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Molecular control of transgene escape from genetically modified plants

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Abstract

Potential risks of gene escape from transgenic crops through pollen and seed dispersal are being actively discussed and have slowed down full utilization of gene technology in crop improvement. To ban the transgene flow, barren zones and 'terminator' technology were developed as GMO risk management technologies in transgenic crops. Unfortunately, the technologies have not protected reliably the transgene migration to wild relatives. The present study offers a novel molecular technique to eliminate gene flow from transgenic plants to wild relatives by recoverable block of function (RBF). The RBF consists of a blocking sequence linked to the gene of interest and a recovering sequence, all in one transformable construct. The blocking sequence blocks a certain molecular or physiological function of the host plant. Action of the blocking sequence leads to the death of the host plant or to an alteration in its phenotype resulting in inability for sexual reproduction in nature. The recovering construct recovers the blocked function of the host plant. The recovering construct is regulated externally by a specific chemical or physical treatment of the plants and does not act under natural conditions. In nature, hybrids of the transgenic plants with its wild relatives carrying the RBF will die or be unable to reproduce because of the blocking construct action. A working model of RBF is described in this report as one example of the RBF concept. This RBF example is based on *barnase* (the blocking construct) and *barstar* (the recovering construct) gene expression in tobacco under sulfhydryl endopeptidase (SH-EP) and a heat shock (HS) promoter, respectively.

Keywords: Control of transgene escape; *Barnase*; *Barstar*; Recoverable block of function; GMO risk management; *Nicotiana tabacum*

1. Introduction

Over the past few years, concern over the safety of transgenic crop production has attracted a lot of attention among the scientific community and the public at large. While the potential risks to human or animal health of a particular transgene and its product can be tested and measured, the impact of gene escape is more complex. On the other hand, the potential of transgenic improvement of crops is so high, that rather than banning the use of transgenesis, it is more productive to find solutions to prevent gene escape.

The idea of preventing gene escape is not new. The arrest of embryo development was achieved in *Brassica napus* by expression of a modified exotoxin A of *Pseudomonas aeruginosa* under the napin promoter [1]. In another approach, NPK15, a tobacco protein-serine/threonine kinase, acting as a 'suicide gene' was used to block the proliferation of the host cells [2]. The main inconvenience of these methods is that the block of plant development is unrecoverable and the transgenic plant can be propagated only vegetatively or in vitro. Yet another approach describing the method for arresting seed germination [3] has the same problem as the previous ones. The transgenic plants developed by this method cannot be produced or propagated in the field after inhibition of germination. In fact, the method does not prevent trans-

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gene escape because if the inhibitory gene is not activated, transgenic seeds can germinate in the field and the resulting transgenic plants can freely hybridise with wild relatives. Engineered male sterility first reported by De Block and Debrouwer [4] does not solve the problem of transgene transfer to natural plant populations. Male sterile plants can be pollinated by wild, non-transgenic plants and the resulting seeds can spread further into the natural populations. Another problem with the method is that plants need to be pollinated every time with non-transgenic or special transgenic lines to produce a seed yield.

To address the shortcomings of existing approaches to control transgene escape, we have developed a new method, the recoverable block of function (RBF), which blocks gene flow from transgenic plants at the molecular level. It prevents hybridization of transgenic plants with sexually compatible plants of close taxa in nature. RBF is a molecular model, which makes transgenic plants dependent on external regulation. The blocking construct comprises a DNA sequence introduced into the plant genome and linked to the gene(s) of interest. The blocking construct blocks a particular molecular or physiological function of the host plant. Its action leads to the death or alteration of the phenotype or physiology of the host plant making it incapable of reproduction. Thus, the blocking construct controls the gene flow from the transgenic plant and spread of the transgene(s) in surrounding natural populations. The recovering construct comprises a DNA sequence introduced into the plant genome separately or together with the blocking construct and the gene(s) of interest. The action of the recovering construct is responsive to external regulation. The expression of the recovering construct removes the block and recovers the blocked function of the plant. The recovering construct does not act during the life cycle of the plant under natural conditions. An example of a working model of the RBF in transgenic tobacco plants is provided to illustrate the concept.

2. Materials and methods

2.1. Bacteria and plant transformation

Escherichia coli strain XL1 was used for the cloning of the DNA constructs. Tobacco plants *Nicotiana tabacum* cv. Samsung were transformed with *A. tumefaciens* strain LBA4404 [5] carrying binary pGPTV-HPT pBIN19-based vector [6,7]. Tobacco plants were transformed by leaf disc inoculation [8], without using nurse culture and with 30 mg/l hygromycin selection instead of kanamycin.

