Absence of plasmids encoding adhesion-related proteins in non-insect-transmissible strains of *Spiroplasma citri*

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In the plant-pathogenic mollicute *Spiroplasma citri*, spiralin is the major lipoprotein at the cell surface and is thought to be one of the components involved in the interactions of the spiroplasma with its insect vector. With the aim of identifying surface proteins other than spiralin, monoclonal antibodies (mAbs) were produced by immunization of mice with the spiralin-defective *S. citri* mutant Gll3-9a2. mAb 10G3 was found to react with several polypeptides of 43–47 and 80–95 kDa, all of which were detected in the detergent phase after Triton X-114 partitioning of proteins. Mass spectrometry (MALDI-TOF) analyses of the two major polypeptides P47 and P80 of Gll3-9a2, reacting with mAb 10G3, revealed that P47 was a processed product and represented the C-terminal moiety of P80. Search for sequence homologies revealed that P80 shared strong similarities with the *S. citri* adhesion-related protein P89 (Sarp1) of *S. citri* BR3, and is one (named Scarp4a) of the eight Scarps encoded by the *S. citri* Gll-3 genome. The eight *scarp* genes are carried by plasmids pSci1–5. Western immunoblotting of proteins with mAb 10G3 revealed that, in contrast to the insect-transmissible *S. citri* strain Gll-3, the non-insect-transmissible strains ASP-1, R8A2 and 44 did not express Scarps. Southern blot hybridization experiments indicated that these strains possessed no *scarp* genes, and did not carry plasmids pSci1–5. However, *S. citri* strain Gll3-5, lacking pSci5, was still efficiently transmitted, showing that, in the genetic background of *S. citri* Gll-3, the pSci5-encoded genes, and in particular *scarp2b*, *3b* and *5a*, are not essential for insect transmission. Whether plasmid-encoded genes are involved in transmission of *S. citri* by its leafhopper vector remains to be determined.

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INTRODUCTION

Plant-pathogenic mollicutes, phytoplasmas and spiroplasmas, are associated with hundreds of diseases that affect a wide variety of plants including ornamentals, vegetables, fruit trees and grape, leading to important economic losses (Lee *et al.*, 2000; Seemüller *et al.*, 2002). The bacteria multiply in the phloem sieve tubes, where they are introduced by phloem sap-feeding leafhoppers or psyllids, which therefore play a crucial role in the spread of these pathogens. *Spiroplasma citri* is the causal agent of stubborn disease of citrus (Saglio *et al.*, 1973) and brittle root disease of horseradish (Fletcher, 1983). However, it infects many other plants including the Madagascar periwinkle (*Catharanthus roseus*), which serves as the experimental host plant for most of the

plant-pathogenic mollicutes. In nature, *S. citri* is transmitted in a persistent manner by the leafhopper vectors *Circulifer tenellus* in the USA (Liu *et al.*, 1983a) and *Circulifer haematocoeus* in the Old World (Fos *et al.*, 1986). *S. citri* can be experimentally transmitted to periwinkle plants by injection into the leafhopper vector (Foissac *et al.*, 1996). Over the years, *S. citri* has become a model for studying several aspects of the relationships of plant-pathogenic mollicutes with their hosts, the plant and the vector insect (Bové *et al.*, 2003; Fletcher *et al.*, 1998). In particular, it has been shown that sugar metabolism, and especially fructose import, is a key factor of spiroplasmal pathogenicity (Gaurivaud *et al.*, 2000; André *et al.*, 2005). *S. citri* has also been used to study the pathway of spiroplasmas within the leafhopper vector (Kwon *et al.*, 1999; Liu *et al.*, 1983b). When ingested by the leafhopper, spiroplasmas must cross two physical barriers before being transmitted to the host plant. First, they cross the gut epithelium to move from the lumen to haemolymph, where they multiply, and then they migrate to the salivary glands, moving across the associated membranes to reach the salivary ducts (Liu *et al.*, 1983b). Mainly based on electron

Abbreviation: MALDI-TOF, matrix-associated laser desorption ionization-time of flight.

The GenBank/EMBL/DDBJ accession numbers of *S. citri* plasmids pSci1–6 are AJ969069, AJ969070, AJ969071, AJ969072, AJ969073 and AJ969074, respectively.

microscopy studies, model mechanisms of spiroplasma movement through these two barriers have been proposed (Fletcher *et al.*, 1998). However, the processes are still not well understood and the genetic determinants involved are not known. In *S. citri*, several surface proteins have been suggested to play a role in transmission. Among these, protein P89, later named Sarp1, has been shown to be implicated in adhesion to insect (*C. tenellus*) cells, suggesting that this protein is directly involved in spiroplasma–insect cell interaction (Yu *et al.*, 2000; Berg *et al.*, 2001). Using a reverse genetic approach, it has been shown that disruption of a gene predicted to encode a surface lipoprotein, with homology to a solute-binding protein of an ABC transporter, strongly reduces transmission of *S. citri* by the leafhopper *C. haematoceps* (Boutareaud *et al.*, 2004). Recently, it has been shown that spiralin, the most abundant membrane protein of *S. citri*, acts as a lectin binding to glycoproteins from the insect vector *C. haematoceps* (Killiny *et al.*, 2005). Besides being the most abundant lipoprotein at the cell surface, spiralin is highly immunogenic. Consequently, monoclonal antibodies (mAbs) raised against *S. citri* cells were all found to react with spiralin. In previous studies, we have shown that insect transmission of a spiralin-disrupted *S. citri* mutant was poorly efficient as compared to the wild-type strain, suggesting that spiralin is required for efficient transmission of the spiroplasma by its leafhopper vector (Duret *et al.*, 2003). However, the finding that this mutant could still be transmitted also suggested that proteins other than spiralin were probably involved. With the aim of identifying new surface proteins, putatively involved in the spiroplasma–vector insect interactions, we used the spiralin-defective mutant as the antigen source to produce mAbs directed against surface proteins other than spiralin. One mAb was found to react with a membrane-associated protein sharing strong similarities with adhesion-related protein Sarp1 of *S. citri* BR3 (Berg *et al.*, 2001). In the *S. citri* wild-type strain GII-3, this protein is one of the eight members of the adhesion-related protein family which we named Scarp (for *S. citri* adhesion-related protein). Interestingly, the eight *scarp* genes are distributed on five plasmids (pSci1–5) of 13–28 kbp (Foissac *et al.*, 2004). Comparison of insect-transmissible and non-transmissible strains of *S. citri* revealed that the non-transmissible strains did not express Scarps and even did not possess *scarp* genes. We showed that the *S. citri* mutant GII3-9a2, which possesses no spiralin, also lacked pSci5, a plasmid carrying three *scarp* genes. Experimental transmission assays were conducted to determine whether pSci5 was required for efficient transmission of *S. citri* via injection into its vector insect *C. haematoceps*.

