BIOLOGIA PLANTARUM (PRAHA)

28 (4) : 265—269, 1986

Binary Plant Vector Based on Ri Plasmid and Part of T-DNA of the Ti Plasmid

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Abstract. A binary vector system in *A. tumefaciens* for the introduction of foreign genes into the plant genome was developed. The first component is the Ri plasmid and the second component is a small vector plasmid, replicating in *Agrobacterium*, which carries the 25 bp terminal sequence of the Ti plasmid, the *Nos* gene as the selectable marker and neighbouring sequences of the Ti plasmid. Functions necessary for integration are provided *in trans* by the virulence region of the Ri plasmid. Transformed cells are selected on the basis of hairy root tumor proliferation and agropine synthesis. They also show *Nos* activity coded by the gene on the small part of Ti T-DNA.

The common way of introducing cloned genes into the plant genome with the aid of the Ti plasmid of *A. tumefaciens* relies on an intermediary plasmid (LEEMANS *et al.* 1981, MATZKE and CHILTON 1981). It is a small plasmid carrying part of T-DNA of the Ti plasmid, which is maintained and genetically manipulated in *E. coli*. The intermediary vector is introduced into an *Agrobacterium* carrying the Ti plasmid and the single or double recombination between the T-DNA and the T-DNA fragments of the intermediary plasmid is selected.

As the last step is often laborious and time-consuming, binary vectors have been developed as a more versatile alternative (DE FRAMOND *et al.* 1983, HOEKEMA *et al.* 1983, AN *et al.* 1985). They are based on the knowledge that all the functions necessary for the introduction of T-DNA into the plant genome are located on the virulence region of the Ti plasmid, outside the T-DNA (KLEE *et al.* 1983). The virulence region consists of seven *loci* (HOOYKAAS *et al.* 1984). The T-DNA of the Ti plasmid is flanked by terminal sequences, *i.e.* direct repeats of a 25 bp consensus sequence (ZAMBRYSKI *et al.* 1982). The right terminal sequence is necessary for the integration of T-DNA into the plant genome (WANG *et al.* 1984).

The first component of the binary vector is a shortened Ti plasmid, deprived of T-DNA, but carrying all the virulence functions. The second component is the T-DNA region carried by the vector plasmid. All the virulence functions necessary for the integration of the T-DNA are provided *in trans* by the first component. The virulence regions of Ti and Ri plasmids are homologous

Received June 19, 1985; accepted September 25, 1985

(HOOYKAAS et al. 1984) and Ri plasmids can be used in binary vector systems to provide virulence functions for T-DNA of the small vector plasmid (HOEKEMA et al. 1984).

In the study presented here we constructed a binary vector consisting of a small part of Ti plasmid, *i.e.* the right T-DNA terminal sequence, the *Nos* gene and neighbouring Ti plasmid sequences of different length, carried by the recircularized Bgl II fragment of pSa (LEEMANS *et al.* 1982, WARD and GRINSTED 1982), as the vector plasmid, which was introduced into *A. tumefaciens* strain carrying the Ri instead of Ti plasmid.

MATERIAL AND METHODS

Table 1 gives the list of bacteria and plasmids used. Plasmid DNA was isolated according to the method of BIRNBOIM and DOLY (1979) and purified by CsCl-ethidium bromide centrifugation. Restriction endonuclease digestion and cloning were performed as described in MANIATIS *et al.* (1982) and *A. tumefaciens* transformation according to HOLSTERS *et al.* (1978).

TABLE 1
Bacterial strains and plasmids used

Bacterial strains	Plasmids	Relevant markers	Source	Reference
E. coli HB101				
	MiniSa	Kn^rSpc^r		(VLASÁK and ONDŘEJ 1985)
	pGV0415	Amp^r	J. Schell*	(LEMMERS *et al.* 1980)
A. tumefaciens C58	pTiC58		M. Bezděk**	
A. tumefaciens C58Cl	pRiA4b	Rf^rSpc^r	A. Petit***	(PETIT *et al.* 1983)
A. rhizogenes 15834	pRi15834a,b,c		J. A. Lippincott****	

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A clone of *Kalanchoe daigremontiana* and one month old *in vitro* cultivated seedlings of *Nicotiana tabacum* cv. White Burley and *Petunia hybrida* cv. Sněženka were used. *Nos* activity in plant tissues was assayed by the method of OTTEN and SCHILPEROORT (1978). A mannopine standard was synthesized according to PETIT *et al.* (1983) and mannopine and agropine production by transformed plant tissues was assayed by the methods referred to in PETIT *et al.* (1983).

