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M. Czakó · R. P. Marathe · C. Xiang · D. J. Guerra
G. J. Bishop · J. D. G. Jones · L. Márton

Variable expression of the herpes simplex virus thymidine kinase gene in *Nicotiana tabacum* affects negative selection

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Abstract The potentials and limitations of negative-selection systems based on the human herpes simplex virus thymidine kinase type-1 (*HSVtk*) gene, which causes sensitivity to the nucleoside analog ganciclovir, were examined in tobacco as a model system. There were great differences between individual HSVtk$^+$ transgenic plants in ganciclovir sensitivity. Inhibition of growth while under selection correlated with *HSVtk*-transcript levels. Negative selection against HSVtk$^+$ transformants at the level of *Agrobacterium*-mediated transformation using a ganciclovir/kanamycin double-selection medium (the positive selection marker neomycin phosphotransferase-II gene was in the transformation vector) resulted in a three- to six-fold reduction in the frequency of kanamycin-resistant shoots. The efficiency of negative selection in this case was limited due to the great variation in *HSVtk* expression, i.e., the frequently occurring transformants with low, or no, ganciclovir sensitivity escaping negative selection. Two independently constructed *HSVtk* genes showed the same variability of the phenotype in *Nicotiana tabacum* transformants. Distinct phenotypes, ranging from no regeneration through abnormal or delayed regeneration, were observed when leaf segments were placed on shoot-inducing medium supplemented with 10^{-6}–10^{-3} M ganciclovir. The highest *HSVtk* mRNA and ganciclovir sensitivity levels were observed in plants which were transformed with the pSLJ882 chimeric construct. The pSLJ882 plant expression vector carried the coding sequence of *HSVtk*, whereas plasmid pCX305.1 carried an *HSVtk* construct retaining the untranslated 5 leader and viral 3 regions. The pCX305.1 transformants showed, at most, a delayed formation of shoots with thin stems and very narrow leaves. Ganciclovir sensitivity showed typical Mendelian segregation. A gene-dosage effect was also seen at the seedling level in the progeny of two transgenic lines.

Key words Negative · selection · HSV thymidine kinase · Transgenic tobacco

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M. Czakó · R. P. Marathe · L. Márton (✉)
Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

C. Xiang[1] · D. J. Guerra
Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83843, USA

G. J. Bishop · J. D. G. Jones
Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich Research Park, Colney, Norwich, NR4 7UH, UK

Present address:
[1]AgBiotech Center, Waksman Institute, Rutgers, P.O. Box 759, Piscataway, NJ 08855, USA

Introduction

The concept of negative selection is based on the expression of a marker gene that causes immediate or conditional inhibition of growth. Negative-selection marker genes can be used in genetic approaches towards understanding biological processes. Such genes are of use for the identification of mutants defective in signalling processes (Karlin-Neumann et al. 1991), for the elimination of a particular class of cells, thus identifying the place and time of promoter activity (Koltunow et al. 1990; Mariani et al. 1990; Thorsness et al. 1991; Czakó et al. 1992), and in studies of gene inactivation. All these applications require that the negative-selection marker be reliably expressed at a sufficient level in the transgenic plant(s) chosen for the analysis.

An important application of negative selection is at the level of transformation. Transformation followed by a combination of positive and negative selection is a strategy for recovering transgenic organisms resulting from homologous recombination-mediated gene targeting (Capecchi 1989). A negative-selection gene controlled by a promoter active during and after the transformation process will suppress or kill cells that have randomly integrated the vector and enrich for cells with the targeted mutation (or gene).

Only the herpes simplex virus thymidine kinase (*HSVtk*) and the diphtheria toxin chain-A genes have been used at the level of *Agrobacterium*-mediated transformation in plants (Czakó and An 1994; Czakó and Márton 1994). *HSVtk*, as a conditional negative selection gene, has the added advantage that negative-selection can be controlled through its dependency on an externally provided substrate, the nucleoside analog ganciclovir. As such, *HSVtk* had proven extremely useful in the enrichment for homologous recombinants (Mansour et al. 1988) in mammalian systems. There are other conditional and non-conditional negative-selection markers for plants but they have not been tested at the transformation level: these include the alcohol dehydrogenase gene (Widholm and Kishinami 1988), the cytosine deaminase gene of *E. coli* (Perera et al. 1993; Stougaard 1993), the RNase T1 gene of *Aspergillus* and the barnase gene from *Bacillus amyloliquefaciens* (Mariani et al. 1990), the yeast *RAS2* gene (Hilson et al. 1990), the *Pseudomonas* gene encoding exotoxin A (Koning et al. 1992), and the anti-*nptII* gene (Xiang and Guerra 1993).

