Fructose Utilization and Phytopathogenicity of *Spiroplasma citri*

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Spiroplasma citri is a plant-pathogenic mollicute. Recently, the so-called nonphytopathogenic *S. citri* mutant GMT 553 was obtained by insertion of transposon Tn4001 into the first gene of the fructose operon. Additional fructose operon mutants were produced either by gene disruption or selection of spontaneous xylitol-resistant strains. The behavior of these spiroplasma mutants in the periwinkle plants has been studied. Plants infected via leafhoppers with the wild-type strain GII-3 began to show symptoms during the first week following the insect-transmission period, and the symptoms rapidly became severe. With the fructose operon mutants, symptoms appeared only during the fourth week and remained mild, except when reversion to a fructose⁺ phenotype occurred. In this case, the fructose⁺ revertants quickly overtook the fructose⁻ mutants and the symptoms soon became severe. When mutant GMT 553 was complemented with the fructose operon genes that restore fructose utilization, severe pathogenicity, similar to that of the wild-type strain, was also restored. Finally, plants infected with the wild-type strain and grown at 23°C instead of 30°C showed late symptoms, but these rapidly became severe. These results are discussed in light of the role of fructose in plants. Fructose utilization by the spiroplasmas could impair sucrose loading into the sieve tubes by the companion cells and result in accumulation of carbohydrates in source leaves and depletion of carbon sources in sink tissues.

Additional keywords: fructose permease, phloem, phosphofructokinase, sugar metabolism.

Mollicutes are wall-less prokaryotes phylogenetically related to gram-positive eubacteria with low content of guanine and cytosine in their DNA (Weisburg et al. 1989). Two groups of mollicutes are phytopathogenic: the spiroplasmas and the phytoplasmas. Spiroplasmas are characterized by helical morphology and motility. Among the 45 spiroplasma species or characterized strains (Williamson et al. 1998), three species are plant pathogens. The most studied spiroplasma, *Spiroplasma*

citri, is the causal agent of citrus stubborn disease (Markham et al. 1974; Saglio et al. 1971, 1973). Even though this spiroplasma was first isolated from sweet orange (*Citrus sinensis*) trees affected by stubborn disease, the agent infects many plant species other than citrus, including periwinkle (*Catharanthus roseus*), a good model plant, in which it induces stunting, leaf yellows, and wilting, leading to periwinkle death, a few weeks after infection (Calavan and Bové 1989). *S. citri* is transmitted from plant to plant by phloem-feeding leafhoppers such as *Circulifer haematocaps* and *Circulifer tenellus* (Calavan and Bové 1989). Phytoplasmas, the second group of plant-pathogenic mollicutes, are also transmitted from plant to plant by phloem-feeding leafhoppers. However, phytoplasmas are not available in culture, making their study more difficult. All phytopathogenic mollicutes are restricted to the sieve-tube elements of the phloem tissue.

The mechanisms involved in the phytopathogenicity of plant mollicutes are poorly understood. Toxins and lactic acid production have been suggested as possible factors (Daniels 1979, 1983). It was also shown that *S. citri* infection caused a shortage of auxins, and it was proposed that utilization of plant sterols by *S. citri* resulted in a deficit in growth regulators (Chang 1998). In spite of these studies, the molecular mechanisms of the interaction between the spiroplasma and the host plant remain unknown. Recently, a presumptive nonpathogenic mutant of *S. citri*, mutant GMT 553, was obtained by random insertion of transposon Tn4001 into the genome of the wild-type strain GII-3 (Foissac et al. 1997a, 1997b). Although wild-type strain GII-3 induced symptoms during the first week following the insect-transmission period, it took 4 weeks for symptoms to appear with the mutant, and symptom development was associated with reversion to wild type by spontaneous excision of transposon Tn4001 from the genome of mutant GMT 553. Also, the rate of multiplication of mutant GMT 553 in the plants was approximately twofold lower than that of the wild-type strain. In mutant GMT 553, transposon Tn4001 was shown to be inserted in the first of the three fructose operon genes (Gaurivaud et al. 2000a). The first gene, *fruR*, probably codes for the putative regulator protein of the operon. The protein deduced from the nucleotide sequence of *fruR* belongs to the DeoR family, which includes bacterial transcriptional regulators of carbohydrate catabolic operons (van Rooijen and de Vos 1990). The second gene, *fruA*, codes for the fructose permease of the phosphoenolpyruvate:fructose phosphotransferase system (fructose PTS). The fructose permease allows

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uptake and concomitant phosphorylation of fructose into fructose-1-phosphate. The third gene, *fruK*, codes for 1-phosphofructokinase (E.C. 2.7.1.56), which uses ATP to phosphorylate fructose-1-phosphate into fructose-1,6-bisphosphate. In the wild-type strain GII-3, the fructose operon is transcribed into two RNAs, one of 2.8 kb (*fruR* and *fruA*) and one of 3.8 kb (*fruR*, *fruA*, and *fruK*). Insertion of transposon Tn4001 at the 5' end of *fruR* abolishes expression of all three genes of the operon (Gaurivaud et al. 2000a). This explains why mutant GMT 553 is unable to use fructose as a carbon and energy source. Functional complementations of mutant GMT 553 with expression vectors harboring different combinations of the fructose operon genes have shown that *fruR+fruA+fruK*, *fruA+fruK*, or *fruA* alone were able to restore fructose utilization (Gaurivaud et al. 2000a). In planta mutant GMT 553 reverted to the wild-type phenotype; therefore, fructose operon mutants, other than transpositional mutants, were produced with the hope that they would be more stable and not revert (Gaurivaud et al. 2000b). They consist of mutants in which the fructose operon was disrupted by homologous recombination (mutants H31, H45, and E38) and of a spontaneous xylitol-resistant mutant (*xyl3*) in which the *fruA* gene was found to be mutated. In the work presented here, strains of mutant GMT 553 complemented with wild-type fructose operon genes were introduced into periwinkle plants, the symptoms produced were compared with those obtained by the noncomplemented mutant, and spiropasma titers in plants were determined. We show that the genes that are able to restore fructose utilization

are also responsible for restored phytopathogenicity. Phytopathogenicity of additional fructose operon mutants confirm involvement of the fructose operon in *S. citri* phytopathogenicity.

RESULTS

Spiroplasmas were injected into *C. haematoceps* leafhoppers, and batches of 10 leafhoppers were caged on periwinkle plants (10 insects per plant) as described previously (Foissac et al. 1997a). The injected insects were allowed to feed on the plants for 2 weeks (transmission period), after which they were killed with insecticide. Symptom development was monitored during observation periods of 7 (Table 1), 6 (Table 2), 11 (Table 3), or 9 (Table 4) weeks, following the 2-week transmission period, and the spiropasma titers (CFU) in representative plants were determined at regular intervals.

