Journal of Plant Physiology 168 (2011) 382–391

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Transcriptome analysis reveals novel genes involved in nonhost response to bacterial infection in tobacco

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ARTICLE INFO

Article history:
Received 13 April 2010
Received in revised form 30 July 2010
Accepted 30 July 2010

Keywords:
cDNA-AFLP transcriptome analysis
Hypersensitive response
Nicotiana tabacum
Nonhost response
Xanthomonas axonopodis pv. *citri*

ABSTRACT

Plants are continuously exposed to pathogen challenge. The most common defense response to pathogenic microorganisms is the nonhost response, which is usually accompanied by transcriptional changes. In order to identify genes involved in nonhost resistance, we evaluated the tobacco transcriptome profile after infection with *Xanthomonas axonopodis* pv. *citri* (Xac), a nonhost phytopathogenic bacterium. cDNA-amplified fragment length polymorphism was used to identify differentially expressed transcripts in tobacco leaves infected with Xac at 2, 8 and 24 h post-inoculation. From a total of 2087 transcript-derived fragments (TDFs) screened (approximately 20% of the tobacco transcriptome), 316 TDFs showed differential expression. Based on sequence similarities, 82 differential TDFs were identified and assigned to different functional categories: 56 displayed homology to genes with known functions, 12 to proteins with unknown functions and 14 did not have a match. Real-time PCR was carried out with selected transcripts to confirm the expression pattern obtained. The results reveal novel genes associated with nonhost resistance in plant–pathogen interaction in tobacco. These novel genes could be included in future strategies of molecular breeding for nonhost disease resistance.

Introduction

Plants are exposed to a variety of pathogens including bacteria, viruses and fungi. In plant–pathogen interactions, when the pathogen is capable of colonizing a susceptible plant and causing disease, it is known as a compatible interaction. On the other hand, if the pathogen enters a resistant or nonhost plant in which no disease can be produced, the interaction is an incompatible one. Incompatible interactions stimulate the activation of defense mechanisms in the plant that delay pathogen growth (Kamoun, 2001; Thordal-Christensen, 2003).

The nonhost interaction is the most common form of resistance exhibited by plants against the majority of potentially pathogenic microorganisms and is considered a general defense mechanism (Nürnberger and Lipka, 2005). Two types of nonhost responses have been described: type I does not produce any visible symptoms, whereas type II results in a hypersensitive response (HR) (Oh et al., 2006). The HR is a rapid and confined reaction characterized by reactive oxygen species (ROS) production and a form of localized cell death (LCD) at the site of infection that results in a necrotic area where the pathogen is eliminated.

During plant–pathogen interactions, the expression levels of many different genes are modified in the plants, allowing for the activation/inhibition of defense mechanisms, depending on the response triggered (Kazan et al., 2001). Genomic-scale assays are revealing a complex network of signaling cascades that are involved in the plant defense response (Ramonell and Somerville, 2002). Transcript profiling plays an important role in annotating and determining gene functions, and different methods have been used to study plant–pathogen interactions such as differential display (DD), serial analysis of gene expression (SAGE), microarrays and cDNA-amplified fragment length polymorphism (cDNA-AFLP) (Donson et al., 2002). Several studies have employed the cDNA-AFLP technique to assess gene expression patterns in plants exposed to biotic stress. This technique allows for the discovery of new genes without prior knowledge of the genomic sequences of the model plant in a highly reproducible way and with little false negative bands (Santaella et al., 2004).

Abbreviations: ACO, aminocyclopropane-1-carboxylic acid oxidase; ADC, arginine decarboxylase; cDNA-AFLP, cDNA-amplified fragment length polymorphism; DD, differential display; EIX, ethylene-inducing xylanase; GLOX, glycolate oxidase; hpi, hours post-inoculation; HR, hypersensitive response; LCD, localized cell death; RRM, RNA recognition motif; ROS, reactive oxygen species; TDFs, transcript-derived fragments; TMV, tobacco mosaic virus; Xac, *Xanthomonas axonopodis* pv. *citri*.

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doi:10.1016/j.jplph.2010.07.014

Previous studies have investigated tobacco gene expression during oxidative stress. Exposure of tobacco leaves to methyl viologen altered at least 95 genes as detected by DD; 85% of them included antioxidant genes, those implicated in abiotic and biotic stress and those involved in cellular protection and detoxification (Vranová et al., 2002). Furthermore, tobacco plants deficient in catalase were exposed to high light intensities, and the transcriptome was analyzed by cDNA-AFLP (Vandenabeele et al., 2003). The transcriptional response was similar to that reported during biotic and abiotic stress (Vandenabeele et al., 2003). However, to date, no study has been carried out to analyze the tobacco transcriptome during the HR nonhost response.

Xanthomonas axonopodis pv. *citri* (Xac) is a Gram-negative bacterium causing the citrus canker (Brunings and Gabriel, 2003). In a previous study, we found that Xac is capable of inducing a HR nonhost response in cotton, bean, tomato and tobacco leaves (Dunger et al., 2005). In all cases, the HR phenotype was demonstrated by the inhibition of bacterial growth, ion leakage, ROS production and LCD (Dunger et al., 2005; Daurelio et al., 2009).

Thus, a transcriptomic analysis of tobacco leaves inoculated with Xac was performed to elucidate the HR nonhost expression pattern. We have detected several tobacco genes previously identified in response to different stresses, including abiotic and biotic stress. Novel genes were also identified in this tobacco nonhost plant-pathogen interaction.

Materials and methods

Plant material and bacterial inoculation procedure

Nicotiana tabacum cv. Petit Havana plants were used to analyze differentially expressed transcripts from the tobacco HR. Plants were grown in a greenhouse at 25/18 °C (day/night temperatures) with a photoperiod of 16 h and controlled relative humidity. Young leaves from one-month-old plants were used. The Xac strain was grown at 28 °C in SB medium (5 g/L sucrose, 5 g/L yeast extract, 5 g/L peptone, and 1 g/L glutamic acid, pH 7) containing 25 μg/mL ampicillin. The Xanthomonas strain was kindly provided by Blanca I. Canteros (INTA Bella Vista, Argentina). For the pathogen treatment, an overnight culture was diluted to 10 mM $MgCl_2$ and 10^7 colony forming units/mL of Xac. The bacterial dilution and 10 mM $MgCl_2$ (control) were pressure infiltrated into the abaxial side of the leaves using a syringe without a needle (Daurelio et al., 2009). Xac-inoculated regions were cut and harvested at 2, 8 and 24 h post-inoculation (hpi). Plants inoculated with the control solution were harvested at 2 hpi. Collected tissues were frozen in liquid nitrogen immediately after harvesting. Different plants were used for each sample preparation. Samples were prepared in duplicate.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen plant tissue samples using the TRIzol® Reagent (Invitrogen), according to the manufacturer's protocol. RNA quality and yield were assessed by spectrophotometry and agarose gel electrophoresis as described by Sambrook et al. (1989). Double stranded cDNA was synthesized and purified as indicated in Blanco et al. (2005). The quality and yield of purified ds cDNA were assessed by agarose gel electrophoresis as described by Sambrook et al. (1989).

cDNA-AFLP analysis

A cDNA-AFLP analysis was performed as described by Bachem et al. (1996) with some modifications (Blanco et al., 2005). Transcript-derived fragments (TDFs) were generated by digestion of the ds cDNAs with a rare-cutting enzyme (*Bst*YI) and a frequent-cutting enzyme (*Mse*I) as indicated in Blanco et al. (2005). The TDFs were then subjected to adaptor ligation and two rounds of amplification.

