Plasmid pSci6 from *Spiroplasma citri* Gll-3 confers insect transmissibility to the non-transmissible strain *S. citri* 44

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The insect-transmissible strain Gll-3 of *Spiroplasma citri* contains plasmids pSci1–6, five of which (pSci1–5) encode adhesin-like proteins and one (pSci6) encodes protein P32, which has been associated with insect transmissibility. In contrast, *S. citri* strains ASP-1 and 44, which cannot be transmitted via injection into the leafhopper vector *Circulifer haematoceps*, lack these proteins and also do not carry plasmids pSci1–6. To further study the apparent relationship between the presence of plasmids and insect transmissibility, plasmids from *S. citri* Gll-3 were introduced into the insect-non-transmissible *S. citri* strain 44 by electrotransformation using the *tetM* gene as the selection marker. Tetracycline-resistant transformants were shown to carry one, two or three distinct plasmids. Plasmids pSci1–6 were all detected in the transformants, pSci1 being the most frequently found, alone or together with other plasmids. Selected *S. citri* 44 transformants having distinct plasmid contents were submitted, separately or in combination, to experimental transmission to periwinkle (*Catharanthus roseus*) plants via injection into the leafhopper vector. The occurrence of symptomatic plants indicated that, in contrast to *S. citri* 44, spiroplasmal transformants were transmitted to the host plant, in which they multiplied. Spiroplasma cultures isolated from these infected plants all contained pSci6, leading to the conclusion that, under the experimental conditions used, transformation by pSci6 conferred insect transmissibility to *S. citri* strain 44. This is believed to be the first report of a phenotypic change associated with transformation of *S. citri* by natural plasmids.

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INTRODUCTION

Spiroplasma citri is a plant-pathogenic bacterium belonging to the class *Mollicutes*, a group of wall-less micro-organisms that have arisen from low-G + C, Gram-positive bacteria by regressive evolution (Weisburg *et al.*, 1989). Plant-pathogenic mollicutes, spiroplasmas and phytoplasmas cause many diseases affecting a large variety of crop plants worldwide (Firrao *et al.*, 2005; Lee *et al.*, 2000; Seemüller *et al.*, 2002). The bacteria inhabit the phloem sieve elements and are transmitted from plant to plant by phloem sap-feeding leafhopper insects, which therefore are responsible for the spread of the diseases. The relationships of plant mollicutes with their hosts have been recently reviewed (Bové *et al.*, 2003; Christensen *et al.*, 2005).

S. citri is the aetiological agent of stubborn disease of citrus (Saglio *et al.*, 1971, 1973) and brittle root disease of horseradish (Fletcher *et al.*, 1981). It is transmitted, in a persistent manner, by the leafhoppers *Circulifer haematoceps* (Fos *et al.*, 1986) and *Circulifer tenellus* (Liu *et al.*, 1983a).

Various studies have addressed the movement of *S. citri* into the intestines and salivary glands of its leafhopper vector (Kwon *et al.*, 1999; Liu *et al.*, 1983b). For transmission to occur, the ingested spiroplasmas must infect the gut epithelial cells before crossing into the haemolymph, where they continue to multiply and then migrate to other organs and eventually invade the salivary glands, from which they are injected into the phloem through the salivary duct during feeding. Thus, successful transmission of *S. citri* by its leafhopper vector relies on the ability of the spiroplasmas to cross two physical barriers, the gut epithelium and the salivary gland membrane. To explain how spiroplasmas pass through these barriers, a model has been proposed (Fletcher *et al.*, 1998), in which spiroplasmas adhere to receptors on the apical membrane of the gut epithelium cell, before being taken into the cell by endocytosis. After migrating within the cell, spiroplasmas are released by exocytosis and reach the haemocoel through the basal lamina. A similar mechanism has been proposed for migration of the spiroplasmas from the haemocoel into the salivary duct through the salivary gland cells. However, the genetic determinants and the molecular mechanisms

Abbreviation: ScARP, *S. citri* adhesion-related protein.

that govern the interactions between spiroplasmas and insect cells are still to be discovered.

Several surface proteins have been implicated in the transmission of *S. citri* by its leafhopper vector. In *S. citri* BR3, protein SARP1 is needed, *in vitro*, for adherence of spiroplasmas to insect cells (Berg *et al.*, 2001; Yu *et al.*, 2000), suggesting that this protein might be involved in the interaction of the spiroplasmas with insect cells. However, genetic evidence of its involvement is lacking. Using a reverse genetic approach, it has been shown that inactivation of a gene encoding a lipoprotein showing limited homology with the solute-binding protein of an ABC transporter resulted in a dramatic decrease of transmission efficiency, indicating that this protein might play a role in the transmission process (Boutareaud *et al.*, 2004). Similarly, a *S. citri* mutant devoid of spiralin, the major lipoprotein at the cell surface, was poorly transmitted as compared to the wild-type strain GII-3 (Duret *et al.*, 2003). In this case, studying interactions between spiroplasma and insect proteins revealed that, *in vitro*, spiralin acted as a lectin, binding glycoproteins of the vector insect (Killiny *et al.*, 2005). However, in spite of the absence of spiralin, the spiralin-less mutant, though at low efficiency, was still transmitted, indicating that insect transmission certainly involved several proteins other than spiralin. The fact that both transmissible and non-transmissible strains possess equal amounts of spiralin supports this idea. Recently, we showed that non-transmissible strains of *S. citri* lacked plasmids encoding adhesin-like proteins, whereas these plasmids were detected in all transmissible strains tested (Berho *et al.*, 2006).

Many *S. citri* strains have been shown to carry natural plasmids (Gasparich *et al.*, 1993; Ranhand *et al.*, 1980) and some of them were mapped and/or cloned in *Escherichia coli* (Archer *et al.*, 1981; Bové *et al.*, 1989; Mouchès *et al.*, 1983). For years, spiroplasma plasmids were designated as cryptic, since no phenotypic trait had been associated with their presence, and none of them had been sequenced until recently. *S. citri* GII-3 (wild-type) contains seven plasmids, pSciA and pSci1–6, which were first identified as circular contigs of 7.8–35.3 kbp during the *S. citri* genome sequencing project (Foissac *et al.*, 2004). In addition to proteins involved in DNA partitioning and conjugative gene transfer, these plasmids also encode proteins that are potentially involved in the interactions of the spiroplasma with its vector insect. Plasmids pSci1–5, in particular, encode eight proteins (ScARPs) sharing high homology with the adhesion-related protein SARP1 from *S. citri* BR3 (Berg *et al.*, 2001). In addition, plasmid pSci6 encodes a hydrophilic protein, P32, which has been associated with insect transmissibility (Killiny *et al.*, 2006). A comparison of protein profiles and plasmid contents of transmissible and non-transmissible strains of *S. citri* revealed that the non-transmissible strains possessed no ScARPs and no P32, and also did not possess plasmids pSci1–6 (Berho *et al.*, 2006; Killiny *et al.*, 2006). Such a correlation between the

occurrence of plasmids and the ability of the spiroplasma strains to be transmitted by the leafhopper vector suggested that plasmids pSci1–6 encoded genetic determinants that might be essential for insect-transmission. To further investigate this hypothesis, plasmids from insect-transmissible strain GII-3 were introduced into the plasmid-free, non-transmissible strain 44 by electroporation. Successful transmission of *S. citri* 44 transformants to the host plant via injection into the leafhopper vector strongly suggests that genetic determinants required for insect transmission of *S. citri* 44 are encoded by plasmid pSci6.

