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Expression of foreign genes in regenerated plants and in their progeny

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Chimeric genes comprised of the nopaline synthase promoter and bacterial coding sequences specifying resistance to kanamycin, chloramphenicol or methotrexate, were inserted into the non-oncogenic Ti plasmid vector pGV3850 by recombination (through homologous pBR322 sequences present in the chimeric gene constructs and pGV3850). These co-integrates in *Agrobacterium* were used to infect single plant protoplasts of *Nicotiana* by co-cultivation. The resistance traits allowed the selection of transformed calli in tissue culture in the presence of the appropriate antibiotic. Furthermore, as a non-oncogenic Ti plasmid was used for the protoplast transformation, phenotypically normal and fertile plants could be regenerated from the resistant calli. We have shown that these fully differentiated plant tissues exhibit functional expression of resistance traits (Km^R and Cm^R). All plants carrying the chimeric genes developed normally, flowered, and set seeds. The inheritance of several of these resistance traits was analyzed and shown to be Mendelian. These results are model experiments to demonstrate that genes of interest can be systematically transferred to the genome of plants using non-oncogenic Ti plasmid derivatives; and that transformed plants are capable of normal growth and differentiation, thus providing a natural environment for the study of gene expression and development of plant cells.
Key words: Agrobacterium/plant cell transformation/selectable markers/Ti plasmid vectors/chimeric genes

Introduction

The Ti plasmid of *Agrobacterium tumefaciens* has long been proposed as a vector for the genetic engineering of plants, and recently this potential has begun to be realized. Normally the transfer of a defined segment of the Ti plasmid, T-DNA, into the genome of many plants and its subsequent expression results in the formation of a crown gall tumor (for reviews, see Kahl and Schell, 1982; Bevan and Chilton, 1982; Caplan *et al.*, 1983; Zambryski *et al.*, 1983a); now, this system has been modified to allow specific assays for gene expression in all parts of fully differentiating plants. On the one hand, expression vectors containing the signals controlling the transcription of the T-DNA-encoded nopaline synthase gene have been used to express bacterial coding sequences in *Agrobacterium*-transformed plant cells (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983; Fraley *et al.* 1983). Furthermore, several of these chimeric gene constructions direct the expression of antibiotic resistance traits allowing the specific selection of transformed cells in tissue culture (Herrera-Estrella *et al.*, 1983b; Fraley *et al.*, 1983). On the other hand, the Ti plasmid itself has been modified to allow efficient DNA transfer without tumor formation. This non-oncogenic Ti plasmid derivative, pGV3850, has the additional property of being able to act as a general acceptor plasmid to transfer any gene of interest cloned in a pBR-like vector plasmid (Zambryski *et al.*, 1983b). We have combined these two developments and report here the transfer of chimeric genes encoding bacterial resistance functions into plants using pGV3850; furthermore, we report the functional expression of these resistance traits in the tissues of whole plants regenerated from the initial transformation event, and that the newly acquired DNA sequences are transmitted as Mendelian factors to the following plant generations.

Results

Introduction of chimeric genes into the non-oncogenic Ti plasmid pGV3850

We have previously reported the construction of chimeric genes containing the transcriptional signals of the *nos* gene and the coding sequence of the neomycin phosphotransferase II [NOS-APH(3')II] from Tn*5*, the chloramphenicol acetyltransferase (NOS-CAT) from Tn*9*, and the methotrexate-insensitive dihydrofolate reductase (NOS-DHFR Mtx^R) from R67 (Herrera-Estrella *et al.*, 1983a, 1983b). Figure 1A shows the relevant restriction enzyme recognition sites for each of these chimeric genes. The present studies were aimed at testing whether these chimeric resistance genes could be transferred to plant cells using the non-oncogenic vector Ti plasmid, pGV3850 (Zambryski *et al.*, 1983b), and whether the resistance traits would be expressed in whole plant tissues regenerated from transformed cells.

To insert the chimeric genes into the T-DNA of Ti plasmid pGV3850, the plasmids harbouring these genes were mobilized to *Agrobacterium* using the Mob function of the plasmid ColE1 and the Tra function of R64*drd*11 as described previously (Van Haute *et al.*, 1983). The kanamycin resistance of the plasmids carrying the chimeric genes (see Materials and methods) was used as a genetic marker to select for co-integration. Since the origin of replication of pBR-like plasmids is not functional in *Agrobacterium*, kanamycin-resistant exconjugants are derived from co-integration of the plasmid carrying the chimeric gene with the homologous pBR sequences present in-between the T-DNA borders of pGV3850 via a homologous recombinant event. The structure of the T-DNA of such co-integrates was confirmed by Southern blot hybridization (data not shown). As can be seen from Figure 1B, the chimeric genes present in these co-integrates are flanked by two pBR sequences in direct orientation. Since a second recombination event through the homologous pBR322 sequences can also occur at a frequency of 2×10^{-2} (Zambryski *et al.*, 1983b), we always grow *Agrobacteria* under antibiotic selection (e.g., kanamycin as described in Figure 1) to maintain co-integrates.

