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PROGRESS REPORT

Exploration of new perspectives and limitations in *Agrobacterium* mediated gene transfer technology.

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Genetic manipulation of plants often involves introducing homologous or partly homologous genes. Ectopic introduction of homologous sequences into plant genomes may trigger epigenetic changes, making transgene expression unpredictable. Problems caused by the nonallelic interaction of homologous sequences could be avoided by homologous gene targeting (HGT). Therefore, the main objective of this project is to examine the feasibility of using *Agrobacterium* mediated gene transfer (AMGT) for HGT in plants.

Our investigations on gene-targeting in a *Nicotiana plumbaginifolia* recipient plant, in which a single T_R -DNA copy was the target sequence, resulted in the recognition of the high frequency of *Agrobacterium*-mediated transformation related mutagenesis (see **Appendix 1** for a manuscript accepted with revision in Transgenic Research). Inappropriate genetics of the recipient system, made us to **switch to *Arabidopsis thaliana***, which became the favored experimental plant species due to its well known advantages for molecular biology.

At the time this proposal was submitted, however, transgenic plants could be obtained only with a low efficiency. **First, we improved the root transformation protocol** and applied it for the transformation of **sustained root cultures** which can provide unlimited amount of starting material for transformation. It also allows clonal propagation and easy, long term storage of individual somaclones. Full totipotency and genetic stability were also characteristic for these long term-and/or stored cultures (see **Appendix 2.** for reprint). Both efficient transformation and vegetative propagation of the resulting clones are instrumental for the development of our homologous gene targeting recipient system. A sustained root culture of a confirmed heterozygous recipient plant was initiated. The G5 mutant *Arabidopsis*, homozygotic for a large deletion removing the entire *nia2* gene, was crossed to wild-type (WT).

Next, **we constructed the core homologous gene targeting vector** from a minimal binary plant transformation vector (pGA768; from Dr. G. An, Washington State University, Pullman). The target gene homology consists of a 5 kb genomic fragment of the *Arabidopsis nia2* gene (from Dr. N. Crawford, University of California, San Diego). Next, a chimeric kanamycin resistance gene was inserted into the *XhoI* site 2 kbp from the left (5' to the coding region) end of the of the *nia2* fragment, disrupting the *nia2* coding region (**Appendix 3.** shows both the core binary plant transformation vector and details of its T-region). The core vector proved to be functional, as expected, in tobacco leaf disc and *Arabidopsis* root culture transformation, as assayed by selection for kanamycin resistance.

From the heterozygous G5 x WT plant, in which there is a single copy of the *nia2* target gene, several chlorate resistant lines were obtained in transformation experiments where the HGT vector was used, more than from cultures without transformation. NR activity in most of these lines were at basic level, the level of the G5 homozygote material that is 5–10% of the heterozygous NR activity. In the kanamycin selected transformation controls, where no chlorate selection was used the NR activity was the same, basic level (G5 homozygote). The NR deficient phenotype may result from mutation, T-DNA insertion and homologous recombination as well as for ectopic interaction of the homologous *nia2* sequences. The very high frequency silencing of NR activity indicated that most probably cosuppression occurs between the introduced and cognate *nia2* sequences. It is in line with the fact that among the kanamycin resistant transgenic plants obtained with the original pGA472 vector (without *nia2* sequences) no NR deficient plants were found.

To find conditions under which cosuppression does not occur, 3' and 5' deletions were introduced into the *nia2* homologous region. It will help determine what regions of the *nia2* sequences are responsible for silencing. The partial haploidy in the *nia2* locus may be responsible for the complete silencing of each individual transformant. Similar observations have been reported recently by the Matzke laboratory: one transgene copy silenced the other partially homologous copy. Actually, the silenced NR activity in every transgenic plant was below the expected G5 residual level: 48.2 ± 10.3 nmol $\text{NO}_2^-/\text{h}/50$ mg tissue. In the silenced plants it was only 18.3 ± 6.7 . The activity in the heterozygous starting material was 320.9 ± 30.1 . Several transgenic plants from these experiments are being investigated to find out the molecular events leading to frequent silencing in the *nia2* gene because the HGT and insertion mutagenesis can also result in complete inactivation.

As an alternative to the initial chlorate selection, negative selection against random integration events can be used at the level of transformation. A combination of different conditional and nonconditional negative selection markers (which are outside the homologous region) offers efficient enrichment for HGT. In these experiments, the internal kanamycin gene provides positive selection for the transformation, and the negative selection marker(s) provide the enrichment for HGT events. Plasmid vectors carrying the diphtheria toxin A fragment gene (**Appendix 3 and 4**) and the Human Herpes Simplex Virus thymidine kinase gene (**Appendix 3, 5 and 6**) have been constructed and used in transformation experiments. The DtxA coding sequence in these constructs is under the control of the seed specific vicilin promoter (**Appendix 4**) and the negative selection takes place at the seed level in the next generation. Under the control of constitutive promoters the DtxA gene caused dramatic mortality even in mixed bacterial infections due to transient expression. Other negative selection markers were also developed such as the chimeric *Rhodococcus* xylene oxidase (**Appendix 7**) and the chimeric *Pseudomonas* xylene oxidase (**Appendix 8**). These genes produce toxic oxidation products from aromatic compounds in the cells. Their toxic effect may be influenced by antioxidants and substrates in the culture medium (manuscript in preparation). The combination of a sense-antisense orientation of the same positive selection marker gene (we developed it from the hygromycin gene) also resulted in a negative selection effect; about 5-fold in our model experiments (manuscript in preparation). The sense-antisense positive/negative selection construct

T-DNA insert independent mutations induced in transformed plant cells during Agrobacterium cocultivation

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Abstract

Transformation frequencies were determined for 1n, 2n, and 4n *Nicotiana plumbaginifolia* protoplast cultures in *Agrobacterium*-mediated gene transfer experiments. An unexpected, large drop (50%) in plating efficiencies was observed in the non-selected (control) 1n populations after transformation treatment with virulent strains. This effect was not observed in the 2n or 4n cultures or in the 1n cultures when treated with avirulent bacteria. The mortality rate was nonproportionally high and could not be explained by the low (0.1-0.5%) transformation efficiency in the 1n population, indicating mutagenesis of the cell populations independently from the T-DNA insertions. Mutagenesis was also indicated in gene tagging experiments where nitrate reductase-deficient (NR⁻) mutants were selected from haploid and diploid heterozygotic (*nia*/wt that is for the NR apoenzyme gene or *cnx*A/wt that is for one of the molybdenum containing cofactor gene) protoplasts and leaf disc cultures of *Nicotiana plumbaginifolia* after *Agrobacterium* cocultivation. The chlorate-resistant isolates were tested for the T-DNA specific kanamycin-resistance trait only after NR-deficiency had been established. Thirty-nine independent NR-deficient mutants were further analyzed by Southern-blot hybridization. There was no indication of integrated "T-DNA" sequences in the mutated NR genes, contrary to the fact that NR-deficient cells were found more frequently in cell populations which became transformed during the treatment than in the populations which did not. These observations suggest that transformation-competent cells undergo mutagenesis during the *Agrobacterium* gene transfer process not only as a result of stable integration events, but also by such accompanying events that do not result in major changes in the mutated loci. The nature of these changes at the molecular level remains to be elucidated.

Introduction

Current models of *Agrobacterium*-mediated gene transfer (AMGT) involve the induction of bacterial transfer (*vir*) functions by phenolic compounds released by wounded plant cells. The *virD1* and *virD2* gene products generate single stranded nicks on the lower strands of the T-region 25 bp flanking repeats (left and right borders). Single stranded linear molecules are believed to be generated according to the strand replacement model predicting termination at the nicks. The right border probably initiates the T-strand synthesis and the left border repeat terminates it. The right border, apart from the 25 bp repeat, also carries a specific enhancer. The "overdrive" sequence is required for efficient transformation. The *VirD2* protein stays tightly bound to the 5' end while the T-strand becomes associated with the *virE2* gene product (single-stranded DNA binding protein) forming a translocation and integration complex according to recent models (Hooykaas and Schilperoort, 1992; Zambryski, 1992). Very limited information is available about the integration events themselves. It is not really known what kind of intermediates are expected besides the completed integration events. Even a double stranded recombination model cannot be excluded (Meyerhofer et al., 1991). T-DNA integration occurs seemingly randomly (Wallroth et al., 1986; Chyi et al., 1988), with the limitation mentioned below, (Koncz et al. 1989). The junction points are typically in or near the T-DNA borders although large numbers of aberrant T-DNA integration events (>40 %) have also been observed by different laboratories (Gheysen et al., 1990; Deroles and Gardner 1988).

High frequency insertional activation of promoterless reporter genes (harbored near the left or right side of the T-DNA) suggests a preferential integration of T-DNA into transcribed regions of the plant genome during *Agrobacterium* mediated gene transfer (Koncz et al., 1989). Thus, a high efficiency gene tagging is expected using the insertion mutagenesis approach (for a review see: Koncz et al., 1992).

Very high efficiency gene tagging (>80% of the recessive mutants) was observed in experiments in which intact *Arabidopsis* seeds were exposed to the transforming agrobacteria (Feldmann 1991). The mechanism involved in this case might be fundamentally different from those involved in somatic cell transformation, because the actual transformation event appears to take place in the flowers after or during gametogenesis (Feldmann, 1991) and not in the somatic cells of the germinating seed.

Several laboratories have observed large numbers of mutations independent of T-DNA insertions when somatic cell tissue cultures were used for *Agrobacterium*-mediated gene transfer. In early experiments with *Nicotiana* species, there was not a single case found, in which T-DNA cosegregated with an induced mutation indicating that the mutations were independent of T-DNA insertions. The use of a promoterless selection marker gene in these experiments presumed integration into actively expressed

genes (Andre et al., 1986; Koncz et al., 1992), and, logically, integration should have been linked to the mutation.

In *Arabidopsis thaliana*, which has a small genome compared to the different *Nicotiana* species, only a few mutants from a large number of transformants were identified as having originated from a T-DNA insertion event (Koncz et al., 1990; Van Lijsebettens et al., 1991). The remaining mutants were explained by "tissue culture effects", i.e. generation of genetic variability by the *in vitro* conditions (Larkin and Scowcroft, 1981; Gould, 1986). There was no attempt to determine whether the mutation was caused by the culture conditions or by the transformation process itself in the affected cells. Only recessive mutations cosegregating with a T-DNA (thus originating from an insertion event) were analyzed further.

We present here results that suggest an accumulation of genetic lesions that occur during the AMGT process. Predominantly, these genetic lesions were not physically linked to the T-DNA insertions introduced into the host genome but preferentially occurred in transformed cells. These observations indicate that mutagenesis is associated with, but not necessarily linked to transformation in the affected cells.

Materials and methods

Bacterial strains and plasmids

A. tumefaciens Ach5 and LBA4013 are both tumorigenic, the latter being a highly virulent (Márton et al., 1979) derivative of the first strain (Hamilton and Fall, 1971). LBA4013 carries the plasmid pAL102 (Klapwijk et al., 1978). The avirulent strain LBA4040 is derived from *A. tumefaciens* Ach5 and its plasmid, pAL209, is derived from pTiB6S3 by deletion of about 75% of its plasmid genome, including the entire T-region (Koekman et al., 1978). *A. tumefaciens* A6044 is a derivative of *A. tumefaciens* A281 (Montoya et al., 1977) containing, besides the binary vector pGA472 (An et al., 1985), the pTiBo542, wild-type Ti plasmid. The avirulent strain C58C3 is a plasmid-free derivative of the wild-type strain C58 (Hamilton and Fall, 1971; from R.A. Schilperoort, Leiden, The Netherlands), and has the same chromosomal background as does A281. LBA4404 is a non-tumorigenic helper strain (Hoekema et al., 1983). *A. tumefaciens* strains were grown on YEP solid medium or in liquid AB minimal medium in the presence of the appropriate antibiotics: tetracycline, 3 mg l⁻¹; kanamycin 7.5 mg l⁻¹; streptomycin 200 mg l⁻¹; rifampicin 25 mg l⁻¹ (An et al., 1988).

