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Study of the expression of defense related protein genes in stolbur C and stolbur PO phytoplasma-infected tomato

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Abstract

Phytoplasma are phloem-restricted plant pathogenic bacteria that cause hundreds of diseases. They are not cultivated *in vitro* and can be transmitted through insect vectors or grafting. Researches have been investigating defense pathways to different pathogens (bacteria, fungi...) but little is known about defense pathways activated in phytoplasma infected plants. In this study, the expression of genes related to salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) pathways have been investigated in stolbur C phytoplasma and stolbur PO phytoplasma-infected tomato. We noted elevated level of expression for Pathogenesis Related (PR) genes. Transcription of SA and ET marker genes was up-regulated in stolbur PO and stolbur C-infected tomato, while transcription of PIN2, a gene regulated by JA, increased only in stolbur C-infected tomato.

Key words: phytoplasma, PR protein genes, aalicylic acid, jasmonic acid, RT-PCR, gene expression.

Introduction

Phytoplasmas are plant pathogenic wall-less bacteria restricted to phloem sieve tubes. They induce plant disorders such as leaf yellowing, growth aberrations and flower malformations. The pathogenicity mechanism of these bacteria are yet not well understood. Recent data revealed that phytoplasmas effectors can induce specific symptoms (Zhang *et al*., 2004, Hoshi *et al*., 2009). However, it is not known if the plant defense pathways are activated in response to the infection. The three major plant defense pathways involve salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Pieterse *et al*., 2001). Here, the expression of marker genes of these pathways was studied in tomato infected with two different isolates of stolbur phytoplasma. In spite of similar multiplication rates in plant, these two isolates, ie stolbur C and PO, induce distinct symptoms. Flowers from stolbur PO-infected plants show hypertrophied sepals, aborted stamens and petals. This is never observed in tomato infected with stolbur C isolate. The activation of the different defense pathways was examined in stolbur C and PO-infected plants. SA dependent pathway was found to be activated in infected tomato, while JA pathway seemed to be activated only in stolbur C-infected tomato.

Materials and methods

Tomato (*Solanum lycopersicum* cv Ailsa Craig) plants were infected with the stolbur phytoplasma by grafting. RNA were extracted from tomato leaves with Tri-reagent (SIGMA) and treated with DNase, following the supplier protocol.

Semi-quantitative RT-PCR was done with primers specific for each studied gene. For each tube of RT, 1 µg of DNA, 0.55 µM of an oligodT18, 0.01 M DTT, 2.2 mM dNTP, 280 U of Rnase OUT were mixed in a final volume of 19 µl. After 5 min at 65°C and 5 min at 45°C, 1 µl (200 U) of Superscript II reverse transcriptase (Invitrogen) was added and incubated for 1 hour. Five min at 75°C allowed the enzyme denaturation. PCR were done with 1 µl of cDNA, 0.35 mM $MgCl_2$, 0.9 mM dNTP, 2 µg/ml BSA, 4.4 µM each primer (3' and 5') and 2.2 U *Taq* DNA polymerase (Promega), in a final volume of 25 µl. Hybridization temperature depends on primers used.

RT-PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The intensity of the bands was measured with a FluorS and the associated software Quantity One (Biorad).

The cDNA obtained after reverse transcription were diluted according to 100 ng/µl. Quantitative real-time RT-PCR SYBRgreen assays were performed on a Roche Light Cycler480. Each reaction tube contained 1X SYBRgreen Fluorescein Mix (Applied Biosystem, Foster city,USA), 250 nM of each primer and 1 µl of RT-PCR product in a final volume of 25 µl. The thermal cycling programme was: 95C° for 15 min, 45 cycles with 95°C for 20 seconds, annealing temperature according to primer tested for 40 seconds, 72°C for 40 seconds. This was followed by a melt curve programme: 95°C for 30 seconds for 1 cycle, 60°C for 10 seconds for 1 cycle, melting step from 60°C to 100°C for 10 seconds for 80 cycles (step of 0.5°C) and final cycle of 72°C for 10 min. All samples were amplified in triplicate from four distinct RNA batches extracted from four distinct tomato samples.

Efficiency of the primer pairs was determined using dilution of the RT-PCR product (pure-1/10-1/100-1/1000-1/10000) and using the Light-Cycler480 software.