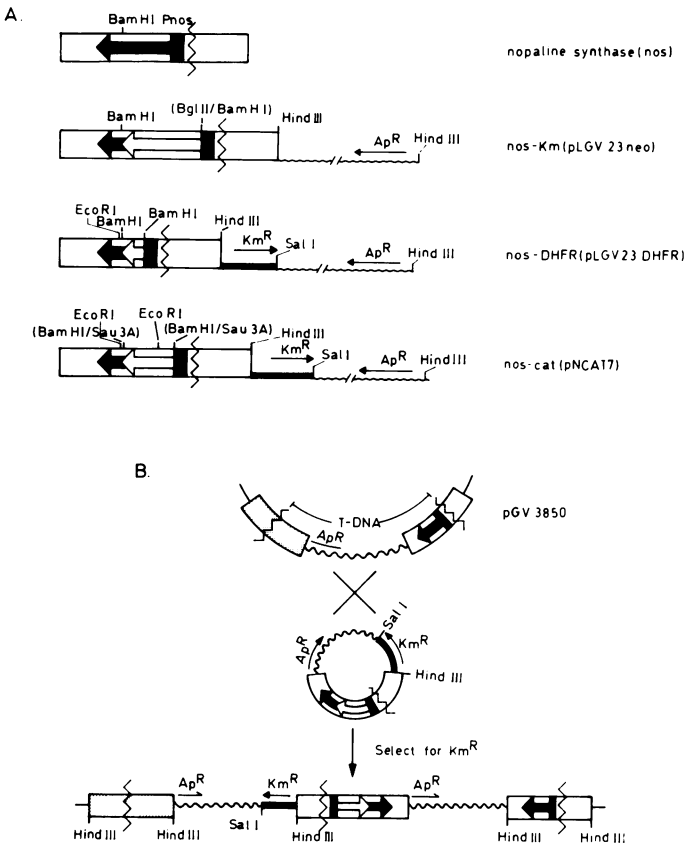

Fig. 1. Schematic representation of chimeric gene constructs before and after co-integration with pGV3850. (**A**) shows the various chimeric genes containing bacterial coding sequences under the control of the *nos* promoter. The first line shows the *nos* gene (in black) within the *Hind*III fragment 23 (white box); the *nos* promoter is the vertical black bar labelled Pnos and the *nos* coding sequence is the black arrow. The right T-DNA border sequence (25-bp repeat) important for T-DNA transfer and/or integration is shown as a jagged line. Three different chimeric gene constructions are shown below the *nos* gene; the white arrows indicate the coding sequences of the inserted bacterial antibiotic resistance genes. The NOS-Km(pLGV23NEO) construct contains the Km resistance coding sequence as a *Bgl*II/*Bam*HI fragment from Tn*5* cloned in expression vector pLGV2381 as described (Herrera-Estrella *et al.*, 1983b). The NOS-DHFR (pLGV23DHFR) contains the Mtx resistance coding sequence from R67 as a *Bam*HI fragment cloned in expression vector pLGV2382 as described (Herrera-Estrella *et al.*, 1983b). The NOS-CAT(pNCAT7) contains the chloramphenicol resistance coding sequence from pBR325 (Bolivar, 1978) as a *Sau*3A fragment in pLGV2382 (this work). pBR322 sequences of these constructs are shown as wavy lines. The kanamycin resistance of the plasmid pLGV2382 or the chimeric kanamycin resistance gene of *Nos*-Km were used in the selection for co-integration with pGV3850. (**B**) shows the structure of one of the co-integrates formed between one of the chimeric genes in **A** and pGV3850. The *Hind*III fragments 10 (grey) and 23 (white) which contain the T-DNA border terminal sequence (jagged line) are indicated in the pGV3850 plasmid above. A single recombination event between pGV3850 and the plasmid carrying the chimeric gene through the homologous pBR322 sequences produces a co-integrate useful to transform plant cells. As indicated in the co-integrate there is an additional T-DNA (border) terminal sequence adjacent to the chimeric gene which is capable of directing transfer in a rightward direction from this point (see text).

Selection of transformed calli

To test whether the chimeric genes in pGV3850 could be used to select directly transformed plant cells growing in tissue culture, two different varieties of *Nicotiana* which are easily manipulated in culture were chosen as model systems. Protoplasts of either *Nicotiana tabacum* cv. Petit Havana (SR1) (Maliga *et al.*, 1973), or the haploid line of *Nicotiana plumbaginifolia* (Pln) (Negrutiu, 1981) were isolated and were co-cultivated with the *Agrobacterium* strains harbouring the engineered co-integrates. The method of co-cultivation used is a modification of that originally published by Márton *et al.* (1979), and the details are described elsewhere (Zambryski *et al.*, 1984). After 3 days of co-cultivation, the protoplast cultures were made bacteria-free by washing and addition of antibiotics (500 μg/ml cefotaximum). The protoplast-derived cells were allowed to grow for 3–4 weeks to form small colonies of ~64 cells each. The colonies were plated on solid medium containing the appropriate antibiotic, kanamycin (50–100 μg/ml), methotrexate (0.1–0.5 μg/ml), or chloramphenicol (5–10 μg/ml), depending on the chimeric gene used. After 14 days of selection, kanamycin-resistant calli could be distinguished from the sensitive ones, as fast-growing compact calli in a background of sensitive, small, brown calli (Figure 2). The chloramphenicol selection gives slow-growing compact calli that are resistant, while the sensitive calli remain small and become brownish and friable after three weeks (see Materials and methods). The methotrexate selection is not as clear cut as the kanamycin or chloramphenicol selection, since even resistant calli often contain necrotic regions; nevertheless, resistant calli are detected on selective medium (see Materials and methods). We should note that the best results for the methotrexate selection are obtained with medium containing 0.1% active charcoal; the active charcoal may remove toxic compounds either present in the methotrexate itself or released by cells growing in its

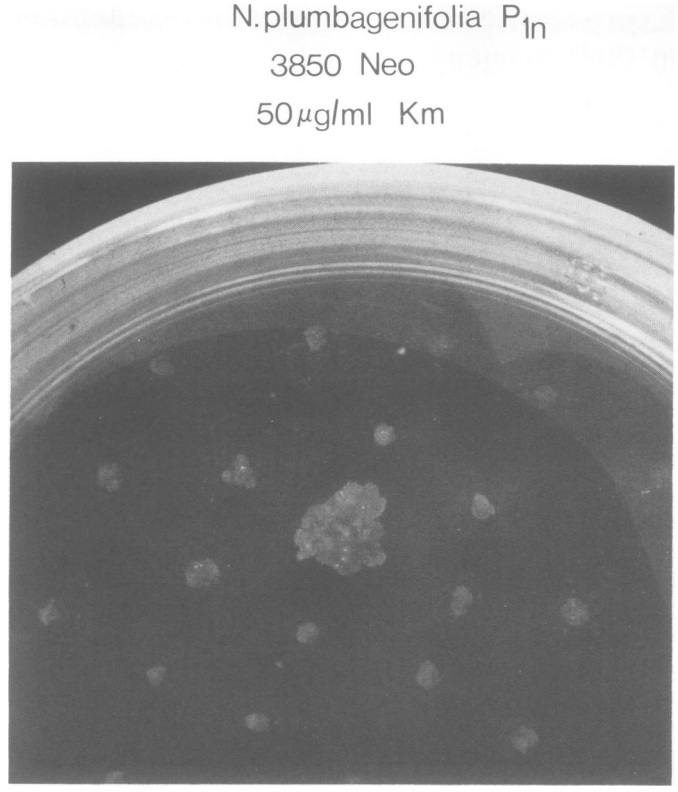

Fig. 2. Selection of Km^R calli from a co-cultivation experiment with *N. plumbaginifolia* Pln and the *Agrobacterium* strain containing the pGV3850::NOS-Km co-integrate plasmid. Fifty calli obtained from co-cultivation were transferred to solid medium containing 50 μg/ml kanamycin. After 3 weeks a clear difference could be seen between the resistant calli, that were green and growing, and sensitive ones, that were brown and dead.

