

INVITED REVIEW

SUGAR METABOLISM AND PATHOGENICITY OF *SPIROPLASMA CITRI*

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SUMMARY

Spiroplasma citri is a plant-pathogenic mollicute, phylogenetically related to Gram-positive bacteria. Spiroplasma cells are restricted to the phloem sieve elements and are transmitted by leafhopper vectors. Recent research has allowed depicting a unique scenario in *S. citri* pathogenicity, where sugar metabolism plays a major role. *In vitro* *S. citri* uses fructose, glucose, and trehalose, which are imported through the Phosphoenolpyruvate phosphoTransferase System (PTS). When both fructose and glucose are present, fructose is used preferentially. The fructose-PTS permease is made of one single polypeptide IIBC^{Fru}, whereas the glucose permease is split into two polypeptides IICB^{Glc} and IIA^{Glc} encoded by two separated genes *ptsG* and *crr*. The glucose IICB^{Glc} and trehalose IIBC^{Tre} permeases function with a single IIA^{Glc} domain, enabling the spiroplasma to rapidly adapt from glucose to trehalose and vice versa. Up-regulation of expression of the PTS permease genes by the relevant sugar also illustrates the adaptive capacity of the spiroplasma. *S. citri* mutants unable to import glucose and trehalose are still highly pathogenic. In contrast, mutants unable to import fructose induce very mild and delayed symptoms, indicating that fructose and glucose play distinct roles in spiroplasmal pathogenicity. While fructose is used as a substrate by the spiroplasma to grow, glucose accumulates in source leaves of the host plant, leading to physiological disorders and down-regulation of photosynthesis genes. Fructose utilization by the spiroplasmas is postulated to deprive companion cells of fructose, thereby impairing sucrose loading in the sieve elements. Alternatively, preferential use of fructose is hypothesized to increase invertase activity, leading to glucose accumulation, and inhibition of photosynthesis. Both model mechanisms may contribute to *S. citri* pathogenicity.

Key words: *Spiroplasma citri*, sugar catabolism, PTS permease mutants, plant-mollicute interactions, carbohydrate partitioning.

INTRODUCTION

Plant pathogenic mollicutes are associated with several hundred diseases affecting a wide variety of crops including ornamentals, vegetables, fruit trees, and grapevine (McCoy *et al.*, 1989; Lee *et al.*, 2000; Seemüller *et al.*, 2002; Firrao *et al.*, 2005). In the plant, the bacteria are restricted to the phloem sieve tubes. They cannot be transmitted mechanically. In nature, they are transmitted between plants by phloem sap-feeding insects (mainly leafhoppers, planthoppers, and psyllids) in a persistent manner (Fletcher *et al.*, 1998). A comprehensive review of insect vectors of plant mollicutes has appeared recently (Weintraub and Beanland, 2006).

Plant pathogenic mollicutes belong to two phylogenetically distinct groups, phytoplasmas and spiroplasmas. Phytoplasmas were first observed by electron microscopy (Doi *et al.*, 1967) but have not been cultured so far. In contrast, spiroplasmas, and in particular *Spiroplasma citri*, have been cultured since 1970 (Saglio *et al.*, 1971). The cellular and molecular properties of *S. citri* have been reviewed previously (Bové *et al.*, 1989; Renaudin and Bové, 1994; Bové, 1997). All spiroplasmas are associated with arthropods, whereas only three, *S. citri* (Saglio *et al.*, 1973), *S. kunkelii* (Williamson and Whitcomb, 1975), and *S. phoeniceum* (Saillard *et al.*, 1987), are pathogenic to plants. *S. citri* is the etiological agent of the stubborn disease of citrus (Saglio *et al.*, 1973) and of brittle root disease of horseradish (Fletcher, 1983). In nature, *S. citri* is transmitted by the leafhoppers *Circulifer haematoceps* in the Old world and *Circulifer tenellus* in the USA (Kaloostian *et al.*, 1979; Liu *et al.*, 1983; Fos *et al.*, 1986), and it can be experimentally transmitted to periwinkle plants (*Catharanthus roseus*) through injection to the insect vector (Foissac *et al.*, 1996). Most phytoplasmas also can be transmitted to periwinkle via their respective insect vectors or by dodder, a parasitic, phloem-feeding plant.

In periwinkle, spiroplasmas and phytoplasmas induce disorders similar to those observed in their original host plant such as leaf yellowing, internode shortening, proliferations, flower and fruit malformations, stunting, and/or decline. However, in spite of a few studies on plant response to phytoplasma infection

(Lepka *et al.*, 1999; Jagoueix-Eveillard *et al.*, 2001; Tan and Whitlow, 2001; Bertamini *et al.*, 2002; Bertamini *et al.*, 2003; Maust *et al.*, 2003; Choi *et al.*, 2004; Favali *et al.*, 2004; Pracros *et al.*, 2006), the mechanisms by which they cause disease in plants are poorly understood, and the genetic determinants involved, virtually unknown.

