Catharanthus roseus, an Experimental Host Plant for the Citrus Strain of *Xylella fastidiosa*

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ABSTRACT

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We verified by pathogenicity tests that the herbaceous plant *Catharanthus roseus* (Madagascar periwinkle) can be used as an experimental host for the strain of *Xylella fastidiosa* that causes citrus variegated chlorosis (CVC). Plants were mechanically inoculated with CVC strain 9a5c, the genome of which was recently sequenced. Plants were inoculated with the virulent 8th passage (9a5c-8) and the 51st passage (9a5c-51). Leaf deformation and stunting were seen 2 months after inoculation on 18 of 21 plants with 9a5c-8 and 8 of 21 plants with 9a5c-51. The plants were infected with *X. fastidiosa* as shown by polymerase chain reaction. The bacterium could be reisolated from all plants tested, showing that CVC-*X. fastidiosa* multiplied and moved systemically in *C. roseus* plants causing dysfunction in plant growth. The disease symptoms evolved within 4 months post-inoculation to a severe leaf chlorosis in all inoculated plants. The localization of *X. fastidiosa* in the xylem was verified by immunofluorescence. Genes coding for proteins with homologies to plant sterol-C-methyltransferase, a transketolase-like protein, subunit III of photosystem I, and a desiccation protectant protein were found to be differentially expressed in symptomatic *C. roseus* plants as a response to infection with *X. fastidiosa* in comparison to healthy plants. A tentative correlation between the pattern of expression of these *C. roseus* genes with the mechanism of pathogenicity of *X. fastidiosa* is discussed.

Xylella fastidiosa is a fastidious Gram-negative, xylem-limited bacterium (28). In many economically important plants, different groups of *X. fastidiosa* cause diseases such as citrus variegated chlorosis (CVC) (4,25), periwinkle wilt (PW), and Pierce's disease (PD) of grapevine, alfalfa dwarf, leaf scorch of almond, coffee, elm, sycamore, oak, plum, mulberry, maple, and oleander (22,23). The full host range of the bacterium is large and includes many asymptomatic herbaceous and woody plant species (10,13). Strains of *X. fastidiosa*, however, are pathogenically specialized. For example it was shown that although both PD and PW strains of *X. fastidiosa* multiply in grapevine and periwinkle, the PW strain does not induce symptoms in grapevine, and the PD bacterium induced only slight chlorosis in periwinkle but not typical PW symptoms (8).

X. fastidiosa isolated from different hosts have been separated into groups by several approaches including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), by polymerase chain reaction (PCR) using tRNA consensus primers or strain specific primers or by subtractive hybridization (1,2,6,9,20,21).

CVC is a major problem in the state of São Paulo, Brazil, where over 60 million trees (30%) are affected. It occurs in Argentina where it is called "pecosita" (7,12). Rapid dissemination of CVC comes from the use of infected nursery trees and transmission of *X. fastidiosa* by several sharpshooter vectors. In Brazil *X. fastidiosa* is also associated with plum leaf scald (11,15) and coffee leaf scorch (3,17,19), and was recently detected in *Catharanthus roseus* (Madagascar periwinkle) showing stunting and dieback symptoms (27). The strains isolated from coffee and citrus are closely related (3,17,19,24), whereas the plum strain is distantly related (24). The strain isolated from *C. roseus* in Brazil is still uncharacterized.

Periwinkle genes are differentially expressed following infection with phloem-restricted pathogens *Spiroplasma citri* or the stolbur phytoplasma (14, Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., Danet, J. L., Bové, J. M., and Garnier, M. *unpublished data*) which cause diseases in many different plants, inducing yellowing, growth aberrations, flower malformation, internode shortening, and stunting. cDNAs corresponding to these genes were used as probes for northern-blot analysis studying the reaction of periwinkle to infection with *X. fastidiosa*, a xylem-restricted pathogen. These cDNAs code for proteins with homologies to plant sterol-C-methyltransferase (SMT), a transketolase-like protein (TLK), subunit III of photosystem I, and a desiccation protectant protein, respectively (14, Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., Danet, J. L., Bové, J. M., and Garnier, M., *unpublished data*). SMT is a rate-limiting enzyme for the phytosterol biosynthesis, which plays multiple roles in plant growth and development. TLK is involved in both the Calvin cycle and the oxidative pentose phosphate pathway (OPP) of higher-plant chloroplasts, synthesizing different sugar phosphate intermediates. The subunit III of photosystem I has a function in the photosynthetic pathways and the desiccation protectant protein is involved in plant cellular protection strategy to tolerate water deficiency.

