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Crown Gall Tumor and Root Nodule Formation by the Bacterium *Phyllobacterium myrsinacearum* after the Introduction of an *Agrobacterium* Ti Plasmid or a *Rhizobium* Sym Plasmid

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Phyllobacterium rubiacearum **and** ***P. myrsinacearum*** **are symbiotic bacteria that can be isolated from the leaf nodules of members of the plant families** ***Rubiaceae*** **and** ***Myrsinaceae*****, respectively. They are classified as belonging to the bacterial family** ***Rhizobiaceae*****. We found that** ***P. myrsinacearum*** **becomes able to induce crown gall tumors upon receipt of a Ti plasmid from** ***Agrobacterium tumefaciens*****. Moreover, the introduction of the Sym plasmid pRL1JI from** ***Rhizobium leguminosarum*** **conferred on this bacterium the ability to nodulate the legume** ***Vicia sativa*****. These results show that the host range of Ti and Sym plasmids includes the genus** ***Phyllobacterium*****. They also indicate that chromosomal genes involved in tumorigenesis and nodule formation are functionally present in** ***Phyllobacterium*****. Our data confirm the classification of** ***Phyllobacterium*** **in the** ***Rhizobiaceae*** **family.**

Members of the bacterial family of *Rhizobiaceae* interact with plants in various ways. *Agrobacterium tumefaciens* strains are pathogenic and cause tumorous outgrowths, crown galls, on a wide range of dicotyledonous plant species, whereas *Agrobacterium rhizogenes* strains evoke the hairy root disease that is characterized by abundant root proliferation from the site of infection (Melchers and Hooykaas 1987). Bacteria of the genus *Rhizobium* can enter into a symbiosis with leguminous plants resulting in the formation of nitrogen-fixing root nodules (Verma and Long 1983; Rossen *et al.* 1987).

Besides these genera, the *Rhizobiaceae* family includes a third genus, called *Phyllobacterium* on the basis of rRNA cistron conservation (De Smedt and De Ley 1977). Phyllobacteria are thought to be responsible for the formation of leaf nodules on many species of the plant families *Rubiaceae* and *Myrsinaceae*. Moreover, the bacteria are essential for a proper development of the plants with which they live in obligate symbiosis. Plants cured of the bacteria by heat treatment are "cripples," which grow slowly, produce abnormal leaves, and die eventually. Under normal situations, cripples occur rarely because the bacteria are transmitted through the seed (Lersten and Horner 1976). To date, the precise role of the bacteria in this symbiosis remains unclear.

In *Agrobacterium* and *Rhizobium*, the bacterial genes involved in tumor formation and nodulation (respectively) have been localized on large plasmids called tumor-inducing (Ti) and symbiosis (Sym) plasmids. In addition, chromosomal genes play a role in the early steps of the processes, such as attachment of the bacterium to the plant cell walls. (Douglas *et al.* 1982; Thomashow *et al.* 1987). Some of these genes (*exo*, *chv* genes) are present in *Agrobacterium* as well as in *Rhizobium* and are essential both for tumor induction and nodulation (Cangelosi *et al.* 1987; Dylan *et al.* 1986; van Veen *et al.* 1987).

In fact, all chromosomal genes necessary for tumor induction are present in *R. leguminosarum* (the symbionts of clover, pea, and bean) as indicated by the fact that the introduction of Ti or Ri plasmids into these bacteria renders them tumorigenic (Hooykaas *et al.* 1977). In contrast, other rhizobia such as *R. meliloti* (the symbiont of alfalfa plants) do not become tumorigenic upon receipt of a Ti or Ri plasmid (Hooykaas and Schilperoort 1984). Reversely, it has been shown that the introduction of Sym plasmids into *Agrobacterium* leads to nodulating *Agrobacterium* strains (Hooykaas *et al.* 1981, 1982; Hirsch *et al.* 1984). This indicates that chromosomal nodulation genes are present not only in *Rhizobium* but in *Agrobacterium* as well. We have extended these analyses to *Phyllobacterium* and report here on the introduction of a Ti plasmid of *A. tumefaciens* and a Sym plasmid of *R. leguminosarum* into two different *Phyllobacterium* strains and on the expression of Ti and Sym plasmid genes in these chromosomal backgrounds.

