 6	3	self-pollinated	ambient	95
35Sp-*barstar* 6	3	crossed with NTS	ambient	88

Abbreviations: NTS: *Nicotiana tabacum* cv. Samsung; HSp: heat shock promoter of soybean (Czarnecka et al., 1989); SH-EPp: cysteine endopeptidase promoter from *Vigna mungo* (Akasofu et al., 1990); CRUp: cruciferin promoter of *Brassica napus* (Rodin et al., 1992).

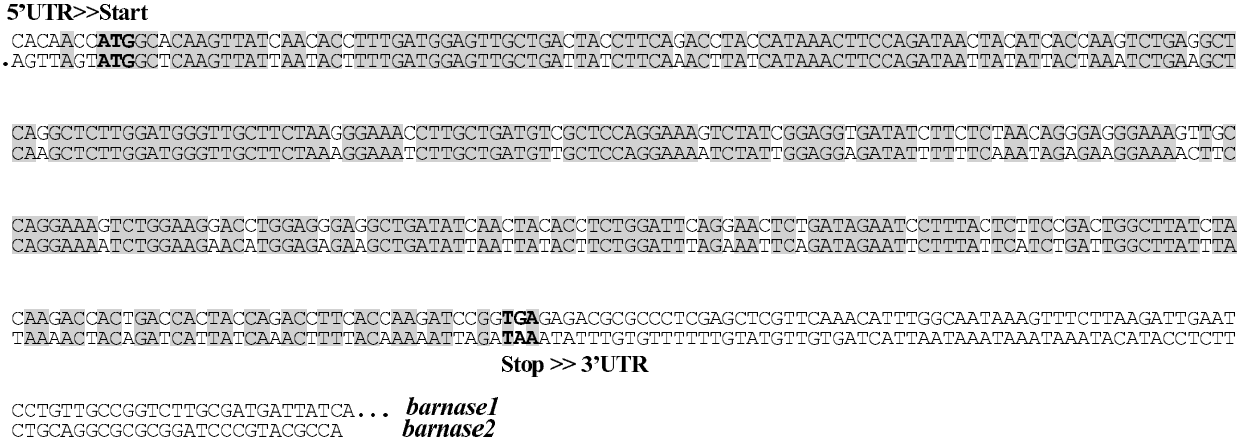

Figure 2. Alignment of the *barnase1* and *barnase2* genes.
Untranslated regions (UTR) and coding sequences of the two *barnase* genes are aligned to show their similarity. Homology between CG-enriched *barnase1* and AT-enriched *barnase2* are marked with gray boxes. The start and the stop of translation are marked as well as the end of the 5'UTR and the beginning of the 3'UTR.

under control of the CRU promoter prevented the carrier plants from developing fruits. The normally developed flowers then dried out and inflorescence suffered senescence. Such a phenomenon has been observed in plants carrying the non-segregating double RBF (Fig. 1C), as well as in those carrying single RBF with BC_2 (Fig. 1D).

The blocking effect of *barnase* expression under CRU promoter have been neutralized by the application of heat shock to plants carrying two BC in the case of double RBF as well as one BC_2 in the case of single RBF. The phenotypical effect of *barnase* expression was similar in segregating and one-insert double RBF systems.

Several lines of transgenic tobacco plants carrying the insert with non-segregating RBF (Fig. 1C) behaved as expected. The molecular data from *barstar* and the two versions of *barnase* coincided with this expected behavior. Following heat shock application, the plants produced seeds with a germination percentage between 90–95%. About three-fourths of the seedlings were GUS positive, which is an indication that the plants carry a single insert of double RBF. Non-transgenic tobacco plants pollinated with transgenic pollens produced seeds with germination percentages close to 100%, provided that heat shock was applied; almost half of these seedlings were GUS positive. In the absence of heat shock, crossed plants (non-transgenic tobacco plants pollinated with transgenic pollen) produced seeds, only about 50% of which germinated. GUS-positive seedlings were not produced among the germinating seeds. Frequencies of the germinated seedlings without heat shock and GUS-positive seedlings after heat shock suggest that most of the analyzed tobacco plants carried a single transgenic insert. Some of the GUS positive seedlings of the second generation were grown to mature plants. These plants showed similar phenotypic traits as their parental lines and produced viable seeds only after heat shock was applied.

