Identification of host genes potentially implicated in the *Malus pumila* and 'Candidatus *Phytoplasma mali*' interactions

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Abstract

Two'Candidatus *Phytoplasma mali*' strains (AP and AT), were studied in experimentally infected apple trees to analyze transcriptional profiles during interaction with phytoplasmas. Three groups of sample combinations were employed: healthy - infected, symptomatic - non-symptomatic, and AP-infected - AT-infected sample. The majority of genes were differently expressed between healthy and infected samples. Changes in gene expression involved a wide spectrum of biological functions, including processes of metabolism, cell defence, photosynthesis, transport, transcription, signal transduction and protein synthesis. The possible effect of phytoplasma infection on these processes and their relationships with disease development, symptom appearance and possible plant defence system is discussed.

Keywords: Apple, phytoplasmas, 'Ca. *P. mali*', gene expression, transcriptome.

Introduction

Phytoplasmas are cell wall-less and phloem-restricted plant pathogenic bacteria. They are known to be associated with diseases in several hundred plant species and have been shown to be transmitted in a propagative manner by sap-sucking insect vectors (Aldaghi et al., 2005). Apple proliferation (AP) is one of the most serious phytoplasma diseases of apple trees in Europe: it is caused by 'Candidatus *Phytoplasma mali*', belonging to the apple proliferation group; this disease causes considerable economic losses mainly by decreasing the size and quality of fruits (Frisinghelli et al., 2000).

Little is known about the genes involved in the phytoplasma-plant host interaction. There is only some information about the increase of phenolic compounds and hydrogen peroxide in host plants infected by phytoplasmas (Musetti et al., 2000; 2004; Junqueira et al., 2004). For more than 10 years, scientists worked toward the development and improvement of methods to study gene expression regulation. Transcriptome analysis is a common way of discovering differences in gene expression because regulation of gene activity occurs primarily at a transcription level. Whether particular genes are over- or underexpressed when comparing diseased with normal tissue provides information with respect to the understanding of the mechanisms of the disease. To date, a number of methods have been successfully developed to identify differential gene expression in various biological systems, including DDRT-PCR, cDNA-AFLP and microarray (Frolov et al., 2003; Venkatesh et al., 2005). cDNA-AFLP is a comprehensive transcript profiling methodology (Donson et al., 2002) for genome-wide expression analysis that does not require any prior knowledge of gene sequences. This PCR-based technique combines the feature of high-throughput with a high sensitivity and specificity, allowing detection of rarely expressed genes and distinguishing between homologous genes (Reijans et al., 2003). The aim of the present research is to study the gene expression differentially regulated by phytoplasma in infected host plant (apple) during compatible interaction with 'Ca. *P. mali*'.

Materials and methods

Biological materials and RNA extraction: Apple AP-infected scions by two different strains of 'Ca. *P. mali*' (AP-N17 and AT2-SO8D) were grafted on healthy apple trees (MM106) in an insect-proof greenhouse. Fifty mg of whole plant tissues from healthy, AP-symptomatic, AP-nonsymptomatic and AT2-symptomatic samples of apple trees maintained in a greenhouse were used for RNA extraction, ground with liquid N_2, and processed with Invisorb® spin plant RNA mini kit (Invitek GmbH, Berlin, Germany) following the manufacturer's instructions. DNA-free™ kit (Ambion Inc., Austin, Texas, USA) was used to eliminate genomic DNA carry-over in RNA samples, according to the manufacturer's instructions.

Transcriptional profile: Double-stranded cDNA was synthesized from 5-10 µg of total RNA according to the instructions for the Superscript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) using oligo dT_{12-18}-contaning primers. Double-stranded cDNA was then incubated with *Eco*RI and *Mse*I for 2 h at 37 °C, according to the instructions of the AFLP® Analysis System I (Invitrogen, Carlsbad, CA, USA). The products were subjected to pre-amplification with combinations of *Eco* and *Mse* primers. After dilution of the PCR fragments, selective amplifications were carried out with combinations of an *Eco* primer and an *Mse* primer containing two or three selective bases at the 3' end following the instructions of the AFLP® Analysis System I kit. Amplification products were separated in a vertical denaturing polyacrylamide gel (6 %).

Interesting cDNA-AFLP fragments were recovered as described previously (Dellagi et al., 2000; Campalans et al., 2001) and amplified with the selective primers used to generate the corresponding cDNA-AFLP profile. The purified cDNAs were directly processed to a sequencing reaction, and fragments without clear alignment in direct sequencing were cloned. Alignment and homology of obtained sequences was then carried out, and the identified protein sequences used in queries against the UniProt database (http://www.expasy.org/cgi-bin/sprot-search-de).

