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Restoration of virulence of *Vir* region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains

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Summary. Three avirulent Tn7 insertion mutants mapping in the *vir* E region of the *Agrobacterium tumefaciens* plasmid pTiB6S3 regain virulence by co-infection with several wild-type strains and with a number of strains carrying mutations in other regions of the Ti plasmid. This finding indicates that during tumour induction normal *Agrobacterium* strains produce a diffusable factor required for transformation and might allow the isolation of such a factor.

Introduction

The initial steps involved in the transformation of plant cells by *Agrobacterium tumefaciens* and leading to Crown gall formation are still largely unknown. Most probably several steps are involved. The analysis of mutants affected in virulence could lead to a better understanding of these processes. Several avirulent mutants have been isolated, and found to map in different regions of the Ti plasmid (Hernalsteens et al. 1978; Holsters et al. 1980; De Greve et al. 1981; Garfinkel et al. 1980; Ooms et al. 1980).

Three regions involved in tumour induction have been defined on Ti plasmids: 1. The T-region, the region of the Ti plasmid which is transferred to and integrated in the plant genome. The T-DNA genes themselves are not needed for the process of DNA transfer and integration, as shown by mutants which lack the T-DNA genes responsible for hormone-autotrophic growth (Leemans et al. 1982; Zambryski et al. 1983). 2. The D-region or *vir* region originally defined as one of the regions of homology between the octopine plasmid pTiB6S3 and the nopaline plasmid pTiC58 (Engler et al. 1981), and 3. A region located near the origin of replication. (De Greve et al. 1981). The *vir* region was studied in detail for the octopine plasmids (Garfinkel et al. 1980, De Greve et al. 1981). Intracellular complementation analysis has demonstrated that this region consists of several complementation groups (Iyer et al. 1982; Klee et al. 1982; Hille et al. 1982). The virulence of the *vir* region mutants used by these authors could not be restored by coinfection with Agrobacteria carrying wild-type Ti fragments.

Using a sensitive and rapid virulence assay, based on the infection of *Kalanchoe daigremontiana* stem fragments (Otten 1982) we show here that the virulence of three of 12 avirulent *vir* region mutants can be restored by the simultaneous presence of other strains of Agrobacterium at the same infection site.

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Materials and methods

Bacterial strains. The bacterial strains used are listed in Table 1. Figure 1 shows a map of the different insertions and deletions in pTiB6S3.

SDS-curing. To cure RP4 from GV3105pGV2027, SDS selection was applied (J.P. Hernalsteens, pers. communication). SDS treatment selects against bacteria expressing the RP4-encoded pili. pGV2027 was first transferred by conjugation to a cured, SDS-resistant rifampicin resistant strain, GV10. Exconjugants were selected on streptomycin rifampicin plates. Because the exconjugants contained pGV2027 with the RP4 sequences which confer SDS-sensitivity upon the host bacterium, they were no longer SDS^R. One of the exconjugants was colony-purified and grown overnight in liquid LB. Dilutions were plated on LB+ 0.05% SDS and SDS^R colonies were isolated and tested for loss of the RP4-encoded carbenicillin and kanamycin resistance markers, and for loss of the RP4-encoded transfer (tra) functions by mating with the cured *Agrobacterium* strain GV3105.

Conjugations. Conjugation between *Agrobacterium* strains was carried out by mixing 0.1 ml of an overnight culture of each partner on a YEB plate and incubating for 7 h at 28° C. The conjugation mix was then streaked out on selective plates and colonies from these plates were colony-purified one more time.

Media and growth conditions. Agrobacterium strains were grown in liquid YEB with aeration at 28° C (Van Larebeke et al. 1977). To test for growth on octopine medium, minimal octopine medium was used as described by Van Larebeke et al. 1977.

Antibacterial drugs. Antibacterial drugs were added to the media at the following concentrations: (μg/ml): rifampicin 100, erythromycin 50, streptomycin 300, spectinomycin 100, kanamycin 25, carbenicillin 100 and chloramphenicol 25.

