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Heritable transformation of tobacco by *Agrobacterium*-mediated transfer of the *Streptomyces*-derived herbicide resistance gene *bar*

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Abstract. The *bar* gene of *Streptomyces hygroscopicus* encodes an enzyme that detoxifies the herbicide Basta. We have transferred the *Streptomyces*-derived *bar* gene to tobacco through the *Agrobacterium tumefaciens* gene delivery system. Expression of *bar* was driven by two different promoters, TR2′ or CaMV 35S, in two DNA constructs. TR2′ is a weak promoter in tobacco. CaMV 35S is, on the other hand, a strong promoter in tobacco, and transformation using the CaMV 35S promoter construct yielded Basta-resistant transgenic plants. Out of the over one hundred transformants obtained, most could be grown to maturity. Four of these were characterized by genetic and molecular methods. Subsequently, one of the four plants was not resistant and did not show presence of *bar* DNA. The remaining three plants contained one or more copies of *bar* DNA at one or two loci. Segregation data were consistent with this observation: we obtained ratios of either 3:1 (single locus) or 15:1 (two loci) Basta-resistant:Basta-sensitive in the F_2 generation. Field-grown plants showed resistance to Basta up to a level of 4000 g of active ingredient per hectare.

Keywords. Transgenic tobacco; *bar* gene; herbicide resistance; vertical transmission.

1. Introduction

Infection of plant tissue by suitably engineered *Agrobacterium tumefaciens* and the resulting transformation of host tissue by agrobacterial T-DNA has proven to be an efficient gene delivery system, specially for dicotyledonous plants. Several agronomically important genes have been transferred to crop plants using this system (see Fraley 1992). Bialaphos, produced by some *Streptomyces* species, is an antibiotic with broad-spectrum herbicidal activity. It consists of phosphinothricin (PPT) and two L-alanine residues. PPT is an inhibitor of glutamine synthetase. Basta 20SL (Hoechst) is the commercial product containing 200 g l^{-1} PPT. The *bar* gene of *Streptomyces hygroscopicus* imparts resistance to Basta (De Block *et al.* 1987). Here we describe production of transgenic tobacco plants containing one or more copies of the *bar* gene using an *Agrobacterium*-based gene transfer method, differential expression from two foreign promoters in tobacco, the nature of T-DNA insertion, and, finally, the inheritance of the transferred gene.

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2. Materials and methods

2.1 *Agrobacterium strain and DNA constructions*

The *bar* DNA (source *Streptomyces hygroscopicus*) was isolated from pIJ4101 (White *et al.* 1989). The plant cloning vector pPCV708 (Koncz and Schell 1986) was digested with *Bgl*II or *Bam*HI and filled in with Klenow enzyme, and the *bar* gene segment was ligated to it. Insertion at the *Bgl*II site placed the *bar* gene downstream of the CaMV 35S gene promoter. Alternatively, insertion at the *Bam*HI site placed the *bar* gene under the control of the TR2′ promoter (figure 1). The recombinant plasmids, p35S*bar* and pTR2′*bar*, were transferred to *Agrobacterium tumefaciens* GV3101 (pMP90RK) (Koncz and Schell 1986) by triparental mating using helper plasmid pRK2013 (Figurski and Helinski 1979).

2.2 *Plant variety*

Nicotiana tabacum cv Petit Havana SR1 was used. This variety is a good host for *Agrobacterium* infection and for sustaining a transferred gene or genes. Regeneration of plants can also be efficiently done from leaf discs in tissue culture (Viegas *et al.* 1987).

2.3 *Leaf disc transformation*

Leaves were sterilized for 5 minutes with 0·1% $HgCl_2$, washed copiously with sterile water, and cut into small pieces (0·5 cm^2). The leaf pieces were floated for 18 h on half-strength MS medium (Murashige and Skoog 1962) containing late-exponential phase culture of the tailored *Agrobacterium*. The explants were then cultured as described in Viegas *et al.* (1987) to regenerate transgenic tobacco plants. For selection of transformants, bialaphos at 2 to 10 $mg\,l^{-1}$ was used.