METHODS

Bacterial strains, transformation and growth conditions. *S. citri* GII-3 wild-type strain was originally isolated from its leafhopper vector *Circulifer haematoceps* captured in Morocco (Vignault *et al.*, 1980). *S. citri* strains R8A2 (Saglio *et al.*, 1973), ASP-1 (Townsend *et al.*, 1977) and 44 (kindly provided by Dr Hosseini Pour, University

of Kerman, Iran; unpublished) were isolated from stubborn-diseased sweet orange trees in Morocco, Israel and Iran, respectively. Unlike R8A2 and ASP-1, strain 44 has not been extensively propagated *in vitro*. However, attempts to infect periwinkle plants via injection into the leafhopper vector *C. haematoceps* repeatedly failed, similar to the situation with R8A2 and ASP-1. The spiralin-disrupted mutant GII3-9a2 was obtained by gene targeting through homologous recombination (Duret *et al.*, 2003). *Spiroplasma melliferum* BC3 (ATCC 33219), *Spiroplasma apis* B31 (ATCC 33834), *Spiroplasma floricola* 23.6 (ATCC 29989), *Spiroplasma phoeniceum* P40 (ATCC 43115) and *Mesoplasma florum* L1 (ATCC 33453) were also used in this study. Spiroplasmas and mesoplasmas were grown at 32 °C in SP4 medium (Whitcomb, 1983) from which fresh yeast extract was omitted. Electrotransformation of *S. citri* has been described previously (Stamburski *et al.*, 1991).

Plasmid constructs. Plasmids pSR2, pBOG, pBSO and pSD4 have been described elsewhere (Lartigue *et al.*, 2002; Renaudin, 2002). To construct pNE6, the spiralin gene of *S. citri* GII-3 was amplified from genomic DNA with primers SR26 and SR27. Then the 1.3 kbp *EcoRI*-restricted PCR product was inserted at the unique *EcoRI* site of pBOG. Genes *scarp4a*, *5a* and *3b* were amplified from genomic DNA of *S. citri* GII-3 with primer pairs 3B1F/4A1R, 5A1F/5A1R and 3B1F/3B1R, respectively (Table 1). After restriction with *Bam*HI and *Bgl*II, the amplification products were inserted into the *Bam*HI site of pSD4 to yield plasmids pNB4F/R, pNB5F/R and pNB3F/R [F and R indicate whether the *scarp* gene has the same (F) or the opposite (R) orientation of *dnaA* in the *oriC* fragment of pSD4].

DNA isolation and Southern blot hybridization. Spiroplasma genomic DNA was prepared from 10 ml cultures using the Wizard genomic DNA purification kit (Promega). After digestion with restriction enzymes, digested DNA fragments were fractionated by agarose gel electrophoresis, blotted to positively charged nylon membranes by the alkali transfer procedure, and hybridized with appropriate [digoxigenin]dUTP-labelled probes using standard stringency conditions (Sambrook *et al.*, 1989). Hybridization signals were detected with anti-digoxigenin–alkaline phosphatase conjugate and HNPP (2-hydroxy-3-naphthoic acid-2'-phenylamidine phosphate) as the substrate, following the supplier's instructions (Roche Diagnostics). Fluorescent signals were detected using a Fluor-S Multimager phosphorimager (Bio-Rad). Probes S4, specific to *scarp4a*, and S235, hybridizing to all *scarp* genes except *scarp4a*, were produced by PCR amplification of genomic DNA with primer pairs S4F/S4R and S235F/S235R, respectively. Probe F consisted of a 191 bp sequence of the hypothetical protein F that is present in all six plasmids (pSci1–6) of *S. citri* GII-3. It was generated by amplification of genomic DNA with primers repF and repR (Table 1).

Production of mAbs. Spiroplasma cells from a 100 ml culture of *S. citri* GII3-9a2 were collected by centrifugation at 20 000 g for 20 min and washed three times in HS buffer (8 mM HEPES pH 7.4, 280 mM sucrose). The final pellet was resuspended in HS buffer to a final protein concentration of 1 mg ml⁻¹ and used as the antigen. BALB/c mice were immunized by injection of 25 µg antigen emulsified with complete Freund's adjuvant by the footpad route. Two weeks later, an additional injection of antigen mixed with the incomplete adjuvant was performed by the subcutaneous route. Three days after the second injection, lymphocytes from ganglions and Sp210 myeloma cells were fused using the PEG procedure and the hybridomas were selected in HAT medium (Ravoet & Bazin, 1990). The hybridomas were grown in RPMI medium (Moore & Hood, 1993) and those producing antibodies specific to spiroplasma antigens were screened by ELISA and cloned by limiting dilution. Immunoglobulins G (IgG) were purified from the hybridoma supernatants by affinity chromatography on protein A-Sepharose columns according to standard procedures (Ausubel *et al.*, 1995). Isotypes of mAbs were determined by ELISA.

Table 1. Primers

Primer	Nucleotide sequence (5'–3')*	Position	Accession no.
S4F	AGTTTATGCAGACGGAAAAC	10123–10142	AJ969072
S4R	ACGCATTCCAGCGTTAGAC	10495–10513	AJ969072
S235F	TAAACATTGATATTGCCAACCCC	3617–3639	AJ969069
S235R	GGTTAAAGTTGCAGAATTATTATC	3973–3996	AJ969069
3B1F	GGAATATAgATcTCAGAAAGGAAATAAG	9528–9555	AJ969073
3B1R	AATAATTGCGATcCAACTGCGATGGC	12132–12158	AJ969073
4A1R	GCGATAGgATCcTTAAACGCC	12246–12266	AJ969072
5A1F	GTGCTAgatCTATTTTTATTTTTAGAAAGG	5997–6027	AJ969073
5A1R	GTTATTTTAACGGaTccATGGTAAATTGG	8699–8727	AJ969073
repF	GCTAATAAATTAACTCGCAA	12313–12332	AJ969069
repR	TTTTCCATTTTTAACTCCCGCTC	12481–12503	AJ969069
SR26	GGTCgAattcCAGCCAGAATTAAGTTAGTGAAC	4141–4174	AF012877
SR27	AGCATgaaTtCCTTGTGAATCACCACCAGA	5380–5409	AF012877

*Bold letters indicate restriction enzyme sites. Lower-case letters indicate mismatched nucleotides.

