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cDNA-AFLP analysis of gene expression changes in apple trees induced by phytoplasma infection during compatible interaction

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Abstract In order to gain insight into molecular and physiological changes in apple trees during compatible interaction with two '*Candidatus* Phytoplasma mali' strains (AP and AT), cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) technique was used. A rootstock of apple (MM106) susceptible to '*Ca.* P. mali' was used to extend the range of the potential host responses by the maximum number of identified genes that will be deregulated by phytoplasma in apple. Gene expression comparisons were studied in three directions: healthy versus infected samples, symptomatic versus non-symptomatic sample, and AP-infected versus AT-infected sample. Forty-five genes whose steady-state levels of expression significantly changed in response to phytoplasma infection were identified. Among their partial cDNA sequences, only 27 showed similarity to DNA or protein data bases; of these, 18 were related to known genes in plants, and the rest were related to unknown or hypothetical proteins. Eighteen out of 45 did not show any similarity with sequences in data bases (potential novel genes). Quantitative real-time RT-PCR (qRT-PCR) was used to confirm differential expression of AFLP identified genes, and showed the similar profile expression for 11 known genes among 18, and for 13 unknown, hypothetical or novel genes among 27. Changes in gene expression involved a wide spectrum of biological functions, including processes of metabolism, cell defence, senescence, photosynthesis, transport, transcription, signal transduction and protein synthesis. This is the first study of global gene profiling in plants in response to phytoplasma infections using cDNA-AFLP, and a model is proposed to explain the mode of action of the '*Ca.* P. mali' in apple.

Keywords Apple · '*Ca.* P. mali' · cDNA-AFLP · Gene expression · Interaction · qRT-PCR

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Introduction

Phytoplasmas are obligate parasites of plant phloem tissues that have diverged from gram-positive eubacteria, and belong to the '*Candidatus* Phytoplasma' genus within the class *Mollicutes* (IRPCM 2004).

They cannot be cultured *in vitro* in cell-free media and this makes slow progress in their study.

Plants infected by phytoplasma exhibit an array of symptoms including virescence/phyllody, proliferation and stunting, that suggests profound disturbances in the normal balance of growth regulators. Phytoplasma diseases occur worldwide; very often associated with economically important plant diseases including coconut lethal yellowing, peach X-disease, and apple proliferation (AP) (Bertaccini 2007).

AP is one of the most serious diseases of apple trees in Europe. It is caused by '*Candidatus* Phytoplasma mali' belonging to the apple proliferation ribosomal group (Seemüller et al. 1998b). AP disease potentially causes considerable economic losses by decreasing size and quality of fruits (Loi et al. 1995; Seemüller et al. 1998a; Frisinghelli et al. 2000). The most typical symptom is witches' broom at the end of shoots; also leaves are generally smaller and more dented, with unusually enlarged stipules (EPPO/CABI 2006).

At the present, little is known about the genes involved in the phytoplasma-plant host interaction that can lead to production of defence proteins, increase of phenolic compounds and hydrogen peroxide overproduction in host plants (Musetti et al. 2000, 2004; Junqueira et al. 2004). The host gene expression in *Catharanthus roseus* after challenge with three different mollicutes, '*Ca.* P. aurantifolia', stolbur phytoplasma and *Spiroplasma citri*, was investigated by differential display RT-PCR, and 24 genes were identified as deregulated (Jagoueix-Eveillard et al. 2001). However, only eight of them had homologies with known proteins in the GenBank, *e.g.* the genes coding for proteins involved in photosynthesis, sugar transport, and response to stress. By the same techinque, Carginale et al. (2004) found only four genes that were differentially expressed in *Prunus armeniaca* infected by phytoplasma. Using semi-quantitative RT-PCR and *in situ* RNA hybridization, Pracros et al. (2006) found some floral development genes to be differentially regulated in a tomato–stolbur phytoplasma interaction.

To date, a number of methods have been successfully developed to identify differential gene expression in various biological systems (Frolov et al. 2003; Venkatesh et al. 2005), however the cDNA-AFLP analysis 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 technique allows detecting rarely expressed genes and distinguishing between homologous genes (Reijans et al. 2003). The weak point of cDNA-AFLP is the presence of false positives (Lievens et al. 2001); for which reason it is necessary to confirm the obtained results by another approach such as real time RT-PCR (Mills et al. 2001).

The aim of the present research is to study the gene expression differentially regulated by phytoplasma in infected host plant (apple) by means of the cDNA-AFLP technique. In other words, the goal of the current study was the analysis of transcriptional profiles of *Malus pumila* (rootstock: MM106) during compatible interaction with '*Ca.* P. mali' in order to gain insight into molecular and physiological changes in diseased plants. After confirmation of cDNA-AFLP results by quantitative real-time RT-PCR (qRT-PCR), a model is proposed to interpret roles of the confirmed genes in apple-phytoplasma interaction.

Materials and methods

Plant materials and phytoplasma strains

Apple AP-infected scion woods were provided by Dr. B. Pradier [Station de Quarantaine des Ligneux (Lempdes, France)], and were grafted on MM106 apple rootstocks (very susceptible to '*Ca.* P. mali'). The two strains of '*Ca.* P. mali' used (infected scions) were AP-N17 and AT2-SO8D. All inoculation experiments were carried out in March-April and inoculated plants were maintained in an insect-proof and climatized-quarantine greenhouse (14 h light, 20–25°C, and high relative humidity [only for 2 weeks after grafting]).