HSVtk proved to be a good model to demonstrate the potentialities and limitations of negative-selection systems based on the accumulation of a gene product, which may be highly variable among individual transformants. Here we report the variability of *HSVtk* phenotypes in tobacco, which reduces the efficiency of negative-selection applied to a transformant population generated by *Agrobacterium*-mediated transformation. Two independently constructed *HSVtk* genes showed the same variability of phenotype in *Nicotiana tabacum* transformants.

Materials and methods

Molecular cloning

The *HSVtk* gene of the macroplaque strain of human herpes simplex virus type I was taken from plasmid pIC19R/MCI-TK (Mansour et al. 1988). The *HSVtk* coding region with flanking untranslated leader and 3′ regions (*Bgl*II-*Sma*I fragment, nucleotides 253–1799, the coding region is from 310 to 1438, McKnight et al. 1981) was subcloned into pUC19 to give pCX303. *HSVtk* was then moved into a binary plant-expression vector (having the multiple cloning site of pKYLX7; Schardl et al. 1987) as a *Hin*dIII fragment and thus placed under the control of the $35S^2$ (duplicated enhancer) promoter of pKYLX71:$35S^2$ (Mogen et al. 1990; Maiti et al. 1993) and the *rbcS*-E9 3′ region (Hunt and McDonald 1989) to give plasmid pCX305.1 (see Fig. 1A).

A *HSVtk* construct which was free of upstream and downstream viral regulatory sequences was also made. Oligonucleotide mutagenesis (Kunkel 1985) was carried out to introduce a *Cla*I site at the ATG (resulting sequence ATCG*ATG*) and a *Bam*HI site 15 bp downstream from the translation termination codon. The resulting plasmid, pSLJ341, was digested with *Bam*HI and *Cla*I, and the fragment carrying *HSVtk* sequences extending from nucleotide position 310 to 1455 (McKnight et al. 1981) was ligated to pSLJ4K1 (Jones et al. 1992). The p35S/*HSVtk* fusion was released from pSLJ751 by *Bgl*II and *Hin*dIII digestion and was cloned into pSLJ456 (Jones et al. 1992). This yielded pSLJ882 (see Fig. 1B), a binary vector plasmid containing a *Agrobacterium* T-DNA gene *1′ nptII* transformation marker and the p35S *HSVtk* fusion. The binary vectors pCX305.1, pSLJ882, and pGA643 (An et al. 1988) were transferred into *Agrobacterium tumefaciens* by the direct transformation method (An 1987) as described by Czakó and Márton (1994).

Conditions for plant growth and transformation

Nicotiana tabacum L. cv 'Xanthii', cv 'Petite Havana', and cv 'Petite Havana' SR1 plants (Márton et al. 1979) were used for *Agrobacterium*-mediated leaf-segment transformation as described (Jones et al. 1992; Czakó et al. 1992).Transformants were selected on 100 mg l^{-1} of kanamycin hemisulfate (SIGMA). Transgenic plants were named as follows. The 'A', 'B', and 'C' series were transformed by pCX305.1, pSLJ882, and pGA643, respectively. Transgenic lines B1-4 are KAN-resistant F1 plants of individual primary transformants of 'Petite Havana', lines A1–2 are 'Xanthii' primary transformants, and lines A3–5, B5–8, and C1 are 'Petite Havana' SR1 primary transformants. GAN sensitivity of callus and shoot regeneration on leaf segments of transgenic plants was tested on solid RMOP medium as described (Czakó and Márton 1994). For an analysis of the segregation of KAN (600 mg l^{-1}) or GAN (10^{-3} M) sensitivity, seeds were poured from the capsules directly onto the germination medium. All germination media contained 400 mg l^{-1} of Timentin (SmithKline), and 50 mg l^{-1} of the fungicide benomyl (as Benlate DF50, DuPont) to prevent microbial growth. GAN sodium salt (M_r = 277, as Cytovene, Syntex, Palo Alto, Calif.) was dissolved in water, filter-sterilized and added to the autoclaved media. Scoring of resistant and sensitive seedlings was performed 4 weeks after germination. KAN-sensitive seedlings were bleached and the shoot apex was inhibited.

