***Catharanthus roseus* Genes Regulated Differentially by Mollicute Infections**

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A differential display of mRNAs was used to isolate periwinkle cDNAs differentially expressed following infection with one of three mollicutes: *Spiroplasma citri*, *Candidatus Phytoplasma aurantifolia*, and stolbur phytoplasma. Twenty-four differentially expressed cDNAs were characterized by Northern blots and sequence analysis. Eight of them had homologies with genes in databanks coding for proteins involved in photosynthesis, sugar transport, response to stress, or pathways of phytosterol synthesis. The regulation of these genes in periwinkle plants infected by additional phloem-restricted bacteria showed that they were not specific to a given mollicute, but correlations with particular symptoms could be established. Expression of transketolase was down regulated following infection with a pathogenic strain of *S. citri*. No down regulation was observed for the nonphytopathogenic mutant GMT553, which is deficient for fructose utilization.

Mollicutes are an important group of plant pathogens that are responsible for hundreds of diseases worldwide (McCoy et al. 1989). They are localized exclusively in the phloem sieve tubes and transmitted by phloem-sap-feeding insects such as leafhoppers or psyllids. Two groups of plant-pathogenic mollicutes are *Spiroplasma* spp., which can be cultured in artificial media (Saglio et al. 1971); and *Candidatus Phytoplasma* spp., which have, so far, resisted in vitro cultivation (Seemüller et al. 1998). Most spiroplasmas and phytoplasmas can be transmitted to periwinkle plants (*Catharanthus roseus*) via dodder (*Cuscuta* spp.), a parasitic phloem-feeding plant, or via their respective insect vectors. Within periwinkle plants, spiroplasmas and phytoplasmas induce disorders similar to those observed in their original host plant such as leaf yellowing, growth aberrations (proliferations, internode shortening, stunting), flower malformations, and/or decline. The mechanisms involved in symptom development are unknown and, currently, the mollicute interactions with their host plants are poorly understood.

The most studied phytopathogenic mollicute is *Spiroplasma citri* because it was the first of the three plant pathogenic spiroplasmas to be cultured and characterized (Saglio et al.

1973). Viral and artificial plasmid vectors have been developed for gene cloning and expression in the spiroplasmas (Renaudin and Bové 1995; Ye et al. 1994). Transposon Tn4001 mutagenesis could be applied to *S. citri*, resulting in the identification of GMT 553, a mutant that is weakly pathogenic (Foissac et al. 1997) and unable to use fructose (Gaurivaud et al. 2000b). Fructose utilization by the wild-type spiroplasma was correlated with symptom expression (Gaurivaud et al. 2000a).

As no phytoplasma cultures are available, studies on phytoplasma interactions with host plants are limited and genetic approaches cannot be envisaged. Phytoplasma-induced modifications of phloem structure and biochemical disturbance of phloem functions have been reported (Lepka et al. 1999; McCoy 1979). The differential display (DD) technique (Liang and Pardee 1992), which allows identification and isolation of eukaryotic cDNAs expressed differentially under various conditions, has been used extensively by animal and plant physiologists to study gene expression during development and recently has been applied to the study of plant-pathogen interactions (Benito et al. 1996; Mareyrat et al. 1998; Seehaus and Tenhaken 1998; Truesdell and Dickman 1997). In the case of phytoplasmas, only one attempt to isolate genes involved in plant-phytoplasma interactions has been reported (Smart and Kirkpatrick 1996), but apparently has not been pursued.

Here we describe for the first time the use of the DD method to identify genes that are up or down regulated in periwinkle plants following infection by one of three mollicutes: *Spiroplasma citri*; stolbur phytoplasma (Fos et al. 1992; McCoy 1989), which induces leaf yellows, internode shortening, and flower aberrations in periwinkle and many other plants; and *Candidatus Phytoplasma aurantifolia*, the agent of witches' broom disease of lime (WDBL) that is responsible for spectacular proliferations and witches' broom in *Citrus* spp. and periwinkle plants (Garnier et al. 1991; Zreik et al. 1995).

The periwinkle genes differentially displayed after infection with the above three mollicutes also were studied in periwinkle plants infected with various other phytoplasmas or phloem-restricted proteobacteria. Expression of a transketolase gene in plants infected by the pathogenic GII-3 strain of *S. citri* or the weakly pathogenic mutant GMT 553 (Foissac et al. 1997; Gaurivaud et al. 2000a; Gaurivaud et al. 2000b) also is described.

MATERIALS AND METHODS

Bacterial strains.

The cultivable mollicute *S. citri* (Saglio et al. 1973) and several phloem-restricted uncultured bacteria were used in this study (Bové et al. 1998). They were maintained in periwinkle plants, grown in an insect-proof greenhouse, and propagated by grafting. Additionally, they consisted of the phytoplasmas responsible for aster yellows (AY), apple proliferation (AP), stolbur phytoplasma (Mc Coy et al. 1979), *Candidatus* Phytoplasma aurantifolia, the WBDL agent (Garnier et al. 1991; Zreik et al. 1995), and the alpha-proteobacterium *Candidatus* Liberibacter sp. that is responsible for citrus Huanglongbing disease (Jagoueix et al. 1994). Symptoms induced by these pathogens are yellowing and stunting (*S. citri*, stolbur phytoplasma, *Candidatus* Liberibacter sp.), yellowing and proliferation (AY), and pale-green leaves and proliferation (*Candidatus* Phytoplasma aurantifolia, apple proliferation).

