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The Herpes Simplex Virus Thymidine Kinase Gene as a Conditional Negative-Selection Marker Gene in Arabidopsis thaliana

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The human herpes simplex virus thymidine kinase type 1 gene (HSVtk) acts as a conditional lethal marker in mammalian cells. The HSVtk-encoded enzyme is able to phosphorylate certain nucleoside analogs (e.g., ganciclovir, an antiviral drug), thus converting them to toxic DNA replication inhibitors. The utility of HSVtk as a conditional negative-selection marker was explored in Arabidopsis thaliana (L.) Heynh. HSVtk was introduced into Arabidopsis by Agrobacterium-mediated transformation. Transgenic plants were morphologically indistinguishable from wild type and exhibited normal fertility. Ganciclovir at 10⁻³ to 10⁻⁴ M drastically reduced shoot regeneration on transgenic, HSVtk⁺ root explants or callus formation on HSVtk⁺ leaf explants but did not affect the wild-type cultures. There was a 35-fold reduction in shoot regeneration 8 d after transfer to shoot-induction medium. Negative selection against HSVtk activity along with kanamycin selection was also efficient in Agrobacterium-mediated gene transfer experiments. Shoot regeneration was 25 times lower on double-selective (ganciclovir plus kanamycin) plates than in the kanamycin control. This regeneration rate in double-selective plates is in the range of the frequency of shoots normally escaping kanamycin selection in Arabidopsis cultures.

Negative-selection marker genes are in increasing demand for genetic approaches toward understanding biological processes and for selecting cell types with desired alterations. The concept of negative selection is based on the expression of a marker gene that causes immediate or conditional cell lethality. It can be used when a particular class of cells needs to be eliminated. These markers may also be part of positive-negative-selection schemes for homologous recombination-mediated gene targeting (Capecchi, 1989). A potent negative-selection gene controlled by a promoter active during and after the transformation process will kill cells that have randomly integrated the vector and enrich for cells with the targeted mutation (or gene).

Expression of such suicide genes in particular cells of an organism may result in dominant "missing pattern" mutations (as discussed by Koncz et al., 1992) that may have special agronomic importance (i.e., male sterility). Suicide genes can be used to identify the place and time of promoter activity (Czakó et al., 1992) and mutants defective in signaling processes (Karlin-Neumann et al., 1992). Several nonconditional negative-selection markers are available for plant studies, such as the RNase T1 gene of Aspergillus (Mariani et al., 1990), the barnase gene from Bacillus amyloliquefaciens (Prior et al., 1991), the yeast RAS2 gene (Hilson et al., 1990), the diphtheria toxin A chain (Koltunow et al., 1990; Mariani et al., 1990; Czakó and An, 1991; Thorsness et al., 1991; Czakó et al., 1992), the Pseudomonas gene encoding exotoxin A (Koning et al., 1992), and the anti-nptI gene (Xiang and Guerra, 1993).

Conditional lethal genes offer an added element of control over the negative selection through their dependency on externally provided substrates (lethal synthesis). The tms2 gene of Agrobacterium tumefaciens T-DNA has been proposed as a tool for fundamental studies of gene inactivation (De-picker et al., 1988) and for identification of mutations in signaling processes in plants (Karlin-Neumann et al., 1992). The analogous aux2 gene of the Agrobacterium rhizogenes T-DNA also has the potential as a negative-selection gene in Brassica at the plant level (Béclin et al., 1993).

Additional negative-selection genes used in other eukaryotic systems are considered for plant systems to develop one that can be used during the plant regeneration step after Agrobacterium-mediated gene transfer. The human herpes simplex virus thymidine kinase enzyme is able to phosphorylate certain nucleoside analogs that are not accepted by the cells' own kinase(s). For example, GAN, an antiviral drug, is converted to a toxic nucleotide analog that blocks DNA replication in mammalian cells (St. Clair et al., 1984). Thus, HSVtk acts as a conditional lethal marker, which has proven extremely useful in the enrichment for homologous recombinants in the mammalian system (Mansour et al., 1988).

Here we report the development of conditions for efficient negative selection with the herpes simplex virus thymidine kinase type 1 gene in Arabidopsis thaliana.

Abbreviations: GAN, ganciclovir or 9-(1,3-dihydroxy-2-propoxy-methyl)guanine; HSVtk, herpes simplex virus thymidine kinase type 1 gene; KAN, kanamycin; MS, Murashige-Skoog; NAA, 1-naphthaleneacetic acid; nptII, neomycin phosphotransferase gene.

