

Identification of genes differentially expressed in the phytopathogenic fungus *Cercospora nicotianae* between cercosporin toxin-resistant and -susceptible strains

Sonia Herrero, Alongkorn Amnuaykanjanasin & Margaret E. Daub

Department of Plant Biology, North Carolina State University, Raleigh, NC, USA

Correspondence: Sonia Herrero, Department of Plant Biology, Box 7612, North Carolina State University, Raleigh, NC 27695-7612, USA. Tel.: +1 919 513 8220; fax: +1 919-515-6987; e-mail: sonia_herrero@ncsu.edu

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Abstract

Plant pathogens from the genus *Cercospora* produce cercosporin, a photoactivated fungal toxin that generates toxic reactive oxygen species. Mechanisms governing toxin auto-resistance in *Cercospora* spp. are poorly understood. In this work, suppressive subtractive hybridization was used to identify genes differentially expressed between the cercosporin-resistant wild-type (WT) *Cercospora nicotianae* and a sensitive strain lacking a transcription factor (CRG1) that regulates resistance. Out of 338 sequences recovered, 185 unique expressed sequence tags (ESTs) were obtained and classified into functional categories. The majority of genes showed predicted expression differences, and 38.5% were differentially expressed at least twofold between the WT and mutant strain. ESTs were recovered with homology to genes involved in detoxification of noxious compounds, multidrug membrane transporters and antioxidant and polyketide biosynthetic enzymes as well as to ATPases and ATP synthases. The findings suggest that CRG1 regulates genes involved in pH responses in addition to those involved in toxin resistance and biosynthesis.

Introduction

The fungal toxin cercosporin is a perylenequinone produced via the polyketide pathway by members of the phytopathogenic genus *Cercospora*. Photoactivation of cercosporin leads to the production of highly toxic reactive oxygen species (ROS), namely singlet oxygen and superoxide (Yamazaki *et al.*, 1975). During the course of fungal infection, cercosporin acts as a virulence factor affecting symptom development and disease severity. In spite of being almost universally toxic, *Cercospora* spp. produce and secrete millimolar concentrations of cercosporin with no observable decrease in growth. These fungi are also resistant to other photoactivated compounds that generate singlet oxygen (Daub & Ehrenshaft, 2000; Daub *et al.*, 2005).

In the authors' laboratory, the tobacco pathogen *Cercospora nicotianae* is used as a model system to study the physiological and genetic mechanisms governing resistance to cercosporin and singlet oxygen. Studies conducted to date indicate that resistance is complex and may be regulated at the transcriptional level. Proposed resistance mechanisms

include maintenance of the toxin in a reduced, nonphotoactive state inside the fungal mycelium (Daub *et al.*, 1992, 2000; Ververidis *et al.*, 2001; Panagiotis *et al.*, 2007) and the production of vitamin B6 (Ehrenshaft *et al.*, 1999; Ehrenshaft & Daub, 2001), shown to function as an antioxidant and a quencher of both superoxide and singlet oxygen (Bilski *et al.*, 2000; Denslow *et al.*, 2005). Membrane transporters have also been reported to be involved in cercosporin resistance in *Cercospora* spp. (Callaham *et al.*, 1999; Choquer *et al.*, 2007) as well as in conferring resistance when overexpressed in sensitive organisms (Ververidis *et al.*, 2001; Hayashi *et al.*, 2002; Upchurch *et al.*, 2002, 2005). Interestingly, common mechanisms known to be effective in quenching superoxide radicals or singlet oxygen such as antioxidant enzymes and carotenoids, respectively, do not play a significant role in protecting *Cercospora* spp. against cercosporin (Daub, 1987; Ehrenshaft & Daub, 1994).

The *C. nicotianae* deletion mutant 'cercosporin resistance gene 1' (*crG1*) is highly sensitive to cercosporin, with growth inhibition ranging from 50% to total growth suppression, depending on the growth conditions and the concentration

of cercosporin. However, this strain is not impaired in its ability to reduce cercosporin or produce normal levels of vitamin B6 (Jenns *et al.*, 1995; Chung *et al.*, 1999). The product encoded by *CRG1* belongs to a family of transcriptional regulators known as zinc cluster proteins found only in fungi (Chung *et al.*, 2003b). This group of transcription factors is known to regulate a diverse number of cellular activities including metabolism of sugars and amino acids, cellular respiration, gluconeogenesis, and multidrug resistance (Akache *et al.*, 2001). *Cercospora nicotianae* mutants deficient in *CRG1* are highly sensitive to cercosporin and can produce significantly lower amounts depending on the media conditions, suggesting that *CRG1* regulates both genes controlling resistance as well as biosynthesis.