Symptoms observed are shown on Figure 1 in comparison to symptomless plants (Fig. 1A, G1, and H2). The first symptom seen was marginal chlorosis (Fig. 1B and C, ± and + on tables, respectively). According to the spiropasma strain involved, the symptoms remained mild (Fig. 1B or C) or became severe with asymmetric, chlorotic young leaves and mild chlorosis on old leaves (Fig. 1D, ++ on tables), followed by systemic leaf yellows (Fig. 1E and F1, +++ on tables). The last stage was lethal wilting (Fig. 1, F2, and I4, ++++ on tables).

Plants with early symptoms becoming severe showed significant stunting (Fig. 1G3, I3, and I4) in comparison to plants in which symptoms developed late and remained mild (Fig.

Table 1. Phytopathogenicity of mutant GMT 553 before and after complementation with fructose operon genes

<i>Spiroplasma citri</i> strain ^a	Periwinkle no.	Symptom evolution ^b							Spiroplasma titer ^c	
		1	2	3	4	5	6	7	2	4
GII-3	p1	+	++	++	+++	++++	++++	++++	4 × 10 ⁶	2 × 10 ⁷
	p2	+	++	+++	+++	+++	++++	++++	2 × 10 ⁶	7 × 10 ⁶
	p3	+	++	+++	+++	++++	++++	++++	3 × 10 ⁶	8 × 10 ⁵
GMT 553	p1	–	–	–	+	+	+	+	4 × 10 ³	4 × 10 ⁵
	p2	–	–	–	+	+	+	+	3 × 10 ⁵	6 × 10 ⁶
	p3	–	–	+	+	+	++	++	1 × 10 ⁵	2 × 10 ⁶
GII3pBOT	p1	–	–	+	+	+++	+++	+++	1 × 10 ⁴	3 × 10 ⁶
	p2	–	+	+	+	+++	+++	+++	8 × 10 ⁵	2 × 10 ⁶
553pBOT	p1	–	–	–	+	+	+	+	1 × 10 ⁴	1 × 10 ⁶
553pBOT-R	p1	–	–	–	–	+	+	+	50	5 × 10 ⁵
	p2	–	–	–	–	+	+	+	3 × 10 ³	7 × 10 ⁵
	p3	–	–	–	+	+	+	+	2 × 10 ²	1 × 10 ⁶
553pBOT-A	p1	+	+	+	++	++	++	+++	4 × 10 ⁷	3 × 10 ⁷
	p2	–	+	++	++	+++	+++	+++	6 × 10 ⁶	1 × 10 ⁷
	p3	–	+	+	++	++	+++	+++	3 × 10 ⁶	1 × 10 ⁷
	p4	+	+	+	++	+++	+++	+++	1 × 10 ⁷	1 × 10 ⁷
553pBOT-K	p1	–	–	–	–	+	+	+	0	ND
	p2	–	–	–	+	+	++	++	3 × 10 ⁶	ND
553pBOT-AK-oriC	p1	+	+	++	+++	++++	D	...	2 × 10 ⁶	5 × 10 ⁷
	p2	+	+	++	+++	++++	D	...	1 × 10 ⁶	1 × 10 ⁷
553pBOT-AK-fru	p1	+	++	+++	+++	++++	D	...	3 × 10 ⁷	2 × 10 ⁷
	p2	+	++	+++	+++	++++	D	...	6 × 10 ⁷	8 × 10 ⁷
553pBOT-RA	p1	–	–	–	–	+	+	++	0	ND
	p2	–	–	–	–	+	+	++	3 × 10 ⁴	ND
553pBOT-RAK	p1	+	+	+	++	+++	+++	++++	5 × 10 ⁶	2 × 10 ⁶
	p2	+	+	+	++	+++	+++	++++	4 × 10 ⁶	2 × 10 ⁶
	p3	+	+	++	++	+++	+++	+++	3 × 10 ⁶	6 × 10 ⁶
	p4	+	+	++	++	+++	+++	+++	5 × 10 ⁶	4 × 10 ⁶

^a pBOT = plasmid pBOT; R, A, and K = *fruR*, *fruA*, and *fruK*, respectively.

^b Symptom evolution in periwinkle plants: – = absence of symptoms; + = marginal chlorosis on young leaves; ++ = asymmetric and chlorotic young leaves, mild chlorosis on older leaves; +++ = stunting and systemic leaf yellows; ++++ = lethal wilting; and D = death of periwinkle at weeks 1 through 7 after the 2-week insect transmission period.

^c CFU per 200 mg of leaf midribs at 2 and 4 weeks after transmission period. ND = not done

1G2, H1, I1, and I2). In some cases, plants having first shown only mild symptoms developed severe symptoms later. Such plants were less stunted than plants with early, severe symptoms, but the change of symptoms from mild to severe was rapid and easily noticeable. As we will show below, this happened when fructose⁻ mutants reverted to fructose⁺ spiroplasmas.

Phytopathogenicity of mutant GMT 553 before and after complementation with fructose operon genes.

Plants infected with wild-type strain GII-3 began to show marginal chlorosis within the first week of the observation period and developed general chlorosis during the fourth week (Table 1; Fig. 1). With the fructose⁻ mutant GMT 553, no symptoms could be seen at the end of the third week (Table 1). Symptoms started to appear at the end of the fourth week but remained at the marginal chlorosis stage. Thus, mutant GMT 553 is essentially characterized by the fact that it induces late symptoms and the symptoms remain mild.

Strains of mutant GMT 553 complemented with various genes of the fructose operon were produced and described previously (Gaurivaud et al. 2000a). In these strains, complementation was obtained with plasmid pBOT carrying various fructose operon genes. Only strains in which the complementing plasmid was integrated within the GMT 553 spiroplasma genome were used. This was important because, during the 2-week transmission period and the 7-week observation period, no tetracycline was present in the insects or the plants, and the spiroplasmas were thus not subjected to tetracycline selection pressure. Under these conditions, integration of the complemented genes in the genome is expected to assure better stability. Integration was shown to be either at *oriC* or the fructose operon or the *infB* region upstream of the fructose operon (Table 5). Integration of the insert-free plasmid pBOT into *oriC* of mutant GMT 553 leading to mutant strain 553pBOT (Table 5) did not change symptom evolution in comparison to that of pBOT-free mutant GMT 553 (Table

1). With wild-type strain GII-3 transformed with pBOT (spiroplasma GII3pBOT) (Table 5), the first marginal chlorosis symptoms appeared in the second (plant P2) or third (plant P1) week of the observation period, and developed rapidly into general chlorosis after 5 weeks (Table 1). The titer of spiroplasma GII3pBOT in periwinkle plants after 2 weeks was lower than that of the wild-type strain GII-3 (10^4 to 8×10^5 and 3×10^6 CFU per 200 mg of midribs, respectively) but, after 4 weeks, the titers were similar. However, integration of plasmid pBOT into GII-3 or mutant GMT 553 did not abolish the difference between the mutant and the wild-type strain for symptom development.