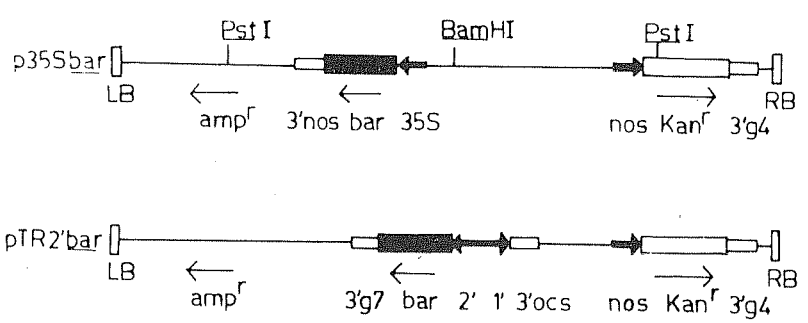

Figure 1. Schematic representation of the DNA constructs p35S*bar* and pTR2′*bar*. LB and RB are the left and right borders of T-DNA. In p35S*bar*, the *bar* gene is downstream of the CaMV 35S promoter and is followed by the nopaline synthase (3′nos) polyadenylation signal. In pTR2′*bar*, the *bar* gene is under the control of the TR2′ promoter and is followed by the T-DNA gene 7 (3′g7) polyadenylation signal. A kanamycin resistance gene (*kan*^{r}) is downstream of the *nos* promoter and is followed by the polyadenylation signal of T-DNA gene 4 (3′g4). The thick arrows indicate promoters; thin arrows show direction of transcription.

2.4 *Southern hybridization*

Total plant DNA was isolated as described by Dellaporta *et al.* (1983) and estimated spectrofluorimetrically. For hybridization 10 μg of DNA cleaved with *Pst*I and *Bam*HI (Bangalore Genei, India) was used. DNA electrophoresed on a 0·8% agarose gel was blotted onto a Hybond-N (Amersham) membrane and the hybridization carried out using a ^{32}P-labelled *bar* DNA probe prepared using a multiprime labelling kit (BRIT, India). Blots were washed with 2 × SSC (1 × SSC = 0·15 M sodium chloride and 0·015 M trisodium citrate) containing 0·1% SDS at 65°C, and with 0·2 × SSC at room temperature.

2.5 *Northern hybridization*

Leaf RNA was isolated as described by Jones *et al.* (1985); 20 μg of total RNA was electrophoresed through a 1·5% formaldehyde/agarose gel and transferred to Hybond-N (Amersham), and the hybridization reaction carried out with the ^{32}P-labelled *bar* gene probe. The blot was washed with 2 × SSC containing 0·1% SDS and 0·1 × SSC containing 0·1% SDS at 60°C.

2.6 *Genetic analysis of progeny*

Seeds obtained by self-pollinating the transformed plants or by crossing transformed plants with untransformed plants as male parent were used. Seeds were placed on moist filter paper in petri dishes for germination. After ten days the germinated seedlings were transferred to paper cups containing sterilized soil. Three weeks later the seedlings were sprayed with 0·5% (1 g active ingredient, PPT, per litre) Basta 20SL (Hoechst). Sensitive and resistant seedlings were classified and counted ten days after the spraying.

2.7 *Evaluation of resistance in the field*

Resistant seedlings (six weeks old) were transferred to an experimental field. The plants were transplanted in rows 30 cm apart. Herbicide was applied as a mist using a hand-held sprayer so that all the leaves were completely wet. The first herbicide treatment of 1000 g a.i. per ha (active ingredient per hectare) was applied on nine-week-old plants. Subsequent treatments of 2000 and 4000 g a.i. per ha were applied at fifteen-day intervals. Basta was used at a concentration of 4 g a.i. per litre of water for the 1000 and 2000 g a.i. per ha treatments and at 8 g a.i. per litre of water for the 4000 g a.i. per ha treatment.