Nucleic acid analyses

Total plant DNA was prepared and probe DNA was labeled as described (Czakó and Márton 1994). For extraction of total RNA (Dr. B. McGurl, personal communication), 3 g of tissue was frozen in liquid nitrogen. The powdered tissue was immediately added to a preheated (95°C) mixture (single phase) of 5 ml of equilibrated phenol and 5 ml of 100 mM Tris-HCl (pH 8.0), vortexed for 1 min, and centrifuged at 3100 *g* for 15 min. The aqueous layer was extracted with saturated chloroform, and RNA precipitated with 2.5 vol of ethanol in the presence of 0.3 M sodium acetate (pH 4.8). RNA was pelleted (10,000 *g*, 20 min at 4°C) and resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Samples were frozen in liquid nitrogen and stored at –80°C. RNA (30 µg per lane) was fractionated on a 1% (w/v) formaldehyde-agarose gel, then transferred onto a Zeta-Probe membrane (BioRad) in 10×SSC using the manufacturer's protocol. Intactness of the total RNA and the accuracy of equal loading between samples were judged from the sharpness and intensity of rRNA bands.

Results

Construction of *HSVtk* type-1 markers and ganciclovir sensitivity of transgenic plants

The *HSVtk* coding region was inserted into binary plant-expression vectors as transcriptional fusions. Two independently constructed plasmids, pCX305.1 and pSLJ882, were compared for negative selection. In plasmid pCX305.1, the *HSVtk* coding region flanked by the untranslated 5′ leader and 360 bp of the 3′ region, encompassing the viral polyadenylation region, was cloned between the $p35S^2$ promoter (cauliflower mosaic virus 35*S* RNA promoter with an enhancer duplication) and the *rbcS*-E9 polyadenylation signals (Fig. 1A).

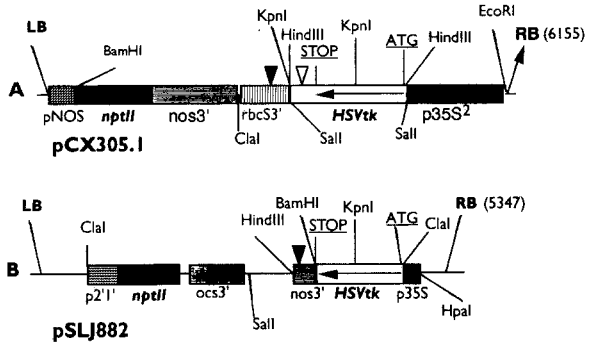

Fig. 1 Physical organization of the T-DNA carrying portions of the *A. tumefaciens* binary plasmid vectors used for introducing *HSVtk* into *Nicotiana* and *Arabidopsis*. **A** In pCX305.1, the *HSVtk* transcriptional fusion is expressed from the $35S^2$ promoter, a cauliflover mosaic virus 35*S* RNA promoter with an enhancer duplication ($p35S^2$), and terminated by the *rbcS*-E9 polyadenylation region (rbcS3′). The *open box* shows the extent of HSV-derived sequence (nucleotides 253–1799, the coding region is from 310 to 1438, McKnight 1980). Black triangle (▼) shows the position of the *rbcS*-E9 polyadenylation site which is 677 bp downstream from the STOP codon. The *open triangle* (▽) shows the position of the viral polyadenylation site. Distance from *HSVtk* translation termination: 64 bp. The *nptII* chimeric KAN-resistance gene is under the control of the *Agrobacterium* nopaline synthase promoter (pNOS) and terminator (nos3′) regions. **B** In pSLJ882, the *HSVtk* transcriptional fusion is expressed from a p35*S* promoter and terminated by nos3′. *Black triangle* (▼): position of the nopaline synthase is: polyadenylation site, distance is 191 bp. The *nptII* chimeric KAN-resistance gene is under the control of *Agrobacterium* gene 2′ promoter (p2′1′) and the *Agrobacterium* octopine synthase terminator (ocs3′) regions. Abbreviations: *ATG* translation start; *LB* left border repeat; *RB* right border repeat; *STOP* translation termination. The length of the T-region is shown in the parentheses next to RB