Virulence assay. Strains were tested for their virulence by measuring LpDH or NpDH activity in an assay using *Ka-*

Table 1. List of bacterial strains and their effect on GV10pGV2027a

Chromosomal Background	Plasmid	Origin or reference	Restoration of GV10pGV2027a virulence	Description
GV3105	pGV2009	De Greve et al. 1981	−	Tn7 insertions in the *vir* region of pTiB6S3
GV3105	pGV2026	De Greve et al. 1981	−	
GV3105	pGV2027	De Greve et al. 1981	−	
GV3105	pGV2029	De Greve et al. 1981	−	
GV3105	pGV2030	De Greve et al. 1981	−	
GV3105	pGV2032	De Greve et al. 1981	−	
GV3105	pGV2033	De Greve et al. 1981	−	
GV3105	pGV2034	De Greve et al. 1981	−	
GV3015	pGV2036	De Greve et al. 1981	−	
GV3105	pGV2037	De Greve et al. 1981		
GV3105	pGV2038	De Greve et al. 1981	−	
GV3105	pGV2074	De Greve et al. 1981	−	
GV3105	pGV2014	De Greve et al. 1981	−	Tn7 insertion in ori of pTiB6S3
GV3101	pGV2237	Leemans et al. (unpublished data)	+	*Δ EcoRI-Ω* fragments of LpDH gene of pTiB6S3
GV3101	pGV2217	Leemans et al. 1982	+	*Δ* T_L of pTiB6S3
LBA4404	pAL4404	Ooms et al. 1982	+	*Δ* from *Sma*I-4 into *Hin*dIII-14 (*Δ* T_L and *Δ* T_R of pTiAch5)
LBA4412	pAL4412	Ooms et al. 1982	+	*Δ* from *Sma*I-6 into *Sma*I-3a (*Δ* T_L and *Δ* T_R of pTiAch5)
GV3105	pGV3143	Holsters et al. 1980	+	Tn7 insertion in pTiC58: tra^- mutant
GV3105	pGV3187	Holsters et al. 1980	−	*vir* region mutant pTiC58
LBA4301	pGV3850	Zambryski et al. 1983	+	*Δ* T-DNA of pTiC58
GV3105	–	Holsters et al. 1980	−	C58C1[a] $Ery^R Cm^R$
GV3101	–	Holsters et al. 1980	−	C58C1[a] Rif^R
GV10	–	Hernalsteens (unpublished data)	C58C1[a] Rif^R SDS^R	
GV5001	pGV4100	Holsters et al. 1978b	−	*E. coli* carrying pTiB6S3::RP4
C58	pTiC58		+	wild-type nopaline strains
T37	pTiT37		+	
281	pTi281		+	null-type strain
15834	pTi15834		+	*Agrobacterium rhizogenes* strain

[a] C58C1 is a cured derivative of C58

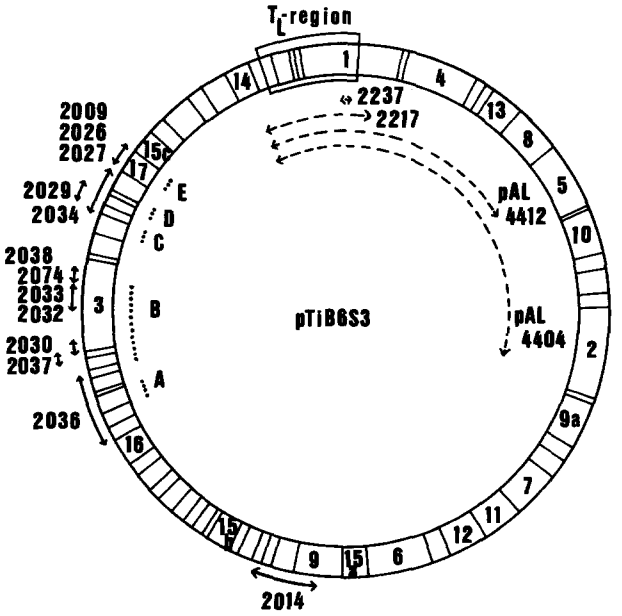

Fig. 1. Map of pTiB6S3 showing the main *Hin*dIII fragments taken from a detailed map according to De Vos et al. 1981, and showing the location of the different insertions and deletions described in Table 1. ⟷: Tn7 insertions, ← – →: deletions, ·····: *vir* complementation groups according to Iyer et al. 1982

lanchoe daigremontiana stem segments (Otten, 1982). All experiments were done in duplo, i.e. by testing two independently infected segments. Alternatively, strains were inoculated on intact *Kalanchoe* plants previously wounded by puncturing the stem or scratching the leaf with a Pasteur pipette.

