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Ti plasmid containing *Rhizobium meliloti* are non-tumorigenic on plants, despite proper virulence gene induction and T-strand formation

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Abstract. We examined the expression of the *vir* genes of the *Agrobacterium tumefaciens* Ti plasmid in *Rhizobium meliloti*, which remains non-tumorigenic on plants after introduction of a Ti- or Ri-plasmid. Both the levels of virulence (*vir*) gene expression, induced by the plant phenolic compound acetosyringone, and of subsequent T-strand formation were comparable to what is observed in *Agrobacterium*. In contrast to the situation in *Agrobacterium*, though, *vir* induction in *R. meliloti* did not require a low pH (5.3) of the induction medium and the optimum temperature for induction in *R. meliloti* was significantly lower than in *Agrobacterium*. At 37°C no induction of the *vir* genes was found both in *Agrobacterium* and *R. meliloti*. We postulate that the lack of tumorigenicity of Ti carrying *R. meliloti* strains is due either to a lack of proper attachment of the bacteria to plant cells, or to an improper assembly of a *vir*B-determined essential structure in the cell wall of *R. meliloti*.

Key words: *Agrobacterium tumefaciens* – *Rhizobium meliloti* – *Vir* gene expression – T-strands – Chromosomal virulence genes

Agrobacterium tumefaciens strains induce tumor formation on dicotyledonous plants by the introduction of DNA sequences (T-region) from their Ti (tumor inducing) plasmid, into the plant genome. The tumor formation process has been dissected into a number of separate events comprising i) recognition and attachment of *Agrobacterium* to plant cells present in wounded tissue, ii) induction of the virulence (*vir*) genes on the Ti plasmid by plant phenolics and subsequent processing in the bacterium of the T-region, iii) transfer of the resulting T-DNA intermediate molecules to the plant cell nucleus and iv) expression of the T-DNA *onc*-genes resulting in proliferation of the transformed tissue. The actual transfer step has not been elucidated but detailed information concerning steps i) ii) and iv) have been reported over the last years (for recent reviews see: Matthysse 1986; Melchers and Hooykaas 1987; Koukolikova et al. 1987; Memelink et al. 1987). Several genes (*chvA*, *chvB*, *exoC*, *att*), localized in the chromosome of *Agrobacterium* have been identified that are involved in the attachment of *Agrobacterium* to the plant cell (Douglas et al. 1982; Thomashow et al. 1987; Matthysse 1987). Concomitant with attachment to the plant cells, the virulence genes on the Ti plasmid are activated by plant phenolics which accumulate at the site of the wound (Stachel et al. 1985). The VirA protein is present in the inner membrane of *Agrobacterium* and acts as a sensor of the plant phenolics (Melchers et al. 1987; Leroux et al. 1987). Transduction of this signal leads to activation of the positive regulatory protein VirG (Winans et al. 1986; Melchers et al. 1986). The products of the other *vir* genes are thought to participate in processing and transfer of the T-region into the plant cells. VirD proteins nick the bottom strand of specific border sequences that delineate the T-region and ss T-DNA copies are generated (Stachel et al. 1986; Yanofski et al. 1986). The VirE2 protein has a ss-DNA binding activity which might protect or direct the T-strands (Gietl et al. 1987). The sequence of the *vir*B operon indicates that most of the VirB proteins are highly hydrofobic and a role as building blocks of a pilus-like structure has been suggested (Thompson et al. 1988; Ward et al. 1988). After transfer and incorporation into the plant genome, the expression of T-DNA *onc* genes encoding enzymes involved in auxin- and cytokinin synthesis leads to unlimited proliferation.