For *S. citri*, several mechanisms have been proposed to explain pathogenicity. Toxins and lactic acid production have been suggested to play a role in disease development (Daniels, 1983). *S. citri* infection was also shown to cause a shortage of auxins, and it was proposed that utilization of sterols by spiroplasmas could result in a deficit in growth regulators (Chang, 1998). However, in the absence of genetic studies, these hypotheses could not be confirmed experimentally. More recently, the development of suitable genetic tools such as plasmid vectors and insertional mutagenesis through transposon insertion or homologous recombination, (Ye *et al.*, 1994; Renaudin *et al.*, 1995; Foissac *et al.*, 1997b; Duret *et al.*, 1999; Renaudin, 2002; Renaudin and Lartigue, 2005), has led to a significant breakthrough, when a *S. citri* mutant affected in its pathogenicity was isolated (Foissac *et al.*, 1997a).

Further genetic studies have led to the identification of spiroplasmal genes associated with biological functions such as motility, insect transmission, and pathogenicity (see Bové *et al.*, 2003 for a review). In particular, it was shown that the pathogenicity of *S. citri*, i. e. the ability to induce symptoms, correlates with its ability to use fructose (Gaurivaud *et al.*, 2000a), indicating that sugar metabolism is certainly an important factor in the relationships of *S. citri* with its two hosts, the plant and the leafhopper vector. Indeed, carbohydrate partitioning was shown to be impaired in *S. citri* infected plants (André *et al.*, 2005).

From these data, models have been proposed to explain how the sugar metabolism of *S. citri* might interfere with the normal physiology of the host plant to cause the disease. Although this review article mainly focuses on pathogenicity of *S. citri*, some data relevant to plant response to phytoplasma infection will also be discussed. A comprehensive review on the interactions of phytoplasmas with their hosts has been published recently (Christensen *et al.*, 2005).

SUGAR CATABOLISM IN *S. CITRI*

Spiroplasmas are fastidious in their nutritional requirements and they are cultivated in various undefined, complex media (Chang, 1989). *S. citri* was first isolated in SMC medium (Saglio *et al.*, 1971) and later grown in various enriched media such as BS, M1A, and SP4, which contain yeast extract, fetal bovine or horse serum, and several added carbohydrates (Whitcomb,

1983). Carbohydrates appear to be the major energy sources for spiroplasmas (Chang, 1989). Fermentative catabolism of carbohydrates by *S. citri* results in lactic and acetic acid production (Miles, 1992). Therefore, the pH decrease of culture medium has been used to determine carbohydrate requirements. However, because the presence of carbohydrates and enzymes such as invertases in the complex media might interfere with fermentation of the added sugar, some contradictory data have been reported (Chang, 1989).

Thereafter, the chemically defined medium CB3 has been used to determine the nutritional requirements of *S. citri* (Chang, 1989; Chang *et al.*, 1994). With respect to carbohydrates, the results indicated that *S. citri* actively ferments fructose, glucose, and trehalose, but not sorbitol, and raffinose. In the case of mannose and sucrose only a very slight pH decrease was observed, indicating that *S. citri* was almost unable to ferment these sugars. These data have been recently confirmed by using the simplified, HSI medium (Whitcomb, 1983), in which the spiroplasma growth is dependent on the added sugar. When *S. citri* GII-3 (Vignault *et al.*, 1980) was grown in HSI, the pH decrease strictly correlated with the ability of the spiroplasma to catabolize the sugar added to the medium. In the presence of fructose, glucose, or trehalose the acidification curves were identical, with a pH variation from 7.6 to 5.3 within 5 days. In contrast, no significant pH variation (less than 0.3) was observed with sorbitol (Gaurivaud *et al.*, 2000b; André *et al.*, 2005).

In the case of sucrose, only a minor pH decrease (0.6) was detected, which could be explained by the presence of traces of contaminant sugars. However, virtually no spiroplasma growth was detected, indicating that *S. citri* was unable to use sucrose as energy source. Sucrose is the most abundant carbohydrate in the phloem sap but cannot be catabolized by *S. citri*. In contrast fructose and glucose, which are present as traces, can readily be used by spiroplasma cells. *In vitro* studies showed that, when grown in HSI medium containing fructose or glucose, *S. citri* GII-3 used fructose and glucose at approximately the same rate. Interestingly, however, when grown in the presence of both two sugars, it was found that *S. citri* GII-3 used fructose preferentially (André *et al.*, 2005). This was further confirmed by the fact that, in the presence of the two sugars, the MIC of α -methylglucopyranose (α MG), a toxic analogue of glucose, was identical to that determined in the presence of fructose alone, indicating that, in the presence of fructose, glucose is virtually not used.

