cDNA-AFLP Analysis of Differentially Expressed Transcripts in Partially Resistant Apple Cultivar Leaves Infected by *Venturia inaequalis*

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Abstract

Apple scab is one of the most important diseases of apple. Although resistance genes governing qualitative resistance have been isolated and characterized, the biological roles of genes governing quantitative resistance are still unknown. This study investigated the molecular and biochemical mechanisms involved in the partial resistance against *Venturia inaequalis* of an old Belgian apple cultivar 'Président Roulin'. Gene expression in both resistant and susceptible apple cultivars after scab inoculation was studied using the cDNA-AFLP method. From about 10,250 cDNA fragments detected, 252 bands were up- or down-regulated in the resistant cultivar 48 hours after scab inoculation. From these amplicons, 135 appeared to be regulated only in the resistant cultivar, but not in the susceptible cultivar 'Gala'. Some 224 fragments were constitutively expressed by the resistant cultivar only, and were not affected by the fungal infection. All the pathogen-responsive fragments and some of the fragments showing constitutive expression were cloned, sequenced and compared with GENBANK accessions using blastx. Homologies to known genes revealed several proteins belonging to the NBS-LRR and LRR-RLK classes of plant R genes. Other defense-related transcripts were also identified and could play a role in a partial scab defense response. Expression of these genes was verified by real-time quantitative PCR.

INTRODUCTION

Apple scab, caused by *Venturia inaequalis* (Cke.) Wint., is one of the most serious diseases of apple. Scab infections have been reported from almost all apple-producing countries and cause huge economic losses. For apple, at least 12 major scab-resistance genes have been identified and mapped in five linkage groups of the apple genome (Gessler et al., 2006). After the recent discovery that some new scab races are able to overcome the resistance conferred by the frequently used major gene, *Rvi6* (*Vf*), efforts have been renewed to identify new sources of polygenic scab resistance which is thought to be more durable. Various mapping populations have been studied for partial resistance against *V. inaequalis*, leading to the identification of QTLs providing broad-spectrum resistance. Although attempts have been made to identify genes or proteins involved in complete scab resistance (Komjanc et al., 1999; Gau et al., 2004; Degenhardt et al., 2005), the function of genes controlling partial resistance remains unknown. This study was conducted to analyze the defense cascade of the partially resistant apple cultivar 'Président Roulin' via the identification of genes that are differentially expressed between resistant and susceptible cultivars, with and without pathogen inoculation. Since 1975, CRA-W has run a research program focusing on the conservation, evaluation and potential use of local old fruit tree cultivars (Lateur and Populer, 1994), especially for breeding (Lateur et al., 1999). One of the program's objectives is to identify new sources of partial resistance to scab, and 'President Roulin' is one of our new parent candidates.

MATERIALS AND METHODS

Plant Material and Inoculation with *Venturia inaequalis*

Grafted plants of the Belgian *Malus* × *domestica* 'Président Roulin', which is partially resistant to scab, and the susceptible cultivar 'Gala' were used in this study. Young leaves from actively growing plants were sprayed with a suspension of conidia from six strains of *V. inaequalis* originating from the INRA collection at Angers (EU.B.04, EU.B.16, EU.F.05, EU.F.11, EU.I.09 and EU.D.49), France and calibrated at 2.5×10^5 conidia/ml. The inoculated plants were incubated at 20°C under maximum relative humidity (RH) for two days and were then transferred to the greenhouse. The control plants were inoculated with sterile water. Levels of scab infection were scored 21 days after inoculation. Two plants per treatment were used.

RNA Extraction and cDNA-AFLP

Total RNA was isolated from the three youngest leaves of each plant, 48 hours after inoculation, using the extraction method described by Gasic et al. (2004). After DNaseI treatment, purification of mRNA was performed with 250 µg total RNA using the Qiagen Oligotex mRNA kit (Qiagen Inc.). Double-stranded cDNA was obtained from 500 ng mRNA following the instructions of the Superscript Double Stranded cDNA Synthesis kit (Invitrogen Inc.). cDNA-AFLP analysis was performed with the AFLP Core Reagent kit (Invitrogen Inc.), using the restriction enzyme pairs EcoRI/MseI and 140 specific primer pairs containing two or three additional bases at the 3' end. The amplification products were separated by electrophoresis on a 5% polyacrylamide gel under denaturating conditions.