Plants infected with mutant GMT 553 complemented with the entire fructose operon (strain 553pBOT-RAK) (Table 5) showed the same symptom development as the wild-type strain (Table 1). This result indicated that the fructose operon was, indeed, involved in *S. citri* pathogenicity.

Complementation of mutant GMT 553 with either *fruR* (strain 553pBOT-R), *fruR+fruA* (strain 553pBOT-RA), or *fruK* (strain 553pBOT-K) did not restore early symptom development (Table 1). These complemented mutants were unable to use fructose (Table 5). Early symptom development was restored when the mutant was complemented with *fruR+fruA+fruK* (553pBOT-RAK), *fruA+fruK* (553pBOT-AK), or *fruA* (553pBOT-A). These results indicated that only *fruA*, but not *fruR* or even *fruK*, was involved in symptom development similar to that obtained with the wild-type strain. The early symptom development seen with the complemented mutants having regained fructose utilization (553pBOT-RAK, 553pBOT-AK-*oriC*, 553pBOT-AK-*fru*, and 553pBOT-A) (Table 5) is not due to reversion to the wild-type phenotype because (i) using the techniques described by Foissac et al. (1997a), no revertants were detected by polymerase chain reaction (PCR) with primers surrounding the integration site of transposon Tn4001, and (ii) all complemented mutants were gentamycin resistant, indicating that transposon Tn4001 was

Table 2. Phytopathogenicity and reversion of *Spiroplasma citri* spontaneous xylitol-resistant mutant xyl3

Strain, plant no. ^a	Symptom evolution ^b						CFU at week ^c		ccu at week ^d			
									2		5	
	1	2	3	4	5	6	2	5	Glu	Fru	Glu	Fru
GII-3												
P1	+	++	+++	+++	++++	++++	4×10^5	ND	10^4	10^5	10^5	10^5
P2	+	++	+++	+++	++++	++++	7×10^5	8×10^5	10^4	10^4	10^7	10^6
P3	+	++	+++	+++	++++	++++	...	...	...	...	...	...
xyl1												
P1	-	+	++	+++	+++	++++	9×10^5	4×10^5	10^6	10^6	10^7	10^7
P2	+	+	++	+++	+++	++++	8×10^5	4×10^5	10^4	10^5	10^7	10^7
P3	-	+	++	+++	+++	++++	...	...	...	...	...	...
GMT 553												
P1	-	-	-	+	+	+	5×10^4	5×10^5	10^3	0	10^7	10^2
P2	-	-	-	+	+	++	1×10^5	5×10^5	10^3	0	10^7	0
P3	-	-	-	-	+	+	...	...	...	...	...	...
xyl3												
P2	-	-	-	+	+	++	2×10^4	1×10^6	10^2	0	10^6	10^6
P3	-	-	+	+	+	++	...	...	...	...	...	...
P4	-	-	+	+	+	++	...	...	...	...	...	...

^a *Spiroplasma citri* strain and periwinkle number.

^b Symptom evolution in periwinkle plants: - = absence of symptoms; + = marginal chlorosis on young leaves; ++ = asymmetric and chlorotic young leaves, mild chlorosis on older leaves; +++ = stunting and systemic leaf yellows; and ++++ = lethal wilting of periwinkle at weeks 1 through 6 after the 2-week insect transmission period.

^c CFU per 200 mg of leaf midribs; ND = not done.

^d Titer of spiroplasmas able to use fructose (Fru) or glucose (Glu) as color changing units (ccu) per 200 mg of leaf midribs. 0 = not detected

still present. It is interesting to note that the two strains with complementing genes *fruA+fruK* integrated either at *oriC* (553pBOT-AK-*oriC*) or at the fructose operon (553pBOT-AK-*fru*) induced early symptoms (i.e., similar to those due to the wild-type strain GII-3 or strain 553pBOT-RAK, even though fructose utilization by strain 553pBOT-AK-*oriC* or 553pBOT-AK-*fru* was twofold lower [Fru/Glu = 0.5; Table 5] than that conferred by GII-3pBOT [Fru/Glu = 1.1] or 553pBOT-RAK [Fru/Glu = 1.2]).

The titers of the complemented mutants in periwinkle plants were determined at weeks 2 and 4 of the observation period. The strains that did not use fructose (553pBOT-R, 553pBOT-K, 553pBOT-RA, and 553pBOT) (Table 5) showed, at week 2, titers of 10^2 to 10^5 CFU per 200 mg of midribs (Table 1), 10 times lower than the titer reached by the wild-type strain (10^6). However, at week 4, the titers were similar (10^6 to 10^7 CFU per 200 mg of midribs) (Table 1). With the strains able to use fructose (553pBOT-RAK, 553pBOT-A, 553pBOT-AK-*oriC*, and 553pBOT-AK-*fru*) (Table 5), the titers were similar to those of the wild-type strain at both weeks 2 and 4 (10^6 to 10^7 CFU per 200 mg of midribs) (Table 1).

In summary, only late and relatively weak symptoms were obtained with complemented mutant strains unable to use fructose, whereas early and severe symptoms were obtained with strains able to use fructose. *FruA* alone was sufficient to restore fructose utilization as well as early, severe symptom development.

Phytopathogenicity of *S. citri* spontaneous xylitol-resistant mutant xyl3.