Despite the broad host range of *Agrobacterium* mediated tumor formation, some plant species can not easily be transformed by *A. tumefaciens* (see van Veen et al. 1988a, for a review). The Ti plasmid can be stably maintained in other members of the *Rhizobiaceae* such as *R. leguminosarum* biovars (Hooykaas et al. 1977), *R. meliloti* (Hooykaas and Schilperoort 1984) and *Phyllobacterium* (van Veen et al. 1988b). The efficiency for tumor formation by *R. leguminosarum* and *P. myrsinacearum* strains harboring Ti plasmids is somewhat less than by *Agrobacterium* but surprisingly *R. meliloti* derivatives are almost completely avirulent (Hooykaas and Schilperoort 1984; van Veen et al. 1988a). In this report, we have investigated *vir* gene induction and T-strand formation in a *R. meliloti* genetic background in order to find out at what point the tumor induction process might fail in this close relative of *Agrobacterium*.

Materials and methods

Bacterial strains, plasmids and microbiological techniques

Agrobacterium tumefaciens strain LBA1010 (*rif*) contains a wild type pTiB6 plasmid; LBA1826 contains pAL1890

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which is a derivative of an R772::pTiB6 cointegrate plasmid of which most of the Ti part is deleted with the exception of *vir*ABG and which contains a *vir*B2:*lac*Z translational fusion and transposon Tn1831 (Sp^r) as marker (Melchers, unpublished); LBA2516 contains an intact R772::pTiB6 plasmid with the same *vir*B2-*lac*Z fusion. *Rizobium meliloti* strain LPR2120 is a *rif* resistant derivative of wild type isolate RCC2001 (Rothamsted Culture Collection); LPR2122 is a derivative of LPR2120 containing plasmid pAL657 (Hooykaas et al. 1979) which is pTiB6::Tn1; LPR2160 is a derivative of LPR2120 containing plasmid pAL1890. Plasmids containing *lac*Z fusions were used to monitor *vir* gene induction whereas pTiB6 and pAL657 containing strains we used to detect T-strands. Plasmid pAL1890 was introduced into *R. meliloti* as described earlier (Hooykaas et al. 1977). Transconjugants were selected on Ty medium (Beringer and Beynon 1978) containing Spectinomycin (Sp 250 mg/l) and Rifampicin (Rif 20 mg/l) and checked for bacteriophage immunity patterns with phages LPB51, 64, 70 and S2, S3, S5, S6 (Hooykaas et al. 1977) and plasmid profiles as described by Kado and Liu (1981).

Induction of virulence genes

Bacteria from freshly prepared plates, were grown overnight in RMM medium pH 7.2 (Hooykaas et al. 1982). After centrifugation they were resuspended to an OD650 of 0.25 in RMM induction medium – pH 5.3 (unless stated otherwise); buffered with 62.5 mM phosphate; 3% sucrose (instead of 0.2% glucose). Acetosyringone (AS) was added to a final concentration of 100 μM. After 6 or 24 h of induction (at 29 °C unless stated otherwise) cells were collected (two 1 ml samples) and β-galactosidase activity was measured as described by Miller (1972).

Isolation and detection of T-DNA intermediates

Bacterial cultures in mineral medium (van Schie et al. 1984) supplemented with biotin, calciumpantothenate and thiamin (0.01 mg/l) were initiated from freshly prepared mineral medium plates. Overnight cultures were diluted 50-fold in mineral induction medium (pH 5.3, 3% sucrose) and AS (100 μM) was added after 6 h of incubation at 25 °C. After 15 h of induction, cells were collected, total DNA was isolated according to Ooms et al. (1981) and 2 μg was loaded onto 0.6% Tris Borate EDTA agarose gels. After electrophoresis (at 20 V, 16 h) the DNA was transferred to nitrocellulose membranes under non-denaturing conditions as described (van Haaren et al. 1987). Single stranded T-DNA intermediates encompassing the T_L-DNA were detected using an RsaI fragment comprising the T-cyt gene of pTiB6 as a probe, while T_C and T_R derived molecules were detected with the pTiB6 BamHI fragment 2. Note that BamHI 2 has approximately 280 bp homology with T_L-DNA as well.