Escherichia coli HB101 (Boyer and Roulland-Dussoix, 1969) was used as a recipient for the binary vector-type plasmid pGA472 (An et al., 1985) reisolated from *A. tumefaciens* A6044 by a published procedure (An et al., 1988). Cloned fragments of the pTiAch5 T-region (pGV0120, pGV0153, pGV0201; DeVos et al., 1981) and the 1.8 kbp *Hind*III-*Bam*HI fragment of the Tn5-derived neomycin

phosphotransferase II gene from the plasmid pKC7 (Rao and Rogers, 1979) were used as probes for T-DNA detection. The 1.6 kbp *EcoRI* fragment of the cDNA clone p13-29 of *Nicotiana tabacum* (Calza et al., 1979) was used as a probe for the nitrate reductase gene.

Plant material, callus culture and plant regeneration

Methods for axenic culture of *N. plumbaginifolia* Viviani plants, and a description of the androgenic haploid line, HNP28 have been published (Sidorov and Maliga, 1982). Chromosomes were counted in root tips by a standard acetocarmine staining technique. Seeds were surface sterilized and germinated as described (CZAKÓ and Márton, 1986).

Protoplast isolation and coculture transformation

Protoplasts were isolated from leaves and transformed by coculture with *A. tumefaciens* as described (Márton et al., 1979; Márton et al., 1985) with several modifications. Naphthaleneacetic acid, 1.0 mg l⁻¹, 2,4-dichlorophenoxyacetic acid, 0.1 mg l⁻¹ and 6-benzyladenine, 0.2 mg l⁻¹ were used as phytohormones in the medium containing 0.4 M sucrose. At the end of the cocultivation period the plant cells were collected by centrifugation after the cultures had been diluted with four volumes of low density, isosmotic W5 solution (Márton et al., 1982b). Washing in W5 was repeated twice. Complete dissociation of the cell aggregates was accomplished by a short cellulase treatment at the end of the cocultivation period: 30 min digestion of the cultures in K₃ medium (Márton et al., 1979) containing 0.5% cellulase R-10. In fresh protoplast cultures (0-24 hrs old), enzyme treatment was not required; aggregation was negligible. Kanamycin-resistant transformants were selected at the colony level by plating in or spreading (5,000 - 10,000 colonies in 2 ml liquid medium for a 10-cm culture dish) onto 0.4 M sucrose K₃ medium containing kanamycin sulfate, Sigma, (100 mg l⁻¹), carbenicillin, Roerig, New York, NY10017, (200 mg l⁻¹), cefotaxime, Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ 08876, (200 mg l⁻¹), and solidified with 0.7% (w/v) agar. Resistant lines appeared as green growing calli against a background of brownish dying colonies within 2-4 weeks.

Isolation of nitrate reductase-deficient mutants

Leaf protoplast cultures were cocultured with bacteria for 36 or 48 hours and after repeated washing, the plant cells were allowed to form small colonies (2-3 weeks) without selection in liquid K₃ medium but in the presence of 200 mg l⁻¹ carbenicillin and 200 mg l⁻¹ vancomycin (Sigma). The resulting colonies were plated onto selective medium containing 50 mM potassium chlorate, 100 mg l⁻¹ kanamycin or both. Kanamycin-resistance and NR-deficiency were established by repeated selection and by *in vivo* NR assays,

respectively.

Chlorate-resistant clones were isolated from protoplast-derived colonies as described (Márton et al., 1982a), except that the KClO_3 (40 mM; autoclaved separately in water) and the succinate buffer (8.25 mM; buffered to pH 5.6 with NH_4OH and filter sterilized) were added to solidified (0.7% agar) K_3 medium containing 0.4 M sucrose. The addition of succinate to the culture medium was required in order to avoid toxicity of the exclusive ammonium assimilation in the NR-deficient cells (Müller and Grafe, 1978). For double selection, kanamycin sulfate at 50 mg l^{-1} was also included. Colonies were plated either into the agar or spread on the surface. Selected clones were maintained on $\text{NH}_4\text{-S-RMOP}$ (Márton et al., 1982a). Ammonium-succinate was replaced by 10 mM disodium-succinate for maintenance of callus cultures. Both $\text{NH}_4\text{-succinate}$ and Na-succinate were autoclaved with the medium.

NR-deficient lines were identified by comparing growth on RMOP to growth on $\text{NH}_4\text{-S-RMOP}$ as well as by assaying "in vivo NR activity" (Márton et al., 1982a).

Classification of clones by XDH activity, molybdate response, and plant regeneration ability

Xanthine dehydrogenase (XDH) activity was detected in crude extracts after polyacrylamide gel electrophoretic separation as described (Mendel and Müller, 1976). Molybdate complementation was tested on RMOP medium supplemented with 0.2 M Na_2MoO_4 . In *cnxA*-type mutants (only the final step of the molybdenum-containing cofactor biosynthesis is impaired: the insertion of molybdenum into the cofactor moiety) NR-activity, growth without succinate and shoot regeneration ability were all restored on molybdate (Márton et al., 1982a). In the other NR mutant classes, shoot regeneration could be induced by AgNO_3 treatment (Purnhauser et al., 1987). Green spots or shoot primordia were transferred to MS salts (Murashige and Skoog, 1962) with sucrose (30 g l^{-1}) and 10 mM Na-succinate for plant regeneration and rooting. For seed-setting, plantlets were grafted on topped wild-type *N. tabacum* var. Petite Havana (SR1; Maliga et al., 1973) plants. Segregation of NR-deficiency in progeny seedlings was analyzed on 'Mn' medium containing 10 mM KNO_3 as the sole nitrogen source (Negrutiu et al., 1983).

Fusion complementation of NR-deficient lines

Protoplast fusion treated cells were cultured in K_3 medium containing 0.4 M sucrose in which only cells with NR-activity could grow (Márton et al., 1982b). Complementation was confirmed by the protoplast fusion disc complementation assay (Biasini and Márton, 1985), an *in vivo* NR assay.

Opine analyses

Octopine synthase was assayed in tissue extracts (Otten and Schilperoort, 1978). Agropine and mannopine was detected in tissue extracts as described (Czakó and Márton, 1986).

DNA analyses

DNA was extracted from plant material as described (Rogers and Bendich, 1988). Binary vector plasmid DNA was isolated from *A. tumefaciens* as described (An, 1987). Standard methods were used for preparation and restriction endonuclease digestion of plasmid DNA from *E. coli*, and for plasmid transformation. The digested DNAs were subjected to horizontal agarose (0.7 %) gel electrophoresis (2 Volts cm^{-1}) in TEA buffer (Sambrook et al., 1989). DNA was blotted either onto nitrocellulose (type BA85; Schleicher & Schuell Co; Southern, 1975) or onto Zeta-Probe membrane (Biorad) according to the instructions of the supplier. Purified DNA fragments were labeled by the random priming technique (Feinberg and Vogelstein, 1983) using a hexanucleotide mixture (Pharmacia) as primer. Hybridization on nitrocellulose was as described (Czakó and Márton, 1986), and on Zeta-Probe according to the instructions of the supplier.

Results and discussion

Enhanced mortality of haploid protoplasts during cocultivation with a virulent A. tumefaciens strain

In *Agrobacterium*-mediated gene transfer experiments (Márton et al., 1979; Márton 1984) a drop in plating efficiency was seen in cell wall regenerating haploid *N. plumbaginifolia* protoplast cultures cocultured with the tumorigenic *A. tumefaciens* strains Ach5 or LBA4013. The plating efficiency in diploid (and in tetraploid) cultures was not significantly affected by either of these two bacterial strains.

The haploid protoplast population was not affected if allowed to replicate prior to cocultivation or when cocultivated with the avirulent strain LBA4040 (same host but carrying the avirulent plasmid pAL209) (Table 1). In experiments with the *A. tumefaciens* Ach5, where this problem has been addressed, the plating efficiency and the transformation efficiency were simultaneously determined in the cocultivated cultures by plating 2.2×10^5 colonies onto hormone-free medium. Transformation efficiencies of 1n, 2n and 4n protoplast cultures selected for tumorous (hormone independent) growth were 0.21%, 0.96% and 2.07%, respectively. Selected colonies were further tested for octopine synthase ('OS') activity (another marker on the pTi-Ach5 T_L -DNA), and 48% proved to be positive. The transformation frequency of the diploid cultures grown without any selection was also determined by screening individual colonies not only for the

above markers but also for the pTiAch5 T_R-DNA markers agropine synthase - 'AS', and mannopine synthase - 'MS'. Out of 488 colonies only 2 tumorous, 4 tumorous/OS+, 1 tumorous/OS+/AS+/MS+, 1 AS+/MS+ and 2 MS+ colonies were found. The combined frequency, 2.25%, indicated that the above selection was very efficient for identifying transformed lines. The presence of MS and AS activities in non-tumorous lines (3 out of 11 T-DNA transfers) suggested the frequent occurrence of independent integration of T_R-DNA (Czakó and Márton, 1986) under these conditions.

The relatively low transformation frequency in haploid cultures (0.21%) does not explain the observed lethal effect of the transformation process (greater than 50% drop in plating efficiency in the nonselected haploid cultures). When transformation treatment was initiated at various time points after protoplast isolation, the cultures gradually escaped this effect. The original plating efficiency was restored when cocultivation was initiated after 36 hours. By this time, the rather well synchronized leaf mesophyll protoplast-derived cell population reached the genome replication (S-)phase and entered the first cell divisions (Table 1). The latter observation and the result that no decreased survival was observed when haploid protoplasts were treated with the same but avirulent bacterial strain, and that plating efficiency in diploid (or in tetraploid) cultures was not significantly affected by the virulent bacterial strains, suggest that the damage could be genetic and caused by mutagenesis that occurs during the transformation process.

Mutagenesis related to Agrobacterium-mediated gene transfer

A similar mutagenesis-like phenomenon was also observed in plant gene tagging experiments involving different nitrate reductase genes. NR-deficient cell lines were isolated from haploid and diploid protoplast cultures as well as diploid leaf-disc cultures after *Agrobacterium*-mediated gene transfer treatment. The leaf-discs were from diploid plants that were heterozygotic for certain NR genes (*N. plumbaginifolia* wild-type was crossed either with *nia*- or *cnxA*-type NR⁻ mutants previously isolated and characterized genetically and biochemically in our laboratory; Biasini and Márton, 1985; Márton et al., 1982a; 1982b; 1985; Purnhauser et al., 1987).

a. Isolation of NR⁻ lines from haploid protoplasts

In some experiments haploid *N. plumbaginifolia* protoplasts were cocultivated with *A. tumefaciens* A6044 carrying the binary vector pGA472 or with strain LBA4404 as a non-transforming control. After removal of most bacterial cells by repeated washings, the plant cells were cultured in liquid medium in the presence of carbenicillin and cefotaxime. Selection for NR-deficiency was carried out on 40 mM chlorate containing medium as described above. The chlorate-resistant colonies were assayed for NR

activity by the *in vivo* NR assay.

The presence of the molybdenum containing cofactor (MoCo) was ascertained by a xanthine dehydrogenase (XDH) assay. As previously observed for haploid starting material (Müller and Grafe, 1978; Márton et al., 1982a; 1982b; Gabard et al., 1987), apoenzyme minus (*nia*) lines were predominantly isolated, possibly because of the negative pleiotropic effects that MoCo deficiency places on the *cnx*-type mutants in a medium supplemented only for NR-deficient mutants. The only MoCo mutant isolated fell into the *cnxA* complementation group. It had the *cnxA*-specific, high molybdate reparable NR⁻ phenotype. In the rest of the fully NR-deficient category (11 cell lines), confirmation of the complementation group (*nia*) was carried out by the protoplast fusion disc complementation assay. This technique allowed for fast sorting of cell lines. The NR-deficient cell lines were also tested for kanamycin-resistance encoded by the vector used for transformation (Table 2). The double selection approach was also tried (simultaneous selection for chlorate- and kanamycin-resistance) but it proved to be inefficient. The kanamycin selection interfered with chlorate selection by causing high chlorate tolerance. On double selection media, transformed cells expressing kanamycin-resistance were scored as chlorate-resistant (putative NR-deficient). Only a few NR-deficient cell lines were recovered from the double selection experiment by individual testing of colonies for NR activity. The result of the double selection suggests that kanamycin directly or indirectly inhibits NR activity under these conditions.