A pre-amplification reaction was performed, using the adaptor ligated ds cDNA fragments as templates and oligonucleotides complementary to the corresponding adaptors as primers. Two reactions were carried out with different BstYI primers, generating two pre-amplified subpopulations. Conditions for the pre-amplification reactions are indicated in Blanco et al. (2005). The quality and yield of pre-amplifications were assessed by agarose gel electrophoresis as described by Sambrook et al. (1989).

In a second round of amplification, independent subpopulations of the pre-amplified cDNA fragments were selectively re-amplified. The selective primers used during this step were identical to the pre-amplification primers but were extended by two nucleotides (in the case of the BstYI primer) or one nucleotide (in the case of the MseI primer) at the 3′ end, respectively. To visualize the reaction products by autoradiography, the BstYI selective primer was end-labeled with [γ^{33}P]ATP and T4 polynucleotide kinase (Gibco), according to the provider's protocol. Conditions for selective amplification reactions are indicated in Blanco et al. (2005). Selective amplification products were then resolved in a 6% polyacrylamide sequencing gel at 100 W for approximately 3 h and detected by autoradiography. The intensities of the detected bands were measured using the Gel-Pro Analyzer Software 3.1 (Media Cybernetics). Oligonucleotides used as adaptors and primers for cDNA-AFLP analyses are given in Supplementary Table 1. All primers were HPLC or gel purified. Two biologically independent samples were analyzed by cDNA-AFLP. Both samples were compared using the same sets of primer combinations. In addition, to assess the reproducibility of the electrophoresis results, two aliquots of several PCR reactions were run in different gels.

Isolation and sequencing of TDFs

Gel slices containing TDFs from differentially expressed genes were identified by alignment with the autoradiogram and excised from the polyacrylamide gel, crushed with a micropipette tip and incubated in 100 μL of TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, pH 8) for 1 h at 65 °C with occasional vortexing. The eluted DNA fragments were then PCR re-amplified using 2.5 μL of the eluted sample as a template, the respective primer combination used during the second round of amplification and the conditions described for the pre-amplification reactions. The resulting PCR products were checked on 2% agarose gels as described by Sambrook et al. (1989). Those presenting with the correct size and quality were sent to High-Throughput Sequencing Solutions for sequencing analysis (University of Washington, www.htseq.org). Sequence data from this article have been deposited in the GenBank database under the accession numbers indicated in Table 1.

Gene function analysis

Database searches were performed using the BLAST Network Service (NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST). The sequence of each TDF was searched against all sequences in the non-redundant databases using the BLASTn, BLASTx and tBLASTx algorithms and in the others-ESTs database using the BLASTn program. For those sequences with homologous only in the others-EST database, the most homologous ESTs were used for a new search with the BLASTx program in the non-redundant databases ("x est"). Putative functions were assigned to the sequences following the classifications used by Mahalingam et al. (2003), considering, for sequences showing an Arabidopsis ortholog, the functional classifications of MIPS (Munich Information Center for Protein Sequences, http://www.mips.gsf.de) and MapMan (Thimm et al., 2004).

Table 1
TDF homologies classified by expression patterns (regulation). Similarities to genes in the database were determined by using different BLAST tools (see Material and Methods). Fragment names are observed together with their respective expression ratio level (ratio). Accession number, putative function (function) and length (bp) of the fragment sequenced also are indicated. The sequence with the highest similarity to each TDF is shown along with the respective *E*-value and BLAST tool utilized (see Materials and Methods).

Regulation	Fragment	(Ratio)	Accession	Function	Length	Homologue	*E*-Value	Blast
2 hpi up	1T13-2-1a	(4.83)	GR557625	S	200	Mitogen-activated protein kinase MAPKAPK5 (*H.sap.* AL356476.13)	8.7E−02	tx
	2T13-3-2a	(7.45)	GR557632	U	265	Conserved hypothetical protein (*R. comm.* EEF29405.1)	5.0E−12	x
	3T13-3-3a	(4.13)	GR557638	U	292	Homologous EST (*N. tab.* FG155856.1)	3.0E−45	est
	4T13-4-2a	(4.61)	GR557645	U	290	Hypothetical protein (*V. vin.* XP_002273068.1)	2.0E−11	x
	5T13-4-5a	(2.11)	GR557651	S	220	Protein kinase 2 (*N. tab.* CAE55204.1)	4.0E−15	x
	6T31-1-1a	(2.13)	GR557658	M	241	HAD-superfamily hydrolase (*M. trunc.* ABE82011.1)	1.0E−19	x
	8T31-3-7a	(2.13)	GR557672	T	224	Homologuos EST (*N. ben.* CK293679.1) codes for a putative	0.004	est
						UDP-galactose transporter (*S. den.*, AAT40483.1)	8E−150	x est
	9T31-4-2a	(3.97)	GR557680	U	156	Unknown protein (*A. tha.* AAM64931.1)	8.0E−04	x
	10T31-4-3a	(3.82)	GR557608	P	159	Homologous EST (*N. tab.* EG650117.1) codes for a Miraculin	1.0E−05	est
						homologue protease inhibitors (*Y. jap.* BAA82840.1)	3.0E−18	x est
	23C23-2-2a	(2.08)	GR557628	U	243	DNA sequence (*H. sap.* AL731561.12)	0.015	tx
	37C24-4-7a	(2.53)	GR557636	R	169	Agenet Tudor-like domain (*M. trunc.* ABE78986.1)	0.007	x
	78T43-4-6a	(7.61)	GR557665	St	264	Polyprotein (*C. par.* AAK70406.1)	2.00E−22	x
	79T43-4-7a	(2.95)	GR557666	D	180	Homologous EST (*S. tub.* BQ505441.2) codes for a Glycoside hydrolase, family 18 chitinase (*M. trunc.*		
						ABD32310.1)	3.0E−74	x est
	98C14-2-1a	(2.18)	GR557679	S	338	Receptor protein kinase (*I. trif.* AAC23542.1)	3.0E−34	x
	103T12-1-5a	(>10)	GR557603	D	194	Cell wall peroxidase PO2 (*C. annuum.* DQ632587.1)	8.0E−09	n
	104T12-1-7a	(4.96)	GR557604	E	235	Homologous EST (*N. tab.* EB683896.1) codes for a putative	3.0E−10	est
						COX VIIa-like protein (*A. tha.* At4g21108)	6.0E−21	x est
	116C42-2-4a	(2.40)	GR557614	M	175	Homologous EST (*N. tab.*EH618685.1) codes for a putative	5.0E−54	est
						UDP-glucose:glucosyltransferase (*L. bab.* BAG80549.1)	3.0E−09	x est
	120C42-4-1a	(3.02)	GR557615	T	293	Cytochrome P450 (*N. tabacum* ABC69396.1)	4.0E−46	x
	121C43-2-2a	(2.63)	GR557616	GD	163	GTP-binding protein (*C. annuum* AAF65513.1)	1.0E−12	x
2 hpi down	15T31-2-3b	(0.13)	GR557621	St	174	Histone H3 family protein (*O. sat.* EEE63859.1)	8.0E−07	x
	24C23-1-1b	(0.44)	GR557629	E	352	Glycolate oxidase (*S. lyc.* CAA63482.1)	1.0E−41	x
	88T21-2-1b	(0.42)	GR557670	E	333	Chlorophyll a-b binding protein 40 (*N. tab.* CAA36958.1)	5.0E−48	x
	107T12-1-3b	(0.48)	GR557605	E	276	Chlo. phot. oxyg.-evol. prot. 33 kDa sub (*N. bent.* AAX53163.1)	4.0E−24	x
8 hpi up	16T31-1-5c	(3.71)	GR557622	GD	364	Homologous EST (*N. tab.* BP533045.1) codes for a arabinogalactan-protein	3.0E−05	est
						precursor (*N. ala.* AAA66362.1)	8.0E−04	x est
	18T31-3-3c	(4.50)	GR557623	T	156	Water channel protein (*N. tab.* DQ914525.1)	4.0E−25	n
	19T31-1-2c	(2.18)	GR557624	St	208	H/ACA ribonucleoprotein complex subunit 2 (*O.sat.* ABR25566.1)	5.0E−11	x

