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Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a *petE* promoter-*bar* gene compared with a CaMV 35S-*bar* gene

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Abstract Inheritance of resistance to herbicide (300 mg/l glufosinate ammonium) up to the third (T3) seed generation was compared in two populations of transgenic lettuce (*Lactuca sativa* L. cv 'Evola') harbouring a T-DNA containing the *bar* gene, linked to either the Cauliflower Mosaic Virus (CaMV) 35S promoter, or a –784-bp plastocyanin promoter from pea (*petE*). Only 2.5% (4/163) of CaMV 35S-*bar* plants, selected by their kanamycin resistance (T0 generation), transmitted herbicide resistance at high frequency to their T3 seed generation compared with 97% (29/30) for kanamycin resistant *petE-bar* plants. In the case of 35S-*bar* transformants, only 16% (341/2,150) of the first seed generation (T1) plants, 22% (426/1,935) T2 plants and 11% (1,235/10,949) T3 plants were herbicide-resistant. In contrast, 63% (190/300) T1 plants, 83% (2,370/2,845) T2 plants and 99% (122/123) T3 *petE-bar* transformed plants were resistant to glufosinate ammonium. The T-DNAs carrying the *petE-bar* and CaMV 35S-*bar* genes also contained a CaMV 35S-neomycin phosphotransferase (*npt*II) gene. ELISA showed that NPTII protein was absent in 29% (45/156) of the herbicide-resistant T2 plants from 8/19 herbicide-resistant *petE-bar* lines. This indicated specific inactivation of the CaMV 35S promoter on the same T-DNA locus as an active *petE* promoter. The choice of promoter and T-DNA construct are crucial for long-term expression of transgenes in lettuce.

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Introduction

Several reports have described the production of transgenic lettuce (Michelmore et al. 1987; Chupeau et al. 1989; Enomoto et al. 1990; Torres et al. 1993; Yang et al. 1993; Curtis et al. 1994a, 1996a,b; Kisiel et al. 1995; Falk et al. 1996; Gilbertson et al. 1996; Pang et al. 1996; Dinant et al. 1997), with a number of useful traits being introduced into this economically important crop. However, genetically engineered lettuce has yet to be released onto the market. This may be related, at least in part, to the high degree of transgene instability in this crop. For example, Gilbertson (1996) reported that 80% of transgenic first seed generation lettuce plants resistant to lettuce mosaic virus (LMV) lost their resistance in the subsequent generation. Falk et al. (1996) produced transgenic lettuce and tobacco plants both carrying the same three genes for resistance to LIYV (lettuce infectious yellows virus). LIYV resistance was observed after analysing a limited number of transgenic tobacco plants but, surprisingly, LIYV resistance was not observed in any transgenic lettuce plants. Dinant et al. (1993, 1997) produced transgenic plants of lettuce and tobacco carrying the same LMV coat protein (LMV-CP) gene. A high degree of heterologous resistance to potato virus Y (PVY) was observed in transgenic tobacco, but in transgenic lettuce, the LMV-CP gene gave only poor resistance to LMV, which was of no agronomic value. Therefore, it is imperative to adopt a strategy to increase the frequency of high, stable and heritable transgene expression in this economically important leafy vegetable.

The promoter is a major factor which influences the level and stability of transgene expression. Pwee and Gray (1993) reported that a truncated pea plastocyanin promoter 784 bp in length (–784-bp *petE*) directed six-fold and 11-fold higher *gus* gene expression in photosyn-