NR-independent chlorate toxicity was seen in *N. plumbaginifolia* cultures (Márton et al., 1982a). In *Chlamydomonas*, chlorate toxicity was found to be primarily dependent on the nitrate transport system and chlorate, itself, caused mutations (Prieto and Fernández, 1993).

b. Isolation of NR-deficient mutants from diploid material

Diploid protoplast and leaf disc cultures, heterozygous for either the *cnxA* or the *nia* allele, were used as above to isolate NR⁻ lines (Table 2 and 3). The *cnxA*/wt and *nia*/wt heterozygotes allowed us to focus on one gene at a time, as well as to avoid the low plating efficiency consistently seen with transformed haploid cultures (see Table 1).

In diploid cultures a higher frequency of NR-deficiency was observed than in haploid cultures. The opposite result was expected because mutations in more genes (8-10 vs. 1 structural genes) could lead to NR-deficiency in the haploid system. In the heterozygous cultures, recessive mutations only in the single *CNXA* or *NIA* locus are likely to be recovered. The above finding - the higher frequency of the NR⁻ cells from the diploid heterozygous cultures - can be explained by different mechanisms:

(i) Diploid cells were intrinsically more vigorous and allowed for significant growth of the cultures during the selection

period. The involvement of much larger cell populations in the selection could contribute to the observed higher frequencies (the actual number of colony forming units did not change, only the number of cells in the colonies). During selection the occurrence of one resistant cell in a cluster could classify the whole colony as chlorate-resistant.

(ii) From the diploid, heterozygous cultures, the NR⁻ cells were recovered more efficiently because of their vigor.

(iii) Tissue culture conditions caused multiple lethal mutations in the haploid cell cultures. The survival of diploid cells, heterozygous for single *nla* or *cnx* mutations (the degree of heterozygosity for other recessive lethal mutations is negligible), is not significantly affected by the mutagenesis because they are much less exposed to mutations leading to lethal auxotrophy.

Therefore, NR-deficient (and any other auxotrophic) mutants could be scored far more efficiently using the heterozygotic diploid cultures rather than the haploids and the observed frequencies may not be directly comparable between the 1n and 2n experiments.

The frequency of NR⁻ mutants increased about two-fold in the haploid and six-fold in the heterozygotic cultures compared to the non-treated in experiments using the strain A281(pGA472) (Table 2). This increase is expected because of the mutagenic effect of T-DNA insertions. At the same time, the proportion of transformed (kanamycin-resistant) colonies in the NR-deficient population also increased dramatically. The transformation efficiency in several experiments was approximately 0.1% for haploid *N. plumbaginifolia* cultures under the same conditions. The same frequency of transformants could be expected among the NR-deficient lines if the two events were independent. The significantly higher (70x) incidence of the transformed genotype in the NR-deficient population indicates a certain link between mutations resulting in NR-deficiency and transformation.

The experiments were repeated using leaf disc transformation of the above heterozygotic plants. Leaf discs, cocultured with bacteria, were placed onto selective callus induction medium containing chlorate, and NR-deficient cell lines were established (as above). When the NR⁻ cell lines were screened for kanamycin-resistance, an even more pronounced linkage between transformation and mutations leading to NR-deficiency became apparent. Without kanamycin selection, two out of three NR-deficient lines from the *nla*/wt and the one from the *cnxA*/wt heterozygous material selected on chlorate medium were found to be transformed. In the rest of the 39 lines, obtained on chlorate containing medium, with wild-type level of NR-activity (escapees of chlorate selection) no other kanamycin-resistant lines were found, suggesting an involvement of the transformation itself in the mutagenesis of the NR gene (Table 3).

Because the host strain (A281) which carried the binary vector construction carried a wild-type Ti plasmid, tumorous

transformants were also seen. Two of the five leaf disc- and one of the protoplast culture-derived NR-deficient cell lines were tumorous. Interestingly, all three carried the binary vector T-DNA (kanamycin-resistance) as well indicating frequent co-transfer of T-DNAs (An et al., 1985).

c. Southern blot analysis of putative insertion mutants

Cell lines with both transgenes and NR deficiency were considered to represent putative gene tagging events from all three experiments. DNA analysis was performed to test this hypothesis.

Genomic DNA from the MoCo deficient category (*cnxA*) could not be analyzed with *N. plumbaginifolia* specific probes because, to our knowledge, none of the several MoCo genes have been isolated; neither cDNAs nor even the protein products are available or known in plants. Therefore, we took an indirect approach in the experiments, in which the *cnxA*/wt heterozygotic plant was used as a recipient. We analyzed the localization of the T-DNAs in the plant genome, and made an attempt to detect a pattern which could indicate T-DNA insertion within the same genomic fragment of different transformants. Common insert-harboring fragments in the plant genome would be indicated by the generation of identical sized bands by enzymes that do not cut the insert, an equal sum of two hybridizing fragments with enzymes that do cut, and confirming patterns in further digests. By digesting the genomic DNA with restriction enzymes that did not cut within the inserted T-DNA, insert-harboring genomic fragments were generated and their size determined by Southern blot hybridization with a T-DNA probe. Five restriction enzymes that do not cut and two that cut only once into the inserted T-DNA (*EcoRI*, *MluI*, *SallI*, *ScaI*, *SmaI*, *XbaI* and *XhoI*, respectively) were used individually and in combination to digest genomic DNA of 11 MoCo-deficient transformed lines. At least 20 T-DNA insertions were analyzed. Six of the lines carried one, four of them two and one of them multiple copies of pGA472 T-DNA; three of them carried Ti-plasmid T-DNA as well (see above). There was no indication of common insertion sites in any of the 11 MoCo deficient/kanamycin-resistant cell lines analyzed to justify further investigation. If rearranged T-DNA was present in the same genomic fragment, different bands would be generated on the southern blots and those lines would appear as ectopic integrations in these experiments. However, in most transformants (apart from minor variations in the border junctions [Meyerhofer et al. 1991]), full size T-DNA integration is expected. The plants regenerated from these cell lines were almost sterile, making genetic analysis of linkage between the kanamycin-resistance and NR-deficiency impossible.

In the apoenzyme-deficient category a direct approach could be taken because the heterologous NR apoenzyme cDNA probe from *N. tabacum* (Calza et al., 1987) hybridized to the single NR apoenzyme gene in *N. plumbaginifolia*. The NR probe allows for direct demonstration of T-DNA insertion into the NR coding region by fragment size shifts and hybridization of NR and T-DNA

specific probes. Surprisingly, direct involvement of a T-DNA insertion into the NR gene was not established in these experiments either. Involvement of T-DNA insertions in the NR deficiency was not indicated even by indirect evidence in five kanamycin-resistant/apoenzyme-deficient lines. The search for silent T-DNA sequences in the NR coding region from 12 additional apoenzyme-deficient (*nia*) cell lines (kanamycin-sensitive), and 11 leaky cell lines (complementation group could not be identified because of residual NR activity, kanamycin-sensitive ones) gave similar results. The large scale screening for restriction fragment length polymorphism (10 six-base recognition enzymes: *Bam*HI, *Eco*RI, *Kpn*I, *Mlu*I, *Nco*I, *Sal*I, *Sca*I, *Sma*I, *Xba*I, *Xho*I and 2 CpG methylation-insensitive four base recognition enzymes: *Mbo*I and *Msp*I) resulted in the identification of two small alterations detected by a four-base recognition enzyme in the tested locus. There was an extra hybridizing band in the higher molecular weight region in *Mbo*I digested DNA from the *cnxA*/wt-derived line #24 (panel A on Fig.1). In *Msp*I cut DNA from the partially NR-deficient #81 line, the uppermost band was displaced to the higher molecular weight region (panel B on Fig.1).

Conclusion

The existence of several NR⁻ mutant lines not carrying a T-DNA insert could be explained by the mutagenic effect of the tissue culture conditions. However, it is difficult to explain why these mutations preferentially occurred in the transformed cell population under conditions where the transformed state did not have any known selective advantage. These findings indicate that the observed mutagenic effect predominantly originated from the transformation process itself and only partly, if at all, from the culture conditions (see haploid cultures without AMGT).

The observations leading us to propose that the transformation process in competent cells is accompanied by mutagenesis, which is related to the transformation process but not caused by the T-DNA insertions, are summarized below. (i) AMGT-treatment of haploid *N. plumbaginifolia* protoplast cultures resulted in a large drop in survival that was not proportional to the relatively low transformation efficiency; (ii) cell death correlated with the use of bacteria with intact gene transfer ability and to the haploid state of the exposed plant cells, indicating an accumulation of gene transfer related-genetic lesions; (iii) more NR-deficient mutants could be obtained in AMGT experiments than in the avirulent *Agrobacterium*-treated control cell population; (iv) although NR-deficiency frequently co-occurred with gene transfer in the AMGT-treated cell population (competent cells), T-DNA sequences were not found in the mutant NR genes by direct or indirect means.

Mutations accompanying the gene transfer process appear to be far more frequent than actually observed (and therefore analyzed)

in marker transfer events (see lethal effect). Most of the non-tagged mutants observed by other authors could very well have originated from the proposed AMGT-related mutagenesis which is neither of insertional nor tissue culture origin. Such a degree of transformation-induced genetic instability is apparently characteristic for the somatic cells, because it has not been seen in seed transformation experiments, where the actual transformation events take place in the flowers during or immediately after gametogenesis and zygote formation (Feldmann, 1991).

The mechanism of *Agrobacterium*-mediated gene transfer related mutagenesis remains obscure. A large number of T-DNA intermediates were seen in cocultivated *Petunia* protoplasts during the the first 24 hours of cocultivation (Virts and Gelvin, 1985) and in *Petunia* leaf disc cocultivation experiment a systemic transient GUS expression in the exposed cells (Janssen and Gardner, 1989) also suggested that the efficiency of gene transfer from *Agrobacterium* is extremely high. One can speculate that the entering T-DNA copies initiate several transformation events in the nucleus of each competent cell but the majority of these intermediates are repaired before replication could stabilize them. Only one or a few integrating T-DNA copies survive and become responsible for the observed transformation. The accompanying mutagenesis might be explained by genetic lesions created at the altered and partially repaired transformation initiation sites by an error-prone repair like mechanism (Walker, 1985) as it was seen in *E. coli* transformants (Lukácsovics and Venetiáner, 1991).

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Table 1. Plating efficiency of protoplasts of different ploidy levels after cocultivation^a initiated at various time points after protoplast isolation, with virulent or avirulent *Agrobacterium* strains.

Age of protoplasts (hours)	Plating efficiency(%) ^b			
	1n		2n	
	vir ^c	avir ^d	vir	avir
0	43	77	73	75
12	31	88	59	85
24	61	79	68	76
36	71	67	74	76
48	78	69	68	73
72	78	87	82	75

^aThe *Agrobacterium* cocultivation treatment was applied for 48 hours.

^bStandard deviation was between 4.04 and 8.02.

^cvir: LBA4013 virulent strain

^davir: LBA4040 avirulent strain

Table 2. Selection of NR⁻ mutants after coculture transformation of *Nicotiana plumbaginifolia* protoplasts

Plant genotype	Bacterial strain	Selection	Number of NR ⁻ kan ^R		Frequency of NR ⁻
1n wt <i>N.plum.</i>	none	chlorate	3	0	1.32 x 10 ⁵
	C58C3	chlorate	2	0	1.90 x 10 ⁵
	A6044	chlorate	42	3	3.47 x 10 ⁵
	none	chl+kan	0	0
	A6044	chl+kan	14	14	1.04 x 10 ⁵
	A6044	kanamycin	ND ^a
2n <i>cnxA</i> /wt	none	chlorate	13	0	8.84 x 10 ⁵
	A6044	chlorate	52	7	52.70 x 10 ⁵
	A6044	chl+kan	3	2	1.65 x 10 ⁵
	A6044	kanamycin	ND

^aND= not determined.