Table 1 (*Continued*)

Regulation	Fragment	(Ratio)	Accession	Function	Length	Homologue	*E*-Value	Blast
	20T13-3-4c	(2.24)	GR557626	U	120	Homologous EST (*S. lyc.* AK323204.1)	0.002	n
	21T13-4-10c	(3.14)	GR557627	T	151	Glyc.rich protease inhib./seed stor./LTP fam. (*N. tab.* BAA95941.1)	0.012	x
	28C23-2-3c	(2.04)	GR557630	M	239	Homologous EST (*N. tab.* DV159906.1) codes for a aminoacyl-tRNA hydrolase (*A. tha.* NP_173279.2)	3.0E−07 2.0E−86	est x est
	29C23-2-5c	(2.67)	GR557631	U	205	Homologous EST (*N. tab.* BP131039.1)	6.0E−14	est
	30C23-4-2c	(2.07)	GR557633	D	462	Lipase class 3 protein-like (*O. sat.* BAD19435.1)	6.0E−29	x
	31C23-4-4c	(6.56)	GR557634	M	320	Palmitoyltransferase ZDHHC9 (*Z. mays* NP_001151207.1)	4.0E−17	x
	41C24-4-4c	(10.2)	GR557639	D	242	Chitinase/lysozyme (*N. tab.* CAA55128.1)	4.0E−35	x
	42C24-3-4c	(2.65)	GR557640	P	294	Homologous EST (*N. tab.* BP531211.1) codes for a putative CAAX prenyl protease (*A. tha.* AAL07084.1)	8E−144 1.0E−41	est x est
	45C14-1-7c	(2.27)	GR557642	D	167	Pathogenesis-related protein (*S. tub.* AAA03020.1)	1.0E−08	x
	48C24-1-1c	(3.93)	GR557643	M	476	Arginine decarboxylase 2 (*N. tab.* AAF42972.1)	1.0E−52	x
	49C24-1-3c	(2.20)	GR557644	M	350	UDP-glucose:protein transglucosylase-like (*S. lic.* AAT44738.1)	1.0E−12	x
	85T32-4-1c	(>10)	GR557668	M	381	Putative RRM-containing protein (*V. vin.* XP_002274164.1)	2.0E−25	x
	89T21-2-5c	(8.69)	GR557671	U	170	Hypotethical protein (*V. vin.* XP_002280988.1)	5.0E−9	x
	95C24-2-3c	(2.71)	GR557676	D	204	EIX receptor 2 LRR_RI (*S. lic.* AAR28378.1)	3.0E−09	x
	108T12-1-1c	(3.03)	GR557606	P	379	Proteasome activator subunit 4-like (*O. sat.* BAD53980.1)	2.0E−33	x
	109T12-2-2c	(>10)	GR557607	P	201	Homologous EST (*N. lan.* x *N. san.* EB698516.1) codes for an armadillo-like helical proteasome/cycl. reg. sub. (*M. tru.* ABE85726.1)	7.0E−69 1.0E−29	est x est
	110T12-4-1c	(2.31)	GR557609	S	353	APK2A Protein Kinase (*A. tha.* NP_172889.1)	3.0E−37	x
	114C34-2-4c	(3.31)	GR557613	M	202	HMG-CoA synthase (*N. lan.* ABV02025.1)	7.0E−16	x
	125C42-2-2c	(2.91)	GR487951	R	240	WRKY transcription factor 6 (*S. tub.* ABU49725.1)	6.0E−20	x
8 hpi down	56T13-1-1d	(0.54)	GR557647	M	269	Putative trehalose-6-phosphate synthase (*R. com.* EEF40113.1)	4.0E−29	x
	57T13-2-2d	(0.39)	GR557648	M	136	CIG1 proline dehydrogenase/oxygenase (*N. tab.* BAB83948.1)	1.0E−29	n
	58T13-4-1d	(0.29)	GR557649	M	363	Auxin repressed/dormancy asso. prot. (*N. tab.* ABY16785.1).	3.0E−26	x
	60T13-4-7d	(0.31)	GR557652	U	187	Homologous EST (*N. tab.* FG639326.1)	2.0E−73	est
	66C23-4-1d	(0.49)	GR557656	P	409	Subtilisin-like protease (*N. tab.* ABQ58079.1)	2.0E−30	x
	68C23-4-5d	(0.50)	GR557657	M	170	Putative amino oxidase (*R. com.* EEF36188.1)	3.0E−13	x
	70C23-2-1d	(0.35)	GR557659	S	282	Conserved hypothetical protein (*R. com.* EEF30425.1), similar to HHP1 protein (A. tha. NP_197527.1)	2.0E−07	x
	96C24-2-4d	(0.24)	GR557677	E	176	NDH dependent flow 6 (*A. tha.* NP_173308.2)	2.0E−17	x
	102C14-2-2d	(0.33)	GR557602	S	295	ACC oxidase (*N. tab.* CAA67119.1)	8.0E−25	x
	112T12-1-2d	(0.18)	GR557611	E	265	Homologous EST (*S. lyc.* AK319582.1) codes for a putative 33 kDa polyp. water-oxidizing complex of PII (*N. tab.* CAA45701.1)	2.0E−09 2.0E−48	est x est

Table 1 (*Continued*)

