Mutagenesis by Insertion of Tn4001 into the Genome of *Spiroplasma citri*: Characterization of Mutants Affected in Plant Pathogenicity and Transmission to the Plant by the Leafhopper Vector *Circulifer haematoceps*

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Two hundred and fifty-seven transposon Tn4001 mutants of *Spiroplasma citri* strain GII3 were used for transmission assays by the leafhopper vector *Circulifer haematoceps* intoperiwinkle (*Catharanthus roseus*) plants. Multiplication of the mutants in the two hosts, the leafhopper and the plant, as well as the symptom expression in the plant were studied. Two mutants, GMT 470 and GMT 553, caused no symptoms on plants. Tn4001 is inserted as a single copy in the genome of these mutants. Mutant GMT 470 did not multiply, or multiplied only poorly, in the leafhopper and was not transmitted by the insect to the plant, nor to culture medium through Parafilm membrane. The growth rate of GMT 470 in SP4 medium was twice as slow as that of wild-type strain GII3. Mutant GMT 553 multiplied in the leafhopper as well as the wild-type spiroplasma, and was transmitted by the leafhoppers into the plants, where it reached the same titers as the wild-type strain but in approximately twice as much time. The plants containing high titers of mutant GMT 553 remained symptomless for several weeks. However, symptoms began to develop at a time when revertants that had lost the transposon were detected.

Among members of the class *Mollicutes* (wall-less gram-positive eubacteria with low guanine plus cytosine DNA) (Weisburg et al. 1989), the genus *Spiroplasma* is one of the largest, with more than 30 described or proposed species (Williamson et al. 1996). The spiroplasmas are characterized by motility and helical morphology, and by their association with arthropod hosts, especially insects (Williamson et al. 1989; Hackett and Clark 1989). The first-cultured and most-studied spiroplasma is *Spiroplasma citri*, one of three plant-pathogenic, sieve-tube-restricted, and leafhopper-vector-transmitted agents (Calavan and Bové 1989; Bové et al. 1989). *S.*

citri is recognized as the first plant-pathogenic mollicute to have been cultured and characterized as the result of etiological studies of citrus stubborn disease (Saglio et al. 1973), and for which Koch's postulates were fulfilled (Markham et al. 1974). Available in culture since 1970, *S. citri* has been the object of many studies concerning its cellular and molecular biology. (Bové et al. 1989; Bové 1993). Viral and plasmid vectors have been developed for gene cloning and expression in the spiroplasma (Renaudin and Bové 1994; Renaudin et al. 1995). In spite of these studies, little was known concerning the genetic determinants that govern the interaction between the two hosts in which *S. citri* multiplies: the leafhopper vector and the plant. Existence of such determinants is indicated by the fact that certain strains of *S. citri* multiply in the leafhopper vector but are not transmitted by the leafhopper to the host plant, under conditions in which other strains are transmitted efficiently (Bové et al. 1988; Wayadande and Fletcher 1995). In addition, variation in symptom severity among stubborn diseased citrus trees has been noted by several investigators and was considered to be due to differences among various strains of the pathogen (Gumpf and Calavan 1981). Spiroplasma genes involved in the interactions between the pathogen and its two hosts can be identified by mutagenesis and adequate screening procedures. We have developed a technique for *S. citri* mutagenesis by random insertion of transposon Tn4001 into the spiroplasmal genome (Foissac et al., in press). Tn4001 transpositional mutagenesis has been previously used to analyze *Mycoplasma pneumoniae* cytoadherence (Hedreyda and Krause 1995). We have obtained several hundred Tn4001 mutants of *S. citri* strain GII3. Two hundred and fifty-seven mutants have been screened for their ability to be transmitted by the leafhopper to the plant and to multiply actively in the plant, without inducing symptoms in the infected plants. One such mutant, GMT 553, has been characterized and described in this paper.

RESULTS

Screening for GMT mutants affected in leafhopper transmissibility and phytopathogenicity.

Each GMT mutant in gentamicin-SP4 medium was micro-injected into 20 female leafhoppers, and these were caged

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onto two periwinkle plants (plants A and B; 10 insects per plant) for a 2-week transmission period at $30 \pm 2^\circ\text{C}$, as described in Materials and Methods. For each transmission experiment with GMT mutants, wild-type strain GII3 and *S. citri*-free SP4 medium were used, at the same time and under the same conditions, as positive and negative controls, respectively. At the end of the 2-week transmission period, the insects were killed, and the plants were observed for symptom development during a 4-week post-transmission period. When all 257 GMT mutants had been screened, the results were as follows. The 32 plants from the *S. citri*-free leafhoppers remained symptomless. The plants from the leafhoppers microinjected with the wild-type strain GII3 began to show mar-

Fig. 1. Periwinkle plants infected with various GMT mutants at the end of the 4-week transmission period. Symptomatic plants correspond to some of the 248 GMT mutants and are indistinguishable from plants infected with wild-type strain GII3. The two symptomless plants correspond to GMT mutant 553. With GMT mutant 470, two similar symptomless plants were obtained.

