IDENTIFICATION OF TOMATO GENES DIFFERENTIALLY EXPRESSED DURING COMPATIBLE INTERACTION WITH *PYRENOCHAETA LYCOPERSICI*

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SUMMARY

Breeding for resistance is the most effective tool for controlling the corky root disease of tomato caused by the fungus *Pyrenochaeta lycopersici*. However, little is known about the molecular bases of tomato-*P. lycopersici* interaction. In order to identify genes involved in the basal defence response activated in a susceptible cultivar and in disease symptom development, a set of cDNA-AFLP fragments derived from a profiling experiment was analysed. A total of 247 differentially expressed TDFs (transcript-derived fragments), identified as putative tomato genes, were characterized by similarity searches, and classified into 11 broad functional classes. Timings ranging between the early [48, 72 and 96 h post-infection (hpi)] and the late infection stages (20 and 27 dpi) were used. The changes of tomato root transcriptional profiles showed large differences in quantity and quality between the early and late stages of infection. Mechanisms of basal defence were most likely activated at early stages, when a gene coding for a receptor-like serine-threonine protein kinase and other genes of the signalling class were upregulated. At 20 dpi some of the mechanisms involved in defence were still activated, while at 27 dpi a general repression of gene expression was observed.

Key words: transcriptomics, cDNA-AFLP, corky root, defence response, host-pathogen interaction.

INTRODUCTION

Plants defend themselves from a variety of pathogens employing both preformed barriers and inducible molecular responses. Various responses are activated by two main branches of their defence system (Jones and Dangl, 2006). The first branch, called basal defence response, is activated when extracellular pathogen-associated molecular patterns (PAMPs) common to many classes of microbes, are recognized by transmembrane pattern recognition receptors (PRRs). Successful pathogens, however, are able to defeat this line of defence leading to a compatible interaction. The second branch of the plant defence system, represented by products of resistance (R) genes, specifically recognizes corresponding pathogen avirulence (Avr) factors, according to the well known gene-for-gene model (Flor, 1971) and triggers a rapid incompatibility resistance response. Defence responses activated by both PRR and R proteins are in part qualitatively similar through the pathway towards effector defence proteins, however, in R gene-mediated resistance, they develop faster and with higher intensity (Tao *et al*., 2003) and are associated with a localized programmed cell death (hypersensitive response) limiting pathogen growth (Nimchuk *et al*., 2003). In the basal PRR-mediated defence, the signalling may be less rapid and less robust, giving the pathogen a greater opportunity to overcome the defences, thus leading to a compatible reaction and disease development (Tao *et al*., 2003). Pathogens have evolved a variety of virulence determinants trying to misdirect host defences and to derive nutrients from the plant.

Transcriptional profiling of plant responses to pathogens revealed in the last 10 years novel aspects in compatible and incompatible interactions between plants and their pathogens. A number of new candidate genes possibly involved in the interactions of wheat and *Puccinia striiformis* f. sp. *tritici* in both compatible and incompatible interactions were identified (Wang *et al*., 2010). These authors found that plant responses in both cases were qualitatively similar, although quantitative differences could be observed soon after infection. Transcriptome analysis of barley/*Blumeria graminis* interactions demonstrated that the expression patterns of PRR-triggered basal defence-related genes were similar in compatible and incompatible interactions during the first hours of infection. However, the suppression of basal defence-related transcripts observed in the compatible interactions after several hours from infection suggests that: (i) the inhibition of basal defence promotes the development of pathogen's haustoria, consequently delaying or preventing the onset of host defence

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responses (Caldo *et al*., 2006); (ii) host-specific resistance could have evolved from the ability of plants to prevent the pathogen from suppressing the plant's basal defence (Caldo *et al*., 2004).

Pathogenic fungi can be divided in biotrophs, necrotrophs and hemibiotrophs. Biotrophic pathogens obtain nutrients from living host tissue, while necrotrophic ones feed on dead or dying cells. Many others, the so-called hemibiotrophs, act as both biotrophs and necrotrophs, depending on the stages of their life cycle (Glazebrook, 2005; Spoel *et al*., 2007). A study on both compatible and incompatible interaction of Arabidopsis and *Golovinomyces cichoracearum* (former *Erysiphae cichoracearum*), constituted an important step towards understanding the molecular mechanisms involved in the establishment of compatible interactions with obligate biotrophs. Transcript profiling experiments indicated that gene expression changes in response to infection might support fungal nutrition by promoting alterations in host metabolism (Fabro *et al*., 2008). In fact, under conditions leading to compatibility, in the obligate biotrophic interaction of *Uromyces fabae* with its host *Vicia faba*, global gene expression analysis revealed significant changes in host gene expression, not only in the immediate vicinity of the primary infection but also in some distant organs, like stems and roots (Wirsel *et al*., 2001). Except for these reports, however, few studies have examined plant defence programs and global host metabolic features leading to the establishment of compatibility; a plethora of other studies have dealt with incompatible interactions.

Tomato corky root rot (CRR), caused by the hemibiotrophic fungus *Pyrenochaeta lycopersici*, is a soilborne disease that represents a serious concern for many tomato-growing areas, including major producers such as China, USA, Italy and Japan, both in the greenhouse and in the field. CRR causes progressive deterioration of the entire root system. Initially, infected roots develop necrotic lesions that continue to expand until small roots are rotted, while larger roots get thicker and darker. In the advanced stages of infection, typical corky lesions develop exposing the vascular bundles and limiting the uptake of water and nutrients. As a result, plants become stunted and suffer severe fruit yield losses (Last and Ebben, 1966; Goodenough and Maw, 1973; Pohronezny and Volin, 1991).

Although the majority of cultivated tomato varieties are susceptible to the fungus, resistance occurs in several wild relatives. For instance: (i) a CRR resistance gene from *Solanum glandulosum* with a low degree of dominance was reported (Hogenboom, 1970); (ii) the resistance of *S. habrochaites*, mediated by a single dominant gene, was used for resistant rootstock development (Smith and Proctor, 1965); (iii) the recessive *pyl* gene was introgressed from *S. peruvianum* into a *S. lycopersicum* background (Laterrot, 1983) and two tomato cultivars, Mogeor and Moboglan, were obtained (Laterrot, 1987). The *pyl* gene was then mapped to the tip of the short arm of chromosome 3 of tomato (Doganlar *et al*., 1998). However, the molecular bases of the tomato resistance/tolerance to *P. lycopersici* are still unknown. Recently, a cDNA-AFLP based approach was chosen for the identification of fungal genes differentially expressed in a compatible interaction of tomato with *P. lycopersici* (Aragona and Infantino, 2008). With the present work, the study of the cDNA-AFLP transcriptional profiles was continued in order to identify and characterize the tomato counterpart, and to further understand the mechanisms that underlie this compatible interaction.