Method of calculation:

$$\text{Relative Gene Expression} = \frac{\text{Efficiency}^{(\text{Ct healthy} - \text{Ct infected})}\ (\text{tested gene})}{\text{Efficiency}^{(\text{Ct healthy} - \text{Ct infected})}\ (\text{control gene})}$$

Phytoplasma detection was carried out also by nested PCR. Primers used were R16mF2/R16mR1 and R16F2n/R16R, yielding a 1250 bp DNA fragment (Gundersen and Lee, 1996). First amplification was as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 60°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. An aliquot (1/40) of the first PCR product was used for the nested amplification. Cycles are as follow: 94°C 4 minutes, 35 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute and 30 seconds, with an extension at 72°C for 10 minutes. Eight µl of product were analyzed on agarose gel and quantified using Quantity One software (Biorad).

Results and discussion

Leaves were collected on healthy tomato plants or on tomato plants infected with stolbur C or stolbur PO phytoplasma. After RNA extraction, the infection was verified by nested RT-PCR with primers U3-U5. Stolbur C and stolbur PO phytoplasmas were detected in leaves of infected tomato at similar levels.

Q-RT-PCR specific to defense genes from SA, ET and JA pathways were carried out. Ten genes were tested: PR1a, PR1b, PR2a, PR2b, PR5, PR10, LOXD, PIN2, PAL, CHS2, that includes genes encoding enzymes for the biosynthesis of SA and JA, transcription factor and Pathogenesis Related protein genes (PR). Each expression was tested on stolbur C and stolbur PO-infected plants. The up- or down-regulation was estimated with the relative gene expression calculation. Examples of data are shown in figure 1. Increased transcripts levels for SA/ET pathways marker genes were observed in stolbur C and stolbur PO-infected tomato (table 1). Wound inducible gene PIN2 regulated by methyl jasmonate was up-regulated in stolbur C tomato leaves and down-regulated in stolbur PO-infected tomato leaves. Same results were observed for the phenylalanine ammonia-lyase gene PAL. Conversely, chalcone synthase gene CHS2 was activated in stolbur PO tomato and repressed in stolbur C tomato leaves.

These results were confirmed from semi-quantitative RT-PCR experiments done with the same RNA preparations.

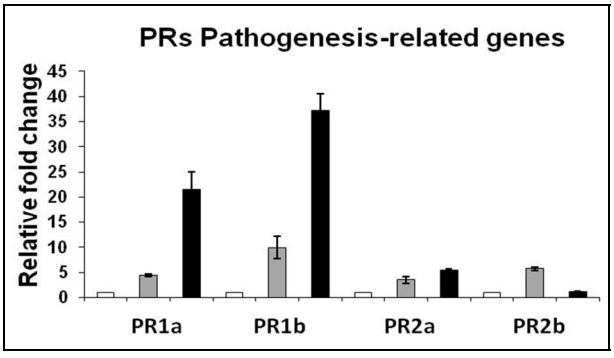

Figure 1. Histogram representing the relative gene expression of PR1a, PR1b, PR2a and PR2b genes, in healthy (white), stolbur C (grey) and stolbur PO (black) infected tomato leaves.

Table 1. Relative gene expression of 10 genes compared with those in healthy plants. RNA lots extracted from stolbur C (C) or stolbur PO-infected tomato leaves (PO).

Genes	RNA lot 1		RNA lot 2		RNA lot 3	
	C	PO	C	PO	C	PO
PR1ac	4,42	21,50	6,54	56,50	1,42	1,98
PR1bas	9,92	37,20	9,05	73,80	1,91	3,12
PR2ac	3,50	5,44	6,94	5,50	0,78	0,46
PR2bas	4,30	0,60	1,98	1,07	0,75	0,03
PR5	5,04	18,40	13,70	39,00	0,92	3,41
PR10	1,84	11,90	2,24	2,95	2,04	1,73
LOXD	1,68	0,07	0,31	0,07	0,85	0,58
PIN2	1,98	0,67	1,58	0,06	2,78	0,13
PAL	1,60	0,05	1,96	0,01	2,75	0,01
CHS2	0,28	6,54	0,19	1,54	0,50	2,25

Conclusions

Stolbur C and PO phytoplasma induced different symptoms on tomato leaves and flowers. They also induced distinct patterns of defense gene expression. SA and ET dependent defense pathways were activated in stolbur PO-infected tomato indicated by the high level of expression of SA/ET related genes. In turn, PIN2 (JA pathway) was repressed. In stolbur C-infected tomato, both SA/ET as well as JA pathways were activated indicated by increased transcription level of PIN2.

The role of these defense pathways on the development of the phytoplasma infection will be further investigated by studying the effect of pre-activation of these pathways on the phytoplasma infection.

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