PHOSPHOENOLPYRUVATE PHOSPHOTRANSFERASE SYSTEMS OF *S. CITRI*

The bacterial phosphoenolpyruvate phosphotransferase system (PTS) is a multiprotein system, which

catalyses the concomitant import and phosphorylation of its sugar substrates (Postma *et al.*, 1996). It consists of two general soluble proteins, enzyme I (EI) and HPr, both of which lack sugar specificity, and one membrane-bound, sugar specific permease (enzyme II).

The enzyme II (EII) complex is generally made of three functional components (IIA, IIB, and IIC), which occur either as protein subunits or domains of a multidomain polypeptide (Reizer and Saier Jr., 1997). The IIC domain is an integral membrane component whereas IIA and IIB are cytosolic proteins. For sugar uptake, the phosphate moiety is transferred from phosphoenolpyruvate (PEP) to the sugar via EI, HPr, EIIA, and EIIB. Then the phosphorylated sugar is imported across the membrane by the integral membrane porter EIIC. Based on the phylogeny of the IIC components, PTS permeases are currently classified in seven families, namely the glucose (Glc), fructose (Fru), lactose (Lac), galactitol (Gat), glucitol (Gut), mannose (Man), and ascorbate (Asc) families (Barabote and Saier Jr., 2005).

As revealed by genomic analyses of bacterial PTS, the *Mollicutes* have only Glc-, Fru-, and Asc-type PTS, with those of the Glc-type being the most frequently encountered. The plant mollicutes, *Mesoplasma florum*, *S. citri*, and *S. kunkelii*, do possess PTS systems of the Glc and Fru families (http://www.broad.mit.edu/annotation/microbes/mesoplasma_florum/; André *et al.*, 2003; (<http://www.genome.ou.edu/spiro.html>). In contrast, the isolates OY (Onion-Yellows) and AY-WB (Aster-Yellows Witches' Broom) of '*Candidatus Phytoplasma asteris*' (Oshima *et al.*, 2004; Namba *et al.*, 2005; Bai *et al.*, 2006) lack PTS homologues.

A survey of the *S. citri* genome sequence has revealed the presence of several CDS related to PTS proteins. Genes *ptsH* and *ptsI*, encoding the general energy-coupling enzyme I (EI) and HPr, *hprK* encoding a ATP-dependent Hpr kinase/phosphorylase, as well as genes encoding components of sugar-specific permeases were identified. In agreement with the ability of *S. citri* to metabolize glucose, fructose, and trehalose, its genome was found to encode the corresponding PTS permeases. In the fructose permease EII^{Fru}, the three domains IIA^{Fru}, IIB^{Fru}, and IIC^{Fru} are fused in a single polypeptide IIABC^{Fru} encoded by gene *fruA* (Gaurivaud *et al.*, 2000b). The permease gene *fruA* is part of the fructose operon, which comprises three genes, *fruR*, *fruA*, and *fruK*, encoding respectively, a transcription regulator, the permease enzyme EII^{Fru}, and the 1-phosphofructokinase (1-PFK) (Gaurivaud *et al.*, 2000b; Gaurivaud *et al.*, 2001).

In contrast to fructose permease EII^{Fru}, the trehalose permease EII^{Tre} (IIBC^{Tre}) only possesses domains IIB^{Tre} and IIC^{Tre}, but lacks its own IIA^{Tre} domain. The trehalose permease gene *treP* is also part of an operon, which consists of the three genes *treR*, *treP*, and *treA*, encoding a putative regulator, the trehalose permease

EII^{Tre}, and the amylase, respectively (André *et al.*, 2003). In contrast to fructose permease EII^{Fru}, the glucose permease EII^{Glc} is split into two distinct polypeptides, IIA^{Glc} and IICB^{Glc}, encoded by two separate genes, *crr* and *ptsG*, respectively, the *crr* gene being located in the vicinity of *ptsI*, but distant from *ptsG*.

As indicated above, *S. citri* uses trehalose, in spite of the lack of a specific, IIA^{Tre} trehalose permease domain. However, it was shown that the trehalose and glucose-PTS permeases share a unique IIA domain, i.e. the trehalose permease functions with the IIA^{Glc} domain. This was first suggested from yeast two-hybrid experiments showing that the IIA^{Glc} domain interacted not only with IIB^{Glc} but also with the IIB^{Tre} domain (André *et al.*, 2003). Furthermore, the finding that a *crr* (encoding IIA^{Glc})-disrupted mutant used neither glucose nor trehalose definitively demonstrated that, in *S. citri*, glucose and trehalose-PTS permeases function with a single IIA component (Duret *et al.*, 2005). This finding is consistent with the occurrence of two distinct genes *crr* and *ptsG* encoding, respectively, the IIA^{Glc} and IICB^{Glc} polypeptides. In agreement with the phylogeny of mollicutes, such an organization of the glucose and trehalose-PTS permeases was also found in the plant mollicutes *S. kunkelii* and *M. florum*, and in the mycoplasmas of the *Mycoplasma mycoides* group (Zhu *et al.*, 1994; Gaurivaud *et al.*, 2004).