Transcript-Derived Fragments (TDFs) Isolation and Sequencing

Bands were positioned on the polyacrylamide gel by autoradiography using X-ray films. Band intensities were digitized using the PhosphorImager tool (Biorad) and quantified using QuantityOne software (Biorad). Bands of interest were cut from the polyacrylamide gels, eluted, reamplified with the selective primers and cloned into pJET 1.2 vector (Fermentas). Two transformed colonies per TDF with an insert of the expected size were subsequently sequenced. Homology searches were conducted in blastx against the GENBANK non-redundant and EST databases (NCBI), taking homologies with an E value <1E10-3 as a significant match. The predicted proteins were classified according to the Gene Ontology Consortium, using the automatic bioinformatic tool Blast2GO.

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

qRT-PCR was performed with specific TDF primers using Biorad CFX96 and Maxima SYBR Green qPCR master mix (Fermentas Inc.). Melting curve analyses were performed to differentiate between the desired amplicons and any primer dimers or DNA contaminants. The individual PCR efficiencies of each primer pair were determined using the method described by Ramakers et al. (2003). The relative expression ratio of the target genes between scab-inoculated and water-treated plants was evaluated using the ΔΔCt method described by Applied Biosystems ($R=2^{\Delta\Delta Ct}$), with the glyceraldehyde 3-phosphate dehydrogenase gene (GADPH) as the internal reference. Individual PCR efficiencies and ΔCt values were then subjected to the ANOVA procedure, using Minitab 13.20 software, at a statistical significant level of $P<0.05$ and $P<0.01$, respectively. Two technical replications were made for each target gene.

RESULTS AND DISCUSSION

Venturia inaequalis Infection and Scab Symptom Evaluation

On both cultivars 'Président Roulin' and 'Gala', scab symptoms appeared between 7 and 12 days after inoculation. After 21 days, 90% of the leaf surface from the susceptible cultivar 'Gala' was covered by sporulated apple scab lesions. However,

typical chlorotic and necrotic lesions with slight sporulation were observed on leaves of the resistant cultivar 'Président Roulin'. These symptoms were considered as resistance responses and were classified in the class 3b, as described by Chevalier et al. (1991). When 'Président Roulin' is crossed with the highly susceptible 'Gala', the resistance can be traced back in a very small proportion of the resulting progenies (data not shown); thus we assume that the partial resistance has a polygenic control.

cDNA-AFLP Fingerprinting

The TDFs analyzed by cDNA-AFLP ranged in size from 30 to 800 base pairs (bp). Some 140 AFLP primer combinations were applied, resulting in a mean of 73 TDFs per primer pair, and a total of about 10,250 gene tags were evaluated. Taking into account that about 40% of the apple cDNA could potentially be visualized with the restriction enzyme EcoRI and MseI used for this study (estimation obtained using the AFLPInSilico program (Rombauts et al., 2003), with 450 full-length apple cDNA from Genebank), we estimate that we analyzed a representative sample of about 20% of the apple genes expressed in the tissues by applying 132 of the 256 possible primer combinations EcoRI/MseI with two additional bp.

Overall, the same profiles were obtained for the two cultivars. In fact, only 2% of the fragments were genotype-specific. These bands were not affected by fungal infection. This probably reflects the fact that equivalent amounts of amplified cDNA were compared and that the two cultivars used in this study were genetically similar. Altered expression patterns after *Venturia* inoculation were observed for 2.7% of the analyzed fragments (pathogen-responsive TDFs). Half of these TDFs displayed differential expression after fungal attack, that also differed between the two genotypes (pathogen-induced and genotype-specific TDFs representing 1.3% of all the analyzed fragments). The other TDFs were differentially expressed in both cultivars. The pathogen-induced and genotype-specific TDFs are considered to be the most interesting ones, as these fragments represent genotype-specific genes that could be involved in plant defense reaction.

All the pathogen-responsive TDFs from the resistant cultivar and some of the genotype-specific TDFs were excised from the gels, re-amplified and cloned into *Escherichia coli*. Two colonies per TDF were then sequenced to verify the presence of identical inserts in the different clones. For 40% of the TDFs, sequences differed between the two corresponding clones. This problem of mis-cloning reflects the major problem of cDNA-AFLP fingerprinting because one band in a gel can correspond to a single DNA fragment or a mixture of several fragments. This illustrates the need for an independent technique to confirm the differential expression of genes corresponding to cloned cDNA-AFLP. Finally, 560 sequences were compared with GENBANK accessions using blastx.

