Expression of a rhizobium phaseoli sym plasmid in R. trifolii and agrobacterium tumefaciens: incompatibility with a R. trifolii sym plasmid

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Expression of a *Rhizobium phaseoli* Sym Plasmid in *R. trifolii* and *Agrobacterium tumefaciens*: Incompatibility with a *R. trifolii* Sym Plasmid

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Identification of the Sym plasmid in *Rhizobium phaseoli* strain RCC3622 is described. Introduction of this plasmid into *R. trifolii* or *Agrobacterium tumefaciens* strains resulted in bacteria capable of forming characteristic spherical root nodules on beans. This Sym plasmid, designated pSym9, was characterized as 275 MDa and nonconjugative. pSym9 was incompatible with the *R. trifolii* Sym plasmid pSym5, and carries genes determining a melanin-like black pigment. A second plasmid of 135 MDa, pRph3622a, was also transferred from *R. phaseoli* to *R. trifolii* and *A. tumefaciens*. Transconjugants carrying this plasmid did not form root nodules on beans. In contrast to other *Rhizobium* plasmids, pRph3622a was unstable in *A. tumefaciens*.

Bacteria of the genus *Rhizobium* are characterized by their ability to induce nodules on the roots of leguminous plants. In these root nodules, nitrogen is fixed by enzymes encoded by the bacterial genome. *Rhizobium phaseoli* forms N-fixing root nodules on beans, which are spherical, in contrast to the cylindrical nodules formed by *R. trifolii*, *R. leguminosarum*, and *R. meliloti* on clovers, peas, and alfalfa, respectively.

It is now well established that genes involved in the induction of root nodules (*nod* genes), in addition to genes involved in nitrogen fixation (*nif* and *fix* genes) are located on large bacterial plasmids designated Sym plasmids (Hombrecher *et al.*, 1981; Hooykaas *et al.*, 1981). So far Sym plasmids have been identified in only two strains of *R. phaseoli* (Beynon *et al.*, 1980; Lamb *et al.*, 1982). The two Sym plasmids have the same size (190 MDa) and are incompatible with *R. leguminosarum* plasmid pRL7. In this paper we describe the identification of a 275-MDa Sym plasmid in *R. phaseoli* strain RCC3622, and the transfer and expression of this plasmid (pSym9) in *R. trifolii* and in the tumor-inducing bacterium *Agrobacterium tumefaciens*. Furthermore, we present evidence that pSym9 is incompatible with a *R. trifolii* Sym plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids are listed in Table 1. The media used are described in Hooykaas *et al.* (1982b).

Bacterial crosses and characterization of transconjugants. Crosses were performed on TY medium as described in Hooykaas *et al.* (1980). Transconjugants were purified three times by single colony isolation on selective medium. Thereafter, they were checked by phage immunity typing. The bacteriophages used, LPB1, LPB51, LPB52, and LPB84 have been previously described (Hooykaas *et al.*, 1982b).

Plasmid identification. This was according to Casse *et al.* (1979). The molecular weight of plasmids was estimated from their relative mobility in agarose gels.

Total DNA isolation, restriction-endonuclease digestion, agarose-gel electrophoresis, Southern blotting, fragment labeling, DNA–DNA filter hybridization, and autoradiography. These methods were as described by Prakash *et al.* (1981).

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TABLE 1

BACTERIAL STRAINS AND PLASMIDS

A. *Rhizobium* strains.

LPR no.	Nodulation	Hosts	Origin or description
5	+	Clover	RCC5 (wild-type)
9	+	Bean	RCC3622 (wild-type)
1107	+	Pea, vetch	JB897 (J. Beringer, U. K.)
1122	+	Pea, vetch	LPR1107 Rif^r
5001	+	Clover	LPR5 Rif^r
5045	−		LPR5001 cured of pSym5
5071	+	Bean	LPR9009 × LPR5001
5073	+	Bean	LPR9009 × LPR5045
9005	+	Bean	LPR9 Rif^r
9006	+	Bean	LPR9 (pRL180)
9008	+	Bean	LPR9006 with Tn*5* in pRph3622a
9009	+	Bean	LPR9006 with Tn*5* in pRph3622b (=pSym9)