Results

Virulence restoration of the Vir-region mutants GV3105pGV2009, GV3105pGV2026 and GV3105pGV2027 by coinfection with GV3101pGV2237

When 12 *vir* region mutants of the octopine strain B6S3, GV3105pGV2009, GV3105pGV2026, GV3105pGV2027, GV3105pGV2029, GV3105pGV2030, GV3105pGV2032, GV3105pGV2033, GV3105pGV2034, GV3105pGV2036, GV3105pGV2037, GV3105pGV2038 and GV3105pGV2074 (De Greve et al. 1981) were used to infect *Kalanchoe* stem segments, no LpDH activity could be detected even after several days of incubation, whereas control segments infected with the wild-type strain B6S3 showed a very high LpDH activity. On the basis of earlier estimations of the minimal LpDH activity detectable in *Kalanchoe* stem segments (Otten 1982) the mutant-induced activities, if any, must lie below 0.5% of the level induced by B6S3.

However, when the same mutants were each mixed in a 1:1 ratio with the B6S3 mutant GV3101pGV2237 (Leemans et al., unpublished, see Table 1), which lacks the LpDH gene, three mutants GV3105pGV2009, GV3105pGV2026 and GV3105pGV2027 regained virulence as shown by the appearance of LpDH activity. In these experiments the activity induced by GV3105pGV2027 was consistently stronger than the activity of GV3105pGV2009 and GV3105pGV2026. Although GV3105pGV2027 showed high LpDH levels in the presence of GV3101pGV2237 it only reached about 25% of the activity seen in a control experiment in which stem segments were infected with a 1:1 mixture of GV3101pGV2237 and B6S3 and the extracts diluted to different degrees (results not shown). The Ti plasmid cured strain GV3105 did not restore the virulence of GV3105pGV2027, which showed that the effect was due to the presence of the Ti plasmid, as expected. For the sake of convenience we will call strains enabling GV3105pGV2027 to induce LpDH activity "helper strains".

The interpretation of the mixed infections is however subject to caution. The pGV2027 mutation was obtained (De Greve et al. 1981) by insertion of Tn7 in a pTiB6S3::RP4 cointegrate, which can be transferred by conjugation to plasmid-less *Agrobacterium* strains with a frequency of $4 \cdot 10^{-2}$ (Holsters et al. 1978a), due to the transfer (tra) functions of the broad host range plasmid RP4. The observed virulence restoration in the case of the GV3105pGV2027/GV3101pGV2237 coinfection could therefore be the consequence of transfer of pGV2027 coding for LpDH to GV3101pGV2237 and subsequent transfer of the pGV2027 derived T-DNA to the plant cell using the intact *vir* region functions of pGV2237.

To exclude this possibility the RP4 functions of pGV2027 were deleted.

Elimination of the RP4 derived tra functions of pGV2027

To eliminate the tra functions of pGV2027 encoded by RP4, SDS curing of RP4 was carried out as described in materials and methods. SDS^R colonies were found at a frequency of 10^{-6}–10^{-7}. Of 65 SDS^R colonies all grew on spectinomycin or on octopine plates indicating that they still contained the Ti plasmid. 11 of 65 colonies did not grow on Km, and 8 of these colonies were also sensitive to Cb. One of the Cb^S clones (called GV10pGV2027a) was selected to test its tra functions, by mating it with the plasmid-less strain GV3105 (Ery^RCm^R) and selecting transconjugants on spectinomycin erythromycin plates. The GV10pGV2027a × GV3105 mating yielded less than 10^{-7} exconjugants. GV10pGV2027a therefore had not only lost the resistance markers coded for by RP4 (Cb and Km) but also the tra functions.