Regulation	Fragment	(Ratio)	Accession	Function	Length	Homologue	*E*-Value	Blast
	128C42-2-1d	(0.42)	GR557618	U	337	Chromosome 2 clone (*S. lyc.* AC215431.2)	7.0E−16	n
	130C43-2-1d	(0.35)	GR557619	M	215	T-protein of glycine decarboxylase complex (*P.sat.* CAA52800.1)	1.0E−13	x
24 hpi up	71T31-2-4e	(4.28)	GR557660	P	154	Homologue EST (*S. tub.* CV473671.1) codes for a putative serine esterase chloroplastic (*A. tha.* NP_194307.2)	4.0E−11	est
							6.0E−53	x est
	92T21-2-2e	(3.68)	GR557674	S	314	Homologue EST (*N. tab.* FG157013.1) codes for a diacylglycerol kinase (*A. tha.* NP_001078791.1)	2.0E−88	est
							4.0E−70	x est
	93T21-4-3e	(3.30)	GR557675	U	184	Conserved hypothetical protein (*M. tru.* ABE82474.1)	3.0E−06	x
24 hpi down	64C23-2-4f	(0.58)	GR557655	R	194	Homologous EST (*N. tab.* FG179771.1) codes for WD-repeat putative protein (*R. com.* EEF28403.1)	1.0E−85	est
							1.0E−39	x est
	75T13-1-3f	(0.50)	GR557662	T	286	Homologue EST (*N. tab.* FG199638.1) codes for a GDP-mannose transporter, putative (*R. com.* EEF36730.1)	6.0E−119	est
							5.0E−12	x est
	76T13-4-4f	(0.39)	GR557663	U	235	Homologous EST (*N. tab.* DV999170.1)	2.0E−69	est
	77T13-3-1f	(0.39)	GR557664	P	475	Ubiquitin-conjugating enzyme family protein-like (*S. tub.* ABA40444.1)	5.0E−64	x
	83T43-4-4f	(0.32)	GR557667	P	185	SM10(DCN1) gene (*N. tab.* DQ885938.1)	2.0E−20	n
	86T32-4-2f	(<0.1)	GR557669	R	267	Put. pollen-specific LIM domain-containing prot. (*S. lyc.* AAX73300.1).	1.0E−39	x
	97C24-2-2f	(0.23)	GR557678	St	327	Beta-tubulin (*N. ate.* AAR37366.1)	4.0E−36	x
	113C34-2-2f	(0.37)	GR557612	T	239	Cytoc.P450-depen. fatty acid hydroxylase (*N. tab.* AAL54887.1)	3.0E−15	x

Real-time PCR

Primer sequences were designed with Primer3 v.0.4.0 software (Rozen and Skaletsky, 2000), and analyzed TDFs and product sizes are indicated in Supplementary Table 2. cDNA was synthesized from 1 μg total RNA from the same samples used in the cDNA-AFLP analysis with the M-MuLV RT enzyme (Promega, USA) and $d(T)_{22}$ oligonucleotide, following the manufacturer's instructions. DNAse treatment was not utilized, since control PCR reactions without the reverse transcription step yielded no products. Moreover, PCR products of the TDF 24C23-1-1b using genomic DNA or cDNA as a template were sized differently, allowing for the detection of genomic DNA contamination. Real-time PCR was performed with a Realplex Instrument (Eppendorf) equipped with Realplex Software version 4.0. Reactions were performed with 1 μL cDNA template and a homemade SYBR green-I reaction mixture (Karsai et al., 2002), containing 1:50,000 diluted SYBR green-I (Invitrogen), 10 pmol of each primer, 0.5 U Platinum-Taq DNA polymerase (Invitrogen), 40 mmol dNTPs, 3.75 mM $MgCl_2$ and 1× Platinum-Taq buffer in a final volume of 20 μL under the following conditions: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 59 °C for 20 s and 72 °C for 40 s. Fluorescent intensity data were acquired during the 72 °C extension step. Specificity of the amplification reactions was assessed by agarose gel electrophoresis and melting curve analyses, which were run at 95 °C for 15 s and 60 °C for 15 s followed by an increase in temperature from 60 to 85 °C (0.2 °C/s) with continuous fluorescence recording. To perform the analysis of relative expression we used the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), normalizing to actin expression levels. Tobacco actin primers were provided by Dr. Nicolás Blanco. All real-time PCR experiments were performed in duplicate.

Results

Analysis of differentially expressed genes during tobacco hypersensitive response to Xac

Xac produces a nonhost plant-pathogen interaction in tobacco plants, causing the HR to occur (Dunger et al., 2005). Tobacco leaves were infiltrated with Xac and 10 mM $MgCl_2$ as a control. Bacterial growth curves were constructed to corroborate the interaction status (data not shown). RNA was extracted at 2, 8 and 24 hpi for Xac and at 2 hpi for control treatments and subjected to the cDNA-AFLP protocol.

A total of 39 primer combinations were used on two biological replicates. The expression profiles were highly reproducible and the replicates showed a high correlation. A section of a typical cDNA-AFLP electrophoresis profile is shown in Fig. 1. Each combination of primers produced patterns of approximately 50 fragments on average, which ranged from 100 to 800 bp (Fig. 1). The primers used in these reactions included the forward primer BstYI-C-AT and three different reverse primers: MseI-A, MseI-G and MseI-T. The four treatments employed are shown with their respective duplicates (Fig. 1).

A total of 2087 TDFs were analyzed, out of which 316 displayed differential expression (Supplementary Table 3). Considering only

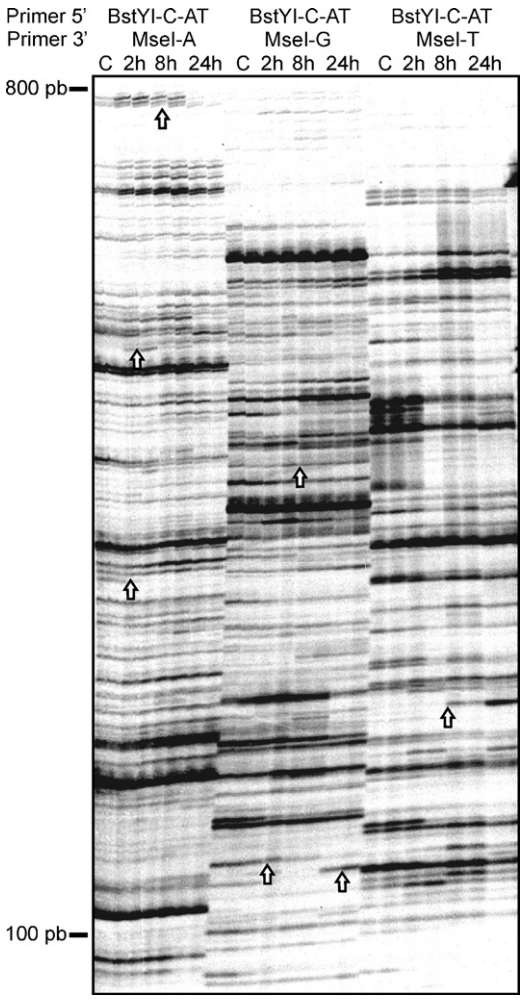

Fig. 1. Example of the results obtained from the cDNA-AFLP analysis. TDFs resulting from selective amplification by using three combinations of primers that are indicated in the top of the figure. Rows correspond to controls (C), infected 2 hpi (2 h), infected 8 hpi (8 h) and infected 24 hpi (24 h) in duplicate. Arrows indicate examples of TDFs that were selected in the analysis: for the primer pair combination BstYI-C-AT/MseI-A, TDFs 24 h down, 8 h up and 2 h down are shown from the top to the bottom, respectively; for the primer pair BstYI-C-AT/MseI-G, TDFs without modification in expression, 8 h down and 24 h up, respectively; and for the primer pair BstYI-C-AT/MseI-T, a TDF 8 h up.