Fig. 2. Southern blot hybridizations between the DNAs of GII3, GMT 470, and GMT 553 and a *Tn4001*-specific probe. DNA from GMT 470 (A, lane 2), GMT 553 (B, lane 3), and wild-type strain GII3 (A, lane 1; B, lane 1) was digested with *Eco*RI. Positive control is plasmid pISM1001 digested with *Bam*HI (B, lane 2). DNA size standards and size of hybridizing *Eco*RI fragments are indicated in margins in kb.

ginal chlorosis within the first week of the post-transmission period, and at the fourth week, symptoms (leaf yellows and stunting) were severe. With 248 of the 257 GMT mutants, the two plants (plants A and B) corresponding to each mutant developed the same symptoms and at the same time as the plants infected with the wild-type strain GII3. For seven of the GMT mutants, one of the two plants was lost, but the second plant showed the same symptoms as those from the other 248 GMT mutants. Finally, for two mutants, GMT 470 and GMT 553, plants A and B of each mutant were both symptomless and indistinguishable from the healthy control plants at the end of the 4-week post-transmission period, as shown in Figure 1 for GMT 553.

Southern blot hybridizations with a *Tn4001*-specific probe were carried out with the *Eco*RI-digested DNAs of the two mutants. A single hybridization signal at 10.2 kb was observed in DNA from mutant GMT 470 (Fig. 2A, lane 2). With DNA from mutant GMT 553, the signal was 9.4 kb (Fig. 2B, lane 3). No hybridization signal was observed with GII3. These results confirm that *Tn4001* is present as a single copy and is inserted at different sites in the genomes of the two mutants.

No symptoms were obtained after inoculation with mutants GMT 470 and GMT 553. This may be due either to the fact that the plants were not infected (no transmission from the leafhoppers to the plant or no multiplication in the plant) or because the mutants did not induce symptoms in the plant. To examine these alternatives, the occurrence of *S. citri* in the plants corresponding to GMT 470 (plants 470A and 470B) and GMT 553 (plants 553A and 553B) was examined by trying to culture *S. citri* from the four plants. The culture assays were carried out 3 weeks after the transmission period. No spiroplasma could be cultured from plants 470A and 470B, but *S. citri* cultures were obtained with plants 553A and 553B. To verify that the spiroplasma isolated from these plants was GMT 553, the genomic DNAs of the spiroplasmas cultured from periwinkles 553A and 553B were digested with *Eco*RI and hybridized with the *Tn4001*-specific probe. As shown in Figure 3, the probe hybridized with a restriction fragment of 9.4 kb (lane 4 and 5) and was the same size as the hybridizing fragment in the GMT 553 mutant (lane 3). No hybridization was obtained with wild-type strain GII3 (lane 2). These results

Fig. 3. Southern blot hybridization between *Eco*RI-digested genomic DNA from various *Spiroplasma citri* isolates and the *Tn4001*-specific probe. DNA from GII3 (lane 2), GMT 553 (lane 3), and *S. citri* isolated from periwinkle plant 553A (lane 4) and 553B (lane 5) 3 weeks after the transmission period. Positive hybridization control is native nondigested plasmid pMUT (lane 1). DNA size is indicated in kb.

indicate that symptomless periwinkle plants 553A and 553B were indeed infected with *S. citri* mutant GMT 553. Infection of these plants by the spiroplasma was also demonstrated at the end of the 4-week post-transmission period by immunofluorescence with *S. citri*-specific monoclonal antibody FD1 (results not shown). Under the same conditions, periwinkle plants 470A and 470B, from which no spiroplasmas could be cultured, gave negative immunofluorescence reactions.

The screening assay has thus revealed two particular GMT mutants of *S. citri*. One mutant, GMT 553, was transmitted by the leafhoppers to the periwinkle plants but did not induce symptoms in these plants during the 4-week post-transmission period. This mutant could be a phytopathogenicity mutant, i.e., a mutant affected in a gene involved in symptom production. However, absence of symptoms would also be obtained if the mutant could not reach high enough titers in the plant for symptom expression. The second mutant, GMT 470, could not be detected in the plants, either because it was not transmitted by the leafhoppers to the plant or because it could not multiply in the plant. To investigate these possibilities, the multiplication of the two mutants in the leafhoppers and the plants and their transmission by the insect were further studied in comparison with the wild-type strain GII3.

Multiplication rate of GMT 470, GMT 553, and GII3 in leafhoppers.