Restoration of virulence of GV10pGV2027a in the presence of a tra⁻ strain

To also exclude the possibility that the wild-type Ti tra functions of a helper strain were involved in the restoration of the virulence of GV10pGV2027a a coinfection experiment was done with a tra⁻ mutant of C58, GV3105pGV3143 (Holsters et al. 1980). Figure 2 shows that GV10pGV2027a did produce LpDH when mixed with GV3105pGV3143. When GV10pGV2027a (but not GV3105pGV2027) was used alone, an extremely weak LpDH activity could occasionally be detected (see discussion). Figure 2 also shows that infection with C58 or GV3105pGV3143 alone only produced a very low NpDH activity, a particularity of this infection system which was noted earlier (Otten 1982). As observed with the GV3105pGV2027/GV3101pGV2237 combination, the level of LpDH activity obtained with the GV10pGV2027a/GV3105pGV3143 combination was lower than with the control B6S3/GV3105pGV3143 combination.

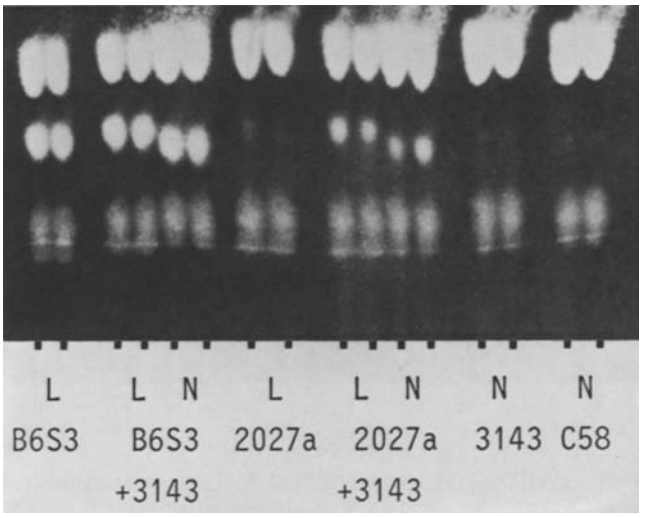

Fig. 2. LpDH assays (L) and NpDH assays (N) of *Kalanchoe* stem fragments infected with B6S3, GV10pGV2027a, GV3105pGV3143, C58 and 1:1 mixtures of B6S3 + GV3105pGV3143, and GV10pGV2027a + GV3105pGV3143. The assays were done 7 days after infection and incubated for 4 h

Virulence restoration of GV10pGV2027a in the presence of other agrobacterium strains

The virulence of GV10pGV2027a could also be restored in the presence of other *Agrobacterium* strains i.e. C58, T37 (nopaline strains), 281 (a null-type strain) and the *Agrobacterium rhizogenes* strain 15834 (data not shown). This indicated that the *vir* function(s) missing in GV10pGV2027a is (are) of general significance and present in different types of *Agrobacterium* strains.

The virulence of GV10pGV2027a was also restored by *Agrobacterium* mutants lacking the T-DNA either in part or completely: GV3101pGV2217 (a B6S3 derivative lacking the T_L-DNA, Leemans et al. 1982), LBA4301pGV3850 (a C58 derivative lacking most of the T-DNA, Zambryski et al. 1983), LBA4404pAL4404 and LBA4412pAL4412 (Ach5 derivatives lacking both the T_L and the T_R regions, Ooms et al. 1982). All these mutants do not induce tumours but have an intact *vir* region. They show that strains do not have to induce tumorous growth or even transfer T-DNA to be effective helper strains. A number of other mutant strains however were not effective. The first group included other octopine *vir* region mutants: GV3105pGV2009, GV3105pGV2026, GV3105pGV2029, GV3105pGV2030, GV3105pGV2032, GV3105pGV2033, GV3105pGV2034, GV3105pGV2036, GV3105pGV2037, GV3105pGV2038 and GV3105pGV2074, and one C58-de-