those expression changes in which the signal intensities were doubled or decreased by half (ratios values $\geq$2 or $\leq$0.5), differentially expressed TDFs were selected and classified by comparing Xac 2 hpi with control 2 hpi, Xac 8 hpi with Xac 2 hpi and Xac 24 hpi with Xac 8 hpi, respectively (Fig. 1). The number of differentially expressed TDFs belonging to each group is shown in Supplementary Table 3. The greatest change in the expression pattern, for both repressed and induced TDFs (down- and up-regulated, respectively), corresponded to 8 hpi, which contained a prevalence of up-regulated transcripts (Supplementary Fig. S1, Supplementary Table 3). At 2 hpi, the number of up-regulated genes was higher than the down-regulated ones, while at 24 hpi, this pattern was reversed (Supplementary Fig. S1, Supplementary Table 3). *In silico* analysis allowed us to calculate that 20% of the whole tobacco transcriptome was covered in this study (Supplementary Table 3). A total of 96 bands were isolated and PCR re-amplified, and fragment sizes were confirmed in agarose gels (data no shown). Out of them, 82 TDFs were successfully sequenced, presenting a size range of 151–522 bp with an average length of 269 bp.

Classification of differentially expressed TDFs

The 82 sequenced TDFs were sorted into 11 categories (Fig. 2, Supplementary Fig. S2). The list of the 68 homology-assigned TDFs with their corresponding protein similarities is given in Table 1. Putative functions were assigned to 56 TDFs (68.3% of the analyzed sequences). Despite the categories "unknown" and "without homologies" (14.6% and 17.1% of the analyzed sequences, respectively), the largest set of genes corresponded to the functional groups involved in primary and protein metabolism (19.9% and 9.8%, respectively). A minor number of TDFs belonged to the disease defense, signal transduction and DNA transcription regulation categories (7.3%, 8.5% and 4.9%, respectively) (Table 1, Fig. 2, Supplementary Fig. S2).

The disease defense category included six TDFs displaying differential expression. The TDFs 79T43-4-7a and 103T12-1-5a, induced at 2 hpi, shared homology with *Solanum tuberosum* (potato) ESTs similar to a chitinase of *Medicago truncatula* and a *Capsicum annuum* (pepper) cell wall peroxidase, respectively (Table 1, Fig. 2, Supplementary Fig. S2). The four remaining TDFs, induced at 8 hpi, included 30C23-4-2c with homology to an uncharacterized lipase class 3, 41C24-4-4c, homologous to a tobacco class V chitinase/lisozyme, 45C14-1-7c, homologous to a potato ribonuclease Bet v I and 95C24-2-3c, homologous to a *Solanum lycopersicum* (tomato) ethylene-inducing xylanase receptor (Table 1, Fig. 2, Supplementary Fig. S2).

Seven TDFs were classified as being involved in signal transduction. These included five TDFs homologous to different protein kinases (TDFs 1T13-2-1a, 5T13-4-5a and 98C14-2-1a induced at 2 hpi; 110T12-4-1c induced at 8 hpi; 92T21-2-2e induced at 24 hpi), one to heptahelical transmembrane protein 1 (HHP1) (70C23-2-1d, repressed 8 hpi) and one to an enzyme involved in ethylene synthesis (102C14-2-2d repressed at 8 hpi) (Table 1, Fig. 2, Supplementary Fig. S2).

Similar to the transcription factors, four TDFs were classified as being involved in DNA transcriptional regulation (Table 1, Fig. 2, Supplementary Fig. S2). The TDF 37C24-4-7a (induced at 2 hpi) presented homology with an Agenet Tudor-like domain, the TDF 125C42-2-2c (induced at 8 hpi) with a WRKY transcription factor and the TDFs 64C23-2-4f and 86T32-4-2f (repressed at 24 hpi) with a WD-repeat protein and a putative pollen-specific LIM domain-containing protein, respectively.

Eight TDFs were classified as being involved in protein metabolism (Table 1, Fig. 2, Supplementary Fig. S2). In this group, four TDFs were related to the proteosomal processing of proteins (108T12-1-1c and 109T12-2-2c induced at 8 hpi; 77T13-3-1f and 83T43-4-4f repressed at 24 hpi), three were similar to proteases (42C24-3-4c induced at 8 hpi; 66C23-4-1d repressed at 8 hpi; 71T31-2-4e induced at 24 hpi) and one was similar to a protease inhibitor (10T31-4-3a induced at 2 hpi).

The transport category clustered 6 TDFs (Table 1, Fig. 2, Supplementary Fig. S2), including those similar to the electron transporter cytochrome P450 (120C42-4-1a induced at 2 hpi), a water channel protein (18T31-3-3c induced at 8 hpi) and a glycine-rich protein/protease inhibitor/seed storage/LTP family (21T13-4-10c induced at 8 hpi).

There were 13 TDFs classified as being involved in metabolism (Table 1, Fig. 2, Supplementary Fig. S2), including those homologous to arginine decarboxylase (ADC) 2 (48C24-1-1c induced 8 hpi), an UDP-glucose transglucosylase-like protein involved in cellulose biosynthesis (49C24-1-3c induced 8 hpi), an amino oxidase (68C23-4-5d repressed 8 hpi) and a HMG-CoA synthase (114C34-2-4c induced 8 hpi). These four TDFs are generally considered as part of the defense response.

Moreover, six TDFs were classified as being involved in energy, four as part of structural cell components and two as being involved

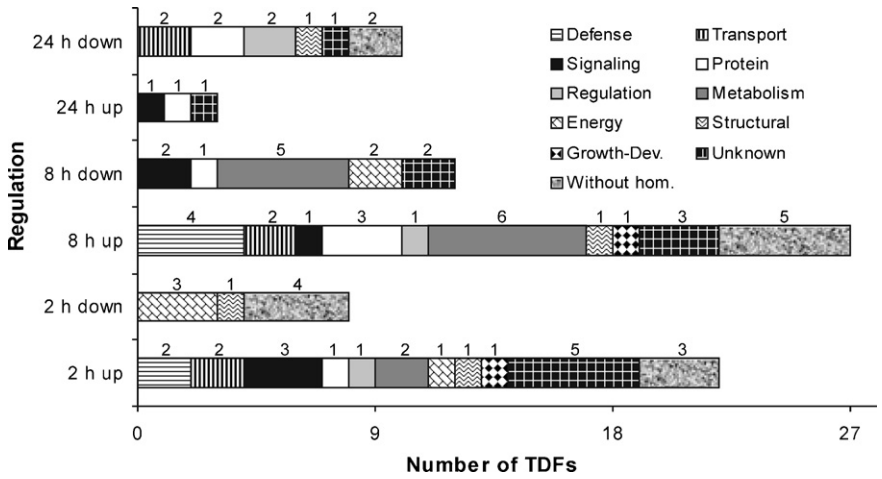

Fig. 2. Distribution of TDFs in functional categories based on putative functions as deduced from BLAST analysis. Classification is shown for each time point (2, 8 or 24 hpi) and type (up or down) of regulation. The numbers above the bars indicate the number of sequences in each category. Defense: disease defense; Signaling: signal transduction; Protein: protein metabolism; Growth-Dev.: growth and development, Regulation: DNA transcriptional regulation; Unknown: unknown function; Without hom.: without homology. The names of the other categories are clearly indicated in the figure.