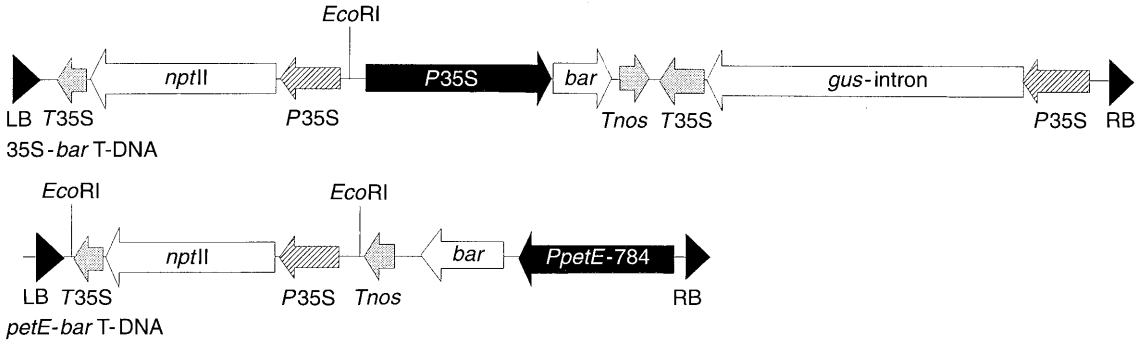

Fig. 1 35S-*bar* and *petE*-*bar* T-DNA cassettes

thetic tissues of transgenic tobacco than the *rbc*S (Aoyagi et al. 1988) and CaMV 35S promoters (Benfey et al. 1989), respectively. Curtis et al. (1994b) compared several promoter-*gus* gene fusions in a limited number of transgenic lettuce plants and found that the *petE* promoter gave higher expression in first seed generation (T1) plants than the MAS (Teeri et al. 1989), Mac (Comai et al. 1990) or CaMV 35S promoters.

In order to assess the frequency of stable inheritance of an agronomic trait in transgenic lettuce, we evaluated plants transformed by T-DNA constructs containing either a *petE*- or a 35S-*bar* gene fusion for herbicide tolerance over three seed generations. The *bar* gene is derived from *Streptomyces hygroscopicus* and codes for phosphinothricin acetyltransferase (PAT) (Keller et al. 1997). PAT confers resistance to the non-selective herbicide bialaphos by acetylation of the free amide group of the active component, phosphinothricin (PPT). The *bar* gene was selected for this assessment because herbicide resistance is an agronomic trait which can be assessed rapidly and economically in large populations of plants under glasshouse and field conditions. Expression of a CaMV 35S-driven *npt*II gene and a *petE*-driven *bar* gene on the same T-DNA were also compared in transgenic lettuce in order to assess the performance of the 35S and *petE* promoters at the same integration site into the plant genome.

Materials and methods

Material for the production of transgenic plants

Lettuce seeds (*Lactuca sativa* L. cv 'Evola') supplied by Leen de Mos ('s-Gravenzande, P.O. Box 54–2690 AB, The Netherlands) were surface-sterilized in 10% (v/v) 'Domestos' bleach (Lever Industrial, Runcorn, UK) for 30 min, followed by three washes in sterile distilled water. The seeds were placed on half-strength agar-solidified (0.8% w/v) Murashige and Skoog (MS; 1962) medium with 1.0% (w/v) sucrose, at pH 5.8 (20-ml aliquots per 9-cm Petri dish; 30–40 seeds per dish). Seeds were germinated at 23°±2°C (16-h photoperiod, 200 µmol m^{-2} s^{-1}, daylight fluorescent tubes). Cotyledons were excised after 7 days for bacterial inoculation.

Bacterial strains and plasmids

The binary vectors pVDH85 and pVDH310 were based on the pBIN19 derivative pMOG18 (Sijmons et al. 1990) and were introduced into *Agrobacterium tumefaciens* LBA4404 (Ooms et al. 1981) by triparental mating. Plasmid VDH85 carried a *nos.npt*II.*nos* gene next to the left border, a 35S-driven *bar* gene and a 35S.*gus*-intron.35S gene (Vancanneyt et al. 1990) adjacent to the right border. This construct was chosen as it gave a high frequency of herbicide resistance in other species. Plasmid VDH310 carried a 35S.*npt*II.*nos* gene next to the left border, with a *petE*-driven *bar* gene adjacent to the right border (Fig. 1).

Bacteria were grown from –70°C glycerol stocks at 28°C on Luria broth (LB) (Sambrook et al. 1989) semi-solidified with 1.5% (w/v) agar and supplemented with kanamycin sulphate (100 mg/l) and rifampicin (50 mg/l). Overnight liquid cultures were incubated at 28°C on a horizontal rotary shaker (180 rpm) and were initiated by inoculating 20 ml of liquid LB medium, containing kanamycin sulphate (50 mg/l) and rifampicin (40 mg/l), into 100 cm^3 conical flasks. Bacterial cultures were grown to an $O.D._{600}$ of 1.0–1.5 prior to inoculation of explants.

Plant transformation

Cotyledons excised from 7-day-old seedlings were inoculated with *A. tumefaciens* and transgenic shoots regenerated using an established procedure (Curtis et al. 1994a). Shoots which regenerated from explants on medium containing kanamycin sulphate (50 mg/l) were rooted *in vitro* in the presence of kanamycin sulphate (50 mg/l) before transfer to the glasshouse, where they were allowed to self-pollinate and to set seed. Seeds were collected and stored at 4°C. Non-transformed (control) plants were regenerated from uninoculated cotyledons on antibiotic-free medium.

