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Genetics

Complementation of *Agrobacterium tumefaciens* tumor-inducing *aux* mutants by genes from the T_R-region of the Ri plasmid of *Agrobacterium rhizogenes*

(hairy root disease/phytohormones)

I. A. OFFRINGA*, L. S. MELCHERS*, A. J. G. REGENSBURG-TUINK*, P. COSTANTINO†, R. A. SCHILPEROORT*, AND P. J. J. HOOYKAAS*‡

*Department of Plant Molecular Biology, Biochemistry Laboratory, University of Leiden, 2333 AL Leiden, The Netherlands; and †Department of Molecular Biology, University of Rome, 00185 Rome, Italy

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ABSTRACT **In this paper we provide information indicating that the agropine-type root-inducing (Ri) plasmid pRi1855 of *Agrobacterium rhizogenes* contains functional genes for auxin production (*aux*) in the right transferred DNA (T-DNA) region (T_R-region). These genes were cloned and introduced into the T-region of the tumor-inducing (Ti) plasmids of mutants of *Agrobacterium tumefaciens* carrying an *aux* mutation. Depending on the Ri *aux* gene present, the oncogenicity of the Ti *aux-1* and/or *aux-2* mutations was restored, showing that the Ri *aux* genes are able to complement the Ti *aux* genes. *Agrobacterium* strains with an agropine-type Ri plasmid not only cause hairy root on certain plant species, but they also induce tumors on other plant species. In this paper it is shown that a mutation in either of the *aux* genes in the Ri plasmid leads to a total loss of tumorigenicity and a strongly diminished rhizogenicity of the host bacterium, revealing that the *aux* genes are important for tumor and root induction. *Agrobacterium* strains containing the T_R-region but not the T_L (left)-region of the Ri plasmid are still tumorigenic on certain plant species but are no longer capable of hairy-root induction.**

Upon infection of plants such as carrot and kalanchoe, *Agrobacterium rhizogenes* strains that carry an agropine-type root-inducing (Ri) plasmid cause hairy root. This plant disease is characterized by abundant root proliferation from the infection sites (1). However, infection of plant species such as sunflower and pea by the same bacterial strains results in the formation of unorganized tumors. The molecular mechanism underlying the interactions between *A. rhizogenes* and plant resembles that of crown gall induction on plants by *Agrobacterium tumefaciens*, which contains the tumor-inducing (Ti) plasmid. In both cases a segment of plasmid DNA, the transferred DNA (T-DNA) is introduced into plant cells at the infection sites (2–4). The T-DNA contains genes that are expressed in the transformed plant cells (5, 6). Amongst these genes are *onc* genes, which are responsible for the unlimited proliferation of the transformed plant cells even in the absence of externally added phytohormones such as auxins and cytokinins (7). In the case of *A. tumefaciens*, three *onc* genes have been identified. Two of these are *aux* genes (*aux-1* and *aux-2*) that encode enzymes involved in the production of the auxin indole acetic acid (8, 9), and the third one is a *cyt* gene, which is responsible for the production of the cytokinin isopentenyl-AMP (10–12). In plant species such as tobacco, the relative proportion of auxins and cytokinins has a strong influence on the differentiation: a relatively high level of auxins leads to root formation, whereas a relatively high level of cytokinins leads to shoot induction (13). In agreement herewith, the inactivation of either of the *aux* genes or the *cyt* gene in the Ti plasmid T-region [region of transferred DNA (T-DNA)] results in bacteria inducing rooty or shooty tumors on tobacco and kalanchoe (14, 15).