Table I. Results of co-cultivation between *Agrobacterium* harbouring pGV3850::chimeric gene co-integrates and *N. tabacum* and *N. plumbaginifolia* protoplast-derived cells

Co-cultivation[a]		Number of tested	Antibiotic concentration	Number of resistant calli	Transformation efficiency	% of resistant calli	
Plant	Bacterial plasmid					nos^{+d}	nos^{-}
SR1[b]	pGV3850::MTX	2000	0.5 μg/ml Mtx	90	4.5%	5%	95%
SR1[b]	pGV3850::Km	6800	100 μg/ml Km	522	7.7%	<1/300	100%
Pln[c]	pGV3850::Km	2400	50 μg/ml Km	110	4.6%	<1/50	100%
SR1[b]	pGV3850::Cm	2400	10 μg/ml Cm	208	8.7%	5%	95%

[a]Co-cultivations were done as described (Herrera-Estrella *et al.*, 1983b; Zambryski *et al.*, 1984). The resistant calli were selected after 2–4 weeks on solid medium containing the indicated concentrations of antibiotics.
[b]SR1: *N. tabacum* cv. Petit Havana (Maliga *et al.*, 1973).
[c]Pln: a haploid line of *N. plumbaginifolia*.
[d]nos: nopaline synthesis (Aerts *et al.*, 1979).

presence. Furthermore, even Mtx^{R} calli do not grow under selective conditions, and a clear distinction between sensitive (dead) and resistant (living) cells can only be observed after 3–4 weeks.

Table I summarizes the results of several co-cultivation experiments using the different co-integrate constructions. The transformation frequency ranges between 4 and 9% of the plated calli. The frequency obtained is approximately the same as when a fully oncogenic Ti plasmid is used to introduce the same chimeric genes (Herrera-Estrella *et al.*, 1983b). These results allow a direct quantitative comparison of the transfer efficiency of pGV3850 compared with the wild-type Ti plasmid. This comparison demonstrates that although pGV3850 has an extensive deletion of most of the internal part of the wild-type T-DNA, it retains full capacity for transferring its modified T-DNA.

Phenotypic expression of chimeric genes in regenerated plants

We have shown that the chimeric gene constructions carrying antibiotic resistant traits can be expressed to provide a selective advantage to transformed cells growing in tissue culture in the presence of the antibiotics. However, it is not known if these resistance traits will also be expressed in whole plants derived from the transformed calli. Since the resistant calli were transformed using a non-oncogenic Ti plasmid, they were transferred to medium with cytokinin [1 mg/l 6-benzylaminopurine (BAP)] to allow regeneration to occur. All colonies containing the different chimeric genes were capable of differentiating to form normal shoots. After 4 weeks, shoots were transferred from the high cytokinin medium to medium without hormones to allow roots to develop. As expected, these shoots have the capacity to form roots and to develop into normal mature plants.

To check whether the transformed plants were derived from only resistant cells rather than a mixture of transformed and untransformed cells, protoplasts were re-isolated from transformed plants and tested for resistance. Three plants called Neo1, Mtx1, and Cat1 were chosen for further analysis (below). Three thousand five hundred calli derived from leaf protoplasts of the pGV3850::NEO plant (Neo1) were tested, and all were found to be Km^{R}; all 400 calli derived from leaf protoplasts of the pGV3850::MTX plant (Mtx1) were Mtx^{R}, and all 1200 similarly derived calli from the pGV3850::CAT plant (Cat1) were Cm^{R}. This suggests that the regenerated plants are homogeneously composed of transformed cells. Thus, we could proceed to test whether the chimeric genes are functionally expressed in the differentiated tissues of the regenerated plants. Several different types of experiments were performed.

First we tested whether shoot cuttings from transformed plants would be able to grow in the presence of antibiotics. A pGV3850::NEO shoot from the Neo1 plant and a control untransformed *N. tabacum* SR1 shoot, both without roots, were placed in agar growth medium containing 100 μg/ml kanamycin. Figure 3A shows that after 3 weeks the pGV3850::NEO shoot was able to form roots whereas the control shoot did not. After further incubation the pGV3850::NEO shoot was able to maintain normal growth, whereas the control did not and eventually died after 2 months. In a similar test, a pGV3850::CAT shoot was able to form roots after 5 weeks in the presence of 5 μg/ml Cm, whereas the control untransformed shoot could not (Figure 3B).

This experiment demonstrates that the nopaline promoter is sufficiently active to allow phenotypic expression of the antibiotic resistance coding sequence in differentiated tissues for root formation to occur. We were not able to perform the rooting test for the pGV3850::MTX shoot, perhaps because methotrexate is too toxic to allow long-term incubation in its presence.

To test whether transformed leaves show phenotypical expression of the resistance traits, individual leaves from the resistant plants were transferred onto callus-inducing medium [Murashige and Skoog (1962) salts, 0.8% agar, 3% sucrose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4 D) and 0.5 mg/l naphthalene acetic acid (NAA)] containing 100 μg/ml kanamycin. After 3 weeks, callus formation was observed in transformed leaves, whereas control non-transformed leaves were not able to form callus (data not shown). This demonstrates that the chimeric genes are sufficiently expressed in leaf cells to allow survival in selective medium until callus formation occurs. In addition, it has also been shown that a leaf extract from a pGV3850::NEO plant contains neomycin phosphotransferase activity. In contrast to the results obtained in animal cells where the NPT(3')II protein is modified probably by glycosylation (Colbère-Garapin *et al.*, 1981; Southern and Berg, 1982), it was found that the protein produced in plants is probably not modified since it co-migrates with the bacterial enzyme both in h.p.l.c. chromatography (Herrera-Estrella *et al.*, 1983b) and in non-denaturing polyacrylamide gels (G. Van den Broeck and P. Schreier, personal communications).