Studying expression of the PTS permease genes revealed that *in vivo* transcription of the wild-type fructose operon (*fruR+fruA+fruK*) was greatly enhanced by the presence of fructose in the growth medium whereas glucose had no effect (Gaurivaud *et al.*, 2001). In the absence of *fruR*, transcription of the truncated operon *fruA-fruK* was not stimulated, indicating that the product of *fruR* was an activator of the fructose operon. Similarly, it was shown that transcription of *ptsG* is stimulated in the presence of glucose and that of the trehalose operon by the presence of trehalose (André, 2003).

The finding that the transcription of each of the three PTS permease genes was stimulated by the respective carbohydrate probably reflects the adaptive capacity of the spiroplasma to multiply in distinct hosts (plant and insect) or host compartments (insect haemolymph or insect cells) having distinct sugar contents. In the plant sieve tubes *S. citri* probably grows on fructose and/or glucose whereas in the leafhopper, the spiroplasma is thought to grow on trehalose, the major sugar in the haemolymph (Becker *et al.*, 1996; Thompson, 2003), or glucose in the gut epithelium and salivary gland cells. In this respect, the fact that glucose and trehalose permeases function with a single IIA domain has been associated with the ability of the spiroplasma to adapt to carbohydrate changes in its environment (André *et al.*, 2003). Regarding the proposed model of spiroplasma movement within the leafhopper vector (Fletcher *et al.*, 1998), the occurrence of a unique IIA domain shared by

the two permeases would help the spiroplasma to rapidly adapt from glucose within the gut epithelium cells to trehalose in the haemolymph, and back to glucose in the salivary gland cells before to reach the salivary duct.

In addition to the general enzymes EI, HPr, and HprK/P, and the fructose-, glucose-, and trehalose-PTS permeases, CDS of which were clearly identified, the *S. citri* genome was also found to encode several other hypothetical PTS permease components, of unknown substrate, most of which were described as truncated products.

FRUCTOSE IMPORT IS A KEY FACTOR OF PATHOGENICITY

Screening *S. citri* insertional (*Tn4001*) mutants by experimental transmission to periwinkle (*C. roseus*) plants through injection to the leafhopper vector *C. haematocaps* has led to the isolation of the so-called non-pathogenic mutant GMT553 (Foissac *et al.*, 1997a). In contrast to the wild-type strain GII-3, this mutant produced very mild symptoms, in spite of multiplication to high titre in the host plant (Table 1). In this mutant, the transposon was inserted into the first gene *fruR* of the fructose operon and, consequently, transcription of all three genes, *fruR*, *fruA*, and *fruK* was completely abolished (Gaurivaud *et al.*, 2000a). As a result, mutant GMT553 was unable to use fructose, indicating that PTS is certainly the major import system of carbohydrates in *S. citri*. Functional complementation of GMT553, by transformation with recombinant *oriC* plasmid carrying genes *fruR*, *fruA*, and *fruK*, alone or in various combinations, revealed that there was a perfect correlation between the ability of the transformed spiroplasmas to import fructose and their ability to produce severe symptoms in the host plant (Gaurivaud *et al.*, 2000a). This was further confirmed by the characterization of *S. citri* mutants resistant to xylitol, a fructose analogue, which is toxic for many bacteria including *S. citri* (Labarere and Barroso, 1989). Indeed, xylitol-resistant mutants were proved unable to import fructose, indicating that, in *S. citri*, both fructose and xylitol are imported via the same PTS. When transmitted to periwinkle, these xylitol resistant mutants multiplied, but did not produce severe symptoms (Table 1) (Gaurivaud *et al.*, 2000c).

In the host plant, *S. citri* cells are restricted to the sieve elements of the phloem. Sucrose is the major form of transported carbohydrate in the phloem sap, but is not used by *S. citri*. In contrast, the concentration of fructose and glucose appears to be very low or undetectable (Chino *et al.*, 1991; Flowers and Yeo, 1992). However, loading sucrose in the sieve elements requires the action of companion cells, into which movement of sucrose occurs via a sucrose/H⁺ cotransporter energized

by the membrane H⁺ATPase within the plasma membrane (Bouché-Pillon *et al.*, 1994).