The xylitol-resistant mutant xyl3 (Gaurivaud et al. 2000b), able to use glucose but not fructose, as well as wild-type strain GII-3, mutant GMT 553, and xylitol-resistant mutant xyl1 (a strain able to use glucose and fructose), were transmitted to periwinkle plants by *C. haematoceps* leafhoppers, as indicated above. Symptom development was recorded during a 6-week observation period, and spiroplasma titers were determined at weeks 2 and 5 by CFU (Table 2). For wild-type strain GII-3, as expected, early and severe symptom development was observed (Table 2). Mutant xyl1, xylitol resistant but able to use glucose and fructose (Gaurivaud et al. 2000b), also produced early and severe symptoms but 1 week later than GII-3. At the end of week 2 and week 5, the spiroplasma titers in planta

Table 3. Phytopathogenicity and reversion of mutants with disrupted fructose operon (H31, E38, and H45) and control strain (H11)

Strain, plant no. ^a	Symptom evolution ^b										
	1	2	3	4	5	6	7	8	9	10	11
H31											
P1	–	–	–	±	± (1×10^4)	±	±/R (1×10^4)	±	±	+/R (3×10^5)	++
P2	–	–	–	±	± (4×10^4)	±	± (1×10^4)	±	±	+/R (1×10^5)	++
E38											
P1	–	–	+	+	+/R (2×10^6)	++	+++/R (8×10^6)	+++	+++	+++/R (5×10^6)	+++
P2	–	+	+ (4×10^5)	+	+++/R (8×10^6)	+++	+++/R (1×10^6)	+++	+++	+++/R (3×10^6)	+++
H45											
P1	–	–	– (3×10^3)	+	+ (4×10^5)	+	+/R (3×10^6)	++	+++	+++/R (6×10^6)	+++
P2	–	–	–	+	+ (2×10^6)	+	+ (2×10^6)	+	+	+++/R (6×10^6)	+++
H11											
P1	+	++	+++/R (6×10^5)	+++	+++	++++	++++/R (1×10^3)	++++	++++	++++	++++

^a *Spiroplasma citri* strain and periwinkle number. Plasmid E and H, see reference Gaurivaud et al. 2000b.

^b Symptom evolution in periwinkle plants: – = absence of symptoms; + = marginal chlorosis on young leaves; ++ = asymmetric and chlorotic young leaves, mild chlorosis on older leaves; +++ = stunting and systemic leaf yellows; ++++ = lethal wilting; and ±: weak marginal chlorosis on young leaves of periwinkle at weeks 1 through 11 after the 2-week insect transmission period. R = presence of spiroplasmas able to use fructose (reversion); figures in parentheses are CFU per 200 mg leaf midribs.

Table 4. Influence of temperature on *Spiroplasma citri* titers and symptom evolution on periwinkle

Plant no.	Temperature ^a	Symptom evolution ^b									CFU at week ^c		
		1	2	3	4	5	6	7	8	9	2	4	7
P1	30/48/30	–	+	++	+++	+++	+++	+++	++++	++++	2×10^7	2×10^7	1×10^7
P2	30/48/30	–	+	++	+++	+++	+++	+++	++++	++++	2×10^7	2×10^7	2×10^7
P3	30/48/23	–	–	–	+	++	+++	+++	+++	+++	4×10^5	5×10^6	1×10^7
P4	30/48/23	–	–	–	–	–	+	+	++	+++	1×10^3	ND	2×10^6
P5	30/24/30	–	+	++	+++	+++	+++	+++	++++	++++	5×10^7	2×10^7	3×10^7
P6	30/24/30	–	+	++	+++	+++	+++	+++	++++	++++	2×10^7	2×10^7	2×10^7
P7	30/24/23	–	–	+	++	+++	+++	+++	+++	+++	2×10^4	1×10^7	2×10^7
P8	23/48/23	–	–	–	+	++	+++	+++	+++	+++	1×10^3	8×10^6	1×10^7
P9	23/48/23	–	–	–	–	–	–	–	–	–	0	0	7×10^5
P10	23/24/23	–	–	–	–	–	+	++	+++	+++	0	195	2×10^7
P11	23/24/23	–	–	–	–	–	–	–	–	–	0	0	114

^a Temperature (°C) at transmission period/duration of transmission period (h)/temperature (°C) during the 9-week observation period.

^b Symptom evolution in periwinkle plants: – = absence of symptoms; + = marginal chlorosis on young leaves; ++ = asymmetric and chlorotic young leaves, mild chlorosis on older leaves; +++ = stunting and systemic leaf yellows; and ++++ = lethal wilting on young leaves of periwinkle at weeks 1 through 9 after the 2-week insect transmission period.

^c CFU per 200 mg leaf midribs; ND = not done.

were similar (4×10^5 to 9×10^5 CFU per 200 mg of midribs) for the wild-type strain and mutant *xyl1* (Table 2). With mutant GMT 553 as well as *xyl3*, symptom development was delayed, the first marginal chlorosis symptoms appearing only between weeks 3 and 5. The CFU titers of mutant GMT 553 and *xyl3*, at week 2 of the observation period, were approximately 10 times lower than those of wild-type strain GII-3 or mutant *xyl1*, whereas, at week 5, the titers were similar for all the strains (Table 2).

At weeks 2 and 5, the spiroplasmas from the plants were analyzed for their ability to use glucose or fructose, and their titers (color-changing units [ccu] per 200 mg of midribs) were determined. The titer of spiroplasmas able to use glucose (glucose⁺) is representative of the whole spiroplasma population (fructose⁺ and fructose⁻ spiroplasmas). However, in the case of fructose⁻ mutants, the titer of spiroplasmas able to use fructose represents the spiroplasma revertants only. For wild-type strain GII-3 or mutant *xyl1* at weeks 2 and 5, the titers of glucose⁺ spiroplasmas were similar to the titer of fructose⁺ spiroplasmas; this was expected because these strains are able to use both sugars. In the case of the plants infected with fructose⁻ strains, no reversion to the fructose⁺ phenotype is expected during the early stages of the observation period, and the whole spiroplasma population should be fructose⁻. However as soon as reversion occurs, fructose⁺ spiroplasma should appear. Indeed, no fructose fermenting spiroplasmas were detected at week 2 in the plants infected with GMT 553 or *xyl3*; however, at week 5, spiroplasmas able to use fructose were found, indicating that reversion had occurred (Table 2). Fur-

thermore, for mutant *xyl3*, at week 5, the titer of glucose⁺ spiroplasmas was similar to the titer of fructose⁺ spiroplasmas, indicating that practically all spiroplasmas were revertants. This is not so at week 5 for mutant GMT 553, because no revertants were detected in plant P2 (Table 2) or represented as little as 0.001% of the total spiroplasma population in plant P1.

Phytopathogenicity of mutants with disrupted fructose operon (H31, E38, and H45).