B. Agrobacterium strains

LBA no.	Tumor induction	Origin or description
202	−	LBA201 cured of pTiC58
288	−	LBA202 Rif^r Nal^r
1010	+	LBA288 (pTiB6)
2708	−	LBA288 (pRL180) (pRph3622a::Tn*5*)
2709	−	LBA288 (pRL180) (pSym9::Tn*5*)
2718	+	LBA1010 (pRL180) (pSym9::Tn*5*)

C. Plasmids	Relevant properties	Reference or description
pJB4JI	GmKmSmSpIncP	(Beringer *et al.*, 1978)
pRL180	CbSmTcIncP	(Hooykaas *et al.*, 1982a)
pSA30	TcNifKDH	(Cannon *et al.*, 1979)
pSym5 (=pRtr5a)	Nod Fix Hsp(clover) (230 MDa)	(Hooykaas *et al.*, 1981)
pSym9 (=pRph3622b)	Nod Fix Hsp(bean) Mel (275 MDa)	(This paper)
pTiB6	Agc Occ Onc Vir Ape Inc Rh-1 (120 MDa)	Octopine Ti plasmid

Note. Agc = agropine catabolism; Ape = phage AP1 exclusion; Cb = carbenicillin resistance; Fix = nitrogen fixation; Gm = gentamycin resistance; Hsp = host specificity; Inc = incompatibility group; Km = kanamycin resistance; Mel = melanin (black pigment) production; Nif KDH = structural genes for nitrogenase; Nod = nodulation; Occ = octopine catabolism; Onc = oncogenicity; Sm = streptomycin resistance; Sp = spectinomycin resistance; Tc = tetracyclin resistance; Vir = virulence.

Tumor induction tests were carried out on tomato and sunflower as described previously (Hooykaas *et al.*, 1977).

Nodulation tests. Tests on small plants (clovers, vetches) were as described by Vincent (1970) and Van Brussel *et al.* (1982). Plants were grown in glass test tubes on agar slopes. Beans were grown in 250-ml Erlenmeyer flasks on agar medium as described by Djordjevic *et al.* (1982) for peas. Nitrogen fixation by the nodules was checked 20 days after inoculation with bacteria using acetylene-reduction tests as described by Planqué and Van Brussel (1976).

RESULTS

Introduction of Transposon Tn5 in Plasmids of R. phaseoli RCC3622

In our experiments, wild-type strain RCC3622 (our collection number LPR9) was used as the parental strain. Like other *R. phaseoli* strains, RCC3622 produced a black pigment on media containing tyrosine. In

order to label the plasmids in *R. phaseoli*, the Tn*5* (Km^r)-carrying[1] suicide plasmid pJB4J1 (Beringer *et al.*, 1978) was introduced into RCC3622. A number of Km^r *R. phaseoli* transconjugants was then tested for their ability to transfer the Km^r marker to *R. trifolii* strain LPR5045, which has been cured of the Sym plasmid. No transfer of Km^r was observed, suggesting that the plasmids in RCC3622 were not self-conjugative. Such a situation has previously been observed in the case of a *R. leguminosarum* Sym plasmid (Hooykaas *et al.*, 1982b). Therefore, we repeated the Tn*5* mutagenesis experiment, using a derivative of RCC3622 carrying the IncP plasmid, pRL180 (Hooykaas *et al.*, 1982a). If any plasmids of RCC3622 (pRL180) received a copy of Tn*5*, subsequent transfer to LPR5045 would be possible from such strains via mobilization by pRL180. Two of five Km^r derivatives of RCC3622 (pRL180), namely LPR9008 and LPB9009, appeared to be able to transfer the Km^r marker to LPR5045 at a frequency of 10^{-7}/ recipient. From these same two strains, transfer of Km^r was observed to *A. tumefaciens* strain LBA288. Plasmid DNA was isolated from the parental *R. phaseoli* strain and from purified *A. tumefaciens* transconjugants and visualized on agarose gels (data not shown). In RCC3622, three plasmids were found of molecular mass 135, 275, and greater than 300 MDa. In *A. tumefaciens* transconjugants from the cross between LBA288 and LPR9008 (such as LBA2708), in addition to pRL180 and the 300-MDa cryptic plasmid pAtC58, the 135-MDa *R. phaseoli* plasmid, pRph3622a, was present.