2.2. DNA synthesis and cloning

Barnase and *barstar* genes originating from *Bacillus amyloliquefaciens* [9] were used for the design of synthetic genes with plant codon preference. The genes were synthesized from 55–59-base long oligonucleotides in high fidelity polymerase chain reaction (PCR). The gene constructs were cloned in pUC19, pBluescript-SKII and pUK21 vectors. Heat shock (HS) [10] and sulfhydryl endopeptidase (SH-EP) [11,12] promoters were cloned by high fidelity PCR from Glycine max and Vigna mungo, respectively. The *uidA* (GUS) gene containing an intron [13] was cloned under the control of 35S promoter [14]. The RBF construct shown in Fig. 1 was compiled and cloned in a modified pGPTV-HPT vector [15].

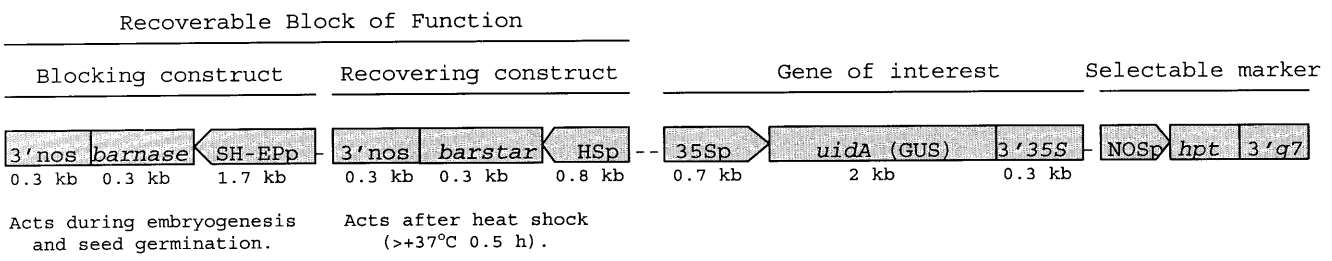

Fig. 1. The RBF DNA construct linked to transgene of interest (*uidA*) and selectable marker gene (*hpt*). Abbreviations, SH-EP, cysteine (sulfhydryl) endopeptidase; HS, heat shock; 35Sp, promoter from CaMV; 3′, polyadenylation signal site; *hpt*, hygromycin phosphate transferase gene; nos, nopalin synthase; p, promoter.

2.3. Molecular analysis of gene expression

Histological GUS assays were performed as described earlier [16]. Northern analysis was performed according to the supplier's recommendations (Boehringer Mannheim — Roche 'The DIG user's guide for filter hybridisation'). The *barnase* and *barstar* sequences were cloned in pBluescript SK II (Stratagene) and used as template for RNA probe (minus strand) and control (plus strand) RNA synthesis. RNA digoxigenin-labeled probe was synthesized by T7 RNA polymerase, and control 'cold' RNA was synthesized by T3 RNA polymerase. For Northern analysis, the 'cold' control RNA was mixed in different concentrations with 10 μg of total RNA of non-transgenic embryos or seedlings. Total RNA preparations were isolated using the Quiagen RNeasy kit. Total RNA (10 μg) was run in an agarose gel and vacuum-blotted on the positive-charged nylon membrane. The membrane was hybridized and developed according to the supplier's instructions.

2.4. Hybridization, germination and heat shock experiments

Greenhouse grown tobacco plants were used in the experiments. Plants for self-pollination were grown in isolation. In hybridization experiments, the transgenic tobacco pollen was used for fertilization of non-transgenic plants. To hybridize the plants, anthers were removed from premature flowers of non-transgenic plants before pollination. The seeds, seedlings and entire greenhouse plants were temperature-treated. The heat shock was performed in Sanyo growth cabinet. The treatment ranged in temperature from 37 to 50°C and in duration from 0.5 h to continuous heat shock. The seeds of the treated plants were germinated in Petri dishes and 12-vessel trays on wet filter paper.

3. Results

3.1. Analysis of expression of SH-EP and HS promoters in GUS assays

To explore the specificity of expression of the HS and SH-EP promoters, we transformed tobacco plants with the *uidA* gene driven by these promoters. Investigations of the transgenic tobacco plants showed that the HS promoter 'leaked' showing GUS enzyme expression at a low level in reproductive organs of some transformed plants. Star expression also peaked on the third day of germination and faded within a few days without outside stimulation. Expression was activated in response to a high temperature in the range of +37–45°C applied for 0.5–3 h. For HS promoter activation, we used 1–2 h at +40°C. The HSp-driven GUS expression increased during the first 2 days and continued for at least 3–4 days after the heat shock. HSp-GUS expression in the germ and young seedpods after 2-h of heat shock treatment at +40°C is illustrated in Fig. 2A. The SH-EP promoter directed the GUS enzyme expression in the embryo and in the seedlings of tobacco. The peak of the GUS activity under the SH-EPp occurred at middle to late stages of embryo development (Fig. 2B) and again appeared on the day 3–5 of germination. There was no GUS expression in the other organs of the tobacco plants.