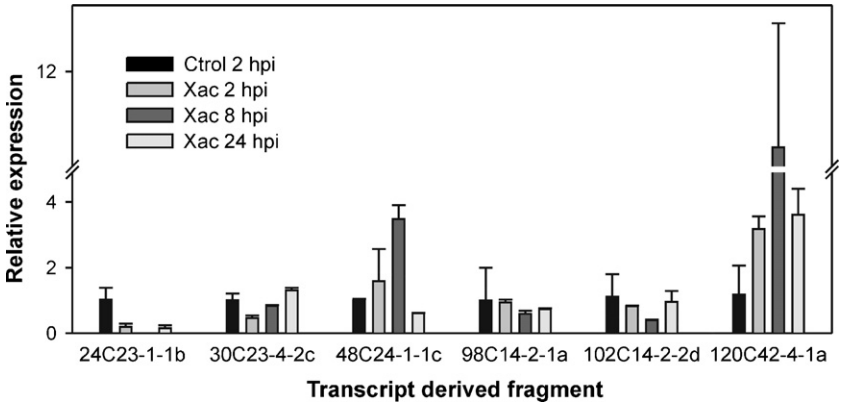

Fig. 3. Real-time PCR showing the expression levels for selected TDFs that are indicated below the axis. Columns show the expression value relative to the control, and bars indicate standard deviations. Treatments are indicated in the figure. The experiment was performed twice with identical RNA samples, obtaining similar results.

in growth and development (Table 1, Fig. 2, Supplementary Fig. S2).

In addition, 12 sequences comprising 14.3% of the TDFs exhibited significant similarities with proteins of unknown function, and thus they were referred to as unknown (Table 1, Fig. 2, Supplementary Fig. S2). Eight of them showed homologous ESTs from stress libraries, including some involved in plant-pathogen interactions (Supplementary Table 5).

The remaining 17.1% of the sequences (14 TDFs) encoded putative proteins with insufficient or no similarity to the GenBank databases and were classified as without homology (Supplementary Table 4, Fig. 2, Supplementary Fig. S2).

Expression analysis

The expression pattern was validated by real-time PCR. The selected TDFs were 98C14-2-1a and 120C42-4-1a (induced at 2 hpi), 24C23-1-1b (repressed at 2 hpi), 30C23-4-2c and 48C24-1-1c (induced at 8 hpi) and 102C14-2-2d (repressed at 8 hpi). These TDFs were putatively involved in pathogen defense (30C23-4-2c or lipase class 3 and 98C14-2-1a or kinase receptor), signaling (102C14-2-2d or ACC oxidase), transport (120C42-4-1a or cytochrome P450) and energy (24C23-1-1b or glycolate oxidase). The TDF 48C24-1-1c (induced at 8 hpi) or ADC, reported previously as being induced in tobacco during incompatible interactions (Sugimoto et al., 2004), was used as an expression profiling control. The expression profiles were confirmed for TDFs 24C23-1-1b, 48C24-1-1c, 102C14-2-2d and 120C42-4-1a (Fig. 3). The expression profile was similar for TDF 30C23-4-2c, which presented higher relative increase at 8 hpi (Fig. 3). The pattern could not be confirmed by real-time PCR for TDF 98C14-2-1a (Fig. 3).

Discussion

The discovery of plant molecular mechanisms responsible for pathogen defense may provide valuable information in the search for disease control alternatives. These mechanisms involve regulation at the transcriptional level, and for this reason, great effort is being made to detect genes that are differentially expressed during this plant response. Although tobacco has been used as a model in different studies including those looking at plant–pathogen interactions, its nonhost response transcriptome has not yet been analyzed. In this work, the tobacco genes involved in nonhost resistance to the bacterial pathogen Xac were detected using cDNA-AFLP. cDNA-AFLP has been used to study host plant responses in several plant–pathogen interactions allowing the detection of many genes related to different functions (Durrant et al., 2000; Santaella et al., 2004; Kemp et al., 2005).

Here, a time course study of the tobacco nonhost response transcriptome was carried out. In previous works, we have shown that the initial symptoms of the tobacco HR against Xac are observed at 8 hpi, while tissue necrosis occurs at 24 hpi. To detect genes that are differentially expressed before, during and after the appearance of these symptoms, samples were taken and analyzed at 2, 8 and 24 hpi, respectively. As it was shown previously, this post-inoculation period thoroughly covers the expression pattern of representative tobacco genes that are part of the nonhost response (Oh et al., 2006), thus increasing the chance of detecting genes that could be putatively involved in the defense response. Previous literature has employed variable control designs in their plant response time course studies. Several authors have chosen just a reference sample, generally non-infected leaves or the earliest pi point (Santaella et al., 2004; Kemp et al., 2005). The specific symptoms associated with the nonhost response such as ROS formation, membrane disruption and LCD have not been observed in tobacco leaves after control treatment at the pi times selected (Dunger et al., 2005; Daurelio et al., 2009; data not published). Consequently, we considered that the control inoculation should not produce major changes in transcription of the nonhost response-related genes. The 2 hpi control allowed us to have a reference point, making it possible to compare the 2 hpi bacterial treatment. In this context, we could assume that the differential expressions detected at 2, 8 and 24 hpi were mainly related to the presence of the bacterial pathogen.

A total of 2087 TDFs were obtained, 316 of which displayed differential expression, and 82 were sequenced. The highest number of modifications in the tobacco transcriptome was observed at 8 hpi, which correlated with symptom occurrence, while the lowest number of modifications was detected at 24 hpi, due to tissue necrosis (Supplementary Fig. S1, Supplementary Table 3). At early time intervals post-infection (2 and 8 hpi) induced TDFs prevailed, which indicated transcriptional activation, while these findings were reversed at late time post-inoculation (24 hpi, Supplementary Fig. S1, Supplementary Table 3). Based on their deduced sequence similarities, TDFs were assigned to different functional categories, including disease defense, metabolism, DNA transcriptional regulation, signal transduction, transport, protein degradation/storage and energy-associated proteins. At 2 hpi, induced genes belonging to the category "without homology" were the most abundant, while at 8 hpi, induced and repressed genes related to metabolism were markedly affected by the infection (Fig. 2, Supplementary Fig. S2).

Among the six induced TDFs from the disease defense group, three were associated with pathogenesis-related proteins (PR). The TDF 79T43-4-7a (2 hpi) exhibited homology to a potato EST similar to a family 18 chitinase, called h-type or class III, which was characterized by having lysozyme activity. These chitinases, known as PR-8, have been shown to be induced in response to pathogens during incompatible interactions in rice (Park et al., 2004). The TDF 45C14-1-7c (8 hpi) showed homology to a potato pathogenesis-related protein (Table 1), also known as PR-10. This protein displays structural homology to the Bet v I allergen family and to proteins whose expression are induced in response to intracellular pathogens, with some showing ribonuclease activity, suggesting a role in the degradation of pathogenic RNA (Sree Vidya et al., 1999). The TDF 41C24-4-4c (8 hpi) showed homology to a class V chitinase/lysozyme from tobacco (Table 1), within the tobacco PR-3 group. This tobacco allele has demonstrated endoglucanase activity against fungal pathogens and induction after treatment with viruses, ethylene or wounding (Melchers et al., 1994). Our finding is the first report of the tobacco PR-8 and PR-10 alleles, revealing their participation together with the PR-3 allele in the tobacco nonhost response against bacteria.