The leafhopper-transmissible strains GII3 (phytopathogenic) and GMT553 (nonphytopathogenic) of *S. citri* (Foissac et al. 1997) also were used in this work. Plants infected with *S. citri*, *Candidatus* Phytoplasma aurantifolia, and *Candidatus* Liberibacter sp. were grown at 30°C during the day and 25°C at night. Plants infected with the other phytoplasmas were grown at 25°C during the day and 20°C at night.

Inoculation of *S. citri* to periwinkle plants.

Healthy periwinkle plants were grown from seeds in an insect-proof greenhouse at 30°C. When they had four to six leaves, either 10 healthy or *S. citri*-infected *Circulifer haematocaps* were put on each plant, as described in Foissac et al. (1997). After 12 days, the insects were removed. Leaves for RNA isolation were taken at different times after insect removal, until plants showed well-developed symptoms.

Differential display (DD) of mRNA was performed with RNA extracted from healthy and infected plants 50 to 60 days after inoculation when infected plants showed well-developed symptoms.

Transmission of the uncultured phloem-restricted bacteria to periwinkle plants.

The uncultured phytoplasmas and *Candidatus* Liberibacter asiaticus were transmitted to periwinkle plants by graft inoculation. For DD analysis, samples were taken when characteristic symptoms were observed approximately 6 weeks after grafting. As controls, healthy plants were grafted similarly with uninfected *C. roseus* shoots.

RNA isolation.

RNA was isolated according to Puissant and Houdeline (1990). Leaf midribs (0.3 to 0.9 mg) were ground in mortar with liquid nitrogen and the resulting powder was transferred into a glass tube. Five milliliters of extraction buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M beta mercaptoethanol) were added. The sample was further ground for 30 s with a type PT 10/35 polytron (Kinematica, Littau-Luzern, Switzerland) and transferred to a 12-ml tube. A volume of 0.5 ml of 2 M sodium acetate, pH 4, 5 ml of water-saturated phenol, and 1 ml of chloroform were added. The tube was vortexed and centrifuged for 10 min at 10,000 × g. Nucleic acids were precipitated from the aqueous

phase by isopropanol for 1 h at -20°C. The sample was then centrifuged for 10 min at 10,000 × g, the pellet was resuspended in 1.5 ml of 4 M LiCl, transferred to an Eppendorf tube and vortexed for 10 to 30 min. The tube was centrifuged for 10 min and the pellet was resuspended in 1 ml of 4 M LiCl, following the same procedure as above. After centrifugation for 10 min, the pellet was resuspended in 500 µl of Tris 10 mM, pH 7.5, 0.5% sodium dodecyl sulfate (SDS), and 1 mM EDTA and alternately submitted to 10 min of vortexing and 10 min of incubations at 65°C, until complete dissolution.

The sample was extracted once with Tris-saturated-phenol and twice with chloroform. Seventy milliliters of 2 M sodium acetate, pH 5, and 700 µl of isopropanol were added to the last aqueous phase and incubated for 1 h at -20°C. After a centrifugation for 15 min at 10,000 × g, the pellet was dried and suspended in 70 µl of water. Subsequently, the RNA sample was treated with RQ1 DNase to remove residual DNA, following the protocol described by the manufacturer (Promega, Madison, WI, U.S.A.).

Differential display.

cDNAs were synthesized with the RNAimage Kit (GenHunter Corp., Brooklyn, MA, U.S.A.) from 0.4 µg of DNA-free RNA for 60 min at 37°C, Malonay Murine Leukemia Virus reverse transcriptase, and reverse transcription (RT) primers H-T₁₁M (H corresponds to the *Hind*III restriction site and M is either deoxyadenosine, deoxyguanosine, or deoxycytidine. Template and primers were denatured for 5 min at 65°C prior to the reaction.

The reaction was heat inactivated for 15 min at 75°C, and one-tenth of each sample was amplified by polymerase chain reaction (PCR), following the manufacturer's instructions, combining the primer used for reverse transcription with arbitrary primers. PCR conditions were 94°C for 30 s, 38°C for 60 s, and 72°C for 90 s for 40 cycles, followed by 7 min elongation at 72°C. Three microliters of the amplified DNA were heated for 3 min at 90°C with 2 µl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and separated by electrophoresis on a 6% denaturing polyacrylamide gel. The gel was then vacuum dried at 80°C for 90 min and exposed to X-ray films overnight. Each reaction was performed at least twice to prevent the selection of false differentially expressed bands.

Differentially displayed DNA bands were excised and eluted from the gel by incubation with 50 µl of water for 2 h at 56°C. Four microliters were used for DNA amplification by PCR, as described above, with 20 µM deoxynucleoside triphosphates (dNTP) instead of 2 µM. DNA was analyzed on 0.7% agarose gels, and the bands at the expected size were excised and purified with the Clean-Mix Kit (Talent, Trieste, Italy). The DNA was cloned in the pGEM-T Easy Vector (Promega), following the manufacturer's instructions.

Northern blot analysis.

Approximately 10 µg of total RNAs were separated on 1% agarose gel containing 3% formaldehyde, 8.8% morpholinepropanesulfonic acid (MOPS), and 0.15 µg of ethidium bromide per ml. RNA samples were treated prior to loading with 47% formamide, 18% formaldehyde, and 9.5% MOPS, and incubated for 10 min at 65°C. One microliter of loading dye was added.