METHODS

Spiroplasma strains. *S. citri* GII-3 wild-type strain was originally isolated from its leafhopper vector *Curculifer haematiceps* captured in Morocco (Vignault *et al.*, 1980). By injection into the leafhopper vector, it can be experimentally transmitted to periwinkle (*Catharanthus roseus*) plants, in which it multiplies and induces severe symptoms (Foissac *et al.*, 1996, 1997). *S. citri* strains ASP-1 (Townsend *et al.*, 1977) and 44 (Hosseini Pour, 2000) (kindly provided by Dr Hosseini Pour, University of Kerman, Iran) were isolated from stubborn-diseased sweet orange trees in Israel and Iran, respectively. In contrast to the situation with GII-3, attempts to infect periwinkle plants by injection of *S. citri* ASP-1 or 44 into the leafhopper repeatedly failed. Accordingly, these strains were described as insect-non-transmissible strains.

Spiroplasmas were grown at 32 °C in SP4 medium (Whitcomb, 1983), from which fresh yeast extract was omitted. Spiroplasma cells were transformed by electroporation as previously described (Stamburski *et al.*, 1991) using 1–2 µg purified plasmid or various ligation mixtures. Spiroplasmal transformants were selected in the presence of 2 µg tetracycline ml⁻¹. The antibiotic concentration was progressively increased from 2 to 15 µg ml⁻¹ during passaging of the transformants.

Construction of *S. citri* strain 44/4. Extrachromosomal DNA was purified from an ASP-1 transformant carrying pSci4NT, in addition to the endogenous plasmid pIJ2000 (Archer *et al.*, 1981). Plasmid pIJ2000 shares the same restriction map as pSciA from *S. citri* GII-3 (GenBank accession no. AJ966734). After digestion by *Xho*I, which cleaves pIJ2000 but not pSci4NT, the digested DNA was used to transform *S. citri* 44 by electroporation. Eight tetracycline-resistant transformants were screened by PCR for the presence of pSci4NT and absence of pIJ2000. The selected *S. citri* 44 transformant carrying pSci4NT was named 44/4.

DNA isolation and Southern blot hybridization. Spiroplasma genomic DNA was prepared from 10 ml cultures using the Wizard genomic DNA purification kit (Promega), whereas plasmid DNA was purified from 25 ml cultures with the Wizard SV minipreps DNA purification kit (Promega). Southern blot hybridization of spiroplasmal DNA with appropriate digoxigenin-dUTP-labelled probes has been described elsewhere (André *et al.*, 2003). Probes S4, specific to *scarp4a*, and S235, hybridizing to all *scarp* genes except *scarp4a*, were produced by PCR amplification of genomic DNA from *S. citri* GII-3 with primer pairs S4F/S4R and S235F/S235R, respectively (Table 1). Gene *scarp4a* is carried by pSci4 whereas *scarp2b*, *3b* and *5a* are carried by pSci5, and *scarp2a*, *3a*, *3c* and *3d* by pSci4, pSci1, pSci3 and pSci2, respectively (Table 1) (Berho *et al.*, 2006). Probe P32 consisted of a 546 bp fragment of gene *p32* generated by PCR amplification with primer pair P32F/P32R (Table 1). Gene *p32* is carried by pSci6. Probe TetM (535 bp) was obtained by PCR amplification of pSRT2 (Lartigue *et al.*, 2002) with primer pair

Table 1. Primers

Primer	Nucleotide sequence (5'–3')	Position	Accession no.
S4F	AGTTTATGCAGACGGAAAC	10123–10142	AJ969072
S4R	ACGCATTCCAGCGTTAGAC	10495–10513	AJ969072
S235F	TAAACATTGATATTGCCAACCCC	3617–3639	AJ969069
S235R	GGTTAAAGTTGCAGAAATTATTATC	3973–3996	AJ969069
Tet1	CTGCAAAAGATGGCGTAC	521–538	X56353
Tet2	CGTAAATGTAGTACTCCAC	1037–1055	X56353
P32F	TACGAATTAATCATTCTAATAGC	24852–24876	AJ969074
P32R	TAGTCCGGCTTGCTCACCA	24332–24351	AJ969074

Tet1/Tet2 (Table 1). The tetracycline-resistance cassette carrying the *tetM* gene under the control of the spiralin gene promoter was retrieved from pSRT2 as a 2.3 kbp *Pst*I restriction fragment.

Direct immunoblotting of colonies. Colony blot immunoassays were conducted as described previously (Duret *et al.*, 2003). Monoclonal antibody 10G3 (Berho *et al.*, 2006), specific to ScARPs, was used as the primary antibody.

Experimental transmission assay. Uninfected leafhoppers *Circulifer haematodeps* were reared on stock (*Matthiola incana*) plants at 28 °C with a photoperiod of 17 h. Micro-injection of *S. citri* into *C. haematodeps* leafhoppers and transmission to the periwinkle host plant (*Catharanthus roseus*) have been described previously (Foissac *et al.*, 1996; Duret *et al.*, 2003). Briefly, the insects were microinjected with 0.2 µl spiroplasma culture (approx. 10⁵ spiroplasma cells) and the injected insects were caged on young periwinkle plants (12 insects per plant, 5–10 plants per spiroplasma strain) for a 2 week transmission period. After insect removal, the plants were kept in the greenhouse at 30 °C for symptom production. Under these conditions, the plant symptoms induced by the wild-type strain GII-3 were produced within 2–4 weeks post-transmission. Alternatively, in the experiments of Table 6, injected insects were first caged on stock plants for a latent period of 10 days, during which the spiroplasmas multiplied in the insects, before being transferred to young periwinkles (five insects per plant) for a transmission period of 10 days. Culture of *S. citri* from plants and insects was carried out as described previously (Duret *et al.*, 2003; Foissac *et al.*, 1996, 1997), except when otherwise stated. Spiroplasma titres in the insects were determined at the end of the transmission period from groups of three to five living insects. Isolation of spiroplasmas from plants was carried out 6–8 weeks post-transmission.