Total RNA extraction and cDNA-AFLP procedure

Four different samples were employed: healthy, AP-symptomatic, AP-non-symptomatic and AT2-symptomatic from the trees maintained in greenhouse for 15 months after grafting. Fifty mg of fresh leaf tissues (from minimum four leaves) was used for each RNA sample preparation. Plant material was ground to a fine powder with liquid N_2, and then 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.

Double-stranded cDNA was synthesized from 5 to 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}-containing primers. For digestion, double-stranded cDNA (400 ng) was incubated with 3U each of *Eco*RI and *Mse*I for 2 h at 37°C. After ligation with *Eco*RI and *Mse*I adaptors, the products were used as primary templates for pre-amplification with combinations of *Eco* (5′-gactgcgtaccaattc-3′) and *Mse* (5′-gatgagtcctgagtaa-3′) primers following the instructions of the AFLP® Analysis System I kit (Invitrogen, Carlsbad, CA, USA).

After 10-fold 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. The *Eco* primers were labelled with [γ-^{33}P] dATP. Amplification products were separated by electrophoresis at 100 W on a vertical denaturing polyacrylamide gel (6%) containing 7 M urea. Gels were dried on Whatman 3MM paper before auto-radiography.

Isolation of cDNA fragments, cloning, sequencing and bioinformatics

cDNA-AFLP fragments of interest were recovered as described previously (Campalans et al. 2001), and amplified by PCR with the selective primers used to generate the corresponding cDNA-AFLP profile. PCR product presence and quality was verified on 1.2% agarose gel, subsequently purified using QIAEX II gel extraction kit (Qiagen, Venlo, The Netherlands), and directly processed to sequencing reaction. Only the fragments without clear alignment in direct sequencing were cloned. These fragments were ligated to pCR® 2.1 and cloned into *Escherichia coli* cells according to the TA cloning® kit (Invitrogen, Carlsbad, CA, USA) instructions. The plasmid vector within bacterial cells was extracted and purified using the GeneJet™ Plasmid Miniprep Kit (Fermentas, Vilnius, Lithuania).

The Big Dye terminator (v3.1) technology (Applied Biosystems, Foster City, CA, USA) was used to amplify and sequence the fragments in both senses with an automated sequencer (DNAVision, Charleroi, Belgium).

The sequences were corrected by means of the software Bioedit Sequence Alignment Editor© developed by Microbiology Department of North Carolina University (USA). Sequence homology was determined using the BLASTX searching engine (Altschul et al. 1997) and the identified protein sequences were used in queries against the UniProt database (http://www.expasy.org/cgi-bin/sprot-search-de).

Quantitative real-time RT-PCR (qRT-PCR)

Four housekeeping genes—Actin, GAPDH, ef1-α and 18S rRNA—were selected among commonly used reference genes to normalize the result of qRT-PCR. The expression stability for these reference genes was evaluated using the geNorm software program (Vandesompele et al. 2002b) where two parameters were defined: M (average expression stability) and V (pairwise variation). A low M value is indicative of a more stable expression, hence, increasing the suitability of a particular gene as a control gene. The 0.15 was proposed as a cut-off value for the pairwise variation (V) below which the inclusion of an additional control gene is not required.

For each sample type (healthy, symptomatic and non-symptomatic), two RNA extracts were independently prepared from two distinct trees. Total RNAs were treated for DNA residue as mentioned for cDNA-AFLP. First strand cDNA synthesis was carried out on approximately 1.5 μg of the total RNA with the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{20}$ primers following the manufacturer's instructions.

Specific primers were designed for all AFLP sequenced cDNAs using Primer Express software Version 5.0 (Applied Biosystems, Forster City, USA). The GeneAmp 5700 Sequence Detection System (Applied Biosystems) was used for amplification and real-time quantification of the first strand cDNAs. Real-time PCR was performed using qPCR MasterMix plus for SYBR green I (Eurogentec, Seraing, Belgium) and 2.5 ng of cDNA. Each sample, non-template control and normalizers was amplified in triplicate in each run. Each experiment was repeated twice.

Calculation of PCR efficiency and relative expression

Individual PCR efficiencies were determined according to Ramakers et al. (2003). PCR efficiencies of each primer pair were subjected to the analysis of variance

(*ANOVA*) procedure of the SAS Version 9.1 software (SAS Institute Inc., Cary, NC, USA). Statistical significance was tested at the $P<0.05$ level.

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. Furthermore, the mean of ΔC_t values for each target gene between the triplicates of two runs, and both samples of each type was determined, distinctly, in normalization by GAPDH and actin. Individual ΔC_t values were further subjected to the SAS software by means of three-way analysis of variance (completely hierarchical) model using multiple comparisons of means (STUDENT-NEWMAN-KEUL's test). Statistical significance level was 0.05. The mean ΔC_t values are as the crude data to calculate the $\Delta\Delta C_t$ in comparisons between infected (symptomatic or non-symptomatic) and healthy samples or between symptomatic and non-symptomatic samples. The relative expression ratio (R) of each target gene between these combination samples was calculated by 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 qRT-PCR) used for studying the gene expression, the Chi-Square test of independence using the SAS software was performed on their data.

Results

Genes deregulated by phytoplasma infection

In cDNA-AFLP analyses, 20 primer combinations during selective amplification produced reproducible banding patterns and amplified approximately 8000 fragments (2000 per sample); in particular 491 fragments (6.1%) were differentially expressed among healthy, symptomatic and non-symptomatic infected samples (Fig. 1). The number of quantitative differences (variability in intensity of bands) was higher than the qualitative ones (presence and absence of bands) (data not shown).