Like plant tissues transformed with the T-DNA of the Ti plasmid, plant lines containing Ri T-DNA grow in the absence of phytohormones (16). The T-DNA present in these lines was found to originate from one segment of the Ri plasmid (3, 4, 17, 18). Therefore, it was likely that this section of Ri DNA contained genes responsible for the unlimited proliferation of the transformed lines. Indeed, it was shown that an insertional mutation in the middle of this T-region leads to the loss of rhizogenicity on kalanchoe leaves (19). However, we have found that the deletion of almost the entire T-region including the middle part does not lead to the loss of rhizogenicity on kalanchoe stems or the loss of tumorigenicity on pea and sunflower (unpublished results), which suggests that other *onc* genes are located elsewhere in the Ri plasmid. Recently it was shown that a second region of the agropine-type Ri plasmids, more than 15 kilobase pairs to the right of the previously described T-region, is occasionally transferred to plant cells during infection and contains genes for the biosynthesis of the opine agropine (20). So far, no *onc* genes had been detected in this region [called T_R (right)-region to distinguish it from the already known T_L (left)-region], but, interestingly, a stretch of DNA showing homology with Ti *aux* genes under low-stringency hybridization conditions was found next to the agropine synthase gene (21–23) (Fig. 1). Recently this "*Aux*" locus of the Ri plasmid was detected in a few transformed lines as part of T_R-DNA (R. Peerbolte, personal communication).

In this paper we show that this Ri *Aux* locus contains genes that can complement both Ti *aux-1* and *aux-2* mutations. Moreover, we demonstrate that these genes are important for the tumorigenicity and rhizogenicity of agropine-type *A. rhizogenes* strains. Finally, using a T_R-DNA clone of the Ri plasmid, it is shown that strains containing the T_R-region but not the T_L-region can induce tumors on certain plant species.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Transposons. Bacterial strains and plasmids are mentioned in the text. The transposable elements used are Tn*3drd* (5-65), a derivative of Tn*3* with a *tnpR* mutation (25), and Tn*1881*, a Tn*3drd* derivative carrying a segment of the octopine Ti plasmid T-region in its

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Abbreviations: *aux*, gene for auxin production; *cyt*, gene for cytokinin production; Ti, tumor-inducing; Ri, root-inducing; T-DNA, transferred DNA; IS, intervening sequence.
‡To whom reprint requests should be addressed.

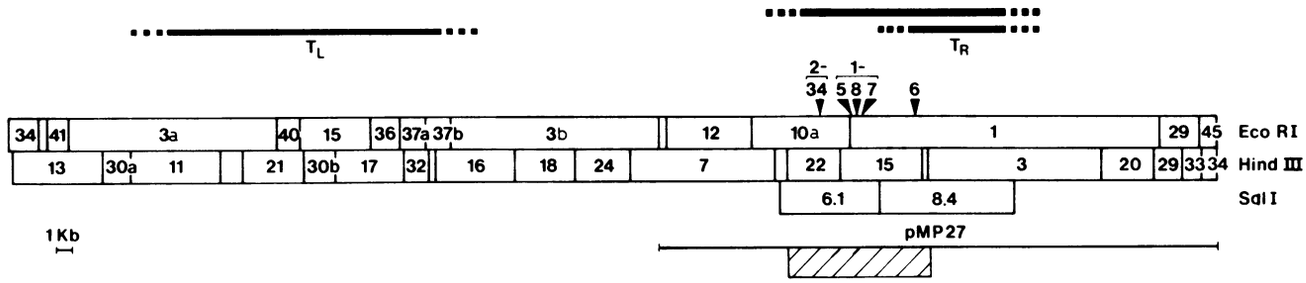

FIG. 1. Restriction map of the pRi1855 T-region. *Eco*RI (24) and *Hin*dIII (22) fragments are numbered according to their decreasing size. Two relevant *Sal* I fragments are indicated by their size in kilobase pairs. The region covered by clone pMP27 is shown. The hatched bar shows the region homologous to the pTiA6 *aux* genes (22). Dark bars indicate the T_L-region as published (4, 19) and the T_R-region as published (20) and, above that, the T_R-region according to R. Peerbolte (personal communication). Small numbered arrowheads symbolize insertions in the *aux* region; 2- and 1- indicate the *aux* gene inactivated by the mutation.

unique *Eco*RI site (26). Intervening sequence 70 (IS*70*) has been described by Hille *et al.* (27).