For acquisition of spiroplasmas through feeding, female, adult leafhoppers were fed on symptomatic, spiroplasma-infected periwinkles (two plants, 60 insects per plant) for 10 days and then transferred to healthy stock plants for a latent period of 20 days to allow spiroplasma multiplication. At the end of the latent period, the living insects were collected and tested for the presence of spiroplasmas. Spiroplasma isolation from insects was performed as described previously (Foissac *et al.*, 1996), except that the leafhoppers were tested individually, each insect being crushed in 1 ml SP4 medium.

RESULTS

Transformation of *S. citri* 44 and ASP-1 with plasmids from *S. citri* GII-3

S. citri GII-3 possesses plasmids pSci1–6, which are absent in the insect-non-transmissible strains 44 and ASP-1 (Berho

et al., 2006). To determine whether *S. citri* GII-3 plasmids could be transferred to strains 44 and ASP-1, the tetracycline-resistance gene *tetM* was introduced into the plasmids and used as the selection marker. The cloning strategy was based on the observation that plasmids pSci1–6 all possessed one *Nsi*I (pSci1 and pSci3–5) or *Pst*I (pSci5–6) restriction site, except pSci2, which has two *Nsi*I sites (Fig. 1). Extrachromosomal DNA purified from *S. citri* GII-3 was digested with *Nsi*I or *Pst*I to yield linear molecules.

Fig. 1. Partial gene organizations and restriction maps of plasmids pSci1–6 from *S. citri* GII-3. Only *mob*, *soj*, *p32*, *scarp* (2a, 2b, 3a, 3b, 3c, 3d, 4a and 5a), *trsE* and *traG* genes are indicated on the maps. Asterisks (*) indicate truncated genes. *arp**, truncated *scarp*-like sequences. Plasmids pSci1–6 were given GenBank accession numbers AJ969069–74.

Fig. 2. Direct immunoblotting of colonies from *S. citri* GII-3 (a), *S. citri* 44 (b) and transformants of *S. citri* 44 (c) with mAb10G3. *S. citri* 44 transformants were those obtained by transformation of *S. citri* 44 with the *Nsi*I ligation mixture.

The *Nsi*I and *Pst*I restriction fragments (approx. 5 µg) were separately ligated to approximately 0.5 µg of the tetracycline-resistance cassette, which was retrieved from pSRT2 (Lartigue *et al.*, 2002) as a 2.3 kbp *Pst*I DNA fragment (*Nsi*I and *Pst*I yield compatible, cohesive ends). Then the ligation mixtures were used to transform *S. citri* strains 44 and ASP-1 by electroporation. After plating the transformation mixtures, both strains (44 and ASP-1) yielded tetracycline-resistant colonies at frequencies ranging from 10^{-8} (*Pst*I) to 5×10^{-7} (*Nsi*I) transformants per c.f.u. and per µg plasmid DNA in the ligation mixture.

Expression of ScARPs in spiroplasmal transformants

Knowing that plasmids pSci1–5 each carried at least one *scarp* gene, putative expression of ScARPs in spiroplasmal transformants was checked by direct immunoblotting of colonies (obtained with the *Nsi*I ligation mixture) with mAb10G3 (Fig. 2). The labelling of most, if not all, of the colonies indicated that these cells did carry plasmids, from which *scarp* genes were expressed to proteins. Production of ScARPs in spiroplasmal transformants was further confirmed by Western immunoblotting, and proteins of the expected molecular mass were detected on the blots (data not shown).

Plasmid content of spiroplasmal transformants

The restriction maps of the various recombinant plasmids predicted from the combination of the *tetM* cassette with the *Nsi*I- or *Pst*I-digested extrachromosomal DNA were determined. The plasmids were named pSciXNT or pSciXPT depending on the restriction site, *Nsi*I (N) or *Pst*I (P), into which the *tetM* cassette (T) was inserted. For each plasmid, the expected sizes of the *Hinc*II restriction fragments hybridizing with probes TetM, S235 and P32, which are specific to *tetM*, *scarp* and *p32* genes, respectively, are indicated in Table 2.

From these data, each plasmid present in the spiroplasmal transformants could be identified by hybridization signals of specific sizes. For example, the presence of pSci1NT was revealed by two specific *Hinc*II fragments, one of 7545 bp hybridizing with the TetM probe, and the other of 763 bp

hybridizing with probe S235. Similarly, plasmid pSci6PT, which does not carry *scarp* genes, was detected as two hybridization signals of 7363 bp (probe TetM) and 1870 bp (probe P32).

To characterize the plasmid content of the *S. citri* 44 transformants, 57 individual colonies (48 plus 9, respectively derived from the *Nsi*I and *Pst*I transformation mixtures) were grown in the presence of tetracycline and

Table 2. Sizes (bp) of the *Hinc*II restriction fragments hybridizing with probes TetM, S235 and P32

Plasmid	Size (bp)	Probe		
		TetM	S235*	P32
pSciA	7790	–	–	–
pSci1	12989	–	763 (3a)	–
pSci2	14407	–	8121 (3d)	–
pSci3	19325	–	664 (3c)	–
pSci4	20224	–	2549 (2a)	–
pSci5	27778	–	556 (3b) 1585 (5a) 3309 (2b)	–
pSci6	35318	–	–	1870
pSci1NT	15291	7545	763 (3a)	–
pSci2N1T†	16709	10423	10423 (3d)	–
pSci2N2T†	16709	3285	8121 (3d)	–
pSci21NT†	10336	10336	–	–
pSci22NT†	8675	3372	3372	–
pSci3NT	21627	5406	664 (3c)	–
pSci4NT	22526	4174	2549 (2a)	–
pSci5NT	30080	4417	556 (3b) 1585 (5a) 3309 (2b)	–
pSci5PT	30080	7950	556 (3b) 1585 (5a) 3309 (2b)	–
pSci6PT	37620	7363	–	1870

*The names of the *scarp* genes hybridizing with the S235 probe are indicated in parentheses. Probes TetM, S235 and P32 are described in Methods.

†Plasmids pSci2N1T and pSci2N2T respectively result from insertion of the *tetM* cassette into the *Nsi*I (1) and *Nsi*I (2) sites of pSci2 (see Fig. 1). Plasmids pSci21NT and pSci22NT respectively result from the combination of the *tetM* cassette (2302 bp) with the larger (pSci21NT) and smaller (pSci22NT) *Nsi*I fragment of pSci2 (8034 and 6373 bp, respectively).

genomic DNAs were analysed by Southern blot hybridization with probe TetM, and a mixture of probes S235 and P32 (Fig. 3). The diversity of the hybridization patterns indicated that the various spiroplasmal transformants carried distinct plasmid contents.