MATERIALS AND METHODS

A. tumefaciens strain LBA691 (C58 cured, *str*-2, *nal*-3) carries plasmid pAL688, which is an R702::pTiB6 cointegrate plasmid (Sp^r; Klapwijk, unpublished). Strain LBA2315 (C58 cured, *ery*-4, *cml*-1) harbors pGV3133, which is a pTiC58 (Tra^c) derivative labeled with Tn*7* (Sp^r; Holsters *et al.* 1980). *R. leguminosarum* RBL5523 (*rif*, *str*) contains a derivative of the *R. leguminosarum* Sym plasmid pRL1JI labeled with Tn*1831* (Sp^r, Sm^r, Hg^r; Priem and Wijffelman 1984; Hooykaas *et al.* 1980). *P. rubiacearum* LMG1 t1, originally isolated from *Pavetta zimmermanniana*, and *P. myrsinacearum* LMG2t2, isolated from *Ardisia crispa*, were kind gifts of J. De Ley. Spontaneous rifampicin-resistant derivatives LAZ17 and LAZ19 were isolated from TYG plates containing 20 mg/L of rifampicin. Agrobacteria were grown in LC medium (Hooykaas *et al.* 1977), rhizobia in TY medium (Beringer and Beynon 1978), and phyllobacteria in TYG medium that consists of 5 g/L of

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tryptone, 3 g/L of yeast extract, 2 g/L of glucose, 5 mg/L of $FeSO_4 \cdot 7H_2O$ and 1 mg/L of trace elements $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, H_3BO_3, and $NaMoO_4$. Media were solidified with 18 g/L of Difco Bacto agar.

Bacteria were conjugated on TYG plates as described previously (Hooykaas *et al.* 1977); 0.09% $CaCl_2$ was added in conjugations with *Rhizobium* and *Phyllobacterium*. Transconjugants were selected on TYG medium containing spectinomycin (250 mg/L) and rifampicin (20 mg/L). Putative transconjugants were checked for immunity against a set of *Agrobacterium*- and *Rhizobium*-specific phages according to Hooykaas *et al.* (1977), as well as for plasmid content following Kado and Liu (1981).

Tumor-induction tests were done on *Kalanchoe daigremontiana*, *Kalanchoe tubiflora*, *Lycopersicon esculentum*, and *Nicotiana glauca*, as described by Hooykaas *et al.* (1977). Opine synthase activity was measured according to Otten and Schilperoort (1978). Nodulation tests were done on *Vicia sativa* and *Vicia hirsuta* seedlings, as described by Van Brussel *et al.* (1982).

RESULTS

Two *Phyllobacterium* strains were used as recipients for the Ti plasmid of *A. tumefaciens*, viz. *P. rubiacearum* (LAZ17), which was isolated from *P. zimmermanniana*, a member of the plant family *Rubiaceae*, and *P. myrsinacearum* (LAZ19), which originated from *Ardisia crispa*, a member of the plant family *Myrsinaceae*. As donor Ti plasmid pAL688, a cointegrate plasmid of pTiB6 with the broad host range, conjugative plasmid R702 was chosen both to maximize transfer frequencies because of possible DNA restriction in *Phyllobacterium* as well as to ensure maintenance of the plasmid via the broad host range replicator. Putative transconjugants, which were not sensitive to *Agrobacterium*-specific phages, were purified and then analyzed for plasmid content. The *Phyllobacterium* recipient strains themselves were found to contain large plasmids of 280, 320, and more than 500 Md in the case of LAZ17 (not shown) and of 200, 280, 320, and more than 500 Md in the case of LAZ19 (Fig. 1, lane 7).

In the transconjugants, an additional band corresponding in size to that of pAL688 was present (Fig. 1, lane 6). Because the Ti plasmid genes involved in tumor formation were present in the *Phyllobacterium* transconjugant strains, we assayed for the expression of the Ti plasmid genes and for the presence of chromosomal virulence genes by infection experiments on *K. daigremontiana*, *K. tubiflora*, *L. esculentum*, and *N. glauca*. The *P. rubiacearum* strains harboring plasmid pAL688 were unable to induce tumors on any of the plants tested. *P. myrsinacearum* itself did not induce any response, but the transconjugant LAZ100 induced tumors containing octopine synthase activity. Tumors were equal in size to those induced by the *Agrobacterium* donor strain on *K. tubiflora* (Fig. 2) and *N. glauca*, but were smaller on *K. daigremontiana* and *L. esculentum* (not shown). These results show that Ti plasmids can indeed be expressed functionally in *P. myrsinacearum*, but that on some plant species, the efficiency of tumor formation is lower than after infection with the corresponding *Agrobacterium* donor.

To investigate the possibility that a Ti replicator is functional in *Phyllobacterium*, we tried to introduce pGV3133, a nopaline Ti plasmid derivative labeled with Tn*7* (Sp^r), which does not contain a broad host range origin of replication. Surprisingly, we found that this plasmid was stably maintained in *Phyllobacterium* (Fig. 1, lane 1).