Mutants H31, E38, and H45, with a disrupted fructose operon, were obtained by homologous recombination involving one crossing over between the fructose genes on the genome of wild-type strain GII-3 and deleted versions of *fruA*+*fruK* carried by plasmid pBOT (Gaurivaud et al. 2000b). They are fructose⁻ and glucose⁺. In mutant H11, recombination occurred at *oriC*, but not at the fructose operon; this mutant is fructose⁺ and was thought to be an even better control than GII-3.

Mutant H11, when transmitted to periwinkle plants, induced early and severe symptoms (Table 3), similar to those obtained with wild-type strain GII-3. The fructose⁻ mutants H31, H45, and E38 showed delayed symptoms and, up to the fifth week of the observation period, symptoms remained weak and were characterized by some stunting and slight marginal chlorosis only on young leaves. Titers of spiroplasmas in plants (CFU per 200 mg of midribs) were determined at weeks 3, 5, 7, and 10 (Table 3). At week 3, strain E38 had a titer similar to that of control strain H11, even though the symptoms were much less pronounced. Plant P1 infected with strain H45 had no symptoms yet at week 3, and its spiroplasma titer (3×10^3

Fig. 1. *Spiroplasma citri*-induced symptoms in periwinkle plants. **A**, Symptomless plants; **B**, weak marginal chlorosis on young leaves; **C**, marginal chlorosis on young leaves; **D**, asymmetric and chlorotic young leaves, mild chlorosis on older leaves; **E**, **F1**, **G3**, and **I3**, stunting and systemic leaf yellows; **F2** and **I4**, lethal wilting; and **G**, **H**, and **I**, stunting of plants with late, mild symptoms (G2, H1, H3, I1, and I2) or early, severe symptoms (G3 and I3) in comparison to healthy plants (G1 and H2).

CFU per 200 mg of midribs) was 100 times lower than that of strain E38 in plant P2 (4×10^5 CFU per 200 mg of midribs). In case of strain H31, symptoms occurred at week 4 and they remained particularly mild for as long as 9 weeks. The titer of spiroplasma H31 in the plants also remained low; it increased only 10-fold from week 5 (1×10^4 to 4×10^4 CFU per 200 mg of midribs) to week 10 (1×10^5 to 3×10^5 CFU per 200 mg of midribs). At weeks 5, 7, and 10, the presence of revertants able to use fructose was studied (Table 3). In the two plants infected with strain E38, revertants were detected as early as week 5, a time when the spiroplasma titer was already high (2×10^6 to 8×10^6) and had reached its maximum value. Also in these two plants, the symptom development, from week 5 on, was fast. Therefore, once revertants appeared, spiroplasma titers were high and symptoms became severe. In plant P1 infected with strain H45, a similar situation was observed from week 7 on. Interestingly, in plant P2 infected with H45, revertants were detected only at week 10; until then, the symptoms remained mild, even though the spiroplasma titer was high. This indicates that fructose⁻ spiroplasmas are phytopathogenic but induce only late symptoms and that, with such spiroplasmas even at high titers, the symptoms remain mild. However, with the occurrence of fructose⁺ revertants, the symptoms quickly become severe.

The reversion phenomenon occurring in planta was studied in more detail by culturing the spiroplasmas from leaf midribs at weeks 5, 7, and 10 (Table 3), followed by Southern hybridization of *Hpa*II restricted spiroplasma DNA with a *fruR*-specific probe, as already described (Gaurivaud et al. 2000b). In this technique, spiroplasmas with a fructose⁻ phenotype are characterized by the 4.2- or 4.5-kbp restriction fragment, whereas fructose⁺ spiroplasmas yield the unique 10-kbp fragment. Spiroplasmas cultured at week 5 (Fig. 2A) from plant P1 infected with strain H31 (lane 1), or plant P1 infected with strain H45 (lane 5), yielded the 4.5-kbp fragment, as expected for the fructose⁻ phenotype. At week 7 (Fig. 2B), the Southern hybridization revealed not only the 4.5-kbp fragment but also the 10-kbp fragment (lanes 1 and 5), indicating that reversion

from a fructose⁻ phenotype to a fructose⁺ phenotype had occurred. At week 10 (Fig. 2C), only the 10-kbp fragment was seen, indicating that the fructose⁺ spiroplasmas had overtaken the fructose⁻ spiroplasmas. These results are in agreement with the presence, in plant P1 infected with strain H45, at week 7 but not at week 5, of spiroplasmas able to use fructose as studied by acidification of the culture medium (Table 3). With plant P2 infected with H31 and plant P2 infected with H45, the 10-kbp fragment was detected at week 10 but not yet at week 7 (Fig. 1, lanes 2 and 6). In other words, it takes only 3 weeks for a fructose⁻ spiroplasma population to change to a population with only fructose⁺ spiroplasmas. This observation indicates that, in the plants, the rate of multiplication of spiroplasmas able to use fructose is higher than that of spiroplasmas unable to use fructose.

Influence of *S. citri* titers on symptom production.

Our results (Tables 1, 2, and 3) show that, in general, early and severe symptoms are shown by plants with high titers of fructose⁺ spiroplasmas and that, conversely, late and relatively mild symptoms are associated with fructose⁻ spiroplasmas, even when they reach high titers. In these experiments (Tables 1, 2, and 3), low spiroplasma titers in planta were obtained early in the observation period with spiroplasma mutants in which the fructose operon was inactivated by various means. The following experiments were designed to decrease the spiroplasma titers in the absence of fructose operon mutations or gene disruption, rather than by growing the infected plants at 23°C instead of 30°C, knowing that *S. citri* has a growth optimum at 32°C (Saglio et al. 1973). In these experiments, the injected leafhoppers were first caged on stock plants (*Mathiola incana*) at 30°C for 2 weeks for multiplication of the spiroplasmas in the insects to occur. Next, the insects were caged on the periwinkle plants for inoculation access for 24 or 48 h at 23 or 30°C. Symptom development during a 9-week observation period occurred at 23 or 30°C. Results are given in Table 4. When insect transmission and symptom development were carried out at 30°C, little variation in spiroplasma titers

Table 5. Properties of strains of *Spiroplasma citri* selected for functional complementation experiments