As the first step, some sucrose must be cleaved by sucrose synthase, into fructose and UDP-glucose, to yield the energy required for sucrose loading. Fructose, glucose, and sucrose are thought to diffuse from companion cells to sieve elements, where the two hexoses, and in particular fructose, can be used by *S. citri*. Therefore, to explain the role of fructose import in pathogenicity, it was hypothesized that fructose utilization by the spiroplasmas deprives the companion cells of the fructose they need (as energy source) for loading sucrose into the sieve elements, leading to an impaired partitioning of carbohydrates in the plant (Gaurivaud *et al.*, 2000a; Bové *et al.*, 2003).

As a result, the probable accumulation of sugars in source leaves and depletion in sink organs, were thought to be responsible for symptoms such as leaf yellowing and stunting, respectively. From these studies, fructose utilization appears as a key factor of spiroplasmal pathogenicity. However, *S. citri* mutants unable to import fructose still produced very mild symptoms, suggesting that factors other than fructose utilization are probably involved. In this respect, lactic acid production has been suggested as a possible factor (Daniels, 1983; Gaurivaud *et al.*, 2000a) but no increase of lactate concentration was detected in *S. citri* infected periwinkle leaves (André *et al.*, 2005).

GLUCOSE AND TREHALOSE IMPORTS ARE NOT ESSENTIAL FOR PATHOGENICITY

In the proposed model, the spiroplasmas present in the sieve elements compete with companion cells for fructose and glucose utilization (Gaurivaud *et al.*, 2000a). Considering that, *in vitro*, *S. citri* metabolizes fructose and glucose equally well, it is likely that, in the phloem sap, the two hexoses play the same nutritional role and, therefore, it was thought that *S. citri* mutants unable to import glucose should be non-pathogenic, similarly to the fructose operon mutant GMT553.

In *S. citri*, in which the PTS seem to be the major sugar import systems, the glucose-PTS permease EII^{Glc} is made of two polypeptides IIA^{Glc} and IICB^{Glc} encoded by genes *crr* and *ptsG*, respectively (André *et al.*, 2003). The glucose permease mutant GII3-glc1 was constructed by inactivation of *ptsG* through homologous recombination, using a replicative *oriC* plasmid as vector. Disruption of *ptsG* completely abolished transcription of the gene, resulting in the absence of a functional glucose-PTS permease. When grown in HSI medium supplemented with various sugars, the *ptsG*-disrupted mutant GII3-glc1 still used fructose and trehalose but was unable to import glucose (Table 1). Unexpectedly, experimental transmission to periwinkle

Table 1. List of *S. citri* mutants with their corresponding phenotypes.

	GII-3 (wt)	GMT553 *	XylB *	GIIβ-glc1 *	GIIβ-gt1 *	GIIβ-arg1 *	G76 *
Target gene	-	<i>fruR</i>	<i>fruA</i>	<i>pIsG</i>	<i>crr</i>	<i>arcA</i>	<i>sc76</i>
Protein	-	Fructose operon regulator	Fructose permease IIABC ^{fru}	Glucose permease IICB ^{Glc}	Glucose permease IIAG ^{Glc}	Arginine deiminase	Putative ABC transporter solute binding protein
Affected function	-	Fructose import	Fructose import	Glucose import	Glucose and trehalose import	Arginine catabolism	?
Fructose catabolism	+	-	-	+	+	+	+
Glucose catabolism	+	+	+	-	-	+	+
Trehalose catabolism	+	+	+	+	-	+	+
Arginine catabolism	+	nd	nd	nd	nd	-	nd
Growth in the vector insect	+	+	+	+	+	+	+
Insect transmission	+	+	+	+	+	+	+
Growth in the host plant	+	+	+	+	+	+	+
Pathogenicity	+	-	-	+	+	+	+
References	Foissac <i>et al.</i> , 1997a; Gaurivaud <i>et al.</i> , 2000a	(very mild symptoms) Foissac <i>et al.</i> , 1997a; Gaurivaud <i>et al.</i> , 2000a	(very mild symptoms) Gaurivaud <i>et al.</i> , 2000c	André <i>et al.</i> , 2005	Duret <i>et al.</i> , 2005	Duret <i>et al.</i> , 2005	Boutareaud <i>et al.</i> , 2004

* *S. citri* mutants were obtained through Tn4001 insertion (GMT553 and G76), spontaneous mutation (XylB) or homologous recombination (GIIβ-glc1, GIIβ-gt1, and GIIβ-arg1).

plants following injection to the leafhopper vector, revealed that, in contrast to the fructose operon mutant GMT553, the glucose permease mutant GIIβ-glc1 induced severe symptoms, undistinguishable from those produced by the wild-type strain GII-3 (André *et al.*, 2005). These results, showing that fructose and glucose utilization are not equally involved in spiroplasmal pathogenicity are consistent with biochemical data showing that in the presence of both sugars, *S. citri* uses fructose preferentially.