Protein isolation, Western immunoblotting and colony blot immunoassay. Spiroplasma cells from a 50 ml culture were collected by centrifugation and washed three times in HS buffer as described above. Protein concentration was determined as described by Bradford (1976) before the pelleted cells were lysed in 0.25 ml of Laemmli solubilization buffer (Sambrook *et al.*, 1989). Protein separation by SDS-PAGE, Western immunoblotting and colony blot immunoassays were conducted as described previously (Duret *et al.*, 2003).

Triton X-114 phase partitioning. Spiroplasma proteins were separated into hydrophobic and hydrophilic fractions by the Triton X-114 partitioning method of Bordier (1981) essentially as described by Cheng *et al.* (1996). Spiroplasma cells from a 150 ml culture (10^{11} – 10^{12} c.f.u.) were collected by centrifugation and washed three times in HS buffer as described above. The pelleted cells were dispersed in 1 ml Tris-NaCl buffer (10 mM Tris/HCl pH 7.4, 154 mM NaCl) containing 1% Triton X-114 and the mixture was maintained at 4 °C for 40 min with gentle shaking. After removal of the insoluble components by centrifugation at 15 000 g for 10 min at 4 °C, the supernatant was incubated at 37 °C for 10 min to allow condensation of the detergent phase. The aqueous and detergent phases were then separated by centrifugation at 15 000 g for 10 min at room temperature. The aqueous phase was transferred to a new tube, chilled at 4 °C, and Triton X-114 was added to a final concentration of 1% in a total volume of 1 ml. The detergent phase was adjusted to 1 ml with Tris-NaCl buffer without adding Triton X-114. After 10 min at 4 °C the mixtures were incubated at 37 °C for 10 min and then centrifuged at 15 000 g for 10 min at room temperature. This cycle was repeated three times to ensure complete partitioning. The final aqueous and detergent phases were adjusted with Tris-NaCl buffer to 1 and 0.5 ml, respectively.

Mass spectrometry (MALDI-TOF). After SDS-PAGE the proteins were stained with colloidal Coomassie blue and the protein bands that were shown to react with mAb 10G3 by Western immunoblotting were excised for analysis. The in-gel trypsin digestion, MALDI-TOF (Applied Biosystems, Voyager DE super STR) analyses and peptide mass fingerprinting database searches were carried out at INRA (<http://www.jouy.inra.fr/unites/proteines/papss/>). The masses of peptides obtained from proteolytic digestion were compared to the predicted masses deduced from the *S. citri* GII-3 protein database. This database was constructed from sequence data of the ongoing *S. citri* GII-3 genome sequencing project (Foissac *et al.*, 2004).

In silico analyses. DNA and protein sequence analyses were performed using the programs proposed by Infobiogen (<http://www.infobiogen.fr/index.html>). Multiple alignments were done with MultAlin (Corpet, 1988). The BLAST program (Altschul *et al.*, 1997) was used to search homologues in general databases (<http://www.ncbi.nlm.nih.gov/blast/>) or in the *Spiroplasma kunkelii* partially sequenced genome (<http://www.genome.ou.edu/spiro.html>).

Experimental transmission assay. *S. citri* was transmitted to periwinkle (*Catharanthus roseus*) plants via injection into its leaf-hopper vector (*Circulifer haematiceps*) by the method of Foissac *et al.* (1996) as described previously (Duret *et al.*, 2003). Briefly, the insects were microinjected with 0.2 µl of the spiroplasma culture (approx. 10^5 spiroplasma cells), and the injected insects were caged on young periwinkle plants (12 insects per plant, 5–10 plants per spiroplasma strain). After a 2 week transmission period, the insects were removed, and the plants were kept in the greenhouse at 30 °C for symptom development. Culture of *S. citri* from plants and insects has been described elsewhere (Foissac *et al.*, 1997; Gaurivaud *et al.*, 2000; Duret *et al.*, 2003). Midribs (~0.2 g) were minced with a razor blade in 2 ml SP4 medium. After 30 min at room temperature, the extract was filtered (0.45 µm) and dilutions were plated on agar medium to determine the number of c.f.u. Titres of *S. citri* in the insects were determined at the end of the transmission period.

RESULTS

mAbs against *S. citri* GII3-9a2

With the aim of characterizing *S. citri* antigenic proteins other than spiralin, the spiralin-defective mutant GII3-9a2 (Duret *et al.*, 2003) was used as the source of antigens to immunize mice. Hybridomas producing immunoglobulins specifically directed against *S. citri* were first screened by ELISA and then by Western immunoblotting. Two hybridomas were selected, both of which produced antibodies of the IgG2b type. One of these, mAb 11A4, was found to react with a polypeptide of 70 kDa (Fig. 1a, lane 2). Unfortunately, subsequent passaging of hybridoma cells repeatedly resulted in loss of antibody production. The other, mAb 10G3, reacted with one major polypeptide of

Fig. 1. (a) Western immunoblotting, with mAb 10G3, of total proteins from *S. citri* GII3-9a2 with hybridoma supernatants 4C10, used as the negative control (lane 1), mAb 11A4 (lane 2) or mAb 10G3 (lane 3). (b) Western immunoblotting of total proteins from *S. citri* GII-3 (lane 1), GII3-9a2 (lane 2), *S. melliferum* (lane 3), *S. phoeniceum* (lane 4), *S. floricola* (lane 5), *S. apis* (lane 6) and *M. florum* (lane 7).

43 kDa and several other polypeptides with apparent molecular masses ranging from 80 to 95 kDa (Fig. 1a, lane 3). These results suggested that distinct proteins shared a common epitope, or that the various signals corresponded to processed products of a single protein. As shown in Fig. 1(b), polypeptides reacting with mAb 10G3 were present in *S. citri* GII-3 and GII3-9a2 (lanes 1 and 2), and in *S. phoeniceum* (lane 4) but not in *S. melliferum* (lane 3), *S. floricola* (lane 5), *S. apis* (lane 6) or *M. florum* (lane 7).

mAb 10G3 reacts with an adhesion-like protein

In Triton-X-114 partitioning the hydrophobic, membrane-associated proteins are incorporated into the detergent phase while hydrophilic proteins are sequestered in the aqueous phase. Western immunoblotting of *S. citri* GII-3

proteins using mAb 10G3 indicated that polypeptides P43 and P80-95 were amphiphilic, as they were completely partitioned in the detergent phase (Fig. 2b, lane 5). No polypeptides were detected in the aqueous phase (Fig. 2b, lane 6). Interestingly, several polypeptides were repeatedly detected in the detergent phase, whereas in total protein preparations, P43 was the major if not the only (depending on the experiment) polypeptide detected (Fig. 2b, lane 4). In mutant GII3-9a2 also, P43 was the major polypeptide detected in total proteins (Fig. 2b, lane 1). In the detergent phase, however, three major polypeptides of 43, 47 and 80 kDa were detected (Fig. 2b, lane 2).