Table 3. Selection of NR⁻ mutants after leaf disc transformation of diploid heterozygote *N. plumbaginifolia* with A281(pGA472) bacteria

Plant genotype	Selection	NR ⁻ /kan ^R
wild-type <i>N.plum.</i>	chlorate	0
	chl+kan	0
<i>nia</i> /wt	chlorate	3/2
	chl+kan	1/1
<i>cnxA</i> /wt	chlorate	1/1
	chl+kan	1/1

Legend to figure:

Figure 1. Hybridization of tobacco nitrate reductase cDNA probe to DNA gel blots of nitrate reductase-deficient lines. DNA was digested with *Mbo*I (panel A) or *Msp*I (panel B). Note the restriction fragment length polymorphism of the *nia* gene of the NR⁻ lines 24 and 81.

A: MboI

B: MspI

bp

1722-

861-

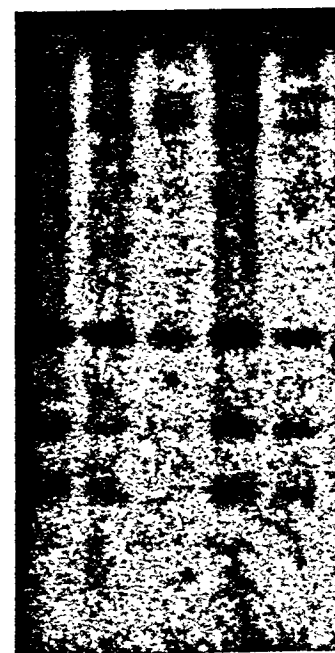
615-

369-

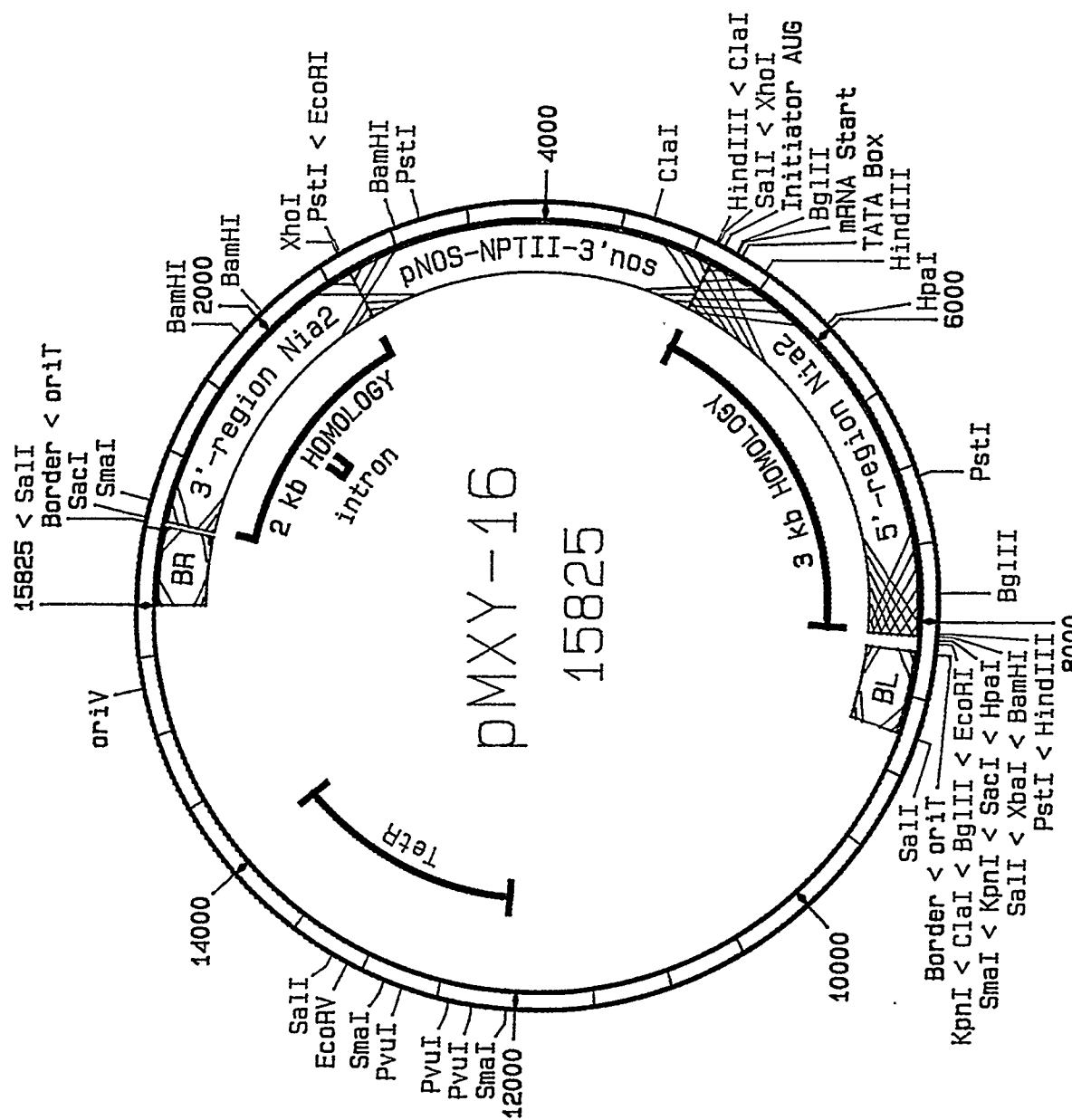
123-

4 24 22 c 178 34 30 36

p13-29 Eco RI



68 77 81 82 83

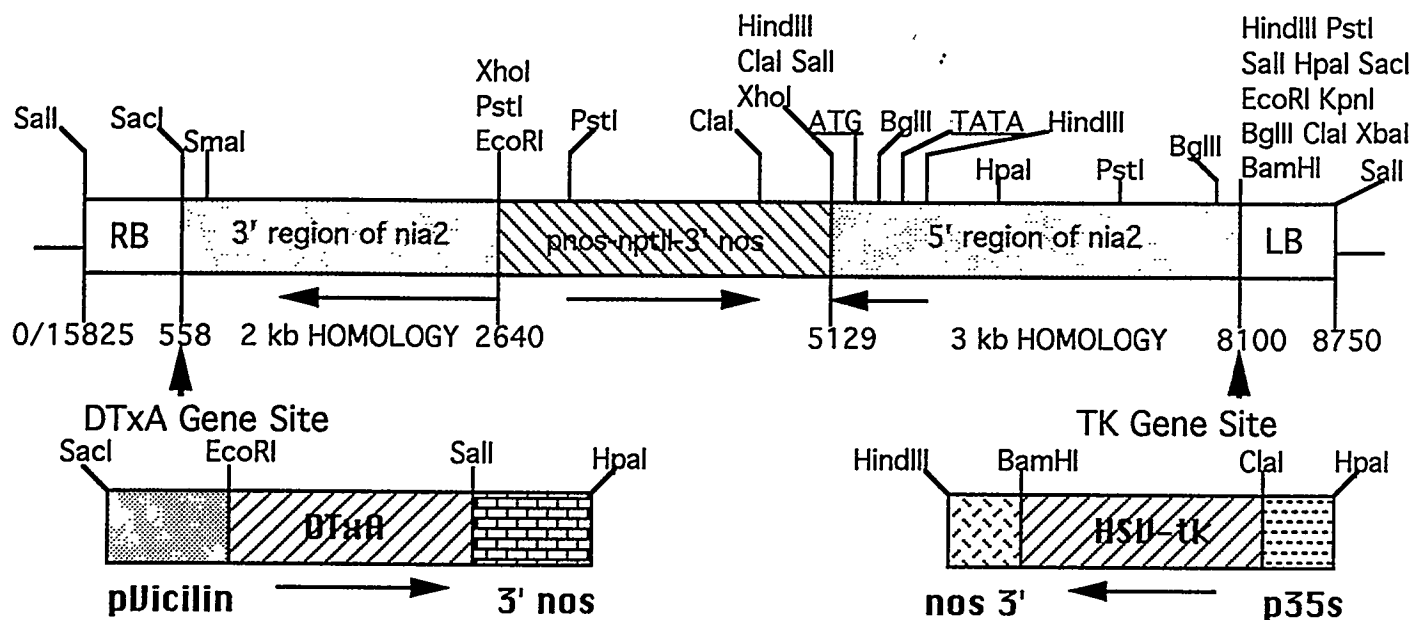


Using enzyme data from: user0: [graduate.xyu.work]ez.dat; 3 FileCheck: 6031

PLASMIDMAP of: pMXY-16.Txt check: 9089 from: 1 to: 15825

G_Xyu February 20, 1994 23:04

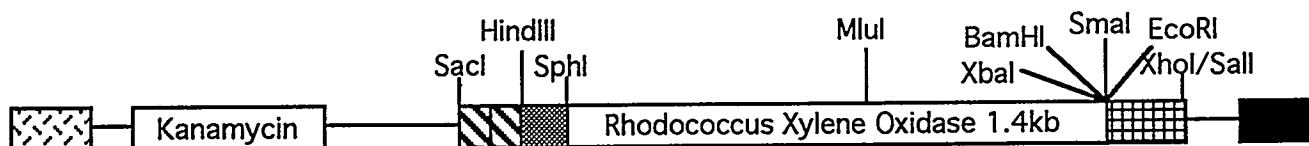
pMXY-16 T-DNA MAP



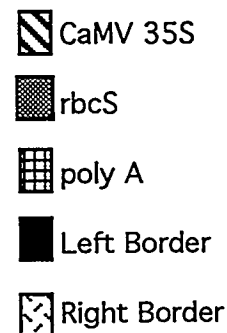
pMXY-16 T-DNA Region: pMXY-16 plasmid was constructed in two steps. First, a 5 kb *SacI* fragment from pAT60 (Crawford, 1989) was inserted into binary vector pGA768 (G. An, unpublished result.) *SacI* site. Then a kanamycin resistant gene cassette from pGA472 (G. An, 1986) was inserted into the *XhoI* site. There is a 2 kb *Arabidopsis thaliana* *nia2* gene homologous sequence near the right border of T-DNA and a 3 kb homologous sequence adjacent to the left border. The transcriptional TATA box and the translation start codon are shown in the map, as well as the normal transcription direction.

Negative Selection Markers: The Herpes Simplex Virus thymidine kinase gene (HSV-tk) and the diphtheria toxin A-fragment gene (DTxA) will be used as negative selection markers. HSV-tk gene from pSLJ882 (Jones, 1992) has been liberated as *HindIII*-*HpaI* fragment and will be inserted into the unique *KpnI* site. The construction of DTxA gene from pAM774 (A. Mitra, unpublished result) under the control of a vicilin promoter (Czako, 1992) is in progress. This gene cassette will be inserted into the *SacI* site next to the right border as indicated above.

Chimeric Rhodococcus Xylene Oxidase

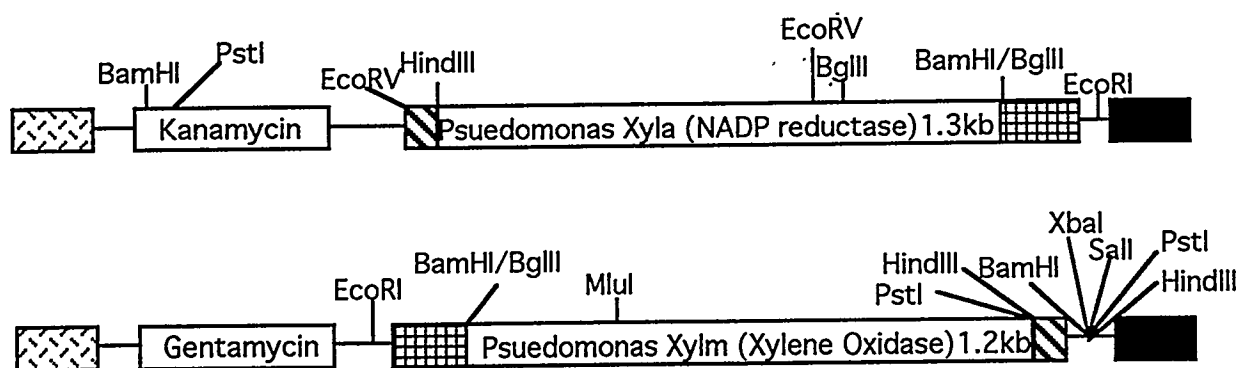


Binary vector plasmid pMAW2007: containing the Rhodococcus Xylene Oxidase gene originally obtained from pSLH8 subcloned as a PstI-XbaI fragment into pOK12 to give pMAW5002. The SphI-BamHI fragment was cloned into pJIT117 containing a double 35S promoter, the chloroplast transit peptide from the rubisco small subunit and the CaMV polyadenylation sequence to give pMAW3006. This was then cloned as a SacI-XhoI fragment into the SacI-SalI site of the binary vector pPZP111 which contains the nptII gene conferring kanamycin resistance giving rise to pMAW2007.