The fragments differentially expressed were divided into three groups (healthy versus infected samples; symptomatic versus non-symptomatic; and AP- versus AT2-infected samples) (Table 1). The majority of fragments (95.3%) were differently expressed (as quantitatively or qualitatively) between healthy and infected samples (symptomatic, non-symptomatic or both). More than 50% of the fragments were differentially regulated between symptomatic and non-symptomatic samples. The 34.2% (168 fragments) of differentially expressed bands were common among the three groups (deregulated in all groups), and some of them were common between two of three groups. Interestingly, there were a considerable number of fragments with identical expression in healthy and non-symptomatic samples and differing from the two symptomatic samples (data not shown). Also, 64 bands were present only in the healthy sample, and 54 were present only in all infected samples (Table 1).

Between 491 transcript derived fragments (TDF) that were differentially expressed, 66 bands were selected on the basis of their strong differential expression, ranging from 130 to 600 bp long. A deliberate choice among TDFs was therefore performed only for the fragments that clearly showed differences between

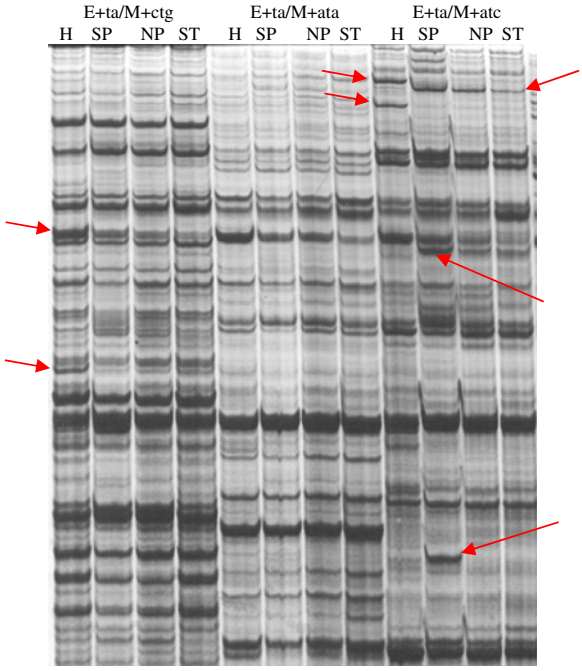

Fig. 1 cDNA-AFLP profiles obtained for healthy (H), symptomatic AP-infected (SP), non-symptomatic AP-infected (NP) and symptomatic AT2-infected (ST) samples using three primer pairs combinations of an *Eco* (E) primer and an *Mse* (M) primer containing two or three selective bases (a, t, c and g) at the 3′ end. Arrows show bands differentially regulated between samples

Table 1 Comparison of gene expression between different combinations of samples obtained by cDNA-AFLP. Gene regulations varying between samples are divided into three groups

Gene expression comparison between	Number of fragments differentially expressed
Healthy and infected samples (group 1)	468 (95.3%)[a]
Symptomatic and non-symptomatic infected samples (group 2)	252 (51.3%)
AP-infected and AT2-infected samples (group 3)	149 (30.3%)
Present or absent genes (qualitative differences)	Number of fragments
Genes present in healthy and absent in all infected samples	64 (13%)
Genes absent in healthy and present in all infected samples	55 (11%)

[a] percentage of differentially expressed genes in each group to total differentially expressed genes (491).

two or more samples; however this selection does not mean that other repressed or induced bands are not involved in interaction. The sequences of 45 out of 66 fragments (68%) were obtained by direct or cloning-sequencing: 18 cDNA had no matches in international databases (novel genes), and 27 fragments showed significant similarities to plant genes (Table 2). In particular among 27, one was matching with a plant gene of unknown function, eight had significant similarity with hypothetical plant proteins, and 18 were previously characterized (known proteins) in plants (an *E* value score equal or below 1e–05 was considered significant). With the information from UniProt Knowledgebase, putative functions were assigned to the 18 known proteins (Table 2).

Quantitative real-time RT-PCR (qRT-PCR)

According to the geNorm data, the M values of all selected reference genes were lower than 1.5 (Table 3), and therefore these genes were concluded to be stably expressed housekeeping genes in our samples. GAPDH and actin had the lowest M value followed by 18S rRNA and ef1-α. Based on this approach, GAPDH and actin are the most suitable reference genes to normalize the amounts of starting cDNA and consequently, the transcript profile of healthy and infected samples.

In order to determine how many reference genes should be included, normalisation factors (NFn) were calculated by stepwise inclusion of an extra, less stable, reference gene according to Vandesompele et al. (2002b). Figure 2 shows the pair wise variation Vn/Vn+1 between two sequential normalisation factors NFn and NFn+1. In three real-time PCR repetitions, the inclusion of a 3rd gene had no significant contribution to the NF (lower V2/3 value than 0.15 as a cut-off). Then in confirmation of cDNA-AFLP results by qRT-PCR, the two control genes described above (GAPDH and actin) are enough for normalisation of qRT-PCR data.