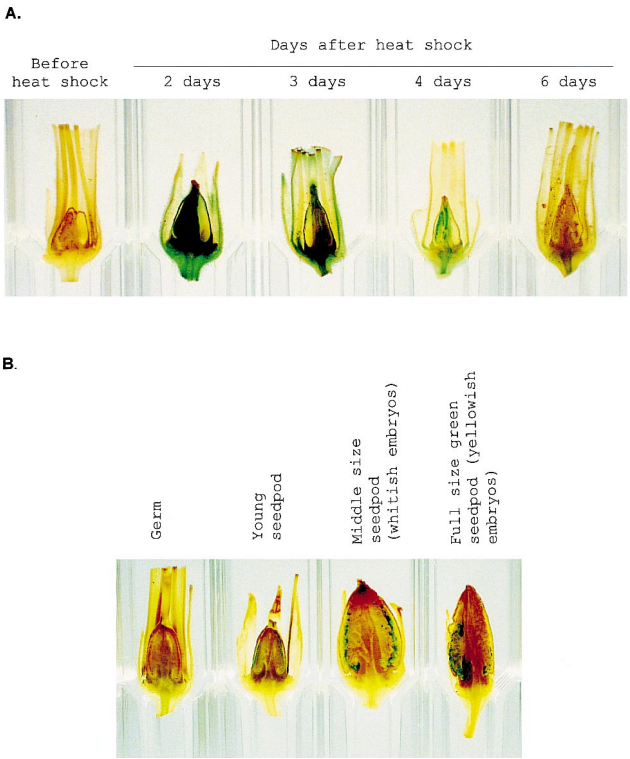

Fig. 2. Expression of the *uidA* gene in transgenic tobacco. (A) Expression of *uidA* gene under Heat Shock promoter in the germ — young seedpods before and after 2 h +40°C heat shock. (B) Expression of *uidA* gene under SH-EP promotoer in germ — seedpods of different stages of development.

3.2. Analysis of RBF action in tobacco plants

Several transgenic tobacco plants were recovered after transformation by the DNA construct containing the *uidA* gene (the gene of interest), *hpt* selectable marker gene and the RBF with *barnase* and *barstar* genes shown in Fig. 1. The transgenic tobacco plants carrying the RBF construct showed normal phenotype — they grew, flowered and produced seedpods after self-pollination. Although the seeds collected from the plants were of normal size, they did not germinate. Heat shock application during germination did not recover the blocked germination function of the transgenic seeds. As we predicted, the embryo development was arrested by the expression of *barnase* at the time of seedpod maturation. As a result of the *barnase* expression, embryos of the seeds were dead.

All of the inflorescences were cut off from the tobacco plants. After this the plants were allowed to flower again. The plants with the new flowers and green seedpods were exposed to heat shock at +40°C for 1 h every second day. Matured seedpods were collected from the tobacco plants. The seeds were germinated and heat shock was applied at least once for 1 h at +40°C on the second or third day of germination. The heat-treated seeds formed normal seedlings (Fig. 3A), which grew into normal tobacco plants. The seeds also attempted to germinate without the heat shock, however, most of them could not expand their cotyledons. They exhibited an etiolated phenotype (Fig. 3B) and died. Some of the seedlings, however, expanded their cotyledons and overcame the block of development. Most likely, the germination of the non-treated seedlings was caused by accumulation of enough Barstar protein during embryo maturation to inhibit the action of *barnase* expressed during seed germination. Supplementary experiments with the heat shock promoter construct showed that only repeated heat shock treatments of the seedpod-carrying plants removed efficiently the block of germination function of the seeds. A short or continuous heat shock applied only once did not remove the block of germination function.

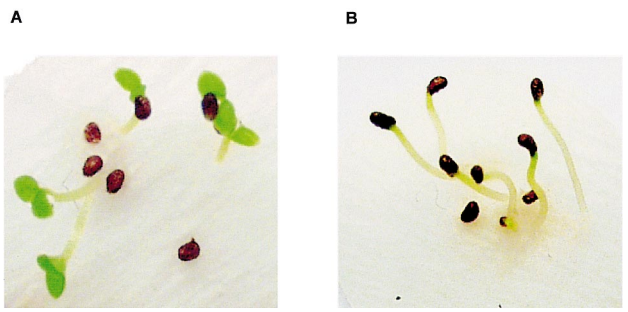

Fig. 3. Seedlings (6-day old) of transgenic tobacco plants carrying the RBF construct. (A) The seedlings treated with high temperature (+40°C) developed normally. (B) The seedling without the temperature treatment could not expand their cotyledons and grew taller.