APPENDIX

Chimeric Psuedomonas Xylene Oxidase System



Binary Plasmid Vectors pMAW2001 and pMAW2010: containing the *Psuedomonas Xyla* and *Xylm* genes respectively. pMAW2001 was created by cloning the *Xyla* (NADP reductase) gene as a *NheI* fragment from pGSH2836 into the *XbaI* site of pUC19 to create pMAW3001. The *HindIII*-*BamHI* fragment was removed and cloned into the *HindIII*-*BglIII* site of pGA643 to give rise to the binary vector pMAW2001 containing the chimeric NADP reductase *Xyla* gene under control of the CaMV35S promoter and poly adenylation signal with the *nptII* gene for Kanamycin selection. The *Xylm* (Xylene oxidase) gene was cloned as a *Sall*-*NheI* fragment from pGSH2836 into the *Sall*-*XbaI* site of pUC19 to create pMAW3003. The *HindIII*-*BamHI* fragment was removed and cloned into the binary vector pGA643 to place it under control of the CaMV35S promoter and poly adenylation signal to create pMAW2002. The *BamHI*-*EcoRI* fragment was then removed and cloned into the *BamHI*-*EcoRI* site of the binary vector pZP122 to give rise to pMAW2010 containing a chimeric xylene oxidase with the proper plant promoter and terminator but with gentamycin resistance as the plant selectable marker.

-  CaMV 35S
-  poly A
-  Left Border
-  Right Border

**Expression of the Herpes Simplex Virus thymidine kinase gene
in *Nicotiana tabacum* L.**

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APPENDIX 6.

Footnotes:

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^cAbbreviations: GAN, ganciclovir or 9-(1,3-dihydroxy-2-propoxymethyl)guanine; *HSVtk*, Herpes Simplex Virus thymidine kinase type 1 gene; KAN, kanamycin; NAA, 1-naphthaleneacetic acid; *nptII*, neomycin phosphotransferase gene.

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Running title: *HSVtk* as a negative selection marker in tobacco

Key words: negative selection - HSV thymidine kinase - transgenic tobacco

Summary

The human herpes simplex virus thymidine kinase type 1 (*HSVtk*) gene, was introduced into tobacco by *Agrobacterium*-mediated gene transfer and its utility as a conditional negative selection marker was investigated. In mammalian cells the HSVtk enzyme is able to phosphorylate certain nucleoside analogues (e.g. ganciclovir, an antiherpetic drug), thus converting them to toxic DNA replication inhibitors. Transgenic tobacco plants carrying the *HSVtk* construct from the pCX305.1 binary vector were phenotypically indistinguishable from wild-type and exhibited normal fertility. On ganciclovir containing medium shoot formation was delayed but otherwise not inhibited and special shoot and leaf morphology (filiform leaves) was induced. The introduction of another *HSVtk* constructs with different regulatory sequences (pSLJ882), however, frequently resulted in inhibition of shoot regeneration and growth in leaf disc cultures under the same conditions on ganciclovir containing medium. These plants expressed the highest levels of *HSVtk* mRNA and in shoot culture or in the greenhouse a paler green color of the young leaves was observed. The ganciclovir sensitivity showed a Mendelian intermedier inheritance when the seed progenies of these plants were germinated on ganciclovir containing medium. Unlike in tobacco, the above *HSVtk* constructs were equally well expressed in *Arabidopsis thaliana* transgenic plants.

Introduction

Genetic approaches to understanding biological processes involve the use of marker genes, the expression of which causes immediate or conditional cell lethality. Such genes can be used as negative selection markers for elimination of a particular class of cells (programmed cell death), and in studies of gene inactivation and deletion formation. These markers can also be incorporated into positive-negative selection schemes for homologous recombination mediated gene targeting (Capecchi, 1989). A potent negative selection gene, when 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). For high enrichment, however, cell death is required, even at very low transgene expression levels, because frequently only residual gene expression is seen (position effect).

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), which may have special

agronomic importance (i.e., male-sterility). Suicide genes can be employed 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 non-conditional 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), or the anti-nptII 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 on gene inactivation (Depicker et al., 1988) and for identification of mutations in signalling processes in plants (Karlin-Neumann et al., 1992). The analogous *aux2* gene of the *A. rhizogenes* T-DNA also has the potential as a negative selection gene in *Brassica*, at the plant level (Béclin et al., 1993). The *Escherichia coli* cytosine deaminase (*codA*) gene was shown to provide substrate-dependent toxicity in transgenic tobacco and *Lotus japonicus* plants (Stougaard, 1993) as well as in *Arabidopsis* (Perera et al., 1993). However, negative selection at the level of transformation was not reported for these genes.

In plants, conditional negative selection at the level of *Agrobacterium*-mediated transformation was described the first time using *HSVtk* in *Arabidopsis* root culture system (Czakó and Márton, 1994). Previously, *HSVtk* had been proved to extremely useful in the enrichment for homologous recombinants (Mansour et al., 1988) in mammalian systems. The mechanism of the negative selection was explained as follows: the HSV thymidine kinase enzyme is able to phosphorylate certain nucleoside analogues 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). *HSVtk* may act as a conditional lethal marker *in planta* presumably by the same mechanism.

Here we report species specific differences, regulatory sequence specific differences and variability of the expression of chimeric *HSVtk* genes. The promoter-*HSVtk*-polyA fusion which was efficient negative selection marker in *Arabidopsis* root culture transformation experiments (Czakó and Márton, 1994) was only marginally efficient in tobacco; produced only delayed shoot regeneration on leaf discs and

the shoots were morphologically different (filiform). Another construct, however provided high level expression of *HSVtk* mRNA and high sensitivity to GAN in transgenic *Nicotiana tabacum* cultures, but inefficient negative selection in *Agrobacterium*-mediated leaf disc transformation.

Results

HSVtk type 1 markers confer sensitivity to GAN of transgenic tobacco

The pCX305.1 and pSLJ882 binary plant expression vectors were used to introduce the human herpes simplex virus type 1 thymidine kinase coding sequence into tobacco. In pCX305.1 the *HSVtk* coding region and 350 bp of the 3' flanking region, encompassing the polyadenylation region, were cloned between a duplicated cauliflower mosaic virus 35S RNA promoter (p35S) and the *rbcS*-E9 polyadenylation signals to give pCX305.1. In the case of pSLJ882 (Fig. 1), the *HSVtk* type 1 gene was inserted into a p35S-nos3' expression cassette. The p35S-*HSVtk* cassette was then inserted into the binary vector. These binary plant expression vectors were used to introduce *HSVtk* into tobacco by *Agrobacterium*-mediated transformation. Kanamycin resistant lines were selected both from NT1 suspension and from regenerating leaf disc cultures. As controls, transgenic lines were generated using also the pGA643 plasmid which carries the same KAN resistance gene but no *HSVtk*. The transgenic tobacco plants and callus lines were further characterized by genomic DNA gel blot analysis with probes covering the *HSVtk* and the *nptII* genes, respectively. The *HSVtk* probe detected the *HSVtk* sequences in all of the kanamycin resistant transformants tested (not shown). Most of the *HSVtk*⁺ transgenic plants were phenotypically indistinguishable from wild-type with the exception of some pSLJ882 transformants. In some of the pSLJ882 transformants the areas between the veins on leaves were noticeably paler green both in shoot culture and in the greenhouse, but the plants were otherwise normal and fully fertile (e.g. '882D' and 'S882-1' that also expressed the highest levels of *HSVtk* mRNA, see below).

Growth of pCX305.1 transformants on GAN medium

A growth assay was used to test if the *HSVtk*⁺ trait was manifested in transgenic plants. GAN was incorporated into shoot inducing (RMOP) medium at concentrations from 10⁻⁸ to 10⁻⁴ M, the latter being completely inhibitory to *HSVtk*⁺ *Arabidopsis* (Czakó and Márton, 1994). Leaf segments were taken from non-transformed plants and from kanamycin

resistant transgenic tobacco lines: (i) a 'HSVtk⁺' (X305-1 line) plant carrying HSVtk from pCX305.1 and (ii) a 'HSVtk⁻' pGA643 transformant. Several shoot primordia appeared on the wild-type and HSVtk⁻ transgenic leaf segments as early as after 9 days of culture, while on leaf segments of HSVtk⁺ plants those started to form only after 14 days when GAN was present in at least 10^{-5} M concentration. The great difference in regeneration frequency between the HSVtk⁻ and HSVtk⁺ explants, observed only around the middle point of this time window, (Table 1). In addition to the delay in shoot initiation, GAN caused an alteration of leaf development, while the number of shoots was not reduced on HSVtk⁺ leaf segments. The slender shoots bore leaves with underdeveloped blades lending a filiform appearance to the shoots (Fig. 2). GAN was already inhibitory at 10^{-5} M concentration - the concentration at which filiform leaves were already observed.

While in the transgenic shoots there was at least a short time window for the identification of the HSVtk⁺ phenotype, GAN had no effect on the growth rate, or other visible characteristics of wild-type and HSVtk⁺ transgenic tobacco cell lines derived from NT1 suspension cultures or from plants even at an elevated - 10^{-3} M (equivalent to 277 mg l⁻¹) - concentration.

Growth of pSLJ882 transformants on GAN medium

The results of the above growth assay on GAN containing media were somewhat different with the pSLJ882 transformed transgenic plants than that with the pCX305.1 transformants. Some of the plants were more sensitive to GAN which was incorporated into shoot inducing (RMOP) medium at concentrations from 10^{-8} to 10^{-3} M.

Along with the pSLJ882 transformants '882A', '882B', '882C', '882D', and 'S882-1', some of the pCX305.1 transformants ('S305-1B', 'X305-1' and 'X411' were retested, too. At 10^{-3} M, total inhibition of any proliferation was observed (Fig. 3). While callus formation was unimpeded on all other transformants and the controls, shoot formation was completely inhibited at this high level of GAN concentration, even in wild-type controls. The minimal concentration causing filiform shoots was also lower, i.e. 10^{-6} M, for the '882D' and 'S882-1' transformants. This symptom occurred only at 10^{-5} M with the '882A', '882B', 'S305-1B', 'X305-1' and 'X411' transformants. Explants taken from '882C' and a kanamycin resistant 'HSVtk⁻' pGA643 transformant behaved as the non-transformed control.

In HSVtk⁺ shoot explants, 10^{-3} M GAN inhibited rooting and shoot apex development. Wild-type plants were not affected.

Negative selection at the level of germinating seed progenies

To test the negative selection *in planta*, transgenic tobacco plants were selfed and the F1 seeds germinated with or without 10^{-3} M (equivalent to 277 mg l⁻¹) GAN in the medium. Segregation for kanamycin resistance at 600 mg l⁻¹ was determined from seeds from the same capsules. The 'S882-1' seedlings exhibited a 3:1 segregation of normal to KAN sensitive development. On the GAN medium, the overall segregation to normal, intermediate and severely inhibited phenotypes was 1:2:1, consistent with segregation of a semidominant trait (Table II). Pigmentation, shoot apex and root growth was severely inhibited in one out of four seedlings; even the cotyledons did not expand completely, and the yellowish seedlings had a bottle shaped, swollen hypocotyl (Fig. 4). Two-fourth of the seedlings displayed a well distinguishable phenotype that was intermediate between wild-type and severely inhibited. The color was yellow green, and the cotyledons were enlarged, swollen. Growth of HSVtk⁻ (wild-type) seedlings was virtually not affected by GAN. The HSVtk⁺ tobacco seedlings from both the severely and intermediately inhibited classes could be rescued by transfer to non-selective shoot inducing (RMOP) medium.