Hybridization with the TetM probe revealed that genomic DNAs from tetracycline-resistant spiroplasmas all contained *tetM* sequences, suggesting that they were transformed by plasmids carrying the *tetM* gene. For example, in clones N5, N11, N27 and P9, the sizes of the TetM-hybridizing fragments (7545, 10336, 4417 and 7363 bp) were indicative of the presence, in these transformants, of pSci1NT, pSci21NT, pSci5NT and pSci6PT, respectively (Fig. 3a, lanes N5, N11, N27 and P9). This was further confirmed by hybridization with probes S235 and P32. Plasmids pSci1NT and pSci5PT were detected as S235-hybridizing fragments of 763 and 556, 1585 and 3309 bp, respectively (Fig. 3b, lanes N5 and N27), whereas pSci6PT was revealed as a 1870 bp fragment hybridizing with the P32 probe (Fig. 3b, lane P9). As expected, pSci21NT did not hybridize with the S235 and P32 probes (Fig. 3b, lane N11).

Interestingly, extrachromosomal DNAs from some transformants yielded additional hybridization signals, indicating that more than one plasmid might be present in these clones. In clone N19 for example, detection of two signals of 7545 and 4417 bp with the TetM probe and four signals of 763, 556, 1585 and 3309 bp with probe S235 strongly suggested that this clone contained the two plasmids pSci1NT and pSci5NT (Fig. 3a, b, lanes N19). Similarly, in clone N21, detection of signals of 7545 and 3285 bp with the TetM probe and 8121 and 763 bp with the S235 probe suggested the presence of pSci1NT and pSci2N2T (Fig. 3a, b, lanes N21). Plasmid pSci2N2T certainly occurred from the ligation of the *tetM* cassette to the linear pSci2 resulting from either partial *NsiI* digestion or combination of the two *NsiI* fragments.

Unexpectedly, in addition to recombinant plasmids carrying the *tetM* gene, some clones were found to contain unmarked plasmids (i.e. plasmids having no *tetM* gene). Clone P3, carrying pSci5PT, was also found to contain unmarked pSci4 as revealed by the presence of the 2549 bp fragment hybridizing with the S235 probe and the absence of the 4174 bp fragment hybridizing with the TetM probe (Fig. 3a, b, lanes P3). Similarly, clone P10 was found to contain unmarked pSci1 and pSci4, in addition to pSci6PT. The three plasmids were detected as hybridization signals of 763, 2549 and 1870 bp, respectively (Fig. 3b, lane P10). Three plasmids, pSci1 (unmarked), pSci2N2T and pSci21NT, were also detected in clone N33 (Fig. 3a and b, lanes N33). In clones N23 and N24, containing pSci1NT and pSci4, as well as in clone N30, carrying pSci5NT, the *tetM* hybridization signals of 7545 bp and 4417 bp were barely detected (Fig. 3a, lanes N23, N24 and N30, respectively). In addition, a few clones yielded hybridization signals of unexpected sizes, suggesting that plasmid rearrangements might have occurred in these clones. In clone N25, for example, the

presence of pSci1NT was detected as the 763 bp fragment hybridizing with the S235 probe (Fig. 3b, lane N25). However, hybridization with the TetM probe did not reveal the expected signal of 7545 bp but, instead, a fragment of approximately 5600 bp (Fig. 3a, lane N25).

In summary, transformants with a wide range of plasmid contents were obtained with both *NsiI* and *PstI* ligation mixtures. Considering the *tetM*-marked plasmids in the *S. citri* 44 transformants, pSci1NT was the most frequently detected (58 %) as compared to pSci21NT (21 %), pSci2N2T (10 %) and pSci4NT and pSci5NT (4 % each) (Table 3). Depending on the transformants, pSci1NT was detected alone (20 clones) or together with other marked or unmarked plasmids (eight clones). Taking into account that cells of *S. citri* GII-3 contain seven plasmids, pSciA and pSci1–6, the finding that *S. citri* 44 transformants carried up to three plasmids within the same cell was not totally surprising. None of the transformants was found to carry either pSci22NT or pSci3NT. It is thought that, in contrast to pSci21NT, which was detected in 23 % of the transformants, pSci22NT probably did not possess the replication origin of pSci2 and therefore could not replicate in spiroplasma cells. In pSci3, the location of the *NsiI* site, downstream of *trsE*, is identical to that in pSci4, and thus cannot explain why pSci3NT, in contrast to pSci4NT, was not detected. Unmarked pSci3 was also not detected in the *S. citri* 44 transformants tested. In the case of transformation with the *PstI* mix, plasmids pSci5PT and pSci6PT were detected at similar frequencies, 44 % and 33 %, respectively. Similar data were obtained with *S. citri* ASP-1 (hybridization data are not shown) except that pSci4NT was detected at a higher frequency (39 % versus 4 % for strain 44) and pSci2N2T was not detected (Table 3). Considering a complete digestion of pSci2 into two *NsiI* fragments, the probability of combination of the two fragments together with the *tetM* cassette to yield pSci2N2T was expected to be very low. Taken as a whole, these results showed that plasmids pSci1, pSci2, pSci4, pSci5 and pSci6, as well as their *tetM*-marked derivatives, were efficiently introduced into *S. citri* strains 44 and ASP-1, in which they replicated.

Transmission of *S. citri* 44 transformants

To determine whether plasmids from *S. citri* GII-3 could confer insect transmissibility to *S. citri* 44, cultures of various *S. citri* 44 transformants were submitted to experimental transmission to periwinkle plants via injection into the leafhopper vector. In a preliminary screening experiment, two distinct spiroplasma cultures were used. One, named Mix44/1256, was made of a mixture of equal amounts of strains 44/1 (clone N6), 44/2 (clone N33), 44/5 (clone N27) and 44/6 (clone P9) carrying plasmids pSci1NT; pSci1, pSci2N2T and pSci21NT; pSci5NT; and pSci6PT, respectively, and the other, named 44/146 (clone P10), containing the three plasmids pSci1, pSci4 and pSci6PT. Strains GII-3 (wild-type) and 44 (insect non-transmissible) were used as controls. As expected, all five plants developed symptoms in the case of GII-3 whereas none (out of 10) was infected by *S.*

Table 3. Summary of transformation experiments of *S. citri* strains 44 and ASP-1 by extrachromosomal DNA from *S. citri* GII-3

Plasmid	<i>S. citri</i> 44		<i>S. citri</i> ASP-1	
	Transformants*	%	Transformants*	%
pSci1NT	28/48	58	26/41	63
pSci2N1T	0/48	0	0/41	0
pSci2N2T	5/48	10	0/41	0
pSci21NT	10/48	21	12/41	30
pSci22NT	0/48	0	0/41	0
pSci3NT	0/48	0	0/41	0
pSci4NT	2/48	4	16/41	39
pSci5NT	2/48	4	0/41	0
pSci5PT	4/9	44	4/8	50
pSci6PT	3/9	33	3/8	38

*Ratio of the number of spiroplasma transformants carrying the relevant plasmid to the total number of transformants tested.

citri 44 (Table 4). In contrast, with both cultures Mix44/1256 and 44/146, four plants out of 10 developed severe symptoms, with spiroplasma titres [$1.2\text{--}3.3 \times 10^7$ c.f.u. (g midribs) $^{-1}$] similar to that [1.2×10^7 c.f.u. (g midribs) $^{-1}$] of plants infected by *S. citri* GII-3.