Microbiological Methods. Bacteria were cultured and selected as described (28), and conjugation experiments were performed on membrane filters as described by Hooykaas *et al.* (29).

Molecular Cloning, Southern Blotting, and DNA Isolation. Molecular cloning and Southern blotting procedures have been described by Maniatis (30). Total *Agrobacterium* DNA was isolated as described by Ooms *et al.* (31). Plasmid DNA was isolated from *Escherichia coli* by the "miniprep" procedure (32) for small-scale isolation and by the procedure of Clewell and Helinski (33) for larger isolation.

Construction of Plasmid pAL1330. The R plasmid R772 was introduced into an *E. coli* strain carrying clone pMP27. The resulting strain was used as a donor in a cross to an *E. coli* recipient. Acceptor strains that had received the tetracycline-resistance (Tc^r) marker but not the carbenicillin-resistance (Cb^r) marker of pMP27 were checked for the presence of an R772::pMP27 cointegrate. One such plasmid, in which the cointegrate had formed via transposition of IS*70* from R772 into the Cb^r marker of the pBR322 part of pMP27, was called pAL1330 and used in further experiments.

Construction of Plasmid pAL1380. R plasmid R772 was introduced into *Agrobacterium* strain LBA1334, which carries pAL1334, a derivative of pRi1855 with a spectinomycin-resistance (Sp^r) marker in a segment that is not involved in virulence. The resulting strain was used in a cross with an *E. coli* recipient. Sp^r colonies were selected in the expectation that these would contain cointegrates between R772 and pAL1334, since the Ri plasmid cannot replicate in *E. coli*. Such Sp^r transconjugants were obtained with a low frequency, and plasmid isolations showed that they contained the desired cointegrates. After retransfer to *Agrobacterium*, they were tested for virulence. A plasmid that had retained all genes necessary for virulence, pAL1380, was selected for further work.

Introduction of Tn*3drd* and Tn*1881* into Clones. A derivative of R772, carrying Tn*3drd*, was introduced into the *E. coli* strains containing the Ri clones and then used for the mobilization of the clones to a third acceptor strain. Transfer of the Ri clones to the acceptor strain only occurs after cointegrate formation with the R plasmid via transposition of Tn*3drd* (or less frequently IS*70*). In the acceptor strain, the cointegrate readily dissociates releasing the R plasmid and a clone with a copy of the transposon or IS*70*. Plasmid DNA was isolated from the transconjugants and analyzed by restriction enzyme analysis, and clones with a Tn*3drd* insert in the required position were selected. An identical procedure using R772::Tn*1881* instead of R772::Tn*3drd* was applied to create Tn*1881* insertions.

Introduction of Tn*3drd* Insertions into pAL1380. Plasmid pAL1380 was crossed to *E. coli* strains containing the mutated Ri clones. Resulting strains were crossed to empty acceptor bacteria. Cb^r (Tn*3drd* marker) colonies that were sensitive to markers from the vector [pACYC184 (34)] part were selected. Such colonies represented strains in which a double crossing-over had taken place.

Tumorigenicity Tests. Stems of tomato, sunflower, *Nicotiana rustica, Kalanchoe daigremontiana* and *Kalanchoe tubiflora* were wounded and infected with a sterile toothpick that had been dipped in a colony of the strain to be tested. Sterile decapitated seedlings were prepared and infected as described by Kurkdjian *et al.* (35).