Analysis of herbicide resistance

Seedlings were sprayed 2 and 3 weeks after seed-germination with a 1:500 (v:v) dilution of *Challenge*® (*Finale*) herbicide (AgrEvo UK Crop Protection, East Winch, King's Lynn, UK) containing a final concentration of 300 mg/l glufosinate ammonium. Plants were scored 5 weeks after seed germination as resistant (green and healthy) or sensitive (bleached and dead).

Double Antibody Sandwich (DAS) ELISA for quantification of NPTII protein in plant tissue

Discs (1 per plant) were punched from randomly selected leaves of 28-day-old glasshouse-grown plants using the lids of 1.5-ml microfuge tubes. Explants within the tubes were frozen immediately in liquid nitrogen. Frozen samples were ground to a fine powder with a plastic microhomogeniser cooled previously in liquid nitrogen. Five hundred microliters of protein extraction buffer [0.25 M TRIS-HCl (pH 7.8), 1 m*M* phenylmethylsulfonylfluoride] was added to each sample; the latter was vortexed (15 s) and then placed on ice. Samples were centrifuged for 10 min at 10,000 *g* in a microcentrifuge at 4°C. The supernatants (200 µl) were removed to new tubes and stored on ice. Protein extracts were quantified (Bradford 1976) before storage overnight at –70°C. The amount of

NPTII protein in 80 µg of each plant protein extract was quantified using an NPTII ELISA kit (5 Prime→3 Prime Inc®, Boulder, Colo., USA) according to the manufacturer's instructions. Colour development of the final reaction in plant protein extracts and NPTII standards was quantified using a microtitre plate reader (A_{405nm}) (Microplate® Reader, Dynatech, Billingshurst, UK).

T-DNA copy number analysis

Total genomic DNA was isolated from approximately 1 g of fresh leaf material (Dellaporta et al. 1983). Ten microliters of plant DNA was digested with 50 units of *Eco*RI restriction endonuclease for 16 h at 37°C. Non-radioactive Southern analysis was performed as described (McCabe et al. 1997).

Results

Southern blot analysis of T1 plants

Hybridization of the *bar* gene probe to *Eco*RI-digested genomic DNA from *petE-bar*-transformed T1 plants resulted in band sizes which varied among T1 plants derived from different individual T0 kanamycin-resistant parents. This was expected, as the *bar* gene probe hybridized to fragments containing the junction between the right side of integrated T-DNA and plant genomic DNA (Fig. 1). Thus, the fragment size depended on the site of integration. One to three bands hybridized to the *bar* gene probe, which indicated the presence of multiple T-DNA copies in some plants.

Following removal of the *bar* gene probe from the filter, hybridization of the *npt*II gene probe to the same filter resulted in bands corresponding to the expected size of 1.6 kb. In some samples, the *npt*II probe hybridized to an extra band of approximately 3.5 kb or 6.0 kb. This indicated the presence of an extra T-DNA insert in which the *Eco*RI site adjacent to the left T-DNA border was either absent or did not cut with the restriction enzyme. These patterns of DNA hybridization in transgenic T1 lettuce plants have been presented in detail by Mohapatra et al. (1999) and, consequently, are not reproduced in the present report.

Herbicide resistance of T0 and T1 plants

Following transfer from culture to the glasshouse, randomly selected kanamycin-resistant 35S-*bar*- and *petE-bar*-transformed T0 plants were sprayed with herbicide. Seventy-five percent (58) of the 77 *petE-bar* T0 plants, derived from 20 independently inoculated cotyledons, were herbicide-resistant. However, only 11% (3/27) of 35S-*bar* T0 plants (derived from 9 independently inoculated cotyledons) exhibited resistance to the herbicide.