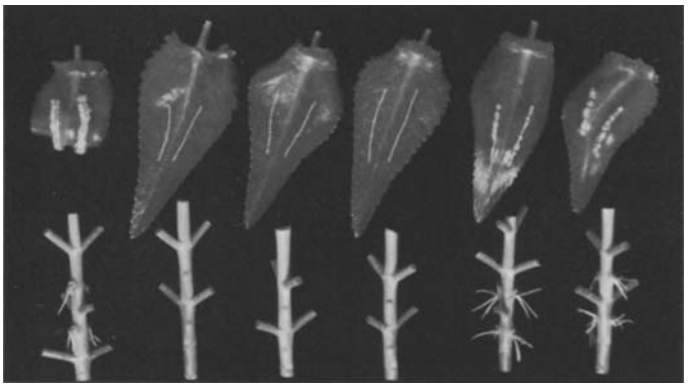

Fig. 3. *Kalanchoe* whole plant infections with B6S3, LBA4301pGV3850, GV10pGV2027a, GV3101pGV2217, GV10pGV2027a + LBA4301pGV3850 (1:1), and GV10pGV2027a + GV3101pGV2217 (1:1). Leaves and stems were cut off and photographed 4 weeks after the infection

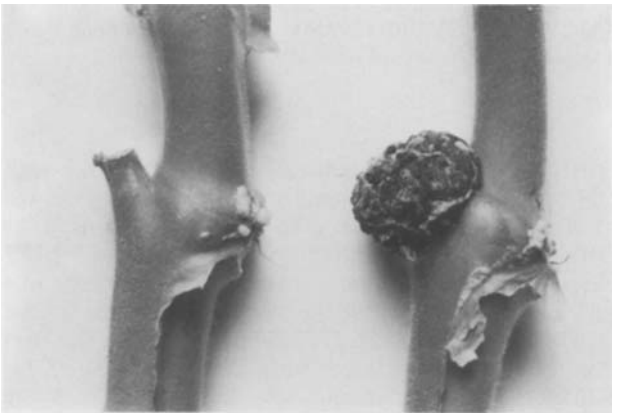

Fig. 4. *Nicotiana tabacum* inoculated with GV10(pGV2027a) (left) and B6S3 (right), 3 months after inoculation

rived *vir* region mutant, GV3105pGV3187 (Holsters et al. 1980).

The second type of ineffective helper strain was represented by the avirulent ori mutant GV3105pGV2014, carrying a mutation close to the origin of replication (Hernalsteens et al. 1978; De Greve et al. 1981) (data not shown).

Influence of GV5001 on GV10pGV2027a

GV5001 (an *E. coli* strain carrying a pTiB6S3::RP4 cointegrate which in *Agrobacterium* is virulent, Holsters et al., 1978b) did not induce LpDH activity on *Kalanchoe* stem segments either when used alone or in a 1:1 mixture with GV10pGV2027a (data not shown). This showed that most likely *vir* region fragments cloned in *E. coli* would be ineffective in restoring the virulence of GV10pGV2027a, when tested in coinfection experiments.

Tumour induction on whole plants

Since in the *Kalanchoe* stem segment assay only LpDH activity is measured we confirmed the general character of the GV10pGV2027a-induced transformation in the presence of helper strains by infection of intact *Kalanchoe* plants with GV10pGV2027a/GV3101pGV2217 and GV10pGV2027a/LBA4301pGV3850. Both stems and leaves were infected. When mixed with either one of the avirulent strains GV3101pGV2217 or LBA4301pGV3850, GV10pGV2027a formed tumours both on stems and on leaves within two weeks (Fig. 3). By contrast, GV10pGV2027a alone formed no or only very few tumours and this only after a period of 4 months. These tumours were white, smooth and spherical and did not induce root formation in the neighbouring stem tissue as do normal B6S3-induced tumours. Also on decapitated tobacco plants GV10pGV2027a occasionally formed very small tumours (see Fig. 4) which might represent a new Crown gall phenotype.