To make sure that symptom production in these plants did result from multiplication of the spiroplasma strains injected into the insects and not from an unexpected contamination, spiroplasmas were isolated from both insects and symptomatic plants, and characterized. Basically, the plasmid contents of the spiroplasma cultures were analysed by PCR amplification and Southern blot hybridization of genomic DNAs with primers and probes specific to genes *scarp* and *p32*. As shown in Fig. 4, the PCR data were in good agreement with the multiplication, in the insects, of the spiroplasma strains that were initially injected. For example, PCR with primer pairs S235F/S235R and P32F/P32R yielded positive amplification with all isolates from insects (Fig. 4a, c, lanes 5–8) except in the case of 44 (Fig. 4a, c, lanes 3 and 4). In agreement with the absence of pSci4 in the Mix44/1256, PCR amplification with primer pair S4F/S4R was positive with isolates GII-3 (Fig. 4b, lane 2) and 44/146 (Fig. 4b, lanes 7 and 8), but not with isolates 44 and Mix44/1256 (Fig. 4b, lanes 3–6). With DNAs from spiroplasma cultures isolated from plants, PCR with primer pair P32F/P32R yielded positive amplification with all isolates, suggesting that they all contained pSci6 carrying the *p32* gene (Fig. 4c, lanes 10–13). As expected, due to the absence of pSci4, PCR amplification specific to *scarp4a* yielded negative results with isolates from Mix44/1256 (Fig. 4b, lanes 10 and 11). The failure to amplify *scarp4a*

from these plants confirmed that symptom production was not due to infection by contaminating, transmissible strains such as GII-3. Interestingly, no *scarp* genes were detected in Mix44/1256 isolated from plant P4 (Fig. 4a, b, lanes 10), suggesting that this isolate only contained pSci6.

These PCR data were further confirmed by Southern blot hybridization of genomic DNA extracted from spiroplasma isolates with a mixture of probes S235 and P32 (Fig. 5). In the case of Mix44/1256, hybridization signals corresponding to pSci1, 2, 5 and 6 were detected in the insects, indicating that all four strains 44/1, 44/2, 44/5 and 44/6 did multiply in the vector insect (Fig. 5, lanes 2 and 3). Unexpectedly, a faint signal, which seemed to correspond to *scarp3c* of pSci3, was detected in the Mix44/1256B isolate (Fig. 5, lane 3). This suggests that one of the four cultures that were mixed prior to injection into the insects might contain a mixed population of cells, a few of them carrying pSci3 or pSci3NT. These plasmids were not detected in the *S. citri* 44 transformants that were initially selected (see Fig. 3). DNAs from plant isolates displayed two distinct profiles. As indicated by the 1870 bp fragment hybridizing with the P32 probe, pSci6 was the only plasmid detected in the isolate from plant P4 (Fig. 5, lane 4). This isolate was named 44/6P4. In contrast, isolate from plant P5 seemed to contain both pSci1 (as revealed by the 763 bp hybridization signal) and pSci6 (Fig. 5, lane 5), suggesting that strains 44/1 and 44/6 were transmitted to the plant whereas 44/2 and 44/5 were not.

In the case of 44/146, all three plasmids, pSci1, pSci4 and pSci6PT, were detected as specific hybridization signals of 763 (*scarp3a*), 2549 (*scarp2a*) and 1870 bp (*p32*) in spiroplasma cultures isolated from both insects and plants (Fig. 5, lanes 6 and 7, and 8 and 9, respectively). These results indicated that strain 44/146 was transmitted to periwinkle plants by the leafhopper vector and that the three plasmids were maintained in the absence of selection pressure. Therefore, it was thought that plasmids pSci1, and/or pSci4, and/or pSci6PT could confer insect transmissibility to *S. citri* 44. Furthermore, the detection of pSci6PT in every spiroplasma culture isolated from plants infected by *S. citri* 44 transformants suggested that this particular plasmid might be involved. To further investigate the correlation between the presence of pSci6PT and the ability of *S. citri* 44/6 to be transmitted via injection into the leafhopper vector, experimental transmission tests were carried out with strains 44/6 (initial clone P9) and 44/6P4 (strain 44/6 isolated from plant P4 after insect transmission of Mix44/1256), both of which contained pSci6PT only (Table 5). In contrast to *S. citri* 44, for which none of the plants was infected, both strains 44/6 and 44/6P4 were transmitted by the leafhopper vector, as indicated by the high proportion of plants showing symptoms, 8/10 and 9/10,

Fig. 3. Southern blot hybridization of *HincII*-restricted DNAs from *S. citri* GII-3, GII-3-9a2 and *S. citri* 44 transformants (lanes N1–48 and P1–12) with probes TetM (a) and S235 plus P32 (b). Sizes of DNA fragments are indicated in base pairs. The names of *p32* and *scarp* genes are indicated in parentheses.

Table 4. Transmission of *S. citri* GII-3 (insect transmissible), *S. citri* 44 (insect non-transmissible) and *S. citri* 44 transformants Mix44/1256 and 44/146 to periwinkle plants via injection into the leafhopper vector