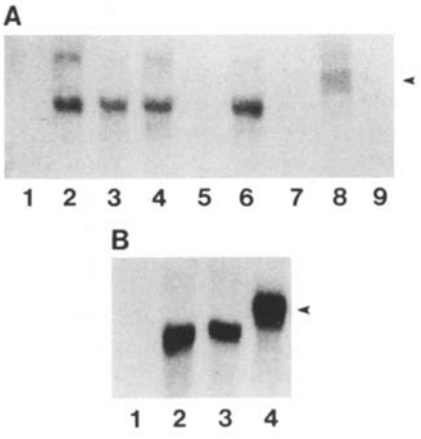

Fig. 2 A, B Northern blots: 30 µg of total RNA isolated from leaves or regenerating-root culture (panel '**A**', *lane 6* only), were hybridized with the ^{32}P-labeled 1.5-kb *Hin*dIII fragment, encompassing the entire coding region of *HSVtk* in the pCX305.1 plasmid (Fig. 1 A). Panel **A**: *1* wild-type (cv 'Petite Havana' SR1), *2* B5, *3* B1, *4* B2, *5* B3, *6* B4, *7* A2, *8* A1, *9* wild-type (cv 'Xanthii'). Panel B: 1 wild-type RLD, 2 RLD 882-1, 3 RLD 882-2, 4 RLD 305-1. The *arrowhead* indicates the position of the 1950-nt RNA standard

Oligonucleotide mutagenesis was carried out to introduce a *Cla*I site at the ATG and a *Bam*HI site 15 bp downstream from the translation *HSVtk* termination codon. *HSVtk* free from upstream leader and downstream viral regulatory sequences was cloned into a p35*S*-nos3′ expression cassette then inserted into a binary vector to make plasmid pSLJ882 (Fig. 1B).

The binary vectors were introduced into *Agrobacterium*. The effective inhibitory concentration of GAN was determined on $HSVtk^+$ transformants. GAN at 10^{-4} M (proven effective in *Arabidopsis*, Czakó and Márton 1994) strongly inhibited (see below) early shoot regeneration from leaf explants of established HSVtk+ plants but did not affect non-transformed tobacco.

Negative selection by ganciclovir against *HSVtk* in *Agrobacterium*-mediated leaf-disc transformation

The negative-selection efficiency against $HSVtk^+$ transformants during transformation was determined by comparing KAN selection in the absence or presence of GAN. Leaf segments were co-cultivated with *Agrobacterium* carrying the $HSVtk^+$ plasmid pCX305.1 or pSLJ882. Shoot regeneration was induced on RMOP medium containing 100 mg l^{-1} of KAN with or without 10^{-4} M GAN. GAN did not significantly reduce the overall number of shoots developing on tobacco-leaf explants cultured on GAN+KAN-containing medium compared to KAN-containing medium. Survivors on GAN+KAN medium included both GAN-insensitive KAN-resistant shoots, and some non-transformed shoots escaping KAN selection. Non-transformed shoots also appeared in the KAN selection plates – as is typical for KAN selection under these conditions. The negative-selection effect became apparent only when the KAN-resistant shoots were scored among those collected from KAN+GAN plates. The number of KAN-resistant shoots was 5.3-times less (7.4% vs. 39.3%) on GAN-containing medium when the pCX305.1, and three-times less (18.5% vs. 57%) when the pSLJ882, binary vector was used for transformation. GAN did not affect transformation by the $HSVtk^-$ pGA643 control plasmid (37% vs 40%).

HSVtk transcript abundance

DNA gel-blot analysis with probes covering *HSVtk* and *nptII*, respectively detected the *HSVtk* and *nptII* sequences in all pCX305.1 and pSLJ882 transformants tested (data not shown). Transcription of *HSVtk* was demonstrated by hybridization of total RNA with the *HSVtk* sequence: 1.4 kb for pSLJ882 and 1.9 kb for pCX305.1. The strongest *HSVtk*-transcript hybridization signals were observed in pSLJ882 transformants, e.g., in 'B4' and 'B5' (Fig. 2A). No hybridization was observed to RNA isolated from 'B3' (Fig. 2A). There were no differences in the intensity between the pSLJ882 (lines 'RLD882-1' and 'RLD882-2') and pCX305.1 (line 'RLD305-1'; Czakó and Márton 1994) *Arabidopsis* transformants used as reference (Fig. 2B).