3. Results

3.1 *Regeneration of transformants*

Following infection of tobacco leaf discs by *Agrobacterium* carrying the p35S*bar* DNA construct, herbicide-resistant shoots were regenerated by selection using 2, 5 or

10 mg l^{-1} bialaphos. These produced roots in the presence of bialaphos (1 to 10 mg l^{-1}). Following cocultivation with *Agrobacterium* carrying the pTR2'*bar* DNA construct, very few shoots could be regenerated on selection medium (2 or 5 mg l^{-1} bialaphos). These shoots remained stunted and failed to produce roots in medium containing bialaphos. They were then rooted in the absence of selection pressure. Shoots were also regenerated by selection with 100 mg l^{-1} kanamycin. These shoots produced roots in the presence of kanamycin but not in presence of bialaphos.

The putative transformants were transferred to soil and grown to maturity. One hundred and four plants were regenerated from explants in the experiment using the 35S*bar* construct, of which 94 grew to maturity. Fifty-six plants were obtained in the experiment using the TR2'*bar* construct.

3.2 *Phenotypic analysis of putative transformants*

A preliminary check for resistance to the herbicide was done by spraying individual leaves with Basta 20SL. Two leaves per plant were sprayed with 0·2, 1, 2, 3, 4 or 5 g a.i. (PPT) per litre. The resistances observed in these plants were as follows.

In the 35S*bar* experiment, 86 of the plants were fully resistant at 4 and 5 g a.i. per litre. Five plants showed some chlorosis at 2 to 5 g a.i. per litre. Three plants were sensitive even to low-concentration sprays (0·2 g a.i. per litre). Four of the plants were analysed further: TPV-1, TPV-4 and TPV-5 were resistant to Basta, but TPV-2 was found to be sensitive when sprayed with the herbicide.

In the TR2'*bar* experiment, the tobacco plants regenerated were tolerant to very low concentrations of herbicide (0·2 to 1 g a.i. per litre) and fared only marginally better than control plants (untransformed).

3.3 *Molecular analysis of the putative transformants*

Southern hybridization: Total DNA isolated from TPV-1, TPV-2, TPV-4 and TPV-5 transformants was digested with *Pst*I and *Bam*HI, electrophoresed and Southern-blotted, and probed with *bar*-specific DNA. A single expected *Pst*I–*Bam*HI 2·65 kb hybridizing DNA band in TPV-1, TPV-4 and TPV-5 indicated presence of the *bar* gene (figure 2). This band was not detected in TPV-2. Comparison of the intensities of the hybridization bands with that of a band containing one copy equivalent of *bar* that was run in the same gel indicated that TPV-1 has a single copy, and TPV-4 and TPV-5 each has more than one copy of the *bar* gene.

Northern hybridization: Northern-blot hybridization of total RNA isolated from leaves of mature TPV-1, TPV-4 and TPV-5 revealed presence of *bar*-specific mRNA (figure 3). No hybridizing band was observed in RNA from TPV-2.

3.4 *Inheritance of bar*

The self-pollination and back-cross progenies of these transformants were treated with herbicide (1 g a.i. per litre) and classified. TPV-1 and TPV-4 gave a segregation ratio of approximately 3 resistant to 1 sensitive in the self-pollination progeny and