From one-dimensional SDS-PAGE, the four protein bands P95, P80, P47 and P43 (boxed in Fig. 2a) corresponding to polypeptides recognized by mAb 10G3 were excised, and

Fig. 2. Coomassie blue-stained SDS-PAGE (a) and Western immunoblotting with mAb 10G3 (b) of proteins from *S. citri* GII3-9a2 (lanes 1–3) and GII-3 (lanes 4–6). Lanes 1 and 4, total proteins; lanes 2 and 5, proteins from the detergent phase; lanes 3 and 6, proteins from the aqueous phase. Gel bands from which proteins were submitted to MALDI-TOF analysis are boxed.

submitted to mass spectrometry (MALDI-TOF) analysis. For protein identification the masses of generated peptides were compared to the predicted masses of peptides from the protein database of *S. citri* (Foissac *et al.*, 2004). The results indicated that 26 % of peptide masses from P47 and 31 % from P80 matched a unique coding sequence (CDS), sharing homology with the adhesion-related protein P89 (Sarp1) of *S. citri* BR3 (Berg *et al.*, 2001), with the matched peptides covering 28 % (203/725) and 36 % (268/725) of the CDS, respectively. The P80 protein was later named Scarp4a (see below). The peptides from P47 and P80 also matched, though to a lesser extent (15 % and 9 %, respectively), the related CDS, Scarp5a. In the case of P95, 21 % of peptide masses matched the CDS Scarp3a, over 28 % (245/866) of the protein. Analysis of P43 yielded no conclusive results. In agreement with the complex Western immunoblotting pattern (mAb 10G3 recognized several polypeptides), these mass spectrometry data suggested that the epitope reacting with mAb 10G3 was probably carried by more than one protein. In addition, the finding that peptides from both P47 and P80 matched the same CDS strongly suggested that P47 was a cleavage product of P80, which was annotated as Scarp4a (theoretical molecular mass 77 040 Da) in the genome of *S. citri* GII-3 (Foissac *et al.*, 2004). Indeed peptides generated from P47 matched the C-terminal moiety of the protein whereas peptides from P80 were distributed all along the protein sequence (Fig. 3). According to these data, the cleavage site would be around and upstream of lysine residue K263. Search for homologies in the databases with the BLAST program revealed that Scarp4a shared 40 % identity and 54 % similarity (19 % gap) with the adhesion-related protein P89 (Sarp1) of *S. citri* BR3 (Berg *et al.*, 2001), and 44 % identity and 54 % similarity (25 % gap) with the related protein Skarp1 of *S. kunkelii* (Davis *et al.*, 2005). A survey of the *S. citri* GII-3 genome revealed that it contained

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1  MKKLLSILTI STLTTSIPAP LLANTPATRT LSSKMDVITG EDIKINEKLN
51  NWEVYADGK PENTVDNKKY EVVWHGLEKT NWKINKNNN AEGIKRIDTVG
101 DLLMMNFGDL TVGEGKSWKD WTNYPSEKRS VYRWGGDGEQ QTPKIDSGNG
151 ITDNLVDKTK FOQGIFALA ENNKTAWSN AGMLTVDGE LNIDIDNKNL
201 EKVYWDGFKQ KILDHMANIN VKPETSEKTH KLLIKYDING KKYTSKDIEV
251 VMAAKIDPPA PSKDDNLSEL IRTTDLGNIF DNNDTIFSA ITQKNARVVD
301 ESQIKITDKT DTQATLSAIE GSKSYHGSVA VTYNVASATT VDLKIDVTSS
351 ASGIQIDSDY LAQLDSSKMT NKVDTFEYSN GASTIKIKQP STGNFITGIV
401 YGCDEKWNKT SQSNNIDLPT GLEIDKQYQ SVDGRLIEL QHKDLPTHK
451 IYLIQISEKQ KIEHYWDEK GKQFEIWAED NGENIRGYS ASQLNLFEI
501 SAIWKQSLKH LDLKLDNEVY DNINKVQDE IYTYTKMLA SVKEQVENVY
551 PGVVENTDYV ININNVLVAGD WTTSKDVKQ AVDGSSTKLS FDSKTIPTVO
601 KEQVPLTST PDNKKDGDGK LWIIGVVGV LAGLGLAYLL FKKFVFDKYF
651 LPKINKRRHD KLVEKVRKEE AEKEENNKG GDE

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Fig. 3. Amino acid sequence of Scarp4a. Peptides identified by MALDI-TOF analyses are indicated. The peptides shared by both P47 and P80 are shaded in grey and the peptides specific to P80 in black. The amino acid sequence of the putative cleavage product P47 is boxed.

eight genes encoding Sarp1-related proteins with sizes ranging from 77 to 95 kDa. Based on their similarities, the *S. citri* GII-3 proteins were classified into four groups (2–5) and named Scarp2a, 2b, 3a, 3b, 3c, 3d, 4a and 5a. Multiple alignments of these proteins together with proteins Sarp1 and Skarp1 revealed that they shared distinctive properties. They possess a highly conserved signal peptide sequence followed by 6–8 tandem repeats consisting of 39–42 amino acids each, a conserved central region of about 330 amino acids, a variable region of about 100 amino acids, and a transmembrane α -helix followed by a charged C-terminal end. As exceptions to the rule, Scarp4a does not possess the amino acid repeats, and Scarp2b and 5a lack the D/E-rich, charged domain at the C-terminal end (Foissac *et al.*, 2004). When compared to Scarp4a as a whole, the cleavage product P47 was found to contain most of the conserved region (more than 75 %). Therefore it is very likely that mAb 10G3, which reacts with P80 (Scarp4a) and its cleavage product P47, as well as P95 (Scarp3a), recognizes an epitope located in this conserved region. Interestingly, in *S. citri* GII-3, all eight *scarp* genes are carried by plasmids (Foissac *et al.*, 2004). Genes encoding Scarp3a, 3d and 3c are carried by plasmids pSci1, pSci2 and pSci3, respectively. Scarp2a and 4a are encoded by pSci4, and Scarp2b, 3b and 5a are encoded by pSci5. Sequences encoding putative C-terminal-truncated Scarp polypeptides of 157 and 429 amino acids have been found in pSci6.