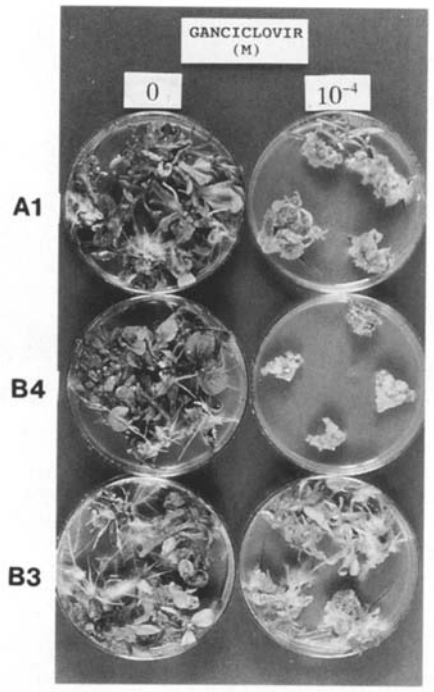

Fig. 3 Effects of GAN at 10^{-4} M on shoot regeneration and callus formation on leaf segments of transgenic tobacco lines after 6 weeks. *1* A1, *2* B4, *3* B3

Transgenic tobacco displays variability in ganciclovir sensitivity

A growth assay was used to test if there was a variation in GAN sensitivity between individual transgenic tobacco plants. GAN was incorporated into shoot-inducing (RMOP) medium at concentrations from 10^{-8} to 10^{-3} M. GAN sensitivity correlated with *HSVtk*-transcript levels. The most severe inhibition was observed among pSLJ882 transformants: total inhibition of proliferation on lines 'B4' and 'B5' at 10^{-3} M GAN. Non-transformed leaf segments, and several transformants, formed callus but did not regenerate shoots at this GAN concentration. GAN at 10^{-4} M still almost completely inhibited growth on 'B4' (Fig. 3) and 'B5' leaf segments, while the non-transformed control regenerated normal shoots. The minimal concentration which caused slender shoot- and narrow leaf-morphology (Fig. 3, right top) was also lower (10^{-6} M), for lines showing high *HSVtk*-transcript levels ('B4' and 'B5'). This symptom occurred at a higher minimum concentration, i.e., 10^{-5} M in the low ('A1' and 'A2') and moderate transcript-level ('B1' and 'B2') transformants, as well as in 'A3' (no RNA data). Explants taken from 'B3', with no detectable *HSVtk* transcript, and the HSVtk$^-$ 'C1' transformants behaved as did the non-transformed control.

The least severe form of GAN inhibition, i.e., the delayed shoot-primordium formation was analyzed quantitatively. Leaf segments were taken from non-transformed plants and from KAN-resistant tobacco lines: a low *HSVtk*-transcript-level (line 'A1') plant carrying *HSVtk* from pCX305.1 and a HSVtk$^-$ pGA643 transformant (line 'C1'). Several shoot primordia appeared on the wild-type and HSVtk$^-$ transgenic leaf segments within 9 days of culture on medium containing 10^{-5} M GAN, while primordia appeared on leaf segments of HSVtk$^+$ plants only after 14 days. The great difference in regeneration frequency between the HSVtk$^-$ and HSVtk$^+$ explants (Table 1) could be seen only around the mid point (12 days) of this time window.

In the high-transcript level 'B5' line, 10^{-3} M GAN in MS medium inhibited rooting and shoot-apex development for 3 weeks. The leaves that slowly developed afterwards were typical for regenerants developing from leaf segments on GAN medium. Wild-type plants were not affected.