Groups of 150 female *C. haematoceps* leafhoppers were microinjected with GMT 470, GMT 553, or GII3 in SP4 medium as well as with *S. citri*-free SP4 medium, and caged on three stock plants, the preferred host of the leafhoppers (50 injected insects per plant). At 0, 3, 6, 9, 12, and 15 days after injection, 10 leafhoppers of each group were collected from the three stock plants and used to determine the number of CFU per insect, as indicated in Materials and Methods. No spiroplasmas were cultured from the leafhoppers injected with *S. citri*-free SP4 medium. GMT 553 multiplied in the leafhopper at the same rate as the wild-type strain GII3 (Fig. 4). Strain GII3 grew from 7×10^4 CFU per insect at time 0 to 5×10^6 CFU per insect at 6 days after injection. For GMT 553, the values were 3×10^4 CFU per insect at time 0 and 5×10^6 CFU per insect, 6 days later. In contrast, GMT 470 never reached a titer higher than that present at time 0 (2×10^4 CFU per insect). Thus, the two GMT mutants, starting with similar titers at time 0, behaved differently in the leafhoppers. GMT

Fig. 4. Multiplication of GII3, GMT 470, and GMT 553 in injected leafhoppers. CFU per insect are average values from 10 insects.

553 grew as well as the wild-type strain, while GMT 470 did not multiply, or multiplied only slightly.

Multiplication of GMT 470 and GII3 in vitro.

Growth of the two mutants was different in gentamicin-SP4 medium. While the growth of GMT 553 in gentamicin-SP4 medium was similar to that of GII3 in gentamicin-free SP4 medium (result not shown), GMT 470 grew in gentamicin-SP4 medium at a slower rate and reached lower titers than GII3 (Fig. 5). The doubling time of GMT 470 was approximately 10 h while that of GII3 was 5 h. On solid 1.5% agar SP4 medium, the colonies of GII3 in the absence of gentamicin and those of GMT 553 in the presence of gentamicin both reached a diameter of 0.12 mm while those of GMT 470 (in the presence of gentamicin) had a diameter of 0.02 mm only.

Ability of leafhoppers to inject GMT 470, GMT 553, and GII3 into SP4 medium.

As indicated above, GMT 470, in contrast to GMT 553, could not be cultured from, and could not be detected by immunofluorescence in, periwinkle plants on which leafhoppers microinjected with GMT 470 were allowed to feed. Failure of GMT 470 to infect periwinkle plants could be due to failure of the leafhopper to inject the mutant into the plant during feeding. To check this possibility, microinjected leafhoppers were kept for 2 weeks on stock plants before they were allowed to feed on SP4 medium through Parafilm membranes as described previously (Foissac et al. 1995), and the presence of *S. citri* in the medium was examined. No CFU were obtained from the media on which leafhoppers injected with SP4 medium alone or GMT 470 had fed, while appreciable numbers of CFU were obtained in the case of GMT 553 and GII3, for which the CFU numbers were similar (Table 1).

These results suggest that failure of GMT 470-injected leafhoppers to infect plants is due to the inability of the mutant to be injected into the plant. In contrast, GMT 553 is inoculated by the leafhoppers into the medium as well as into the plants since the spiroplasmas can be recovered from both the medium and the plants. The fact that these plants are symptomless could be due to the failure of GMT 553 to reach titers high enough for symptom expression. Therefore, experiments were undertaken to evaluate the titers reached in the plants by GMT 553 in comparison with GII3 (positive control)

Fig. 5. Multiplication of GII3 in SP4 medium and GMT 470 in gentamicin-SP4 medium. Solid boxes, wild-type strain GII3; open boxes, mutant GMT 470.

and GMT 470 (negative control). Also, symptom expression was followed for periods longer than the 4-week post-transmission period used in the screening procedure.

Multiplication of GMT 470, GMT 553, and GII3 in periwinkle plants, and their relationship to symptom expression.

Microinjected leafhoppers were immediately caged for a 2-week transmission period on young periwinkle plants (10 insects per plant). Periwinkle plants were submitted to infection with GII3, GMT 553, and GMT 470 through leafhopper transmissions, and were followed for symptom expression (Table 2). The number of CFU present in 0.2 g of leaf midribs was determined in four plants of each group, 2 and 4 weeks after the transmission period. In the case of leafhoppers microinjected with SP4 medium or GMT 470, no spiroplasmas could be cultured from the relevant plants. Two weeks after the transmission period, the titer of GII3 in the corresponding plants was already high and averaged 5×10^6 CFU per 0.2 g

of midribs, while the titer of GMT 553 was lower than that of GII3 and ranged from 0.5×10^2 to 10^5 according to the considered periwinkle plant. However, 4 weeks after the transmission period, the titers for the two spiroplasmas were more similar: an average of 2×10^7 for GII3 and 4.3×10^6 for GMT 553. In periwinkle plant 1 infected with GMT 553, the titer reached 10^7 and was similar to that of GII3 in plants 1, 3, and 4. Symptom expression was followed up to 24 weeks after the transmission period (Table 2). In the case of noninjected leafhoppers or leafhoppers microinjected with SP4 medium alone or GMT 470, no symptoms were observed, and the plants were indistinguishable from healthy plants. With GII3, the nine periwinkle plants began to show symptoms within the first week after the transmission period. After 2 to 3 weeks, all plants were severely affected, and by week 10 they had died. In the case of GMT 553, all 15 plants remained symptomless for 4 weeks; symptoms appeared in week 5, but on only six of 15 plants and these six plants had severe symptoms by week 8, by which time seven other plants had no symptoms or only mild symptoms. All plants, except two, were severely affected by week 16, and six plants were dead by week 24. With some of the GMT 553 plants (plants 5, 14, and 15), symptoms appeared much later, during week 10 for plant 5, week 16 for plant 14, and week 19 for plant 15. Also, these three plants had symptoms on only one or two old shoots while the young shoots and the apical parts of the plants were still symptomless. Spiroplasma could be cultured from the symptomless as well as the symptomatic leaves of these plants at week 24.