Evaluation of gene differentially expression by real-time RT-PCR: Actin, GAPDH, ef1-α and 18S rRNA housekeeping genes were selected as reference genes to normalize the result of real-time RT-PCR. The expression stability for these reference genes was evaluated using the geNorm software program (Vandesompele et al., 2002b).

After new RNA extraction for each sample type (healthy, symptomatic and non-symptomatic infected plants) and elimination of DNA carry-over, cDNA synthesis was carried out on approximately 1.5 µg of the total RNA solution with the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{20}$ primers. Specific primers were designed for all sequenced cDNAs (Primer Express V.5, PE Applied Biosystems, Forster City, USA). Real-time PCR quantification of fragments was performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, USA) in 25 µl volumes containing 1× qPCR MasterMix plus for SYBR green I, 200 µM of each primer, and 5 µl of cDNA (0.5 ng/µl). The thermal cycle consisted of 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

PCR efficiency for each primer pair were determined according to Ramakers et al. (2003) due to $E=10^{slope}$ formula. Expression levels were determined as the number of cycles (C_t) needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (Walker, 2002). For each repetition of samples in every run, the level of gene expression was normalized to that of GAPDH and actin, separately, by means of "C_t of target – C_t of reference gene" formula namely ΔC_t. Individual ΔC_t values were further subjected to the SAS V9.1 (SAS Institute Inc., Cary, NC, USA) by means of three-way analysis of variance (completely hierarchical) model using multiple comparison of means. Statistical significance level was 0.05. The relative expression ratio (R) of each target gene between infected (symptomatic or non-symptomatic) and healthy samples or between symptomatic and non-symptomatic samples was calculated by means of the $\Delta\Delta C_t$ method described by Applied Biosystems ($R = 2^{-\Delta\Delta Ct}$). To test the similarity of two methods (cDNA-AFLP and real-time RT-PCR), the Chi-Square test of independence (SAS V9.1) was performed on their data.

Results

Transcriptional profile: The 20 primer pairs used in cDNA-AFLP for cDNA amplification amplified ~8,000 fragments. Of these, 491 (6.1 %) transcript derived fragments (TDF) were differentially expressed among healthy, symptomatic and non-symptomatic infected samples (Figure 1). The fragments differentially expressed were classified into 3 groups (healthy versus infected samples; symptomatic versus non-symptomatic; and AP- versus AT2-infected sample) (Table 1). 34.2 % of the differentially expressed bands were common among the three groups of samples tested and were differentially regulated in the three groups under comparison. Interestingly, more than 50 % of the fragments were differentially regulated between symptomatic and non-symptomatic samples. The majority of fragments (95.3 %) were differently expressed between healthy and infected samples. Among 491 TDFs, 66 bands that clearly showed differences between two or more samples were selected for further analyses. After isolation from gel and re-amplification, the sequences of 45 out of 66 fragments were obtained by direct or cloning-sequencing and 27 of them showed significant similarities to different plant genes present in the databases, while 18 had no matches (novel genes). With the information from UniProt Knowledgebase, putative functions were assigned to 18 out of 27 fragments (others were related to unknown or hypothetical proteins).

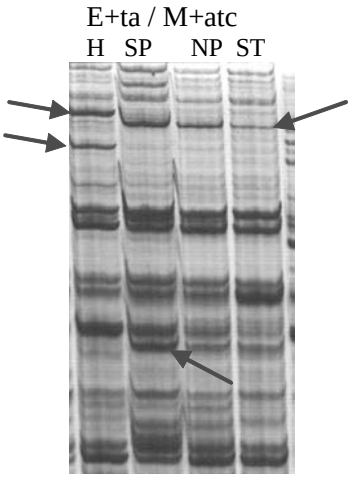

Fig. 1 A profile of cDNA-AFLP obtained by a primer pair [*Eco* (E) / *Mse* (M) primers + added nucleotides] for healthy (H), symptomatic AP-infected (SP), non-symptomatic AP-infected (NP) and symptomatic AT2-infected (ST) samples. Arrows show bands differentially regulated among samples.

Tab. 1 Comparison of cDNA-AFLP gene expression between different combinations of samples, and number of fragments for which the expression patterns were confirmed by real-time RT-PCR.

cDNA-AFLP gene expression comparison between	**Number of fragments differentially expressed**
Healthy sample and infected samples (Group 1)	468 (95.3 %)*
Symptomatic and non-symptomatic samples (group 2)	252 (51.3 %)*
AP-infected sample and AT2-infected sample (group 3)	149 (30.3 %)*
Confirmation of cDNA-AFLP data by real-time PCR	**Number of confirmed fragments**
Fragments confirmed by both GAPDH and actin	11 among 18 known genes
Fragments confirmed by both GAPDH and actin	7 among 25 unknown, hypothetical or novel genes
Fragments confirmed only by GAPDH	6 among 25 unknown, hypothetical or novel genes
Total confirmation	24 fragments out of 43 (56%)

*: percentage of differentially expressed genes in each group to total differentially expressed genes (491).