The *A. tumefaciens* transconjugants from the cross between LPR9009 and LBA288 (such as LBA2709) carried in addition to pRL180 and pAtC58, the 275-MDa *R. phaseoli* plasmid, pRph3622b. These results indicate that in LPR9008, the smallest plasmid pRph3622a carries Tn*5*, but that in LPR9009, Tn*5* is located on pRph3622b.

[1] Abbreviations used: Fix, nitrogen, fixation; Km, kanamycin; Mel, melanin-like pigment; Nod, nodulation; Rif, rifampicin.

Plasmids pRph3622a and pRph3622b were stable in a *R. trifolii* and a *R. phaseoli* background. pRph3622b was also stably maintained in *A. tumefaciens* LBA288, but pRph3622a was very unstable in this background. After growth for 18 generations on nonselective rich medium, the plasmid was lost from 70% of the cells.

Symbiotic Properties of Parental Strains and Transconjugants

Strains RCC3622, RCC3622 (pRL180), LPR9008, and LPR9009 were capable of inducing N-fixing, spherical root nodules on beans. Apparently, neither the introduction of pRL180, nor that of Tn*5* altered the Nod$^+$Fix$^+$ phenotype of the wild type. Purified derivatives of *R. trifolii* LPR5045 and *A. tumefaciens* LBA288 carrying pRph3622a::Tn*5* or pRph3622b::Tn*5* were checked for markers (Tables 2, 3) and then tested for nodulation of beans. Strains LPR5045, LBA288 and derivatives with

TABLE 2

COMPARISON OF THE PROPERTIES OF THE *R. phaseoli* DONOR STRAIN WITH THOSE OF *R. trifolii* STRAINS WITH AND WITHOUT THE *R. phaseoli* Sym PLASMID pSym9.

	LPR[a] 5045	LPR 5073	LPR 9009	LPR 5001	LPR 5071
Resistance to:					
Kanamycin	−	+	+	−	+
Rifampicin	+	+	−	+	+
Tetracyclin	−	+	+	−	+
Sensitivity to phage:					
LPB1	+	+	−	+	+
LPB51, LPB52	+	+	±	+	+
Colony type	Clear	Clear	Turbid	Clear	Clear
Nodulation of clover	−	−	−	+	−
Nodulation of bean	−	+	+	−	+
Presence of:					
pSym5	−	−	−	+	−[b]
pSym9	−	+	+	−	+

[a] LPR9009 *R. phaseoli* (donor); LPR5045, LPR5001 (*R. trifolii* recipients); LPR5073 (from the cross between LPR9009 and LPR5045); LPR5071 (from the cross between LPR9009 and LPR5001).

[b] pSym5 was lost due to incompatibility with pSym9.

TABLE 3

COMPARISON OF THE PROPERTIES OF THE *R. phaseoli* DONOR STRAIN WITH THOSE OF *A. tumefaciens* STRAINS CARRYING AND LACKING THE *R. phaseoli* Sym PLASMID pSym9

	LBA[a] 288	LBA 2709	LPR 9009	LBA 1010	LBA 2718
Resistance to:					
Kanamycin	−	+	+	−	+
Rifampicin	+	+	−	+	+
Tetracyclin	−	+	+	−	+
Sensitivity to phage:					
LPB51, LPB52	−	−	±	−	−
LPB84	+	+	−	+	+
Growth on:					
TY	+	+	+	+	+
TY − Ca[b]	+	+	−	+	+
Nodulation of bean	−	+	+	−	+
Tumor induction on tomato	−	−	−	+	+
Presence of:					
pSym9	−	+	+	−	+
pTiB6	−	−	−	+	+

[a] LPR9009 (*R. phaseoli* donor); LBA288, LBA1010 (*A. tumefaciens* recipients); LBA2709 (from LPR9009 × LBA288); LBA2718 (from LPR9009 × LBA1010).