Molecular analysis of expression

Plants that were positive in GUS assay and that revealed biological symptoms of *barnase* and *barstar,* were analyzed using Northern blot, RT-PCR, and Real-Time PCR analysis. RNA was isolated from heat shock treated embryos of lines carrying the non-segregating double RBF and single RBF (BC_2). To ensure that each of the two versions of *barnase* used were expressed in the double RBF, the total RNA samples were hybridized with probes developed separately for *barnase1* and *barnase2* (Fig. 3A and 3B). To discriminate authentic signals from unspecific ones, 10 pg of alternative synthetic *barnase* RNA mixed with carrier total embryo RNA, was added to the 8th lane in each blot. The cross hybridization signals from the alternative *barnase* did not exceed 3% of the signal of the test *barnase*. Embryonic mRNA expression levels of *barnase1* and of *barnase2* reached up to 0.05 pg and up to 0.03 pg per μg of total embryo RNA, respectively.

RT-PCR analysis confirmed that both *barnase* mRNA were expressed in the transgenic embryos. Nucleotide sequence data of the amplified product shared complete homology with the respective coding regions of *barnase1* and *barnase2*. Real-Time PCR analysis performed on embryo and ovary total RNA of lines carrying the one-insert (non-segregating) double RBF showed that the level of expression of both *barnases* corresponded to those determined by Northern blot analysis. The result of this analysis indicated that the mRNA level of *barnase1* in embryos was twice as high as in ovaries. In contrast, the level of *barnase2* expression was about 3-fold higher in ovaries than in embryos. These results suggest that *barnase2* driven by CRU promoter (BC_2) was responsible for drying flowers in the transgenic tobaccos carrying double as well as single (BC_2) RBFs.

Expression of *barstar* was determined in the same samples of embryo total RNA that were used for analyzing *barnase* expression. Northern blot analysis of these samples showed that heat shock induced expression of *barstar* ranging from 0.04 to 1.0 $pg.\mu g^{-1}$ of total RNA. The Northern blot hybridization data were consistent with the biological effects of RBF demonstrated by several transgenic lines. The lines S7.1, S7.12, S7.19 harbored the double RBF and the line S20.3 harbored the single RBF (Fig. 3C). Low level of *barstar* expressed by the line S20.2 did not coincide with a positive heat shock effect. This inconsistency can be explained by the application of insufficient heat just before the samples were harvested. In contrast, the weak *barstar* signals of lines S7.4 and S7.8 (Fig. 3C) agree with the poor heat shock effect on germination of seeds from these lines, which never exceeded 75%.

DISCUSSION

RBF systems function satisfactorily well (Kuvshinov et al., 2001), although reliability can still be improved. The main reason for development of double RBF is to address the concern that the transgene of interest (TGI) may freely spread in population in the case of inactivation of BC through either nucleotide mutation, DNA rearrangement, or epigenetic silencing such as DNA methylation and RNA-mediated silencing (Horvath et al., 2001; Tax and Vernon, 2001; Windels et al., 2001). The main goal of the present study was to construct RBF using two BC to overcome these issues. Nucleotide mutation events and epigenetic silencing could be minimized by having two BC, which were flanking the TGI and differing in nucleotide sequence. There are data suggesting that the frequency of mutations is significantly increased in the region surrounding and within the transgene, because of presence of the left border of T-DNA (Noguchi et al., 1999; Tax and Vernon, 2001).

In an attempt to increase reliability, we have earlier introduced a BC into an intron of a transgene of interest (TGI), thus increasing the genetic linkage of the two genes