As indicated above, the *S. citri* mutant GIIβ-gt1, in which gene *crr* encoding the IIA^{Glc} component of the glucose-PTS permease was disrupted, used neither glucose nor trehalose (Table 1). Interestingly enough, when transmitted to the host plant by the leafhopper vector, this mutant proved to be highly pathogenic indicating that, in contrast to fructose, glucose and trehalose utilizations are not essential for pathogenicity (Duret *et al.*, 2005). In addition, the *S. citri* mutant GIIβ-gt1, unable to import both glucose and trehalose, was transmitted, indicating that it multiplied to high titre in the leafhopper (Duret *et al.*, 2005).

Even though trehalose is the main sugar in the insect haemolymph, fructose and glucose also are present (Florkin and Jeuniaux, 1974). Therefore, multiplication of GIIβ-gt1 in the leafhopper vector probably relies on fructose. Nevertheless, fructose utilization is not an absolute requirement for *S. citri* multiplication in the insect, since the fructose operon mutant GMT553 also, multiplies to high titre in the leafhopper (Gaurivaud *et al.*, 2000a). These results indicate that, when fructose cannot be used, *S. citri* has the capability to grow on glucose and/or trehalose, not only *in vitro* but, also, in the insect haemolymph.

Arginine metabolism leading to ATP synthesis through the arginine deiminase (ADI) pathway is the primary energy source in non-glycolytic mollicutes. However, carbohydrates and arginine can be metabolized concomitantly by glycolytic mollicutes, including *S. citri* (Townsend, 1976; Pollack *et al.*, 1997). The *S. citri* ADI operon comprises four genes, *arcA*, *arcB*, *arcD*, and *arcC*, encoding respectively the arginine deiminase (ADI), ornithine carbamoyltransferase (OTC), carbamate kinase (CK), and arginine-ornithine antiporter. Knowing that *S. citri* lacks the ability to synthesize most of the amino acids (Chang *et al.*, 1994), the import of arginine from the phloem sap could provide the spiroplasma with a nitrogen and/or energy source. However the *arcA*-disrupted mutant GIIβ-arg1, unable to use arginine, was shown to multiply and induce symptoms in the host plant, similarly to the wild-type strain (Table 1), indicating that, arginine catabolism is not an important pathogenicity factor (Duret *et al.*, 2005).

FRUCTOSE AND GLUCOSE PLAY DISTINCT ROLES

From the finding that fructose and glucose import are not equally involved in pathogenicity of *S. citri*, a model mechanism has been proposed to explain how the preferential use of fructose over glucose by the spiroplasmas could lead to symptom production (André *et al.*, 2005). In higher plants, sucrose represents the major carbohydrate in the phloem sap and its use relies on cleavage into hexoses by specialized enzymes such as sucrose synthase and invertases. These sucrose-cleaving enzymes are essential for maintaining the balance between sugar signals and metabolic paths (Koch, 2004). Alkaline invertase functions in the cytosol and acidic invertases in both the vacuole and the cell wall (Dennis and Blakeley, 2000). In transgenic plants, over expression of invertases, hence hexose accumulation, reduces the photosynthesis rate, leading to symptom development, i.e., leaf yellowing and internode shortening (von Schaeven *et al.*, 1990; Heineke *et al.*, 1992; Heineke *et al.*, 1994; Lerchl *et al.*, 1996), which are reminiscent of those observed in *S. citri*-infected plants. The proposed hypothesis (André *et al.*, 2005) was primarily based on the negative regulation of invertases by the products of sucrose hydrolysis and, in particular, fructose (Sampietro, 1995; Walker *et al.*, 1997).

In mature leaves of healthy plants, due to inhibition of invertase activity by fructose, sucrose hydrolysis is limited (Walker *et al.*, 1997), and low amounts of fructose and glucose, diffuse from the companion cells into the phloem sieve elements. In *S. citri*-infected plants, the spiroplasmas use fructose (preferentially to glucose), leading to a reduced fructose concentration in companion cells and, consequently, to an increase of invertase activity. As a result, more fructose and glucose are produced. While fructose is used by the spiroplasmas to grow, glucose is not used and, instead, accumulates. In agreement with this model, proton-NMR analyses of carbohydrates in plants revealed a significant accumulation of glucose in mature leaves of *S. citri*-infected periwinkles, as compared to healthy ones (André *et al.*, 2005). Interestingly, glucose accumulation was observed in plants infected by *S. citri* GII-3 (wt) or the *ptsG*-disrupted mutant GII3-glc1, but not in symptom-less plants infected with the fructose operon mutant GMT553.