Scarps in transmissible and non-transmissible strains

To assess the putative role of Scarps in transmission of *S. citri* by its leafhopper vector *C. haematoceps*, we searched for the presence of Scarps in transmissible (GII-3, GII3-9a2) and non-transmissible (44, R8A2 and ASP-1) strains of *S. citri* by Western immunoblotting with mAb 10G3 (Fig. 4). As expected, a 43 kDa polypeptide was detected in total proteins from *S. citri* GII-3 and GII3-9a2 (Fig. 4a, lanes 1 and 2). In contrast, no signal was detected with non-transmissible strains 44, R8A2 and ASP-1 (lanes 3–5). Similar data were obtained with proteins from the Triton phase (Fig. 4b). While polypeptides of various sizes were detected in *S. citri* GII-3 (lane 1), none of them were detected in non-transmissible strains (lanes 3–5). Fig. 4 also shows that GII-3 and GII3-9a2 displayed different patterns, in that three major high molecular mass polypeptides were detected in GII-3 (lane 1) and only one in GII3-9a2 (lane 2).

Expression of Scarps in *S. citri* ASP-1

To make sure that mAb 10G3 did react with all Scarps, three of them (Scarp3b, 4a and 5a) were expressed by cloning in *S. citri* ASP-1. The relevant genes were inserted into the *oriC* plasmid vector pSD4 downstream of the *oriC* fragment, and the resulting plasmids pNB3F, pNB4F and pNB5F were introduced into *S. citri* ASP-1 by electroporation. Spiroplasmal transformants were selected on SP4 agar plates containing tetracycline. For all three plasmids, direct immunoblotting of colonies revealed that each

Fig. 4. Western immunoblotting, with mAb 10G3, of total proteins (a) and membrane fraction proteins (b) from *S. citri* GII-3 (lanes 1), GII3-9a2 (lanes 2), 44 (lanes 3), R8A2 (lanes 4) and ASP-1 (lanes 5).

tetracycline-resistant colony reacted with mAb 10G3 (Fig. 5e–j) similarly to those of GII-3 (Fig. 5a, b), whereas no signal was detected with spiroplasmas transformed by the insert-free vector pSD4 (Fig. 5c, d). These data clearly indicated that the epitope detected by mAb 10G3 was present in Scarp3b, 4a and 5a. Based on their similarities, it is very likely that all eight Scarps carry this epitope.

Fig. 5. Colonies (a, c, e, g, i) and direct immunoblotting, with mAb 10G3, of colonies (b, d, f, h, j) from *S. citri* GII-3 (a, b) and ASP-1 transformed by plasmids pNB3F (e, f), pNB4F (g, h), pNB5F (i, j) or insert-free vector pSD4 (c, d).

scarp genes in transmissible and non-transmissible strains

To determine whether the absence of Scarps in non-transmissible strains was due to the non-expression or to the absence of *scarp* genes, genomic DNAs of *S. citri* strains were analysed by Southern blot hybridization with *scarp* probes. The combination of DNA digestion by *HincII* with the use of two distinct probes, one of which is specific to *scarp4a* (S4) and the other (S235) to all *scarp* genes except *scarp4a*, made it possible to detect each *scarp* gene as a DNA fragment of specific size (Fig. 6a, b). In *S. citri* GII-3 (Fig. 6a, b, lanes 1), the sizes of the *HincII* restriction fragments corresponding to *scarp2a*, *2b*, *3a*, *3b*, *3c*, *3d*, *4a* and *5a* were 2549, 3309, 763, 556, 664, 8121, 2509 and 1585 bp, respectively. No signal was detected in non-transmissible strains 44, R8A2 and ASP-1 (Fig. 6a, b, lanes 3–5), indicating that no recognizable *scarp* genes were present in these strains. Interestingly, GII3-9a2 lacked *scarp2b*, *3b* and *5a*, as indicated by the absence of the 3309, 556 and 1585 bp signals (Fig. 6a, b, lanes 2). Because these three genes are carried on a unique plasmid (pSci5), it was hypothesized that the spiralin mutant GII3-9a2 might have lost this plasmid. Indeed, hybridization of unrestricted DNA from GII-3 and GII3-9a2 with the F probe confirmed that GII3-9a2 lacked pSci5 (Fig. 6c, lane 2). None of these plasmids were detected in the non-transmissible strains 44, R8A2 and ASP-1 (data not shown).

Transformation of GII3-9a2 with wild-type spiralin gene

In previous studies, we found that insect transmission of the spiralin-defective mutant GII3-9a2 was much less efficient than that of the wild-type strain GII-3, leading to the conclusion that spiralin is probably required for efficient transmission of *S. citri* by its leafhopper vector (Duret *et al.*, 2003). To test this hypothesis, the spiralin gene was introduced into GII3-9a2 by using the *oriC* plasmid pBOG as the vector. Transformation of GII3-9a2 cells by the recombinant plasmid pNE6 yielded gentamicin-resistant colonies which were shown to contain the plasmid (data not shown). As expected in the light of previous studies (Renaudin & Lartigue, 2005), passaging of the transformants resulted in plasmid integration into the host chromosome as revealed

Fig. 6. (a, b) Southern blot hybridization between *HincII*-restricted DNAs of *S. citri* GII-3 (lanes 1), GII3-9a2 (lanes 2), 44 (lanes 3), R8A2 (lanes 4) and ASP-1 (lanes 5), and probes S235 (a) or S4 (b). Names of *scarp* genes and sizes of the corresponding DNA fragments (in bp) are indicated. (c) Southern blot hybridization between unrestricted DNAs of *S. citri* GII-3 (lane 1), GII3-9a2 (lane 2), and the F probe.