Among the sequenced 45 TDFs, no primer combination was designed for the two fragments 1–22 and 9–13′ (novel genes) because of their shortness. The specificity of PCR amplification of each primer pair was confirmed by analyzing PCR products on agarose gel and by melting curve analysis during real-time PCR (data not shown). qRT-PCR analyses were carried out for all 43 remaining TDFs identified by cDNA-AFLP corresponding either to known, unknown and hypothetical proteins, or to proteins without any similarity to databases. The number of TDFs differentially expressed between AP- and AT2-infected samples in cDNA-AFLP were less than other groups (Table 1), and also because of large number of manipulations in qRT-PCR, only AP symptomatic samples were chosen for qRT-PCR experiments to reduce cost and handlings.

Amplification efficiencies during different PCR runs varied between 90 and 99%. For the same PCR run, efficiencies obtained for normalizer genes and each gene of interest were never significantly different ($P<0.05$). The R^2 values for the reference and target gene transcripts varied from 0.975 to 0.999. These make the normalized expression ratios reliable and accurate. qRT-PCR showed that TDFs were down regulated between 1X (without deregulation) to 17X; or up regulated between 1X to 11X; among healthy, symptomatic and non-symptomatic infected samples.

Table 2 Similarity of TDFs differentially expressed during the apple – phytoplasma interaction with sequences in databases and their annotation detected by BLASTX (Altschul et al. 1997)

cDNA fragment	Size (bp)	NCBI accession	Change(s)[a]	Homology	Annotation	Organism origin	*E*-value[b] (% similarity)
Metabolism							
1-11	290	FL591238	1,2,3	BAA83349.1	Formiminotransferase-cyclodeaminase-like	*Oryza sativa*	2e-20 (73%)
10-17	133	FL591255	1	ABE87232.1	UDP-glucuronosyl/UDP-glucosyltransferase	*Medicago truncatula*	2e-12 (81%)
13-7	428	FL591265	1	ABO83398.1	Cell wall-associated hydrolase, putative	*Medicago truncatula*	2e-10 (96%)
17-29	159	FL591274	1,2	CAB46084.1	Fructose-1,6-bisphosphatase	*Pisum sativum*	4e-12 (97%)
Disease/defence/stress							
8-17	253	FL591248	1,2,3	ABE84888.1	Heavy metal transport/detoxification protein	*Medicago truncatula*	1e-05 (90%)
11-13	295	FL591260	1,2,3	AAV50009.1	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	*Malus* x *domestica*	5e-08 (87%)
17-24	214	FL591273	1,2	XP_002283457.1	Universal stress protein (USP) family protein	*Vitis vinifera*	5e-12 (94%)
Senescence associated							
2-8	172	FL591240	1	BAB33421.1	Putative senescence-associated protein	*Pisum sativum*	1e-05 (100%)
7-9	354	FL591245	1,2,3	ABN50032.1	Putative senescence-associated protein	*Trichosanthes dioica*	8e-31 (98%)
7-25′(8,10)	258	FL591247	1	AAR25995.1	Putative senescence-associated protein	*Pyrus communis*	4e-45 (100%)
Photosynthesis/Energy							
13-20	164	FL591266	1,2	CAN82922.1	Proteins related to photosystem II	*Vitis vinifera*	3e-11 (93%)
20-4′	343	FL591278	1,2,3	NP_051069.1	Photosystem I subunit VIII	*Arabidopsis thaliana*	2e-11 (94%)
Transport							
11-3	58	FL591259	1,3	ABD28324.1	Cellular retinaldehyde-binding/triple function, C-terminal	*Medicago truncatula*	6e-05 (100%)
12-13	197	FL591264	1,2,3	Q9FFD0.2	Putative auxin efflux carrier component 8	*Arabidopsis thaliana*	6e-05 (79%)
Transcription							
14-9	182	FL591268	1,2	NP_565984.1	RNA polymerase II transcription factor SIII (Elongin) subunit A	*Arabidopsis thaliana*	1e-09 (71%)
15-2	370	FL591270	1,2,3	ABO83512.1	No apical meristem (NAM) protein	*Medicago truncatula*	2e-26 (67%)
Signal transduction							
10-2	293	FL591254	1,3	ABF70116.1	Dual specificity protein phosphatase (DSP) family protein	*Musa balbisiana*	2e-13 (97%)
Protein synthesis							
8-26(3)	140	FL591250	1	XP_002511579.1	Glycyl-tRNA synthetase 2	*Ricinus communis*	2e-07(100%)
Unknown or hypothetical protein							
4-25	135	FL591243	1,2,3	ABE84186.2	Unknown protein	*Medicago truncatula*	1e-05 (81%)
2-8′	172	FL591240	1	ABA98651.1	Hypothetical protein	*Oryza sativa*	9e-05(95%)
4-7	264	FL591242	1	ABE85508.2	Hypothetical protein	*Medicago truncatula*	8e-22 (85%)
7-19	223	FL591246	1,3	EAZ17008.1	Hypothetical protein	*Oryza sativa*	3e-05 (75%)

Table 2 (continued)

cDNA fragment	Size (bp)	NCBI accession	Change(s)[a]	Homology	Annotation	Organism origin	*E*-value[b] (% similarity)
10-19	124	FL591256	1,2	ABE87182.2	Hypothetical protein	*Medicago truncatula*	1e-05 (90%)
12-6	90	FL591262	1	AC092750_8	Hypothetical protein	*Oryza sativa*	4e-06 (100%)
13-7′	428	FL591265	1	ABE87182.2	Hypothetical protein	*Medicago truncatula*	2e-20 (100%)
16-14	281	FL591271	1,2,3	EAY93033.1	Hypothetical protein	*Oryza sativa*	3e-29 (86%)
19-15	156	FL591277	1,3	EAZ10858.1	Hypothetical protein	*Oryza sativa*	2e-12 (87%)
Without significant similarity or low similarity[c]							
1-22[FL591239], 3-10[FL591241], 6-16[FL591244], 8-26(1,4)[FL591249], 9-3[FL591251], 9-5[FL591252], 9-13′[FL591253], 10-27(7)[FL591257], 10-27(10)[FL591258], 11-19′[FL591261], 12-10[FL591263], 13-21[FL591267], 14-16[FL591269], 17-15[FL591272], 18-7[FL591275], 18-8[FL591276], 20-9[FL591279], 20-12[FL591280]							