MATERIALS AND METHODS

Sequence analysis. A total of 673 putatively differentially expressed transcripts were obtained from the cDNA-AFLP experiment (Aragona and Infantino, 2008). Briefly, young tomato plants (second leaf stage) of cv. Corbarino, grown in greenhouse at 28°C, were inoculated by dipping for 60 sec the entire roots in a homogenate of ISPaVe ER-1211 *P. lycopersici* isolate grown on potato dextrose agar (PDA), then transplanted in pots containing sterile vermiculite and maintained in a greenhouse until evaluation. Control plants were treated similarly but their roots were dipped in PDA without the fungus. For cDNA-AFLP analysis, the taproots were sampled and total RNA was extracted using the RNeasy midi kit (Qiagen, Germany) at five times ranging between the early [48, 72 and 96 h post-infection (hpi)] to the late infection stages (20 and 27 dpi). The cDNA-AFLP protocol applied (Breyne *et al*., 2003) was as follows: double stranded cDNA was synthesized from 200 µg total RNA, digested with *Bst*YI and the 3' ends were captured on streptavidin magnetic beads (DynaI). After digestion with *Mse*I, the released fragments were ligated to *Bst*YI and *Mse*I adapters. After preamplification, a 1:20 (v/v) dilution was amplified with 32 selective primer combinations, carried out with two selective bases on the *Bst*YI primer and one selective nucleotide on the *Mse*I primer. In each combination, one of the primers was labeled with ^{33}P-γ-ATP for detection. Amplified fragments were separated in 6% polyacrylamide gels and the bands corresponding to expressed transcripts were visualized after autoradiography. Four hundred and nine bands that were present only in control samples, or whose intensity in controls was higher than in inoculated samples, were classified as downregulated; 264 bands present only, or with higher intensity, in infected samples were classified as upregulated. The upregulated transcript-derived fragments (TDFs) corresponding to infected samples, thus of plant and fungal origin, had been sequenced in a previous

work by Aragona and Infantino (2008), giving origin to 216 good quality sequences. These authors had focused on the fungal genes expressed during the interaction with tomato, while TDFs corresponding to plant genes had not been further analysed. The downregulated TDFs were sequenced in the present work; eight already sequenced, randomly chosen, upregulated TDFs were also re-sequenced for confirmation.

Sequence information was obtained either by direct sequencing of the re-amplified PCR products using the selective primers, or after cloning into pGEM-T Easy (Promega, USA) and transformation into $CaCl_2$ competent *Escherichia coli* DH5α cells. In the latter case, five independent clones per fragment were amplified using T7 and SP6 primers. When fragments of homogeneous size were obtained, one clone out of five was selected. Sequencing was performed on the ABI Prism 3730 (Applied Biosystems, USA) using ABI Prism™ BigDye v3.1 Terminator Cycle Sequencing kit (Applied Biosystems, USA) and sequence analysis was done using the Sequence Scanner Software v1.0 (Applied Biosystems, USA). Good quality sequences were obtained for 157 downregulated TDFs, while the eight re-sequenced upregulated TDFs nucleotide sequences were compared for similarity with the previously obtained upregulated dataset. After excluding the short reads (<60 bp), 154 out of 157 downregulated and 199 out of the previously obtained 216 upregulated TDFs (in total 353) were analysed for similarity, using BLAST sequence alignment tools (Atschul *et al*., 1997), for identifying tomato genes differentially expressed during the interaction with *P. lycopersici*. Sequences were evaluated for homology to other gene/protein sequences using BLASTx and/or BLASTn against the following databases: UniProt (http://www.uniprot.org/), DFCI gene index for tomato and potato (http://compbio.dfci.harvard. edu/tgi/), Sol Genomics Network (SGN) (http://solgenomics.net/), and NCBI (http://www.ncbi.nlm.nih.gov/).

The Cogeme database (http://cogeme.ex.ac.uk) was additionally used to identify and exclude any putative fungal sequence from analysis of the upregulated dataset. The following criteria were adopted: e-value cut-off $<e^{-10}$; sequence identity cut-off >80%. After BLAST similarity search, the annotated tomato sequences were manually assigned to broad functional classes as described by Polesani *et al*. (2010) on the basis of literature evaluation and gene ontology. UniProt gene ontology was taken when available; if not, the data from the DFCI tomato/potato gene indices and the SGN databases were used. Defence-related genes were functionally classified as resistance, secondary metabolism, stress response, and cell wall. The other functional classes were as follows: signal transduction, transport, proteolysis, metabolism, electr/energy, and cell component. Differentially expressed genes that exhibited similarity to tomato/potato/plant genes annotated as unknown or with unknown gene ontology were classified under the class unknown and uncharacterized. Classification calls for genes that could fit multiple functional classes were attributed giving priority to the classes with direct or indirect involvement in defence responses.

Validation of differentially expressed transcripts by RT-PCR analysis. Artificial inoculation of roots was repeated, and the cDNA-AFLP data for six TDFs corresponding to tomato transcripts with different expression patterns were validated. Two genes modulated during all timings (one up- and one downregulated), two genes modulated only in the later stages of infection (one up- and one downregulated) and two genes upregulated during all early timings and at 20 dpi were chosen and validated. Semi-quantitative RT-PCR was conducted on total RNA extracted from roots inoculated with the fungus and from non inoculated controls, retrotranscribed using the Superscript II reverse transcriptase (Invitrogen, USA). To remove genomic DNA from RNA preparations, total RNA was treated with RNase-free DNase (Promega, USA). First strand cDNA was amplified with 0.3 μM specific primers. Primer pairs for the selected TDFs were designed on cDNA sequences; primers for actin used for normalization as the constitutive control gene were designed on tomato actin gene sequence GI:1498365 (left primer: CAGCAACTGGGATGATATGG; right primer: ATTTCCCGTTCAGCAGTGGT). All primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) available at http://frodo.wi.mit.edu/primer3.

RESULTS

The homology search of the entire set of 353 down- and upregulated TDFs conducted in the present work enabled the identification and annotation of 247 putative tomato sequences. The number of downregulated tomato genes (143) was higher than that of the upregulated genes (104). All putative genes were assigned to 11 broad functional classes as described by Polesani *et al.* (2010).

Being the present work aimed at the identification of differentially expressed tomato genes, all TDFs putatively classified as *P. lycopersici* genes, the 36 sequences unclassified because of the high similarity to both fungus and plant transcripts, as well as the 38 sequences for which no match was found were excluded from further bioinformatic analysis. As to the eight upregulated TDFs, re-sequenced for validation, the nucleotide sequence data fully confirmed previous results. The highest number of differentially expressed transcripts was identified in the late stages, while early induced genes were significantly less (Fig. 1).

Plant genes modulated at the early stage of infection. Only 19 out of a total of 247 transcripts classified as tomato genes were modulated in the early stages of infection. Among these, seven were exclusively "early" genes, while 12 were also detected as modulated in the late stages of the infection. As shown in Fig. 1, that summarizes the distribution of the "early" and "late" genes among functional classes, the "early" genes belong to only 8 of 11 functional classes. Table 1 reports the putative annotations of early-induced genes. Only two of them, namely a gene coding for cytochrome P450-like protein classified as electr/energy and an uncharacterized putative protein were repressed during all early timings, the former being downregulated also during the later stages of infection (20 and 27 dpi). Interestingly, most of the remaining 17 upregulated genes belonged to signal transduction (6), metabolism (4) and stress response (3) classes (Fig. 1 and Table 1).

At 48 hpi, nine genes were induced and only one of them, encoding the glycine-rich protein LeGRP1, was not modulated at later timings. The remaining eight were induced also at other stages; not only at 72 and 96 hpi, but also in advanced phases of infection. Interestingly, two genes, coding for a putative receptor-like serine-threonine protein kinase and a glutathione S-transferase, respectively, were induced over the entire infection course. Seven genes were newly induced at 72 h and four of them, coding, respectively, for a protease inhibitor, a GTP-binding protein, a 14-3-3 protein and the tRNA synthetase class I (C) family protein, were only transiently induced at this time point (Table 1). Only one gene, coding for a peroxidase classified as stress response, was newly induced at 96 hpi and remained induced over throughout.