These experiments show that 4 weeks after the transmission period, a time at which some GMT 553-infected plants had reached titers similar to those of GII3, the plants infected with

Table 1. Spiroplasmas present in culture medium after inoculation through Parafilm membranes by leafhoppers infected by injection

Inoculant	CFU ^a	
	Exp. 1 ^b	Exp. 2 ^c
GI3	115 ± 65	243 ± 34
GMT 553	105 ± 25	300 ± 25
GMT 470	0	0
SP4 medium	0	0

^a CFU inoculated per 10 insects in 24 h, 2 weeks after injection.

^b Average of two experiments performed with 10 insects during 24 h.

^c Average of two experiments performed with 20 insects during 48 h.

Table 2. Symptom development and spiroplasma titers in periwinkle plants at various weeks post transmission

Inoculant	Plant (no.)	Symptoms ^a									CFU ^b	
		Week no.									2	4
		1	2	3	4	5	6	8	10	24		
<i>Spiroplasma citri</i> GII3	1	++	+++	+++	+++	+++	+++	++++	Dead	...	4.2×10^6	10^7
	2	++	+++	+++	+++	+++	+++	++++	Dead	...	ND	4.5×10^7
	3	++	+++	+++	+++	+++	+++	++++	Dead	...	6.6×10^6	1.2×10^7
	4	++	+++	+++	+++	+++	+++	++++	Dead	...	ND	1.5×10^7
	5 to 7	+	++	+++	+++	+++	+++	++++	Dead	...	...	...
<i>S. citri</i> GMT 553	8 and 9	++	+++	+++	+++	+++	+++	Dead	...	...	...	...
	1	-	-	-	-	+	++	+++	+++	Dead	9.2×10^3	10^7
	2	-	-	-	-	+	++	+++	+++	Dead	0.5×10^2	10^6
	3	-	-	-	-	+	+	+++	+++	Dead	10^5	4×10^6
	4	-	-	-	-	+	+	+++	+++	Dead	3.7×10^4	2.3×10^6
	5	-	-	-	-	-	-	-	++ ^c	+++ ^c	...	...
	6	-	-	-	-	-	-	+	++	+++	...	...
	7	-	-	-	-	-	+	+++	+++	Dead	...	...
	8	-	-	-	-	-	-	+	++	+++	...	...
	9	-	-	-	-	-	-	++	++	+++	...	...
	10	-	-	-	-	-	-	+	+++	+++	...	...
	11	-	-	-	-	+	++	+++	+++	Dead	...	...
	12	-	-	-	-	+	+	++	+++	+++	...	...
	13	-	-	-	-	-	-	+	++	+++	...	...
	14	-	-	-	-	-	-	-	-	+++ ^c	...	...
	15	-	-	-	-	-	-	-	-	++ ^c	...	...
<i>S. citri</i> GMT 470	1 to 4	-	-	-	-	-	-	-	-	-	0	0
	5 to 20	-	-	-	-	-	-	-	-	-	...	...
<i>S. citri</i> -free SP4 medium	1 to 4	-	-	-	-	-	-	-	-	-	0	0

^a -, no symptom; +, mild, marginal chlorosis on young leaves; ++, assymetric and chlorotic young leaves, mild chlorosis on older leaves; +++, stunting and systemic leaf yellows; +++++, lethal wilting.

^b CFU per 0.2 g of leaf midribs; ND = not done.

^c Symptoms were present only on one or two old shoots.

GMT 553 were still symptomless, while plants infected with GII3 showed severe symptoms. However, the GMT 553–infected plants did not remain symptomless. They eventually developed symptoms after week 5, although some plants remained symptomless for 16 weeks.

In a second experiment, titers of GMT 553 and GII3 were determined in three different periwinkle plants, 2, 3, 4, 5, and 6 weeks after the transmission period. GMT 553 and GII3 had different titers only at week 1: an average of 2×10^3 CFU per 0.2 g of midribs for GMT 553 and 10^6 for GII3. From week 2 on, the titers of GMT 553 were as high as those of GII3 and had an average plateau value of 1.5×10^6 . In this experiment, the three GII3–infected periwinkle plants showed symptoms during the first week after transmission, but the GMT 553–infected plants showed symptoms only during the fourth week, a time at which the titer of GMT 553 had already peaked at 10^6 for 2 weeks.

