

DEVELOPMENT OF A TRANSFORMATION SYSTEM IN WHITE CLOVER USING MODIFIED TI-PLASMID VECTORS

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ABSTRACT

A system has been developed for introducing cloned foreign genes into the forage legume, white clover (*Trifolium repens* L.). The system utilises modified Ti-plasmid binary vectors containing a chimaeric gene which confers kanamycin resistance, the natural gene transfer mechanism of *Agrobacterium* and a high regeneration white clover genotype as recipient. Transformed white clover cells expressed an unselected marker gene (nopaline synthase) and had the expected bacterial neomycin phosphotransferase II activity. Integration of vector DNA sequences into white clover DNA has been confirmed by Southern hybridisation. Transformed white clover cells retained their plant regeneration capacity.

KEYWORDS

Legume transformation, binary vectors, *Agrobacterium*, gene transfer.

INTRODUCTION

Recently, efficient methods for introducing cloned genes into plants have been developed (Caplan *et al.*, 1983). The ability to manipulate genes and make defined additions or alterations to the genome of crop plants could lead to improvements in agronomic performance. These techniques are also important for studying gene expression in plants.

The most widely used method for the transformation of dicotyledonous plants is based on the natural gene-transfer capacity of *Agrobacterium tumefaciens*. *A. tumefaciens* causes tumorous growths on susceptible plants by the transfer and integration into the plant nuclear genome of a specific segment (T-DNA) of the large Ti-plasmid (Chilton *et al.*, 1977). This T-DNA contains genes for hormone independent growth and the synthesis of novel metabolites called opines. Transfer of the T-DNA is mediated by a separate locus (*vir*) on the Ti-plasmid which contains several genes which encode *trans* acting products. The two functional units, *vir* and T-DNA, can be placed on

separate plasmids to form a binary system (Hoekma *et al.*, 1983). In order to regenerate plants from transformed cells the tumour-inducing genes have been deleted from T-DNA and replaced with a chimaeric gene which utilises regulatory regions from the nopaline synthase gene and the coding sequence of a bacterial neomycin phosphotransferase to confer resistance to kanamycin (Bevan, 1984; An *et al.*, 1985). Efficient *Agrobacterium* vectors have been developed by placing the modified T-DNA into a broad host range plasmid to form shuttle or binary vectors.

If gene manipulation and introduction techniques are to be used for plant improvement, transformation systems will have to be established for a wide variety of crop plants, including the important legumes. A major component of current transformation methods is a requirement for plant regeneration from cultured cells. An efficient method for regenerating an important forage legume, white clover, from cultured cells, has been developed by selecting a high-regeneration genotype (White, 1984). Here we describe the development of a system for introducing cloned genes into white clover utilising this genotype, binary vectors and the *Agrobacterium* system of transfer.

MATERIALS AND METHODS

A list of bacterial strains and plasmids used is given in Table 1. Vectors pGA470 and pGA472 were mobilised from *E. coli* MC1000 to *A. tumefaciens* GV3850 in a triparental mating with *E. coli* HB101 containing pRK2013. Transconjugants were selected on minimal medium containing 10 mg/L tetracycline and 10 mg/L kanamycin to give GD102 and GD103 respectively. The binary vectors used are described by Bevan (1984) (pBin6, pBin19) and An *et al.* (1985) (pGA470, pGA472).

In preliminary experiments approximately 20 different white clover genotypes were inoculated with *A. tumefaciens* strains C58 and C58-C1 to test for tumour formation. Stolon internodes of glasshouse-grown plants were wounded and inoculated, using a toothpick, with *A. tumefaciens* grown on agar medium. For *in-vitro* transformation studies stolon internode segments from the high-regeneration genotype, WR8 (White, 1984), were

Table 1. Bacterial strains and plasmids used.