[a] Differentially expressed change(s) present in 3 groups of comparisons between samples (referred to Table 1)

[b] The E value was used to indicate the significance of sequence similarity

[c] NCBI accession numbers of fragments in this category are found in bracket

Comparison of cDNA-AFLP and qRT-PCR results

In qRT-PCR studies, statistical analysis comparing ΔC_t values showed 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 (data not shown). In other hand, the Chi-Square test was performed to test the similarity of cDNA-AFLP and qRT-PCR methods based on the numbers of fragment-samples comparisons between 129 (43 fragments×3 levels of comparison between healthy, symptomatic and non-symptomatic samples) that show the same profile by both methods. GAPDH and actin respectively confirmed the differentially expression of 78 and 54 (out of 129) fragment-samples comparisons (Table 4).

Only when results of cDNA-AFLP assays were confirmed by qRT-PCR tests using both reference genes, was it considered as complete confirmation and 48 out of the 129 comparisons showed this agreement. The expressions of 30 and 6 out of the 129 comparisons were confirmed (partial confirmation) only by one of the two reference genes, GAPDH and actin, respectively (Table 5). AFLP differential expression patterns of the remaining 45 comparisons could not be confirmed by qRT-PCR. The qRT-PCR and cDNA-AFLP results were not conflicting since in a considerable number of cases (38 out of 45), the difference between TDF expression pattern among samples was not statistically significant by one or both reference genes.

In another way, among 18 differentially expressed genes with known function (Table 2), qRT-PCR confirmed the expression pattern of eleven TDFs [fragments 2-8, 8-26(3), 11-3, 11-13, 12-13, 13-7, 13-20, 15-2, 17-24, 17-29 and 20-4′] by both reference genes. Besides, seven [fragments 2-8′, 4-7, 9-3, 9-5, 12-10, 13-7′ and 16-14] and six [10-19, 12-6,, 18-7, 18-8, 19-15, 10-27(10), 11-19′ and 20-9] TDFs between 25 unknown, hypothetical or novel genes were confirmed for their deregulated expression profile, respectively, by both reference genes and only GAPDH.

Discussion

The molecular mechanisms involved in symptom development and interaction between phytoplasmas and

Table 3 Calculating average expression stability (M) and normalization factor by means of ΔC_t values using geNorm approach to choose most stable reference genes. Normalization factor will be necessary for careful choice of the number of reference genes

Sample	Actin	EF1-α	GAPDH	18S rRNA	Normalisation Factor (NF)
Healthy1	7.80E−01	3.60E−01	7.10E−01	9.60E−01	0.9540
Infected- symptomatic1	1.00E + 00	5.10E−01	9.50E−01	1.00E + 00	1.2033
Infected- nonsymptomatic1	5.00E−01	4.20E−01	7.90E−01	7.80E−01	0.8651
Healthy2	5.70E−01	5.30E−01	8.30E−01	6.50E−01	0.9164
Infected- symptomatic2	6.80E−01	7.40E−01	7.40E−01	7.20E−01	1.0379
Infected- nonsymptomatic2	9.60E−01	1.00E + 00	1.00E + 00	5.20E−01	1.2124
M value <1.5	0.470	0.582	0.412	0.541	

their hosts are largely unknown. Using the cDNA-AFLP technique we identified a series of plant genes whose expression is altered between healthy and infected, between symptomatic and non-symptomatic, and between plants infected by different strains of the phytoplasma. It means, different strains of the pathogen may differently deregulate the gene expression in their host; and so different strains of '*Ca.* P. mali' probably differ (to some extent) from each other for their pathogenicity manners in host. Some of the differentially-expressed fragments did not show any similarity with sequences in databases, representing therefore potential novel proteins important in interaction and may be related to the specific plant responses to phytoplasmas.

The cDNA-AFLP is a simple, rapid and very powerful tool for genome-wide expression analysis of organisms, where little information is available on the genome sequences (Breyne and Zabeau 2001). This method largely overcomes the limitations of arbitrarily primed PCR and DDRT-PCR such as problems with reproducibility and difficulty in representing very rare messages (Bachem et al. 1998). Different techniques can be employed for confirmation (to detect false-positive) of cDNA-AFLP results. qRT-PCR was privileged for its rapidity, simplicity, high sensitivity, low RNA quantity needed, and high reproducibility as intra- and inter-assay variation (Vandesompele et al. 2002a; Massart and Jijakli 2005; Goossens et al. 2005). In the current study, the expression of 24 (56%) out of 43 AFLP-identified genes [or 84 out of the 129 comparisons of fragment-samples] was reproducibly confirmed to be deregulated by phytoplasma using qRT-PCR.