These data clearly indicate that fructose and glucose play distinct roles in spiroplasmal pathogenicity. While fructose is used as a substrate (carbon and energy source) by the spiroplasma to grow, glucose accumulates in the mature leaves of infected plants, inducing physiological disorders such as inhibition of photosynthesis genes. Accordingly, differential display analyses of mRNAs revealed that periwinkle genes encoding a transketolase, a chlorophyll binding protein, the subunit III of photosystem I, and the ribulose 1,5-biphosphate carboxylase-oxygenase were down-regulated in *S. citri*-

infected plants (Jagoueix-Eveillard *et al.*, 2001). It is noteworthy that NMR analyses of *S. citri*-infected periwinkles did not reveal an increase of lactate concentration in infected plants and, therefore, do not sustain the hypothesis that high lactate concentrations produced by *S. citri* would be responsible for disease symptom production (Daniels, 1983; Bové *et al.*, 2003). Although to a lesser extent, sucrose concentration was also found to increase in *S. citri*-infected plants. This result does not fit the model, in which an increased invertase activity should lead to a decrease in sucrose concentration, and seems more likely related to either a deficient sucrose loading, as previously proposed (Gaurivaud *et al.*, 2000a), and/or to a limited sugar transport in the phloem, as described in phytoplasma-infected plants (Maust *et al.*, 2003). Therefore, both mechanisms, i. e., reduction of sucrose loading with subsequent impairment of carbohydrate partitioning (Gaurivaud *et al.*, 2000a; Bové *et al.*, 2003) and preferential use of fructose by the spiroplasma leading to increased invertase activity, glucose accumulation, and inhibition of photosynthesis (André *et al.*, 2005) might contribute to spiroplasmal pathogenicity. In this respect, whether or not invertase activity actually increases in *S. citri*-infected plants must be investigated.

IMPAIRED CARBOHYDRATE PARTITIONING IN PHYTOPLASMA-INFECTED PLANTS

Phytoplasmas cause many plant diseases, with a wide variety of symptoms including yellowing, stunting, proliferations and flower malformations, some of which (witches' broom, virescence) are specific to certain phytoplasma groups or isolates (Lee *et al.*, 2000). These symptoms are indicative of a disturbed hormone balance and disorders in phloem function. Flower abnormalities in stolbur phytoplasma-infected tomatoes have been associated with deregulations of floral development genes (Pracros *et al.*, 2006). However, the underlying mechanisms of phytoplasma pathogenicity are poorly understood (Christensen *et al.*, 2005).

In spite of the recent determination of complete phytoplasma genome sequences (Oshima *et al.*, 2004; Bai *et al.*, 2006) functional genomics of these non-cultivable organisms is still out of reach. Nevertheless, investigating the host plant response has revealed that, in most cases, phytoplasma infection affects carbohydrate translocation and carbohydrate levels in various tissues, with a marked increase of soluble carbohydrates and starch in source leaves, and a decrease in sink organs, i. e. young leaves and roots. For example, a considerable accumulation of both soluble carbohydrates and starch was detected in leaves of pear decline-diseased pear trees (Catlin *et al.*, 1975). Impaired translocation of photosynthates was also reported in phytoplasma-infected peri-

winkle and tobacco plants (Lepka *et al.*, 1999), in which high levels of carbohydrates were detected in source leaves, regardless of partial bleaching and, hence, lower rate of photosynthesis. Whereas accumulation of carbohydrates could explain the bleaching of source leaves through repression of photosynthesis genes, the lower levels of carbohydrates in the sink organs could be responsible for growth impairment (Lepka *et al.*, 1999). Similar data, reduction of photosynthesis (CO₂ exchange) rate, increased carbohydrate levels in mature leaves, and decreased levels in stem and root tissue were reported in various phytoplasma/host plant pathosystems, such as papaya infected by the dieback phytoplasma (Guthrie *et al.*, 2001), periwinkle infected by the ash yellow phytoplasma (Tan and Whitlow, 2001), and coconut palm infected by the lethal yellowing phytoplasma (Maust *et al.*, 2003). In field-grown grapevine leaves infected by the stolbur phytoplasma, the contents of chlorophyll and carotenoids, as well as the ribulose-1,5-bisphosphate activity markedly decreased (Bertamini *et al.*, 2002). Similar data were observed in apple leaves infected by 'Ca. P. mali' (Bertamini *et al.*, 2003). Therefore, impaired translocation of carbohydrates with a subsequent inhibition of photosynthesis seems to be a common feature of plant mollicute infection. In agreement with a reduced photosynthetic activity, periwinkle genes involved in photosynthesis were found to be down-regulated in *S. citri*- and stolbur phytoplasma-infected periwinkle plants (Jagoueix-Eveillard *et al.*, 2001).

In addition to carbohydrates, phytoplasma infection of periwinkle also leads to an increase of metabolites related to the biosynthetic pathways of terpenoid indole alkaloids or phenylpropanoids (Choi *et al.*, 2004; Favali *et al.*, 2004). Basically, it was hypothesized that high levels of carbohydrates would induce the infected plants to use sucrose and glucose that accumulate in the source leaves in secondary metabolic pathways and, in particular, those leading to terpenoid indole alkaloids or phenylpropanoids (Choi *et al.*, 2004).