In the leafhoppers and the plants, the Tn4001 *S. citri* mutants are not subjected to gentamicin pressure and could revert to the wild-type strain. The symptoms eventually observed on the GMT 553–periwinkle plants could be due to such revertants. The following experiments were conducted to examine this possibility.

Reversion of *S. citri* mutant GMT 553 to wild-type strain GII3 in periwinkle plants.

The two periwinkle plants 1 and 3 infected with GMT 553 (Table 2) were used 18 weeks after the transmission period, for spiroplasma isolation by culture in SP4 medium free of gentamicin. Aliquots of the primary cultures were directly plated on SP4 medium containing 100 µg of gentamicin per ml. No gentamicin-resistant colonies were detected, indicating that the spiroplasmas isolated from the plants at week 18 were gentamicin-sensitive. Aliquots of the primary cultures obtained from the two plants were also microinjected into leafhoppers; at the same time, additional leafhoppers were injected with GMT 553, GII3, or spiroplasma-free medium. The leafhoppers were used to infect three periwinkle plants per injected material. While the three plants infected with GMT 553 developed no symptoms during the first 4 weeks after the

transmission period, the plants infected with the spiroplasmas from the two primary cultures developed symptoms within the first week of the post-transmission period, and thereafter they were indistinguishable from the plants infected with GII3. These results suggested that gentamicin-sensitive phenotypic revertants were present in the plants 18 weeks after their infection with gentamicin-resistant GMT 553.

A second experiment was initiated to search for genotypic revertants of GMT 553, i.e., *S. citri* cells having lost Tn4001 at the site of insertion. Three periwinkle plants infected with GMT 553 through leafhopper transmission were used to isolate *S. citri* by culture in liquid medium each week from week 1 to week 7 after the 2-week transmission period (Table 3). The presence of Tn4001 in the spiroplasma was determined by polymerase chain reaction (PCR), as described in Materials and Methods. Plants infected with GII3–injected leafhoppers were used as controls. Figure 6 and Table 3 show that indeed *S. citri* revertants could be detected. The revertants appeared between week 4 and week 6, at a time when the plants showed early, mild symptoms (Table 3). Thereafter, the percentage of revertants, as determined from the number of colonies obtained in the presence and absence of gentamicin, increased rapidly; for instance, in the case of plant 553 p8, it reached 66% at week 4 and 96% at week 7. These results show that in plants infected with GMT 553, revertants do occur, and that there is concomitance between appearance of symptoms and detection of revertants.

DISCUSSION

Two hundred and fifty-seven gentamicin-resistant mutants of *S. citri* strain GII3 were obtained by random insertion of transposon Tn4001 into the spiroplasmal genome (Foissac et al., in press). For two of these mutants tested, the transposon was stably maintained over 80 generations in a nonselecting medium (Foissac et al., in press). This period is sufficient to screen for mutants affected in their interaction with the two spiroplasmal hosts, the leafhopper vector and the periwinkle plant, in which no gentamicin selection pressure can be maintained. After microinjection into the leafhoppers, each

Table 3. Symptom development and reversion of mutant GMT 553 to wild-type spiroplasma in infected periwinkle plants

Plant ^a	Weeks post transmission period						
	1	2	3	4	5	6	7
GII3 p5	– ^b	+	+++	+++	+++	+++	+++
GII3 p6	+	++	+++	+++	Dead	...	...
GII3 p8	+	++	+++	+++	+++	++++	++++
GII3 p9	+	++	+++	+++	++++	Dead	...
GII3 p10	+	++	+++	+++	++++	Dead	...
T1	–	–	–	–	–	–	–
T2	–	–	–	–	–	–	–
553 p1	–/NR ^c	–/NR	–/NR	+/NR	+/R	+/R	++/R
553 p7	–/NR	–/NR	–/NR	–/NR	–/NR	+/R	+/R
553 p8	–/NR	–/NR	–/NR	+/R ^c	+/R	++/R	++/R

^a T1, T2: plants from leafhoppers injected with *Spiroplasma citri*–free SP4 medium; GII3 pX: plants from leafhoppers injected with *S. citri* GII3; 553 pX: plants from leafhoppers injected with mutant GMT 553.

^b –, no symptom; +, mild, marginal chlorosis on young leaves; ++, asymmetric and chlorotic young leaves, mild chlorosis on older leaves; +++, stunting and systemic leaf yellows; +++++, lethal wilting.

^c NR = no revertants detected by polymerase chain reaction (PCR); R = revertants detected by PCR.