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of wild-type Arabidopsis thaliana (L.) Heynh. genotype RLD (Lingrige and Griffing, 1959) were originally provided by Dr. C.R. Somerville (Michigan State University, East Lansing). Since then, they have been harvested in a growth chamber at 24°C under 16 h of illumination with a mixture of fluorescent and incandescent light. Irradiance, 80 μmol m⁻² s⁻¹, and 70% RH were measured by a steady-state porometer (LI-1600; Li-Cor, Inc., Lincoln, NE). Media for seed germination, root pretreatment (ARMI), and shoot induction (ARMIr) were the same as used by Márton and Browse (1991), except the regenerated plantlets were directly transferred to MS medium containing 0.2 mg L⁻¹ of NAA and no vitamins, omitting the shoot multiplication step on ARMIr. The phytohormone composition for ARMIr was 3 mg L⁻¹ of IAA, 0.15 mg L⁻¹ of 2,4-D, 0.6 mg L⁻¹ of BA, and 0.3 mg L⁻¹ of isopentenyl adenine; for ARMIr the phytohormone composition was 0.2 mg L⁻¹ of NAA and 4 mg L⁻¹ of isopentenyl adenine. All phytohormones were filter sterilized and added to precooled (45°C) media.

Transgenic A. thaliana plants were obtained by the root transformation protocol of Márton and Browse (1991), except that acetosyringone was not used in the bacterial culture medium and the subculture on ARMIr was omitted (see above). About 40 cm of roots were cultured in a plate. Putative transformants were selected on ARMIr containing 50 mg L⁻¹ KAN. A commercial formulation (Timentin; SmithKline Beecham Pharmaceuticals, Philadelphia, PA) of the penicillin ticarcillin and the β-lactamase inhibitor clavulanic acid was incorporated in the medium at 400 mg L⁻¹ to eliminate Agrobacterium. Resistance to KAN was verified by rooting shoots on MS medium containing 0.2 mg L⁻¹ of NAA. MS medium is MS salts (Murashige and Skoog, 1962), 3% (w/v) Suc, and 3 mL L⁻¹ of Miller's solution (6% [w/v] KH₂PO₄). KAN-resistant leaf segments were able to form callus on 50 mg L⁻¹ of KAN-containing C medium (similar to MS, except for 1.0 mg L⁻¹ of NAA, 100 mg L⁻¹ of myo-inositol, and 1 mg L⁻¹ of thiamine hydrochloride). Individual transgenic plants were vegetatively propagated as root cultures (Czakó et al., 1993).

GAN-free base or its sodium salt (as Cytovene; Syntex Laboratories, Palo Alto, CA) was dissolved in water, filter sterilized, and added to the autoclaved media. GAN sensitivity of transgenic plants was tested on solid RMOP medium (same as C except for 1.0 mg L⁻¹ of BA and 0.1 mg L⁻¹ of NAA [Maliga, 1984]).

Bacterial Strains and Plasmids Used

The pGA643 (An et al., 1988), pGA972 (Czakó et al., 1992), and pCX305.1 (Fig. 1; M. Czakó, R.P. Marathe, C. Xiang, D.J. Guerra, G.J. Bishop, A. Jones, L. Mártón, unpublished data) binary vectors were transferred into Agrobacterium tumefaciens strains LBA4404 (Hoekema et al., 1983) and EHA105 (Hood et al., 1993), both carrying a disarmed helper Ti plasmid, by the direct transformation method (An, 1987).

Agrobacterium strains were grown on YEP solid medium or in liquid AB minimal medium (An, 1987) in the presence of appropriate antibiotics: 3 mg L⁻¹ of tetracycline, 7.5 mg L⁻¹ of KAN, 200 mg L⁻¹ of streptomycin, and 25 mg L⁻¹ of rifampicin.

Determination of Bacterial Counts and GAN Sensitivity

The inhibitory effect of GAN was tested by adding 10⁻⁴-, 10⁻⁵-, 10⁻⁶-, and 10⁻⁷ M GAN into wells made in YEP nutrient agar plate (1.5% agar) that had been inoculated by overlaying with the Agrobacterium strain resuspended (100 μL of an overnight suspension diluted to an A₆₀₀ of 1.0) in soft agar (0.7%) YEP medium. The plates were examined for inhibition zones representing minimal inhibitory concentrations after 24 h.