Plant genes modulated at the late stages of infection. The largest number of transcripts (240) was modulated in cv. Corbarino at the late infection stage. Twelve of these genes were modulated already at the earlier stages of infection, while 228 were exclusively modulated at a later stage. The distribution of the 240 genes among the 11 functional classes is summarized in Fig. 1. At the later stages of infection, the five classes with the highest number of modulated genes were those denoted as metabolism (27.5%), unknown and uncharacterized (17.5%), signal transduction (16.5%), stress response (9.5%) and transport (9%). Signal transduction and metabolism, the two most modulated classes at the early infection stage, ranked third and first at the late stages (Fig. 1).

Fig. 2 highlights the different behaviour of tomato in this compatible interaction during the last two timings: a larger number of genes was modulated at 27 than at 20 dpi, the majority of the genes were repressed (134) and only 49 were induced. By contrast, the situation was opposite at 20 dpi, the majority of the genes being induced (80) while only nine were repressed. At 20 dpi, metabolism, signal transduction and unknown and uncharacterized were the functional classes with the highest number of upregulated genes while, only two of the downregulated genes were present in each most represented class (metabolism, unknown and uncharacter-

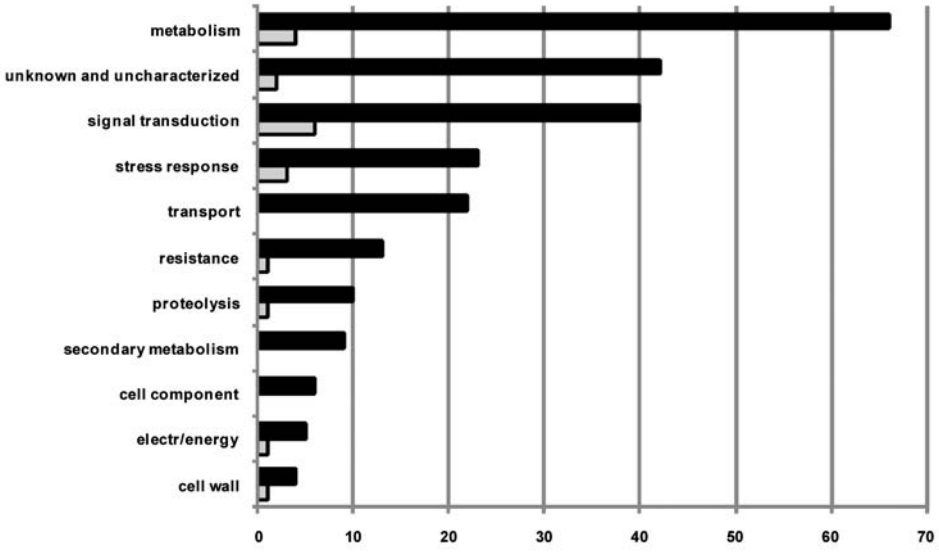

Fig. 1. Distribution of modulated genes among functional classes. Nineteen genes (17 up- and 2 downregulated) whose expression changed at early stages of infection (from 48 to 96 hpi) are shown in grey; 240 genes (98 up- and 142 downregulated) whose expression changed at the late stages of infection (20 and 27 dpi) are shown in black. Early modulated genes that remained modulated at the late stages of infection have been included in 240 late regulated genes as well. The number of genes in each class is given on the X-axis.

Table 1. Tomato genes modulated during the early three timings (48, 72, 96 h post inoculation).

Transcript-derived fragments	Annotation	Hours post inoculation		
		48	72	96
Metabolism				
CCA10	Q6ELF9 Poly(A)-binding protein C-terminal interacting protein 6 (*Cucumis sativus*)	+[a]	+	+L_{20}[b]
CAG1	SGN-U581447 similar to AT2G31170.1 tRNA synthetase class I (C) family protein		+	
TCC2	A5BJX2 Putative uncharacterized protein (*Vitis vinifera*)		+	+
CAG19	tomato|TC242974 homologue to A1BVT0 Cluster: Putative ROX1 (*Nicotiana tabacum*)		+	+L
Unknown and uncharacterized				
CGC1	A5BPV7 Putative uncharacterized protein (*Vitis vinifera*)	-	-	-
TGT20	tomato|TC237367 homologue to Q0IT26 Cluster: Os11g0425600 protein (*Oryza sativa*)	+	+	+L_{20}
Signal transduction				
CCT7	potato|TC223645 similar to Q9XET9 Cluster: Ethylene receptor homolog (*Solanum lycopersicum*)	+	+	+L_{20}
CGC4	A2Q0R1 Putative uncharacterized protein WAKL1 (*Nicotiana tabacum*)	+	+	+L_{20}
CAA30	P2C09 Probable protein phosphatase 2C 9 (*Arabidopsis thaliana*)	+	+	+L_{20}
CAC11	Q8VX53 Putative receptor-like serine-threonine protein kinase (*Solanum tuberosum*)	+	+	+L
CAG3	B4FUE0 GTP-binding protein PTD004 (*Zea mays*)		+	
CAT26	A1E0X8 NAC domain protein NAC2 (*Solanum tuberosum*)		+	+L
Stress response				
CAT23	potato|TC218491 homologue to A5YWI8 Cluster: Glutathione S-transferase (*Solanum commersonii*)	+	+	+L
CCT5	SGN-U592647 14-3-3 protein (*Solanum tuberosum*)		+	
CGT60	Q9XIV9 Peroxidase (*Nicotiana tabacum*)			+L
Resistance				
TCT14	potato|TC218614 weakly similar to Q1G2Y5 Cluster: AT1G05450 Protease inhibitor/seed storage/LTP family protein (*Arabidopsis thaliana*)		+	
Proteolysis				
CAT9	Q93X76 Putative carboxyl-terminal proteinase (*Gossypium hirsutum*)	+	+	+L_{20}
Electr/energy				
CAT15	B9MVI3 Cytochrome P450 (*Populus trichocarpa*)	-	-	-L
Cell wall				
CGG25	Q94CI8 Glycine-rich protein LeGRP1 (*Solanum lycopersicum*)	+		

[a] The symbols "+" and "-" stand for "induced" and "repressed", respectively.
[b] Modulation at the late stage, if any, is reported as well, coded as "L" (late), when present at both 20 and 27 dpi, and with "L_{20}" when present only at 20 dpi.

ized, and stress response). Genes assigned to the class resistance, stress response, transport, and proteolysis were also induced (Fig. 2A). At 27 dpi, the classes containing the highest number of repressed genes were metabolism, signal transduction, unknown and uncharacterized, transport and stress response, while the classes with the highest number of induced genes were unknown and uncharacterized, metabolism, and stress response (Fig. 2B). For each functional class, genes were mostly (or exclusively) upregulated at 20 dpi and mostly (or exclusively) downregulated at 27 dpi (Tables 2-4, Fig. 2A and 2B).