The RNAs were transferred to Hybond C extra (Amersham Corp., Arlington Heights, IL, U.S.A.) membranes in 10× SSC (1.5 M NaCl plus 0.15 M sodium citrate) and dried at 80°C for 2 h.

Prehybridization was performed at 42°C in a solution containing 5× SSC; 50% formamide; 0.5% SDS; 5× Denhardt; 0.02 M Tris, pH 7.5; and 0.3 mg of yeast RNA per ml.

Hybridization was performed in the same buffer containing alpha-³²PdATP-labeled probes obtained from the pGEM-T easy vectors.

Membranes were washed three times for 10 min with 2× SSC and 0.5% SDS at room temperature, then with 0.1× SSC and 0.1% SDS at 50°C for 45 min before exposure to X-ray films.

Isolation of the transketolase gene (Q) by RT-PCR.

Ten micrograms of RNA from healthy periwinkle plants were mixed with 4 µl of 5 µM Q2 primer (5'-TCATGAACACCTGATC-3'), defined at the 5' end of the Q cDNA, heated 10 min at 75°C, and immediately put on ice. Five microliters of 5× buffer (Promega), 2 µl of DTT, 2 µl of 200 µM dNTPs, and 1 µl of MMLV reverse transcriptase were added, and the tube was incubated for 90 minutes at 37°C. The enzyme was heat inactivated for 10 min at 70°C. The PCR was done following the same protocol as that of the differential display PCR with primers Q2 and Q1 (5'-AACTACCCAATCCACATGAA-3') defined in a consensus 3' end sequence of *Oriza sativa* AF024512 and *Arabidopsis thaliana* Y14333 transketolase sequences.

DNA sequencing and analysis.

Clones were sequenced with the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Sequences were compared with those from the National Center for Biotechnology Information (NCBI) databases with the Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments were performed by CLUSTAL and MAP algorithms.

RESULTS

Identification of mRNA differentially expressed in periwinkle plants following infection with mollicutes.

Plant RNA was submitted to reverse transcription and the DNA obtained was amplified by PCR, yielding cDNAs. The cDNAs from healthy periwinkle plants were compared on polyacrylamide gels with those obtained from periwinkle plants infected either by *S. citri*, the stolbur phytoplasma, or *Candidatus* Phytoplasma aurantifolia. Approximately 80 bands were seen on each lane of the gels, and the amplified products were 100 to 1,200 bp. Figure 1 illustrates an area of a polyacrylamide gel where a band (arrow) that was prominent in the profiles from healthy periwinkle plants (lanes 1 and 2) was faint in those infected with *S. citri* (lanes 3 and 4).

Seventeen differentially expressed DNA bands were selected following infection by *S. citri* and 26 and 23 bands following infection with the stolbur and WBDL phytoplasmas, respectively, totaling 66 bands. The selected bands were cut out of the dried polyacrylamide gel and the DNAs were eluted, reamplified with the same primer combinations, and cloned for sequence determination and verification of the differential expression by Northern blots.

Of the 66 bands, only one could not be reamplified, whereas 38 bands have so far been analyzed. Four bands gave no hybridization signals and nine bands yielded equivalent signals with RNAs extracted from healthy and infected periwinkle plants, thus corresponding to false positives. The results obtained from the currently analyzed bands are summarized in Table 1 and Northern blots for 14 bands are shown in Figure 2. Northern blots agreed with the differential display gels for all the bands tested, except band J, which was isolated in the case of *S. citri* infection. Band J also appeared to be up regulated on the gel, but hybridization revealed very strong signals with RNAs extracted only from healthy periwinkle plants, showing that it corresponded to a down-regulated gene. All three pathogens resulted in the obtention of up- and down-regulated genes. Until now, however, a majority of bands corresponded to down-regulated genes, in the case of *S. citri* and stolbur phytoplasma, whereas the predominance of up-regulated genes was found in periwinkle plants infected with *Candidatus* Phytoplasma aurantifolia.

Sequence analysis of the cDNA clones.

The nucleotide sequences of 24 cDNA clones were determined and compared with those in databanks to reveal putative homologies. Sixteen of them did not show homology with known proteins. In particular, none of the bands analyzed from *Candidatus* Phytoplasma aurantifolia-infected periwinkle plants had equivalents in the databanks. On the contrary, DNA from bands I, Q, C1, A11-1, C9-1 and G17-4, isolated

Fig. 1. Polyacrylamide gel electrophoresis of cDNA isolated from healthy (lanes 1 and 2) or *Spiroplasma citri*-infected (lanes 3 and 4) periwinkle plants. Arrow shows a differentially expressed cDNA.

from *S. citri*-infected periwinkle plants, had strong homologies with previously sequenced protein genes. Band I showed strong homology in its derived amino-acid sequence (68 amino acids) with sterol-C-methyltransferase from *Synechocystis* sp. and *A. thaliana*. This enzyme is involved in the synthesis of phytosterols, which play multiple roles in plant growth and development as they are precursors of steroid growth regulators (Haughan et al. 1988; Shi et al. 1996). The putative product of band Q had a strong homology (70% identity, 80% similarity) with a transketolase-like protein from *O. sativa* (AF024512), an enzyme involved in sugar transport (Bernacchia et al. 1995). In plants, transketolases also play important roles in the Calvin cycle, catalyzing two reactions for the regeneration of ribulose-1,5-biphosphate (Lange et al. 1998). The band C1-deduced protein had homology with a wall-associated kinase (He et al. 1999), A11-1 product with a desiccation protectant protein (Maitra and Cushman 1994) and the G17-4 protein with a chlorophyll a/b binding protein (Falconet et al. 1993). Homology with a gene of unknown function also was evidenced (band C9-1). In the stolbur phytoplasma infection, two of the repressed bands (C2 and Z1) had homologies with genes involved in photosynthesis (subunit III of photosystem I and ribulose 1,5-biphosphate carboxylase–oxygenase).