To determine viable bacterial cell density in different stages of co-cultivation and selection, samples were collected from the liquid medium and from the roots. From the root samples, bacteria were liberated by squashing with a glass rod in a conical tube in liquid YEP medium. Colony-forming units were determined by plating aliquots from serially diluted samples on YEP medium.

Genomic DNA Analysis

Total DNA was prepared from leaf tissue by the cetyltrimethyl-ammonium bromide method (Rogers and Bendich, 1988). DNA samples digested with HindIII or KpnI were fractionated on a 0.7% (w/v) agarose gel in 0.5 x Tris-borate-EDTA buffer and then transferred onto a Zeta-Probe membrane (Bio-Rad) by alkaline blotting using the manufacturer’s protocol. Probe DNA was labeled with ³²P using the random priming procedure of the manufacturer (Amersham) and hybridized to the membrane according to standard procedures (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Callus and Shoot Formation Is Inhibited by GAN on Explants of Herpes Simplex Virus Thymidine Kinase Transformants

The feasibility of HSVtk as a conditional negative-selection marker was explored in a plant system. HSVtk was fused to a cauliflower mosaic virus 35S RNA promoter derivative that is present on a binary vector carrying a plant-selectable KAN-resistant nptII (Fig. 1). The chimeric construct was stably transformed into A. thaliana by Agrobacterium-mediated gene transfer. KAN-resistant plants were subjected to genomic DNA gel blot analysis with probes covering HSVtk or nptII. The HSVtk probe detected the HSVtk sequences in most KAN-resistant transformants (Fig. 2, lane 2).

Transgenic plants harboring the HSVtk construct (HSVtk⁺) were phenotypically indistinguishable from wild-type plants and exhibited normal morphology and fertility. Conditions were sought under which the plant material carrying HSVtk exhibited a distinct phenotype upon addition of the toxiogenic substrate, GAN. GAN sensitivity was not manifested at the seedling level. Self-progeny of a randomly picked HSVtk⁺ (RLD305-1, Fig. 2, lane 2) plant and wild-type seeds were germinated in the presence of 10⁻⁴ M GAN, 50 mg L⁻¹ KAN, or both. Seedlings of the RLD305-1 transgenic plant were...
indistinguishable from wild-type seedlings on GAN-containing medium. Segregation for KAN resistance (approximately 3:1) was not affected either. Explant growth assays were also used to test whether GAN had any adverse effects on normal tissues and to determine whether the HSVtk trait was manifested in the transgenic plants. Callus growth assay was carried out on wild-type plants and a randomly picked HSVtk~ plant on GAN-containing medium. Leaf segments were incubated on callus-inducing (RMOP) medium containing GAN at concentrations from $10^{-7}$ to $10^{-4}$ M. GAN ($10^{-4}$ M) proved to be completely inhibitory to callus initiation on HSVtk~ leaf segments, whereas callus formation on wild-type leaf segments was not affected (Table I).

These observations prove that the GAN-HSVtk gene system fulfills the following conditions for utility as a conditional negative-selection marker: (a) the substrate used in "lethal synthesis" should not be toxic to normal plant cells, (b) there should be an absence of any intrinsic enzyme activity capable of converting the substrate into a toxic metabolite in plants, and (c) the reaction carried out by the transgene should be sufficiently specific to minimize interference with normal cellular metabolism.

Sensitivity of the shoot-regeneration process from root segments to $10^{-4}$ M was tested, because this is the procedure of choice for mass transformation of Arabidopsis. To simulate transformation conditions, KAN was also included in the media, and a KAN-resistant HSVtk~ plant (line RLD972-1; transformed by pGA972 carrying the same KAN-resistance gene but without HSVtk) was also used as a control.

Explants taken from amplified root cultures (Czakó et al., 1993) of HSVtk~ (line RLD305-1), HSVtk+ (line RLD972-1), and wild-type RLD plants (line RLD-1, Czakó et al., 1993) were induced to regenerate shoots by a two-step protocol (Márton and Browse, 1991). Either $10^{-4}$ M GAN, 50 mg L$^{-1}$ of KAN, both, or neither was included in ARMIr medium during shoot induction. GAN drastically reduced shoot regeneration from HSVtk~ root explants.

The difference between the wild-type and HSVtk~ cultures was particularly striking at the earliest stages of shoot regeneration (8 d after transfer to the ARMIr medium), with 371 versus 11 dark green foci per plate (not shown). The few shoots that eventually developed on the HSVtk~ roots appeared to be normal. Since no rearrangement could be detected in HSVtk~ as judged from DNA gel blot hybridizations with an HSVtk-specific probe, it may be assumed that these plants escaped negative selection by silencing the foreign DNA, e.g. by methylation (Renckens et al., 1992).