Tables 2 to 4 describe in detail the putative annotations of differentially expressed genes belonging to the classes denoted signal transduction (divided into subclasses: hormone responsive, transcription factors, kinases and phosphatases, GTP-binding, and calcium-binding), resistance and secondary metabolism, stress response and cell wall. Table 2 shows that the group of protein kinases involved in signal transduction, that were modulated at the late stage of infection, is composed of both repressed and induced elements, with repression observed at 27 dpi. Among other putative genes involved in signalling, the genes coding for GTP-binding proteins were also both repressed and induced, while those encoding calcium-binding proteins were all repressed at 27 dpi. The expression levels of transcription factors were modulated during infection. Interest-

ingly, most of them were repressed during late infection (27 dpi), while just one, coding for a NAC2 protein, was induced both in the early and in the late infection stages (Table 1 and 2). Members of the bHLH and MYB families were repressed at 27 dpi, while the AP2/ERF, WRKY, zinc finger, NAC and heat shock factor families showed some induced elements, mostly at 20 dpi (Table 2).

Genes putatively involved in defence mechanisms, modulated at the late stage of infection, were classified after Polesani *et al.* (2010) as resistance, secondary metabolism, stress response, and cell wall (Table 3 and Table 4). The latter class was included since modulation of cell wall-related genes could be consistent with involvement in plant-pathogen interaction (Hematy *et al.*, 2009). Genes in the resistance class comprised those coding for different pathogenesis-related proteins (PR), other antifungal proteins and enzyme inhibitors. As far as the lipid transfer protein (LTP) family of PR proteins is concerned, two genes were modulated at the late

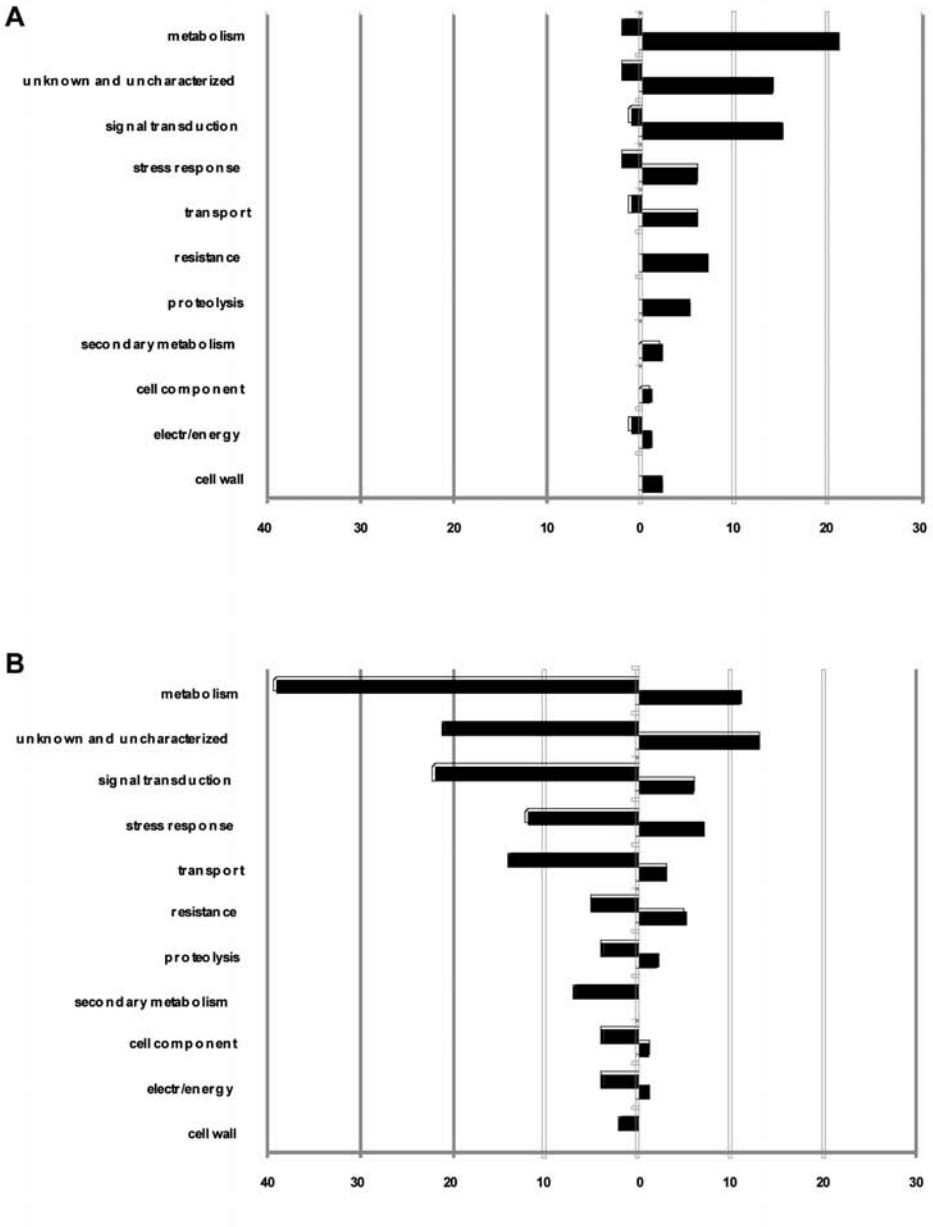

Fig. 2. Distribution among functional classes of up- and downregulated genes, identified in the late stage of infection. Histograms on the left of the Y-axis indicate down-; those on the right indicate upregulated genes. A, 89 genes (80 up- and 9 downregulated) whose expression was modulated at 20 dpi; B, 183 genes (49 up- and 134 downregulated) whose expression was modulated at 27 dpi. Genes modulated at 20 dpi that remained modulated at 27 dpi are also included. The number of genes in each class is given on the X-axis.

Table 2. Tomato genes modulated at the late stage of infection with *Pyrenochaeta lycopersici* attributed to the functional class signal transduction.