C. roseus is a symptomatic host for the citrus strain of *X. fastidiosa* and may be useful for experimental studies. We have used strain 9a5c, whose genome was recently sequenced (26). This strain was triply cloned and shown to be phytopathogenic in sweet orange seedlings (16). For post-genomic studies, it was essential to obtain an experimental host that is easier to use than citrus and which develops symptoms faster than sweet orange seedlings. We tested the 8th passage of strain 9a5c (9a5c-8), known to be phytopathogenic, and also the 51st passage (9a5c-51), to evaluate the effect of prolonged subculturing of the CVC strain of *X. fastidiosa* on phytopathogenicity.

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MATERIALS AND METHODS

Subculturing CVC-*X. fastidiosa* 9a5c strain. The triply cloned strain 9a5c (16) was grown in PW-broth medium at 28ºC in the dark with 100 rpm rotatory agitation. The initial culture was passaged weekly by serial transfers at 1/100 dilution. Dilution plating on PW agar medium (six 10-fold dilutions) was used to estimate the number of viable bacterial cells (colony forming units, CFU) and confirm freedom from contamination. The plates, sealed with Parafilm to prevent desiccation, were incubated at 28ºC in the dark for 6 to 14 days. Cultures were stored at –20ºC in PW broth containing 20% glycerol.

Mechanical inoculation of plants. *Catharanthus roseus* (L.) G. Don (cv. Peppermint Cooler) was used as the experimental host plant. Plants were propagated by seeds and grown in an insect-proof greenhouse. Three days before inoculation, the plants were transferred from the greenhouse to the culture room, with a photoperiod of 14 h light at 28ºC and 10 h dark at 18ºC. Inoculations were done after 5 h of light (at 28ºC) ensuring xylem tension and good liquid uptake.

A 7-day-old broth culture (late log phase) of *X. fastidiosa* was centrifuged at 4,000× *g* for 15 min and the resulting pellet resuspended in sterile phosphate-buffered saline (PBS) (20 mM sodium phosphate, pH 6.8, 0.85% NaCl) at an OD_{560nm} of 0.1, corresponding to approximately 10^8 CFU/ml. This correlation was obtained from a growth curve of *X. fastidiosa* in liquid medium, in which the number of CFU was obtained by dilution plating and A_{560nm} readings were taken at different phases of the culture as a function of time.

Two 1-cm-long inverted-U-shaped incisions were made with a scalpel on opposite sides in the second internode from base of the stem of 11-week-old periwinkle plants (15 cm high). A unit of 20 µl of the prepared suspension of *X. fastidosa* was deposited on each incision. The drop was then allowed to enter in the xylem by 6 repeated punctures with a needle. An addi-

Fig. 1. *Catharanthus roseus* plants 2 months after inoculation with 9a5c-8 (left) and 9a5c-51 (middle) in comparison with the phosphate-buffered saline-mock inoculated plant (right). Symptoms are stunting and leaf deformation.

Fig. 2. A, Mature leaves from diseased *Catharanthus roseus* plants shown in **B,** 4 months post-inoculated with 9a5c-8 and 9a5c-51 in comparison with healthy phosphate-buffered saline-mock inoculated plant.

tional 20 µl aliquot of cell suspension was added on the wounded stem and allowed to be absorbed. After inoculation, the bark strips were flipped back on the scion and sealed by gentle finger pressure. In addition, the closest pair of leaves immediately above the inoculation site was also inoculated; a unit of 20 µl of *X. fastidosa* suspension was pipetted on the upper surface of the petioles, and allowed to be absorbed by pin-holes, punched into the petioles. Control plants were inoculated in the same way with PBS or left untreated. Inoculated and control plants were kept in the culture room and watered every 3 days. Twenty-one plants were mechanically inoculated with the 8th and 51st passages of CVC strain 9a5c, originally obtained from the axenic culture 8.1b by triple cloning (4).