by Southern blot hybridization with the spiralin probe (Fig. 7a). DNA digestion by *HpaI*, *HindIII*, *EcoRI* plus *BamHI*, and *PstI* plus *HindIII*, yielded hybridizing fragments of sizes consistent with integration of pNE6 within the pBS sequences, as indicated in Fig. 7(b). In particular the 14.1 kbp *HpaI* fragment of GII3-9a2 (Fig. 7a, lane 2) was replaced by two fragments of 10 and 13 kbp in the pNE6 transformant (Fig. 7a, lane 3). Once integrated into the host chromosome, plasmid sequences were stably maintained, regardless of the presence or absence of selection pressure. Expression of spiralin in two of these transformants was demonstrated by direct immunoblotting of colonies (data not shown) and was further confirmed by Western immunoblotting (Fig. 8). Large amounts of spiralin, similar to that in GII-3 (Fig. 8, lane 1), were detected in the pNE6-transformed cells (lanes 4 and 5) but not in cells transformed with the insert-free pBOG (lane 3). In the experiments reported in Table 2, transmission of the GII3-9a2 pNE6 transformant, which we named GII3-9a2S, was compared to that of the spiralin-defective mutant GII3-9a2. The wild-type strain GII-3 was used as the control. In the case of GII3-9a2S, a higher proportion of plants (6/10 vs 4/10 in expt 1 and 9/10 vs 4/9 in expt 2) were infected (as compared to GII3-9a2). In addition, a majority of infected plants (4/6 in expt 1, and 5/9 in expt 2) developed severe symptoms, concurrently with plants infected by the wild-type strain GII-3, and in contrast to those infected by GII3-9a2, for which a 2–4 week delay was observed. However, in spite of the high-level expression of spiralin in GII3-9a2S, the transmission efficiency was not fully restored, as indicated by the observation that a few plants (4/10 in expt 1 and 1/10 in expt 2) were not infected (Table 2). The fact that, in addition to

spiralin, GII3-9a2 also lacks pSci5 would explain why functional complementation could not be achieved by transformation with the spiralin gene alone. Therefore, to assess the putative role of pSci5 in transmission, attempts were made to produce a *S. citri* GII-3 variant lacking pSci5, in order to compare its transmission efficiency to that of GII3-9a2.

Plasmid curing of *S. citri* GII-3

With the aim of obtaining *S. citri* strains lacking one or several plasmids, serial dilutions of a *S. citri* GII-3 culture were grown either at normal growth temperature (32 °C) or at sublethal temperature (37 °C) for 5 days. After plating the highest dilutions with visible growth, the plasmid contents of individual clones were determined by Southern blot hybridization of *HincII*-digested genomic DNA with the *scarp* probe S235. At 32 °C, 100 % (14/14) of the tested clones displayed plasmid patterns identical to that of the parental, wild-type strain GII-3. They all possessed the six plasmids pSci1–6. In contrast, when grown at 37 °C, 7/17 clones had plasmid patterns distinct from that of the initial strain. Five of them lacked pSci5, one lacked pSci3, and one lacked both pSci3 and pSci5 as indicated by the absence of the relevant hybridization signals (3309, 1585 and 556 bp for pSci5, and 664 bp for pSci3). One additional propagation at 37 °C yielded new variants and one particular clone lacked pSci1, pSci3 and pSci5 (Fig. 9, lane 8). The various hybridization patterns are illustrated in Fig. 9. In particular, this figure shows that the *S. citri* variant GII3-5 (lane 3) exhibited the same hybridization pattern as GII3-9a2 (lane 2). The absence of the 556, 1585 and 3309 bp fragments corresponding to *scarp3b*, *5a* and *2b* indicates that this variant has lost pSci5.

Fig. 7. (a) Southern blot hybridization between restricted DNAs from *S. citri* GII3-9a2 and GII3-9a2S, and the spiralin probe (SR14-SR16). Purified pNE6 (lanes 1, 4, 7 and 10), and genomic DNAs from GII3-9a2 (lanes 2, 5, 8 and 11) and the pNE6 transformant GII3-9a2S (lanes 3, 6, 9 and 12) were restricted by *HpaI* (lanes 1–3), *HindIII* (lanes 4–6), *EcoRI* plus *BamHI* (lanes 7–9) and *PstI* plus *HindIII* (lanes 10–12). Sizes of DNA fragments are indicated in kbp. (b) Partial restriction maps and gene organization of the spiralin gene regions of *S. citri* GII3-9a2 and GII3-9a2S, and schematic representation of recombination between pNE6 and pBS sequences present in the chromosome of GII3-9a2. The maps are not to scale. Ori, *S. citri* chromosomal replication origin (*oriC*); O, reduced *oriC* fragment; p, spiralin gene promoter; spi, spiralin gene; Δ spi, 5'-truncated spiralin gene; gfp, GFP gene; Gm^R, gentamicin-resistance gene *aacA-aphD* of Tn4001; tetM, tetracycline-resistance gene *tetM* of Tn916; B, *BamHI*; E, *EcoRI*; H, *HindIII*; Hp, *HpaI*; P, *PstI*.

Experimental transmission of *S. citri* GII3-5

To determine whether pSci5 was required for efficient transmission, we compared the transmission efficiency of *S. citri* GII3-5 lacking pSci5 with those of *S. citri* strains GII-3 (wild-type), used as the control, and GII3-9a2, lacking both spiralin and pSci5. In these transmission tests, spiroplasmas were injected directly into the insect haemolymph, bypassing the passage through the alimentary canal. Hence, the

Fig. 8. Western immunoblotting, with anti-spiralin mAb 12G9, of total proteins from *S. citri* GII-3 (lane 1), GII3-9a2 (lane 2), and GII3-9a2 transformed by the insert-free vector pBOG (lane 3) or by pNE6 (lanes 4 and 5).

efficiency of transmission to the host plant reflects the ability of the spiroplasmas to cross the salivary gland barrier. The results in Table 3 indicate that all strains multiplied to high titres in the insect, regardless of the presence or absence of pSci5. Interestingly, in the case of GII3-5, all plants (7/7 in expts 1 and 2) developed severe symptoms within 2 weeks post-transmission, similarly to GII-3. In contrast, symptoms in the few plants (4/9) infected by GII3-9a2 could only be observed after a 2–4 week delay. These results suggested that pSci5 was not essential for transmission. Taken as a whole, the data suggest that spiralin rather than pSci5 is required for efficient transmission of *S. citri* to periwinkle by its leafhopper vector.