Most HSVtk$^+$ plants were phenotypically indistinguishable from wild-type except for certain pSLJ882 transformants which were noticeably paler green both in shoot cul-

Table 1 Effect of GAN on shoot induction on the A1. HSVtk$^+$ tobacco-line leaf explants after 12 days

Ganciclovir concentration (M)	Shoot primordia (% of 0 M GAN)	
	HSVtk$^+$	HSVtk$^-$
0	100	100
10^{-7}	92	93
10^{-6}	71	98
10^{-5}	1	88
10^{-4}	0	93

Table 2 Mendelian segregation of resistance to KAN (600 mg l^{-1}) and GAN sensitivity (10^{-3} M) in the self progeny of transgenic plants carrying the chimeric *HSVtk* either from pCX305.1 or pSLJ882

Line	No. of seedlings			No. of seedlings			
	KAN		Ratio[b]	GAN			Ratio[c]
	R[a]	S[a]		R	I[a]	S	
A1	542	183	3.0:1	415	0	0	N/A[d]
A2	410	146	2.8:1	507	0	0	N/A
A3	569	207	2.7:1	324	0	0	N/A
A4	200	75	2.7:1	210	0	0	N/A
A5	298	86	3.5:1	225	0	0	N/A
B2	74	20	3.7:1	NT[e]			
B3	627	17	36.9:1	593	0	0	N/A
B4	1151	182	6.1:1	25	0	161	1:6.4
B5	298	103	2.9:1	99	209	93	1:2.1:0.9[c]
B6	276	96	2.9:1	67	170	70	1:2.5:1[c]
B7	200	75	2.7:1	252	0	0	N/A
B8	155	49	3.2:1	NT			

[a] R=resistant; S=sensitive; I=intermediate sensitive
[b] 3:1 or 35:1 segregation with one exception. Expected and observed segregation ratios were compared by a χ^2 test ($P \leq 0.05$)
[c] 1:2:1 segregation
[d] N/A=does not apply
[e] NT=not tested

ture and in the greenhouse (5 out of 18). For example, lines 'B4' and 'B5', (the latter expressing the highest levels of *HSVtk* transcript; Fig. 2). The pale color was evident on young leaves, and persisted after spraying with benzyladenine (1 mg l^{-1}), or after grafting to wild-type tobacco.

Negative selection at the level of germinating-seed progenies

To test the negative selection *in planta*, seeds were plated on 10^{-3} M or no GAN. Wild-type seedlings were not affected. In the progeny of lines 'B5' (high transcript level) and 'B6' (Table 2) the overall segregation to normal, intermediate, and severely inhibited phenotypes was 1:2:1, consistent with segregation of a semi-dominant trait. The same lines exhibited a 3:1 segregation of KAN resistant (600 mg l^{-1}) to sensitive (normal) seedlings. On the GAN plate, pigmentation, shoot-apex and root growth was severely inhibited in one out of four seedlings; the cotyledons did not expand completely, and the yellowish seedlings had a bottle-shaped, swollen hypocotyl (Fig. 4). In 50% of the seedlings the yellowish-green cotyledons were swollen. One in every four seedlings was virtually not affected by GAN. The $HSVtk^+$ seedlings from both the severely and intermediately inhibited classes could be rescued by transfer to non-selective shoot-inducing (RMOP) medium. The sensitivity of transgenic seed germination to GAN provides an excellent possibility for negative selection at the seedling level in these individual transformants.

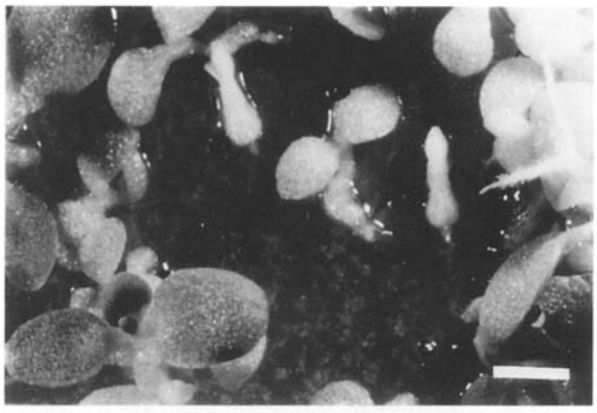

Fig. 4 Segregation of GAN-sensitive and wild-type seedlings in the progeny of the B5 transgenic plant after 6 weeks on 10^{-3} M GAN containing medium. Severe inhibition results in a bottle shape in some seedlings. Others show an intermediate phenotype: arrested development and paler green color as compared to the unaffected, wild-type seedlings. Bar represents 2 mm

Discussion

Two *HSVtk* constructs have been compared. The *HSVtk* gene carried on plasmid pSLJ882 is devoid of the upstream leader and downstream viral sequences still present in plasmid pCX305.1. *HSVtk* expression resulted in distinct phenotypes in the presence of 10^{-6}–10^{-3} M GAN.