The other three disease defense TDFs possessed similarities with proteins of diverse function in the plant response. The TDF 103T12-1-5a (2 hpi) showed similarity to cell wall peroxidases. The peroxidase genes are likely up-regulated in response to pathogen elicitors and play a role in pathogen-induced cell wall modifications, including lignification, suberization and oxidative crosslinking (Passardi et al., 2004). The TDF 95C24-2-3c (8 hpi) displayed homology to tomato ethylene-inducing xylanase (EIX) receptor 2 LeEIX2 (Table 1). *LeEix*-induced gene silencing in tobacco suppressed the response to xylanase (Ron and Avni, 2004), while the LeEix2 allele in tomato regulated the induction of defense responses, including the HR (Bar and Avni, 2009). The TDF 30C23-4-2c (8 hpi) showed homology to a rice lipase class 3 (Table 1), which is different from the alleles reported in tobacco EDS1 and the calmodulin-binding heat-shock protein, but similar to an EST from a pathogen-challenged potato library. Although its role is still unknown, EDS1 is fundamental to the plant-pathogen response (Hu et al., 2005), indicating the significance of this lipase family in situations of biotic stress. Consequently, novel tobacco alleles from the cell wall peroxidase, EIX receptor and lipase class 3 involved in the nonhost response were reported.

Another group of noteworthy induced TDFs is reported. WRKY transcription factors constitute a protein superfamily that regulates the expression of many plant genes during biotic stress (Eulgem et al., 2000), and the TDF 125C42-2-2c (8 hpi) showed homology to potato WRKY6 (Table 1) and tobacco WRKY4. Tobacco WRKY4 was induced by salicylic acid and in host resistance to the tobacco mosaic virus (TMV) (Chen and Chen, 2000). The participation of polyamine metabolism in plant–pathogen interactions has also been reported, and the TDF 48C24-1-1c (8 hpi) displayed high homology to tobacco ADC2. Tobacco ADC1 has been shown to be induced during the host HR (Yoda et al., 2002) in necrotrophic interactions (Marina et al., 2008) and nonhost bacterial interactions (Yoda et al., 2009). The TDF 85T32-4-1c (8 hpi) showed similarities to a Vitis putative protein containing a RNA recognition motif (RRM) (Table 1). RRM proteins are involved in pre-mRNA splicing via interactions with specific RNA sequences, mainly through the RRM domain, and their function in plant-pathogen interactions has not yet been reported. The TDF 120C42-4-1a (2 hpi) showed similarities to the pepper cytochrome P450 CaCYP1 (Table 1). CaCYP1 was induced during the HR, and its silencing resulted in enhanced bacterial growth and reduced expression of defense-related genes, suggesting it is a fundamental part of the pepper defense response (Oh et al., 2007). The current analysis reveals the participation of the WRKY4 transcription factor, the ADC2 allele, a novel tobacco cytochrome P450 allele and a novel tobacco RRM-containing protein in the tobacco nonhost response during bacterial attack.

In addition, three significantly repressed TDFs were detected. The TDF 102C14-2-2d (8 hpi) showed homology to NT-ACO1, a tobacco aminocyclopropane-1-carboxylic acid oxidase allele (ACC oxidase or ACO). After inoculation with HR-inducing TMV, the tobacco ACO genes EFE-26, EFE-27 and DS321 showed increased expression (Ohtsubo et al., 1999), but one ACO gene from *Nicotiana glutinosa* was suppressed (Kim et al., 1998). The TDF 24C23-1-1b (2 hpi) showed similarities to the H_2O_2-producing enzyme glycolate oxidase (GLOX) (Table 1). GLOX was strongly down-regulated in the barley host-incompatible response to *Bipolaris sorokiniana* (Schäfer et al., 2004), suggesting that photorespiration was not the main source for the observed H_2O_2 accumulation. The TDF 70C23-2-1d (8 hpi) presented similarities to Arabidopsis HHP1. Five HHP genes similar to human AdipoRs were studied in Arabidopsis that showed HHP1 repression during syringolin treatment and temperature stress (Hsieh and Goodman, 2005). Here, we report NT-ACO1 as a repressed ACO allele with ROS formation that is independent of photorespiration and the involve-

ment of a novel tobacco HHP1 allele in tobacco HR athwart bacteria.

A group of TDFs with unknown function showed homology to ESTs from stressed Solanaceae libraries, including five ESTs detected in pathogen-challenged plants (Supplementary Table 5). Two TDFs (2T13-3-2a, 4T13-4-2a) showed sequences and induced expression patterns similar to TDFs detected in hydrogen peroxide-stressed tobacco (Vandenabeele et al., 2003). The above mentioned similarities suggest that these new proteins might be involved in the plant defense response. Further investigation will be necessary to know their function.

One of the most important advantages of the cDNA-AFLP technology is the possibility of discovering novel genes. In this work, several tobacco TDFs showed no similarity to proteins in databases and likely represented novel plant defense proteins. Furthermore, other TDFs did not exhibit homology with any previously reported tobacco sequences, thus constituting putative novel tobacco defense proteins. On the other hand, since fragments were rather small, TDFs presenting with homology to the same protein but corresponding to alternatively spliced products could not be distinguished with this technique. Nevertheless, each TDF assigned in this study presented with a particular homology, and no similarities between them were observed.

Although there is a high level of reproducibility associated with cDNA-AFLP, TDF profile corroboration using alternative techniques is recommended. The manner of validating TDF pattern repeatability varies greatly between different reports. Here, to have a broad idea of the results obtained, 4 expression patterns categories were validated (2 hpi up, 2 hpi down, 8 hpi up and 8 hpi down) using real-time PCR. A total of 5 TDFs out of the 6 analyzed were confirmed, spanning all categories (1 of 2, 1 of 1, 2 of 2 and 1 of 1, respectively) and showing a good correlation between both results. The putative function of corroborated TDFs has been discussed previously. The discrepancy observed may be due to the techniques employed or to the gene family complexity.

Our transcriptome-analytical data contribute to a better understanding of the molecular mechanisms involved in the nonhost defense response to bacterial infection of tobacco and may also apply to other species under similar stress. In summary, in this work we have identified PR-8 and PR-10 chitinases, a peroxidase, an ethylene-inducing xylanase receptor, a lipase class 3, a protein with a RRM domain, a cytochrome P-450 and a HHP receptor as novel genes involved in the nonhost response. We suggest these novel genes as possible targets in transgenic breeding for the improvement of plant resistance to pathogen attacks.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT PICT 01-12783 to EGO) and from CONICYT, Chile (FONDECYT 1060494 to L.H.). EGO and JO are staff members and LDD and SP are Fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

The authors particularly thank the AMSUD Pasteur program for funding LDD's stay at the Pontificia Universidad Católica de Chile. We are also grateful to Dr. Estela M. Valle and Dr. Daniela V. Rial for providing corrections and making very important contributions to this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.07.014.