Functional Categories of Apple Transcripts Modulated by Apple Scab Infection

Among the 560 sequences obtained, 334 were homologous to known expressed sequences, either as tentative consensus sequences or EST, whereas 45 fragments were homologous with predicted proteins with unknown functions. Some 181 sequences had no matches in the NCBI database. Apart from 15 sequences which found homology with the apple stem pitting virus, all the sequences derived from the recently completed apple sequencing project. No *V. inaequalis* transcripts were found.

The putative functions of the apple TDFs were then classified according to the Gene Ontology Consortium. About 30% of the annotated sequences have metabolic roles and 27% are involved in the cellular process. Other relevant groups, accounting for 10 and 8% of the TDFs, respectively, include response to stimulus (particularly response to stress and defense response) and biological regulation.

Table 1 shows the most interesting TDFs differentially expressed in the partially resistant 'Président Roulin' compared with the susceptible 'Gala' after scab inoculation. These TDFs correspond to annotated proteins reported to have a potential role in defense responses as reported by the Gene Ontology Consortium and through careful analysis of

the scientific literature. Three TDFs differentially expressed in our partially resistant cultivar encoded for the NBS-LRR family protein of resistance gene. Other TDFs encoded for proteins such as gluthatione-S-transferase, MAP kinase, ABC transporter, transcription factor, for proteins involved in photosynthesis, in protein metabolism such as cystein proteases, ubiquitin ligase and SUMO ligase, and for a pectin methylesterase inhibitor acting in the cell wall metabolism.

We then compared the localization of these selected TDFs, mapped in silico using the GBrowse on the Genome Database for *Rosaceae* (http://www.rosaceae.org/), focusing on genomic regions already known to be involved in the control of scab resistance (QTL and major resistance genes) (Gessler et al., 2006). Interestingly, this analysis showed that half of our fragments co-localized with these regions of interest. These fragments could be involved in the defense system of the partially scab resistant 'Président Roulin'.

Validation of Representative Gene Expression by qRT-PCR

To verify the results obtained from the cDNA-AFLP analysis, differential gene expression of 14 TDFs out of the 24 TDFs presented in Table 1 were further confirmed by qRT-PCR (Table 2). The TDFs were chosen on the basis of their co-localization with known apple QTLs for scab resistance and homologies with proteins widely shown to act in plant defense systems against the pathogen. No PCR amplification product was obtained for 44DU105' and 2EU169. The PCR efficiencies of all TDFs analyzed never differed from the PCR efficiency of the housekeeping gene used for normalizing the data. The differential expression of most of the cDNA-AFLP fragments (83%) was confirmed by qRT-PCR. Inoculation by *V. inaequalis* significantly induced or repressed genes with a ratio from 2 to 28 in the inoculated resistant cultivar compared with the non-inoculated control plants. In addition, apart from the TDFs 44GU169 and 44EU122, no significant differential expression was observed in the susceptible cultivar after pathogen inoculation. In all cases, the ratio of expression was higher in the resistant cultivar than in the susceptible one. Except for two TDFs (1AU61' and 43CU118), qRT-PCR expression data accorded with the cDNA-AFLP results, this illustrates the need for an independent technique to confirm the differential expression of genes corresponding to cloned cDNA-AFLP.

CONCLUSION AND PERSPECTIVES

In conclusion, by applying the cDNA-AFLP method, we identified genes differentially expressed in the partially resistant cultivar 'Président Roulin', but not in the susceptible cultivar 'Gala', in response to *V. inaequalis*. This method was effective in leading to gene discovery while we had no a priori on the gene sequence involved in partial resistance to apple scab. Some of these genotype-specific, pathogen-responsive TDFs showed sequence homologies with proteins related to plant defense response, suggesting they could play an important role in the partial resistance mechanism of 'Président Roulin' against *V. inaequalis*. The expression patterns of 12 TDFs were analyzed by qRT-PCR. For 83% of the TDFs analyzed, the results from the real-time PCR accorded with the cDNA-AFLP findings. The differential expression of these genes of interest will be further confirmed in a biological replication. The kinetic expression will then be studied using qRT-PCR for the whole developmental stage of *V. inaequalis*.

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Tables

Table 1. Selection of differentially expressed TDFs in the partially resistant cultivar 'Président Roulin' and not in the susceptible cultivar 'Gala' after scab inoculation.