Strains ^a	<i>S. citri</i>	Plasmid		Fructose operon genotype	Sugar utilization ^b		
		Complementing	Integration site ^c		Glu	Fru	Fru/Glu
553pBOT-RAK (13.1.1)	GMT 553	pBOT-RAK	Fru operon	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruRAK</i>]	+	+	1.2
553pBOT-R (221)	GMT 553	pBOT-R	<i>oriC</i>	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruR</i>]	+	—	...
553pBOT-A (Fru21)	GMT 553	pBOT-A	<i>oriC</i>	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruA</i>]	+	+	0.5
553pBOT-K (FruK7)	GMT 553	pBOT-K	Fru operon	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruK</i>]	+	—	...
553pBOT-AK- <i>oriC</i> (Fru31)	GMT 553	pBOT-AK	<i>oriC</i>	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruAK</i>]	+	+	0.5
553pBOT-AK- <i>fru</i> (Fru36)	GMT 553	pBOT-AK	Fru operon	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruAK</i>]	+	+	0.5
553pBOT-RA (TXA4)	GMT 553	pBOT-RA	<i>infB</i> region	(<i>fruRAK</i>)::Tn4001 pBOT[<i>fruRA</i>]	+	—	...
553pBOT	GMT 553	pBOT	<i>oriC</i>	(<i>fruRAK</i>)::Tn4001 pBOT	+	—	...
GII3pBOT	GII-3	pBOT	<i>oriC</i>	<i>fruRAK</i> pBOT	+	+	1.1
Xyl1	GII-3	...	...	<i>fruRAK</i>	+	+	...
Xyl3	GII-3	...	...	<i>fruRaK</i> ^d	+	—	...
H11	GII-3	plasmid H	<i>oriC</i>	(<i>fruRAK</i>)::pBOT[<i>fruAA</i> (5')KΔ(3')]	+	+	...
H31	GII-3	plasmid H	Fru operon	(<i>fruRAK</i>)::pBOT[<i>fruAA</i> (5')KΔ(3')]	+	—	...
H45	GII-3	plasmid H	Fru operon	(<i>fruRAK</i>)::pBOT[<i>fruAA</i> (5')KΔ(3')]	+	—	...
E38	GII-3	plasmid E	Fru operon	(<i>fruRAK</i>)::pBOT[<i>fruAA</i> (5'3')KΔ(5'3')]	+	—	...

^a pBOT = plasmid pBOT; R, A, K = *fruR*, *fruA*, and *fruK*, respectively. Plasmid E and H, see reference Gaurivaud et al. 2000b. For several strains, the name appearing in reference Gaurivaud et al. 2000a is indicated in parentheses.

^b Sugar (Glu = glucose, Fru = fructose) is fermented (+) or not fermented (—). Flu/Glu: for each strain able to use both glucose and fructose, the maximal rate of acidification was calculated for fructose (V_{fru}) and glucose (V_{glu}) and the ratio V_{fru}/V_{glu} (Fru/Glu) was deduced.

^c Fru operon = fructose operon; *infB* (gene for translation initiation factor IF2) is upstream of the fructose operon; *oriC* = origin of DNA replication.

^d The *fruA* gene of mutant xyl3 was found to be mutated.

was observed between duplicate plants (see, for example, periwinkle P1 and P2). In contrast, at 23°C, a great variation in spiroplasma titers was observed (see periwinkle P3 and P4). Nevertheless, Table 4 shows clearly that symptoms developed when *S. citri* titers reached the maximum value (i.e., approximately 10^6 to 10^7 CFU per 200 mg of midribs in this experiment). The earlier this titer was obtained, the earlier the symptoms occurred. For instance, in plant P1 (30/48/30), where symptom expression was allowed to occur at 30°C, the titer of 2×10^7 CFU per 200 mg of midribs was already obtained at week 2, and the first symptoms were observed at this time. In contrast, in plant P3, where symptom expression occurred at 23°C, it took 4 weeks to reach the titer of 5×10^6 and symptom development. This experiment shows that, when multiplication of wild-type strain GII-3 is decreased by growing the infected periwinkle plants at 23°C instead of 30°C, the symptoms were late but rapidly became severe.

In this experiment (Table 4), the results between duplicate plants where spiroplasma multiplication occurred at 23°C (P3 and P4, for instance) were quite different. This is probably due to the fact that the number of spiroplasma cells injected by leafhoppers into plants varied from plant to plant. At 30°C, in spite of this variation, the spiroplasmas grew quickly, and rapidly (within 2 weeks) reached the critical titer of 10^6 to 10^7 CFU per 200 mg of midribs at which symptoms occur. At the suboptimal temperature of 23°C, the spiroplasma multiplication was slower and the time required to reach the critical titer was much longer and differed from one plant to the other. For example, in plants P9 and P11, the critical titer was not even reached at 7 weeks, and no symptoms were present at week 9.

DISCUSSION

In the phytopathogenicity tests reported here, plants were injected via leafhoppers for a 2-week transmission period with one strain of *S. citri* (Table 5). Plants infected with the fructose⁻ spiroplasmas showed symptoms occurring as late as 4 weeks after the 2-week insect-transmission period, in contrast to plants infected with the fructose⁺ spiroplasmas, which showed symptoms as early as 1 week after the transmission period. The plants infected with the fructose⁺ spiroplasmas always became severely affected; whereas, on plants infected with fructose⁻ mutants, symptoms remained mild until revertants, with a fructose⁺ phenotype, eventually occurred. This was the case with the disrupted fructose operon mutants, in which symptoms remained mild as long as no revertants appeared. This is especially true for mutants H31 and H45, in which revertants were detected as late as 7 to 10 weeks; whereas, in the case of mutant E38, revertants occurred earlier, at week 5. Interestingly, we have found recently (Gaurivaud et al. 2000b) that, in mutant H31, and probably also in mutant H45, there are in fact two mutations rendering the strains fructose⁻: (i) fructose operon disruption and (ii) a spontaneous mutation in the fructose permease *fruA* gene. This probably explains why, with two mutations, it takes longer for these strain to revert to a wild-type fructose⁺ phenotype. These experiments would have been even more conclusive if the fructose⁻ mutants had not reverted. Nevertheless, the results establish, as a whole, the fact that late, mild symptoms are associated with the fructose⁻ phenotype, even at high spiroplasma titers.

In the above experiments, it is assumed that the occurrence of fructose⁺ spiroplasmas in plants initially infected with fructose⁻ strains is due to reversion. It could also result from a contamination of the fructose⁻ strains with fructose⁺ organisms. However, this is not the case for the following reasons: (i) all strains, and in particular the fructose⁻ mutants, were triply cloned according to the classic mollicute cloning procedure (Tully 1983); (ii) the cloned strains could always be shown to have the expected phenotype; (iii) in cultures of fructose⁻ strains, fructose⁺ spiroplasmas were never detected, even though such fructose⁺ spiroplasmas, if present, would have quickly overgrown the fructose⁻ organisms; and (iv) in planta, it takes only 3 weeks to pass from a fructose⁻ spiroplasma population to a spiroplasma population entirely fructose⁺ (Fig. 2). If such a passage was due to the presence of fructose⁺ contaminants in the triply cloned fructose⁻ strains injected in the plants, fructose⁺ spiroplasmas would have been detected relatively early in infection and symptoms would have become severe. This was not the case (see Table 3 in particular).