Results

Induction of the virulence genes

Introduction of a Ti or Ri plasmid into *Rhizobium meliloti* does not render this bacterium tumorigenic on plants (Hooykaas and Schilperoort 1984). To investigate whether this was caused by the lack of proper *vir*-gene expression in this bacterial background, we introduced plasmid pAL1890 into *R. meliloti* (resulting in LPR2160) which carries a *vir*B2-*lac*Z fusion to monitor the induction of the *vir*B promoter by the *vir*A/*vir*G regulatory system in *R. meliloti*. *Agrobacterium* strain LBA2516, which carries the same *vir*B2-*lac*Z fusion was used as a positive control. Growth of agrobacteria in the induction medium (pH 5.3) is slow with a doubling time of about 6 h, *R. meliloti* strains did not grow at all in this medium even after prolonged incubation. Growth of the bacteria however is not required for induction of the virulence genes as shown by the results in Table 1. After 6 h of incubation with acetosyringone (AS), the *vir*B promoter was induced 38-fold in the *Agrobacterium* background (LBA2516). In *R. meliloti* LPR2160, a 16-fold induction was found.

Table 1. Virulence induction in *Agrobacterium tumefaciens* and *Rhizobium meliloti*

Bacterial strain[a]	AS	β-Galactosidase activity
LBA2516	–	70U
LBA2516	+	2700U
LPR2160	–	210U
LPR2160	+	3450U

The induction of the *vir* genes was measured in *R. meliloti* under the conditions optimized for *Agrobacterium* (i.e. pH 5.3 and 29 °C) (Alt-Moerbe et al. 1988; Melchers et al. 1989). Since both *vir*-specific features and chromosomal features could be responsible for the effects of pH and temperature, it was interesting to see whether the same or other conditions were optimal for *vir*-induction in *R. meliloti* as compared to *Agrobacterium*. It is known that *R. meliloti* can grow well at temperatures up to 42 °C, whereas *Agrobacterium* grows slowly at 37 °C and not at all at higher temperatures. We tested *vir* induction at different temperatures and the results are shown in Fig. 1. In our assay-conditions the optimum temperature for *Agrobacterium vir*-induction was around 29 °C, while the induction decreased more strongly at higher than at lower temperatures. No induction was found at 37 °C. While *R. meliloti* LPR2160 like *Agrobacterium* showed decreased *vir*-induction at 33 °C and no induction at all at 37 °C, unexpectedly and in contrast to what happens in *Agrobacterium*, *vir*-induction in *R. meliloti* was significantly better at 25 °C than at 29 °C and reached levels twice as high as *Agrobacterium* at 25 °C. These results indicate that the temperature sensitivity of *vir*-induction is at least partially chromosomally determined.

The effects of the pH of the induction medium on *vir*-induction in *Agrobacterium* and *R. meliloti* were compared and the results are shown in Fig. 2. Induction media were buffered with five times the normal amount of phosphate buffer (62.5 mM phosphate final concentration). Under these conditions the pH of the *R. meliloti* cultures remained constant during the first 6 h of incubation and fell at the most 0.2 pH units after 24 h of incubation (in the case initial pH was 6.8 no significant acidification was detected after 24 h incubation). As can be seen in Fig. 3, an initial pH above 6.4 gave strongly attenuated *vir*-induction in an *Agrobacterium* background. By comparison, the induction of the *R. meliloti* derivatives was virtually independent of the pH of the induction medium. These results indicate that the chromosomal background at least partly determines the