Fig. 6. Detection of GMT 553 revertants by polymerase chain reaction. Lanes 1 and 12: 100-bp molecular marker; lane 2: no DNA; lane 3: GII3 DNA; lane 4: GMT 553 DNA; lanes 5 to 11: DNAs from spiroplasmas cultured from leaves taken on plant 553 p8 (Table 3), at weeks 1, 2, 3, 4, 5, 6, and 7, respectively.

one of the 257 mutants was examined over a 6-week screening period for the following properties: (i) multiplication in the insect and transmission from the insect to the plant over a period of 2 weeks (transmission period); (ii) multiplication in the plant; and (iii) induction of symptoms over a period of 4 weeks (post-transmission period).

Two hundred and fifty-five mutants induced symptoms during the first 2 weeks of the post-transmission period as fast and as severe as those induced by the wild-type strain, and these mutants were not studied further. With two mutants only, GMT 470 and GMT 553, no symptoms could be seen at the end of the 4-week post-transmission period. Southern blot hybridization with a *Tn4001*-specific probe confirmed that the two mutants had a single copy of the transposon inserted at different insertion sites. GMT 470 could not be detected in or cultured from the plants on which the leafhoppers microinjected with GMT 470 had fed. In fact, these plants were probably not infected, because the mutant was not transmitted by the leafhoppers to the plant. The following three arguments support this view.

First, leafhoppers separated from sucrose-supplemented SP4 culture medium by a Parafilm membrane are able to feed on the medium by puncturing the membrane with their stylets. This artificial feeding procedure resembles the natural situation in which leafhoppers feed on plants. When feeding through the Parafilm membrane, leafhoppers infected with the wild-type strain GII3 or mutant GMT 553 were able to inject the spiroplasma into the medium and the number of spiroplasmas (CFU) present was determined by plating on solid medium and counting the colonies. With GMT 470, no colonies were obtained, indicating that the leafhopper did not inject the mutant into the medium.

Second, the number of GMT 470 spiroplasmas present in the leafhoppers did not increase during the 15 days following microinjection of the mutant. In certain experiments, no spiroplasmas could be recovered. These results indicate that GMT 470 did not multiply, or multiplied only poorly, in the leafhoppers. Under the same conditions, GMT 553 multiplied as well as the wild-type strain GII3 in the leafhopper.

Third, GMT 470, in liquid SP4 medium, grew less well than GII3 or GMT 553; the doubling time was approximately twice as long, and the growth curve reached a plateau value of 5×10^8 CFU per ml, lower than the 10^{10} CFU per ml for GII3. On solid SP4 medium, the GMT 470 colonies had a diameter 6 times smaller than those of GII3 or GMT 553.

These results show that GMT470 is affected in its growth capacity in the leafhoppers and also, even though at a lower level, in the culture medium. We have recently shown that, in GMT 470, *Tn4001* is inserted into a gene encoding a putative product that has appreciable homology with a calcium-transporting ATPase (C. Paré, C. Saillard, and J. M. Bové, *unpublished results*). The fact that GMT 470 is not injected by the leafhoppers into the plants or into growth medium through Parafilm membranes is probably related to the very poor multiplication of GMT 470 in the leafhoppers. To be injected by the leafhopper into plants or through Parafilm membranes, *S. citri* must penetrate into the salivary glands. *S. citri* variants that are not able to achieve this last step after multiplication in the insect have been obtained by extensive culture in artificial medium or long presence in herbaceous hosts (Bové et al. 1988; Wayadande and Fletcher 1995; Lallemand 1987). Efforts to determine the presence of GMT 470 in the salivary

glands were not undertaken, as the spiroplasma grew poorly or not at all in the insect. Moreover, as gentamicin selection cannot be maintained for a long time in the injected insects, transmission experiments, performed after 4 weeks of incubation, could be altered by the appearance of revertants.