DISCUSSION

In this study, we produced mAbs against a *S. citri* mutant lacking spiralin. mAb 10G3 was found to react with several polypeptides, indicating that these polypeptides shared a common epitope. Mass spectrometry (MALDI-TOF) analyses of the polypeptides reacting with mAb 10G3 led to the characterization of adhesion-related protein Scarp4a, which shares strong similarities with the previously described

Table 2. Transmission of *S. citri* GII-3 (wild-type), GII3-9a2 and GII3-9a2S to periwinkle (*Catharanthus roseus*) plants via injection into the leafhopper vector (*Circulifer haematocephus*)

Inoculant (c.f.u. ml ⁻¹)	C.f.u. per insect*	Plants	Symptoms†	C.f.u. (g midribs) ^{-1‡}
Expt 1				
SP4 medium	—	P1	—	—
		P2	—	ND
		P3	—	ND
		P4	—	ND
<i>S. citri</i> GII-3 (8·6 × 10 ⁸)	7·8 × 10 ⁵	P1	+	ND
		P2	+	ND
		P3	+	1·2 × 10 ⁶
		P4	+	ND
		P5	+	ND
GII3-9a2 (1·2 × 10 ⁹)	7·7 × 10 ⁵	P1	+ (4)	ND
		P2	—	—
		P3	+ (2)	ND
		P4	—	—
		P5	—	ND
		P6	—	ND
		P7	—	—
		P8	+ (2)	4·2 × 10 ⁶
		P9	+ (3)	ND
		P10	—	—
GII3-9a2S (7·8 × 10 ⁸)	6·2 × 10 ⁵	P1	+	ND
		P2	—	—
		P3	+ (2)	3·5 × 10 ⁶
		P4	+	ND
		P5	—	—
		P6	—	ND
		P7	—	ND
		P8	+	ND
		P9	+ (3)	ND
		P10	+	ND
Expt 2				
SP4 medium	—	P1	—	—
		P2	—	ND
		P3	—	ND
		P4	—	ND
<i>S. citri</i> GII-3 (1·3 × 10 ⁹)	8 × 10 ⁵	P1	+	ND
		P2	+	ND
		P3	+	ND
		P4	+	ND
		P5	+	1·4 × 10 ⁶
GII3-9a2 (1·5 × 10 ⁹)	7·6 × 10 ⁵	P1	+ (4)	ND
		P2	—	ND
		P3	—	ND
		P4	—	ND
		P5	+ (2)	4·4 × 10 ⁶
		P6	o	—
		P7	—	ND
		P8	+ (4)	ND
		P9	+ (2)	ND
		P10	—	—
GII3-9a2S (7·6 × 10 ⁸)	6·2 × 10 ⁵	P1	+	ND
		P2	—	—
		P3	+	ND

Table 2. cont.

Inoculant (c.f.u. ml ⁻¹)	C.f.u. per insect*	Plants	Symptoms†	C.f.u. (g midribs) ^{-1‡}
		P4	+ (2)	4.2 × 10 ⁶
		P5	+	3.7 × 10 ⁶
		P6	+ (4)	ND
		P7	+ (3)	ND
		P8	+ (2)	ND
		P9	+	ND
		P10	+	ND

*Spiroplasma titres in the insects were determined at the end of the transmission period. Each value, indicated as c.f.u. per insect, represents the mean of two independent determinations, each from a set of eight insects.

†Numbers in parentheses indicate the delay (weeks) in symptom appearance compared to that for the control strain GII-3. o, dead plants (from something other than *S. citri* infection).

‡Spiroplasma titres in plants were determined 7 weeks post-transmission; ND, not determined.

Sarp1 of *S. citri* BR3 (Berg *et al.*, 2001) and Skarp1 of *S. kunkelii* (Davis *et al.*, 2005). In the *S. citri* GII-3 genome, *scarp4a* is one of the eight *scarp* genes, all of which are carried on plasmids: *scarp3a*, *3d* and *3c* on pSci1, pSci2, and pSci3, respectively; *scarp2a* and *4a* on pSci4; and *scarp2b*, *3b* and *5a* on pSci5. Genes *arp1* of *S. citri* BR3 and *skarp1* of *S. kunkelii* also are carried by plasmids, pBJS-O (accession no. NC_007101) and pSKU146 (Davis *et al.*, 2005), respectively. In addition to genes encoding adhesion-related proteins, the *S. citri* and *S. kunkelii* plasmids carry genes encoding proteins involved in DNA partitioning and transfer such as Soj, TraE/TrsE, TraG and Mob. TraE proteins are known to be involved in pilus biogenesis, and

structures that appeared to be conjugation pili were observed to connect *S. kunkelii* cells to each other and to insect cells (Ozbek *et al.*, 2003). Moreover, the finding of *oriT*-like regions in the *S. citri* GII-3 plasmids, as in pSKU146, suggests that these plasmids might be involved in conjugative gene transfer between strains. An extensive description of the plasmids from *S. citri* GII-3 will be published elsewhere.

Search for the presence of Scarps by Western immunoblotting of proteins with mAb 10G3 in various spiroplasmas suggested that Scarps were restricted to plant-pathogenic spiroplasmas, since they were detected in *S. citri*, *S. kunkelii* (Davis *et al.*, 2005) and *S. phoeniceum* P40, but not in *S.*

Fig. 9. Southern blot hybridization between probe S235 and *Hinc*II-restricted DNAs of *S. citri* GII-3 (lanes 1, 7 and 9), GII3-9a2 (lane 2), and of the distinct variants having lost pSci5 (GII3-5, lane 3), pSci3 (lane 6), pSci3 plus pSci5 (lane 4), pSci1 plus pSci5 (lane 5), and pSci1 plus pSci3 plus pSci5 (lane 8). Names of *scarp* genes and sizes of the corresponding DNA fragments (in bp) are indicated.

Table 3. Transmission of *S. citri* GII-3 (wild-type), GII3-9a2 and GII3-5 to periwinkle (*Catharanthus roseus*) plants via injection into the leafhopper vector (*Circulifer haematoceps*)

Inoculant (c.f.u. ml ⁻¹)	C.f.u. per insect*	Plants	Symptoms†	C.f.u. (g midribs) ^{-1‡}		
Expt 1						
SP4 medium§	—	P1	—	—		
		P2	—	ND		
		P3	—	ND		
		P4	—	ND		
<i>S. citri</i> GII-3§ (1·3 × 10 ⁹)	8 × 10 ⁵	P1	+	ND		
		P2	+	ND		
		P3	+	ND		
		P4	+	ND		
GII3-9a2§ (1·5 × 10 ⁹)	7·6 × 10 ⁵	P5	+	1·4 × 10 ⁶		
		P1	+ (4)	ND		
		P2	—	ND		
		P3	—	ND		
		P4	—	ND		
		P5	+ (2)	4·4 × 10 ⁶		
		P6	o			
		P7	—	ND		
		P8	+ (4)	ND		
		P9	+ (2)	ND		
GII3-5 (1·3 × 10 ⁹)	4·6 × 10 ⁵	P10	—	—		
		P1	o			
		P2	+	ND		
		P3	+	ND		
		P4	+	ND		
		P5	+	6·3 × 10 ⁶		
		P6	+	ND		
		P7	+	ND		
		P8	o			
		P9	+	ND		
Expt 2		P10	o			
		SP4 medium				
		<i>S. citri</i> GII-3 (7·6 × 10 ⁸)	1·4 × 10 ⁶	P1	—	—
				P2	—	ND
				P3	—	ND
				P1	o	
		GII3-5 (1·2 × 10 ⁹)	0·9 × 10 ⁶	P2	+	ND
				P3	+	ND
				P4	+	2·6 × 10 ⁶
				P5	o	
P1	o					
P2	+			ND		
P3	+			ND		
P4	o					
P5	o					
P6	+			3·8 × 10 ⁶		
		P7	+	ND		
		P8	+	ND		
		P9	+	ND		
		P10	+	ND		

* † ‡See Table 2 for details.