Bacterial strain	Plasmid (s)	Origin
<i>A. tumefaciens</i>		
C58	pTiC58	
C58-C1, a derivative cured of pTiC55		
GV3850	pGV3850	Zambryski <i>et al.</i> (1983)
LBA4404	pAL4404	Hoekma <i>et al.</i> (1983)
LBABin6	pAL4404, pBin6	Bevan (1984)
LBABin19	pAL4404, pBin19	Bevan (1984)
GD102	pGV3850, pGA470	this study
GD103	pGV3850, pGA472	this study
<i>E. coli</i>		
MC1000 (GA470)	pGA470	An <i>et al.</i> (1985)
MC1000 (GA472)	pGA472	An <i>et al.</i> (1985)
HB101 (RK2013)	pRK2012	Ditta <i>et al.</i> (1980)

surface sterilised in 2.5% sodium hypochlorite for 25 min, washed in sterile dH₂O and the bleached ends were cut off. Inoculation was effected by touching the cut ends on to *A. tumefaciens* grown on agar medium. Segments were incubated in a moist petri dish at 28 °C for 3 days, then 1 mm terminal slices were cut off and placed on media containing 500 mg/L cefotaxime. Segments inoculated with strains C58 or C58-C1 were placed on B, medium lacking hormones, while those inoculated with strains LBA4404, LBABin6, LBABin19, GV3850, GD102 and GD103 were transferred on to callusing medium containing 100 mg/L kanamycin. Media and conditions for callus initiation, maintenance, suspension culture and shoot regeneration were as previously described (White, 1984).

Aliquots (100 mg) of callus material were crushed and assayed for nopaline accumulation by paper electrophoresis with octopine and nopaline standards and stained using phenanthrenequinone (Otten and Schilperoot, 1978).

Extracts from kanamycin resistant cell lines were submitted to electrophoresis on a 10% non-denaturing polyacrylamide gel together with extracts of *E. coli* containing the neomycin phosphotransferase II (NPT II) gene. NPTII activity was assayed according to the procedure of Reiss *et al.* (1984). Plant DNA was isolated from suspension cultured cells of transformed cell lines using the procedure of Sutton (1974). Integrated DNA was detected by DNA/DNA blot hybridisation according to Southern (1975), using an isolated 3.5 kilobase Hind III fragment of DNA encoding NPTII as a probe. DNA manipulation procedures were as described in Maniatis *et al.* (1982).

RESULTS

In-vivo and in-vitro tumour formation

Preliminary experiments established that a range of white clover genotypes, including WR8, form tumours *in-*

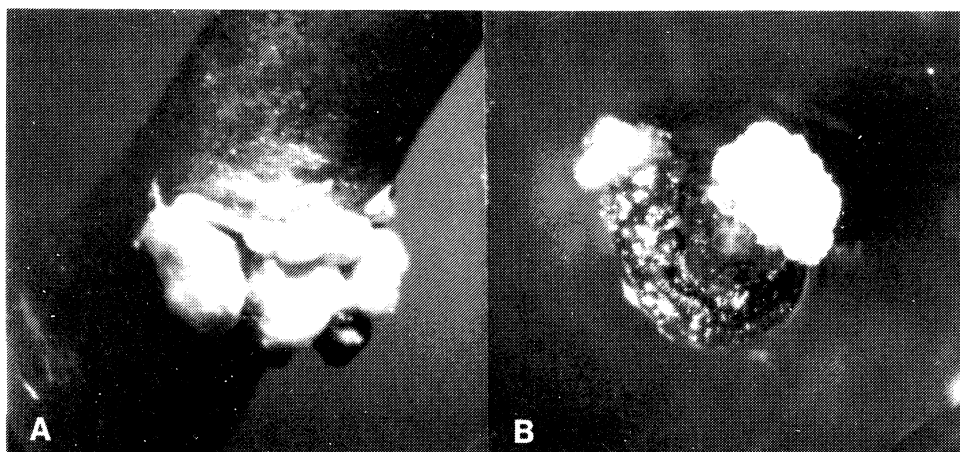


Figure 1. (a) *In-vivo* and (b) *in-vitro* tumour formation on white clover stolon tissue inoculated with *A. tumefaciens* C58.