Reisolation of *X. fastidiosa* from inoculated *Catharanthus roseus*. The four terminal leaves of 4 months post-inoculated plants were washed in water, and dried with blotting paper. The subsequent procedures were done under aseptical conditions in a laminar-flow hood. Periwinkle leaf petioles and midribs were excised and drops of melted paraffin deposited at both ends. Plant material was surface sterilized by immersion in 70% ethanol for 4 min and 6% sodium hypochlorite for 15 min under agitation. Midribs were rinsed five times with sterile deionized water. For extraction of the xylem sap, the edges of the disinfected petioles and midribs were cut off, removing the paraffin, and tightly packed into a 0.5 ml sterile eppendorf tube with a 1 mm hole in the bottom and inserted into a 1.5 ml eppendorf tube. Each set of two eppendorf tubes was transferred aseptically to a 15 ml tube. A unit of 200 µl of PW medium was added on top of the plant material, the tubes were centrifuged at 4,000× *g* for 15 min at room temperature extracting the xylem sap. The pellet was resuspended in 1 ml PW medium, 200 µl were plated on PW agar medium and the remaining suspension was incubated at 28ºC. Plates were observed for colony development at 4-day intervals for 15 days with a binocular microscope (×10 magnification). The characteristic appearance, growth rate, and growth pattern of colonies on PW agar medium and the characteristic growth in liquid PW were regarded as preliminary indications of *X. fastidiosa* growth. Final identification of the bacteria as *X. fastidiosa* was by PCR and immunoblotting as described below.

PCR. We followed the conditions for PCR as described (21), using primers CVC-1 and 272-2 *int* for the specific detection of the CVC strain of *X. fastidiosa.* For the detection of *X. fastidiosa* in the xylem sap of inoculated plants, bacterial cells were collected by centrifuging the petioles and midribs of three leaves situated two or three nodes above the inoculation sites (15,000× *g* for 10 min), as described above for the culturing of *X. fastidiosa*. After centrifugation, the pellets were air-dried under vacuum for 5 min and resuspended in 100 µl of TE (10 mM Tris, 10 mM EDTA, pH 8.0). The suspension was incubated at 65°C for 15 min and 1 µl was transferred directly to 20 µl of PCR reaction mixture. For the identification of