§Data for controls (SP4, GII-3 and GII3-9a2) in Expt 1 of Table 3 are the same as for Expt 2 of Table 2 and are from the same experiment.

melliferum, *S. floricola*, *S. apis* or *M. florum*. Interestingly, in spite of the ability of mAb 10G3 to recognize the various Scarps, none of them was detected in the non-transmissible strains R8A2, ASP-1 and 44 of *S. citri*, all of which produce spiralin. Southern blot hybridizations also revealed that these strains do not possess *scarp* genes and do not even carry plasmids pSci1–6. This apparent correlation between transmissibility and the presence of plasmids pSci1–6 strongly suggests that genetic determinants of transmissibility are carried by these plasmids. Spiroplasma adhesion-related proteins, in particular, might be involved in the insect transmission process by interacting with insect cell receptors. When injected into the leafhopper, all *S. citri* strains (insect-transmissible and non-transmissible) multiplied to equal extents in the haemolymph. Therefore, it is likely that adhesion-related proteins are required for the spiroplasmas to cross the salivary gland barrier. In agreement with this hypothesis, it has been shown that most of the Sarp1 (or P89) protein is exposed at the spiroplasma cell surface and that this protein is implicated in adherence of spiroplasmas to cells from the leafhopper vector *C. tenellus* (Yu *et al.*, 2000). In this respect, the possibility that adhesion-related proteins may also be required for crossing of the midgut epithelial layer cannot be excluded.

Previously, we showed that the spiralin-disrupted *S. citri* mutant GII3-9a2 was poorly transmitted, leading to the conclusion that spiralin was required for efficient transmission of *S. citri* by the leafhopper *C. haematoceps* (Duret *et al.*, 2003). The putative role of spiralin in the insect transmission process was further documented by the finding that, *in vitro*, this protein binds to glycoproteins from the leafhopper (Killiny *et al.*, 2005). Thus, spiralin seems more likely to be involved in specific spiroplasma–insect cell interactions rather than playing a role in protecting the spiroplasma cell membrane within the insect (Castano *et al.*, 2002). However, in spite of the increased transmission efficiency, transformation of GII3-9a2 with the wild-type spiralin gene failed to fully restore the wild-type phenotype. Therefore, the finding that the spiralin mutant GII3-9a2 had lost pSci5, encoding Scarp2b, 3b and 5a, suggested that, in addition to spiralin, pSci5 might also be required for efficient transmission. Unexpectedly, the transmission efficiency of *S. citri* GII3-5, a *S. citri* GII-3 strain having lost pSci5, was found to be quite similar to that of the wild-type strain GII-3, indicating that pSci5 was not essential for transmission. One possibility to explain the lack of an effect of pSci5 curing is that function(s) encoded by pSci5 could be complemented by pSci1–4 and/or pSci6. Indeed, most of the CDS of pSci5, including Scarps, are also present in at least one of the other plasmids. Thus, the reason why introduction of the wild-type spiralin gene into GII3-9a2 did not completely restore the transmission efficiency remains unclear. However, it should be mentioned that the spiralin mutant GII3-9a2 was obtained through two successive recombination events, leading to the insertion of 10 kbp foreign sequences into the spiroplasmal chromosome (Duret *et al.*, 2003). Consequently, the possibility cannot be excluded that, in this mutant, expression of

genes other than, and in the vicinity of, spiralin might be altered.

Hitherto, all *S. citri* plasmids were cryptic in that they were not associated with a given phenotypic character. The finding that non-insect-transmissible strains do not possess plasmids pSci1–6 suggested that these plasmids encode genetic determinants of transmissibility. In this respect, plasmid curing of *S. citri* GII-3 would be of primary importance to assess the role of plasmid-encoded genes in a given chromosomal background. In this study, growing *S. citri* GII-3 at sublethal temperature (37 °C) led to spiroplasma strains lacking one, two or three plasmids (pSci1, pSci3 and pSci5). However, in spite of multiple propagations at 37 °C, we were unable to produce a *S. citri* GII-3 strain having no plasmids. Unexpectedly, the use of curing agents such as acridine orange, acriflavine and novobiocin did not improve plasmid loss. Such results have been previously reported in chlamydiae, in which curing agents led to a paradoxical increase in plasmid copy number (Pickett *et al.*, 2005).

The presumed implication of plasmid-encoded genes in insect transmission has also been hypothesized in the case of the plant mollicute '*Candidatus* Phytoplasma asteris' (Nishigawa *et al.*, 2002). Comparison of plasmids pOYM, from an insect-transmissible line, and pOYNIM, from a non-insect-transmissible line of the onion yellows phytoplasma, revealed that pOYNIM lacked two ORFs that exist in pOYM. In this case, it was thought that the two ORFs encoding, respectively, an SSB protein and an unknown protein might confer some selective advantages to the plasmid or to the phytoplasma in the insect cell environment.

In summary, our data suggest a correlation between the presence of plasmids pSci1–6 and the ability of *S. citri* strains to be experimentally transmitted via injection into the leafhopper vector *C. haematoceps*. In agreement with this hypothesis, plasmids related to pSci1–6 were also detected in all three transmissible *S. citri* strains (other than GII-3) that were tested in our laboratory. In addition, the insect-transmissible strain BR3-3X has also been found to carry a plasmid (pBJS-O; accession no. NC_007101) related to pSci1–6. However, the possibility that loss of plasmids and loss of transmissibility could be independent events cannot be fully rejected. Obviously, further experiments, such as plasmid transfer between strains, are needed to further confirm the correlation between the presence of plasmids pSci1–6 and the spiroplasmal transmissibility. The *scarp* genes represent putative genetic determinants of transmissibility. However *S. citri* plasmids encode many other proteins of unknown function. Genetic studies are now required to further investigate the role of the various plasmid-encoded genes in the biology of *S. citri*.

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