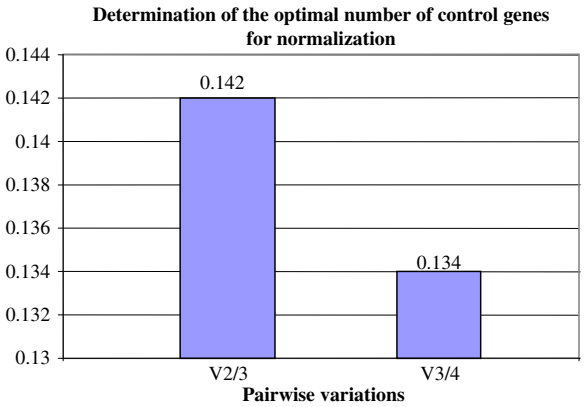

Fig. 2 Pairwise variation analysis between the normalisation factors NFn and NFn+1, to determine the optimal number of control genes for normalisation. The V2/3 below than 0.15 as a cut-off value for the pairwise variation (V) indicates that the inclusion of third additional control gene is not required

Gene expression is not a stable and monotonous process in cells, it depends on condition and environmental factors; also in stressed cells the gene expression pattern can be different from time to time. Moreover, although the samples were collected randomly from plants, it is clear that the gene expression pattern is different before and after colonisation of tissue by pathogen. On the other hand, false positives have been reported to constitute a significant portion of the differentially expressed bands in transcriptome techniques (Lievens et al. 2001). These may explain the different results in the expression patterns of some genes obtained by AFLP and qRT-PCR. As reported by Tan and Whitlow (2001), leaves formed before inoculation remained visually and physiologically asymptomatic, this phenomenon can also explain the differences obtained in this research for the gene expression patterns between symptomatic and non-symptomatic samples.

Based on the function of the confirmed genes (11 out of 18 known genes), a relationship is here

Table 4 Three examples of calculation for expression ratios of target genes (fragments) normalized by GAPDH using $\Delta\Delta C_t$ method in qRT- PCR. The results are compared with cDNA-AFLP results in a statistical analysis manner ($P<0.05$). Shaded boxes mean complete similarity of qRT-PCR results with ones in cDNA-AFLP

	Mean ΔC_t			$\Delta\Delta C_t$			Expression ratio ($2^{-\Delta\Delta Ct}$)			Statistical analysis of ΔC_t			qRT-PCR gene deregulation			cDNA-AFLP gene deregulation		
Fragment	Heal	Sym	Nsym	Sym – Heal	Nsym – Heal	Sym – Nsym	Sym to Heal	Nsym to Heal	Sym to Nsym	Heal	Sym	Nsym	Sym to Heal	Nsym to Heal	Sym to Nsym	Sym to Heal	Nsym to Heal	Sym to Nsym
11-13	6.56	9.84	8.96	3.29	2.40	0.89	0.10	0.19	0.54	A	B	B	-	-	0	-	-	0
12-10	5.20	2.79	2.49	-2.42	-2.71	0.30	5.33	6.54	0.82	A	B	B	+	+	0	+	+	+
12-13	0.29	2.88	4.27	2.59	3.98	-1.39	0.17	0.06	2.62	A	B	B	-	-	0	-	0	-

Heal: healthy sample, Sym: symptomatic sample, Nsym: non-symptomatic sample, 0: no deregulation, +: upregulation, -: downregulation

proposed in a model to interpret the role of these genes in the apple-phytoplasma compatible interaction that cause symptom expression and plant responses to phytoplasma infection (Fig. 3).

Transient changes in the ion (Ca^{2+}, K^+, H^+) permeability of plasma membrane appear to be a common early event in stress signalling (Wan et al. 2002), this is generally followed by the synthesis and release of second messengers, e.g. reactive oxygen species (ROS) (Neill and Burnett 1999) (Fig. 3). ROS (e.g. H_2O_2) play a central role in the defence of plants against pathogens (Jabs et al. 1997; Durrant et al. 2000), and the key enzymes involved in removing ROS are antioxidants (e.g. peroxidases). So, we speculate that like other stresses the cellular ROS concentration increased after challenge with phytoplasma [although any gene related to their biosynthesis was not identified in the present study], however the antioxidants levels were likely increased in the compatible interactions (susceptible hosts) by the cell to reduce oxidative damage. In our case, the ROS reduction was probably resulted to the suppression of defence pathways in this susceptible (MM106) infected host.

Table 5 Summary of confirmation of cDNA-AFLP results by qRT-PCR. This table shows the distribution of confirmed induced or repressed fragments among 129 comparisons of fragment-samples

Confirmation of cDNA-AFLP data by qRT- PCR	Number[a] (percentage)
Complete confirmation by GAPDH and actin	48 (37%)
Complete confirmation by GAPDH	30 (23%)
Complete confirmation by actin	6 (5%)
Total	84 (65%)

[a] Confirmation no. among 129 combinations of 43 fragments and 3 levels of comparison between healthy, symptomatic and non-symptomatic samples

The second messengers can initiate a protein phosphorylation cascade [kinases and phosphatases have roles in plant responses to stress] (Xiong et al. 2002); therefore the upregulation pattern of DSP (member of kinase-phosphatase proteins family) is not unexpected in the current study, and this component mediates phytoplasma-induced signal transduction pathways (Fig. 3). These signals may activate the expression of genes encoding transcription factors (TFs). The TFs, e.g. NAM identified in present study (Fig. 3), activate the expression of stress-inducible genes during symptoms expression and physiological changes stimulated by infection (Neill and Burnett 1999; Yang et al. 2003). Hereafter in the proposed model, the identified genes are classified in three groups based on their role in the regulation of plant responses to phytoplasma infection: 1) genes related to photosynthesis pathways; 2) genes involved in symptom expression; and 3) genes involved in the regulation of plant defence mechanisms (Fig. 3).