Inoculant (c.f.u. ml ⁻¹)	Plants	Living insects*	C.f.u. per insect†	Symptoms	C.f.u. (g midribs) ^{-1‡}
SP4 medium	P1	7-2-1	—	—	—
	P2	7-1-1	—	—	ND
	P3	8-8-8	—	—	ND
	P4	8-5-5	—	—	ND
<i>S. citri</i> GII-3 (1.4 × 10 ⁹)	P1	7-2-1	8.2 × 10 ⁵	+	ND
	P2	10-10-10		+	ND
	P3	7-4-4		+	ND
	P4	6-2-2		+	1.2 × 10 ⁷
	P5	8-3-1		+	ND
<i>S. citri</i> 44 (1.4 × 10 ⁹)	P1	8-4-4	1.9 × 10 ⁵	—	ND
	P2	8-3-2		—	ND
	P3	8-4-4		—	ND
	P4	8-3-3		—	—
	P5	7-5-2		—	ND
	P6	6-4-2		—	—
	P7	9-5-5		—	ND
	P8	6-4-4		—	ND
	P9	8-5-3		—	ND
	P10	8-7-6		—	ND
Mix44/1256	P1	9-9-9	1.9 × 10 ⁴	+	ND
	P2	7-3-2		—	ND
	P3	5-3-0		—	ND
	P4	9-6-6		+	1.3 × 10 ⁷
44/1 (1.2 × 10 ⁹)	P5	8-8-8		+	3.3 × 10 ⁷
44/2 (6.5 × 10 ⁸)	P6	8-2-0		—	ND
44/5 (9.5 × 10 ⁸)	P7	7-4-0		—	ND
44/6 (1.3 × 10 ⁹)	P8	5-4-1		—	—
<i>S. citri</i> 44/146 (3.3 × 10 ⁸)	P9	7-3-2		—	ND
	P10	7-7-7		+	ND
	P1	9-6-3	6.3 × 10 ⁴	—	ND
	P2	8-7-7		+	ND
	P3	9-8-8		+	2.8 × 10 ⁷
	P4	9-5-5		—	—
	P5	8-4-2		—	ND
	P6	8-6-5		+	1.2 × 10 ⁷
	P7	9-3-3		—	ND
	P8	6-6-6		—	ND
	P9	9-2-0		—	ND
	P10	7-7-7		+	ND

*Number of living insects 2, 8 and 15 days after injection.

†Mean of two independent determinations each from a pool of eight insects captured at the end of the transmission period.

‡Spiroplasma titres were determined 6 weeks post-transmission; ND, not determined.

respectively. As expected, spiroplasmas isolated from these plants were shown to contain pSci6PT, as revealed by the restriction pattern of the purified plasmid and the detection of the 1870 bp fragment hybridizing with the P32 probe (data not shown). These results indicated that plasmid pSci6PT alone conferred insect transmissibility to *S. citri* strain 44. To determine whether pSci4NT, like pSci6PT, could confer transmissibility, experimental transmission assays were conducted to compare transmissibility of strain

44/4, carrying pSci4NT, and strain 44/6, carrying pSci6PT. The results, presented in Table 6, showed that, in spite of multiplication of both strains in the insects, strain 44/6 (initial clone P9) was transmitted, as revealed by the occurrence of symptomatic plants (4/10), whereas strain 44/4 was not (0/10). In the case of strain 44/4, the detection of pSci4NT in the spiroplasmas isolated from insects at the end of the transmission period (Fig. 6, lane 6) indicated that the absence of transmission was not due to plasmid loss.

Fig. 4. PCR amplification of genomic DNAs from *S. citri* GII-3, 44 and spiropalasmal transformants Mix44/1256 and 44/146 isolated from insects (lanes 2–8) and plants (lanes 9–13) with primer pairs S235F/S235R (a), S4F/S4R (b) and P32F/P32R (c). Lanes 1 and 2, negative (without DNA) and positive (DNA from *S. citri* GII-3) controls. For each strain, spiropalasmal transformants were isolated from two distinct groups (A and B) of eight insects. Lanes: 3 and 4, *S. citri* 44; 5 and 6, Mix44/1256; 7 and 8, 44/146; 9, *S. citri* GII-3; 10 and 11, Mix44/1256 isolated from plants P4 and P5, respectively; 12 and 13, 44/146 isolated from plants P3 and P6, respectively.

However, the possibility that strain 44/4 may actually be transmissible, but at a rate lower than would be detected in our experiments, cannot be excluded. Nevertheless, these results strongly suggest that pSci6 from *S. citri* GII-3 encodes genetic determinants that are essential for transmission of *S. citri* 44 via injection into the leafhopper *C. haematops*.

Spiroplasma acquisition through feeding

To assess the ability of *S. citri* strains to cross the gut epithelium barrier of the insect vector, healthy leafhoppers were fed on periwinkle plants infected with *S. citri* strains GII-3, 44/6 and 44/146 and, after a latent period, were tested for the presence of spiropalasmal transformants. In two independent experiments, 86 % (24/28 and 12/14) of the insects fed on GII-3 infected plants (control) yielded positive cultures. In contrast, less than 5 % of the insects yielded positive cultures when fed on plants infected with strains 44/6 (0/25 and 1/36) and 44/146 (0/12 and 2/40). Since strain 44 cannot be transmitted to periwinkle, it could not be tested in these experiments. Nevertheless, these data suggest that components other than those encoded by pSci6 are probably required for *S. citri* 44 to efficiently cross the gut epithelium barrier. The finding that strain 44/146, carrying *scarp2a*, *3a* and *4a*, was not acquired more efficiently by the leafhopper also suggests that these components might not be the ScARPs.

DISCUSSION

Plasmids provide their hosts with a wide variety of phenotypes: resistance to antibiotics, ability to use specific

Fig. 5. Southern blot hybridization of genomic DNAs from *S. citri* GII-3 (lane 1) and *S. citri* 44 transformants Mix44/1256 (lanes 2–5) and 44/146 (lanes 6–9) isolated from insects and plants. Lanes: 2 and 3, Mix44/1256 isolated from insect groups A and B, respectively; 4 and 5, Mix44/1256 isolated from plants P4 and P5; 6 and 7, 44/146 isolated from insect groups A and B, respectively; 8 and 9, 44/146 isolated from plants P3 and P6.

compounds as carbon and energy sources, and many other traits offering a selective advantage to the bacteria for colonizing specific biotopes. In plant-pathogenic bacteria, many genes involved in pathogenicity and host specificity are carried on plasmids. Although many strains of *S. citri* have been shown to carry natural plasmids, their biological functions were not reported and plasmid transfer among *S. citri* strains has not been demonstrated hitherto. The previous report of the involvement of pM42 in the erythromycin-resistance transfer is still questionable, since no free plasmid was detected in the spiropalasmal transformants and no plasmid-encoded determinants were characterized (Salvado *et al.*, 1989). Recently, sequencing data revealed that plasmids pSKU146 from *Spiroplasma kunkelii*, pBJS-O from *S. citri* BR3 and pSci1–6 from *S. citri* GII-3 encode proteins that are thought to be involved in the interactions of the spiropalasmal transformants with their insect hosts (Davis *et al.*, 2005; Foissac *et al.*, 2004; Joshi *et al.*, 2005).