CONCLUSION

Most plant pathogenic bacteria are exogenous, colonize the apoplast, and are potentially necrogenic. However, their ability to multiply and kill the plant cells depends on secreted enzymes that degrade the cell-wall and on proteins that are introduced into the plant cells through a type III secretion system (Boucher, 2001; Alfano and Collmer, 2004). In contrast, plant pathogenic mollicutes (phytoplasmas and spiroplasmas), which are injected directly into the phloem sieve tubes by their vector insects, have a direct access to the nutrients of the photosynthesis-enriched phloem sap. Accordingly, the phytoplasma and spiroplasma genomes do not possess genes encoding plant cell-wall degrading enzymes

and type III secretion systems (Namba *et al.*, 2005). Except for genes encoding glucanase and hemolysin-like proteins, which are described as possible virulence factors in the genome of 'Ca. P. asteris', no other known virulence genes were identified in the genomes of plant pathogenic mollicutes.

The ability of pathogenic bacteria to multiply *in vivo* is essential for pathogenicity. In the case of plant mollicutes, the ability to multiply in the host plant mainly relies on their capability to use the nutrients released from companion cells into the phloem sap. Housekeeping genes such as transport systems may therefore be considered as potential virulence factors.

In vitro, *S. citri* uses fructose, glucose, and trehalose, but not sucrose, the major sugar in the phloem sap. Glucose is the most stable of the hexose isomers and is often described as the universal, preferential fuel (Bruckner and Titgemeyer, 2002). However, in contrast to most bacteria, when both fructose and glucose are present, *S. citri* uses fructose preferentially.

Sugar uptake by spiroplasma cells is essentially through the PTS, as indicated by the finding that PTS permease mutants were unable to import the relevant sugar. Whether in *S. citri* glucose uptake could also occur via a ABC transporter is still questionable (Boutareaud *et al.*, 2004). The 'Ca. P. asteris' genome encodes nine glycolytic genes suggesting that phytoplasmas possess a functional glycolysis pathway (Namba *et al.*, 2005). However, even though the absence of PTS genes suggests that these organisms import phosphorylated hexoses, the sugar catabolism of the phytoplasmas is unknown (Christensen *et al.*, 2005).

Mollicute infection of plants usually results in an impaired translocation of carbohydrates, with accumulation of sugars in source leaves and depletion in sink organs. In *S. citri*, genetic studies showed that fructose and glucose import are not equally involved in pathogenicity. Whereas both fructose and glucose can be used by *S. citri* to grow *in vivo*, in the host plant only the use of fructose is essential for pathogenicity. To better understand the link between sugar catabolism and symptom production, two model mechanisms, both of which might contribute to spiroplasmal pathogenicity, have been proposed: (i) competition between spiroplasmas and companion cells for fructose, with reduction of sucrose loading and subsequent impairment of carbohydrate partitioning (Gaurivaud *et al.*, 2000a), (ii) increased invertase activity, due to a preferential use of fructose (over glucose) by the spiroplasma, with subsequent glucose accumulation and inhibition of photosynthesis (André *et al.*, 2005).

In higher plants, sucrose and its cleavage products fructose and glucose are central molecules for carbohydrate translocation, metabolism, and sensing, which are known to be affected by exogenous factors, including pathogen infection (Koch, 2004; Roitsch and Gonzalez,

2004). The sucrose cleaving enzymes, sucrose synthase and invertases, are crucial to maintain the balance between sugar signals and metabolic paths. Considering the hypothetical models of *S. citri* pathogenicity, it will be of interest to compare the expression level and activity of these enzymes in healthy and *S. citri* infected plants. While fructose import has been clearly identified as a pathogenicity factor, the question still stands, is fructose uptake by the spiroplasma the only pathogenic signal? The finding that the *S. citri* mutant GMT553, unable to use fructose, still produces symptoms (though very mild) suggests that additional factors might be involved in virulence. Besides the import of nutrients, the identification in the genome of genes *secA* and *secY* of the Sec translocase system suggests that *S. citri* might also have the capacity for secreting proteins at the spiroplasma cell surface or in the phloem sap.

Plant pathogenic mollicutes, spiroplasmas and phytoplasmas, all inhabit the phloem sieve tubes in which they multiply to cause diseases. However, in addition to yellowing and reduced photosynthesis, they induce a wide variety of symptoms, including stunting, proliferations, and flower abnormalities, which are specific to the pathogen. These observations clearly indicate that different plant mollicutes display distinct pathogenicity mechanisms, causing a wide panel of physiological disorders in the host plants. Obviously, both functional genomics studies of the bacteria and careful analyses of changes of the metabolic profiles in the infected plants will be required for identifying the bacterial genetic determinants and deciphering the molecular mechanisms of pathogenicity of plant mollicutes.

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