1) Two genes related to photosystems I and II (PSI & PSII) that were down-regulated only in the symptomatic part of plants were identified, and under-expression of these genes is probably due to carbohydrate accumulation in symptomatic leaves (Maust et al. 2003). Moreover, the expression of a gene coding for fructose-1,6-bisphosphatase (FBPase) was reduced in the infected plants. Since this is a key enzyme in Calvin cycle, part of photosynthesis reduction under phytoplasma infection may be due to changes in FBPase gene expression. Since Glycyl-tRNA synthetase (GlyRS) responds to stresses through proteins synthesis (Szymanski et al. 2000) and chloroplast development (Uwer et al. 1998), its down-regulation in this study probably deregulates photosynthesis enzymes (PSI, PSII and FBPase) and impairs chloroplasts (Fig. 3). A non-confirmed

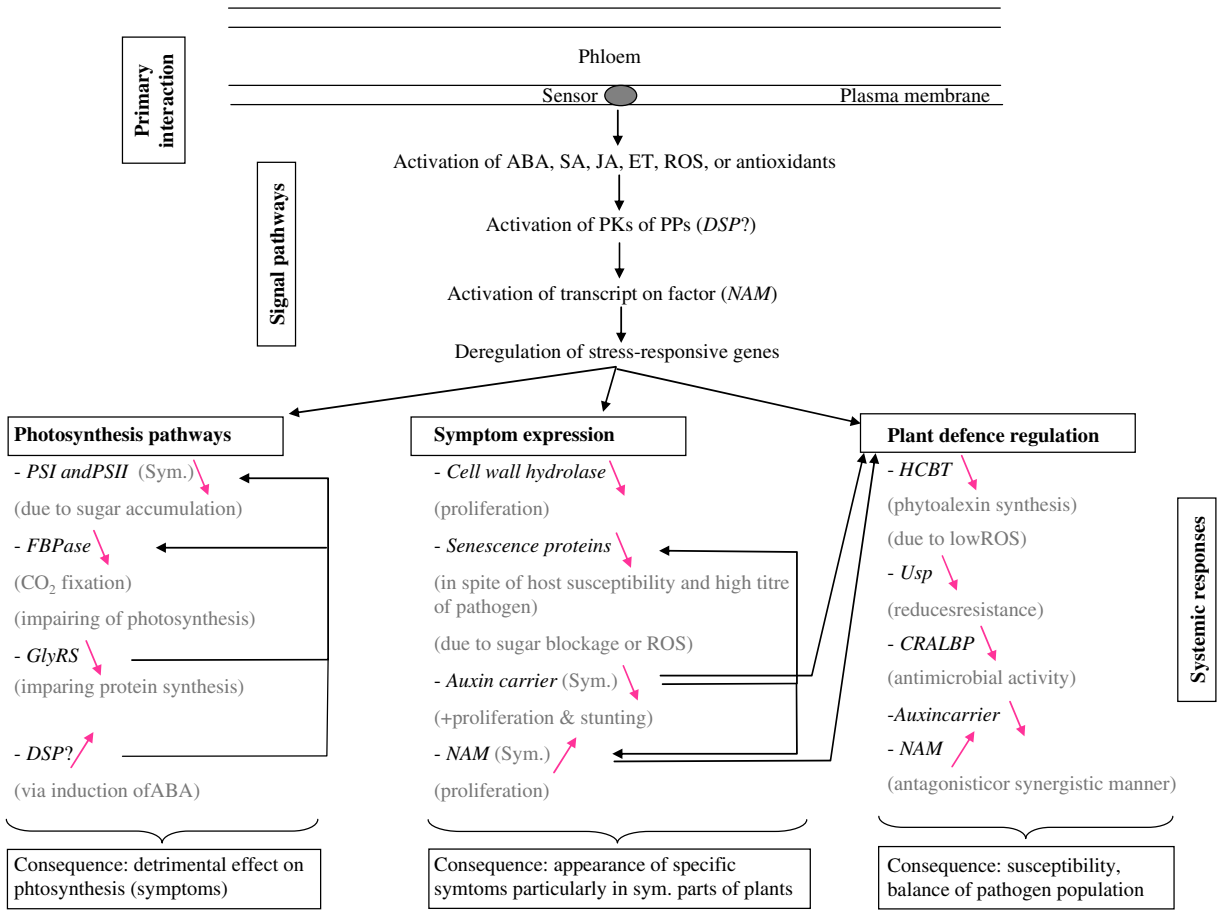

Fig. 3 A preliminary scheme of symptom expression and other physiological changes stimulated by the phytoplasma infection during a compatible interaction with apple. Upward and downward arrows indicate increased and decreased protein levels, respectively. The interactions between the genes products were shown by broken arrows. The genes in italic were identified in the present study. Question marks means no-confirmation of gene by qRT-PCR (abbreviation: ABA, abscissic acid; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ROS, reactive oxygen species; PKs, protein kinases; PPs, protein phosphatases; DSP, dual specificity phosphatase; NAM, no apical meristem; PSI and PSII, photosystem I and II; FBPase, fructose-1,6-bisphosphatase; GlyRS, Glycyl-tRNA synthetase; HCBT, hydroxycinnamoyl benzoyltransferase; Usp, universal stress protein; CALRBP, cellular retinaldehyde-binding protein; Sym., in symptomatic part)

gene, DSP also down-regulates photosynthesis enzymes through induction of abscissic acid (Rodriguez 1998; Schweighofer et al. 2004). Together, these data suggest detrimental effect of phytoplasma infection on photosynthesis pathways (Fig. 3). Deregulation of the key genes in photosynthesis could participate to AP symptom expression as already reported (Jagoueix-Eveillard et al. 2001; Maust et al. 2003; Bertamini et al. 2004).