TDF	Expression[a]	QTL[b] (Chr)	Length (bp)	Annotation (blastx)	Blast score
				Defense response	
43DU149'	+	yes (chr2)	169	cc-nbs-lrr resistance protein	3.1e-7
56AU33'	+	yes (chr1)	208	cc-nbs-lrr resistance protein	3.2e-20
43CU118	-	yes (chr17)	148	TMV resistance protein	5e-5
44AU9	+	no (chr9)	415	LRR receptor kinase-like protein	2e-22
44GU169	+	no (chr12)	356	2-cys peroxiredoxin	4.6e-59
54CU21	+	no (chr8)	190	Phi class glutathione transferase	9.8e-9
				Signal transduction	
2EU181	+	yes (chr13)	211	Putative MAP kinase	1e-30
39AU13	+	no (chr13)	217	MAP kinase phosphatase	5.4e-12
				Transporter	
46EU122	+	yes (chr5)	693	ABC transporter	2e-86
				Photosynthesis	
51DU17	+	no (chr15)	512	Cytochrome P450	1e-86
53DU34	+	no (chr2)	666	Cytochrome P450	2.6e-77
56AU5'	+	yes (chr1)	220	Uroporphyrinogen decarboxylase	1e-16
				Response to environmental stress	
43DU149	+	yes (chr10)	168	Peroxidase 12	9.7e-9
51HU129'	+	no (chr15)	418	Tocopherol cyclase	6e-72
				Metabolism	
44EU122	-	no (chr16)	377	Cysteine protease	5e-67
44EU118	-	no (chr16)	377	Cysteine protease	5e-67
44DU105'	+	no (chr12)	351	Skp1-like protein	5.5e-20
2EU169	+	yes (chr15)	260	E3 ubiquitin protein ligase	8e-35
1AU61'	+	no (chr3)	242	Sumo ligase	4.5e-22
				Transcription factor	
53HU89	+	yes (chr15)	331	Zinc finger homeodomain protein	7e-05
				Cell wall organization	
44GU173	+	yes (chr2)	179	Pectin methylesterase inhibitor	8.6e-26
				Unknown functions	
55FU102	+	yes (chr3)	117	No homology	-
55HU125'	+	yes (chr2)	211	No homology	-
44GU182	-	no (chr11)	106	Predicted protein	1.3e-8

[a] Induced (+) and repressed (-) expression in the cDNA-AFLP fragments after scab infection.
[b] Co-localization (yes, no) and chromosome position of TDF with QTL or major resistance genes against apple scab.

Table 2. Confirmation of the TDFs expression by qRT-PCR.

TDF	Expression[a]	QTL	Annotation	Fold induction/repression	
				Resistant cultivar	Susceptible cultivar
43DU149'	+	yes	cc-nbs-lrr resistance protein	+7.9±2.6*[b]	+1.9±0.0
56AU33'	+	yes	cc-nbs-lrr resistance protein	+2.6±0.1*	+1.5±0.1
44GU169	+	no	2-cys peroxiredoxin	+10.3±0.1*	+2.2±0.1*
43DU149	+	yes	Peroxidase 12	+3.4±0.2*	+1.2±0.1
44EU122	-	no	Cysteine protease	-12.7±4.5*	-4.1±1.2*
44GU173	+	yes	Pectin methylesterase inhibitor	+3.3±0.8*	+1.1±0.1
55FU102	+	yes	No homology	+3.0±0.1*	+1.4±0.5
55HU125'	+	yes	No homology	+4.8±0.0*	-1.5±0.1
44GU182	-	no	Predicted protein	-28.4±4.9*	+ 1.4±0.1
51DU17	+	no	Cytochrome P450	+2.0±0.3*	+1.1±0.3
1AU61'	+	no	Sumo ligase	+1.5±0.3	+1.6±0.1
43CU118	-	no	TMV resistance protein	+1.6±0.1	+1.5±0.2
44DU105'	+	no	Skp1-like protein	-	-
2EU169	+	yes	E3 ubiquitin protein ligase	-	-

[a] Induced (+) and repressed (-) expression in the cDNA-AFLP fragments after scab infection.
[b] Means and SD of fold induction (+) or repression (-) calculated by the ΔΔCt method applied using qRT-PCR. Individual ΔCt values and statistical significance (*) were judged at the $P<0.01$ level.