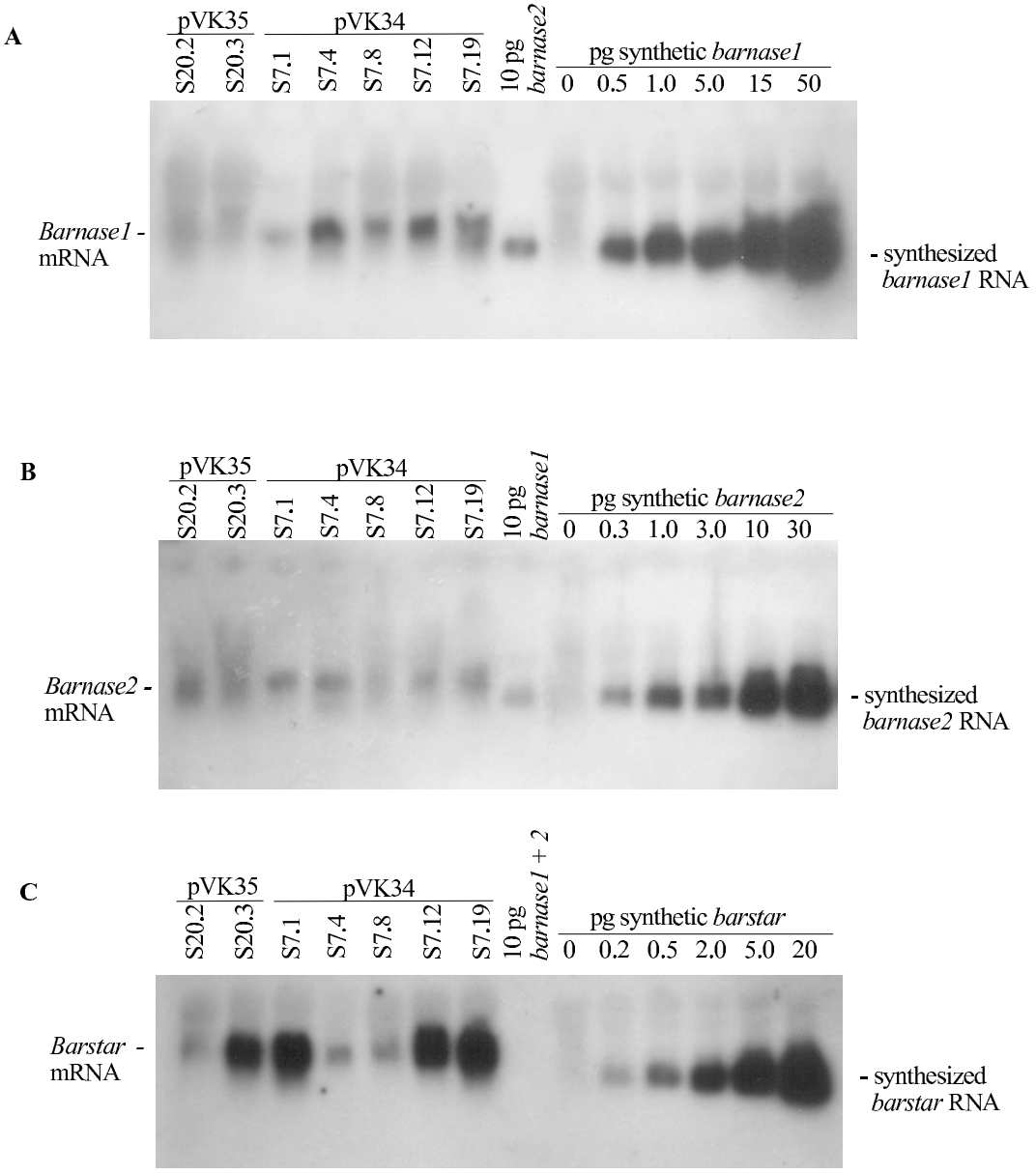

Figure 3. Northern blot analysis of high-CG *barnase1* and high-AT *barnase2* and *barstar* genes in embryos of heat shock treated tobacco plants carrying the double and single RBF constructs.

A. Expression of *barnase1* in tobacco embryos: 10 µg of total embryo RNA isolated from transgenic plants carrying the non-segregating double RBF (pVK34, Fig. 1C) and simple RBF (pVK35, Fig. 1D) were hybridized using a digoxygenin-labeled *barnase1* RNA as a probe. 10 pg of unlabeled *barnase2* RNA were loaded in lane 8. Background caused by cross-hybridization between *barnase1* probe and unlabeled *barnase2* is estimated to be under 3%. *Barnase* signals from lines carrying the non-segregating simple RBF (pVK35) were close to background levels.

B. Expression of *barnase2* in tobacco embryos: The same preparations from transgenic tobacco embryos were compared to similar amounts of *barnase2* RNA. mRNA estimates of *barnase2* were close to 0.03 pg per µg of total embryo RNA. 10 pg of unlabeled *barnase1* RNA were loaded in lane 8.

C. Expression of *barstar* in tobacco embryos: 5 µg samples of total RNA were hybridized with *barstar* probe and compared with 0–20 pg of unlabeled *barstar* RNA. Estimates of *barstar* mRNA ranged from 0.04 to 1.0 pg per µg of total embryo RNA. All samples for control experiments were mixed with 10 µg of total embryo RNA of wild type tobacco.

(Kuvshinov et al., 2004). Mutation in BC would almost surely result in inactivation of TGI because of the overlapping of their sequences. The tandem mitigation concept suggested by Gressel (1999) was an attempt to increase reliability through the introduction of two negative selection factors. His evaluation of possible mutation frequency of one mitigation gene would be 1×10^{-6}. Mutation frequency of two mitigation genes would thus be 1×10^{-12}. These calculations suggest that the probability of nucleotide change to take place is extremely low, particularly when small genes such as *barnase* and *barstar* are used. However, the absence of a recovering means in Gressel's mitigation approach, in contrast to our present alternative, would require several generations for the transgene to be removed from population.