RESULTS

The MiniSa plasmid (6.0 Md) used is the recircularized Bgl II fragment of pSa (LEEMANS *et al.* 1982, WARD and GRINSTED 1982, VLASÁK and ONDŘEJ 1985),

Abbreviations used: Nos = nopaline synthase; Amp^r = ampicilin resistance; Kn^r = kanamycin resistance; Rf^r = rifampicin resistance; Spc^r = spectinomycin resistance.

which carries Kn^{r}Spc^r markers. Plasmids pCB139 (11.2 Md), pCB140 (9.9 Md) and pCB141 (8.9 Md), carrying parts of nopaline Ti T-DNA cloned in the MiniSa, were constructed as shown in Fig. 1. They consist of the right part of T-DNA with the *Nos* gene, the right 25 bp terminal sequence of pTiC58 and neighbouring regions of different lengths integrated into the single Pst I site of MiniSa. The EcoR I site is the only site useful for insertion of foreign genes, either with their own promotors or with the whole pLGV2381 plasmid (HERRERA-ESTRELLA *et al.* 1983) in vectors pCB139 and pCB140. In pCB141, HindIII site can also be used.

These three plasmids were introduced into *A. tumefaciens* C58Cl Rf^{r}Spc^r (pRiA4b) by transformation. The presence of small plasmids in transformant strains was confirmed by agarose gel electrophoresis (Fig. 2). We have already

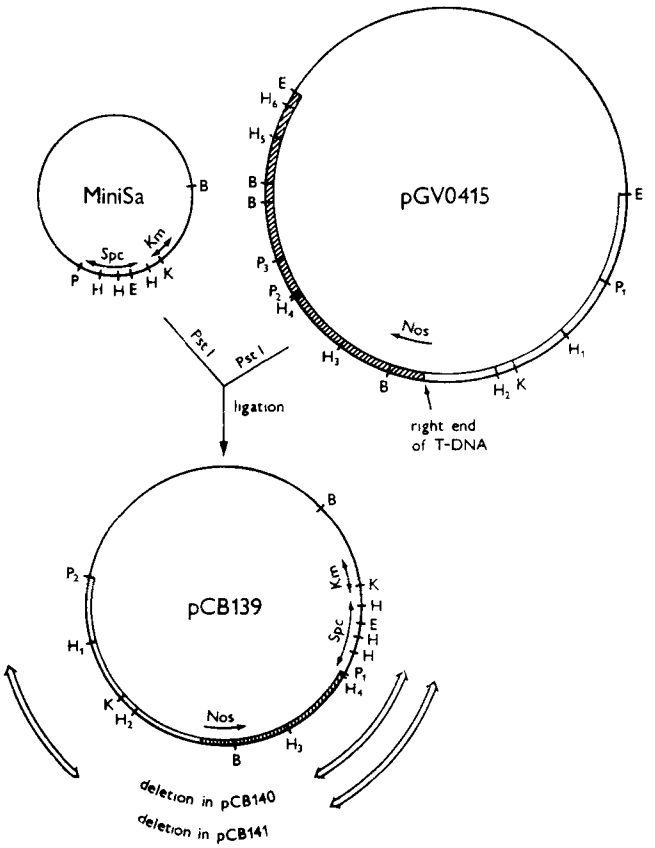

Fig. 1. Construction of plasmids pCB139, pCB140 and pCB141. A Pst I 5.2 Md fragment from the right part of T-DNA, containing *Nos* gene as well as the right part of T-DNA, originating from pGV0415, was cloned into Pst I site of MiniSa in the pCB139. Plasmids pCB140 and pCB141 were derived from pCB139 by Hind III cleavage and ligation, followed by screening for the conservation of H2—H3 fragment. Double line represents Ti DNA, double hatched line T-DNA. Arrows marked Spc, Km and *Nos* indicate genes for resistance to spectinomycin (streptomycin), kanamycin and the gene for nopaline synthase, respectively. Only relevant restriction sites are shown. H — Hind III, E — EcoR I, P — PstI.

shown that MiniSa in *Agrobacterium* does not inhibit virulence (VLASÁK and ONDŘEJ 1985). *A. tumefaciens* C58Cl Rf^rSpc^r strains containing pRi and one of the three small plasmids were used for the induction of tumors in *Kalanchoe*, *Petunia* and tobacco. Within two weeks tumors appeared, from which roots proliferated. Both tumors and roots synthesized mannopine and agropine and showed *Nos* activity (Figs. 3 and 4). The mannopine and agropine synthesis is the consequence of integration of T-DNA of the Ri plasmid, and *Nos* activity indicates the integration Ti T-DNA from the second, smaller component of the binary vector. Results in *Kalanchoe* are shown; tobacco and *Petunia* tumors and roots gave similar results.