RESULTS

Complementation of Ti *aux-1* and *aux-2* Mutations by Genes of the Ri Plasmid. In order to see whether the agropine-type Ri plasmid pRi1855 contains genes that can complement Ti *aux* mutations, plasmid pAL1020 [pRi1855 with a Tn*5* insert (36)] was introduced into strains LBA1513 and LBA1538 containing Ti plasmids with Tn*1831* insertions in *aux-2* and *aux-1*, respectively (37). Transconjugants carrying both the Ti and the Ri plasmid were tested for oncogenicity on tomato, since both parental strains are very weakly tumorigenic on this plant species. The transconjugant strains had a tumor-forming ability similar to *Agrobacterium* strains carrying the wild-type Ti plasmid, showing that genes in the Ri plasmid can complement both *aux-1* and *aux-2* mutations (data not shown). In order to find out whether this was due to genes located in the region of the Ri plasmid showing weak DNA homology to the Ti *aux* genes (see Fig. 1), we used plasmid pMP27 (24), a pBR322 derivative containing the T_R-region of the Ri plasmid, which also embraces this *Aux* locus. A cointegrate between the wide host range R plasmid R772 (38) and pMP27 was isolated (pAL1330) and crossed to Ti mutants LBA1513 and LBA1538 carrying the aux mutations. As can be seen in Fig. 2*A*, pAL1330 complemented both the *aux-1* and the *aux-2* mutation on tomato.

Isolation of Ri Clones Containing the *aux*-Complementing Genes. To localize the Ri genes involved in the complementation of the Ti *aux* mutations more accurately and to find out whether two different genes are responsible for the complementation of *aux-1* and *aux-2* mutations, we performed *in cis* complementation experiments of mutant Ti plasmids with pRi1855 fragments *Hin*dIII 15 (H15), *Hin*dIII 22 (H22), *Eco*RI 10a (E10a), and *Sal* I 6.1 (S6.1). To make insertion of the Ri fragments into the Ti T-DNA possible, we created homology between the plasmids carrying the Ri fragments and the Ti plasmids by introducing transposon Tn*1881* [which is a derivative of Tn*3drd* containing a nonessential part of the Ti T-region (26)] onto the vector [pACYC184 (34)] part of these plasmids. Subsequently, the plasmids were transferred to LBA1513 and LBA1538 by mobilization with plasmid pRK2013 (39). Colonies resistant to pACYC184 markers

were selected. As pACYC184 cannot replicate in *Agrobacterium*, resistance to its markers can only be obtained by recombination of the plasmids carrying the Ri fragments into the Ti T-region. The resulting strains were tested for tumorigenicity on pea seedlings and on tomato, since LBA-1513 and LBA1538 are virtually nononcogenic on these plants. As can be seen in Table 1 the S6.1 fragment complemented both the *aux-1* and the *aux-2* mutations (see Fig. 2*B*), while fragments E10a and H22 only complemented the *aux-2* mutation. Fragment H15 failed to complement either mutation. Thus, it can be concluded that the Ri region, with weak homology to the Ti *aux* genes, contains two genes, one of which complements the *aux-2* mutation and lies in fragment H22. The other complements the *aux-1* mutation and lies in the right half of fragment S6.1 and crosses the H22–H15 junction (see the following section and Fig. 1). It is known that Ti *aux* genes code for proteins involved in the biosynthesis of indole acetic acid, probably via indole acetamide (8, 9), in which the *aux-1* gene product probably takes care of the first step and the *aux-2* gene product determines the second step. The results from our complementation experiments suggest that the *Aux* locus of the Ri plasmid contains two genes (hereafter called Ri *aux-1* and Ri *aux-2*), which determine functions similar or identical to those of the Ti *aux-1* and *aux-2* genes.