T1 plants, derived from self-pollinated T0 plants, were sprayed with herbicide to assess stable inheritance of *bar* gene expression. The 35S-*bar* T1 plants were derived from 163 kanamycin-resistant T0 plants (regenerated from 57 independently inoculated cotyledon explants). Of these T0 plants 34% (55 individuals derived from 30 independent cotyledons) gave ≥10% herbicide-resistant T1 progeny, but only 9% (14 plants from 10 independent cotyledons) produced ≥50% herbicide resistant T1 progeny. T1 plants transformed with *petE-bar* derived from 30 kanamycin-resistant T0 plants (regenerated from 16 indepedently inoculated cotyledons) were tested for herbicide resistance. In contrast to 35S-*bar* plants, 97% (29) of the *petE-bar* T0 plants gave ≥10% herbicide resistant T1 progeny, with 90% (27) producing ≥50% herbicide-resistant R1 progeny. This demonstrated that the *petE-bar* gene was stably expressed at a higher frequency than the 35S-*bar* gene in both T0 and T1 plants.

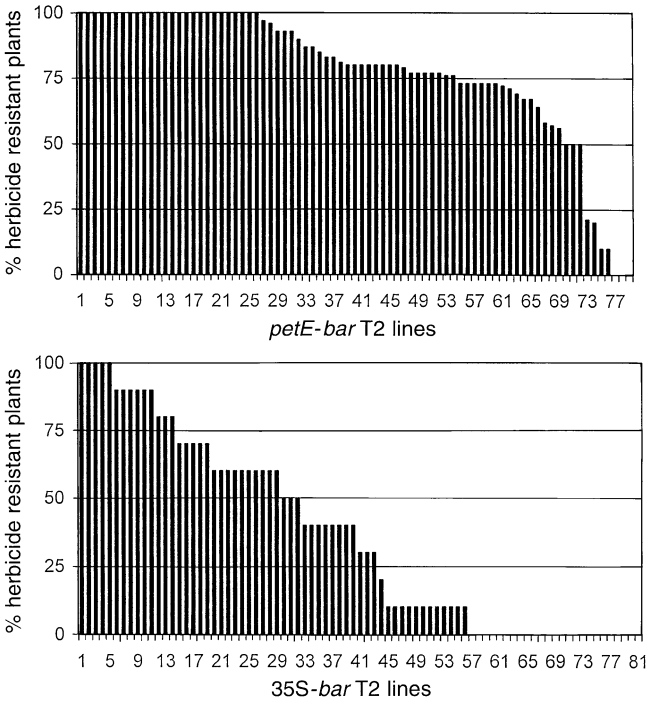

Fig. 2 Herbicide resistance in transgenic lettuce (cv 'Evola') T2 plants derived from 79 and 81 herbicide-resistant *petE-bar* and 35S-*bar* T1 parents, respectively. Thirty *petE-bar* plants and 10 35S-*bar* plants were tested for each T2 line

Transmission of herbicide resistance from T1 to T2 seed generations

Seeds were collected from 79 herbicide-resistant *petE-bar* T1 plants (derived from 22 T0 plants regenerated from 13 independent cotyledons) and 81 herbicide-resistant 35S-*bar* T1 plants (derived from 31 T0 plants regenerated from 16 independent cotyledons) in order to compare the frequency of inheritance of 35S-*bar* and *petE-bar* gene expression from T1 to T2 generations. T2 seeds were germinated and the resulting T2 plants were tested for their herbicide resistance (Fig. 2). Only 69% (56/81) herbicide-resistant 35S-*bar* T1 plants gave herbicide resistant T2 progeny. However, 96% (76/79) of the herbicide-resistant *petE-bar* T1 plants produced herbicide-

Table 1 Percentage of herbicide-resistant plants in T1, T2 and T3 seed generations of 35S-*bar* and *petE-bar* transformed lines of lettuce[a]

[a] Data recorded 5 weeks after germinating seeds in compost in the glasshouse and after spraying with herbicide (300 mg/l glufosinate ammonium) at 14 days and 21 days post-germination

	35S-*bar* Seed generation			*petE– bar* Seed generation		
	T1	T2	T3	T1	T2	T3
Total no. plants tested	2,150	1,935	10,949	300	2,845	123
Total no. plants resistant	341	426	1,235	190	2,370	122
Percentage plants resistant	16	22	11	63	83	99
Total no. lines tested	163	116	379	30	79	19
Total no. lines resistant	56	58	69	29	75	19
Percentage lines resistant	34	50	18	97	95	100

resistant T2 progeny. These data demonstrated that a high degree of transgene inactivation occurred in 35S-*bar* plants, as 31% of the T1 plants failed to transmit herbicide resistance to the T2 seed generation. In contrast, only 4% of the T1 *petE-bar* plants failed to transmit herbicide resistance to their progeny.