In *S. citri* GII-3, plasmids pSci1–5 and pSci6, encoding respectively ScARPs and the hydrophilic protein P32, have been associated with insect transmissibility as they were detected in all insect-transmissible strains tested, but not in insect-non-transmissible ones (Berho *et al.*, 2006; Killiny *et al.*, 2004, 2006). Such an apparent correlation between the presence of plasmids and the ability of *S. citri* strains to be transmitted by the leafhopper vector is reminiscent of the situation found in *S. citri* ASP-1. In this particular strain, the initial culture, which possesses three plasmids of 7.8, 14.8 and 35.4 kbp, could be experimentally transmitted via injection into the leafhopper *Euscelidius variegatus*

Table 5. Transmission of *S. citri* GII-3 (insect transmissible), *S. citri* 44 (insect non-transmissible) and *S. citri* 44 transformants 44/6 and 44/6P4 to periwinkle plants via injection into the leafhopper vector

Inoculant (c.f.u. ml ⁻¹)	Plants	Living insects*	C.f.u. per insect†	Symptoms	C.f.u. (g midribs) ^{-1‡}
SP4 medium	P1	7-4-0	—	—	—
	P2	6-5-4	—	—	ND
	P3	4-4-0	—	—	ND
	P4	11-8-3	—	—	ND
<i>S. citri</i> GII-3 (7.9×10^8)	P1	6-5-1	1.5×10^6	+	ND
	P2	5-4-4		+	ND
	P3	6-6-6		+	ND
	P4	6-4-4		+	6.5×10^6
	P5	6-4-4		+	ND
<i>S. citri</i> 44 (1.2×10^9)	P1	6-3-1	3.7×10^5	—	ND
	P2	5-2-1		—	ND
	P3	6-5-5		—	—
	P4	6-5-5		—	ND
	P5	5-3-3		—	ND
<i>S. citri</i> 44/6 (4.2×10^8)	P1	7-4-2	2.4×10^5	+	ND
	P2	3-2-1		+	ND
	P3	7-5-5		+	ND
	P4	7-5-2		—	—
	P5	7-3-2		+	3.9×10^6
	P6	7-3-2		—	ND
	P7	5-4-2		+	9.9×10^6
	P8	8-4-1		+	ND
	P9	6-4-4		+	ND
	P10	5-4-1		+	ND
<i>S. citri</i> 44/6P4 (8.7×10^8)	P1	7-1-1	2.7×10^5	+	ND
	P2	7-6-1		+	8.3×10^6
	P3	6-3-1		—	—
	P4	6-3-4		+	ND
	P5	6-5-4		+	ND
	P6	8-5-5		+	ND
	P7	7-5-5		+	ND
	P8	7-3-1		+	ND
	P9	8-6-4		+	2.9×10^6
	P10	6-5-2		+	ND

*, †, ‡See Table 4.

(Townsend *et al.*, 1977). However, due to extensive *in vitro* propagation, loss of the two larger plasmids resulted in loss of insect transmissibility (Archer *et al.*, 1981). Interestingly enough, the estimated sizes of these two plasmids (14.8 and 35.4 kbp) are similar to those of *S. citri* GII-3 plasmids pSci2 (14.4 kbp) and pSci6 (35.3 kbp) carrying *scarp3d* and *p32*, respectively. Taken together, these studies support a role of plasmid-encoded determinants in transmission of *S. citri* by its leafhopper vectors.

In the present study, we transformed the non-transmissible *S. citri* strain 44 (having no plasmids) with plasmids from *S. citri* GII-3 and screened the spiroplasmal transformants for insect transmissibility. Transformation of *S. citri* 44 was achieved through electroporation of spiroplasma cells with a

ligation mixture of the extrachromosomal DNA from *S. citri* GII-3 with the *tetM* gene, used as the selection marker. Detection of the expected recombinant plasmids pSci1–5NT and pSci5–6PT in the spiroplasmal transformants indicated that insertion of the *tetM* gene at the *NsiI* or *PstI* sites did not prevent plasmid replication. For clarity, we will not discriminate the natural plasmids pSci1–6 from their *tetM*-marked counterparts (pSci1–5NT and pSci5–6PT) in the subsequent discussion. The five plasmids pSci1–2 and pSci4–6 were each detected by Southern blot hybridization in at least one of the transformants, whereas pSci3 was not detected in the first screen of the transformants. With the *NsiI* mix, a large majority of transformants was found to contain pSci1, alone or together with one or two additional plasmids. The occurrence of several plasmids in a single cell

Table 6. Transmission of *S. citri* GII-3 (insect transmissible), *S. citri* 44 (insect non-transmissible) and *S. citri* 44 transformants 44/4 and 44/6 to periwinkle plants via injection into the leafhopper vector

In this experiment the injected insects were fed on stock plants for 10 days (latent period) and then transferred to periwinkle plants (five insects per plant) for 10 additional days (transmission period).

Inoculant (c.f.u. ml ⁻¹)	Plants	Living insects*	C.f.u. per insect†	Symptoms	C.f.u. (g midribs) ^{-1‡}
SP4 medium	P1	5-1-1	—	—	—
	P2	5-1-0	—	—	ND
<i>S. citri</i> GII-3 (1.3 × 10 ⁹)	P1	5-2-2	9.8 × 10 ⁵	+	ND
	P2	5-2-1		+	1.6 × 10 ⁶
	P3	5-3-5		+	ND
	P4	5-2-1		+	ND
	P5	5-2-2		+	ND
<i>S. citri</i> 44 (1.2 × 10 ⁹)	P1	5-4-2	3.9 × 10 ⁵	—	ND
	P2	5-1-1		—	—
	P3	5-3-2		—	ND
	P4	5-2-1		—	ND
	P5	5-3-3		—	ND
<i>S. citri</i> 44/4 (2.8 × 10 ⁸)	P1	5-3-2	1.4 × 10 ⁵	—	ND
	P2	5-4-3		—	ND
	P3	5-2-2		—	ND
	P4	5-3-2		—	ND
	P5	5-2-1		—	ND
	P6	5-2-2		—	ND
	P7	5-1-0		—	—
	P8	5-2-1		—	ND
	P9	5-2-2		—	ND
	P10	5-2-1		—	—
<i>S. citri</i> 44/6 (4.8 × 10 ⁸)	P1	5-5-2	7.8 × 10 ⁴	+	1.3 × 10 ⁶
	P2	5-3-2		—	ND
	P3	5-3-1		—	—
	P4	5-3-2		—	ND
	P5	5-4-4		+	7 × 10 ⁶
	P6	5-1-0		—	ND
	P7	5-3-3		—	ND
	P8	5-2-1		+	1.3 × 10 ⁶
	P9	5-2-1		+	ND
	P10	5-3-4		—	ND

*, †, ‡See Table 4.

indicates the absence of incompatibility between these plasmids, in agreement with the situation in *S. citri* GII-3, in which each plasmid (pSci1–6) was shown to occur at approximately 10 copies per cell (Foissac *et al.*, 2004). The high proportion of *S. citri* 44 transformants carrying pSci1 probably resulted from the high proportion of small plasmids in the DNA preparation, due to high recovery during the isolation process. In addition, transformation experiments with isolated plasmids revealed that transformation of *S. citri* 44 with pSci1 was approximately ten times more efficient (0.5–1.5 × 10⁵ transformants µg⁻¹) than with pSci6 (0.5–1 × 10⁴ transformants µg⁻¹).