vivo on wounded stolon internodes after inoculation with *A. tumefaciens* strain C58 (Riddiford and White, unpublished). Tumours were not formed after inoculation with strain C58-C1, a derivative of C58 cured of pTiC58. Four to six weeks after inoculation with strain C58, most genotypes formed compact outgrowths similar to the tumour illustrated in Fig. 1(a). Surface sterilized tumour tissue exhibited hormone-independent growth on B₂ medium. This capacity for hormone-independent growth was used to develop a simple *in-vitro* method for transforming white clover. The wounded ends of surface sterilised stolon internode segments were inoculated and incubated for 3 days to allow for a plant wound response and transformation. The proportion of potential transformed cells per segment was then increased by cutting off and transferring only the terminal 1 mm containing the inoculated wound surface to selection medium. Approximately 15-20% of the segments inoculated with strain C58 developed hormone-independent outgrowths on B₂ medium 3-4 weeks after inoculation (Fig. 1b). No growth was obtained from 200 segments inoculated with strain C58-C1.

A further characteristic of plant cells transformed with strain C58 is the expression of a T-DNA gene, nopaline synthase, which results in the accumulation of nopaline. Most, but not all of the cell lines obtained after inoculation with strain C58 and hormone-independent selection, accumulated nopaline (Fig. 2).

Transformation with binary vectors

Four different binary vectors, pBin6, pBin19 (Bevan, 1984) and pGA470, pGA472 (An *et al.* 1985) were tested using *in-vitro* inoculation of WR8 internode segments. Transformed cell lines were selected by transferring the terminal 1 mm of the segments to callusing medium containing 100 mg/L kanamycin. Control inoculations were made with strains LBA4404 and GV3850 which contain only pAL4404 and pGV3850 respectively which supply the *vir* functions. Kanamycin-resistant outgrowths developed only on segments inoculated with *A. tumefaciens* strains containing binary vector plasmids (Table 2). Kanamycin-resistant cell lines were first visible 12-14 days after transfer to selective medium and grew rapidly when transferred to fresh medium (Fig.3). The shoot

Table 2. Transformation of white clover using *A. tumefaciens* containing binary vectors. WR8 stolon segments were inoculated, incubated for 3 days, then placed on selection medium containing 100 mg/L kanamycin. Kanamycin-resistant growth was scored after 3 weeks of culture.

<i>A. tumefaciens</i> strain	Plasmids	Number of segments treated	% Kanamycin-resistant
LBA4404	pAL4404	208	0
LBABin6	pAL4404, pBin6	50	44
LBABin19	pAL4404, pBin19	99	41
GV3850	pGV3850	75	0
GD102	pGV3850, pG470	72	24
GD103	pGV3850, pGA472	24	33

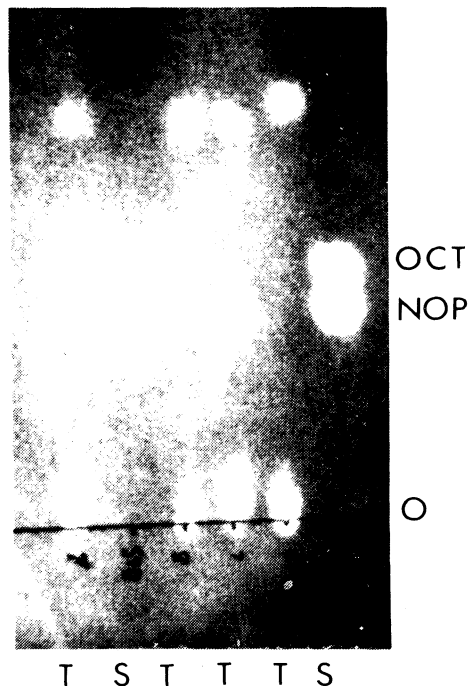


Figure 2. Nopaline assay of white clover cells selected for hormone-independent growth *in-vitro* after inoculation with *A. tumefaciens* C58. OCT = octopine, NOP = nopaline, S = standard, O = origin, T = from hormone-independent tissue.

regeneration capacity of WR8 cells transformed by *A. tumefaciens* strains containing pBin6, pBin19 or pGA470 was similar to that of unselected WR8 cells and occurred both in the presence and in the absence of kanamycin selection.