Negative selection in Agrobacterium-mediated leaf disc transformation

With the 10^{-4} M concentration having been recognized as strongly inhibitory to early shoot regeneration of leaf discs explants of the pCX305.1-1 transgenic plant, an experiment was set up to test the potential efficiency of the negative selection against HSVtk⁺ transformants during the leaf segment transformation procedure. Tobacco leaf segments were cocultivated with *Agrobacterium* carrying the pCX305.1 and pSLJ882 HSVtk⁺ or the pGA643 (HSVtk⁻) plasmid, or none. Shoot regeneration was induced on RMOP medium containing 100 mg L⁻¹ of kanamycin with or without 10^{-4} M GAN. GAN had no visible effect at the shoot initiation in any of the cultures, as shoot primordia appeared at wound sites on leaf segments in every cultures uniformly throughout the whole culture period. However, the number of true kanamycin resistant shoots (the ones which were able to root on kanamycin) was about five times less on GAN containing medium when the pCX305.1 and two times less when the pSLJ882 binary vector was used for transformation. GAN did not affect transformation by the HSVtk⁻ pGA643 control plasmid (see Table III. for a typical experiment).

Transcription of HSVtk constructs

We set out to investigate the reasons for the HSVtk of the pCX305.1 construct conferring on tobacco only moderate sensitivity to GAN. Transcription of HSVtk was demonstrated by hybridization of total RNA from leaves with the HSVtk sequence. The transcript hybridization signal corresponding to the expected approximately 1700 nucleotides was detected in the HSVtk⁺ 'RLD305-1' *Arabidopsis* plant (Czakó and Márton 1994) used as a positive control (Fig. 5). In tobacco, the hybridizing band was extremely weak as compared to that in *Arabidopsis*. Negligible hybridization was also observed in one cell suspension-derived line 'NT1TK' and its single-cell subclone 'NT1TK-1' (not shown). No cross-hybridizing RNA species were detected in wild-type *Arabidopsis* and tobacco plants.

Total RNA from the leaves of pSLJ882 transformed plants were also analyzed and was found that the higher level of GAN sensitivity indeed corresponded to higher levels of mRNA in the transgenic plants. The hybridization signal was very strong in the '882A', '882B', '882D', and 'S882-1' tobacco transformants suggesting that the p35S-HSVtk-nos3' cassette can provide higher levels of expression than the other cassette where 350 bp of the original 3'-region of HSVtk is retained and the p35S enhancer region is duplicated. Practically no hybridization was observed to RNA isolated from '882C' (Fig. 5), which also tested positive for HSVtk DNA in Southern blot. In *Arabidopsis*, in the 'RLD882-1' and 'RLD882-2' transformants the transcript hybridization signal corresponding to the expected approximately 1400 nucleotides was nearly as strong as the expected at least 1925 nt signal in the 'RLD305-1' *Arabidopsis* transformant (Czakó and Márton, 1994) used as a reference.

Discussion

HSVtk has been shown to provide a convenient negative selection in *Arabidopsis thaliana* by channeling the harmless GAN towards nucleic acid metabolism, where it can exert its cytotoxic effect (Czakó and Márton, 1994). Negative selection applied during the *Agrobacterium*-mediated transformation also proved to be possible.

In tobacco the expression of different HSVtk constructs in the presence of various levels of GAN results in distinct phenotypes. The phenotypes ranged, in increasing severity, from delayed shoot formation on leaf segments, through altered leaf morphology, retarded shoot development, inhibition of shoot formation to complete inhibition of proliferation on shoot inducing medium, and inhibition of

root formation and shoot apex development in shoot tip explants. The absence of shoot and root development in the most sensitive plants indicates the inhibition of cell division. Nevertheless, rescue of all growth-inhibited HSVtk⁺ cultures was possible. It will be interesting to test whether the negative effect is cell autonomous and HSVtk therefore usable in programmed cell death and gene targeting experiments. These applications will rely on GAN translocation throughout the plant and requires that phosphorylated GAN is not exported. The effect on shoot meristem, and developing leaves indicated good translocation, but the experiments reported here can not exclude effects from exported phosphorylated GAN.

HSVtk in the pCX305.1 binary vector caused only altered leaf morphology in tobacco. The reasons for this HSVtk construct to confer on tobacco only moderate sensitivity to GAN could be explained by the low level of transcription, which was demonstrated by hybridization of total RNA from leaves with the HSVtk probe. In tobacco, the hybridizing band was extremely weak as compared to that in *Arabidopsis* (Fig. 5). It was assumed that the retention of the original polyadenylation region (350 bp) of HSVtk between the coding region and the *rbcS*-E9 plant polyadenylation cassette, which places the plant polyA signal approximately 700 bp from the translation terminator codon in the pCX305.1 plasmid, was not optimal for tobacco. It is likely to influence the mRNA stability as indicated by the fuzzy nature of the overall weak bands on the Northern blots. The other chimeric HSVtk type 1 gene construct in the pSLJ882 binary vector (with different 3') was also tested and the transcript hybridization signal in these transformants was much stronger and sharper than in the pCX305.1 transformants; nearly as strong as that in the *Arabidopsis* 'RLD305-1' transformant (Czakó and Márton, 1994) that was used as reference. No cross-hybridizing RNA species were detected in wild-type *Arabidopsis* and tobacco plants, demonstrating the potential use of HSVtk as a reporter gene for measuring transcript levels.

The results of the growth assays for GAN sensitivity correlated to the mRNA levels. The level of sensitivity in some of the transformants reached that in *Arabidopsis* transformants. GAN, incorporated into shoot inducing (RMOP) medium at concentrations 10^{-3} M caused total inhibition of any proliferation on leaf segments the transformants which showed the highest level of mRNA expression. The other difference was, that in the pCX305.1 construct, a doubled p35S enhancer element was used to control the coding region HSVtk. Such enhancer region duplications resulted in high(er) levels of gene expression in other systems (Kay et al., 1987; Odell et

al., 1988).

The fact that the filiform leaf symptom appeared in the pSLJ882 transformed population as well, but at a lower threshold GAN concentration, indicated that this morphological marker is indeed related to the low level expression of the *HSVtk* transgene, but not to other sequences of the vectors.

The GAN sensitive phenotype of transgenic plants may not translate to an efficient negative selection at the level of *Agrobacterium*-mediated transformation because of reasons discussed below. KAN sensitive (non-transformed) that escape KAN selection (confirmed by DNA-DNA hybridization, data not shown), seem to be typical for tobacco leaf segment transformation. In order to unmask the difference in transformation frequencies, individual testing of shoots was required, which made negative selection in the tobacco leaf disc system impractical under the conditions applied here. The difference in time window, that could be seen during the shoot primordia formation and development of the transgenic and wild-type plant, was observed only in the GAN treated leaf disc explant cultures of transgenic plants where there was a uniform level of *HSVtk* expression. During transformation, in the new independent transformants, a broad range of *HSVtk* expression occurs, resulting in a broad range of GAN sensitivity, thus explaining the overlapping appearance of shoot primordia from *HSVtk*⁺ and *HSVtk*⁻ sectors.

The efficiency of negative selection in *Agrobacterium*-mediated gene transfer procedure was very poor. The wide range of the level of *HSVtk* expression in individual transgenic plants resulted in many transformants escaping the negative selection by GAN. These relatively GAN insensitive but KAN resistant transgenic shoots with satisfactory levels of *nptII* expression (KAN resistance) could 'cross-protect' KAN sensitive escapees which simply "took the place" (overgrew) of most of the true transformants on the leaf discs, since the number of shoots on a leaf disc is limited by competition. It remains to be proven whether this phenomenon is due to the KAN resistance gene acting non-cell-autonomously as reported for other chimeric resistance genes (Jones et al., 1993). Regional inhibition of shoot formation by developing shoots also occurs; a phenomenon similar to apical dominance. Escapees are seldom seen in non-transformed leaf disc cultures on KAN containing medium, only in the presence of KAN resistant transformants.

GAN did not reduce the number of shoots developing on tobacco leaf explants cultured on GAN+KAN containing medium compared to KAN containing medium after cocultivation with the *A. tumefaciens* strains carrying the pSLJ882 or the PCX305.1 binary vector, respectively. However, a significant

counterselective effect on KAN+GAN medium (from two- to five-fold) became apparent when true transgenic shoots, that is stable kanamycin resistant ones, were scored among the shoots collected from KAN+GAN plates (Table III). Although the total number of shoots regenerated on the GAN+KAN selective medium was essentially the same as that on KAN plates, only the proportion of the non-transformed "escapees" increased. In the case of the pGA643 plasmid, neither the efficiency of transformation, nor the recovery of transformants was affected by the selection on the GAN+KAN plates.

From the marker segregation data, namely that both the kanamycin resistance and the GAN sensitivity showed typical monogenic Mendelian segregation, a normal transmission of the transgene can be deduced. A gene dosage effect was also seen at the seedling level in the progeny of transgenic plants expressing *HSVtk* at a level high enough to result in GAN inhibition in the leaf proliferation assay.

The pale phenotype of the shoots (this appearance coincided with high mRNA) cosegregated with the GAN sensitivity, suggesting a physiological interference of *HSVtk* expression in the plant cells apart from growth inhibition and disturbance of morphological development by GAN. The pale appearance of the tobacco shoots may be explained by an interference of the excess nonspecific (deoxy)nucleoside kinase activity with cytokinin activity. Spraying exogenous cytokinin on the effected leaves could only partly restore the normal pigmentation (data not shown).

Some questions concerning the negative effects of *HSVtk* expression and the positive effects of GAN on plant regeneration in *Arabidopsis* root cultures, which seem to be related to cytokinin effects, are yet to be answered.

Experimental procedures

Bacterial strains used and molecular cloning

Construction of pCX305.1: *Escherichia coli* strains JM101 (Yanish-Perron *et al.*, 1985) and MC1000 (Casadaban and Cohen, 1980) were used as recipients for pUC-type, and binary vector-type plasmids, respectively, constructed by standard procedures (Sambrook *et al.*, 1989). The *HSVtk* type 1 gene was taken from the pIC19R/MCI-TK plasmid, a pUC-derivative (Marsh *et al.*, 1984) carrying the *HSVtk* cassette engineered for expression in mammalian cells (Mansour *et al.*, 1988). The *HSVtk* coding and 3' region that extends from nucleotide position 263 to 1799 (McKnight 1980) was isolated from a *Bgl*III-*Sma*I partial digest of pIC19R/MCI-TK (Mansour *et al.*, 1988), and was subcloned into pUC19 (Norranders *et al.*, 1983). From the resulting pCX303 plasmid *HSVtk* was moved as a

*Hind*III fragment into a pKYLX7 type (Scharl et al., 1987) binary plant expression vector, pKYLX7S2, to give pCX305.1 (Fig. 1). *HSVtk* was thus placed under the control of an enhanced derivative of the Cauliflower mosaic virus (CaMV) 35S RNA promoter in which the *Hinc*II-*Eco*RV fragment that extends from -417 to -90 with respect to the 35S RNA transcription start site (Guilley et al., 1982) was duplicated (personal communication by Dr. A.G. Hunt, University of Kentucky, Lexington). The polyadenylation signals were provided by the *rbcS*-E9 3' region (Hunt and McDonald 1989). The pCX305.1 and pGA643 (An et al., 1988) 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.

Construction of pSLJ882: The *HSVtk* type 1 gene was inserted into a p35S-nos3' expression cassette. The p35S-*HSVtk* cassette was then inserted between the *Hpa*I and *Hind*III sites in the pSLJ456 binary vector (Jones et al., 1992).