Pollen from transgenic tobacco plants was used to fertilize non-transgenic plants. The hybridization experiments with non-transformed plants showed that carrying the RBF construct hybrids exhibited the same traits as parental tobacco plants. The transgenic lines of tobacco carrying one or two copies of transgenes exhibited normal phenotype. The plants carrying three or more copies of RBF suffered from non-specific expression of *barnase* and they were incapable of self-pollination and did not produce seedpods. However, the pollen from these plants was able to fertilize non-transgenic plants, which produced seeds that developed normal GUS-positive seedlings after heat shock treatment. We suppose that this happened as a result of segregation of the transgenic construct copies in the hybrid progeny.

The action of the blocking and recovering genes of the RBF was confirmed by Northern analysis (Fig. 4). *Barnase* mRNA expression under the control of SH-EP promoter peaked at mid-stage of embryogenesis (whitish embryos) at the level of 0.1 pg/1 μg of total RNA and decreased to 0.03 pg/1 μg of total RNA during late embryogenesis (yellowish embryos) (Fig. 4A). The second peak of expression started from the third day of seed germination, reaching 0.04 pg/1 μg of total RNA on the fourth day and disappeared during fifth day of germination (Fig. 4B). According to Northern analysis, the HS promoter 'leaked' as it expressed *barstar* mRNA during both stages of development — embryo and seed germination at levels of 0.05–0.2 pg/1 μg of total RNA (Fig. 4C). High temperature treatment activated *barstar* expression, reaching levels of 1–3 pg/1μg of total RNA. The HS promoter activity rose substantially after repeated high temperature treatments. Although Northern analysis showed that the HS promoter 'leaked', *barstar* expression was not enough to inhibit *barnase* action without the high tempera-