Transcript-derived fragments	Annotation	Days post inoculation	
		20	27
Hormone responsive			
CAG10	D5L142 AP2 domain class transcription factor (*Malus domestica)*	-[a]	
CAT18	SGN-U566716 AT3G24500.1 Ethylene-responsive transcriptional coactivator, putative (*Solanum lycopersicum*)		-
CAC10	Q9SWW1 Ethylene-responsive transcriptional coactivator (*Solanum lycopersicum*)		-
TGA5	SGN-U571612 AT1G59750.3 similar to transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related (*Arabidopsis thaliana*)		-
CAT49	SGN-U562646 AT5G67190.1 encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family (*Arabidopsis thaliana*)		-
CCT7	potato|TC223645 similar to Q9XET9 Cluster: Ethylene receptor homolog (*Solanum lycopersicum*)	E[b]+	
CAG15	Q8LGR9 Transcription factor JERF1 (*Solanum lycopersicum*)	+	
CAA28	SGN-U580955 Auxin and ethylene responsive GH3-like protein (*Capsicum chinense*)	+	
CCT9	SGN-U578033 sp|Q9LYC1|GI1L2_ARATH Probable gibberellin receptor GID1L2 (*Arabidopsis thaliana*)	+	
Transcription factors			
TCA4	Q700C0 MYC transcription factor (*Solanum tuberosum*)		-
TCC10	Q0WVU3 Putative uncharacterized protein At5g06800 (myb-like HTH transcriptional regulator family protein) (*Arabidopsis thaliana*)		-
CAA29	Q8LRM1 Nam-like protein 4 (*Petunia hybrida*)		-
TGT3	SGN-U576530 AT2G40200.1 Basic helix-loop-helix (bHLH) family protein (*Arabidopsis thaliana*)		-
TGG1	D9ZIP0 BHLH domain class transcription factor (*Malus domestica*)		-
CCG10	Q9ATD1 GHMYB9 (*Gossypium hirsutum*)		-
CTT2	A7UGD3 WRKY transcription factor 6 (*Solanum tuberosum*)		-
CAT26	A1E0X8 NAC domain protein NAC2 (*Solanum tuberosum*)	E+	+
TGG2	Q8LRL4 Nam-like protein 11 (*Petunia hybrida*)	+	
CCA14	B9T565 Nuclear transcription factor, X-box binding, putative (*Ricinus communis*)	+	
TCG15	SGN-U563446 AT5G67480.2 TAZ zinc finger family protein / BTB/POZ domain-containing protein (*Arabidopsis thaliana*)	+	
CGT48	B6VB04 WRKY transcription factor-30 (*Capsicum annuum*)		+
TAA7	B9S4N6 DNA binding protein, putative (*Ricinus communis*) (heat shock transcription factor family)		+
Kinases and phosphatases			
CGG11	PSKR1 Phytosulfokine receptor 1 (*Daucus carota*)		-
CGG24	tomato|CD002826 weakly similar to Q7XEV8 Cluster: Serine/threonine-protein kinase NAK putative expressed (*Oryza sativa*)		-
CGT21	C6ZRR5 Protein kinase family protein (*Glycine max*)		-
CTA14	B9S785 Calcium-dependent protein kinase, putative (*Ricinus communis*)		-
CTA2	Q38HW2 Adenylate kinase family-like protein (*Solanum tuberosum*)		-
CAA30	P2C09 Probable protein phosphatase 2C 9 (*Arabidopsis thaliana*)	E+	
CGC4	A2Q0R1 Putative uncharacterized protein WAKL1 (*Nicotiana tabacum*)	E+	
CAC11	Q8VX53 Putative receptor-like serine-threonine protein kinase (*Solanum tuberosum*)	E+	+
CAT41	B9SE02 Serine/threonine-protein kinase Nek8, putative (*Ricinus communis*)	+	
CTT12	Q9AXM2 Pto-like protein kinase D (*Solanum habrochaites*)	+	+
GTP-binding			
TCT1	Q38JF9 Ran protein/TC4 protein-like (*Solanum tuberosum*)		-
CGC14	SGN-U578879 AT1G02130.1 Ras-related protein (ARA-5) / small GTP-binding protein, putative (*Arabidopsis thaliana*)		-
CGT20	SGN-U578879 AT1G02130.1 Ras-related protein (ARA-5) / small GTP-binding protein, putative (*Arabidopsis thaliana*)		-
CGT42	SGN-U581467 AT1G02130.1 Ras-related protein (ARA-5) / small GTP-binding protein, putative (*Arabidopsis thaliana*)	+	+
CGT50	Q8W3J4 Ras-related protein RAB8-1 (*Nicotiana tabacum*)	+	
Calcium binding			
CGC7	Q9SQI5 Centrin (*Nicotiana tabacum*)		-
TCA8	Q53U37 Putative uncharacterized protein (*Solanum lycopersicum*)		-
TCG3	Q93VL8 Calmodulin (*Phaseolus vulgaris*)		-

[a] The symbols "+" and "-" stand for "induced" and "repressed" , respectively.

[b] E: genes modulated also at early stages of infection.

Table 3. Tomato genes modulated at the late stages of infection with *Pyrenochaeta lycopersici* attributed to the functional class resistance and secondary metabolism.

Transcript-derived fragments	Annotation	Days post inoculation	
		20	27
Resistance			
TGC5	SGN-U580641 AT3G12500.1 Basic endochitinase, identical to basic endochitinase precursor SP:P19171 from (*Arabidopsis thaliana*)		-[a]
CAA10	SGN-U578441 Pathogenesis-related protein 10 (*Solanum virginianum*)		-
TGG5	B9R9Q7 21 kDa protein, putative (*Ricinus communis*)		-
CAA8	B5M9E5 Beta-glucosidase 08 (*Solanum lycopersicum*)		-
CAA5	SGN-U576740 AT3G18280.1 Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein, similar to TED4 (*Zinnia elegans*)		-
CAT27	Q00782 Proteinase inhibitor type-2 (*Solanum tuberosum*)	+	
CGC19	B9R9E9 Nonspecific lipid-transfer protein, putative (*Ricinus communis)*	+	
CAC28	Q9SBH8 Metallocarboxypeptidase inhibitor IIa (*Solanum tuberosum*)	+	
CAG8	Q8W2B2 Antifungal protein (*Capsicum annuum*)	+	+
CTT6	Q05539 Acidic 26 kDa endochitinase (*Solanum lycopersicum*)	+	+
TGT1	SGN-U580641 AT3G12500.1 Basic endochitinase (*Arabidopsis thaliana*)	+	+
CAC27	SGN-U573166 Metallocarboxypeptidase inhibitor (*Solanum lycopersicum*)	+	+
TTT13	tomato|TC236707 similar to UniRef100_Q9ZS49 Cluster: Avr9 elicitor response protein (*Nicotiana tabacum*)		+
Secondary metabolism			
CCA12	Q8GSM7 Hydroxycinnamoyl transferase (*Nicotiana tabacum*)		-
TAA2	Q9SWH8 4-coumarate:coenzyme A ligase (*Solanum tuberosum*)		-
TCT10	SGN-U578302 AT5G17230.1 Phytoene synthase (PSY) / geranylgeranyl-diphosphate geranylgeranyl transferase (*Arabidopsis thaliana*)		-
CAT34	Q42958 Catechol O-methyltransferase (*Nicotiana tabacum*)		-
CGC15	D7LTV0 Strictosidine synthase family protein (*Arabidopsis lyrata* subsp. *lyrata*)		-
CGT52	Q4A570 Putative squalene epoxidase (*Solanum lycopersicum*)		-
CAA6	SGN-U570023 AT5G54160.1 Quercetin 3-O-methyltransferase 1 / flavonol 3-O-methyltransferase 1 / caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT1) (*Arabidopsis thaliana*)		-
CAT17	B9RC29 (S)-N-methylcoclaurine 3'-hydroxylase isozyme, putative(*Ricinus communis*)	+	
CAG9	Q9M567 Phenylalanine ammonia-lyase 2 (*Rubus idaeus*)	+	

[a] The symbols "+" and "-" stand for "induced" and "repressed", respectively.

stage of infection, one being induced at 20 dpi and the other repressed at 27 dpi, while a third member of the family, coding for protease inhibitor/seed storage/LTP protein was transiently induced only at 72 hpi during the early stage of infection (Table 1 and Table 3).

Genes coding for enzyme inhibitors (metallocarboxypeptidase inhibitor, proteinase inhibitor type 2) as well as those encoding acidic and basic endochitinases were mainly induced at 20 (some also at 27 dpi), while genes coding for PR-10 and beta-glucosidase were repressed at 27 dpi. Differences were observed in the expression profiles of genes classified in the resistance and secondary metabolism classes. As far as resistance class is concerned, more genes were induced than repressed; even at 27 dpi five genes were still induced, while only two genes involved in the secondary metabolism were induced at 20 dpi, all the others being repressed at 27 dpi.