If it is assumed that the original T0 parental plants contained a single active T-DNA locus, herbicide resistant T1 plants would be expected to produce either 75% or 100% herbicide-resistant T2 plants, depending on whether the T1 plants were hemizygous or homozygous for the *bar* gene. However, 60% (49/81) of the herbicide-resistant 35S-*bar* T1 plants gave fewer than 50% (<5/10) herbicide-resistant T2 plants, indicating transgene silencing. This situation did not occur to such an extent in *petE-bar* plants, since only 9% (7/79) of herbicide-resistant *petE-bar* T1 plants produced fewer than 50% (<15/30) herbicide-resistant T2 plants.

Table 2 Simultaneous analysis[a] of *petE* promoter-driven herbicide (*bar* gene) resistance and 35S promoter-driven NPTII protein expression in individual *petE-bar* T1 and T2 transgenic lettuce plants

	Seed generation	
	T1	T2
Total no. lines tested	7	19
Total no. plants tested	54	186
Total no. plants resistant	44	156
Total no. plants NPTII+ve	44	111
Percentage herbicide resistant plants containing NPTII protein	100	71

[a] Fourteen days after seed germination, one 8-mm-diameter leaf disc was taken from each plant for NPTII ELISA. NPTII positive, NPTII negative and control samples showed an OD_{600} >0.1, <0.5 and 0.03–0.04, respectively. Plants were sprayed with herbicide (300 mg/l glufosinate ammonium) 14 days after seed germination; spraying was repeated 7 days later

Transmission of herbicide resistance from T2 to T3 seed generations

Table 1 summarizes the transfer of herbicide resistance from the T1 to T3 seed generations. Only 5% (17/339) of the herbicide-resistant 35S-*bar* T2 plants (derived from 3 primary transformants) gave herbicide-resistant T3 progeny (data not shown). When data from T3 progeny derived from unsprayed T2 plants were included, it was found that 11% of T3 plants were herbicide-resistant (Table 1). Several 35S-*bar* lines showed a dramatic reduction in the number of herbicide-resistant T3 plants compared to T2 plants. For example, 35S-*bar* line 2C5 produced 70% (21/30) herbicide-resistant plants in the T2 generation, but only 1.7% (3/180) in the T3 generation. However, 99% (122/123 derived from 19 T2 plants) of the *petE-bar* T3 plants were herbicide-resistant.

Differential expression of the 35S-*npt*II and *petE-bar* genes at the same T-DNA locus

In order to determine whether the 35S promoter was more susceptible to positional effects than the *petE* promoter, we analysed simultaneously the expression of the 35S-*npt*II and *petE-bar* genes in 240 T1 and T2 plants derived from 7 T0 parental lines carrying the *petE-bar* gene (Table 2). The herbicide resistance of T2 plants from a hemizygous line and a homozygous line is shown in Fig. 3. At least 45 T2 plants derived from 3 T0 parental lines were herbicide-resistant but did not contain detectable levels of NPTII protein (Table 2). This differen-

Fig. 3 Non-transgenic and transgenic lettuce plants (cv 'Evola') 5 weeks after seed germination and 14 d after the second application of *Challenge*® herbicide at a concentration of 300 mg/l glufosinate ammonium. *Left* 10 non-transgenic plants; *middle* 10 *petE-bar* T2 plants derived from the homozygous line 16A5.2, *right* 10 *petE-bar* T2 plants derived from the hemizygous line 15C1.1

tial expression of both transgenes on the same T-DNA locus suggests that the 35S-*npt*II gene was more susceptible to inactivation than the *petE-bar* gene. As the T0 parents were selected on medium containing kanamycin sulphate, the 35S-*npt*II gene must, initially, have been active in the T0 plants. Interestingly, differential *petE-bar*/35S-*npt*II gene expression was not observed in any of the T1 plants, which indicates that inactivation of the 35S-*npt*II gene occurred, predominantly between the T1 and T2 seed generations.