The most direct evidence for phenotypically detectable expression of resistance genes in all plant tissues is derived from

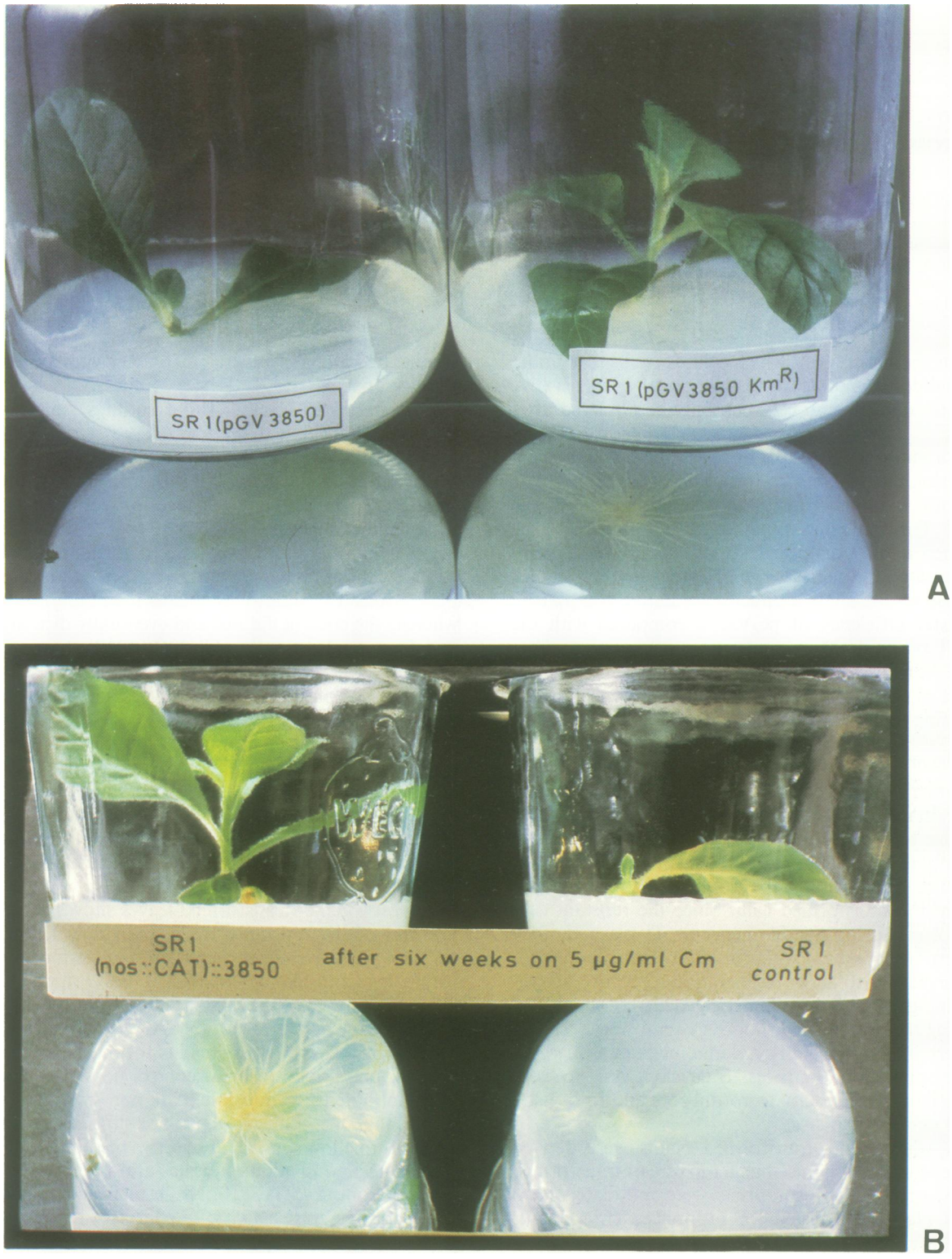

Fig. 3. Rooting test of the pGV3850::NOS-Km (Neo1) and pGV3850::NOS-CAT (Cat1) plants. A piece of the stem containing one or a few leaves of the regenerated plants were placed on plant growth medium containing 100 µg/ml kanamycin (**A**) or 5 µg/ml chloramphenicol (**B**). The pGV3850::NOS-Km plant formed roots after 1 weeks, while the pGV3850::NOS-CAT plant only started to form roots after 3–4 weeks. The sensitive control plants never formed roots in these antibiotic-containing media.

testing the germination and growth of seeds produced from a transformed antibiotic-resistant plant. For example, pGV3850::NEO seeds from the self-fertilized Neo1 plant and control SR1 untransformed seeds were germinated in the presence of 100 µg/ml Km. Both resistant and sensitive seeds were able to germinate in the presence of Km. However, the resistant seedlings continued to grow normally, whereas the sensitive seedings did not grow further and became complete-

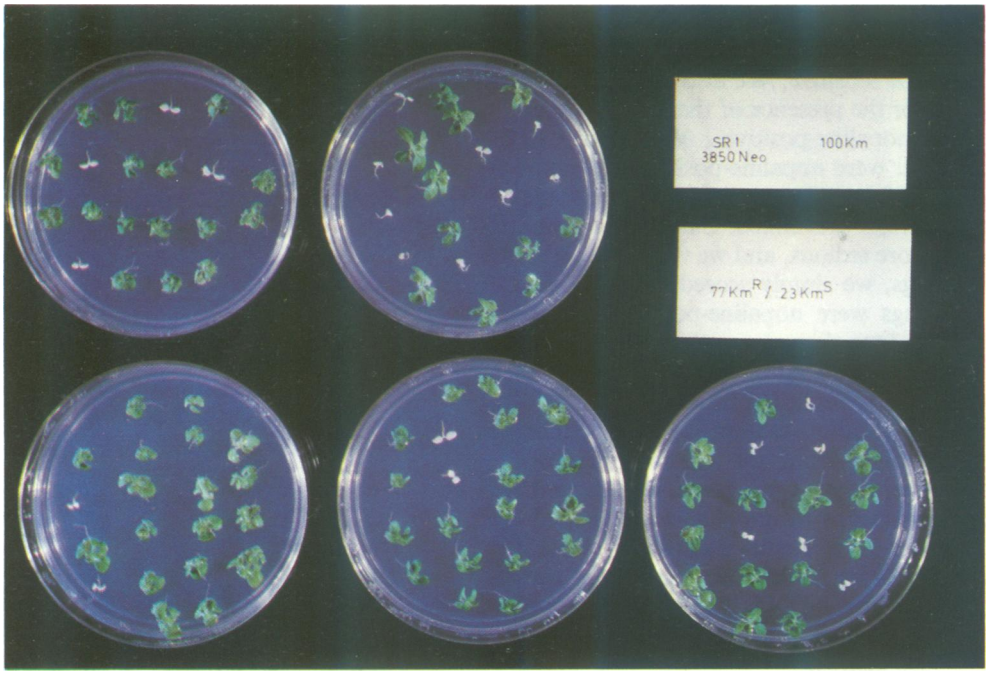

Fig. 4. Resistance test of 100 F1 seeds of a self-fertilized pGV3850::NOS-Km plant (Neo1). One hundred seeds of a self-fertilized pGV3850::NOS-Km plant (Neo 1) were placed on plant growth medium containing 100 μg/ml kanamycin. The resistant seeds germinated and formed plantlets after 3 weeks. The sensitive seeds also germinated, but after 1 week the seedlings did not grow further, and after 2 more weeks, they etiolated and died. We found a perfect 3:1 resistance ratio.

ly etiolated after 3 weeks. Figure 4 compares resistant and non-resistant seedlings derived from the Neo1 plant growing in the presence of Km.