[b] TY-Ca, TY medium from which calcium chloride was omitted.

pRph3622a::Tn*5* were Nod$^-$ on beans, but derivatives of LPR5045 and of LBA288 with pRph3622b::Tn*5* produced a number of characteristic spherical nodules on beans. The number of nodules induced per plant by these strains was variable. On some plants only a few nodules were present, but on others as many nodules were found as after inoculation with RCC3622. Whereas nitrogen fixation occurred in the nodules induced by *R. phaseoli* strains, no nitrogen was fixed in the nodules induced by the *A. tumefaciens* transconjugants with pRph3622b::Tn*5*.

Reisolation experiments showed that in all cases the inoculated strains were responsible for the Nod$^+$ phenotype. Moreover, the reisolated strains were found to be capable of nodulating beans like the parental strains. These experiments, therefore, unequivocally show that the 275-MDa pRph3622b plasmid is the Sym plasmid of strain RCC3622 (our collection number LPR9). Therefore, we propose to call this plasmid pSymRCC3622 or pSym(LPR)9. *R. phaseoli* strains RCC3622, RCC3622 (pRL180), and LPR9008 produce a black pigment after growth on rich medium plates. However, we found that LPR9009 no longer produces this melanin-like pigment (Mel$^-$). Therefore, insertion of Tn*5* in pSym9 probably led to the inactivation of a gene involved in the production of this pigment. In order to verify this, LPR9009 was crossed with a black-pigment producing Rif^r *R. phaseoli* strain (LPR9005). The Km^{r}Rif^r transconjugants obtained were no longer capable of producing the melanin-like pigment, confirming that a pSym9 gene determining this property had been inactivated by the insertion of Tn*5*. Black-pigment production is therefore a marker for pSym9 as for the two *R. phaseoli* Sym plasmids described by others (Beynon *et al.*, 1980; Lamb *et al.*, 1982).

Incompatibility Properties of pSym9

When LPR9009 was used as a donor in a cross with the Sym plasmid-containing *R. trifolii* strain LPR5001, transfer of Km^r occurred at a frequency of $1–5 \times 10^{-8}$ recipient, lower than to the homologous *R. trifolii* strain lacking the Sym plasmid. Derivatives of LPR5001 with pSym9::Tn*5* were purified and then tested for nodulation of clovers and beans. Although the strains were nodulation proficient on bean plants, unexpectedly they had become Nod$^-$ on clover. The simplest explanation for this result would be that the *R. trifolii* transconjugants had lost the 230-MDa *R. trifolii* Sym plasmid pSym5 after introduction of the *R. phaseoli* plasmid pSym9. In the plasmid DNA preparations of the transconjugants however, a plasmid of 230 MDa was present (data not shown). As LPR5001 carries a cryptic plasmid of the same size as its Sym plasmid (our unpublished results), this did not exclude that pSym5 was lost from the transconjugants. To resolve this ambiguity, total DNA was isolated from *R. phaseoli* LPR9009, from *R. trifolii* LPR5001, and from LPR5001 derivatives with pSym9:: Tn*5*. The total DNAs was digested with *Eco*RI, blotted onto nitrocellulose, and hybridized with plasmid pSA30, which carries the *nif* KDH genes from *Klebsiella pneumo-*

niae. As the *K. pneumoniae nif* DH genes are homologous to the corresponding *nif* genes in the Sym plasmids of *Rhizobium* species, a characteristic set of bands appears on an autoradiogram after hybridization of *Eco*RI-digested Sym plasmid DNA or total *Rhizobium* DNA with a ^{32}P-labeled pSA30 probe (Nuti *et al.*, 1979). We found three bands of 2.8, 2.6, and 2.5 MDa in the lane containing *R. phaseoli* LPR9009 DNA, and one band of 3.4 MDa in the lane with *R. trifolii* LPR5001 DNA. As can be seen in Fig. 1, in the lanes corresponding to the DNA preparations from a LPR5001 (pSym9::Tn*5*) transconjugant, bands of 2.8, 2.6, and 2.5 MDa were found, but not a band of 3.4 MDa. This shows that the structural *nif* genes of *R. phaseoli* LPR9009 are located on pSym9. Moreover, from the Nod$^-$ character of the LPR5001 (pSym9::Tn*5*) transconjugants on clovers, together with the absence of the 3.4 MDa. *nif* DNA band and the absence of plasmids smaller than 230 MDa. in these strains (data not shown), it can be concluded that plasmid pSym5 was lost from these *R. trifolii* strains after the introduction of pSym9. This indicates that the *R. trifolii* plasmid pSym5 is incompatible with the *R. phaseoli* plasmid pSym9.