Specificity of the selected bands.

The bands we selected were isolated from periwinkle plants at late stages of infection when severe symptoms were observed on the plants. Leaf yellows and stunting are induced by many, but not all, phloem-restricted plant mollicutes or walled-bacteria. Thus, these symptoms are not highly specific, and it is likely that the down regulation of some of the genes we identified, particularly those involved in photosynthesis,

also occurs with other pathogens. This was verified for bands Q, I, Z1, and PaB1 on Northern blots with RNAs extracted from periwinkle plants infected with one of various phloem-restricted bacteria. Table 2 and Figure 3 show that none of the four genes were regulated specifically by a given mollicute. Gene Q (transketolase) (Fig. 3, first panel) was down regulated in periwinkle plants showing strong leaf yellows whether they had proliferation (aster yellows) or not (*S. citri*, stolbur, *Candidatus Liberibacter* sp.), but down regulation was much less in plants showing proliferations without severe leaf yellows (apple proliferation and WBDL). In contrast, gene I (Fig. 3, second panel) coding for a putative sterol-C-methyltransferase was down regulated in periwinkle plants showing leaf yellows and stunting, but did not appear in those with proliferations. Gene Z1 (ribulose 1,5-biphosphate carboxylase–oxygenase) (Fig. 3, third panel) was down regulated in plants with strong yellowing, whereas gene PaB1, which was activated in *Candidatus* Phytoplasma aurantifolia-infected periwinkle plants, also was activated by the apple proliferation phytoplasma.

Expression of the transketolase-like mRNA following infection of periwinkle plants.

Gene Q, which showed homologies with transketolase, an enzyme involved in sugar transport, was studied further, particularly because the weakly pathogenic mutant GMT 553 of *S. citri* is defective in fructose utilization (Gaurivaud et al. 2000a; Gaurivaud et al. 2000b). The homology of the Q fragment with a transketolase gene was confirmed by isolating a larger DNA fragment by RT-PCR, as described above. A sequence of 2,087 bp was obtained, corresponding to 673 amino acids, or approximately 90% of the transketolase protein. Figure 4 shows the multiple sequence alignment of the amino

Table 1. Differential display and sequencing results

Bands	Primer combinations	Infecting mollicutes	Northern blots results	GenBank homologies
I	T11GC–U2	<i>Spiroplasma citri</i>	R ^a	Delta-C-methyltransferase
J	Id ^b	<i>S. citri</i>	R	N ^c
M	Id	<i>S. citri</i>	A ^d	N
Q	T11CC–U2	<i>S. citri</i>	R	Transketolase
B1	T11A–9	<i>S. citri</i>	R	N
C1	T11A–11	<i>S. citri</i>	A	Wall-associated kinase
C5	T11G–3	<i>S. citri</i>	R	N
A9-1	T11A–9	<i>S. citri</i>	R	N
A11-1	T11A–1	<i>S. citri</i>	R	Desiccation protectant
C9-1	T11C–1	<i>S. citri</i>	R	Unknown <i>Arabidopsis thaliana</i>
C9-12	T11C–9	<i>S. citri</i>	A	N
A13-4	T11A–13	<i>S. citri</i>	R	N
G17-4	T11G–17	<i>S. citri</i>	R	Chlorophyll a–b binding protein
PaB1	T11A–2	<i>Candidatus</i> Phytoplasma aurantifolia	A	N
C1	Id	<i>Candidatus</i> Phytoplasma aurantifolia	A	N
J2	Id	<i>Candidatus</i> Phytoplasma aurantifolia	A	N
X2	T11A–6	<i>Candidatus</i> Phytoplasma aurantifolia	R	N
T1	Id	<i>Candidatus</i> Phytoplasma aurantifolia	A	N
U3	Id	<i>Candidatus</i> Phytoplasma aurantifolia	A	N
C2	T11C–1	Stolbur Phytoplasma	R	Subunit III of photosystem I
A1	T11C–1	Stolbur Phytoplasma	A	N
Z1	T11C–6	Stolbur Phytoplasma	R	Ribulose 1,5-biphosphate carboxylase – oxygenase
F1	T11C–1	Stolbur Phytoplasma	R	N
H2	T11C–2	Stolbur Phytoplasma	R	N

^a Repressed.

^b Idem.

^c No homology.

^d Activated.

acid sequence deduced from the periwinkle transketolase-like gene with four transketolase or transketolase-like amino acid sequences: *Craterostigma plantagineum* (Z46648), *Solanum tuberosum* (Z50099), *A. thaliana* (Y14333), and *O. sativa* (AF024512). A similarity of 83% was observed with the transketolase from *A. thaliana*. Out of 50 invariant residues, as defined by Schenck et al. 1997 (underlined bold letters), 27

were present in the periwinkle transketolase sequence. The thiamin pyrophosphate (TPP) binding motif (cofactor) and the transketolase motif (Schenk et al. 1997) also were identified.