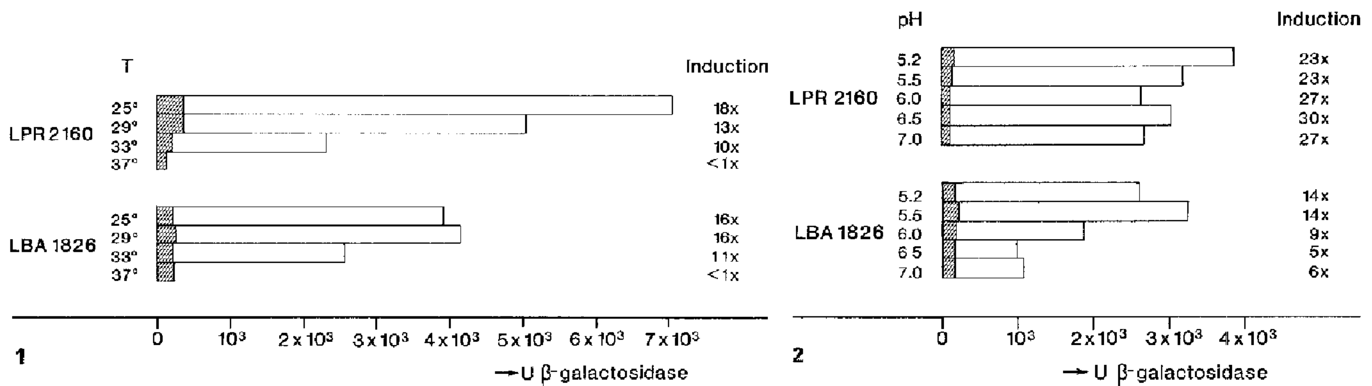

Fig. 1. Virulence gene induction in *Rhizobium meliloti* (LPR2160) and *Agrobacterium tumefaciens* (LBA1826) at different temperatures. Samples were taken after 24 h of induction. ❏ 100 μM AS, ▨ no AS added

Fig. 2. Virulence gene induction in *Rhizobium meliloti* (LPR2160) and *Agrobacterium tumefaciens* (LBA1826) at different pH (values of phosphate buffer 1.25 M stock). Samples were taken after 24 h of induction. ☐ 100 μM AS, ▨ no AS added

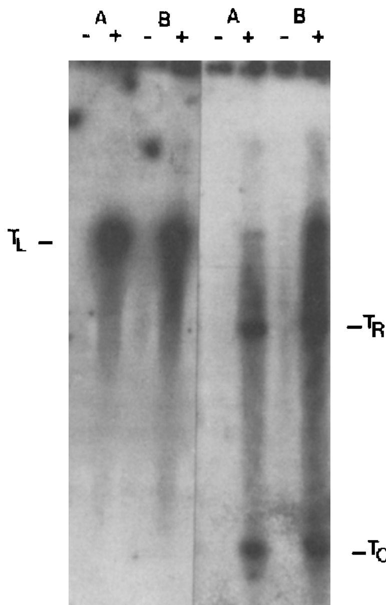

Fig. 3. T-strand formation in *Rhizobium meliloti* (LPR2122, *panels B*) and *Agrobacterium tumefaciens* (LBA1010, *panels A*) On the left T_L-DNA (probe T-cyt) and on the right T_R and T_C DNA (probe BamH1-2) lanes + induced with AS, lanes – no AS added

effect of pH on the efficiency of *vir* gene induction. Moreover, the results show that the lack of tumor induction by *R. meliloti* strains harboring Ti plasmids can not be attributed to inefficient *vir*-induction in this genetic background.

Detection of ss T-DNA intermediates

After induction of the virulence genes by AS the VirD products are responsible for the specific nicking in the bottom strand of the border sequences that delineate the T-region (Yanofski et al. 1986). By strand displacement the bottom strand of the T-region is probably liberated which can be detected on blots of undigested total DNA of AS-induced bacteria. We assayed for the production of T-strands in acetosyringone induced cultures of *Agrobacterium* (LBA1010) and *Rhizobium meliloti* (LPR2122). In both these strains a complete octopine virulence region and both T_L (containing the *onc* genes) and T_R (that is not required for tumor formation) DNA are present. The T_C-DNA is a small piece of DNA inbetween T_L and T_R on the Ti plasmid that has never been detected as a separate unit in tumor cells. In Fig. 3 single stranded intermediates encompassing T_L, T_C and T_R DNA respectively are shown. In *Agrobacterium* strain LBA1010 T_L, T_C and T_R were detected in AS-induced cultures (panels A) as ss-T strands. In a *R. meliloti* background, similar amounts of T-strands were present after induction (panels B). The fact that T-strands were detected in strains harboring a Ti plasmid shows that the Vir products involved in T-DNA processing are present and functional in this genetic background, and these results suggest that the lack of tumour formation by these bacteria is caused by a deficiency either in the initial steps of tumor induction (i.e. recognition and attachment) or in the transfer process of the T-DNA intermediate molecules to the plant cells.

