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Gene expression analysis and cytochemical investigations in '*Candidatus* Phytoplasma mali'-resistant and -susceptible *Malus* genotypes grown *in vitro*

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Abstract

A gene expression study was carried out in '*Candidatus* Phytoplasma mali'-resistant and -susceptible apple genotypes infected with '*Ca*. P. mali'. Genes involved in the general response against stress and pathogens were found to be differentially expressed in the '*Ca*. P. mali' resistant wild genotype *Malus sieboldii*. Furthermore, cellular modifications and ultrastructural features were investigated through cytochemical analysis performed on healthy and '*Ca.* P. mali'-infected *in vitro* plants of *Malus sieboldii* and the '*Ca.* P. mali'-susceptible *Malus* x *domestica* cv. Golden Delicious. Preliminary results showed that while the cellular organisation in the susceptible genotype was deeply affected by the presence of '*Ca.* P. mali' only few localised structural modifications were observed in the resistant genotype.

Key words: '*Candidatus* Phytoplasma mali', cytochemical analysis, gene expression, TEM.

Introduction

Apple proliferation (AP) disease is the most important graft-transmissible and vector-borne disease of apple in Europe and is caused by '*Candidatus* Phytoplasma mali'. Genetic resistance against '*Ca*. P. mali' was observed in the wild genotype *Malus sieboldii* (MS) and in few hybrids derived from the crossings of MS with the '*Ca*. P. mali'-susceptible *Malus x domestica* (Kartte and Seemüller, 1991). So far, the resistance mechanism against '*Ca*. P. mali' is still poorly understood and only little information is available. A form of induced resistance, characterised by the stable remission of AP symptoms and disappearance of the phytoplasma from the canopy, has been observed in old susceptible apple trees known to be infected since several years (Musetti *et al.*, 2004). This phenomenon called "recovery" seems to occur stochastically while the resistance in MS was shown to be a reproducible and inheritable trait (Bisognin *et al.*, 2008a; Seemüller *et al.*, 2008; Jarausch *et al.*, 2010). Recently, cytochemical studies showed that in recovered plants there is a localised production of reactive oxygen species (ROS) with structural changes in the intracellular composition of the phloem tissue (Musetti *et al.*, 2010). However, in both resistance and "recovery" the mechanisms that act in the plant response against the pathogen are still largely unknown.

In this study, a gene expression analysis was carried out through real time quantitative RT-PCR (qRT-PCR) on differentially expressed genes previously individuated through cDNA-Amplified Fragment Polymorphism (cDNA-AFLP) (Moser *et al*., 2007). Moreover, in order to investigate if resistance in MS could be characterised by features similar to that observed in the "recovery" phenomenon a cytochemical analysis was performed in healthy and '*Ca*. P. mali'-infected *in vitro* plants.

Materials and methods

Plant material

Healthy and infected plants were those described in Bisognin *et al.* (2008b). Micropropagation was done as described in Ciccotti *et al.* (2008). The genotypes used in this study were the '*Ca*. P. mali'-susceptible *Malus* x *domestica* cv. Golden Delicious and the '*Ca*. P. mali'-resistant *Malus sieboldii*.

RNA extraction

Total RNA of the *in vitro* plants was extracted according to the protocol described in Moser *et al.* (2004).

Gene expression analysis

The gene expression level was measured through reverse transcription quantitative PCR using the Invitrogen One-step qRT-PCR kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The reactions were performed on a Chromo4 instrument (Biorad, Germany). The data analysis was performed as described in Peirson *et al.* (2003).

Cytochemical analysis

The material was collected from healthy and '*Ca*. P. mali'-infected *in vitro* plants and treated as described by Musetti *et al.* (2004). Visualization of the samples was performed under a Transmission Electron Microscope (Philips CM10, Eindhoven, The Netherlands) operating at 80 kV.

Results

In a previous work (Moser *et al*., 2007), several differentially expressed genes were individuated through cDNA-AFLP analysis on healthy and '*Ca*. P. mali'-infected *in vitro* plants. Based on their putative function a subgroup of genes related to stress and pathogen response was chosen for the real time qRT-PCR analysis. Among these targets, the expression levels of three genes putatively involved in electron transport and H_2O_2 production and signaling were found to be differentially regulated in the '*Ca*. P. mali'-resistant MS but not in the susceptible Golden Delicious (GD). In order to gain more information on the type of response a cytochemical analysis was carried out on the *in vitro* material to investigate the infected plants at a cellular level. The comparison between healthy and '*Ca*. P. mali'-infected MS and GD showed that while the cellular organisation in the susceptible genotype was deeply affected by the presence of '*Ca.* P. mali' in the resistant genotypes only few limited structural modifications were observed. Interestingly, by comparison of healthy tissues from both resistant and susceptible *in vitro* apple plants, it appeared that the lumen of the cell was reduced in different sieve tube members by nacreous layers. In addition, sieve plates tended to present callose accumulation, and vacuolar phenolics were present in the parenchymal cells.

Discussion

In this study we adopted an integrated approach. Our objective was to verify the indications obtained from the analysis at a molecular level with an analysis at a cellular level. The study of the gene expression in '*Ca*. P. mali'-resistant and –susceptible genotypes after the infection with '*Ca*. P. mali' showed that there is a broad range of metabolisms that are affected by the phytoplasma presence. In the resistant genotype it seems that a general response against stress and pathogen is activated. This resembles a type of reaction that was also observed in the "recovery" phenomenon. In order to gain more information on ultrastructural features and modifications at cellular level a cytochemical analysis was performed. Preliminary results on healthy and '*Ca*. P. mali'-infected MS and GD *in vitro* plants showed that in GD there is a dramatic change in the cellular organisation while in MS this was localised to very few cells. Structural features of MS phloem cells, already evident in the healthy tissue, could be correlated to the ability of the genotype to contain the phytoplasma spread.

So far, the indications obtained from the gene expression analysis were not confirmed by the cytochemical analysis of the phloem tissues. Further investigation will be necessary to better integrate the two approaches. Nevertheless, the data obtained from the TEM analysis showed once more the power of this technique in plant-pathogen interaction studies.

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