Comparison of cDNA-AFLP and real-time RT-PCR results: By geNorm analysis, GAPDH and actin were respectively evaluated as the most suitable reference genes to normalize the amounts of starting cDNA in real-time RT-PCR; so, GAPDH was chosen as the principal reference gene and actin as the second in line with the cDNA-AFLP results. Among the sequenced 45 TDFs, no primer combination was designed for two fragments (novel genes), and real-time RT-PCR analyses were carried out for 43 remaining TDFs identified by cDNA-AFLP. For the same PCR run, efficiencies of amplification obtained for normalization genes and each interested genes were never significantly different ($P<0.05$); making the interpreted normalized expression ratios reliable and accurate. In real-time RT-PCR, statistical analyses comparing ΔC_t values showed that using GAPDH and actin, respectively, 27 and 28 out of 43 TDFs were significantly ($P<0.05$) differentially expressed between healthy, symptomatic and/or non-symptomatic samples. Also, the Chi-Square test of independence showed 60 and 42 % of concordance between the two methods (cDNA-AFLP and real-time RT-PCR) by GAPDH and actin, respectively (data not shown). On the other hand, among 18 differentially expressed genes with known function, real-time RT-PCR confirmed the expression pattern of eleven TDFs by both reference genes. Besides, seven, and six TDFs among 25 unknown, hypothetical or novel genes (without similarity) confirmed their deregulated expression profile, respectively, by both reference genes and only GAPDH (Table 1).

Discussion

Using the cDNA-AFLP technique, a series of plant genes whose expression is altered during phytoplasma infection was identified. Some of differentially-expressed fragments did not show any similarity with sequences in the databases, representing therefore potential novel proteins important in interaction that may be related to specific plant responses to

phytoplasmas. Based on the function of each identified gene, a relationship between these genes and their role in pathogen-host interaction is proposed. In the model, the identified genes are classified into three groups to interpret the role of identified genes in symptom expression and regulation of plant responses to phytoplasma infection:

i) Genes related to photosynthesis pathways. Two genes related to photosystems I and II and a gene coding for fructose-1,6-bisphosphatase that were down-regulated only in the symptomatic part of plants were characterized. Underexpression of these photosynthesis genes is probably due to carbohydrate accumulation in infected (symptomatic) leaves (Maust et al., 2003). So, 'Ca. *P. mali*' infection has a detrimental effect on photosynthesis, and the deregulation of key genes in photosynthesis could contribute to symptom expression.

ii) Genes involved in symptom expression. Down-regulation of cell wall-associated hydrolase induces proliferation symptoms since this enzyme is involved in cell wall disassembly; necessary for elongation (Hernandez-Nistal et al., 2006). Moreover, the down-regulation of auxin efflux carrier in this study causes accumulation of auxin in certain cells. The auxins have been implicated in growth, morphology (Brown et al., 2001), and also in apical dominance (Hoshi et al., 2009). So, under-expression of an auxin-transport related gene within MM106 plants inhibits apical dominance, consequently, inducing specific phytoplasma symptoms (proliferation). The only gene which was over-expressed in this group was the one coding for no apical meristem (NAM). Up-regulation of this protein in symptomatic parts of infected plants induces adventitious shoots and proliferation symptoms. On the other hand, the family to which this protein belongs is induced by auxin (Ooka et al., 2003). This means that the accumulation of auxin in certain cells increases the expression of NAM in the same cells. Together, these gene deregulations stimulate the appearance of the specific symptoms of apple proliferation disease.

iii) Genes involved in plant defence mechanisms. Anthranilate hydroxycinnamoyl benzoyltransferase catalyses the first committed reaction of phytoalexin biosynthesis (Yang et al., 1997). Consequently, down-regulation of this enzyme is responsible for the reduction of phytoalexins and high phytoplasma titres in this susceptible host. Also, under-expression of universal stress protein (Usp) increases susceptibility of the host to phytoplasmas as stress agents. On the other hand, the expression of cellular retinaldehyde-binding protein with a potential antimicrobial activity (Molina et al., 1993) or protection property against stresses (Kearns et al., 1998) is reduced in the infected MM106 apples. Together, the plant defence mechanism is thus repressed and higher susceptibility of the host (MM106) and increased multiplication of pathogen occurs.

Taken together, cDNA-AFLP analysis showed that the plant gene expression is modulated in apple in response to phytoplasma colonisation. These results provide a valuable first step towards the understanding of the 'Ca. *P. mali*'–apple interaction. Except for the effect of phytoplasma on photosynthesis, other pathways identified in the current study are presented for first time as target pathways of phytoplasma infection in plants. However, the expression level of the identified genes must be compared in susceptible and resistant (or tolerant) varieties or genotypes in order to find molecular markers and genes important in resistance or tolerance.

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