Discussion

The increase in stable inheritance of herbicide resistance in lettuce transformed with *petE-bar* T-DNA compared with 35S-*bar* T-DNA is most likely to be related to the promoter directing *bar* gene expression. This is supported by the fact that the CaMV 35S-*npt*II gene was inactivated on the same integrated T-DNA as an active *petE-bar* gene. Therefore, the CaMV 35S promoter is, apparently, more prone to inactivation than the *petE* promoter following integration into the lettuce genome. Poor expression of herbicide resistance in plants transformed with the 35S-*bar* T-DNA could also be related to the presence of CaMV 35S sequences linked to the *gus* gene on the same T-DNA (Finnegan and McElroy 1994). In order to confirm, unequivocably, the influence of the 35S and *petE* promoters on *bar* gene expression in lettuce, it would have been preferable to introduce T-DNA constructs which consisted of only the 35S-*bar* or the *petE-bar* gene between the T-DNA borders in the absence of any marker gene. However, because of time constraints, a T-DNA construct was used for analysis of 35S-*bar* gene expression which had already shown high levels of herbicide resistance in tobacco, oilseed rape and sugarbeet. High percentages of herbicide-resistant plants were obtained in these crops, while stable expression over four seed generations was obtained for the majority of transgenic lines in sugarbeet. These results also emphasize that the low number of stable transformants obtained in lettuce in the present investigation was related to a species-specific response.

Several reports have described that the methylation of promoters directing transgene expression in transgenic plants is related to loss of transgene activity. For example, Meyer et al. (1992) observed that the CaMV 35S promoter was hypermethylated in transgenic petunia plants carrying a 35S promoter-driven maize *A1* gene which failed to exhibit the expected red flower colour. In contrast, methylation of the CaMV 35S promoter was not observed in those transgenic petunia plants which produced red flowers. Similarly, Ulian et al. (1996) reported a correlation between the methylation of *Sst*II sites on the *nos* promoter of a *nos.npt*II.*nos* gene with loss of NPTII protein activity in transgenic petunia. Therefore, one possible reason for the difference in susceptibilty to inactivation of the CaMV 35S and *petE* promoters may be the difference in their susceptiblity to methylation (K. van Dun, personal communication), since the CaMV 35S promoter has approximately three times as many methylation sites (59/kb) as the *petE* –784-bp promoter (18/kb). Further studies of the methylation status of the CaMV 35S and *petE* promoters in herbicide-resistant and silenced transgenic lettuce carrying the *bar* gene may confirm this hypothesis.

Loss of herbicide resistance may also be related to the difference in the strength of the –784-bp *petE* and CaMV 35S promoters (Pwee and Gray 1993), as it is probable that a certain threshold of expression of the *bar* gene has to be reached to attain resistance to 300 mg/l glufosinate ammonium. Therefore, partial inactivation of both promoters may have occurred over several generations, but loss of herbicide resistance may have occurred to a higher degree in 35S-*bar* plants because the initial level of expression was lower than in *petE-bar* plants. Northern analysis would confirm whether *petE-bar* plants maintained the same level of *bar* gene expression between generations.

The loss of transgene expression from the T1 to the T2 seed generations in transgenic lettuce has been described previously (Gilbertson et al. 1996). In their report, 39 transgenic T1 plants carrying a T-DNA encoding either a lettuce mosaic virus coat protein gene (LMV-CP) or an untranslatable LMV-CP gene (LMV-UT) were highly resistant to LMV following mechanical inoculation with the virus. However, after self-pollination, only 8 of the 39 LMV-resistant T1 plants produced LMV-resistant T2 progeny, indicating that 80% of the T1 plants failed to transmit the transgene-encoded LMV resistance to the T2 generation. Unfortunately, details of the promoter used to drive LMV-CP and LMV-UT genes were not given in the report by Gilbertson et al. (1996).

Transgene silencing in lettuce with a CaMV 35S-driven tomato spotted wilt virus N (TSWV N) protein was described by Pang et al. (1996). TSWV N protein accumulation decreased rapidly to undetectable levels in homozygous plants 20–40 days after seed germination. In hemizygous plants, a steady decrease of TSWV N protein to undetectable levels was observed over a period of 80 days after seed germination. High transcription rates of the TSWV N gene were observed in the silenced plants in which TSWV N protein was undetectable, indicating that silencing, in this case, was post-transcriptional and was not related to an inactive CaMV 35S promoter.

In the investigation presented here, it is also possible that the loss of herbicide resistance in 35S-*bar* plants may have been due to a post-transcriptional silencing mechanism and not to inactivation of the CaMV 35S promoter. Northern analysis was not performed on these plants and, consequently, this could not be confirmed. However, as the CaMV 35S and the *petE* promoters clearly had a different effect on expression of the same herbicide resistance trait and NPTII ELISA failed to detect 35S-directed NPTII protein expression in some *petE-bar* plants, it is likely that silencing must have occurred, at least at the level of translation.

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