The nopaline fluorescent spots on electropherograms of hairy root tumors, induced by binary vectors, however, were often weaker than those induced by *A. tumefaciens* C58. In a small proportion of tumors (less than 10 %), *Nos* activity was not found at all, suggesting that the MiniSa carrying *Nos* activity probably integrated in the genome of a part of the plants transformed by pRiA4b only.

DISCUSSION

The Sa plasmid is known to carry sequences which suppress the virulence of *A. tumefaciens* and *A. rhizogenes* (FARRAND *et al.* 1981, VLASÁK and ONDŘEJ 1985), but most of the sequences which suppress virulence are deleted in MiniSa plasmid (TAIT *et al.* 1982, VLASÁK and ONDŘEJ 1985).

The *Nos* activity was found after tumor production by the *Agrobacterium* binary vector containing the Ri plasmid, as the first component, and the right part of the nopaline Ti T-DNA integrated in MiniSa as the second component. It confirms the demonstration that the right 25 bp terminal T-DNA sequence (Hind III 23 fragment of pTiC58) without the left terminal sequence and one region is sufficient for independent integration into the plant genome, when the various virulence functions are provided in trans (AN *et al.* 1985). Functions necessary for integration were provided by the virulence region of pRIA4b. Cells transformed by T-DNA of the Ri plasmid are selected by tumor and/or root proliferation. Here we showed that a considerable proportion of hairy root tumors also possess *Nos* activity. The question whether these tumors consist of double transformed cells or chimeric tissues is being studied in regenerating flowering *Petunia* plants, which synthesize both agropine and nopaline, by outcrossing.

Presumably, any *A. rhizogenes* Ri plasmid can be used in this binary vector system. The main advantage is the high ability of tissues transformed by *A. rhizogenes* to regenerate plants (ACKERMANN *et al.* 1977, TEPFER 1983). Plants derived from the tissues transformed by *A. rhizogenes* show complex phenotypic changes which could possibly be useful in crop plant improvement.

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Figs. 2—4 are at the end of the end of the issue.

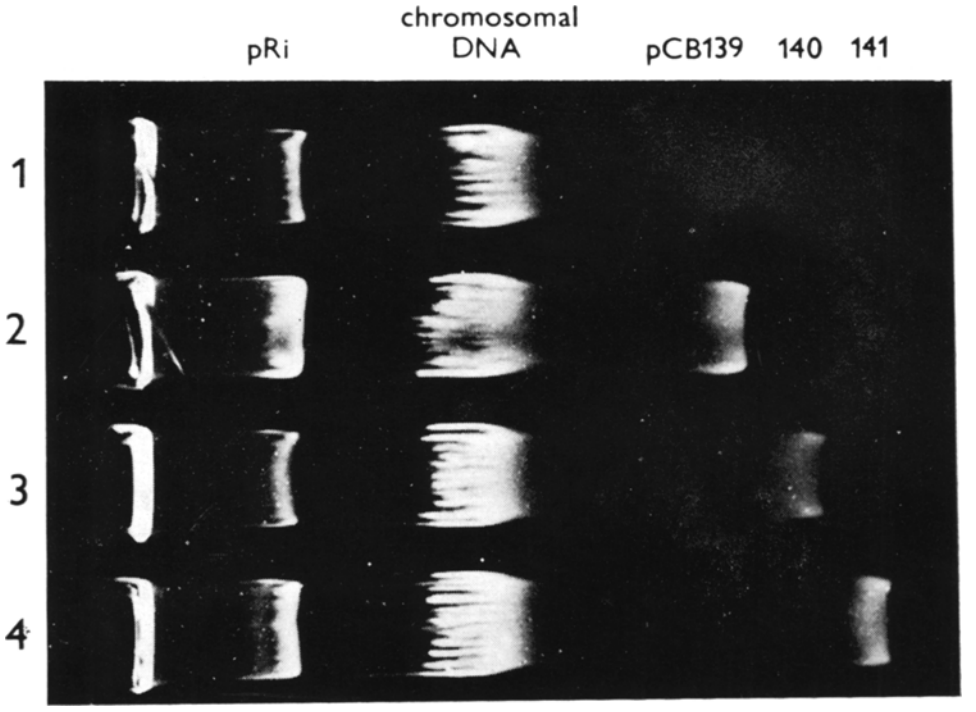

Fig. 2. Agarose gel electrophoresis of plasmids of *A. tumefaciens* strains containing the T-DNA fragment. — Lane 1: C58Cl Rif^rSpc^r (pRiA4b). Lane 2: The same with pCB139. Lane 3: the same with pCB140. Lane 4: the same with pCB141.