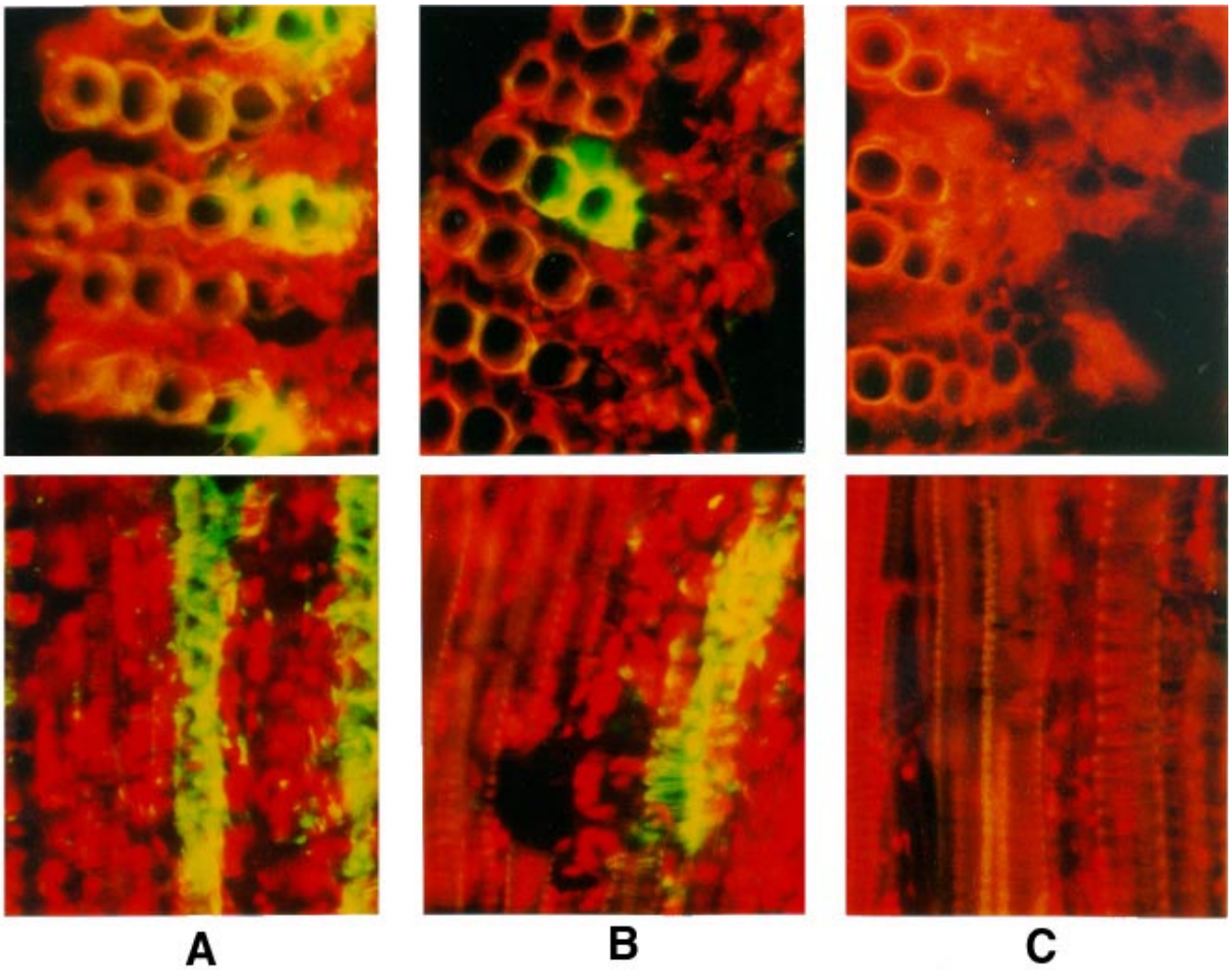

Fig. 3. Immunofluorescence with polyclonal antibody anti-citrus variegated chlorosis strain of *Xylella fastidiosa* on transversal (top) and longitudinal (bottom**)** sections of leaf midribs from *Catharanthus roseus* plants infected with **A,** 9a5c-8; **B,** 9a5c-51; and **C,** and mock inoculated with phosphate-buffered saline (×1,000).

X. fastidiosa in culture, 1 µl of the suspension or a single colony was added directly to the PCR reaction mixture. As positive control, we used 50 ng of *X. fastidiosa* DNA prepared as described (5) with the difference that, after the lysis with lysozyme, the preparation was treated with 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/ml proteinase K for 1 h at 60ºC.

Immunoblot detection of *X. fastidiosa*. The colonies were transferred to a nitrocellulose membrane and treated with a 1/10,000 dilution of a polyclonal antiserum produced against the CVC strain 8.1.b of *X. fastidiosa* (12). A commercial anti-rabbit IgG/alkaline phosphatase conjugate (Sigma) was used as the secondary antibody. For development, we followed the instructions of the conjugate supplier. Colonies of *E. coli* were used as the negative control.

Immunofluorescence. Longitudinal or transversal 30 µm thick sections of midribs cut from healthy or symptomatic young leaves were mounted on microscope slides, fixed by heating at 45ºC for 45 min, and incubated for 30 min at room temperature with a 200-fold dilution of an immunoglobulin fraction purified from the CVC-strain specific polyclonal antiserum according to Garnier et al. (12). After incubation, the slides were rinsed with PBS-Tween and incubated in the dark with a 10,000-fold dilution of anti-rabbit mouse-IgGs labeled with fluorescein isothiocyanate (IgG-FITC). The diluted IgG-FITC solution was prepared by adding 10 µl of the commercial IgG-FITC (Diagnostic Pasteur) to 1 ml of PBS containing 10 µl of commercial solution of Evans blue (Diagnostic Pasteur). After washing with PBS-Tween, a mounting solution (22.2 mM citric acid, 55.6 mM Na_2PO_4, 50% glycerol) was added and the slides were observed using a Zeiss III RS fluorescent microscope with the filter combination PB 455/490 FT510/LP 520.