In contrast to mutant GMT 470, GMT 553 grew to the same high titers as GII3 in the leafhoppers, and was transmitted by the leafhoppers to the plants or through Parafilm membranes. In liquid or on solid SP4 medium, and in the insect, the growth of GMT 553 was indistinguishable from that of GII3. As the plants infected with GMT 553 showed no symptoms at the end of the 4-week post-transmission period of the screening assay, the growth of the mutant in the plant was of special interest. The mutant reached approximately the same titers as GII3 (10^7 CFU per 0.2 g of midrib) but it took longer to do so: i.e., 2 to 4 weeks instead of 1 to 2 weeks. All plants infected with GII3 began to show symptoms during the first week of the post-transmission period and symptoms became severe during the second week, at a time when the spiroplasma titer in the plant had not yet reached its highest value. With GMT 553, plants remained symptomless for 4 weeks post-transmission, and were still symptomless at a time when the spiroplasma titer was high. However, the plants did not remain symptomless. The first symptoms appeared approximately during week 5 and became severe from week 8 on. The development of symptoms in plants infected with GMT 553 is probably not due to the mutant itself, but to the reversion of the mutant to the wild-type strain by excision of the transposon, for the following reasons. (i) Spiroplasmas isolated 18 weeks post-transmission from plants that remained symptomless for 4 weeks, and then developed severe symptoms thereafter, are phenotypically indistinguishable from the wild-type strain. In particular, they were gentamicin-sensitive and when used to infect plants through microinjected leafhoppers, they induced symptoms as early and as severe as GII3. (ii) The gene of GMT 553 in which *Tn4001* is inserted, which has been identified and sequenced, belongs to the fructose operon and codes for a protein that has appreciable homology with the repressor (DeoR) of the *Escherichia coli* deoxyribonucleotide operon (P. Gaurivaud, F. Laigret, and J. M. Bové, *unpublished results*). By PCR, *Tn4001*-free revertants of GMT 553 could be detected in the plants at the time when the first symptoms were seen. When, according to the periwinkle plant considered, symptoms appeared 4, 5, or 6 weeks post-transmission, revertants also appeared at week 4, 5, or 6. (iii) The percentage of revertants to GMT 553 increased with time; so did the severity of the symptoms. At week 7 post-transmission, 96% of the spiroplasmas isolated from the severely affected plants were gentamicin sensitive; at week 18, no gentamicin-resistant GMT 553 spiroplasmas could any longer be detected. These results strongly suggest that the symptoms eventually shown by the initially symptomless plants infected by GMT 553 are due to reversion of the mutant to the transposon-free wild-type spiroplasma. The revertant outgrows the GMT 553 mutant because its multiplication rate is twice as high. In this reversion process, excision of the transposon occurs as shown by PCR, and no reinsertion of the transposon takes place, as indicated by the gentamicin sensitivity of the revertants. Such a reversion process has already been observed in *S. aureus* for *Tn4001* auxotrophic mutants, at frequencies between 10^{-7} and 10^{-6} (Mahairas et al. 1989).

In conclusion, GMT 553 seems unable to induce symptoms in periwinkle plants. We have now identified the genome region of GMT 553 where Tn4001 is inserted. This region is part of the fructose operon. How insertion of Tn4001 in this operon results in symptom abolition remains to be understood.

MATERIALS AND METHODS

Spiroplasmas, plants, and leafhoppers.

S. citri strain GII3 was originally isolated from its leafhopper vector, *Circulifer haematoceps*, captured in Morocco (Vignault et al. 1980). Strain GII3 was selected as the wild-type strain because it is easily transmissible in the glass-house to periwinkle (*Catharanthus roseus*) plants by *C. haematoceps*, and it produces severe symptoms (stunting, leaf yellows) in these plants. All spiroplasmas were grown at 32°C in SP4 medium (Tully et al. 1977). None of the SP4 medium used in this work contained fresh yeast extract. Transposon insertion mutants of *S. citri* strain GII3 were obtained by electroporation of plasmid pMUT containing Tn4001 into spiroplasmal cells as previously described (Foissac et al., in press). These mutants were detected on the basis of their resistance to gentamicin resulting from the presence of Tn4001 in the genome (Rouch et al. 1987). In this way, 257 Tn4001 insertional mutants of strain GII3, called GMT, were obtained and distinguished from spontaneous gentamicin-resistant clones lacking Tn4001, by dot-blot hybridization with a Tn4001-specific probe (Foissac et al., in press). The colonies from the 257 GMT mutants were grown for one passage in liquid SP4 medium containing 100 µg of gentamicin per ml, and aliquots of each first-passage culture were kept at -20°C. For microinjection into leafhoppers, the first-passage culture of each GMT mutant was passaged again in gentamicin-SP4 medium, and this second-passage culture was injected into the insects. The presence of gentamicin in the SP4 medium has no effect on the injected leafhoppers, as previous experiments have indicated (results not shown).

Periwinkle and stock (*Mathiola incana*) plants were grown from seeds in a glasshouse at 25 ± 3°C with a photoperiod of 16 h. Periwinkle plants with 6 to 8 leaves were used for leafhopper-transmission assays. They were transferred from 25 to 30°C for a 2-day acclimatization period before being exposed to the insects.

The leafhopper *C. haematoceps* was used for transmission of *S. citri* to periwinkle plants. The initial insects were captured in Corsica (Fos et al. 1986). They were transferred to the Bordeaux laboratory and caged on 4- to 6-month-old stock plants for egg production and multiplication. Nymphs were transferred onto new stock plants. Each progeny leafhopper colony was raised on 4- to 6-month-old stock plants maintained in an insect-proof cage at 30 ± 2°C with a 16-h photoperiod. New colonies were initiated with insects collected in several old colonies so as to maintain the diversity of the leafhopper population. Colonies were frequently tested for accidental contamination with *S. citri*; such contamination was never observed.

Microinjection of leafhoppers and transmission assay.