The modulation of genes in the stress response and cell wall classes is reported in Table 4. In general, the same trend of repression at 27 dpi can be observed for these two classes, especially for the group of eight early response to dehydration/late embryogenesis abundant (ERD/LEA) proteins, while only for heat shock proteins more genes were induced than repressed during the latest stage of infection (Table 4). Genes related to metabolism of reactive oxygen species (ROS) were both induced and repressed at the late stages of infection. It was particularly interesting that the two genes coding for peroxidase and glutathione S-transferase that were induced both at 20 and 27 dpi were already induced during the early stages of infection (Table 1 and Table 4).

The majority of genes involved in transport, secretion and protein degradation, general metabolism, and genes putatively related to energy and electron transport and those coding for cell components were downregulated at 27 dpi. A strong repression was observed especially for the genes involved in protein synthesis and amino acid and nucleic acid metabolism. As far as electr/energy class is regarded, several genes coding for cytochromes were repressed at 27 dpi (data not shown),

one of them coding for cytochrome P450 being down-regulated over the entire infection course.

RT-PCR validation of transcriptional profiling. Six TDFs corresponding to tomato genes were examined in a new independent infection experiment by RT-PCR, to validate the differential expression resulted from the cDNA-AFLP experiment. Transcripts were selected among the upregulated and the downregulated genes, and among those modulated already in the early stages and those induced/repressed exclusively in the late stages of infection. They showed patterns identical to those observed in the first experiment of AFLP transcript profiling. Data were normalized against the constitutive plant actin gene (Fig. 3).

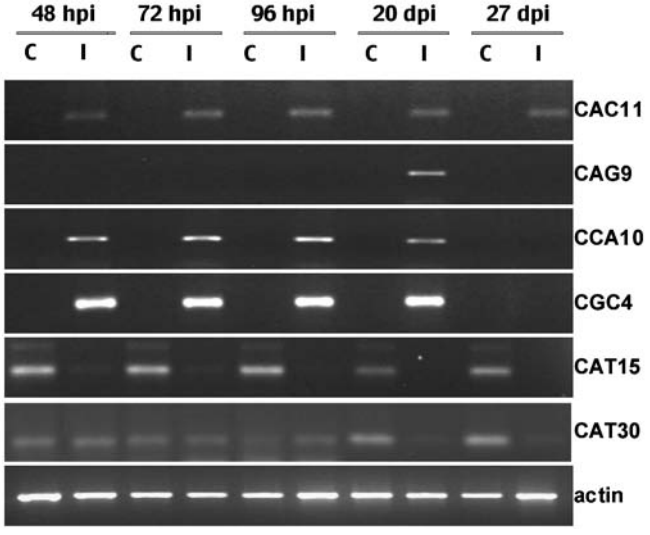

Fig. 3. RT-PCR analysis of selected six tomato genes differentially expressed during tomato-*Pyrenochaeta lycopersici* interaction. RNA was extracted from inoculated (I) or PDA treated, control (C) roots at 48, 72, 96 hpi, 20 and 27 dpi. RT-PCR was performed with specific primers for the following up-regulated: CAC11 - Putative receptor-like serine-threonine protein kinase; CAG9 - Phenylalanine ammonia-lyase 2; CCA10 - Poly(A)-binding protein C-terminal interacting protein 6; CGC4 - Putative uncharacterized protein WAKL1; and the following down-regulated: CAT15- Cytochrome P450; CAT30 - F9L1.21 protein. The plant actin gene was used to normalize the cDNA loading.

DISCUSSION

Tomato corky root rot is a disease of worldwide concern, and only few sources of resistance are available for breeding. Moreover, only little is known about the interaction of tomato with *P. lycopersici*, the causal agent of the disease, and in particular, nothing is known about the modulation in host gene expression. Ekengren (2008) has recently reviewed some quite old papers dealing with the physiological studies of this pathosystem, and the only work dealing with modulation of gene expression during the interaction published until now focused on the genes induced in the fungus (Aragona and Infantino, 2008).

The present work represents the first insight into tomato gene expression profiling of the compatible interaction with *P. lycopersici*. cDNA-AFLP profiles were analysed in order to identify the tomato genes involved in defence mechanisms, and to investigate the physiology of the visible disease symptom development. cDNA-AFLP is a PCR-based technique for genome-wide expression profiling that does not require prior knowledge of gene sequences and combines a high sensitivity with a high specificity, allowing distinction between homologous genes and detection of rarely expressed genes (Bachem *et al*., 1998; Breyne *et al*., 2003). cDNA-AFLP has been used to study the transcriptional profiling of different tomato and fungal pathogen interactions giving an insight into the molecular bases of the defence response (Li *et al*., 2006; Hong *et al*., 2007). Because the regulation of gene expression is a dynamic process, the expression profiling was performed by cDNA-AFLP over a time course, which allowed studying the dynamic behaviour of gene expression and characterizing its changes over time. Time points of 48, 72, 96 hpi during the early stage of infection, and of 20 and 27 dpi at the late stage, were chosen on the basis of the observation of roots infected with a virulent strain of *P. lycopersici* expressing a gene coding for β-glucuronidase (GUS) (Aragona and Infantino, 2008). As reported by these authors, a susceptible (cv. Corbarino) and a resistant (cv. Moboglan) tomato genotypes were artificially inoculated and the taproots sampled at different times (from 24 hpi until 30 dpi), stained in a 1 mM X- Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) solution, destained in a graded ethanol series (Jefferson, 1987) and analysed under a stereomicroscope to monitor the progress of infection. Due to the slow growth of *P. lycopersici* only at 48 hpi the first small necrotic areas, distributed as spots on the taproot, became evident. However differences between the two genotypes started to be noticeable only after 96 hpi and were clear after 7 dpi when the taproot of the susceptible cultivar was already strongly affected whereas in the resistant genotype only scattered stained areas could be observed. Starting from 7 until 30 dpi, the differences were more evident. Roots of the susceptible cultivar were entirely colonized by the fungus, showing the typical symptoms of the corky root disease, while those of the resistant cultivar were still only weakly affected (Fig. 4). GUS fluorimetric assay was performed on the roots of both cultivars to quantify the fungus in the plant tissue expressing the β-glucuronidase activity as nanomoles of 4-methylumbelliferone (MU) produced per minute per milligram of protein (Jefferson, 1987). The activity of β-glucuronidase in the susceptible genotype was about 25 times higher than in the resistant one at 9 dpi (Aragona and Infantino, 2008).

Table 4. Tomato genes modulated at the late stages of infection with *Pyrenochaeta lycopersici* attributed to the functional classes stress response and cell wall.