Ri *aux-1* and *aux-2* Mutations. After identification of fragments carrying the Ri *aux* genes, site-directed mutagenesis was performed to map these genes in more detail and to determine their role in tumor and hairy-root induction. To this end, transposition of Tn*3drd* onto Ri fragments H15, H22, and E10a (cloned in vector pACYC184) was induced as described. The Tn*3drd* insertions were introduced into the Ri plasmid by homologous recombination. As double-recombination events occur with an extremely low efficiency in *Agrobacterium*, a cointegrate between the wide host range plasmid R772 and a derivative of pRi1855 with a Sp^r marker was constructed, enabling us to perform the recombination in *E. coli*. [The cointegrate used, pAL1380, conferred normal virulence on *Agrobacterium* (Fig. 3 *Top*).] After having verified the insertion of Tn*3drd* into the desired location of the Ri plasmid by Southern blotting (data not shown), the ability of the mutated plasmids to complement Ti *aux* mutations on pea seedlings and tomato was checked. Mutation *34* appeared to have inactivated the Ri gene that was able to complement the Ti *aux-2* gene, and mutations *5*, *7*, and *8* had inactivated the Ti *aux-1*-complementing gene, while mutation *6* left both complementing genes intact (see Fig. 1). After having identified Ri plasmids with *aux-1* and *aux-2* mutations in this way, we introduced the plasmids into an *Agrobacterium* strain free of Ti plasmids to study the effect of the mutations on the process of tumor and root formation. The behavior of Ri mutants carrying the *aux-1* and *aux-2* mutations was similar: they no longer induced tumors on sunflower (not shown) and decapitated pea seedlings, they induced only slight proliferations on *N. rustica*, and they did not (or only weakly) induce roots on *K. daigremontiana* and *K. tubiflora* (Fig. 3). Apparently inactivation of either *aux* gene of the Ri plasmid leads to a greatly diminished rhizogenicity and to a complete loss of tumorigenicity, demonstrating the importance of these genes.

Table 1. Complementation of Ti *aux* mutations by fragments of the Ri-plasmid

Introduced fragment	Tumorigenicity of Ti mutant strains*	
	LBA1513†	LBA1538‡
None	−	−
*Hin*dIII 15	−	−
*Hin*dIII 22	+	−
*Eco*RI 10a	+	−
Sal I 6.1	+	+

*Tumorigenicity was tested on decapitated pea seedlings.
†Mutated in *aux-2* gene.
‡Mutated in *aux-1* gene.

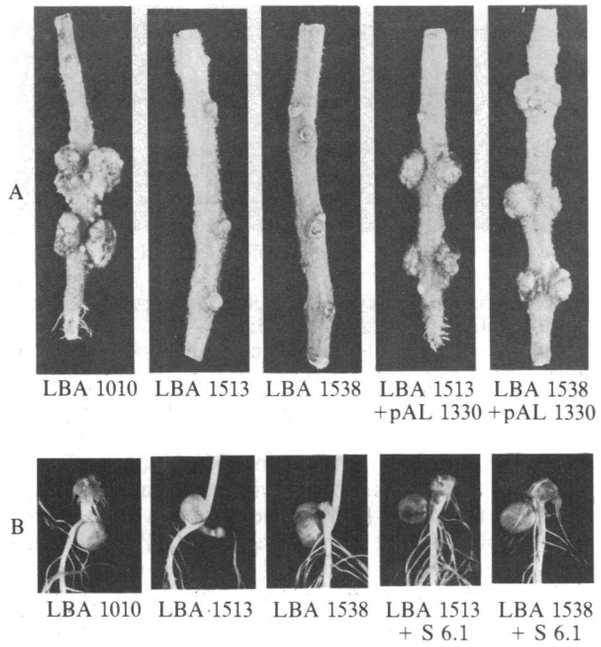

FIG. 2. Complementation of Ti *aux* mutations by Ri T-DNA. (*A*) *In trans* complementation by T_R-region on tomato. (*B*) *In cis* complementation by *Sal* I 6.1 fragment on pea seedlings. Strains: LBA1010, strain containing wild-type octopine Ti plasmid; LBA 1513 and LBA 1538, strains containing Ti plasmid with an *aux-2* and an *aux-1* mutation, respectively; pAL1330, R772 cointegrate with pMP27 (Ri T_R clone). Tumors on tomato and pea were scored after 4 weeks and 2 weeks, respectively.