Finally, expression of *cat* coding sequence can be easily monitored by a simple test for enzymatic activity of chloramphenicol acetyltransferase, providing a useful tool to test the expression of this chimeric gene. Thus, *cat* activity was assayed in stems, roots, leaves, and callus tissue derived from the plant Cat1; all these tissues were found to be positive compared with a control untransformed plant (Figure 5). Previous results had demonstrated that another opine gene, octopine synthase, is expressed in all tissues of transformed plants (Otten *et al.*, 1981; De Greve *et al.*, 1982) and that the nopaline synthase gene is expressed in the leaves of transformed plants (Zambryski *et al.*, 1983b). The results presented here are the first direct evidence that the *nos* gene can be constitutively expressed in all plant tissues; furthermore the signals for this expression reside in the 5′-flanking sequences used in the chimeric gene construction.

Genetic transmission of the resistance traits

The regenerated transformed plants described above were found to be fully fertile, to produce normal amounts of seed, and the seeds are fully capable of germinating (~100%). As mentioned above, seeds containing the kanamycin-resistant chimeric genes can undergo normal germination on kanamycin-containing medium, while normal seeds germinate into etiolated plantlets that stop growing after 10 days. We used this test to examine the genetic transmission of the resistance trait. Seeds obtained from the self-fertilized plant Neo1 were germinated in the presence of 100 μg/ml of kanamycin. From 380 seeds tested, 295 were found to germinate normally in the presence of kanamycin, while 85 were found to be sensitive (Figure 4). This 3:1 ratio of resistance demonstrates that the resistance marker is dominant, that the

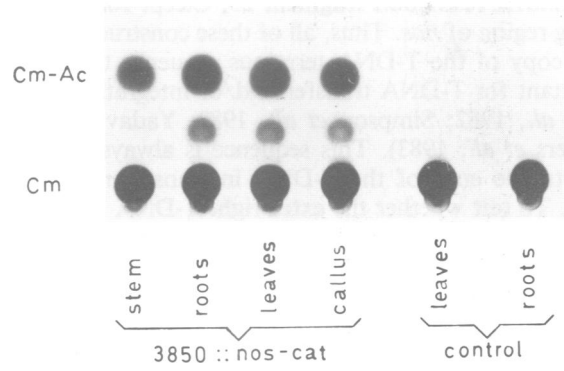

Fig. 5. Assays of *cat* activity in Cat1 plant tissues. 100 mg of leaf, root, stem, or callus tissue were extracted by grinding; *cat* activity was determined as previously described (Herrera-Estrella *et al.*, 1983a). The autoradiogram shown was exposed for 64 h, and illustrates the activity found in the following tissues: **lane 1**, Cat1 stem tissue; **lane 2**, Cat1 root tissue; **lane 3**, Cat1 leaf tissue; **lane 4**, pGV3850::NOS-CAT primary callus tissue; **lane 5**, SR1 non-transformed leaf tissue; **lane 6**, SR1 non-transformed root tissue. When autoradiograms were exposed for 5 or more days, a low background of non-specific chloramphenicol acetylation was detected in non-transformed tissues.

mother plant is hemizygous for this trait, and that the resistance is transmitted as a single Mendelian factor. Southern blotting analysis confirmed that this gene was present at a single locus (data not shown).

The genetic transmission of the methotrexate resistance marker in seedlings derived from the self-fertilized Mtx1 plant cannot be directly assayed on methotrexate-containing medium. Thus, the Mtx1(R1) seeds were germinated into seedlings and callus tissue was obtained from a leaf of each of these individual seedlings. These calli were placed on medium containing 0.5 mg/ml Mtx and active charcoal (see Materials

and methods) to test for resistance. Sixty calli derived from the individual seedlings were tested in this way; 40 were found to be resistant and 20 to be sensitive. We also checked these same seedlings (or calli) for the presence of the nopaline trait as the Mtx1 plant is nopaline-positive. All of the 40 methotrexate-resistant calli were nopaline-positive and none of the methotrexate-sensitive calli were positive; thus the Mtx^R and the nopaline traits are linked. Since the methotrexate resistance test is more tedious, and we wished to test a larger number of seedlings, we simply scored 200 seedlings for nopaline; 144 seedlings were nopaline-positive and 56 were negative. These data (not shown) together suggest that there is a single chromosomal location of the inserted T-DNA which segregates in a normal Mendelian fashion.

We also studied the segregation pattern of the nopaline trait in R1 seedlings derived from the chloramphenicol-resistant, nopaline-positive plant, Cm1 (data not shown). Here we found a lower frequency of nopaline-negative seedlings (6 out of 113). These latter results conform to a 1:16 segregation ratio expected for two independent insertions and suggest the nopaline-containing T-DNA is inserted into two different linkage groups. These results agree with the Southern blot analysis (see below).

Structure of the T-DNA in the transformed plants

The transformed plants Mtx1 and Cat1 were chosen to contain the nopaline trait to facilitate the analysis of the genetic transmission of the newly acquired DNA sequences. However, as shown in Figure 1, the chimeric genes contain the entire *Hind*III restriction fragment 23, except for most of the coding region of *nos*. Thus, all of these constructs contain an extra copy of the T-DNA terminus sequence thought to be important for T-DNA transfer and/or integration (Zambryski *et al.*, 1982; Simpson *et al.*, 1982; Yadav *et al.*, 1982; Holsters *et al.*, 1983). This sequence is always found at or close to the ends of the T-DNA in transformed plant cell DNA. To test whether the extra right T-DNA border linked to the selectable marker is functional when placed in-between the normal T-DNA borders, we assayed for the linkage between the resistance trait and the unselected nopaline synthase marker in the transformed calli. The results summarized in Table I indicate that the nopaline trait is infrequently linked to the resistance marker. Less than 5% of the Mtx^R and Cm^R calli contain nopaline and we did not detect a nopaline-positive callus in the population of the Km^R-transformed calli analyzed here. These results suggest that the internal right T-DNA border can be used for integration at a high frequency.