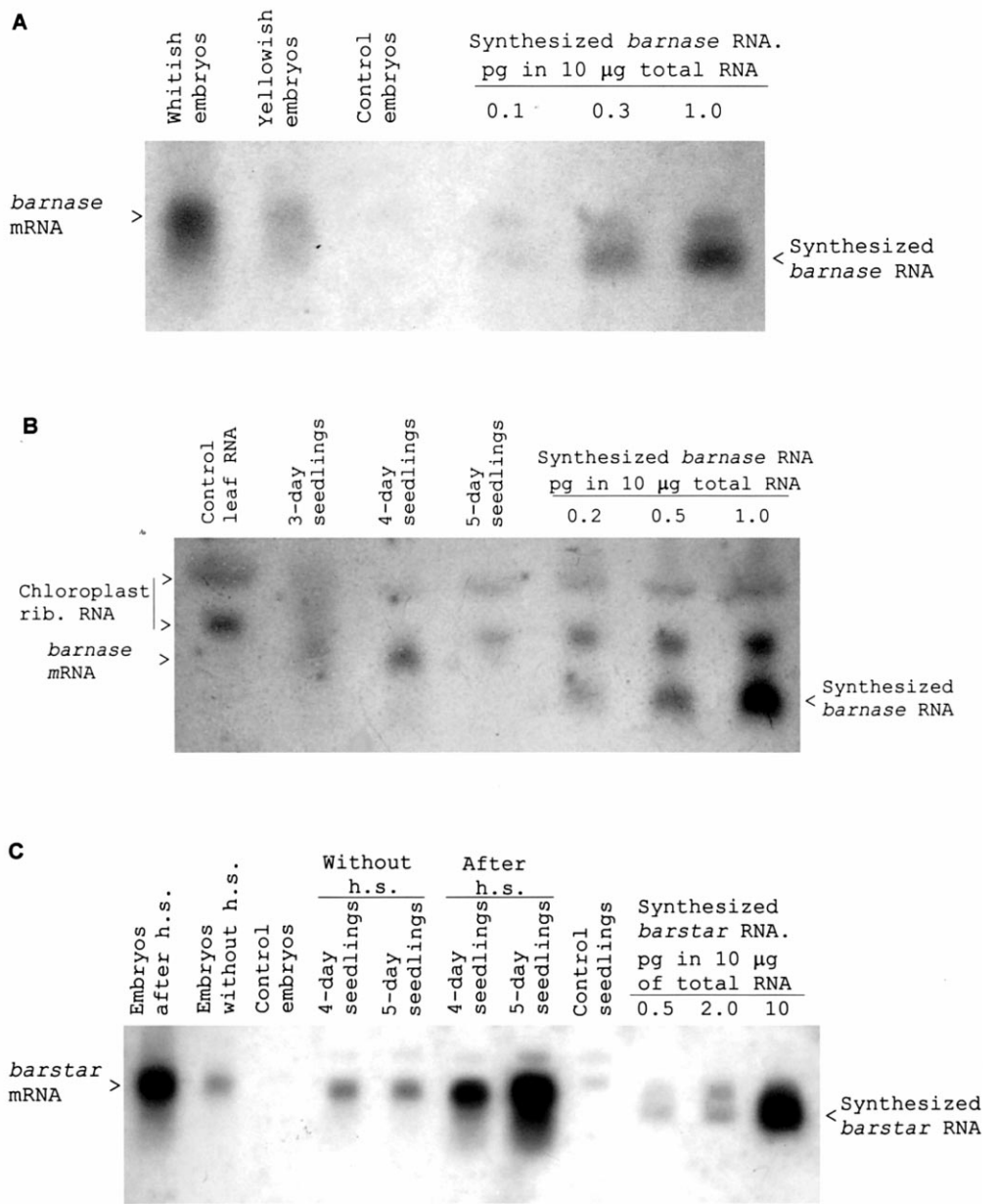

Fig. 4. Northern analysis of RBF expression in transgenic tobacco. (A) Expression of *barnase* mRNA in tobacco embryos at the stages of middle (whitish embryos) and late (yellowish embryos) embryogenesis. Total RNA (10 μg) was loaded in each lane. As controls non-transgenic embryo total RNA and 0.1–0.3–1.0 pg of synthesized *barnase* RNA mixed with 10 μg of total non-transformed tobacco embryo RNA were used. (B) Expression of *barnase* mRNA tobacco seedlings. Total RNA (10 μg) was loaded in each lane. As controls non-transgenic leaf total RNA and 0.2–0.5–1.0 pg of synthesized *barnase* RNA mixed with 10 μg of total non-transformed tobacco leaf RNA were used. Two non-specific bands of chloroplast ribosomal RNA were also detected on the blot. (C) Expression of *barstar* mRNA after heat shock (HS) and without heat shock. Total RNA (10 μg) was loaded in each lane. 0.5–2.0–10 pg of synthesized *barstar* RNA mixed with 10 μg of non-transgenic control tobacco embryos RNA were used as positive control.

ture treatment, especially at the stage of embryogenesis. In general, the Northern analysis confirmed the RBF construct action as it was predicted in the GUS expression experiments.

4. Discussion

In practice, recovering of the RBF of germination requires an application of 1 h +40°C heat

shock treatment once in 2 days during seedpod development and at least one treatment during germination. A single temperature treatment could not restore seed germination. Thus, the RBF of germination linked to the gene of interest (*uidA*) used in this study controlled transgene escape. Effective heat shock treatment of mature plants is possible only in controlled environments, e.g. in greenhouse conditions. In the field, especially in the temperate region, even if the ambient temperature would occasionally rise to +40°C, it is highly unlikely that this would be repeated in subsequent growing seasons. Without the required temperature treatment, the transgenic plants carrying the RBF produce seeds, which do not germinate. Pollen carrying the RBF can pollinate non-transgenic plants, but the seeds produced after the pollination do not germinate under natural conditions. Therefore, the RBF prevents transgene-flow to natural plant populations.

The above example is only one particular model of RBF. Different genes and DNA constructs can be used to assemble other types of RBF. The blocking construct can act at the RNA, protein, metabolic or hormone level. Besides *barnase* and other above-mentioned 'suicidal' genes, other blocking constructs can be used, silencing mRNA synthesis of a vital gene by expressing antisense RNA; enzyme, which overproduces a metabolite or hormone, or produces it with abnormal development- or organ specificity. Recovery can be mediated by an external compensation of a deficiency caused by the blocking or by an outside induction of a responsive promoter of the recovering construct. The induction can be chemical (e.g. salicylic acid or tetracycline) or physical (e.g. temperature or light treatment). The recovering gene can act on DNA (e.g. *tet-tn10*), RNA (e.g. antisense RNA), protein (e.g. *barnase*) or metabolite/hormone level (e.g. enzyme). The most important feature of the RBF is its reliability. The RBF can be lost only by an unlikely event of silencing of the blocking gene expression, mutagenesis caused by crossingover, or DNA deletion. Development of the RBF concept and constructs are ongoing to enhance the versatility, reliability and convenience of the technology.

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