The positive results concerning the expression and the maintenance of Ti plasmids in LAZ19 prompted us to examine whether Sym plasmids of *Rhizobium* could be expressed in this bacterium as well. We used as a donor an *R. leguminosarum* strain containing a Sym plasmid (pRL1JI) labeled with Tn*1831* ($Sp^rSm^rHg^r$). Putative *Phyllobacterium* transconjugants (immune to *Rhizobium* phages) were purified, and their plasmid profiles showed that they had acquired a plasmid of the size of the Sym plasmid (Fig. 1, lane 3). Thus, not only the Ti plasmid but also this Sym plasmid was able to replicate in *Phyllobacterium*. In addition, we assayed for the expression of the Sym plasmid encoded nodulation genes and the presence of chromosomal nodulation genes by inoculation of *V. sativa* and *V. hirsuta* plants (which are the natural hosts of *R. leguminosarum*) with the transconjugant LAZ102. On *V. hirsuta* no nodules were formed, but the *V. sativa* plants were nodulated (Figs. 3 and 4). Cytological examination of the *Phyllobacterium*-induced nodules showed that these structures are true nodules (Fig. 4a). In Figure 4, b–d, details of electron micrographs of some infected cells are shown. In Figure 4b, a heavily infected cell is shown, whereas the cell depicted in Figure 4c contains a number of bacteria that can be found in *Rhizobium*-infected cells. The cell in Figure 4d contains limited numbers of bacteria. We have not detected y-shaped bacteroid structures in nodule cells infected with *P. myrsinacearum*. In addition, the *V. sativa* root system formed thick and short roots due to the expression of the Sym plasmid Tsr trait that is correlated with nodulation on this plant (Van Brussel *et al.* 1982). Bacteria were reisolated from the nodules and turned

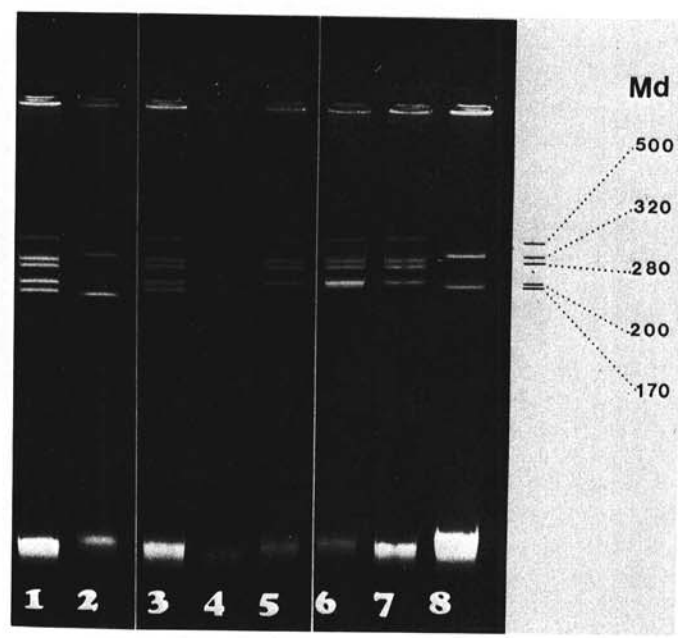

Fig. 1. Total plasmid preparations were separated on 0.8% agarose gels. Lane 1, LAZ19 containing pGV3133; lane 2, LBA2315 (pGV3133); lane 3, LAZ19 containing pRL1JI::Tn*1831*; lane 4, RBLS523 (pRL1JI::Tn*1831*); lane 5, LAZ19; lane 6, LAZ19 containing pAL688; lane 7, LAZ19; lane 8, LBA691, which contains pAL688.