Tumorigenicity of Strains Carrying Only the T_R-Region. Having demonstrated the importance of the Ri *aux* genes, we questioned whether the T_R-region in itself could convey transforming ability upon *Agrobacterium* that was indepen-

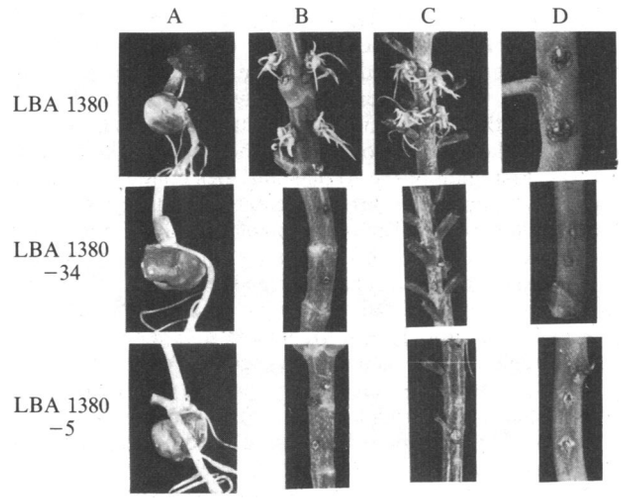

FIG. 3. Effect of *aux* mutations on tumorigenicity and rhizogenicity. (*A*) Pea seedlings. (*B*) *Kalanchoe daigremontiana* stems. (*C*) *K. tubiflora* stems. (*D*) *Nicotiana rustica* stems. Strains: LBA 1380, strain containing pAL1380; LBA 1380-34, Ri aux-2 mutant 34; LBA 1380-5, Ri aux-1 mutant 5. Tumors in *A* were scored 2 weeks after inoculation. Tumors or hairy roots in *B–D* were scored after 5 weeks.

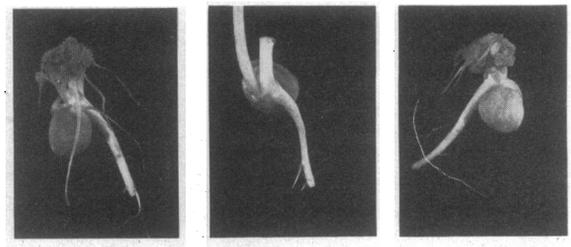

FIG. 4. Tumorigenicity of T_R-region on pea seedlings. Strains: LBA 1060, strain containing pRi1855; LBA 4404, strain containing pAL4404; LBA 4460, strain containing pAL4404 and pAL1330. Tumors were scored after 2 weeks.

dent of the T_L-region. To test this we used the R772::pMP27 cointegrate pAL1330, which contains the whole T_R-region (see Fig. 1). To provide the *vir* (virulence) functions that are required to transfer T-DNA to the plant and that are present on the Ri plasmid but are missing on pAL1330, the cointegrate was crossed into an *Agrobacterium* strain containing pAL4404 (31), a severely deleted Ti plasmid possessing an intact *vir*-region but lacking the entire Ti T-region. [Previous studies (21, 22, 28, 40, 41) and the complementation of Ti strains carrying the *aux* mutation by pAL1330 had already shown that Ti *vir* genes are functional in transferring Ri T-DNA to plant cells.] The resulting strain LBA4460 was used for the infection of various plant species. Tumors arose on pea (Fig. 4) and sunflower (not shown), plants that also react to infections with Ti mutants carrying the *cyt* mutation by tumor formation. No response at all was seen on tobacco, and no or only very small proliferations arose on *K. daigremontiana* and *K. tubiflora* (not shown), showing that while the T_R-region alone is sufficient to induce tumors on some plant species, it is not enough to induce tumor or root formation on other hosts; apparently, additional genes present in the T_L-DNA are required on these plants.