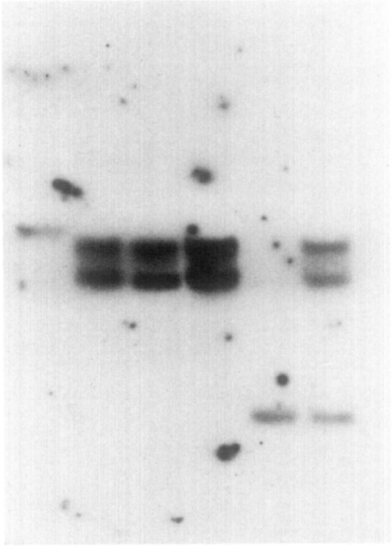

FIG. 1. Autoradiogram of ^{32}P-labeled pSA30 DNA hybridized to *Eco*RI digests of total DNA isolated from left to right: LPR5001, LPR5071, LPR5073, LPR9009, LPR1122, and LPR1122 (pSym9::Tn*5*).

Plasmids pSym5 and pSym9 appeared to be compatible with the IncRh-1 plasmid pTiB6 (Table 3). Strains carrying a Ti plasmid together with pSym5 or pSym9 were capable of inducing tumors (on sunflower and tomato) and root nodules (on their proper host).

DISCUSSION

The results obtained corroborate previous findings that a single Sym plasmid in *R. phaseoli* carries genes involved in nodulation and nitrogen fixation (Lamb *et al.*, 1982). Our data show that the *R. phaseoli* Sym plasmid is not only expressed in a *Rhizobium* background, but also in an *Agrobacterium* background, resulting in *Agrobacterium* strains capable of inducing root nodules on bean plants. Expression of Sym plasmids in an *Agrobacterium* background has already been observed for Sym plasmids from *R. trifolii* (Hooykaas *et al.*, 1981), *R. leguminosarum* (Hooykaas *et al.*, 1982b; Van Brussel *et al.*, 1982) and *R. meliloti* (Kondorosi *et al.*, 1982).

The *R. phaseoli* plasmid pSym9 identified in our work is clearly different from the previously characterized *R. phaseoli* Sym plasmid pRP2JI. pRP2JI is a conjugative plasmid of 190 MDa, whereas pSym9 is a nonconjugative plasmid of 275 MDa. Other results described in this paper show that the *R. phaseoli* plasmid pSym9 belongs to the same incompatibility group as the *R. trifolii* plasmid pSym5 described previously (Hooykaas *et al.*, 1981). At the Nitrogen Fixation Conference in Noordwijkerhout, another case of incompatibility between a *R. trifolii* Sym plasmid and *R. phaseoli* Sym plasmid was reported (O'Connell *et al.*, 1984). These data suggest that symbiotic genes have become linked to different replicons during evolution. It can therefore be speculated that the plasmid-borne symbiotic genes have spread to different types of plasmids via legitimate or illegitimate recombination. It is worth noting that a gene for black-pigment production is present on all the *R. phaseoli* Sym plasmids

so far identified. It is likely that this gene for black-pigment production is closely linked to the symbiotic genes and has spread to different plasmids together with the *R. phaseoli* symbiotic genes. A close linkage of the genes for melanin production and nodulation has indeed been observed for pRP2JI (Lamb *et al.*, 1982). Such a close linkage of these genes could also explain why black-pigment production is characteristic for *R. phaseoli* strains and has not been observed for *R. trifolii* and *R. leguminosarum* strains.

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