Only female *C. haematoceps* leafhoppers were used for microinjection of *S. citri* into the insects, because of their strong resistance to the microinjection procedure (usually, more than 95% survival). Also, female injected leafhoppers live longer than similarly treated males when caged on plants

for transmission assays. CO₂-anaesthetized female leafhoppers were injected between the two last abdominal sternites, with hand-drawn glass needles, with approximately 0.1 µl of gentamicin-SP4 culture medium (about 10⁸ spiroplasmas per ml), as previously described (Foissac et al. 1995). Injected leafhoppers were caged on young periwinkle plants (6- to 8-leaf stage) for a period of 2 weeks at 30 ± 2°C (transmission period). For each one of the 257 GMT mutants, 20 female leafhoppers were used for microinjection of the spiroplasma, and the injected insects were caged on two separate plants (10 insects per plant). At the end of the transmission period, the leafhoppers were killed with insecticide (dichlorvos), and the plants were kept in the greenhouse at 30 ± 2°C. Under these conditions, with wild-type *S. citri* GII3, symptoms appear within the first week following the transmission period. Two weeks after the transmission period, symptoms are severe; 90 to 100% of the plants are found to be infected, and they die within 8 to 10 weeks after the transmission period.

Culture of *S. citri* from plants and insects.

Spiroplasma infection of plants submitted to infection by injected leafhoppers was determined as follows. Aliquots (0.2 to 0.5 g) of midribs from a mixture of young and old periwinkle leaves were minced with a razor blade in 2 ml of SP4 medium. After 20 min of incubation at room temperature, the extracts were passed through filter membranes (pore diameter: 0.45 µm). The number of spiroplasma CFU was determined by plating 10-fold serial dilutions of the filtrate on solid SP4 medium (3 plates per dilution).

In the case of insects, groups of 2 anaesthetized leafhoppers were ground in 2 ml of SP4 medium, in small conical Potter grinders. After 20 min of incubation at room temperature, the extracts were filtered and the filtrate was treated as above for CFU determination.

Spiroplasma detection in plants by immunofluorescence in situ.

The detection of *S. citri*, in 30- to 40-µm-thick longitudinal and cross sections of leaf midribs with monoclonal antibody FD1 specific to *S. citri*, was according to Martin-Gros et al. (1987).

Detection of revertants.

The presence of gentamicin-sensitive revertants in plants infected with GMT 553 was determined in two ways: (i) Extracts from leaf midribs were prepared as indicated above (culture of *S. citri*) and 10-fold dilutions in SP4 medium were plated on solid SP4 medium; colonies obtained in the presence or absence of gentamicin in the medium (100 µg/ml) were counted. (ii) Spiroplasmas were cultured from the first 10-fold dilution of the above extract. Their DNA was extracted and used for PCR amplification (35 cycles of 92°C, 1 min; 62°C, 30 s; 72°C, 1 min) with primers (5' TAACGGCTTGACGAGC 3', 5' GGAGCTGAATGTGGCC 3') designed in the regions surrounding the insertion site of Tn4001 in the genome. With spiroplasmas lacking Tn4001, an amplicon of 437 bp was obtained; in the presence of Tn4001, no amplification occurred, because of the large size of the transposon (4.7 kbp).

Transmission of *S. citri* through Parafilm membranes.

Female leafhoppers injected with *S. citri* were caged on healthy stock plants for 14 days at 30 ± 2°C, and transferred to

small, room temperature cages on which a Parafilm membrane separated the leafhoppers from 5% sucrose-supplemented SP4 medium deposited on the membrane (Foissac et al. 1995). When feeding through the Parafilm membrane, infected leafhoppers injected *S. citri* into the culture medium. After 24 or 48 h, at 20°C, SP4 medium from the Parafilm membrane was plated on solid SP4 medium for CFU counts. These experiments were conducted at 20°C to minimize multiplication of the injected spiroplasmas in the SP4 medium, since the optimal growth temperature for *S. citri* is 32°C.

Spiroplasma DNA isolation.

S. citri cells from a 2-ml culture were harvested by centrifugation (14,000 × g, 30 min) and resuspended in 200 µl of GTE buffer (25 mM Tris-HCl, pH 8.3; 50 mM glucose; 10 mM EDTA). Cells were lysed by heating at 65°C for 10 min in the presence of 0.5% sodium dodecyl sulfate. The lysate was treated with 10 µg of proteinase K at 37°C for 1 h. Nucleic acids were purified by phenol-chloroform extraction, ethanol-acetate precipitation, and resuspension in 20 µl of TE buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA).

Southern blot hybridizations.

DNAs were digested with *EcoRI*, blotted onto charged nylon membranes by alkali transfer, and probed according to standard procedures (Sambrook et al. 1989). The probe was a digoxigenin dUTP-labeled PCR fragment, extended from nucleotide 2023 to nucleotide 2144 of the published sequence of the central *HindIII* fragment of Tn4001 (Rouch et al. 1987). Hybridization signals were detected by the alkaline phosphatase conjugated antibody-CSPD luminescent system (Boehringer, Mannheim, Germany). Plasmid pISM1001 (Mahairas and Minion, 1989), kindly provided by C. Minion, and plasmid pMUT derived from pISM1001 (Foissac et al. 1996), were used as positive controls for hybridization.

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