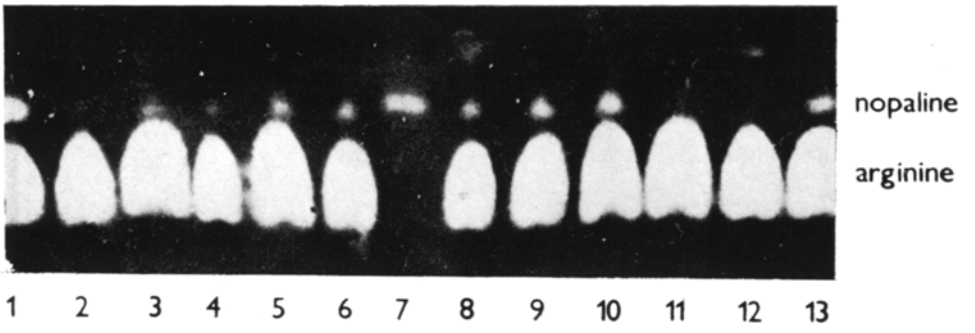

Fig. 3. Nopaline synthase activity in different *Kalanchoe* tumors induced by the following strains of *A. tumefaciens*.

Samples 1, 13 : *A. tumefaciens* C58
Samples 2, 12 : *A. tumefaciens* C58Cl Rif^rSpc^r(pRiA4b)
Samples 3, 6, 8, 11 : *A. tumefaciens* C58Cl Rif^rSpc^r(pRiA4b) (pCB139)
Samples 4, 10 : *A. tumefaciens* C58Cl Rif^rSpc^r(pRiA4b) (pCB140)
Samples 5, 9 : *A. tumefaciens* C58Cl Rif^rSpc^r(pRiA4b) (pCB141)

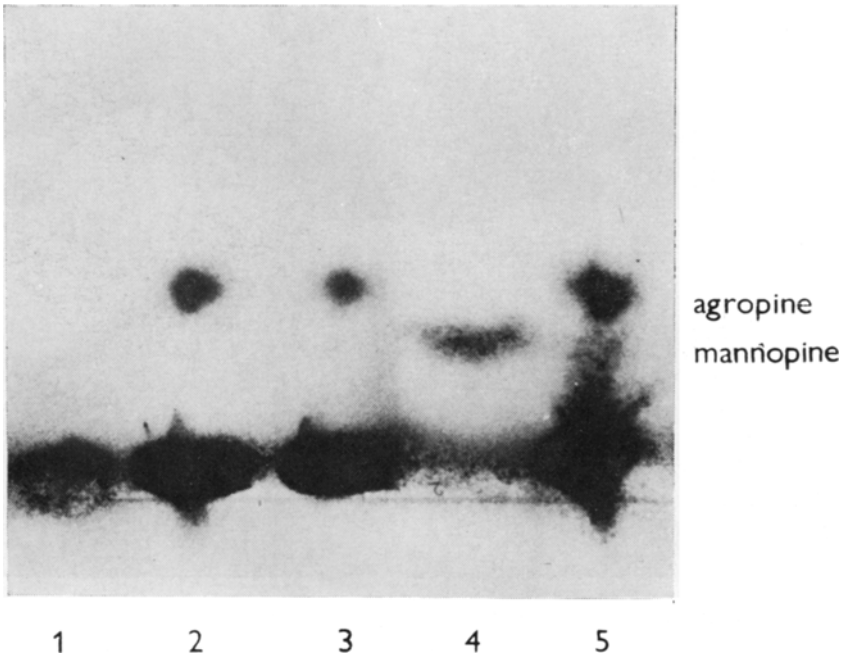

Fig. 4. The detection of agropine and mannopine, by paper electrophoresis and $AgNO_3$ staining, in *Kalanchoe daigremontiana* tumor extracts.
1 — tumor induced by *A. tumefaciens* C58
2 — tumor induced by *A. tumefaciens* C58Cl (pRiA4b)
3 — tumor induced by *A. tumefaciens* C58Cl (pRiA4b) (pCB139)
4 — mannopine standard
5 — *Petunia* roots, transformed by *A. rhizogenes* 15834, which are used as biological standard because of their rich opine content.