DISCUSSION

The T-region of the octopine Ti plasmid is split into a T_L- and a T_R-region (42). *Onc* genes are only present in the T_L-region (31), while the T_R-region contains genes involved in agropine and mannopine biosynthesis (43, 44). The T-region of the agropine Ri plasmid is also divided into two parts (20). It was initially thought that the T_R-region of the Ri plasmid only contained genes involved in agropine biosynthesis and that it lacked *onc* genes. Therefore, it was proposed that this segment of the Ri plasmid could be used as a disarmed gene vector for plants (45). However, in this paper we have shown that the T_R-region of the Ri plasmid does contain *onc* genes, which by themselves are sufficient to induce tumors on certain plant species. The situation of a divided T-region with *onc* genes located in both T-regions is not unique for agropine Ri plasmids; it has also been described recently for the limited host range Ti plasmid (46, 47). In the case of the limited host range plasmid, one T-region contains *aux* genes, while the other T-region carries genes homologous to other octopine Ti plasmid *onc* genes. A defective *cyt* gene is one of the causes of the limited host range of this Ti plasmid (48, 49). The situation of the agropine Ri plasmid is somewhat different: although here *aux* genes are also present in a separate T_R-region, the genes in the T_L-region do not show any DNA homology with the other well-known Ti *onc* genes (6, 21, 22, 40), and the introduction of a Ti *cyt* gene into the Ri plasmid (either in the T_L- or the T_R-region) does not lead to strains that have a tumor-inducing ability identical to strains carrying a wild-type Ti plasmid (unpublished results). This shows that the Ri plasmid does not simply represent a Ti plasmid from which the *cyt* gene is mutated, but forms a separate class of transforming plasmids. Additional evidence for this is (*i*) the fact that Ti and Ri plasmids belong to different incompatibility groups (50) and (*ii*) our localization in the T_L-region of a gene that suppresses root formation (in preparation). In spite of this, the processes of crown gall induction and hairy-root formation are related, as is shown by the finding that genes in the Ri T_R-region can complement the Ti *aux*-genes and by the fact that Ti *vir*-genes can mediate transport of Ri T-DNA to plant cells (shown in this paper) just as Ri *vir*-genes can cause transfer of Ti-DNA (41).

Agropine-type and mannopine-type *A. rhizogenes* strains differ in that the former strains contain a T_R-region with *aux* genes in their Ri plasmid, whereas the latter strains lack such a region. Both types of strains induce hairy root on plants such as carrot and kalanchoe, but the agropine strains are somewhat more vigorous. In contrast, however, only agropine strains are able to induce tumors on sunflower and pea (unpublished results). This is in agreement with our finding that the inactivation of the *aux* genes in the T_R-region of the agropine Ri plasmid results in strains that are no longer tumorigenic on sunflower and pea but are still capable of inducing hairy root—albeit weakly—on kalanchoe. The region covering the Ri *aux* genes is lacking in many hairy-root lines studied (refs. 4, 17, and 18; R. Peerbolte, personal communication). Obviously it might be argued that the Ri *aux* genes do not form part of the T_R-region, but in fact are introduced into plant cells only accidentally. However, we have found that the deletion of the right border sequence of the Ri T_R-region results in strains that are equally diminished in virulence as Ri mutants with the *aux* mutation even if the deletion leaves the *aux* genes and other genes of the T_R-region intact (unpublished results). This indicates that the Ri *aux* genes become active after transfer to the plant cells as part of the T_R-region. If this is the case, it is hard to understand why the T_R-region is absent from many established hairy-root lines. An explanation might be that auxin is essential for the differentiation into root cells of cells transformed by the Ri T_L-region—i.e., for root initiation but not for the proliferation of the initiated roots. If cells transformed by the Ri T_R-region would produce and excrete large quantities of auxin, this might be sufficient to induce cells transformed by the Ri T_L-region alone to differentiate into roots. No root lines have been observed carrying only the T_R-region. This suggests that cells transformed by the T_R-region alone cannot differentiate into roots. The low frequency with which hairy-root lines carrying the T_L- and the T_R-region have been isolated might be due to a low frequency of cotransformation of cells by both of these T-regions.

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