References

Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG. Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 1996;9:745–53.

Bar M, Avni A. EHD2 inhibits ligand-induced endocytosis and signaling of the leucine-rich repeat receptor-like protein LeEix2. Plant J 2009;59:600–11.

Blanco F, Garreton V, Frey N, Dominguez C, Perez-Acle T, Van der Straeten D, et al. Identification of NPR1-dependent and independent genes early induced by salicylic acid treatment in Arabidopsis. Plant Mol Biol 2005;59:927–44.

Brunings AM, Gabriel DW. *Xanthomonas citri*: breaking the surface. Mol Plant Pathol 2003;4:141–57.

Chen C, Chen Z. Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. Plant Mol Biol 2000;42:387–96.

Daurelio L, Tondo ML, Dunger G, Gottig N, Ottado J, Orellano EG. Hypersensitive response. In: Narwal SS, Catalán AN, Sampietro DA, Vattuone MA, Polyticka B, editors. Book on Plant Bioassays. Houston: Studium Press; 2009. p. 187–206.

Donson J, Fang Y, Espiritu-Santo G, Xing W, Salazar A, Miyamoto S, et al. Comprehensive gene expression analysis by transcript profiling. Plant Mol Biol 2002;48:75–97.

Dunger G, Arabolaza LN, Gottig N, Orellano EG, Ottado J. Participation of *Xanthomonas axonopodis* pv. *citri hrp* cluster in citrus canker and in non-host plant responses. Plant Pathol 2005;54:781–8.

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE, Jones JD. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell 2000;12:963–77.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. Trends Plant Sci 2000;5:199–206.

Hsieh MH, Goodman HM. A novel gene family in Arabidopsis encoding putative heptahelical transmembrane proteins homologous to human adiponectin receptors and progestin receptors. J Exp Bot 2005;56:3137–47.

Hu G, deHart AK, Li Y, Ustach C, Handley V, Navarre R, et al. EDS1 in tomato is required for resistance mediated by TIR-class R genes and the receptor-like R gene Ve. Plant J 2005;42:376–91.

Kamoun S. Nonhost resistance to Phytophthora: novel prospects for a classical problem. Curr Opin Plant Biol 2001;4:295–300.

Karsai A, Muller S, Platz S, Hauser MT. Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques 2002;32:790–6.

Kazan K, Schenk PM, Wilson I, Manners JM. DNA microarrays: new tools in the analysis of plant defence responses. Mol Plant Pathol 2001;2:177–85.

Kemp B, Beeching J, Cooper R. cDNA-AFLP reveals genes differentially expressed during the hypersensitive response of cassava. Mol Plant Pathol 2005;6:113–23.

Kim YS, Choi D, Lee MM, Lee SH, Kim WT. Biotic and abiotic stress-related expression of 1-aminocyclopropane-1-carboxylate oxidase gene family in *Nicotiana glutinosa* L. Plant Cell Physiol 1998;39:565–73.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-(\Delta\Delta CT)}$ method. Methods 2001;25:402–8.

Mahalingam R, Gomez-Buitrago A, Eckardt N, Shah N, Guevara-Garcia A, Day P, et al. Characterizing the stress/defense transcriptome of Arabidopsis. Genome Biol 2003;4:R20.

Marina M, Maiale SJ, Rossi FR, Romero MF, Rivas EI, Garriz A, et al. Apoplastic polyamine oxidation plays different roles in local responses of tobacco to infection by the necrotrophic fungus *Sclerotinia sclerotiorum* and the biotrophic bacterium *Pseudomonas viridiflava*. Plant Physiol 2008;147:2164–78.

Melchers LS, potheker-de GM, van der Knaap JA, Ponstein AS, Sela-Buurlage MB, Bol JF, et al. A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. Plant J 1994;5:469–80.

Nürnberger T, Lipka V. Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol 2005;6:335–45.

Oh SK, Lee S, Chung E, Park JM, Yu SH, Ryu CM, et al. Insight into types I and II nonhost resistance using expression patterns of defense-related genes in tobacco. Planta 2006;223:1101–7.

Oh BJ, Ko MK, Kim YS, Kim KS, Kostenyuk I, Kee HK. A cytochrome P450 gene is differentially expressed in compatible and incompatible interactions between pepper (*Capsicum annuum*) and the anthracnose fungus, *Colletotrichum gloeosporioides*. Mol Plant Microbe Interact 2007;12:1044–52.

Ohtsubo N, Mitsuhara I, Koga M, Seo S, Ohashi Y. Ethylene promotes the necrotic lesion formation and basic PR gene expression in TMV-infected tobacco. Plant Cell Physiol 1999;40:808–17.

Park CH, Kim S, Park JY, Ahn IP, Jwa NS, Im KH, et al. Molecular characterization of a pathogenesis-related protein 8 gene encoding a class III chitinase in rice. Mol Cells 2004;17:144–50.

Passardi F, Longet D, Penel C, Dunand C. The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 2004;65:1879–93.

Ramonell KM, Somerville S. The genomics parade of defense responses: to infinity and beyond. Curr Opin Plant Biol 2002;5:291–4.

Ron M, Avni A. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. Plant Cell 2004;16:1604–15.

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.

Santaella M, Suárez E, López C, González C, Mosquera G, Restrepo S, et al. Identification of genes in cassava that are differentially expressed during infection with *Xanthomonas axonopodis* pv. *manihotis*. Mol Plant Pathol 2004;5: 549–58.

Schäfer P, Huckelhoven R, Kogel KH. The white barley mutant albostrians shows a supersusceptible but symptomless interaction phenotype with the hemibiotrophic fungus *Bipolaris sorokiniana*. Mol Plant Microbe Interact 2004;17:366–73.

Sree Vidya CS, Manoharan M, Lakshmi Sita G. Cloning and characterization of salicylic acid-induced, intracellular pathogenesis-related gene from tomato (*Lycopersicon esculentum*). J Biosci 1999;24:287–93.

Sugimoto M, Yamaguchi Y, Nakamura K, Tatsumi Y, Sano H. A hypersensitive response-induced ATPase associated with various cellular activities (AAA) protein from tobacco plants. Plant Mol Biol 2004;56:973–85.

Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, Kruger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 2004;37:914–39.

Thordal-Christensen H. Fresh insights into processes of non-host resistance. Curr Opin Plant Biol 2003;6:351–7.

Vandenabeele S, Van Der Kelen K, Dat J, Gadjev I, Boonefaes T, Morsa S, et al. A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proc Natl Acad Sci USA 2003;100:16113–8.

Vranová E, Atichartpongkul S, Villarroel R, Van Montagu M, Inzé D, Van Camp W. Comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. Proc Natl Acad Sci USA 2002;99: 10870–5.

Yoda H, Ogawa M, Yamaguchi Y, Koizumi N, Kusano T, Sano H. Identification of early-responsive genes associated with the hypersensitive response to tobacco mosaic virus and characterization of a WRKY-type transcription factor in tobacco plants. Mol Genet Genomics 2002;267:154–61.

Yoda H, Fujimura K, Takahashi H, Munemura I, Uchimiya H, Sano H. Polyamines as a common source of hydrogen peroxide in host- and nonhost hypersensitive response during pathogen infection. Plant Mol Biol 2009;70: 103–12.