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; Czako et al., 1992).

The leaf segments were placed on RMB solid (0.7 % [w/v] agar) shoot induction medium lower epidermis up to lessen detachment from the medium due to nastic twisting. RMB is the same as MS-H but with 1 mg L⁻¹ of benzyladenine. 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*.

Tobacco NT1 suspension cells were transformed essentially according to An (1988). Suspension cells were subcultured (25 X dilution) in 'NT1' medium containing MS salts (Murashige and Skoog, 1962); 3% (w/v) sucrose; *myo*-inositol, 100 mg/l; thiamine hydrochloride, 1 mg/l; Miller's solution (6 % (w/v) KH₂PO₄), 3 ml/l and 2,4-dichlorophenoxyacetic acid; 0.1 mg/l, in the dark on the shaker at 150 R.P.M. Two-ml-volume packed cells (sedimentation at 1 x g) of a 3-day-old suspension were resuspended in 4 ml NT1 liquid medium and inoculated with 100 μ l *Agrobacterium* grown as described above. After a 2-day

incubation at 28 °C in the dark, the cells were sedimented by centrifugation (80 x g), then washed twice with NT1 medium, and resuspended in 6 ml NT1 medium. Two ml of the suspension was plated on solid (0.7 % agar) NT1 medium containing kanamycin 100 mg L⁻¹ and 400 mg L⁻¹ of Timentin.

Regenerants were transferred to the growth chamber. Capsules were collected when completely dry. The seeds were poured out from the capsules directly onto the germination medium in the sterile workplace. All germination media contained 400 mg L⁻¹ of ticarcillin, and 100 mg L⁻¹ of the fungicide Benlate to prevent microbial contamination. The KAN resistance of seedlings was tested by germinating seeds on MS plates (MS salts; 3% (w/v) sucrose; Miller's solution [6 % (w/v) KH₂PO₄]) containing 600 mg L⁻¹ of KAN. The GAN sensitivity of seedlings was tested by germinating seeds on MS plates containing 10⁻³ M of GAN. Scoring of resistant and sensitive seedlings was performed four weeks after germination. KAN sensitive seedlings were chlorotic (eventually completely bleached after eight weeks) and shoot and apex development was inhibited.

Arabidopsis plant material and growth conditions were the same as in Márton and Browse (1991) and Czakó & Márton (1994). The HSVtk⁺ 'RLD305-1' transgenic plant was maintained as a root culture as described (Czakó et al. 1993).

GAN sodium salt (M_r=277, 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, 0.1 mg L⁻¹ of NAA, [Maliga 1984]).

Nucleic acid analyses

Total DNA was prepared from leaf tissue by the cetyltrimethyl-ammonium bromide method (Rogers and Bendich, 1988). DNA samples, digested with *Hind*III or *Kpn*I, were fractionated on a 0.7% (w/v) agarose gel in 0.5 x TBE buffer, then transferred onto Zeta-Probe membrane (BioRad) by alkaline blotting using the manufacturers protocol.

Total RNA was extracted from roots, leaves and callus tissue essentially according to McGurl et al., (1992; and personal communication). Three grams of tissue was harvested and rapidly frozen in liquid nitrogen and ground to powder. The powdered tissue was immediately added to a preheated mixture (at 95 °C it is a single phase) of 5 ml of equilibrated phenol (AMRESCO, Solon, OH) and 5 ml of 100 mM Tris-HCl (pH 8.0), vortexed for 1 minute, and centrifuged at 3100 x g for 15 minutes. The aqueous layer was extracted with saturated chloroform (AMRESCO), precipitated with 2.5 volumes of 95% (v/v) ethanol in the presence of 0.3 M sodium acetate

(pH 4.8). The tubes were spun in a microcentrifuge for 20 minutes at 4 °C. RNA was resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0 with sodium hydroxide). Samples were frozen in liquid nitrogen and stored at -80 °C. RNA was fractionated on a 1 % (w/v) agarose gel with formaldehyde (Sambrook et al., 1989), 30 µg per lane, then transferred onto Zeta-Probe membrane (BioRad) in 10X SSC using the manufacturer's protocol. Calf liver 18S (1950 nt) and 28S (4700 nt) RNAs (United States Biochemical, Cleveland, OH) were used as size standards.

Probe DNA was labeled with ^{32}P using the random priming procedure of the manufacturer (Amersham) and hybridized to the membrane according to standard procedures (Sambrook et al., 1989).

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LEGENDS to FIGURES:

Figure 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, *HSVtk* is expressed from a duplicated cauliflower mosaic virus 35S RNA promoter (p35S) and terminated by the *rbcS*-E9 polyadenylation region (*rbcS3'*). The *nptII* chimeric KAN-resistance gene is under the control of *Agrobacterium* nopaline synthase promoter (pNOS) and terminator (*nos3'*) regions. (B) In pSLJ882, *HSVtk* is expressed from a p35S promoter and terminated by *nos3'*. The *nptII* chimeric KAN-resistance gene is under the control of *Agrobacterium* gene 1' promoter (*p2'1'*) and the *Agrobacterium* octopine synthase terminator (*ocs3'*) regions. Abbreviations: LB, left border repeat; RB, right border repeat. The length of the T-region is shown in the parentheses next to the RB.

Figure 2. Shoots, regenerated on non-transformed tobacco leaf segment, have expanded, rounded leaves (left) on 10^{-3} M GAN containing medium. Filiform shoot with underdeveloped leaf blades regenerated on a leaf segment of 'X305-1' plant carrying *HSVtk* (right). Bar represents 2 mm.

Figure 3. Effects of GAN at 10^{-4} M on shoot regeneration and callus formation on leaf segments of tobacco after six weeks. 1. X305-1B. 2. 882D. 3. 882C.

Figure 4. Segregation of GAN sensitive and wild-type seedlings in the progeny of the S882-1 transgenic plant after six weeks on 10^{-3} M GAN containing medium. Severe inhibition results in bottle shape in some seedlings. Other seedlings show an intermediate phenotype: arrested development and paler green color as compared to the unaffected, wild-type seedlings. Bar represents 2mm.

Figure 5. Autoradiographs of RNA transfer blots containing 30 μ g total RNA isolated from leaves or regenerating root culture (panel 'A', lane 6 only). The blots were hybridized with the 32 P-labeled 1.5 kbp *HindIII* 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. S882-1. 3. 882A. 4. 882B. 5. 882C. 6. 882D. 7. X411. 8. X305-1. 9. Wild-type (cv. 'Xanthii'). Panel B: 1. Wild-type RLD. 2. RLD882-1. 3. RLD882-2. 4. RLD305-1. The arrowhead indicates the position of the 1950 nt RNA standard.

Table I. Effect of GAN on shoot induction on 'X305-1' HSVtk⁺ tobacco leaf explants after 12 days.

Ganciclovir concentration (M)	Growth foci HSVtk ⁺	(% of control) HSVtk ⁻
0	100	100
10 ⁻⁷	92	93
10 ⁻⁶	71	98
10 ⁻⁵	1	88
10 ⁻⁴	0	93

Table II. Mendelian segregation of resistance to kanamycin (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.

Clone	No. of seedlings			No. of seedlings			
	KAN			GAN			
	R	S	Ratio ^a	R	I	S	Ratio ^b
<u>cv. 'Petite Havana' SR1</u>							
S305-3	298	86	3.5:1	225	0	0	NA ^c
S305-1B	317	111	2.9:1	324	0	0	NA
S882-1	298	103	2.9:1	99	209	93	1:2.1:0.9
<u>cv. 'Petite Havana'</u>							
882B	74	20	3.7:1		NT ^d		
882D	246	32	7.7:1 ^e		NT		
<u>cv. 'Xanthii'</u>							
X305-1	542	183	3.0:1	415	0	0	NA
X411	135	40	3.4:1	410	0	0	NA

^a3:1 segregation with one exception. Expected and observed segregation ratios were compared by an χ^2 test. Category assignment of segregants was based on the decision to reject an hypothesis at the 5% risk ($P < 0.05$).

^b1:2:1 segregation.

^cNA= does not apply.

^dNT= not tested.

^eAberrant segregation.

Table III. Effect of GAN on the recovery of KAN resistant tobacco shoots after transformation with agrobacteria carrying *HSVtk*.

	SELECTION ON				NEGATIVE SELECTION FACTOR
	KAN		KAN + GAN		
	Number of shoots ^a				
	Kan ^R	Kan ^S	Kan ^R	Kan ^S	
Cocultivation with:					
pGA643	10	15	9	15	1.00
pCX305.1	13	20	2	25	5.01
pSLJ882	16	12	5	22	2.02

^aThe number of shoots (3< leaf) per culture after 3 weeks.

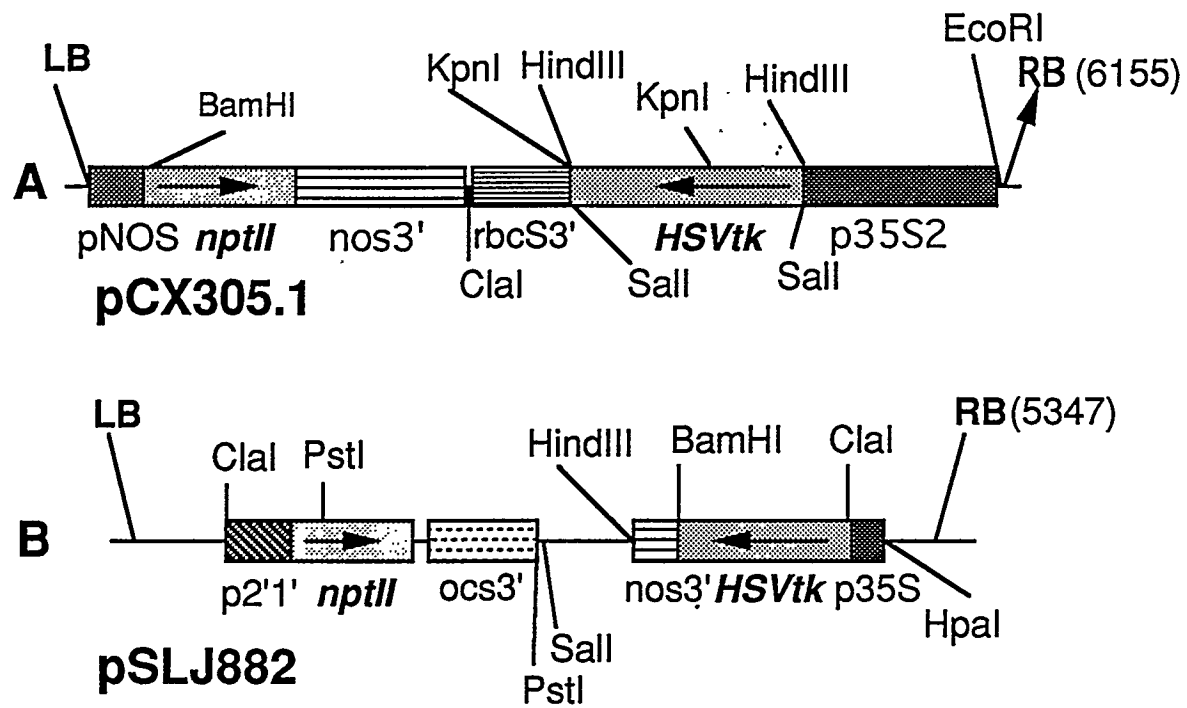


Fig. 1.



Fig. 2.