Discussion

The present study showed that of 12 mutants with mutations in the *vir* region of pTiB6S3 three, GV3105pGV2009, GV3105pGV2026 and GV3105 pGV2027 could regain virulence by coinfection with other *Agrobacterium* strains. The mutations in all three mutants are caused by the insertion of transposon Tn7 in the 2.55 kb *EcoR*-I fragment 23 (De Greve et al. 1981), which corresponds to the *vir* E region described by Iyer et al. 1982. Whether the three mutants map at exactly the same location has not yet been determined, but it is interesting to note that the virulence of GV3105pGV2027 can be restored to a higher level than the virulence of GV3105pGV2009 or GV3105pGV2026.

Because of some concern that plasmid transfer might occur from GV3105 pGV2027 to the helper strain (leading to intracellular complementation of the mutated vir region), the RP4-encoded tra constitutivity of pGV2027 was eliminated by SDS curing. The resulting pGV2027 derivative called pGV2027a was shown to have lost the tra functions encoded for by RP4. Strain GV10pGV2027a was very weakly virulent on *Kalanchoe* and on tobacco: after a delay of several months, only a few plants showed small tumours. Iyer et al. found that their single vir E mutant (probably comparable to pGV2027) gave a weak reaction on *Kalanchoe fedtschenkoi* but not on *Kalanchoe daigremontiana, Nicotiana tabacum, Nicotiana glauca* or *Daucus carota* (Iyer et al. 1982).

In our LpDH assay GV10pGV2027a occasionally gave a very weak activity and showed the same ability to give a high activity in the presence of a helper strain as the original strain GV3105pGV2027. This, together with the fact that other *vir* region mutants did not produce LpDH activity when coinfected with GV3101pGV2237 or with GV10pGV2027a (see also below) indicated that plasmid transfer was not the reason for the observed virulence restoration. Our findings can be explained by the existence of a diffusable factor needed for transformation which is lacking in GV10pGV2027a and is provided by the helper strain. Whether this factor acts on the mutant strain or on the plant cells can not yet be distinguished. However, such a factor might be isolated and purified by using the *Kalanchoe* stem segment assay as a functional test. Three other wild-type strains, T37, 281 and *Agrobacterium rhizogenes* strain 15834 were also able to act as helper strains. However, an *E. coli* strain carrying a pTiB6S3::RP4 cointegrate (GV5001) did not restore the virulence of GV10pGV2027a, which might be due to lack of expression of the Ti plasmid in *E. coli*. This finding predicts that *E. coli* strains carrying

cloned fragments of the Ti plasmid cannot be used to analyze the observed phenomenon.

Surprisingly, 12 avirulent mutants with insertions in the different subregions of the *vir* region of pTiB6S3 (as defined by Iyer et al. 1982) or pTiC58 were unable to restore the virulence of GV10pGV2027a (or have their own virulence restored by GV10pGV2027a). Iyer et al. found a similar negative result with a *vir* E mutant when it was mixed with other *vir* mutants (Iyer et al. 1982). We conclude therefore that a mutation in any one of the other *vir* subregions prevents the proper functioning of the region mutated in pGV2027, in other words, the function of this latter region is apparently dependent on intact *vir* subregions present within the same helper cell. This finding unfortunately excludes the possibility to use *Agrobacterium* strains carrying small *vir*E fragments as helper strains to further define the extent of the region involved. The same finding could explain why Klee et al. (Klee et al. 1982) could not restore the virulence of a *vir* E mutant in a coinfection experiment with a helper strain which carried only part of the *vir* region.

Strains lacking the T-DNA-encoded tumour growth functions, GV3105pGV2217, LBA4404pAL4404, LBA4412pAL4412 and LBA4301pGV3850 were found to act as helper strains, showing that the T-DNA is not involved in the helper effect. Interestingly, LBA4404pAL4404 which carries a large deletion extending into *HindIII*-14 (about 15 kb to the right of the region mutated in the Ti plasmid of GV10pGV2027a) still showed full helper activity. At present, pAL4404 thus represents the smallest Ti plasmid still capable of restoring the virulence of GV10pGV2027a. Experiments are now in progress to define more precisely the region mutated in GV10pGV2027a and to establish the nature of the factor which we propose is excreted by helper strains and enables GV10pGV2027a to introduce its T-DNA stably into plant cells.

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