From the observations that GAN inhibited callus initiation on leaf segments and shoot regeneration on transgenic leaf segments but not the growth of established transgenic callus (not shown), it appears that the initiation step is sensitive to GAN. Because of the large nucleotide pools observed in cultured callus cells (Márton et al., 1978), the nucleotide analog GAN is probably more efficiently removed from the DNA or not even incorporated frequently enough to cause inhibition of replication.

**HSVtk as a Negative Selectable Marker in Arabidopsis**

Direct shoot regeneration from root explants is a frequently used method for the generation of transgenic Arabidopsis,

**Table 1. Effect of GAN on callus induction on transgenic Arabidopsis leaf explants**

<table>
<thead>
<tr>
<th>GAN Concentration</th>
<th>Callus Wt Gain</th>
<th>Callus Wt Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control</td>
<td>HSVtk~</td>
<td>HSVtk~</td>
</tr>
<tr>
<td>M</td>
<td>HSVtk+</td>
<td>HSVtk~</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>94</td>
<td>99</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>74</td>
<td>85</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>22</td>
<td>85</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0</td>
<td>108</td>
</tr>
</tbody>
</table>

**Figure 1.** Physical organization of T-DNA of the *A. tumefaciens* pCX305.1 binary vector used for introducing HSVtk into Arabidopsis. HSVtk (from nucleotide positions 263 to 1799; McKnight, 1980; Mansour et al., 1988) is expressed from a cauliflower mosaic virus 35S RNA promoter (p35S) and terminated by the rbcS-E9 polyadenylation region (3′rbcS; Hunt and McDonald, 1989) in a pKYLX7-type (Schardl et al., 1987) binary plant expression vector, pKYLX752. The 35S RNA promoter in pKYLX752 (Dr. A.G. Hunt, University of Kentucky, Lexington, personal communication) is enhanced by a duplication of the region that extends from −417 to −90 with respect to the 35S RNA transcription start site (Guillew et al., 1982). The nptII chimeric KAN-resistance gene is under the control of the Agrobacterium nopaline synthase promoter and terminator regions (An, 1987). Abbreviations for restriction enzymes: B, BamHI; C, Clal; E, EcoRI; H, HindIII; K, Kpnl. Scale is in kb.

**Figure 2.** Detection of HSVtk sequences in Arabidopsis plants transformed by pCX305.1. Southern blots of EcoRI-digested DNA samples were probed with the 1.58-kb HindIII fragment covering HSVtk (Fig. 1). Lane 1, RLDTK1-9 (kan~); lane 2, RLD305-1 (kan~); lane 3, RLDTKG14/15-2 (kan~); lane 4, RLDTKG6/7-1 (kan~); lane 5, RLDTKG4-4 (kan~); lane 6, RLDTKG2-1 (kan~); lane 7, RLDTKG4-1 (kan~); lane 8, RLD wild type. Sizes (kb) of phage λDNA HindIII fragments, separated on the same gel, are shown on the right.
because the resulting transgenic shoots can be immediately subjected to genetic analysis. HSVtk was, therefore, tested in model transformation experiments under a negative-negative-selection regime using conditions established on HSVtk transgenic lines.

Arabidopsis root explants were co-cultivated with Agrobacterium carrying either pCX305.1 (HSVtk+) or pGA643 (HSVtk−, empty vector) plasmids or with neither plasmid. GAN selection (10−4 M) was started 3 d after inoculation, at the shoot-induction stage on ARMIr medium, simultaneously with KAN selection (50 mg L−1). By this time the gene transfer from Agrobacterium to the plant cells is considered to have already taken place.

GAN reduced shoot regeneration from root explants cultured on KAN-containing medium after transformation with pCX305.1 (Fig. 3), where the HSVtk and the KAN-resistance markers are linked on the T-DNA. The inhibitory effect was as striking at the earliest stages of shoot differentiation as in the model experiments with established transgenic root cultures: 2 versus 50 KAN-resistant shoots (>3 leaves) were observed per five plates. A 4-fold difference in the number of shoots remained even after 4 weeks on shoot-regeneration medium (not shown), at which time shoots developed from KAN-sensitive cells that escaped selection (Márton and Browse, 1991). The low number of regenerating shoots on the double-selection (i.e. GAN and KAN) plates indicates

Figure 3. GAN inhibits regeneration of shoots on Arabidopsis root explants transformed with Agrobacterium carrying the HSVtk+ binary vector. Top, Strain EHA105(pCX305.1). Bottom, EHA105 binary vector-free control strain. Twelve days after transfer to the ARMIr shoot-induction medium containing 10−4 M GAN and 50 mg L−1 of KAN. Bar represents 1 cm.

efficient negative selection against HSVtk+ transformants. This inhibition is consistent with the effect on root explants of a pure HSVtk+ transgenic line (RLD305−1). In the case of the empty-vector (pGA643) plasmid, the regeneration frequency was not affected (85 versus 85 shoots).