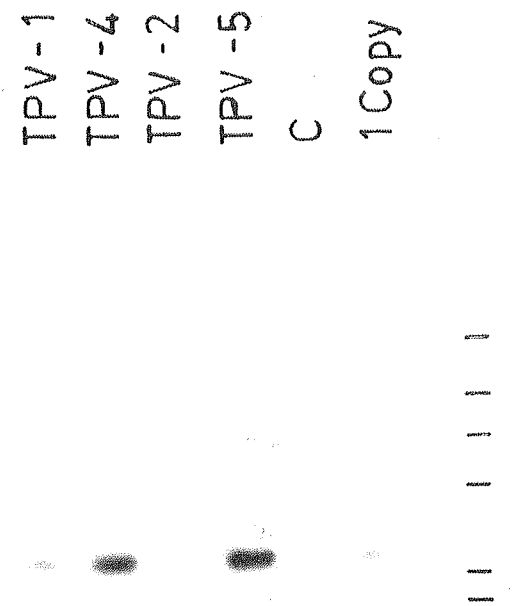

Figure 2. Southern blot analysis of four putative transformants of tobacco. Total DNA digested with *Bam*HI and *Pst*I, electrophoresed and Southern blotted was probed using a ^{32}P-labelled purified *bar* gene fragment. C, Control (untransformed tobacco plant); 1-copy, reconstruction of p35S*bar* DNA equivalent to one copy per genome and 10 μg control DNA digested with *Pst*I and *Bam*HI; lambda *Hin*dIII fragments are shown on the right.

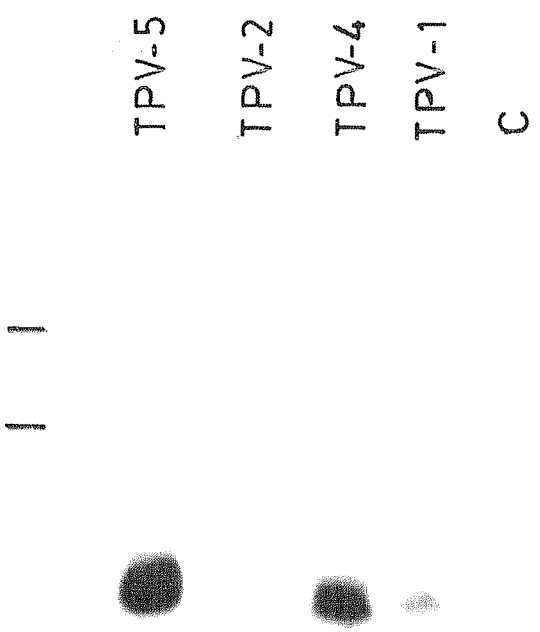

Figure 3. Northern-blot hybridization of RNA extracted from four putative transformants. The positions of 18S and 28S RNA are indicated on the left; C is control RNA from an untransformed tobacco plant.

Table 1. Segregation of resistant and sensitive seedlings in the progeny of transgenic plants.

Pedigree number	No. of seedlings R	S	Expected ratio (R:S)	Probability P corresp. to χ^2
TPV-1	142	41	3:1	0·48
wt × TPV-1	79	90	1:1	0·47
TPV-4	123	45	3:1	0·67
wt × TPV-4	64	71	1:1	0·63
TPV-5	208	12	15:1	0·72
wt × TPV-5	185	69	3:1	0·48

Progeny seedlings were sprayed with Basta 20SL at 1 g a.i. per litre to identify resistant plants.
R, Resistant; S, sensitive
wt, Wild-type parent *N. tabacum* cv Petit Havana

1:1 in the back-cross progeny. This indicated that the T-DNA containing the *bar* gene is present at a single locus in TPV-1 and TPV-4. However, TPV-5 gave a segregation ratio of 15:1 in the self-pollination progeny and 3:1 in the back-cross progeny. This suggests that two or more copies of T-DNA in TPV-5 are distributed in two loci. The segregation data are given in table 1.

The resistant progeny (identified by spraying 1 g a.i. per litre on six-week-old plants) were transplanted to plots in an experimental field. Untransformed wild-type tobacco was also transplanted in every sixth row as control. Ten-week-old plants were sprayed with the dose equivalent of 1000 g a.i. per ha. All control plants turned yellow within 48 h of spraying and eventually died. The transgenic plants remained green, grew in size, and flowered (figures 4 and 5). Two additional herbicide applications of 2000 and 4000 g a.i. per ha were sprayed at 15-day intervals. All three lines TPV-1, TPV-4 and TPV-5 were resistant to these higher doses. However, some mature leaves showed necrosis at 4000 g a.i. per ha. These three lines, with different copy numbers of *bar*, in general did not show any perceptible differences in the level of resistance.