HSVtk proved to be a good model to demonstrate certain features of negative-selection systems based on the accumulation of a gene product or RNA. So far only one conditional negative selection marker, the *HSVtk* gene, has been used for negative selection at the level of *Agrobacterium*-mediated transformation in *Arabidopsis* (Czakó and Márton 1994) where it provided a 25-fold reduction on transformant recovery. In tobacco, the reduction in the frequency of KAN-resistant shoots in the presence of GAN was only 3–5.3-fold with both *HSVtk* constructs in both cultivars because shoots that escaped selection masked the differences.

GAN sensitivity correlated to the transcript levels. Not all transformants reached a level of *HSVtk*-transcript and GAN sensitivity sufficient to cause complete inhibition. The broad range of GAN sensitivity in individual transformants explains the overlapping emergence of shoot primordia both from $HSVtk^+$ and $HSVtk^-$ sectors. It is in contrast with the situation in leaf explants taken from an established $HSVtk^+$ individual where *HSVtk* expression was uniform, so there was a clear delay in shoot-primordia formation as compared to the wild-type (Table 1).

Variation in negative selection-marker expression, abortive integration, inactivation by methylation (Renckens et al. 1992), deletion (Depicker et al. 1988), or inactivation by mutation, in the regulation pathway (Karlin-Neumann et al. 1991) will result in transgenic shoots escaping selection pressure, and consequently, reduce the efficiency of negative selection when a population of transformants is subjected to selection. These are difficulties that everybody has to face when working with negative selection at the level of transformation. The very same variation in the case of positive selection would only result in fewer transformants and only one type of escapee. Those that fail to show high-enough resistance simply will not appear, so will not 'contaminate' the outcome as they do in the case of negative selection.

The reasons for the pCX305.1 *HSVtk* construct to confer less GAN sensitivity on individual plants than the transgene in pSLJ882 could be explained by the lower transcript abundance. The weak *HSVtk*-transcript bands indicated low mRNA stability, rather than low expression because the $35S^2$ promoter in plasmid pCX305.1 yielded greater levels of expression of foreign genes than the 35*S* promoter (Maiti et al. 1993). Both pSLJ882 and pCX305.1 *Arabidopsis* transformants had high *HSVtk*-RNA levels. The retention of the original polyadenylation region (360 bp) of *HSVtk* between the coding region and the *rbcS*-E9 plant-polyadenylation cassette, which placed the plant polyA signal 677 bp from the translation terminator codon in plasmid pCX305.1, was not optimal for tobacco. However, regardless of the vector or recipient tobacco cultivar used, a wide range of *HSVtk*-transcript levels and corresponding GAN sensitivity was observed.

The filiform leaf symptom could also be correlated with the transcript level. This phenotype was apparent with both

pCX305.1 and pSLJ882 transformants, but at a much-lower GAN concentration for the higher-expressing plants.

Negative selection was possible also at the seedling level in three out of ten transgenic plants. A gene-dosage effect was also seen at the seedling level in the progeny of two of them (1:2:1 segregation, Table 2.). Both GAN sensitivity and KAN resistance showed typical Mendelian segregation in most plants. The pale phenotype observed in some of the transgenic plants (with high transcript abundance) co-segregated with the GAN sensitivity, suggesting a physiological interference of *HSVtk* expression, i.e., the excess non-specific (deoxy)nucleoside kinase activity.

Variability in the intensity of phenotypic consequences among the individual transformants may limit the use a particular negative-selection gene to specific applications. Negative selection using *HSVtk*/GAN at the level of *Agrobacterium*-mediated transformation proved to be less efficient than in mammalian cells (Capecchi et al. 1989) and *Arabidopsis* (Czakó and Márton 1994). Therefore, *HSVtk* is probably not suited for tobacco gene-targeting experiments, but the high *HSVtk* expression-level individuals can be used in negative selection at the seedling and plant level, and will also be a useful tool for the fundamental studies on gene inactivation and for identification of mutations in signalling pathways.

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