DNA isolated from transformed and non-transformed white clover cells was analysed for the presence of introduced vector DNA by DNA/DNA blot hybridisation using an isolated fragment encoding bacterial NPT II as a

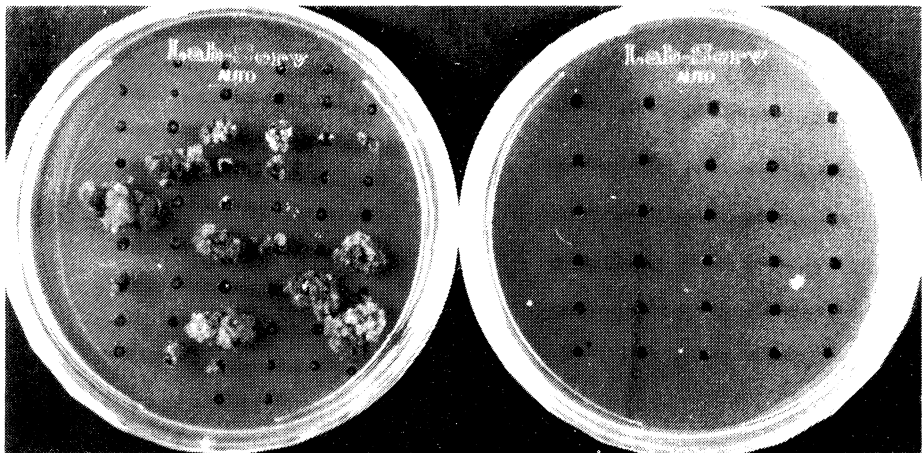


Figure 3. White clover stolon segment transformation and selection for kanamycin-resistant cells. The cultures were photographed 42 days after transfer to medium containing 100 mg/L kanamycin. Segments transformed with pBin6 are shown on the left and segments inoculated with strain LBA4404 (which lacks a binary vector) are shown on the right.

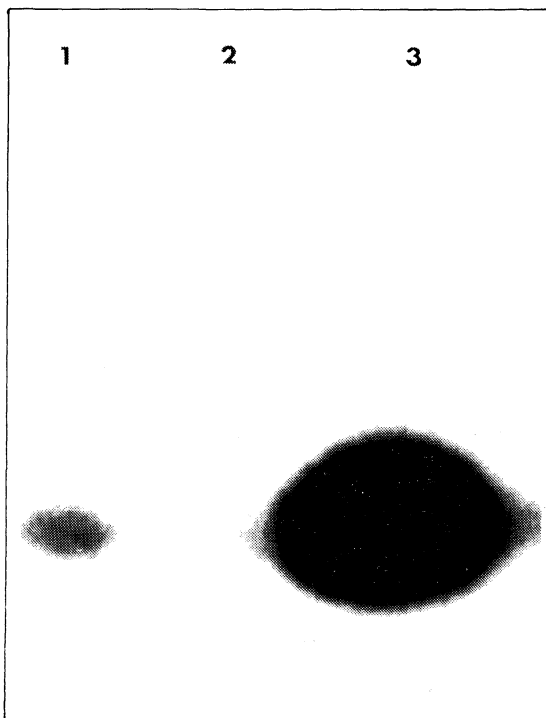


Figure 4. Comparison of NPT (II) activity of transformed (lane 3) and non-transformed (lane 2) white clover cells, together with an extract from *E. coli* cells containing a cloned NPT (II) gene (lane 1).

probe. As expected the probe hybridised to DNA from transformed cells but not to DNA from non-transformed cells (data now shown).