An interference of GAN with the transforming bacteria is not likely. By the time GAN selection started, the majority of bacterial cells were removed by rinsing the roots at the end of the 2-d co-cultivation, and the remaining cells were subjected to the presence of an antibiotic. In addition, the Agrobacterium strains with or without the HSVtk+ plasmid (pCX305.1) were not inhibited by GAN even at 10−4 M in a standard agar diffusion assay. Nor was the residual Agrobacterium titer reduced by GAN in the presence of root tissues (i.e. after 5 d on ARMIr medium) in the co-cultivation experiment. These results suggested that background from HSVtk expression by the cauliflower mosaic virus 35S RNA promoter (Janssen and Gardner, 1989) of pCX305.1 would have negligible effect on the bacteria.

Typical for a population of plants collected in a leaf-segment transformation experiment, DNA-DNA hybridization with radioactively labeled probes revealed different marker combinations: nptII/HSVtk+ positive lines, nptII- positive lines, and nontransformed lines. A 1.58-kb HindIII probe (Fig. 1) containing HSVtk hybridized with up to four bands in EcoRI-digested DNA isolated from transgenic plants, whereas DNA from the wild-type plant (Fig. 2, lane 8) and from KAN-sensitive plants that escaped selection showed no hybridization signal (lanes 3, 5, and 6). The HSVtk probe also detected the 1.0-kb internal fragment of the HSVtk gene (Fig. 1) as well as up to three border junction fragments with Kpn1-digested DNA. On the same blot the nptII-specific probe (2.1-kb ClaI-BamHI fragment) showed hybridization only to samples from KAN-resistant plants corresponding to lanes 1, 2, 4, and 7 (not shown).

The experiments presented here demonstrate that the herpes simplex virus thymidine kinase gene fulfills the requirements for a conditional negative-selection marker in Arabidopsis. With appropriate culture conditions and optimal timing, the inhibitory effect of GAN on transgenic HSVtk+ plants may translate into a positive-negative-selection regime (Mansour et al., 1988) that could enrich for homologous recombination events in gene-targeting experiments. A positive-negative-selection-targeting vector would contain a region of DNA homologous to a gene, interrupted within an exon by the nptII-positive-selection element, with the HSVtk-negative-selection element ligated onto the end. This marker arrangement is designed to enrich for targeted clones, since cells with a randomly integrated targeting vector have a high probability of retaining both positive and negative-selection elements, whereas replacement-type homologous recombination at the targeted locus often excludes the negative-selection element. Double selection for the presence of nptII and the absence of HSVtk (by culture in the presence of KAN and GAN) would result in a reduced number of stably transfected clones with a randomly inserted positive-negative-selection vector and a concomitant enrichment for homologously recombined clones. Unlike with the nonconditional negative-selection markers, the time and level of negative selection can be controlled. From these experiments
negative selection against transgenic Arabidopsis shoots carrying an intact HSVtk may reduce (up to 25-fold) the number of transformed plants one has to deal with in a gene-targeting experiment. GAN toxicity was observed also with HSVtk transgenic tobacco, but there was considerable variability in GAN sensitivity between individual transformants (M. Czakó, R.P. Marathe, C. Xiang, D.J. Guerra, G.J. Bishop, A. Jones, L. Márton, unpublished data).

During the revision of this manuscript, it came to our attention that the Escherichia coli cytosine deaminase gene (codA) was also shown to provide substrate-dependent toxicity in tobacco, Lotus japonicus (Stougaard, 1993), and Arabidopsis (Perera et al., 1993). However, negative selection at the level of transformation has not been reported with this marker.

ACKNOWLEDGMENTS

We are grateful to Dr. John Fried, Syntex Research Inc., for a gift sample of GAN and technical information, Dr. Daniel J. Guerra for providing the pCX305.1 plasmid, and Dr. Arthur G. Hunt for providing information concerning the pKYLX752 plasmid. We thank Allan R. Wenck for critical reading of the manuscript.

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