The expression of the periwinkle transketolase mRNA following infection by the wild-type GII3 strain or the GMT 553 mutant of *S. citri* was studied on Northern blots of RNAs extracted at various times after infection. The outline of the experiment is given in Table 3 and Northern blots in Figure 5. The data showed that the transketolase gene was weakly expressed only in very young (4 weeks old) periwinkle plants at the time when healthy or *S. citri*-infected leafhoppers were put on the plant (lane 0). After a 12-day transmission period, the leafhoppers were removed and the gene was found to be strongly expressed in plants inoculated with the phytopathogenic strain GII3 (Fig. 5A, lane 1) or the weakly pathogenic mutant GMT 553 (Fig. 5A, lane 1'). Control plants that received no insects had the same pattern of transketolase gene expression (data not shown). The presence of spiriplasmas in the inoculated plants was followed by hybridization on Northern blots probed with a gene coding for a constitutive membrane protein of the spiriplasma spiralin (Bové et al. 1993) (Fig. 5B). No spiriplasmas could be detected in the plants when the insects were removed (Fig. 5B, lanes 1 and 1'). At day 22, when faint symptoms were observed on the periwinkle plants inoculated with the wild-type strain spiriplasma (Table 3), down regulation of the transketolase gene was observed and a strong spiralin signal was present (Fig. 5A and B, lane 2). Expression of the transketolase gene was totally abolished after day 22 and symptom severity increased, whereas the spiriplasma titer remained constant (Fig. 5A and B, lanes 3 to 6). In the case of periwinkle plants infected with the non-pathogenic mutant GMT 553, expression of the transketolase gene remained unaffected up to 56 days postinoculation (Fig. 5A, lanes 2' to 7'). This correlated with a low spiriplasma titer in the plants from day 22 to 47 (Fig 5B, lanes 2' to 5'). Forty-nine days after the transmission period, symptoms started to develop in agreement with the increase of the spiriplasma titer, but the transketolase gene expression remained unmodified up to 56 days posttransmission. From there on, however, as was the case with the wild-type spiriplasma, expression of the transketolase gene was abolished (Fig. 5A, lanes 8' and 9'). Previously it was shown that symptom development in infected periwinkle plants occurs when the spiriplasma titer reaches 10^6 to 10^7 organisms per 200 mg of leaf midribs (Foissac et al. 1997). In plants infected with the mutant strain, this titer is reached much later than it does in plants infected with the wild-type strain because the wild-type spiriplasma has a multiplication rate that is higher than the mutant. When the titer of the *S. citri* mutant is high, however,

Fig. 2. Northern blot hybridizations of RNAs extracted from infected periwinkle plants with 32 P-labeled cDNA. Healthy (first two lanes), *Spiroplasma citri* (A, last two lanes), *Candidatus Phytoplasma aurantifolia* (B, last two lanes), or stolbur phytoplasma (C, last two lanes). One healthy lane and one infected lane are shown for ScM.

Table 2. Gene expression in periwinkle plants infected with various phloem-restricted bacteria

Probes	Periwinkle infections						
	None	Aster yellows phytoplasma	<i>Spiroplasma citri</i>	<i>Liberibacter</i> sp.	Apple proliferation phytoplasma	Stolbur phy- toplasma	<i>Candidatus</i> <i>Phytoplasma aurantifolia</i>
Q	+	— ^b	—	—	+	—	+
I	+	+	—	—	+	—	+
Z1	+	—	—	—	+	—	+
PaB1	—	—	—	—	+	—	+

^a Hybridization signal.

^b No or faint hybridization signal.

transketolase is not repressed (Fig. 5B, lanes 6' and 7'). In addition, it was shown that after 8 weeks in the plant, reversion of GMT 553 to the wild-type phenotype Spiroplasmas occurs and is correlated with symptom development (Foissac et al. 1997; Gaurivaud et al. 2000a). This also is in agreement with the down regulation of the transketolase gene (Fig. 5A, lanes 8' and 9').

DISCUSSION

The mechanisms involved in the pathogenicity of plant mollicutes are beginning to be studied. As most of these plant pathogens are not available in culture, we used the DD method to identify genes whose expression is modified following infection. The host plant chosen for this approach was periwinkle because it is used worldwide for mollicute maintenance and studies because mollicutes reach high titers in this species. Our results demonstrate that the DD methodology is suitable to study plant-mollicute interactions, even though, as reported by others, up to 30% of the bands were false positives. With the use of a limited number of primer combinations, we isolated and characterized 24 cDNA clones corresponding to mRNAs that are up or down regulated in periwinkle plants following infection with phytopathogenic mollicutes. Twenty-eight additional differentially expressed bands remain to be studied. Only one of the bands, band J, which appeared up regulated on the gel, turned out to correspond to a down-regulated gene after hybridization. Certainly this is because several genes were present in the band recovered from the DD gel and the clone selected for hybridization was representative of one part of the population.

Because the DNA sequences obtained from the DD gels were short and corresponded to the 3'-untranslated sequence, we were only able to identify similarities with genes in the databases for eight of the DNA clones. Screening of genomic and cDNA libraries are under way to isolate the entire genes in order to increase the chances to identify orthologs in databanks. PCR amplification of most of the putative transketolase gene of periwinkle allowed us to find the characteristic motifs of this enzyme and confirm the homology initially found with transketolases from other plant species.