Expression of introduced foreign genes

The expression of both the selected gene activity (NPT II) and an unselected gene activity (nopaline synthase) in transformed cells was assayed. Six cell lines transformed using the pBin6 vector which contains both a chimaeric NOS-NPT II gene and the nopaline synthase gene all accumulated nopaline, whereas a cell line transformed using pBin19, which lacks nopaline synthase, did not (data not shown). Cell lines transformed using pBin19 and selected on kanamycin medium were assayed for the presence of bacterial NPT II activity. Only selected kanamycin-resistant cell lines expressed a NPT II activity which co-migrated with the bacterial enzyme activity (Fig.4).

DISCUSSION

A simple and effective method for introducing foreign genes into a selected white clover genotype has been developed. This method, and all other methods so far developed for introducing cloned genes into plants, relies on a capability to regenerate plants from cultured cells. Only a portion of the white clover genotypes examined have this capability at present (White, 1984). However, since white clover is an outcrossing species a single recipient genotype could be used to introduce new or altered genes. The gene could then be mobilized into an appropriate genetic background by conventional breeding procedures. The availability of a linked selectable marker (kanamycin-resistance) would assist this process.

During the development of our transformation method it was found that not all hormone-independent cell lines obtained after inoculation with strain C58 accumulated detectable levels of nopaline. It is possible that these cell lines were not clonal and were composed of both transformed hormone-producing and cross-feeding non-transformed hormone-dependent cells. It is noteworthy that all of the cell lines examined after transformation with *A. tumefaciens* containing pBin6 and selection on kanamycin accumulated nopaline. An alternative explanation is that the expression of the nopaline synthase gene has been suppressed. Similar results have been reported by others (see Horsch *et al.*, 1985) and it has been suggested that lack of expression is due to surrounding DNA at the site of insertion.

The development of a white clover transformation system will allow the introduction of foreign genes which in turn may lead to improvements in agronomic performance. Examples of potential alterations are the introduction of single genes conferring herbicide resistance or enhanced nutritional value. Also a legume transformation system could be used to study the expression of plant genes involved in nodule formation and function.

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SYMPOSIUM DISCUSSION

- Dr A. Thomson, Botany Division, DSIR
Does *Agrobacterium tumefaciens* occur naturally in N.Z.? What sort of disease does it cause?
- White
Yes. It causes crown gall, and I think it is a problem in roses.
- Thomson
Could you envisage gene transfer naturally?
- White
Yes, there is some evidence to suggest that there are sequences from a related species *Agrobacterium rhizogenes*. However in the case of *A. tumefaciens* I do not think anybody to date has found any of these sequences.
- Mr H.K. Hall, Crop Research Division, DSIR
We find that crown gall is quite a problem in *Rubus* species, and the product Dygall which is *Agrobacterium radiobacter* was introduced to combat the problem.
- Mr G.J. Piggott, MAF
Could you comment on the hazard of putting potential herbicide resistance into forage species?
- White
I do not see any hazard. We are essentially talking about single genes for specific herbicides. Although we might envisage having to introduce over a period of time a number of genes and combinations of genes, obviously each new cultivar that is produced has to have a different herbicide resistance. I still think there is a wide selection of good herbicides, so there is never going to be a problem in killing these plants.

Mr D.F. Cameron, CSIRO

You mentioned that you had quite a lot of difficulty finding a plant that would regenerate well. Have you got any plans to make a study of regeneration capacity?

White

No — it is not a high priority. We would certainly like to be able to regenerate at will any genotype. But our experience is that, even with extensive modifications to the media, this is not at present possible, so we just use one genotype. While this may create problems in the short term, I think there are breeding strategies to mobilise any gene into the appropriate background.

Cameron

I am wondering if anybody might do inheritance studies on this capacity?

White

I think there would be problems with highly heterozygous and outcrossing species. While you can think of taking a number of genotypes that regenerate, and producing a population which has a higher potential to regenerate you are still faced with the fact that some genotypes will and some genotypes will not. So my preference is to select a really good genotype and just work with that.