2) The second group encloses genes involved in the symptom expression (Fig. 3). Down-regulation of cell wall-associated hydrolase induces short internode distances and proliferation symptoms since this enzyme is involved in cell wall disassembly, necessary for elongation (Hernandez-Nistal et al. 2006). Down-regulation of the senescence-associated protein indicates the decrease of the senescence phenomenon in MM106 infected plants, which is in agreement with the fact that the chlorosis and mortality (as senescence symptoms) are not observed in this host when infected by phytoplasma (Jarausch et al. 1996). Under-expression of this gene is probably due to either low concentration of ROS or to blockage of nutrient transport. Down-regulation of auxin

efflux carrier detected in symptomatic parts cause auxin accumulation (Fig. 3). The auxins, as senescence inhibiting hormones (Quirino et al. 1999), 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 could inhibit apical dominance and induce phytoplasma symptoms as also recently shown by the down-regulation of two auxin efflux carrier proteins in transgenic plants presenting proliferation symptoms (Hoshi et al. 2009). Flavonoids, have been suggested to be auxin transport inhibitors (Brown et al. 2001); therefore under-expression of auxin efflux carrier can be connected with flavonoides synthesis induced by phytoplasmas infection (Choi et al. 2004). The only over-expressed gene in this group is the one coding for no apical meristem (NAM): up-regulation of this protein, determining the position of shoot apical meristem (Souer et al. 1996) 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), therefore auxin accumulation increases the expression of NAM (Fig. 3). Together, these gene deregulations in the symptomatic parts of plants stimulate the appearance of the specific proliferation symptoms in apple.

3) The most important group of the genes identified in this study is the ones involved in the regulation of plant defence mechanisms. Anthranilate hydroxycinnamoyl benzoyltransferase (HCBT) that catalyses the reaction of phytoalexin biosynthesis (Yang et al. 1997) is down-regulated in infected apples. H_2O_2 and other ROS play a role in phytoalexin synthesis. Reduction of H_2O_2 and consequently down-regulation of HCBT is responsible for high phytoplasma titres in this susceptible host. Also, under-expression of universal stress protein (Usp) involved in stress-related responses (Kvint et al. 2003), increases susceptibility of the host to phytoplasmas as stress agents. The level of cellular retinaldehyde-binding protein with a potential antimicrobial activity (Molina et al. 1993) or protection property against stresses (Kearns et al. 1998) is also reduced in infected apples. Together, the plant defence mechanism is thus repressed and higher susceptibility of host and increased multiplication of pathogen in MM106 occurs.

Auxin carrier and NAM mentioned in symptom expression group also interfere in plant defence regulation. Auxin homeostasis is one of the components participating in the regulation of the defence response (Mayda et al. 2000). So, deregulation of auxin efflux carrier in this study suppresses the defence response pathways. Overproduction of the NAM could also regulate in antagonistic manner the genes important in defence through salicylic or jasmonic acid (Spoel et al. 2003) and consequently increases host susceptibility to the disease (Fig. 3).

All genes presented in third group are notably involved in suppression of the plant defence systems possibly leading to high phytoplasma titre and then the death of diseased host. However, as phytoplasma titre in diseased MM106 never exceeds a certain level, and no mortality of this susceptible host is observed (Jarausch et al. 1996), some defence pathways are certainly active in diseased MM106 that prevent very high multiplication of the pathogen. In the current study, a non-confirmed gene, UDP-glucosyltransferase (UGT), showed up-regulation pattern in infected MM106. UGT accumulates flavonoid, polyphenols and other phytoalexins against pathogens. These components are involved in local and systemic defence responses (Chong et al. 2002). Moreover, an alternative possibility for NAM function is the overproduction of NAM in a synergistic manner (Glazebrook 2001; Spoel et al. 2003) stimulates plant defence pathways considering that the family (NAC) to which this protein belongs mediate viral or fungal resistance (Xie et al. 1999; Ren et al. 2000; Collinge and Boller 2001). Thus, the overproduction of UGT and NAM (and probably of some other genes related to defence that were not identified in this study) balances the multiplicative capacity of phytoplasma to a level sustainable for this susceptible host (against other proteins mentioned beforehand that reduce plant defence mechanisms) (Fig. 3).

Taken together, cDNA-AFLP analysis shows a modulation of the apple gene expression in response to phytoplasma colonisation, and provides a first step towards the understanding of the '*Ca.* P. mali'–apple interaction. To our knowledge, there has been no such extensive study on transcriptome about interactions between plants and phytoplasmas other an initial study of the gene

expression patterns in '*Ca.* P. mali'-infected micropropagated *Malus* genotypes (Moser et al. 2007). Also, the pathways proposed in the current study are presented for first time as target pathways of phytoplasma infection in plants except the effect of phytoplasma on photosynthesis. The further comparison of expression level of identified genes in susceptible and resistant (or tolerant) varieties or genotypes will enable the identification of molecular markers and genes relevant in resistance or tolerance to the apple proliferation disease.

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