For the first time, we have shown that a gene coding for a pathogen-induced protein, namely a wall associated kinase (He et al. 1999), was activated by *S. citri* infection. In *Arabidopsis* spp., this gene also is induced by salicylic acid and is involved in the plant's defense response. At this stage, no function could be assigned to the other up-regulated genes that we detected. It is worth noting that most of the up-regulated

genes identified were from plants infected with *Candidatus* Phytoplasma aurantifolia, which induces abundant witches' broom proliferations, an active developmental process compared with leaf yellows and stunting.

The genes we isolated were from late-infection stages and concerned several metabolic pathways. They were not specific to a given phloem-restricted bacterium but, even though the number of pathogens of each symptom type was small, we could associate the regulation of some of the genes with the type of symptoms observed on the plant.

Indeed, sugars seem to play an important role in the pathogenicity of certain mollicutes. Fructose utilization by *S. citri* is a key factor for symptom development (Gaurivaud et al. 2000a). Lepka et al. (1999), showed that infection of tobacco or periwinkle plants with various phytoplasmas results in a marked increase of soluble carbohydrates in source leaves and lower levels of carbohydrates in sink organs. Thus, it has now been proposed that *S. citri* competes with the companion cells for fructose utilization and impairs sucrose loading by the

Fig. 3. Northern blot hybridizations of cDNAs Q (first panel), I (second panel), Z1 (third panel), and PaB1 (fourth panel) with RNAs extracted from healthy periwinkle plants (lane 1) or periwinkle plants infected by aster yellows phytoplasma (lane 2), *Spiroplasma citri* (lane 3), *Candidatus* Liberibacter sp. (lane 4), apple proliferation phytoplasma (lane 5), stolbur phytoplasma (lane 6), or *Candidatus* Phytoplasma aurantifolia (lane 7).

Table 3. Outline of the experiment illustrated in Figure 5

Sampling for Northern blots days after insect addition	GII 3		GMT 553	
	Lanes on Northern blots	Symptoms	Lanes on Northern blots	Symptoms
Day (D) + 12	1	None	1'	No
D + 22	2	Faint	2'	No
D + 32	3	+	3'	No
D + 39	4	+	4'	No
D + 47	5	++	5'	No
D + 49	6	++	6'	Faint
D + 56	Not done (ND)	+++	7'	+
D + 67	ND	+++	8'	+
D + 91	ND	+++	9'	++

companion cells into the phloem sieve tubes (Gaurivaud et al. 2000a). Impairment of sucrose loading also is mentioned by Lepka et al. (1999) for some phytoplasmas. It was thus not surprising to find that in plants infected by certain mollicutes,

there was repression of a gene involved in sugar transport (gene Q, transketolase). Our experiments comparing expression of the transketolase gene in plants infected with wild-type or 553 mutant spiroplasmas confirmed that there is a link be-