Frequency of transgene silencing may be even higher than the frequency of mutation. To avoid the homology-dependent and post-transcriptional gene silencing (Vaucheret et al., 2001), it is prudent to take into account inverted repeats that often induce gene silencing through DNA methylation. DNA methylation and chromatin structure are the most common regulators of post-transcriptional and transcriptional gene silencing in plants and animals (Morel et al., 2000), which is mediated by small double-stranded RNA (Mette et al., 2000). Hence, to avoid the disadvantages above, we had to use BC that differed in DNA and RNA sequences and driven by heterologous promoters. The different DNA sequences may still encode for the same protein product.

The use of two functionally and structurally different BC may also require two different RC, according to scheme: BC_1-RC_1-TGI-RC_2-BC_2. Placing of two RC in the construct may be less convenient because of larger size of construct and complicated induction of RC. It is thus more convenient and reliable to use functionally similar BC differing in DNA sequences but coding for the same amino acid sequence. In this case, the double RBF can consist of single RC according to scheme: BC_1-TGI-RC-BC_2. Our one-insert double RBF construct cloned in one-insert RBF is representing this kind of system (Fig. 1C). Another realization of the concept could be a system where the RC encodes a repressor for the promoters of two BC, and the promoters of BC contain repressor-binding sequence such as *tet* operator (Gatz et al., 1992). In such a case, BC can be both functionally and structurally different. Recovering process would be an external induction of RC followed by expression of the Tet repressor for the promoters of the BC, and thereby repression of both BC.

Double RBF systems may be constructed in segregating and in single-insert cassettes. The results of the present study show that the segregating RBF with a constitutive 35Sp-*barstar* as well as the inducible HSp-*barstar* (Fig. 1A and 1B) worked as expected. Segregating RBF with constitutive expression of *barstar* is convenient and self-controllable system. However, unlike the inducible RC, the segregating RC requires several generations to remove the TGI from plant population. Another limitation of the segregating RBF, in contrast with the non-segregating (one-insert) RBF, is the uncontrollable spread of the RC. The one-insert double RBF removes the TGI from population in the first generation, because of the tight genetic linkage between the blocking and the recovering constructs.

We did not observe silencing phenomenon in the present study. However, the frequency of epigenetic and mutational silencing should be as low as possible to enable the use of transgenic plants in a large population size, a situation common in practical farming. To reconstruct a double RBF comprising of different DNA sequences of BC, we needed promoters equally active, but with different nucleotide sequences. Known from previous studies – SH-EPp and new in the technology – CRUp were a well-coordinated pair working in double RBF.

We were eager to find out whether the expression level of one Barstar would be sufficient to neutralize Barnase RNase expressed from the two *barnase* genes. Data from Northern blot hybridization showed that *barstar* expression was 10-fold higher than expression of both *barnases* combined, bearing in mind that Barstar binds and inactivates Barnase in an equimolar ratio (Hartley, 1989). The morphology of transgenic plants also proved the possibility to use one RC against two BC in the same RBF.

Evaluating possible limitations and restrictions of the developed technology we have to note that the most narrow feature could be expression of *barnase* in embryos. Embryo specific expression of BC and heat shock induction of RC limit the application of technology to plants producing high amount of small seeds, such as tobacco. Production of viable seeds in an industrial scale under greenhouse conditions looks impossible for plants with larger seeds, such as oilseed rape or soybean. Another limitation is that heat shock conditions occasionally could take place in the open field during fruit maturation. This could lead to the partial production of viable transgenic seeds in open field. Changing expression of BC from embryo stage to another stage of development, *e.g.* early germination, could overcome these limitations.

In conclusion, segregating and one-insert double RBF constructs can be effective in preventing transgene flow. Double RBF can solve the problem of occasional

inactivation of transgene escape control caused by mutagenesis or silencing of blocking construct. Beside two blocking constructs, the main features of the double RBF are as follows:

- blocking constructs on both sides of the TGI that preferably consist of different promoters and gene DNA sequences to prevent silencing;
- blocking constructs differing in DNA sequences that may encode for the same blocking protein; in this case, the double RBF may have a single recovering construct;
- ease of expanding this technology to various plant species using different promoters.