The spiroplasma titers in plants with early symptoms reached 10^6 to 10^7 CFU per 200 mg of leaf midrib tissue within 1 week postinfection whereas, in plants with late symptoms, it took approximately 4 weeks to reach similar titers. This indicates that, in plants with late symptoms, the rate of spiroplasma

Fig. 2. Southern blot hybridization between a *fruR* probe and *Hpa*II-digested genomic DNA from spiroplasmas isolated from the following periwinkle plants (Table 3): lane 1, H31P1; lane 2, H31P2; lane 3, E38P2; lane 4, E38P1; lane 5, H45P1; and lane 6, H45P2. Spiroplasmas were isolated from plants A, 5, B, 7, and C, 10 weeks after the 2-week transmission period.

multiplication is lower than that in plants with early symptoms, and that the fructose⁻ phenotype is probably responsible for the low rate of multiplication and, therefore, late and mild symptom development. These correlations are supported by the complementation experiments of mutant GMT 553 with one or several fructose operon genes: *fruR* coding for a putative regulatory protein; *fruA*, the fructose permease gene; and *fruK*, the 1-phosphofructokinase gene (Gaurivaud et al. 2000a). The following gene or gene combinations are able to restore a fructose⁺ phenotype as well as early and severe symptom induction: *fruR+fruA+fruK*, *fruA+fruK*, and *fruA*. The following genes or gene combination did not restore the fructose⁺ phenotype and symptom development remained late: *fruR+fruA*, *fruR*, and *fruK*. These experiments show that the gene involved in fructose utilization as well as in early symptoms induction is essentially *fruA*. Products of housekeeping genes, such as those for sugar metabolism, are not usually considered to be virulence factors in the same way that toxins or degrading enzymes are. However, carbohydrate catabolism has already been shown to be involved in virulence of pathogenic bacteria. For example, mutants of *Erwinia amylovora* unable to use galactose were shown to be avirulent (Metzger et al. 1994).

The optimal growth temperature of *S. citri* is 32°C (Saglio et al. 1973). At 23°C, the growth rate of this organism is sig-

nificantly lower. In plants maintained at 23°C, the wild-type spiroplasma should multiply less rapidly and induce symptoms later than in plants grown at 30°C. This is precisely what was found (Table 4). In infected plants grown at 30°C, spiroplasma titers reached 10⁶ to 10⁷ CFU per 200 mg of leaf midrib tissue within 1 or 2 weeks after the transmission period; whereas, at 23°C, similar titers were generally obtained after 4 weeks. Therefore, whether spiroplasma multiplication was decreased by growing the infected plants at 23°C rather than 30°C, or by inactivating the spiroplasma fructose operon, the effect on symptom appearance was the same: symptoms were delayed. There is, however, one major difference. Even though symptoms occur late at 23°C, they became much more severe than in the case of plants infected with fructose⁻ spiroplasmas, in which the symptoms remain mild unless fructose⁺ revertants appear. Therefore, fructose utilization might be more directly involved in pathogenicity than first thought.

In the plant, the spiroplasmas are restricted to the sieve tubes of the phloem tissue. Pathogenic spiroplasmas that induce early and severe symptoms are fructose and glucose utilizers which must find these sugars within the sieve tubes. Less-pathogenic spiroplasmas, inducing late and mild symptoms, do not use fructose, but do use glucose. Strangely, the concentration of fructose and glucose in the sieve tubes seems to

Fig. 3. Schematic representation of sucrose loading into the sieves tubes, and competition between *Spiroplasma citri* and the companion cell for fructose and glucose utilization. 1 = H⁺ATPase and 2 = sucrose/H⁺ cotransporter.

be very low (Ziegler 1975). Reducing sugars, such as glucose and fructose, could not be detected in phloem sap collected by stylectomy (Chino et al. 1991; Flower and Yeo 1992). Hence the question arises: where does the fructose used by the pathogenic spiroplasmas come from? In many plants, the major form of transported carbohydrates in the sieve tubes is sucrose. Loading of sucrose into a sieve tube requires the action of companion cells. Movement of sucrose into the companion cell is via a sucrose/H⁺ cotransporter within the plasma membrane (Fig. 3). It occurs against a sucrose concentration gradient and is energized by the H⁺ATPase, also localized in the plasma membrane of the companion cell (Bouché-Pillon et al. 1994; Delrot and Bonnemain 1981). For a proton gradient to become established, some sucrose must be metabolized in the companion cell, the first step being cleavage of sucrose by sucrose-synthase into UDP-glucose and fructose. Fructose, glucose, and sucrose are supposed to diffuse from the companion cell into the sieve tube, where the two hexoses can be used by *S. citri*. Furthermore, because the fructose concentration is low, even *S. citri* strains with a low rate of fructose utilization will induce early and severe symptoms, similar to those produced by *S. citri* strains with a high rate of fructose utilization. Two hypotheses, not exclusive of one another, could explain the role of fructose utilization in the phytopathogenicity of *S. citri*. The first hypothesis is based on the production of lactic acid by *S. citri*. Strains able to use glucose and fructose might produce much more acid than strains able to use only glucose, and strong acidification of the phloem sap by the wild-type strain could affect photoassimilate partitioning. The second hypothesis is that *S. citri* and companion cells compete for fructose. Depletion of fructose by the spiroplasmas could result in less active companion cells, modified distribution of photoassimilates, and depletion of carbon sources in sink tissues. Lerchl et al. (1995) demonstrated that transgenic tobacco plants expressing a pyrophosphatase in the phloem were stunted due to reduced sucrose utilization by companion cells and sucrose loading into phloem (Lerchl et al. 1995). Earlier studies showed that phytoplasma infections reduced translocation of photoassimilates (Catlin et al. 1975; Braun and Sinclair 1978). Recently, Lepka et al. (1999) observed a modification of carbohydrate partitioning in periwinkle plants infected by phytoplasmas. Infection led to an increase of soluble carbohydrates in source leaves and a decrease in sink leaves. The lower level of carbohydrates in sink organs could lead to growth impairment as observed for young leaves and roots. Chlorosis of older leaves might be a consequence of accumulation of carbohydrates in source leaves (Krapp et al. 1993; Geigenberger et al. 1996). Preliminary studies in healthy and *S. citri*-infected periwinkles show a modification in soluble sugar concentration in source and sink leaves. Furthermore, it is interesting to note that expression of the gene coding for transketolase is inhibited in periwinkle plants infected with wild-type strain GII-3 but not with mutant GMT 553 (S. Jagoueix-Eveillard, *personal communication*). These results seem to indicate that pathogenicity of sieve tube-restricted plant mollicutes may involve a novel mechanism in which fructose utilization by the pathogen interferes with the normal physiology of the plant. However, even though the fructose⁻ strains induce only mild symptoms, they are still pathogenic, indicating that mechanisms of pathogenicity other than fructose utilization, are probably involved.