	1	15	16	30	31	45	46	60	61	75	76	90
1 tkclcr	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
2 tkipo	-----	-----	MASSSSLT	LSQVIFSPSLPRHGS	SSSSSPSLSFSTFSG	LKST---	PFTSSHR-	---	RILPSTTVTKQQ	---	---	---
3 proper	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
4 TKLOs	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
5 tklat	ILICFTYIPKVFNFI	ELQKVPFMASAF	AF	PSYIITKGLSTD-S	CKSTSLSSSRSLVTD	LPSCLKPNNNSHSN	RRAKVCASLAEKGEY	---	---	---	---	---
1 tkclcr	91	105	106	120	121	135	136	150	151	165	166	180
2 tkipo	-----	MAPKTTLIA	EPFLVSKSVNTIRFL	A-----	IDAVEKA	NSGHPGMPMGCAPMG	HVLYDEFMRFNPKNP	YWFN--	RDRFVLSAG	---	---	---
3 proper	FSVRASAAVETLEKT	DAAIKSVNTIRFL	A-----	IDAVEKA	NSGHPGMPMGCAPMG	HVLYDEFMRFNPKNP	YWFN--	RDRFVLSAG	---	---	---	---
4 TKLOs	HGGRG-----	NSINY	PIHMKNLSQLQQL	ADELREEVIVSVAKT	-GGHLSSSLGVAELT	VALH-----	HVFETPDKILWDVG	---	---	---	---	---
5 tklat	YSNRPPPTPLDITNY	PIHMKNLSQLQQL	ADELRSDFIVFVSKT	-GGHLSSSLGVAELT	VALH-----	YVFNTPDKILWDVG	---	---	---	---	---	---
1 tkclcr	181	195	196	210	211	225	226	240	241	255	256	270
2 tkipo	HGCMLOQYALLHLSGY	DSVKEEDLKSIRQWG	SRTPAHPENFETPGV	EVTTPGLQGQIASAV	GLAVAEKHLAARYNK	PGFEIVDHYTYVILG	---	---	---	---	---	---
3 proper	H-----	QTYGKILTG-	---RRSKMTMRQTC	G-LAGFPRKDESAHD	AFGVGHSSTTSISAGL	GMAIG-RDLGKKNH	-----	VITVIG	---	---	---	---
4 TKLOs	H-----	QSYPHKILTG-	---RRDKMPTMRQTN	G-LSGFTKRSESEYD	SFGTGHSSTTISAAAL	GMAVG-RDLGKGNH	-----	VVAVIG	---	---	---	---
5 tklat	H-----	QSYPHKILTG-	---RRGKMPTMRQTN	G-LSGFTKRGESEHD	CFGTGHSSTTISAGL	GMAIG-RDLGKGNH	-----	VVAVIG	---	---	---	---
1 tkclcr	271	285	286	300	301	315	316	330	331	345	346	360
2 tkipo	DGCQMEGVSNEACSL	AAHWGLGKLIAYDD	NHITIDGDT-----	-----	-----	-----	-----	-----	-----	-----	-----	DVAFTEDV
3 proper	DGCQMEGVSNEACSL	AAHWGLGKLIAYDD	NHISIDGDT-----	-----	-----	-----	-----	-----	-----	-----	-----	EIAFTEDV
4 TKLOs	DGAMTAGQAYEAMNN	AGYLDNLITILNDN	KQVSLPTATIDGPAP	PTGALSNALTRLQSS	RKFRHFGRLQK-LPK	QLGGQVHEMAAKVDT	---	---	---	---	---	---
5 tklat	DGAMTAGQAYEAMNN	AGYLDSDMIVILNDN	KQVSLPTATIDGPSP	PVGALSSALSRLQSN	PALRELREVAKGMTK	QIGGPMHQVAAKVDV	---	---	---	---	---	---
ThDp binding motif												
1 tkclcr	361	375	376	390	391	405	406	420	421	435	436	450
2 tkipo	DKR-----	FD	ALGWHVIVWKNNGNDG	CDEIRAAIEEAKSVK	DR-PTMIKVTITIGY	GAP-SKANTYGVHGN	ALGPKAEATRNKLG	---	---	---	---	---
3 proper	SAR-----	FE	SLGWHVIVWKNNGNTG	YDEIRAAIEEAKSVK	DK-PTMIKVTITIGY	GSP-NKANSYSVHGS	GLGAKEVEATRNKLG	---	---	---	---	---
4 TKLOs	YMRGMNSQRAS-LFE	DLGMYIIGPLDGHN	MEDLVDILEKVSVP	ATGPVLIIHITTEKGR	GYPFAEAAADKMVG	V---KFEPMTGKQ--	---	---	---	---	---	---
5 tklat	YARGMISGSGSLFE	ELGLYYIGPVDGHN	IDDLITILREVKSTK	TGTPVLIVHVTEKGR	GYPFAEAAADKMVG	V---KFEPMTGKQ--	---	---	---	---	---	---
1 tkclcr	451	465	466	480	481	495	496	510	511	525	526	540
2 tkipo	WPYEPFHVDPDVKKH	WSRHIAEGAALESAS	NAKFAEFQKKFPPEEA	ADLKSIIITGELPTNW	ESIFPTTYTPENGLP	TRTSLHQILNGLGDV	---	---	---	---	---	---
3 proper	WPYEPFHVDPDVKKH	WSRHIAEGAALESAS	NAKFAEFQKKFPPEEA	ADLKSIIITGELPAGW	EKALPTYTPESPAD	TRNLSQQNLNALAKV	---	---	---	---	---	---
4 TKLOs	-----	FKSKSKTKSY	-----	TNYFAE---SLISEA	REDDKIIA-----	-----	-----	-----	-----	-----	-----	-----
5 tklat	-----	FKTNNETQSY	-----	TTYFAE---ALVAAE	EVDDKVVA-----	-----	-----	-----	-----	-----	-----	-----
1 tkclcr	541	555	556	570	571	585	586	600	601	615	616	630
2 tkipo	LPGLLGGSDADLTLN	MAFLKNSGDFQKKSP	GERNVKFGAREHAMG	SICNGLALHSPGLLP	YCATY--FVFTDYMR	AAMRISALSARVLY	---	---	---	---	---	---
3 proper	LPGLLGGSDADLTLN	MTLLKMFQDFQKNTP	EERNLRFQVREHGMG	AICNGIALHSLGLIP	YCATF--FVFTDYMR	GAMRISALSARVLY	---	---	---	---	---	---
4 TKLOs	IHAAMGGGTGLNLY--	-----	FQKHFP	-ERCDFVGIAEQHAV	TFAAGLA--SEGLKP	FCATYSSFLQRGYDQ	VVHDVD-LQKIPVRF	---	---	---	---	---
5 tklat	IHAAMGGGTGLNLY--	-----	FQRRFP	-TRCFDVGIAEQHAV	TFAAGLA--CEGLKP	FCATYSSFLQRGYDQ	VVHDVD-LQKIPVRF	---	---	---	---	---
1 tkclcr	631	645	646	660	661	675	676	690	691	705	706	720
2 tkipo	MTHTDSIGL-GEDEP	THQPVHSLASFRAMP	NILTLRPAD	GNETAG	AYRAAVQNGERPSIL	VLARQKLPQLPGTSTI	EGVSKGGVVISDNNSR	---	---	---	---	---
3 proper	MTHTDSIGL-GEDEP	THQPLEHLASFRAMP	NILMFRPAD	GNETAG	AYKVAVLKRRTPSIL	ALSRQKLPQLAGTSTI	EGGAAGGYIVSDNS	---	---	---	---	---
4 TKLOs	AM--DRAGLVGADGP	-HCGAFDVTTFMACLP	NMVMAPSD	ETELMH	MVATAAIDDRLVAF	DIPEEM---ALVQFF	HQTTKELHYLRVKEE	---	---	---	---	---
5 tklat	AM--DRAGLVGADGP	THCGAFDVTTFMACLP	NMVMAPSD	EALICH	MVATAAIDDRPSCF	RYPRGN---GIGVPL	PPNYKGVPLEVGKGR	---	---	---	---	---
Transketolase motif												
1 tkclcr	721	735	736	750	751	765	766	780	781	795	796	810
2 tkipo	GGNSKPDVILIGTGS	ELEIAARAGDELKKE	GKTVRVVSL-VCWEL	FAEQSEKYRETIVLPS	GVTARVSVIAGSTFG	W-----	QKFGVQKGA	---	---	---	---	---
3 proper	YCW-ERVAILGYQA	MVQCNLMQAQLLEIN	GISATVVADARFCKPL	--DGLIRK---	LAQ	QHEVLITVEEGSIGG	FGSHVAQFLALNGLL	---	---	---	---	---
4 TKLOs	VLEGERVALLGYGS	AVQYCLAAASLVERH	GLKVTVADARFCKPL	--DQTLIR---	LAS	SHEVLLITVEEGSIGG	FGSHVAQFLALNGLL	---	---	---	---	---
5 tklat	ILKEGERVALLGYGS	AVQYCLAAASLVERH	GLNVTVADARFCKPL	--DRALIRS---	LAK	SHEVLLITVEEGSIGG	FGSHVQVFLALDGLL	---	---	---	---	---
1 tkclcr	811	825	826	840	841	855	856	870	871	885	886	900
2 tkipo	VGIDRFGASAPAEAL	FKEFGITVEAVVAAA	KEIC-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
3 proper	IGIDGFGASAPADKI	YKEFGITAEAVVAAA	KQVS-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
4 TKLOs	DGNLKWRAAMLPDR	YIVMELKMTKLKRLA	NSKAYCWHCVFSTHA	KTR-ILFKFSSFRQL	NNNYWILVSIHDSF	NLLINILIEKESLN	---	---	---	---	---	---
5 tklat	DGKLKWRPMLVDR	YIDHGAPADQLAEAG	LMPSHIAATLNLIG	APREALF-----	-----	-----	-----	-----	-----	-----	-----	-----
1 tkclcr	901	915	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
2 tkipo	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
3 proper	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
4 TKLOs	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
5 tklat	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