Discussion

The use of bacteria other than *Agrobacterium* for the study of Ti plasmid mediated virulence properties might aid in revealing so far unknown chromosomally encoded virulence functions. Moreover the use of bacteria, more adapted to specific plant hosts might improve the applicability of the Ti gene transfer system for plant species not efficiently transformed by *Agrobacterium*. In this report, we have shown that a *Rhizobium meliloti* (a natural symbiont of alfalfa plants) genetic background allows efficient *vir*-gene induction and T-strands formation. The conditions under which *vir*-induction takes place in *R. meliloti* are less stringent than those required in *Agrobacterium* indicating that the chromosomal background plays a role in at least the pH and temperature dependance of *vir*-induction. Using mutant *vir*A genes it was shown recently (Melchers et al., in preparation) that both pH dependance and temperature sensitivity of *vir*-gene induction are also determined by intrinsic features of the VirA protein. One might hypothesize that the positioning and/or the context of the VirA protein in the inner membrane of the bacterium is slightly different in *Agrobacterium* and *Rhizobium meliloti*, or that a difference in the physiological state of these bacteria under certain conditions, might influence the functioning of VirA.

Besides *vir*-induction we found T-strand production in *R. meliloti* strains containing Ti plasmids, which shows that proper DNA processing takes place in this genetic background. We note that, contrary to the results obtained by Stachel et al. (1987), we did not find detectable amounts of composite single-stranded T-DNA's ($T_L + T_C$, $T_R + T_C$, $T_L + T_C + T_R$) in our DNA preparations. In view of the fact that composite T-DNA's incorporated in the plant genome are rare we assume that suboptimal induction conditions are the cause of these composite T-strands.

The lack of tumor formation by *R. meliloti* strains containing Ti plasmids can, in view of the results presented in this paper not be attributed to a lack of *vir* genes expression or T-strand formation. The results show that all essential virulence genes (*vir*ABDG) of the Ti plasmid are expressed under the conditions used. Proper induction of the *vir*B promoter shows that both the regulatory system *vir*A, *vir*G, and the *vir*B promoter are functional in *R. meliloti*. T-strand formation in this background shows that the *vir*D operon is properly expressed as well. We cannot exclude that the *vir*C, *vir*E and *vir*F host-range loci are down-regulated in *R. meliloti*. However, since pTi containing *R. meliloti* strains are avirulent even on plants that do not need *vir*CEF for tumor formation e.g. sunflower and *Kalanchoe tubiflora*, we do not think it likely that the lack of expression of the host-range *vir* loci is the reason for the avirulence of these strains. *Rhizobium meliloti* does contain functional counterparts of the chromosomal (*chv*) genes which are involved in the attachment of *Agrobacterium* to plant cells; but nevertheless *R. meliloti* bacteria have been found to be deficient in attachment in a number of assays. *R. meliloti* does not attach to cell-wall regenerating protoplasts of tobacco (Krens et al. 1985), and these bacteria (in contrast to *Agrobacterium*) are unable to aggregate *Asparagus* cells (Draper et al. 1983). Moreover, the attachment properties of *R. meliloti* to pea root hair cells is markedly different from the attachment of *R. leguminosarum* biovars and *Agrobacterium* (Smit, Thesis, Leiden University 1988). Specific information about the attachment of *R. meliloti* to alfalfa root hairs is lacking but indirect evidence suggests non-specific as well as specific modes of binding (Caetano Anolles and Favelukes 1986; Lafreniere et al. 1984). It has to be noted that pT_i carrying *R. meliloti* strains are non-tumorigenic on alfalfa plants (our unpublished results) indicating that the attachment mechanism employed on the root-hairs of alfalfa in the nodulation process, can not be used for crown gall induction. An alternative cause for the lack of tumor formation by *R. meliloti* (pTi) strains might be that differences in the cell wall or membranes properties between *Agrobacterium* and a *R. meliloti* hamper the assembly of a structure (e.g. a pilus) which is involved in T-strand transfer to the plant cell in the latter bacterium.

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