Finally, because gene disruption for mutant production is now possible with *S. citri* (Duret et al. 1999; Gaurivaud et al. 2000b), it will be most interesting to study the role of glucose⁻ mutants on *S. citri* phytopathogenicity.

MATERIALS AND METHODS

Bacterial strains and plasmids.

S. citri wild-type strain GII-3 was originally isolated from *C. haematoceps*, a *S. citri* leafhopper vector (Vignault et al. 1980). Mutant GMT 553 was obtained by mutagenesis of wild-type strain GII-3 with transposon Tn4001 (Foissac et al. 1997a, 1997b). Strains used in complementation experiments, spontaneous xylitol-resistant mutants xyl3 and xyl1, strain H11, and mutants with disrupted fructose operon (H31, H45, and E38) were obtained previously (Gaurivaud et al. 2000a, 2000b) (Table 5). *S. citri* was grown in SP4 medium (Tully et al. 1977). Mutant GMT 553 was grown in the presence of gentamycin at 100 µg/ml. HSI medium (mycoplasma broth base at 15 g/liter; PPLO serum fraction, 1% [vol/vol]; sorbitol, 7% [wt/vol]; phenol red at 30 mg/liter; and penicillin at 2 × 10⁵ U/ml) was used for carbohydrate fermentation experiments (Whitcomb 1983). Carbohydrates (glucose, fructose, or sorbitol) were added at the final concentration of 0.5% (wt/vol). Plasmid pBOT carrying the *S. citri* *oriC* and tetracycline resistance gene (*tetM*) was described elsewhere (Renaudin et al. 1995; Ye et al. 1994) and was used for gene expression experiments in *S. citri*.

DNA methods.

Total DNA from the *S. citri* wild-type strain GII-3 and from mutants were isolated as follows. Spiroplasma culture (30 ml) was collected by centrifugation and resuspended in 270 µl of phosphate-buffered saline buffer. Cells were lysed by adding 30 µl of 10% sodium dodecyl sulfate and incubated 30 min at 37°C. The DNA was further purified by phenol-chloroform deproteinization and ethanol-acetate precipitation. Restricted DNA fragments were separated by agarose gel electrophoresis, blotted onto charged nylon membranes by the alkali transfer procedure, and hybridized with [α-³²P]dATP-labeled probes using standard stringency conditions (Sambrook et al. 1989). Probe *fruR* was the 0.4-kbp *HpaI* fragment obtained from plasmid pRAK (Gaurivaud et al. 2000a).

Carbohydrate fermentation.

Carbohydrate catabolism in *S. citri* results in lactic acid and acetic acid production. Acidification of the growth medium was used as an indicator of carbohydrate utilization. *S. citri* growth requires complex media supplemented with fetal calf or horse serum, bringing into the media carbohydrates and enzymes such as invertase, which may interfere with fermentation of sugars added to the medium. For these reasons, we used the sugar-free HSI medium for monitoring carbohydrate fermentation. *S. citri* cells exponentially grown in SP4 medium were harvested by centrifugation (20 min, 12,000 × g, 20°C), washed twice, and resuspended with HEPES-sorbitol buffer (HEPES, 8 mM, pH 7.4; and sorbitol, 10% [wt/vol]). HSI medium supplemented with glucose, fructose, or sorbitol (each at a final concentration of 0.5%) was inoculated with the washed cells. Decrease of pH was followed by color changes of the phenol red.

Experimental infection of periwinkle plants.

Microinjection of *S. citri* into *C. haematoceps* leafhoppers and transmission to the periwinkle host plant (*Catharanthus roseus*) were described elsewhere (Foissac et al. 1997a). Briefly, CO₂-anaesthetized adult female leafhoppers (*C. haematoceps*) were injected between the two last abdominal sternites with hand-drawn glass needles with approximately 0.1 µl of SP4 culture medium (approximately 10⁸ spiroplasma per ml). Injected leafhoppers were caged on young periwinkle plants (six- to eight-leaf stage, 10 insects per plant) for a period of 2 weeks at 30 ± 2°C (transmission period). After this period, the leafhoppers were killed with insecticide (dichlorvos) and the plants were kept in the greenhouse at 30 ± 2°C for symptom development (observation period) and *S. citri* analysis.

To study the relationship between symptom development and the number of spiroplasmas in plants, 300 females of *C. haematoceps* were injected with wild-type strain GII-3 and caged on stock plants (*Mathiola incana*). After 14 days at 30 ± 2°C, the insects were caged on young periwinkle plants for a transmission period of 6, 24, or 48 h at 23 ± 2°C or 30 ± 2°C. The insects were killed with insecticide after the transmission period and the plants were kept in the glasshouse at 23 ± 2°C or 30 ± 2°C.

Titers (CFU and ccu) of *S. citri* in periwinkle plants.

At 2 and 4 weeks after the insect transmission period, aliquots of midribs from a mixture of young and old periwinkle leaves were minced with a razor blade in 2 ml of liquid SP4 medium. After 30 min at room temperature, the extracts were passed through filter membranes (0.45-µm pore diameter). The number of CFU per 200 mg of midrib was determined by plating 10-fold serial dilutions of the filtrate on solid SP4 medium (three plates per dilution).

To determine the concentration (ccu per 200 mg of midribs) of spiroplasmas able to use glucose (glucose⁺) or fructose (fructose⁺), 200 mg of midribs were minced with a razor blade with 2 ml of HSI medium supplemented with 0.5% fructose (wt/vol). After 30 min at room temperature, the extract was passed through filter membranes (0.45-µm pore diameter). Ten-fold dilutions of the filtrate, in HSI medium with glucose or fructose, were used for the determination of ccu per 200 mg of midribs. The ccu per 200 mg was the last 10-fold dilution giving acidification of the medium after 1 week at 32°C. An aliquot of the filtrate was also used for the determination of colony forming units as described above.

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