Northern blot analysis. For RNA isolation, eight young terminal leaves of each control or infected plant 4 months after inoculation were ground in liquid nitrogen with a mortar and pestle, the powder was treated with Tryzol (Gibco-BRL) according to the supplier, and 15 µg of total RNA was submitted to electrophoresis on a 1.2% agarose gel containing 5% formaldehyde and 10% 3-(*N*-Morpholino) propanesulfonic acid (MOPS). RNA samples were prepared in 65% formamide, 22% formaldehyde, 13% MOPS containing 10 µg/ml ethidium bromide, and incubated for 10 min at 65ºC. One microliter of loading dye was added. After electrophoresis, the RNA was transferred to Hybond C-extra nitrocellulose membrane (Amersham) in 20× SSC (3 M NaCl, 0.30 M Na citrate) and fixed by heating at 80ºC for 2 h. Prehybridization was performed at 42ºC in a solution containing 5× SSC, 50% formamide, 0.5% SDS, 5× Denhardt's solution, 0.02 M Tris pH 7.5, and 0.3 mg/ml yeast tRNA. Hybridization was done in the same buffer containing α-^{32}PdATP-labeled probes. The probes obtained as DNA inserts from recombinant pGEM-T-easy plasmids were labeled by random priming according to the kit supplier (Gibco BRL). Membranes were washed 3 times in 2× SSC, 0.5% SDS at room temperature for 10 min and in 0.1× SSC, 0.1% SDS at 50ºC for 45 min, before exposure to X-ray films.

The cDNAs probes code for the following putative periwinkle proteins: sterol-C-methyltransferase (probe Sc 1), transketolase-like protein (probe Sc Q), subunit III of photosystem I (probe St C2), and desiccation protectant protein (probe Sc A11-1) (14, Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., Danet, J. L., Bové, J. M., and Garnier, M. *unpublished data*).

RESULTS AND DISCUSSION

Pathogenicity of *X. fastidiosa* CVC strain to *C. roseus*. Symptoms were observed two months after inoculation of the plants with 9a5c-8 or 9a5c-51. The first symptoms were noticeable as small and deformed young leaves as well as plant size reduction (Fig. 1). Eighteen of 21 plants inoculated with the 8th passage showed these symptoms; whereas, only 8 of 21 plants inoculated with the 51st passage developed the symptoms. The presence of *X. fastidiosa* was detected by PCR in petioles and midribs of leaves collected from nodes, 10 cm distant from the inoculation sites in all inoculated plants, both symptomatic and non symptomatic (results not shown). These results indicate that the early 8th passage as well as the later 51st passage of 9a5c are able to multiply and become systemic in *C. roseus* within 2 months after inoculation. However, the percentage of symptomatic plants was higher with 9a5c-8 than with 9a5c-51. The 8th and the 15th passages of strain 9a5c were already shown to have kept the phytopathogenicity of the initial isolate 8.1b to sweet orange seedling (16).

Four months after inoculation all inoculated plants, including those that did not show symptoms two months post-inoculation, started showing marginal and chlorotic zones along the veins, most frequently in the older leaves (Fig. 2A and B). These leaves abscised early and, in extreme cases, most shoots retained only the most apical leaves. The severity of the symptoms was the same whether the plants were inoculated with 9a5c-8 or 9a5c-51 (Fig. 2). Immunofluorescence, however, showed that the number of xylem vessels containing green-fluorescing bacteria was higher in the plants infected with 9a5c-8 (Fig. 3A) than with 9a5c-51 (Fig. 3B). No green fluorescing material was observed in the xylem tissue of mock inoculated plants (Fig. 3C).

Six months post-inoculation, the infected plants were highly stunted. Young leaves in the apical shoots were highly deformed with rippled veins. Plants have been maintained in the culture room for longer than 12 months, but some plants wilted and died. This wilting occurs very late and is different from that reported for the PW strain (18). In addition, the PW strain has not been reported to induce young leaf deformation (18).

X. fastidiosa was reisolated by plating xylem sap extracted from plants inoculated with either 9a5c-8 or 9a5c-51 on PW-agar plates. Slow-growing bacterial colonies characteristic of *X. fastidiosa* were observed 13 days after plating. The colonies gave positive PCR reactions with the primers CVC1 × 272-2 *int* and were recognized on immunoblots by the specific polyclonal antibody.

X. fastidiosa was recently isolated from naturally infected, diseased plants of *C. roseus* in Brazil showing stunting, dieback symptoms but no chlorosis (27). These symptoms differ from those described here.

The origin of the strain of *X. fastidiosa* infecting citrus is still unknown in Brazil. Alternative hosts of *X. fastidiosa* could act as sources for insect vector acquisition. Whether *C. roseus* and other weed hosts make a significant contribution to the incidence of CVC in Brazil has not been established. Genetic diversity analysis of *X. fastidiosa* isolated from *C. roseus* and other hosts would provide a clue for the mechanism of spread of CVC in Brazil. This information would be useful to forecast and potentially control the spread of this pathogen.