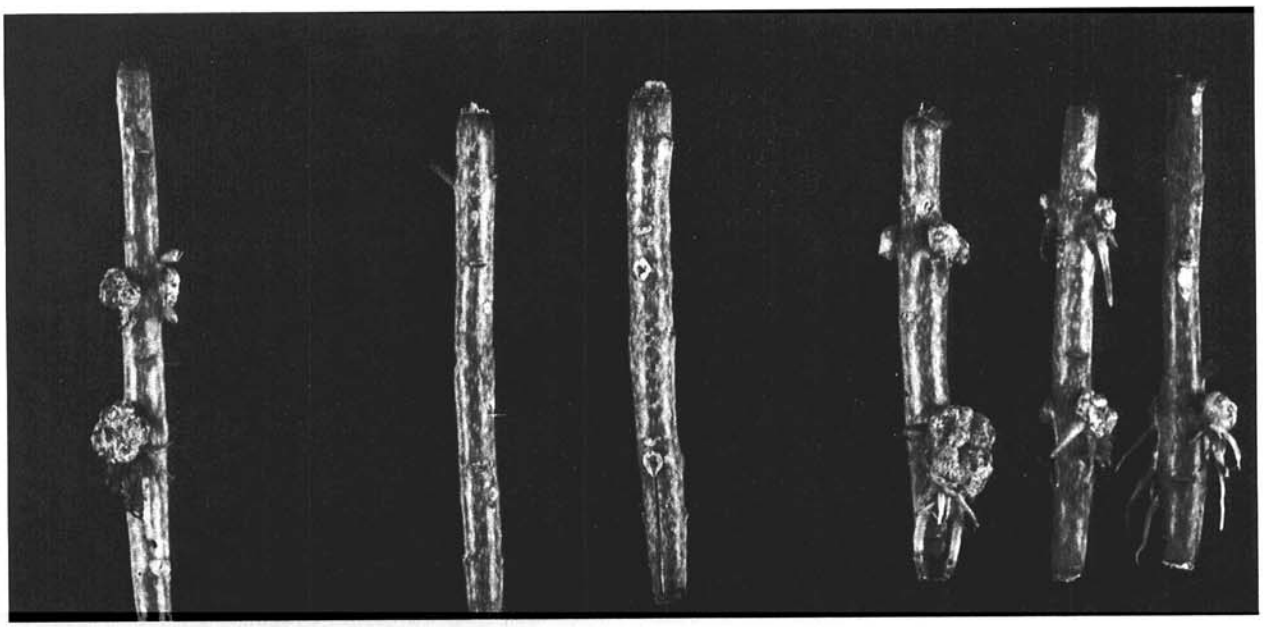

Fig. 2. Tumor induction on *Kalanchoe tubiflora* stems inoculated with strain LBA691 (left), the *Agrobacterium* donor strain harboring the R702::Ti cointegrate plasmid pAL688; strain LAZ19 (center in duplo), the *Phyllobacterium myrsinacearum* recipient, and (on the right in triplo) the transconjugant *Phyllobacterium* strain LAZ100 containing pAL688. Pictures were taken 4 wk after infection.

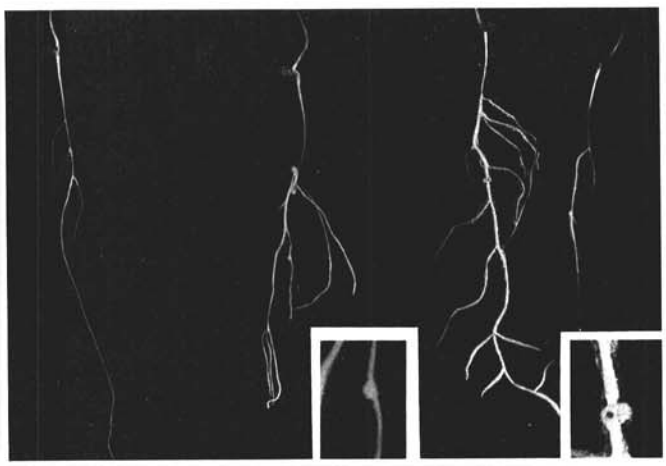

Fig. 3. Root nodule induction on *Vicia sativa*. On the left, seedlings were inoculated with *Phyllobacterium myrsinacearum* (LAZ19); in the center with RBL5523, and at the right with the LAZ19 transconjugant harboring the pRL1JI Sym plasmid (LAZ102 in duplo). Plants were photographed after 3 wk of incubation.