MATERIALS AND METHODS

Bacteria and plant transformation

Escherichia coli, strain XL-1, was used for cloning the DNA constructs. Tobacco plant (*Nicotiana tabacum* cv. Samsung) leaf discs were transformed by *Agrobacterium*-mediated gene transfer as described by Kuvshinov et al. (2001). Transformations with one-insert construct were conducted using a pGPTV-HPT vector (Becker et al., 1992) and transformants were selected on the basis of resistance to hygromycin and GUS activity. For segregating two-insert systems, tobacco plants were first transformed with *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) carrying pGPTV-KAN vector (Becker et al., 1992) with constitutively expressing 35Sp-*barstar* or heat inducible HSp-*barstar* as recovering constructs (RC). The transformed, then regenerated, plants were thereafter subjected to heat shock treatment to induce the HSp-*barstar* transcription. Plants that were positive in Northern blot hybridization against *barstar* probe were chosen for the second transformation with pGPTV-HPT vector (Becker et al., 1992) carrying a GUS gene flanked by two blocking constructs (BC). The two BC expressing *barnase* genes were regulated each by a unique promoter, as in Figure 1A. Tobacco shoots, recovered on hygromycin selection were analyzed in a histological GUS assay. GUS-positive hygromycin resistant plants were transferred from *in vitro* culture to the greenhouse for further studies.

DNA cloning

Two modified versions of a *barnase* gene, originating from *Bacillus amyloliquefaciens,* were used (Hartley, 1989). One version was modified by increasing the CG content (Kuvshinov et al., 2001) and the other by increasing the AT content (Kuvshinov et al., 2004), referred to as *barnase1* and *barnase2*, respectively. Sequence homology of *barnase1* and *barnase2* is depicted in Figure 2. A heat shock promoter from *Glycine max* (Czarnecka et al., 1989) and 35S promoter of CaMV were used to drive the recovering gene, whereas the cysteine endopeptidase promoter of *Vigna mungo* – SH-EPp (Akasofu et al., 1990; Yamauchi et al., 1996) was used to drive *barnase1*, and a cruciferin promoter from *Brassica napus* – CRUp (Rodin et al., 1992) to drive the expression of *barnase2*. The three promoters have been cloned using the high fidelity PCR. BC_1 consisted of *barnase1* regulated by SH-EPp and *nos* polyadenylation signal (Kuvshinov et al., 2001). BC_2 consisted of *barnase2* regulated by CRUp and a short artificial polyadenylation signal (Kuvshinov et al., 2004). In this way, we are able to reconstitute a one-insert RBF with two BC having different nucleotide sequences, but nevertheless, encoding identical Barnase protein. To demonstrate biological action of either BC we developed the vector shown in Figure 1D by removing the SH-EPp-driven blocking construct (BC_1) from the construct depicted in Figure 1C. A GUS gene, containing an intron at the start of the coding sequence (Vancanneyt et al., 1990), was placed under the control of the 35S promoter (Odell et al., 1985), and used to model a transgene of interest.

Analysis of gene expression

Histological GUS assays were conducted according to Gallagher (1992). To ascertain transformation events, a PCR analysis of genomic DNA was performed. Oligonucleotide primers were designed to amplify separately the two versions of *barnase* as well as *barstar* gene. Northern and Southern blot analyses were performed according to the recommendations of Boehringer Mannheim-Roche: the DIG user's guide for filter hybridization. The gene sequences were amplified using primers tailed with the bacteriophage T7 promoter from the 5' end and SP6 promoter from the 3' end. The resulting PCR products were then used as templates for the synthesis of a Digoxigenin-labeled RNA probe as opposite strand and unlabeled control as a full-size sense strand. Serial dilution of picogram amounts of unlabeled control RNA of the genes were mixed with 10 µg of carrier total RNA isolated from the respective organs of non-transgenic plants, and processed in parallel with the sample RNA in Northern blot analysis to estimate transcription levels of the test genes.

Reverse Transcription followed by PCR and real time PCR were performed as described earlier (Kuvshinov

et al., 2004) using specific primers developed for 5'UTR and 3'UTR of *barnase1* and *barnase2*.

Pollination, germination, and heat shock experiments

The experiments were conducted with greenhouse-grown tobacco plants. Heat shock was applied, soon after floral buds were formed, by incubating the plants at 42 °C for 2 hours every second day (Kuvshinov et al., 2001; 2005). The harvested seeds were germinated on moist filter paper at room temperature. Some GUS-positive plants of the second generation of self-pollinated or test-crossed with wild type parents, were grown to maturity and allowed to flower.

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