Fig. 4. Amino acid comparisons of the deduced protein product of gene Q with transketolase or transketolase-like protein sequences from databanks. tkclcr = transketolase from *Craterostigma plantagineum* (Z46648); tkipo = transketolase from *Solanum tuberosum* (Z50099); proper = protein product of Q fragment of *Catharanthus roseus*; tklos = transketolase-like protein from *Oriza sativa* (AF024512); tklat = transketolase from *Arabidopsis thaliana* (Y14333). Underlined amino acids indicate invariant residues; areas surrounded by black lines indicate a thiamin pyrophosphate binding motif and a transketolase motif.

tween fructose utilization by the spiroplasma and deregulation of transketolase expression. Interestingly, in *A. thaliana*, mutation of the transketolase gene causes a highly pleiotropic phenotype affecting the expression of several photosynthetic genes and pigment production (albino phenotype). In general, albino phenotypes are the result of a blockage in the photosynthetic process, affecting chloroplast development either directly or indirectly, and photosynthetic genes are regulated by carbohydrates (Mandel et al. 1996). Inhibition of the transketolase gene might therefore be responsible for the repression of the genes involved in photosynthesis (C2, Z1, G17-4). Indeed, we showed that Z1 followed the same inhibition pattern as gene Q in plants infected with various bacteria and showing yellowing symptoms. Such deregulations explain the leaf's yellow symptoms induced by *S. citri*, the stolbur phytoplasma, and other phloem-restricted agents but not the proliferations induced by several other phytoplasmas. It also is speculated that growth aberrations induced by phloem-restricted bacteria could be the result of perturbations in the plant hormonal balance (Chang 1998). Gene I coding for a putative sterol C-methyl transferase, an enzyme involved in phytosterol biosynthesis, can play such a role. Indeed, sterols are structural membrane components as well as precursors of steroid growth regulators (Haughan et al. 1988; Shi et al. 1996). This gene is down regulated in *S. citri*, stolbur phytoplasma, and liberibacter infections, all characterized by stunting and internode shortening.

This work represents the first identification of plant genes that are deregulated following infection by mollicutes. More work has to be carried out to identify the metabolic pathways modified during plant-mollicute interactions. The genes deregulated during early stages of plant-mollicute interactions also are yet to be identified. The development of expressed sequence tags (EST) on high-density filters for plants such as tomato, a host for the stolbur phytoplasma, and celery, a host for stolbur and *S. citri*, will allow in the near future a more complete analysis of the plant response to mollicute infections. The genes that we isolated will be useful as markers of infection and as controls for the EST filters.

Fig. 5. A, Northern blot hybridizations with transketolase or **B,** spiralin genes with RNAs extracted from healthy periwinkle plants (lane 0) or periwinkle plants infected with *Spiroplasma citri* GII3 wild-type (lanes 1 to 6) or *S. citri* mutant GMT 553 (lanes 1' to 9').

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