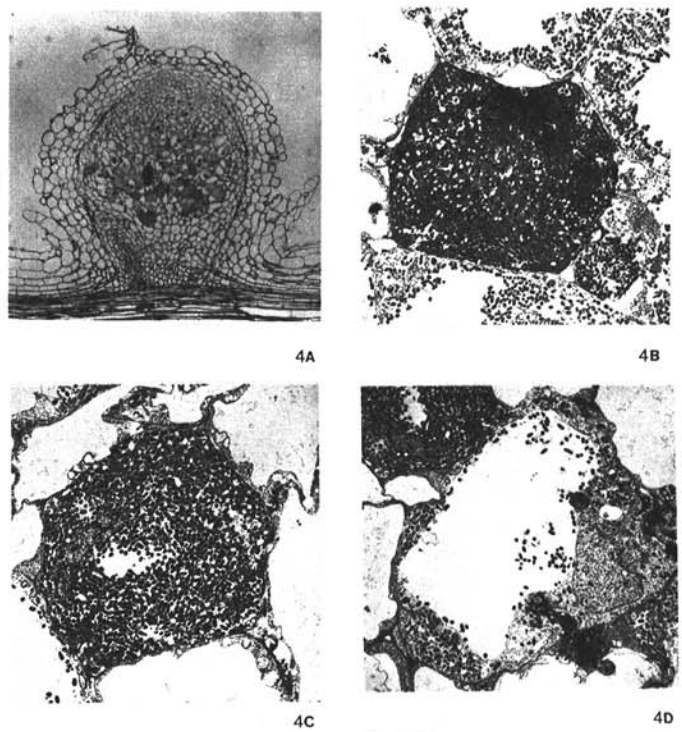

Fig. 4. Light micrograph (**A**) of a nodule induced by *P. myrsinacearum* containing the Sym plasmid pRL1JI on *Vicia sativa* and electron micrographs of different types of infected cells in this nodule tissue (**B–D**).

out to have properties identical to LAZ102, which was used for nodulation; no contaminating rhizobia were found in the nodules. The nodulation genes of the *Rhizobium* Sym plasmid are expressed in *P. myrsinacearum*, and our results thus indicate that essential chromosomal genes involved in tumor formation and nodulation are functionally present in *P. myrsinacearum*.

DISCUSSION

Phyllobacteria have been isolated from leaf nodules of members of the plant families *Rubiaceae* and *Myrsinaceae*. On the basis of rRNA cistron conservation, these bacteria have been classified as members of the bacterial family of *Rhizobiaceae*. To investigate the relationship between *Phyllobacterium* and *Agrobacterium* and *Rhizobium*, we introduced a Ti plasmid of *A. tumefaciens* and a Sym plasmid of *R. leguminosarum* bv. *viceae* into two representative *Phyllobacterium* strains and assayed for the expression of the plasmids by infection tests. An indication of the close relationship between *Phyllobacterium* and other members of the family of *Rhizobiaceae* was the fact that the replication systems of both the pTi and the pSym plasmid were functional in *Phyllobacterium*, which is not the case in

more distant bacteria such as *E. coli* or *Pseudomonas* spp. Plasmids of *Agrobacterium* replicate in *Rhizobium* strains, and Sym plasmids in general replicate in agrobacteria. The Sym plasmid pRL1JI is an exception, however, that can survive in *Agrobacterium* only as a cointegrate plasmid with another replicon (Wijffelman *et al.* 1983). In *Phyllobacterium*, however, pRL1JI replicates stably by itself, although the possibility cannot be excluded that one of the endogenous plasmids provides transacting functions for pRL1JI replication. Whether any of the endogenous plasmids in *Phyllobacterium* plays a role in leaf nodule induction is unclear at the moment, but seems plausible in view of the situation in *Agrobacterium* and *Rhizobium*.

The expression of Ti and Sym plasmids in *Phyllobacterium* further extends previous reports on the behavior of these plasmids in heterologous chromosomal backgrounds. The strain isolated from *P. zimmermanniana* was unable to induce plant tumors after introduction of the Ti plasmid of *A. tumefaciens* and thus behaves similar to *R. meliloti* transconjugants. Because both the octopine-degrading enzymes and the virulence genes are expressed in *R. meliloti* and *Phyllobacterium*, the lack of tumor formation probably is due to the absence of certain chromosomal virulence genes. Interference of functions determined by the endogenous *R. meliloti* and *P. rubiacearum* plasmids with the tumor-induction process is another possible explanation for the lack of tumorigenesis by these strains.

The pTi and pSym harboring transconjugants of LAZ19 were able to induce tumors and nodules, respectively, indicating that the essential chromosomal genes required for tumorigenesis and root nodule formation are present in this bacterium. We do not know whether these genes are essential for leaf nodule formation by *Phyllobacterium*. Like *R. trifolii* strains harboring Ti plasmids, the *Phyllobacterium* LAZ19 (pTi) transconjugants induced attenuated tumors on some plants (Hooykaas and Schilperoort 1984). Furthermore, *Agrobacterium* strains harboring pRL1JI induce nodules on *V. sativa*, but are nodulation deficient on *V. hirsuta* plants (Van Brussel *et al.* 1982) as is the case with *P. myrsinacearum* containing this Sym plasmid. These results underscore the importance of the bacterial chromosome in the host range for tumor formation (Van Veen *et al.* 1988) and nodulation and indicate that, apart from the known chromosomal loci involved in plant bacteria interactions (*chv*, *exo*, *att*), additional genes are present in the chromosome of *Agrobacterium* to optimize tumor formation, whereas *Rhizobium* contains specific genes to optimize the nodulation process.

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