Experimental transmission assays via injection into the leafhopper vector *C. haematiceps* showed that, in contrast to

S. citri 44 (wild-type), *S. citri* 44 transformants were transmitted to the host plant, in which they multiplied and induced symptoms. When introduced into the insects by intra-abdominal injection, both transmissible and non-transmissible strains multiplied in the leafhopper (Bové *et al.*, 2003; Wayadande & Fletcher, 1995). Accordingly, *S. citri* 44 and each one of the *S. citri* 44 transformants (regardless of their plasmid content), multiplied in the insects, in which they were detected at the end of the transmission period. In contrast, after feeding the infected leafhoppers on periwinkles, only spiroplasmas carrying pSci6 were isolated from every infected plant, suggesting that this plasmid could confer transmissibility to *S. citri* 44. Indeed, independent transmission of strains 44/4 and 44/6 clearly indicated that pSci6 alone conferred insect

Fig. 6. Southern blot hybridization of genomic DNAs from *S. citri* GII-3 (lanes 1 and 8), *S. citri* 44 (lanes 2 and 5) and *S. citri* 44 transformants 44/4 (lanes 3 and 6) and 44/6 (lanes 4, 7 and 9). DNAs were extracted from spiroplasma cultures before injection into the insects (lanes 1–4), from insects at the end of the transmission period (lanes 5–7) and from infected plants (lanes 8 and 9).

transmissibility to strain 44, whereas pSci4 did not. Therefore, it is likely that pSci6 encodes genetic determinants that are essential for insect transmission of *S. citri* 44. In agreement with these data, *S. citri* strain GII3-Δ5, having lost pSci5 but which still carried pSci6, has been shown to be transmitted at high efficiency, similarly to the wild-type strain GII-3 (Berho *et al.*, 2006). In addition, strain GII3-Δ135, lacking pSci1, 3 and 5, could also be experimentally transmitted (*S. Duret & J. Renaudin*, unpublished data).

It is noteworthy that pSci6, which was shown to confer insect transmissibility to strain 44, encodes the hydrophilic protein P32, which has been associated with insect transmissibility of *S. citri* (Killiny *et al.*, 2006). However, expression of P32 in *S. citri* 44 through transformation with a recombinant, *oriC* plasmid carrying *p32* failed to restore transmissibility (Killiny *et al.*, 2006). This result suggests that pSci6-encoded determinants other than P32 might be essential for the spiroplasmas to invade, multiply in, or be released from the salivary glands of the leafhopper vector. Plasmids pSci1–5 encode eight adhesin-like proteins closely related to each other (Foissac *et al.*, 2004) and to SARP1 of *S. citri* BR3 (Berg *et al.*, 2001). Plasmid pSci4, in particular, encodes ScARP2a and 4a. In contrast, pSci6 contains no full-length *scarp* gene. Instead, it possesses short *scarp*-like sequences that putatively encode truncated products with limited homology to ScARPs. Since no *scarp* sequences were

detected in the genomic DNA of *S. citri* 44 (by Southern blot hybridization), the finding that pSci6 alone conferred transmissibility to this particular strain suggests that ScARPs are not essential for the spiroplasma cells present in the haemolymph to cross the salivary gland barrier. However, in natural transmission (in contrast to experimental transmission, in which spiroplasmas are directly injected into the haemolymph) the ingested spiroplasmas must cross the gut epithelium before reaching the haemolymph. The involvement of ScARPs at this point of the transmission process cannot be excluded. The fact that SARP1 of *S. citri* BR3 seems to be involved in adhesion *in vitro* of spiroplasmas to insect cells is in good agreement with this hypothesis (Yu *et al.*, 2000). The finding that *S. citri* 44/6, which does not possess ScARPs, was poorly acquired by insects fed on 44/6-infected plants also fits the hypothesis. However, the presence of ScARPs 2a, 3a and 4a in strain 44/146 seems not to improve spiroplasma acquisition by the leafhoppers, when compared to strain 44/6. Therefore the involvement of ScARPs is still uncertain. In addition to P32, pSci6 putatively encodes many other hypothetical proteins of unknown function, some of which are specific to this plasmid. Their possible involvement in the transmission of *S. citri* by its leafhopper vector is being investigated.

Even though pSci6 conferred insect transmissibility to *S. citri* 44, transmission of *S. citri* 44/6 was repeatedly less efficient than that of *S. citri* GII-3, as indicated by the fact that the proportion of infected plants never reached 100%. Two distinct hypotheses could explain these results. In contrast to the situation in GII-3, pSci6 could not be stably maintained in the absence of selection pressure (in the leafhopper and/or in the plant), resulting in a low proportion of cells carrying the plasmid. Indeed, whereas attempts to cure *S. citri* GII-3 of pSci6 were unsuccessful (Berho *et al.*, 2006), *in vitro* propagation of *S. citri* 44/6 in the absence of selection pressure resulted in loss of plasmid pSci6 in more than 90% of the cells after 20 successive propagations. Alternatively, genetic determinants other than those carried by pSci6 might be required for efficient transmission, and these determinants might be absent in the genome of *S. citri* 44, as compared to GII-3. However, equal amounts of spiralin, a protein that is required for efficient transmission of *S. citri* (Duret *et al.*, 2003), were detected in *S. citri* strains 44 and GII-3 (Killiny *et al.*, 2006).

In mycoplasmas, attachment to the host cells is by a specialized structure, which is formed through the recruitment of several proteins that interact between the mycoplasma and the host cell (Balish & Krause, 2005). Similar structures have been observed in cells of *S. kunkelii* attached to the gut epithelium of the leafhopper vector (Ammar *et al.*, 2004; Özbek *et al.*, 2003). However, whether particular proteins are specifically involved in these structures remains to be determined. In phytoplasmas, the immunodominant membrane protein of '*Candidatus* Phytoplasma asteris' has been shown to interact specifically with microfilaments of the leafhopper vector intestine,

suggesting that this complex formation is part of the transmission mechanism (Suzuki *et al.*, 2006). The presumed implication of plasmid-encoded proteins has also been suggested (Nishigawa *et al.*, 2002).

While transformation of *S. citri* with artificial *oriC* plasmids has been extensively used for molecular genetic studies (Bové *et al.*, 2003; Renaudin & Lartigue, 2005), this study is, to our knowledge, the first report of successful transformation of spiroplasma cells by natural plasmids, and opens the way to investigate the role of plasmid-encoded determinants in the biology of the spiroplasma. As an example, transformation of *S. citri* 44 by pSci6 from *S. citri* GII-3 represents the first clear-cut evidence of a link between plasmids and insect transmissibility.

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