To confirm whether the internal T-DNA border was capable of independent integration, the structure of the transferred DNA in transformed plant cells was analyzed by Southern blotting hybridization. Figure 6 shows this analysis for DNA prepared from a pGV3850::Cm^R-transformed plant (Cat1). The hybridization pattern shown in Figure 6B reveals that this particular plant contains more than one copy of the chimeric gene.

All the expected internal bands illustrated in Figure 6A can be seen in the hybridization pattern shown in Figure 6B. For example, *Hind*III fragments of 5.9 kb and 8.5 kb, and an *Eco*RI fragment of 7.8 kb are detected using pBR322 or *Hind*III fragment 23 as probes. When *Hind*III fragment 10 is used as a probe the expected 3.1-kb internal band is detected following digestion of the plant DNA with *Eco*RI. Similarly, the internal 0.9-kb *Eco*RI band homologous to CAT-coding

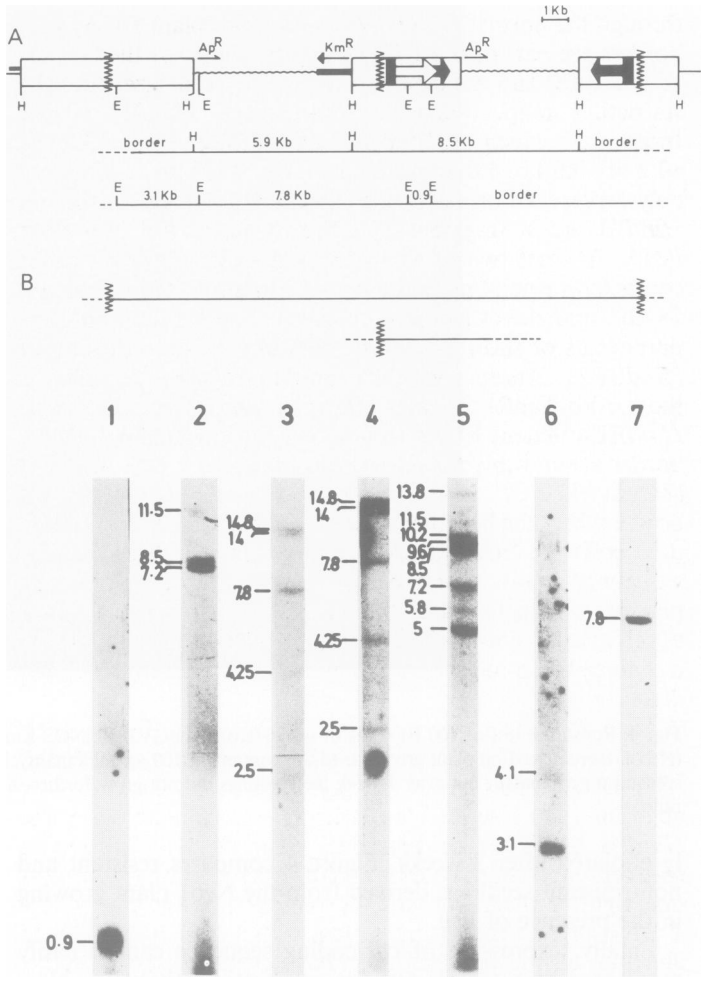

Fig. 6. Analysis of the structure of the T-DNA in the chloramphenicol-resistant plant Cat1. **(A)** The top line presents a schematic representation of the T-DNA region of the Ti plasmid pGV3850:NOS-CAT. The sizes of the expected internal *Hind*III and *Eco*RI fragments after integration into the plant genome are shown below; the fragments adjacent to the expected internal fragments are labeled 'border'. **(B)** Southern blot hybridization analysis of DNA prepared from the plant Cat1. 10 μg of Cat1 DNA was digested either with *Eco*RI **(lanes 1, 3, 4, 6, 7)** or *Hind*III **(lanes 2, 5)**, electrophoresed, and blotted onto nitrocellulose. The DNA was hybridized with radioactive probes and washed as previously described (Zambryski *et al.*, 1983b). The probes used were pBR322 **(lane 3)**, purified nopaline Ti plasmid *Hind*III fragment 23 **(lanes 4, 5)** or *Hind*III fragment 10 **(lane 6)**, 1.1-kb purified *Sma*I/*Hind*III Km fragment from pKC7 (Rao and Rogers, 1979) **(lane 7)** or purified 0.9-kb *Eco*RI fragment homologous to chloramphenicol acetyltransferase from Tn*9* (Marcoli *et al.*, 1980) **(lanes 1** and **2)**. The relative migration of the hybridizing bands in the different lanes cannot be directly compared as the DNA samples were electrophoresed in different gels at different times; thus, the sizes of the hybridizing bands is given in kb, alongside each lane to facilitate comparison of the bands. A diagrammatic representation of the various T-DNAs corresponding to the hybridization bands is shown above.

sequences is detected. All the other hybridizing bands are border fragments which (i) either are contiguous with the normal T-DNA borders of the vector Ti plasmid pGV3850, or which (ii) utilize the border recognition sequence adjacent to the selectable marker gene, i.e., the internal border. Thus, there are two T-DNAs which utilize internal borders; these are recognized as *Hind*III fragments of 11.5 kb and 7.2 kb, or *Eco*RI fragments of 14.8 kb and 14 kb, which hybridize with CAT and *Hind*III-23 probes. The two large *Eco*RI fragments of 14.8 kb and 14 kb likely represent borders which start at the *Eco*RI site adjacent to CAT and continue

through the normal *Hind*III-23 border into plant DNA; such borders are part of the T-DNA copies which use the borders of pGV3850 and are expected to be >8 kb in size from the restriction map. There are five other *Hind*III border fragments which hybridize with *Hind*III-23, 13.8 kb, 10.2 kb, 9.6 kb, 5.8 kb, and 5 kb (lane 5). These bands probably represent normal border fragments starting at the left *Hind*III site in fragment 23 extending rightward into plant DNA. At least two of these are part of full-length T-DNA copies (corresponding to the *Eco*RI fragments of 14.8 kb and 14 kb), and the others may represent borders from integration events of short T-DNAs which stop close to the end of *Hind*III-23. Another expected border fragment observed is the 4.1-kb *Eco*RI fragment (lane 6) which hybridizes with *Hind*III fragment 10. As variation of the normal left T-DNA border around this *Eco*RI site has been previously observed (Zambryski *et al.*, 1982), this fragment probably represents a border where the homology with fragment 10 ends just before the *Eco*RI site frequently found to be internal. We note that we cannot account for all the hybridization bands; for example, there are eight *Hind*III bands and only five *Eco*RI bands which hybridize with *Hind*III fragment 23. This result can be explained by co-migration of some of the bands in the *Eco*RI digest.