C. roseus is a symptomatic host plant for the CVC strain of *X. fastidiosa*. *C. roseus* should be a convenient experimental host plant, since symptoms develop in 100% of infected plants which was not reported to be accomplished with mechanical inoculated citrus. In addition, the symptoms develop faster in *X. fastidiosa* infected *C. roseus* than in sweet orange seedlings (16). Pathogenicity tests in *C. roseus* plants should be useful for the screening of non-pathogenic mutants of CVC-*X. fastidiosa*.

Pattern of gene expression in *C. roseus* infected with *X. fastidiosa* CVC strain. The technique of differential display can be used to study how expression of plant genes is affected by infection with a given pathogen. In this way, several *C. roseus* cDNAs were identified which are differentially expressed following infection by the phloem restricted mollicutes *Spiroplasma citri* or the stolbur phytoplasma (14, Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., Danet, J. L., Bové, J. M., and Garnier, M., *unpublished data*).

Four of these cDNAs were used as probes in northern blot analysis to test the expression of the corresponding genes in *C. roseus* plants, 4 months post-inoculation with *X. fastidiosa* strain 9a5c. By sequence comparisons, these genes were shown to correspond respectively to a sterol-C-meth-

yltransferase, a transketolase-like protein, subunity III of the photosystem I and a desiccation protectant protein. All four genes were down regulated following infection by *S. citri* or the stolbur phytoplasma (14, Jagoueix-Eveillard, S., Tarendeau, F., Guolter, K., Danet, J. L., Bové, J. M., and Garnier, M., unpublished).

In the case of the plants infected with *X. fastidiosa* 9a5c, as shown on Fig. 4, the gene coding for sterol-C-methyltransferase (gene Sc I) was found to be repressed, the genes coding for transketolase (gene Sc Q) and subunity III of photosystem I (gene St C2) were down regulated, while the gene coding for the desiccation protectant protein (gene Sc A11-1) was up-regulated. The down regulation of gene Sc I involved in sterol synthesis and genes Sc Q and St C2 involved in sugar transport and photosynthesis respectively, could be related to the symptoms of stunting and chlorosis often observed on periwinkle plants infected with vascular restricted pathogens. The up-regulation of the Sc A11-1 gene could be the result of the water stress induced by *X. fastidiosa* in the plants. The kinetics of the expression of the genes Sc I, Sc Q, St C2 and Sc A11-1 will be further evaluated in *C. roseus* plants infected with *X. fastidiosa* in order to establish the correlation of their expression patterns with the development of disease symptoms.

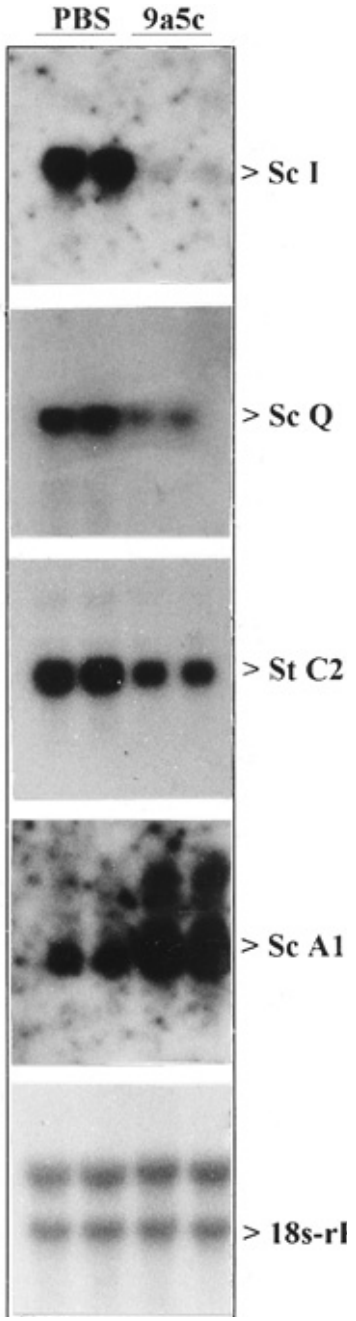

Fig. 4. Northern blot analysis of the expression of the host genes Sc I, Sc Q, St C2 and Sc A11-1 in healthy (phosphate-buffered saline-mock inoculated) or diseased *Catharanthus roseus* plants infected with 9a5c-8. Hybridization with 18s-rRNA probe indicates that the samples were equally loaded.

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