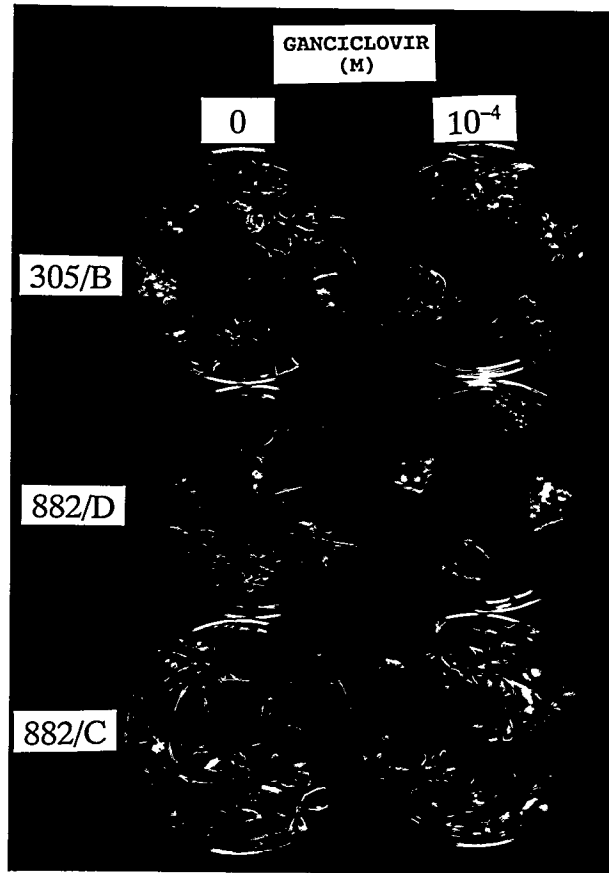


Fig. 2.



Fig. 4.

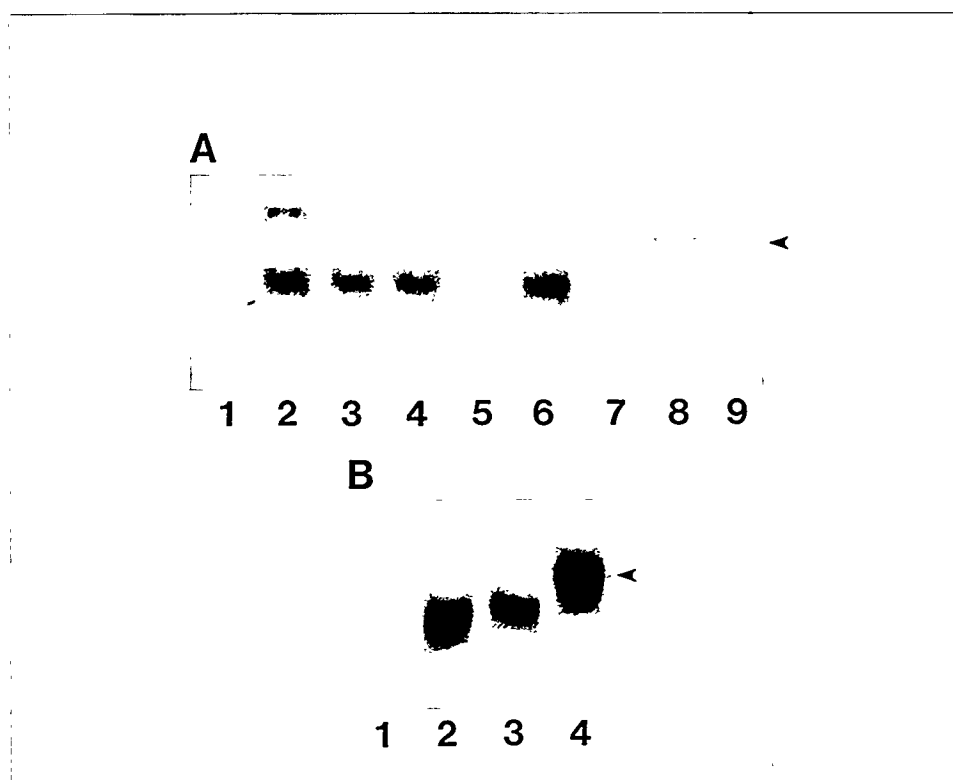
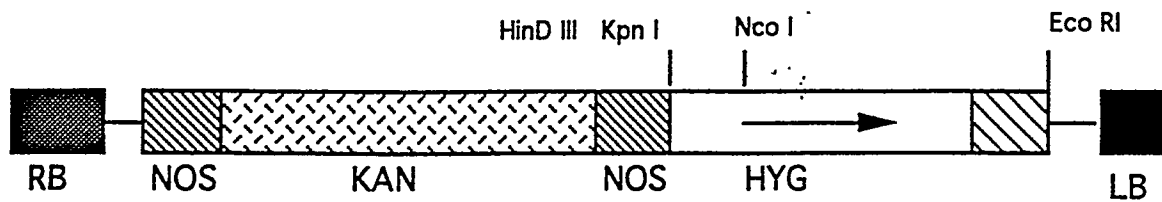


Fig. 5.

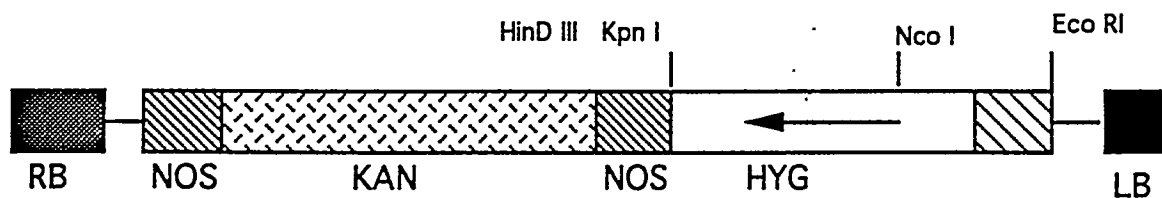
HYGROMYCIN SENSE CASSETTE



The above figure shows T-region of MRM2003. It was made by introducing 1.1Kb Hygromycin resistance gene in sense orientation as BamHI fragment from pTR151 into BglII site of binary plasmid pGA642. The construct contains kanamycin resistance gene as selection marker under the control of Nos promoter. Hygromycin gene is also under the control of Nos promoter.

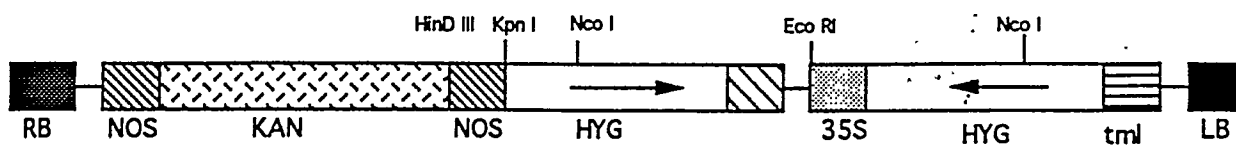
APPENDIX

HYGROMYCIN ANTISENSE CASSETTE



The above figure shows T-region of MRM2010. It was made by introducing 1.1Kb Hygromycin resistance gene in antisense orientation as BamHI fragment from pTR151 into BglII site of binary plasmid pGA642. The construct contains kanamycin resistance gene as selection marker under the control of Nos promoter. Hygromycin gene is also under the control of Nos promoter.

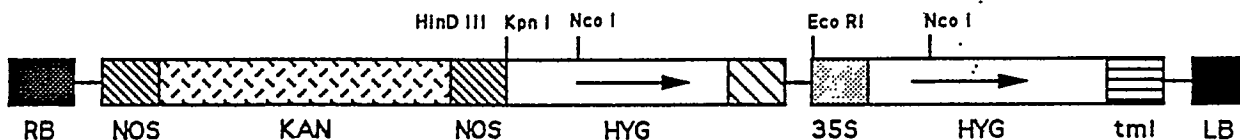
HYGROMYCIN SENSE-ANTISENSE CASSETTE



The above figure shows T-region of MRM2004. It was constructed as follows:
1.1Kb Hygromycin resistance gene was obtained as BamHI fragment from PTR151 and introduced into BglII site of binary plasmid pGA642 in sense orientation. It is placed under the control of Nos promoter. To obtain antisense orientation the BamHI fragment containing Hygromycin resistance gene from pTR151 was inverted. It is under the control of 35S promoter. This cassette was introduced into the above construct as 1.7Kb EcoRI fragment.

APPEND

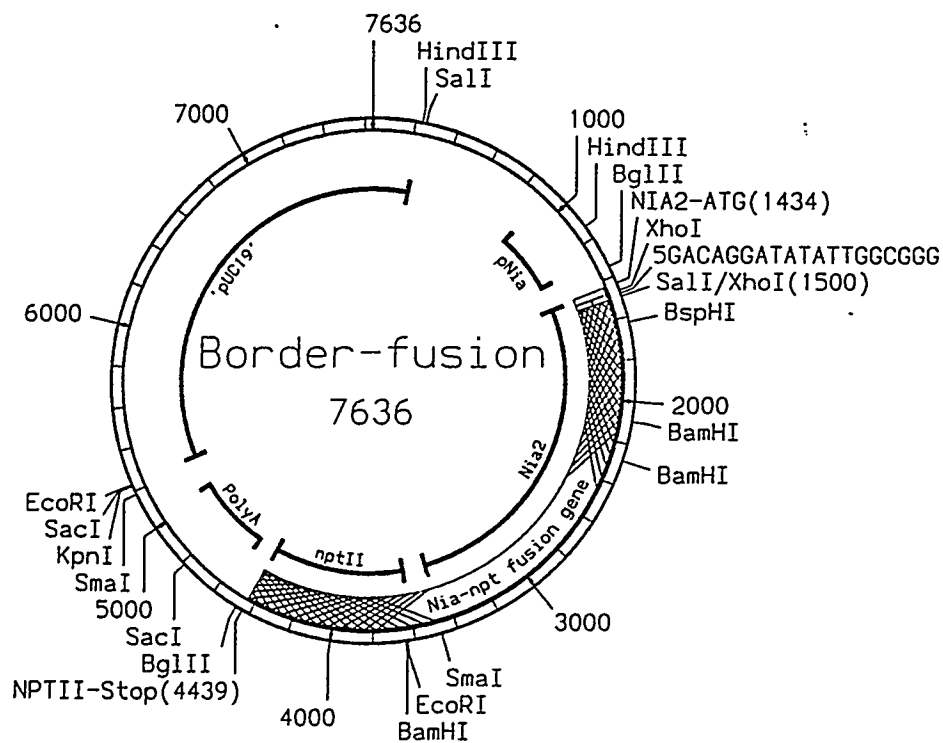
HYGROMYCIN SENSE-SENSE CASSETTE



The above figure shows T-region of MRM2013. It was constructed as follows:
1.1Kb Hygromycin resistance gene was obtained as BamHI fragment from pTR151 and introduced into BglII site of binary plasmid pGA642 in sense orientation. It is placed under the control of Nos promoter. Further, a 1.7Kb fragment of pTR151 containing Hygromycin resistance gene under the control of 35S promoter was introduced in the unique EcoRI site.

The left T-DNA border/*nia2*/kanamycin resistance gene fusion.

A synthetic *Agrobacterium* T-DNA border repeat oligonucleotide is inserted into the *nia2* coding region. The *nia2* gene is also fused to the kanamycin resistance gene. This chimeric gene is inserted into a binary plant vector so that the synthetic border sequence will function as the left border of the T-region.



and the control constructs are shown in **Appendix 9** and **10**. The latter antisense cassette allows a convenient second enrichment cycle if introduced outside the homologous region, because it can inactivate the same, but sense cassette within the homologous region. Therefore the positive selection can work only if the antisense cassette is not integrated, that is, the two cassettes are separated e.g. by homologous recombination before integration. Development of new homologous gene targeting vectors carrying two negative selection markers on both sides are in progress.

Homology between the left end and the target sequence is an interesting but overlooked aspect of *Agrobacterium*-mediated integration, although a scattered, short, left side homology was mentioned by the Köln MPI group in a recent publication. A thorough examination of the left border region's influence on the target search during integration has not been done. Ignorance to important factors involved in the T-DNA integration may be the reason for the general lack of success when using *Agrobacterium* as vector for homologous recombination. In order to examine the effect of left border homology on the targeting of T-DNA integration, new binary vectors are constructed with different length of sequence homology to the *nia2* gene directly at left border (**Appendix 11**). We expect that this approach may not require the use of long stretches of homologous sequences, and could help solve a cosuppression problem. This part of the proposal is based on a new idea, but we have already started working on it, and plan to continue it through the third year.