Thus, to summarize there are: (i)full-length T-DNA copies where the normal left and right T-DNA borders are used; and (ii) T-DNA copies which utilize the internal T-DNA border adjacent to the selectable marker gene. In addition, some *Hind*III bands which hybridize with *Hind*III-23 fragment may represent short T-DNA copies which occur near the normal right T-DNA border. We present the analysis of the T-DNA organization from a plant with a complex pattern to illustrate all the possible integration events observed in the transformed plants. We have also observed simple patterns; for example, the Neo1 plant analyzed above for phenotypic and genetic properties has only one copy of the Km^R gene and this gene was inserted into the plant genome from the internal T-DNA border sequence (data not shown). This latter T-DNA is also short such that it ends within the pBR322 sequences and excludes the *nos* gene.

Thus, these results suggest that the internal T-DNA border sequence is capable of directing independent transfer and/or insertion of the adjacent DNA. Furthermore, when similar constructions where the internal T-DNA border sequence is deleted were used for co-cultivation experiments, a 100% linkage between the resistance trait and the nopaline synthase marker was observed (Herrera-Estrella *et al.*, in preparation). Other recent results of the laboratory have also independently demonstrated that the right T-DNA border sequence is sufficient to promote integration of sequences adjacent to it (A. Caplan, E. Van Haute, K. Wang, and P. Zambryski, unpublished results).

Although the Southern blot analysis strongly suggests that the internal T-DNA border is functional, it is still unknown why it does not always co-transfer the nopaline synthase gene; however, perhaps the following can occur. Since a second recombination event in *Agrobacterium* releases the plasmid carrying the chimeric gene, there is a certain probability that the internal portion containing the chimeric gene would exist as an independent circle. For example, saturated cultures (normally used for co-cultivation) stored at 0°C for 3–4 days in minimal medium or cultures mixed with plant cell protoplasts for several days in plant growth medium did produce circles of the appropriate size (data not shown). Most probably these circles are lost in an actively growing culture of *Agrobacteria* as pBR322 cannot replicate; however, such circles could persist in *Agrobacteria* which are not dividing rapidly. These circles may be capable of independent integration analogous to the binary Ti vector system (de Framond *et al.*, 1983; Hoekema *et al.*, 1983), if a T-DNA border sequence is provided.

Discussion

We have presented results which demonstrate that: (i) foreign DNA sequences can be readily transferred to plant cells using the Ti plasmid vector pGV3850; (ii) whole plants can be easily obtained from the transformed cells, and these plants are phenotypically normal and fertile; and (iii) bacterial coding sequences are functionally expressed in all tissues of regenerated plants under control of the *nos* promoter. Analysis of the genetic transmission of the antibiotic resistance markers showed that they behave as Mendelian factors and are dominant in hemizygous plants. Three different chimeric genes carrying antibiotic resistance traits were studied. All three are capable of providing a selective advantage to transformed cells growing in tissue culture; however, the best results are obtained using the kanamycin resistance marker. It is easy to prepare and store selective media containing kanamycin, and kanamycin-resistant transformed cells are quickly and easily scored. As described, the primary selection of Mtx^R or Cm^R-transformed calli is less straightforward. We have also presented evidence that suggests that the right T-DNA border is independently functional when inserted in-between the normal T-DNA borders. Furthermore, these results demonstrate that the functionality of this sequence is not dependent on its orientation within the Ti plasmid, since in the co-integrates described this internal right border was inserted in the opposite orientation to that of the normal right T-DNA border.

Recently, we have constructed similar co-integrates in pGV3850 containing the Km^R selectable marker, but now lacking the internal T-DNA border sequence; when these constructions were used in co-cultivation experiments we found that the linkage between the selectable marker and the nopaline synthase is restored and that the integration proceeds using the normal T-DNA borders (Herrera-Estrella *et al.*, in preparation). This demonstrates that the extra right T-DNA border present in the construction described in this paper is responsible for the integration events observed. Furthermore, it has been recently shown that the 25-bp terminus sequence itself, normally found at the right T-DNA border, is required for transfer to occur (K. Wang and P. Zambryski, unpublished results). These results, which show that a single border sequence is capable of directing DNA transfer, will be useful in the design of simplified Ti plasmid-derived vectors.

The results presented here open the possibility to study the mechanisms of plant-specific gene regulation in whole plant tissues. For example, we have recently studied the regulation of the light-inducible small subunit (*ss*) gene of ribulose-1,5-bisphosphate carboxylase (*rbpc*) in tumor callus tissue; the regulation was studied by constructing a wild-type Ti plasmid containing a chimeric gene composed of the 5′ sequences of the *ss-rbpc* gene linked to chloramphenicol acetyltransferase (*cat*) (Herrera-Estrella *et al.*, 1984). Tumor tissue grown in the light exhibited *cat* activity, whereas tissue grown in the

dark did not. Now, we have inserted the same chimeric construction into pGV3850 and have obtained plants which express this chimeric gene specifically in leaf tissue, and this expression is highly influenced by the light conditions under which the plant is grown (L. Herrera-Estrella, unpublished results). These model experiments can now be extended to assay for the expression of other DNA sequences which encode functions that may promote plant growth, resistance to pathogens, or increase the nutritive or medicinal value of plants. While this manuscript was in preparation, Horsch *et al.* (1984) reported the regeneration of plants from plant cells transformed with a Ti plasmid containing a similar chimeric gene for kanamycin resistance.

Materials and methods

Bacterial strains

The NOS-Km(pLGV23NEO) and the NOS-DHFR(pLGV23DHFR) constructs have been described before (Herrera-Estrella *et al.*, 1983b). The NOS-CAT(pNCAT7) was constructed as follows: a 950-bp *Sau*3A fragment from pBR325 (Bolivar, 1978) was isolated from a 1% agarose gel by electroelution and cloned into the *Bam*HI site of pLGV2382 (Herrera-Estrella *et al.*, 1983b). All three chimeric genes were transferred from *Escherichia coli* to *Agrobacterium* by the method of Van Haute *et al.* (1983), and co-integrates with the non-oncogenic acceptor Ti plasmid pGV3850 (Zambryski *et al.*, 1983b) were selected on kanamycin-containing medium. The bacterial Km^R gene of pLGV2382 in the NOS-DHFR and the NOS-CAT constructs was used for genetic selection of co-integrate formation with pGV3850. The *nos*-Km^R gene confers resistance to kanamycin both in plants and in bacteria as the *nos* promoter allows a low level of transcription in *E. coli* and *Agrobacterium*; thus, this chimeric gene can also be used for genetic selection of co-integrate formation with pGV3850.