4. Discussion

Our results show that tobacco plants resistant or tolerant to herbicide Basta have been produced by transferring to them one or more copies of the *bar* gene driven by either the CaMV 35S gene promoter or the TR2′ promoter. Transgenic plants carrying the 35S*bar* construct were resistant to high levels of herbicide (4000 g a.i. per ha). On the other hand, preliminary experiments indicate that plants carrying the TR2′*bar* construct show low tolerance to the herbicide. Thus the choice of promoter is very important. Unpublished observations cited in De Greef *et al.* (1989) of tobacco plants transformed with the *bar* gene also indicated that with the TR2′ promoter a ten-fold lower level of enzyme was obtained. In alfalfa too, D'Halluin *et al.* (1990) demonstrated that plants carrying a TR2′*bar* construct generally exhibited only a tolerance under field conditions.

Four transformed plants were analysed in some detail. From Southern hybridization results it appears that TPV-1 has one copy, while TPV-4 and TPV-5 have more than

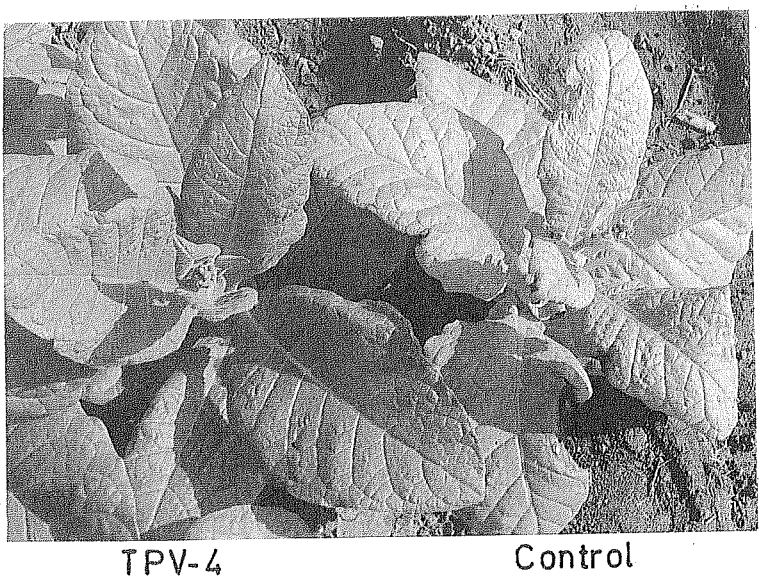

Figure 4. A plant from the F_2 progeny of TPV-4 (left) along with an untransformed control (right) four days after spraying with Basta 20SL (1000 g a.i. per ha).

Figure 5. Evaluation of herbicide resistance in transgenic tobacco plants under field conditions. The picture shows F_2 progeny of TPV-1 ten days after treatment with Basta 20SL (1000 g a.i. per ha).

one copy of the *bar* gene. Moreover, the results of the crosses show that in TPV-5 the T-DNA is present in two loci. Northern hybridization results show differences in the amount of *bar*-specific mRNA in these three plants. However, the independent lines obtained from TPV-1, TPV-4 and TPV-5 did not show significant differences in expression of herbicide resistance at the phenotypic level. In at least one case, Southern hybridization results indicated that the insertion of two T-DNA copies appeared to have occurred in tandem and in inverse orientation (data not shown). It is not clear if such a duplication occurred before or after insertion. The inverse orientation could result from an event that is part of the DNA replication mechanism. Spielmann and Simpson (1986) and Jorgensen *et al.* (1987) have also reported such insertions in tobacco and tomato.

Although tobacco is a major crop in India, Petit Havana is not a commonly grown variety. Transfer of *bar* gene to the tobacco varieties commonly grown can possibly be effected by conventional crossing and selection. DNA constructs and *Agrobacterium*-based delivery can be used to transfer the *bar* gene to other crops.

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