Transcript-derived fragments	Annotation	Days post inoculation	
		20	27
Stress response			
CAT39	Q8H6E7Dehydrin-like protein (*Solanum sogarandinum*)	-[a]	
CAT29	SGN-U573764 AT5G41210.1 Glutathione S-transferase (GST10), identical to glutathione transferase AtGST 10 (*Arabidopsis thaliana*)	-	
CAC12	Q07446 Peroxidase (*Solanum lycopersicum*)		-
TCG7	SGN-U583027 ABA 8'-hydroxylase CYP707A1 (*Solanum tuberosum*)		-
CAT7	tomato|TC232782 similar to Q6ETC4 Cluster: Putative CLB1 protein (*Oryza sativa*)		-
CAA33	Q40159 Late embryogenesis (Lea)-like protein (*Solanum lycopersicum*)		-
CCA7	Q9LKW3 Dehydration-induced protein ERD15 (*Solanum lycopersicum*)		-
CCG2	Q9LKW3 Dehydration-induced protein ERD15 (*Solanum lycopersicum*)		-
TGC13	SGN-U579730 AT4G02380.1 Late embryogenesis abundant 3 family protein / LEA3 family protein (*Arabidopsis thaliana*)		-
CGG53	SGN-U573714 AT2G17840.1 Senescence/dehydration-associated protein-related (ERD7) (*Arabidopsis thaliana*)		-
CTT1	SGN-U563766 AT4G22120.2 ERD (early-responsive to dehydration stress) family protein (*Arabidopsis thaliana*)		-
TAA4	SGN-U579266 101 kDa heat shock protein; HSP101 (*Nicotiana tabacum*)		-
TGA10	Q9AU16 Metallothionein-like protein (*Typha latifolia*)		-
TCT11	SGN-U579724 AT3G17790.1 Acid phosphatase type 5 (ACP5) (*Arabidopsis thaliana*)		-
CGT60	Q9XIV9 Peroxidase (*Nicotiana tabacum*)	E[b]+	+
CAT23	potato|TC218491 homologue to A5YWI8 Cluster: Glutathione S-transferase (*Solanum commersonii*)	E+	+
CAA17	Q8H6E7 Dehydrin-like protein (*Solanum sogarandinum*)	+	
CCA15	tomato|TC234070 P22240 Cluster: Abscisic acid and environmental stress-inducible protein TAS14 (*Solanum lycopersicu*m)	+	
TCG13	Q9SYV0 17.6 kD class I small heat shock protein (*Solanum lycopersicum*)	+	+
CGG45	B9SU43 Heat shock factor protein, putative (*Ricinus communis*)	+	+
CAG23	Q84TA1 Heat shock cognate 70 kDa protein 2, putative, expressed (*Oryza sativa*)		+
TGC14	tomato|TC233899 similar to Q9SL02 Cluster: DNA repair protein RAD50 (*Arabidopsis thaliana*)		+
TTC10	Q9AY51 Putative peptide methionine sulfoxide reductase (*Oryza sativa*)		+
Cell wall			
TGG7	B8Q691 Glycine-rich cell wall protein (*Oryza grandiglumis*)		-
CCA6	A2V888 Hydroproline-rich glycoprotein like protein (*Nicotiana tabacum*)		-
TCC9	SGN-U590411 AT4G17030.1 Expansin-related (*Arabidopsis thaliana*)	+	
TGT12	Q9ZP39 Alpha-expansin (*Nicotiana tabacum*)	+	

[a] The symbols "+" and "-" stand for "induced" and "repressed", respectively.
[b] E: genes modulated also at early stage of infection.

Based on the above results, suggesting that the fate of interaction was determined between 48 and 96 hpi, the timings for the cDNA-AFLP were chosen, i.e. three timings at the early stages, before clear differences between the susceptible and resistant variety became evident, to monitor the mechanisms that lead to a compatible interaction, and two timings corresponding to the largest differences between the susceptible and resistant variety, to study corky root symptom development.

The cDNA-AFLP experiment enabled the identification and characterization of a catalogue of 247 putative tomato genes modulated in this pathosystem and useful as a starting point for future studies of functional genomics. Short sequences, TDFs with unclear species identification (as resulted from the BLAST searches) and fungal genes were intentionally excluded from further analysis. It cannot be excluded that other interesting tomato upregulated transcripts could be either not tagged or be part of the discarded set either because they were difficult to classify (homologous to both tomato and fungal sequences) or showed no homology to known transcripts.

Some aspects of tomato early response to *P. lycopersici* are of particular interest. First of all, because in many plant-pathogen interactions the recognition of specific or general pathogen elicitors originates a signal transduction cascade that may involve protein phosphorylation, ion fluxes, ROS, and other signalling events (Wang

et al., 2010), some of such signal transduction components were identified in the present study. These proteins include two receptor-like kinases, i.e. a putative receptor-like serine-threonine protein kinase and a putative protein WAKL1 (wall-associated kinase like 1) which are induced already at the early stage of infection (Table 1). Interestingly, a member of the NBS-LRR gene family was found to be the key gene responsible for susceptibility in a compatible interaction Arabidopsis-*Cochliobolus victoriae* (Lorang *et al.,* 2007). This finding, together with the discovery of other susceptibility genes reviewed by Eckardt (2002), shed new light on the mechanisms that may lead to the establishment of a compatible interaction.

Secondly, a significant number of genes modulated in the tomato-*P. lycopersici* interaction were related to transcription, at both early and late stages, in agreement with the notion that plant resistance response is mediated by a complex interplay of activating and repressing transcriptional regulators (Eulgem, 2005).

As a general observation, signalling pathways were activated at the beginning of the infection (48-96 hpi) when the plant tried to mount the defence response to the pathogen attack, and some were induced at 20 dpi as well, while a large part of the genes classified as signal transduction were downregulated at 27 dpi (Table 1 and 2, Fig. 2). Differential activation of kinases is also an indicator of the wide physiological remodelling triggered by fungal attack (D'Ippolito *et al*., 2010). These results suggest that *P. lycopersici* may interfere with signalling components and transcription factors involved in the host basal defence and that suppression of signalling pathways may lead to the compatible interaction, as already reported (Polesani *et al*., 2008).

In addition to activation of primary defence responses, pathogen signals may be amplified by secondary signal molecules and can trigger plant defence-related genes, such as those encoding glutathione S-transferases (GST), peroxidases, cell wall proteins, proteinase inhibitors, and PR proteins (Wang *et al*., 2010). Several genes modulated in the present study encoded proteins involved in the generation and scavenging of oxidative stress, but only two genes coding for proteins involved in ROS metabolism, glutathione S-transferase and peroxidase, were induced at early stage of infection and remained activated during the later stage of infection as well. ROS accumulation during the early stages of plant defence is known as oxidative burst. Although ROS accumulation alone is insufficient to trigger disease resistance (Heath, 2000), they contribute to plant defence by reinforcing the cell walls, by their toxicity to invading pathogens and by signalling further defence responses. Differential expression of these genes indicates the activation of defence pathways even in compatible interactions between tomato and the fungus. However, ROS can cause severe cellular damage so are thus tightly regulated and detoxified by complex enzymatic and non-enzymatic mechanism, such as DNA repair protein RAD50 that was identified in the present study as induced at the later stage of infection.