Plant cell culture methods

The co-cultivation method used here is based on that originally published by Márton *et al.* (1979) and our own modifications to this method recently described (Herrera-Estrella *et al.* 1983b). The details of the preparation of single cell protoplasts, co-cultivation with *Agrobacterium*, selection of resistant calli, and regeneration of transformed plants and all media compositions are described elsewhere (Zambryski *et al.*, 1984). The antibiotics used in the culture procedure are cefotaximum (Claforan® , Hoechst), kanamycin (Sigma), chloramphenicol (Sigma), and methotrexate (Aldrich).

The procedure can briefly be summarized as follows: protoplasts are prepared and allowed to regenerate for 4 days. *Agrobacteria* are added and co-cultivated with the protoplasts for 3 days. The protoplasts are washed free of the bacteria and cefotaximum (Claforan® , Hoechst) is added to a final concentration of 500 μg/ml to inhibit any contaminating bacterial growth. The protoplasts are allowed to grow for a further 3 weeks before screening on selective media. The selective procedures used were different for each antibiotic.

For selecting Km^R-transformed calli, one of the following three methods was used: (1) liquid selection; (2) direct selection on solid agar; or (3) pregrowth on solid agar without selection followed by selection on solid agar. The first method uses liquid K3 (Nagy and Maliga, 1976) medium containing 0.1 mg/l NAA, 0.2 mg/l BAP, 0.2 M sucrose, 50 μg/ml kanamycin, and calli at 10^3/ml. Resistant calli can be seen after 2 weeks; these calli are large, white, and compact compared with the small, brown, sensitive calli. For the second method, 1 ml liquid medium containing 10^3 calli are spread on a 9 cm plate containing 0.8% agar, B5 salts (Gamborg, 1968), hormones as above, 3% sucrose, and 50 μg/ml kanamycin. Resistant calli can be distinguished as above after 2 weeks. For the third method, calli in liquid medium are spread on an agar plate as in the second method, but without kanamycin; after 2 weeks the growing calli are removed individually onto an agar plate as above (with kanamycin). This latter method is used to calculate the transformation frequency as the ratio of the number of resistant calli to the total number of calli plated. It should be noted that Km^R calli can be grown and maintained on kanamycin-containing media; this is in contrast to Cm^R or Mtx^R calli which cannot be grown continuously under antibiotic selection.

For selecting Cm^R-transformed calli, liquid selection does not work well. If necessary, a liquid selection can be done using 10 μg/ml chloramphenicol. After 2 weeks resistant calli can be recognized as slightly larger and more compact than sensitive calli; the sensitive calli do not die as a result of this treatment. Any putative resistant calli should be rechecked on solid media (as above for kanamycin selection) containing 10 μg/ml chloramphenicol, but only after a prior treatment of at least 3 weeks growth under non-selective conditions. Resistant calli are visible after 3 weeks as white, compact, and slightly larger than the sensitive calli which are brown and friable. A better selection is to use the direct selection on agar medium (as in the second method for kanamycin) containing 5 μg/ml chloramphenicol. Resistant calli are distinguished as above after 3 weeks. The frequency of transformation is determined as in method 3 for kanamycin using 5 μg/ml chloramphenicol. Cm^R calli can be maintained on chloramphenicol-containing media for no longer than 2 months.

For selecting Mtx^R-tranformed calli either method 1 or method 3 (analogous to the kanamycin selection) are best. The conditions for the liquid selection are as described above for the kanamycin liquid selection except that the medium contains 0.1 μg/ml methotrexate. Resistant calli are detected after 1 week by their compact structure compared with sensitive calli which are friable. The resistant candidates are transferred to non-selective agar medium (as described above) for 3 weeks and then retested on solid media. For the solid medium selection the best results are obtained using medium containing 0.1% active charcoal; this charcoal chelates toxic products which can inhibit growth of even Mtx^R cells. These toxic products are either contained in the methotrexate itself, or are decomposition products released by cells growing in its presence. Thus the solid medium has the following composition: B5 salts, 0.8% agar, 3% sucrose, 0.1% active charcoal, 0.5 μg/ml methotrexate, 1 mg/l NAA and 2 mg/l BAP. Note the higher concentration of the last three components; these increases are necessary to compensate for the relative lowering of their effective concentration due to the presence of the active charcoal. After 4 weeks the calli can be scored: resistant calli are white to greenish in color, while sensitive calli remain brown. Neither type of calli grows significantly on methotrexate-containing media. The frequency of transformation is determined using this solid active charcoal-containing medium using calli pre-grown under non-selective conditions as described above for the kanamycin or chloramphenicol selections.

Rooting test

To assay for expression of resistance traits in whole plants, a rooting test was developed. The expression of Km^R was tested by placing a cutting of a piece of stem containing a leaf and node into a pot (10 cm x 10 cm) containing MS salts (Murashige and Skoog, 1962), 1% sucrose, 0.8% agar, and 100 μg/ml kanamycin. The kanamycin concentration can be varied between 25 and 150 μg/ml for expression from the chimeric genes presented here; the time for root formation to occur varies directly with the kanamycin concentration. With 100 μg/ml kanamycin, root formation (with a Km^R shoot) starts to occur after 1 week; after 2–3 weeks, many roots are observed.

The expression of Cm^R was tested as above by placing a cutting of a piece of stem containing a leaf and a leaf node into a pot of medium (as above) containing 5 μg/ml chloramphenicol. This test is more variable than the kanamycin resistance rooting test. For example, the shoot cutting should not contain the apical shoot since the auxin produced there will enhance root formation and decrease the effectiveness of the test. The concentration of chloramphenicol is critical for the rooting test. There is no selection below 3 μg/ml. Using 100 ml of medium containing 5 μg/ml chloramphenicol, root formation (using a resistant shoot) begins after 3–4 weeks; after 5 weeks many roots are formed. If the chloramphenicol concentration is raised to 10 μg/ml, root formation does not begin until 5 weeks.

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