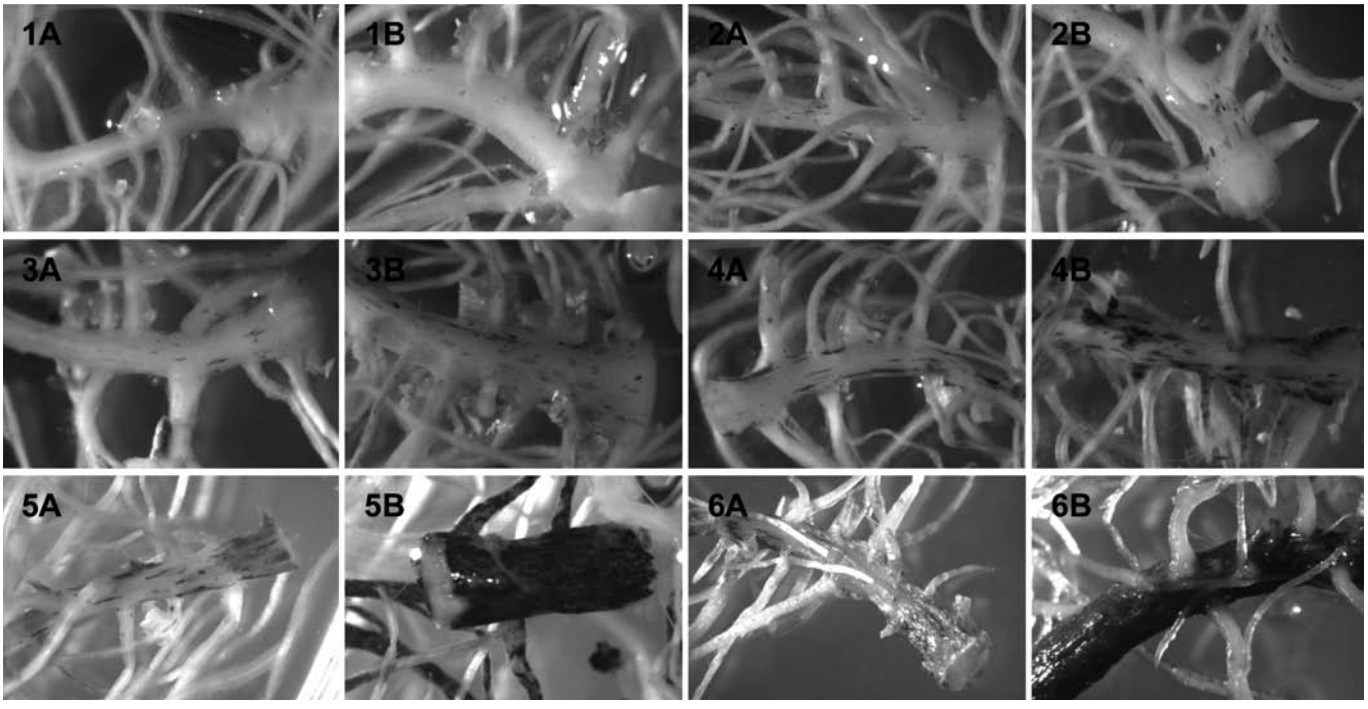

Fig. 4. Macroscopic views of tomato roots artificially infected with the GUS-transformed *Pyrenochaeta lycopersici* isolate ER-1211 after GUS staining at different timings after infection: 1 - 48 hpi; 2 - 72 hpi; 3 - 96 hpi; 4 - 7 dpi; 5 - 20 dpi; 6 - 27 dpi. A, resistant tomato genotype; B, susceptible tomato genotype.

Several genes coding for antifungal proteins, chitinases, and enzyme inhibitors were induced at the later stages of infection during corky root symptom development. Similar results have already been reported for a susceptible grapevine-*Eutypa lata* interaction which showed that the genes known to be involved in plant defence towards fungal pathogens, like those coding for PR proteins, were among the responsive genes at the time of symptoms development (Rotter *et al*., 2009). Chitinase protein was detectable by Western analysis only in tissues with visible disease symptoms in susceptible pine seedlings challenged with *Fusarium subglutinans* f. sp. *pini*, while the resistant plants accumulated it much earlier (Davis *et al*., 2002). All those results confirm the theory that defence responses are induced both in compatible and in incompatible interactions, the main differences being the intensity of the response and its onset time (Rotter *et al*., 2009).

A group of genes modulated at the later stages of infection could be involved in processes linked to the deterioration of the root system as a result of *P. lycopersici* infection. Genes responsible for cell wall loosening (expansin family) were induced. Expansin proteins are known to be involved in cell expansion and other developmental events during which cell wall modification occurs and their upregulation may be associated with response to abiotic stress. Expansins were also reported to be abundantly expressed in the most apical region of adventitious roots (Colmer *et al*., 2004) and to the upregulation of these genes at the later stage of infection obtained in the present study may be attributed the emission of adventitious roots by tomato plant in response to *P. lycopersici*-induced main root system deterioration.

Among the upregulated transport-related genes were those coding for aquaglyceroporin and a protein similar to aquaporin. The upregulation of those genes at later infection stages may reflect the major water requirements for pathogen growth, as already reported by Gibly *et al*. (2004). One class of functional proteins, termed dehydrins, were both upregulated (20 dpi) and downregulated (27 dpi). Several genetic studies have indicated their role in water deficit or cold tolerance. The activation of drought-inducible genes followed by their repression at later stages of infection might reflect the emission of adventitious roots by the plant in response to osmotic stress related to main root system deterioration caused by *P. lycopersici*. Dehydrins were identified among rewatering repressed and drought inducible transcripts (Wong *et al.,* 2006). Downregulation of the transcripts encoding water stress-inducible proteins when adventitious roots develop and become functional has already been reported by Brinker *et al.* (2004).

The gene expression profiles obtained during the late infection stages resembled a typical response to various pathogens, which is qualitatively similar in compatible and incompatible interactions, and includes a shift from housekeeping to defence metabolism (Scheideler *et al*., 2002). Moreover, in the case of compatible interactions the host metabolism is redirected to accomplish the needs of the pathogen (Panstruga, 2003; Fabro *et al*., 2008). A strong downregulation of the genes mainly involved in general metabolism as observed in the present study was reported for *Arabidopsis* inoculated with either the non-host agent of barley powdery mildew *Blumeria graminis* f. sp. *hordei* or the virulent powdery mildew pathogen *Golovinomyces cichoracearum* (former *Erysiphae cichoracearum*) (Zimmerli *et al*., 2004). *Medicago truncatula* genes involved in lipid metabolism and transcription were repressed during interaction with the biotroph *Erysiphae pisi* together with genes in the categories response to stress, calcium-mediated signalling, cell wall organization and biogenesis and flavonoid biosynthesis (Foster-Hartnett *et al*., 2007). According to our results, at 27 dpi also genes assigned to signal transduction, transport, stress response, secondary metabolism and resistance classes were strongly repressed. This could mean that the plant metabolism was definitely compromised and shifted to the pathogen needs, even if some defence-related genes (like those encoding endochitinases and enzyme inhibitors) were still induced even at the latest stage of infection (Fig. 2, Table 2-4).

The present paper represents a contribution to unravel the modifications of gene expression in a non simple experimental pathosystem (tomato-*P. lycopersici*) characterized by slow progression of the disease and the difficult early diagnosis. It seems therefore of utmost importance that this study allowed us to identify a significant set of gene expression modifications through the entire disease time course, from infection to symptom development.

The overall results indicate that the network of signalling, involved in tomato response to *P. lycopersici* infection may be very complex and further studies are necessary to unravel the mechanisms that govern the resistant response. Functional characterization of these genes will assist in the dissection of the molecular mechanisms and cellular processes involved in tomato susceptible and resistant response to the pathogen.

ACKNOWLEDGEMENTS

This work was carried out within the framework of the Project "RESPAT" funded by the Agricultural Research Council - CRA.

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Received June 19, 2011
Accepted December 22, 2011