In this work, PCR-based suppressive subtractive hybridization (SSH) has been used to identify genes regulated by *CRG1* and involved in cercosporin self-resistance. This technique has been successfully used to identify genes involved in pathogenesis by *Alternaria brassicicola* (Cramer & Lawrence, 2001) or in cercosporin biosynthesis in *Cercospora zea-maydis* (Shim & Dunkle, 2002). Here, the isolation, sequence and classification of 185 unique expressed sequence tags (ESTs) recovered in two libraries, representing genes up-regulated in the wild type (WT) and in the *crg1* mutant strain, respectively, were reported. The data obtained through this work constitute the basis to pursue the isolation and characterization of individual genes and further investigate their role in cercosporin and singlet oxygen resistance.

Materials and methods

Sample preparation and mRNA isolation

A WT strain of *C. nicotianae* (ATCC 18366) and the *crg1*-null mutant (205C3) provided by Dr K.R. Chung (University of Florida) (Chung *et al.*, 2003b) were grown in 100 mL of liquid complete medium (CM) at room temperature with shaking (200 r.p.m.) for either 3, 4, or 5 days. These time points were selected to span the period just preceding through active production of cercosporin by *in vitro* cultures. Mycelium was collected by filtration at each time point, washed and lyophilized. For each strain, 0.1 g of lyophilized mycelium collected from each time point was combined, and RNA was extracted with TRI-REAGENT (Sigma-Aldrich, St Louis, MO) following the manufacturer's recommendations. Total mRNA was isolated using the commercially available kit Poly (A) Purist™ MAG (Ambion, Austin, TX).

cDNA subtractive library construction

SSH was used to construct a cDNA subtractive library between the WT *C. nicotianae* and the *crg1* mutant strain

205C3. Library construction was performed using the PCR-select cDNA subtraction kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's directions. Subtraction was performed in both directions, thus allowing selection for genes differentially expressed in either the WT (Forward Library-FLib) or the *crg1* mutant (Reverse Library-RLib). After subtraction, secondary PCR products were cloned using the Topo TA Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA) and maintained in *Escherichia coli* DH5 α cells.

To test for subtraction efficiency, a vitamin B6 biosynthetic gene (*PDX1*) expressed constitutively in both strains was amplified from secondary PCR products. A 5 μ L aliquot of PCR reactions from each subtracted and unsubtracted samples were taken after 15, 20, 25, and 30 cycles and electrophoresed in a 1% agarose gel.

Sequencing and analysis

Sequencing reactions were prepared with 7 μ L plasmid DNA, 2 μ L of BigDye dye (Applied Biosystems, Somerville, MA), and 1 μ L of the sequencing primer M13 (3.3 μ M). The mix was incubated at 96 °C for 10 s, 50 °C for 5 min, and 60 °C for 2 min for a total of 30 cycles. Subsequently, reactions were cleaned with Edge Biosystems Performa DTR 96-well plates (Edge Biosystems, Gaithersburg, MD) following the manufacturer's recommendations. Samples were sequenced at the Genomics Research Lab facility at North Carolina State University.

Before sequence analysis, each expressed sequence tag (EST) was edited to correct sequencing ambiguities and remove vector- and primer-specific sequences. BLAST algorithms were used to identify protein (BLASTX or TBLASTX) homologous sequences using GenBank's nonredundant databases, and the COGEME database (Consortium of Functional Genomics of Microbial Eukaryotes). Hits with *E*-values $\leq 1 \times 10^{-5}$ were considered to be significant matches. When possible, ESTs were assigned to functional categories according to the classification proposed by KEGG (Kyoto Encyclopedia of Genes and Genomes) (<http://www.genome.jp/kegg/>).

Quantitative reverse transcription-PCR (q-RT-PCR)

q-RT-PCR was performed to confirm the differential expression of selected clones. Sequence-specific primers were designed using PRIMER EXPRESS (Applied Biosystems, Foster City, CA) (Table 1). Total RNA isolated as described above for library construction from either WT or mutant *crg1* was used as a template for cDNA synthesis. First-strand synthesis was carried out in a total of 50 μ L using Taqman Reverse Transcription Reagents (Applied Biosystems, Somerville, MA) according to the manufacturer's recommendations. The reaction was incubated at 25 °C for 10 min, 48 °C for

Table 1. Sense and antisense PCR primers used for q-RTPCR to evaluate differential expression of clones selected from the forward (F) or reverse (R) subtracted cDNA libraries (LIB)

Putative function	Lib	Sense primer	Antisense primer
Cysteine synthase	F	CCGTTCTGCAGAGCTCAAG	GCCATTGACCATGTTGAGCAT
Flavoheomoprotein	F	CAGCCGGAACACTACGAGATC	ATGTTGGCGAGTTGCCAGTAT
Cercosporin transporter	F	ATGTTGGCGAGTTGCCAGTAT	TGCGGTGTGTCGAGTGTGA
Cyanide hydratase	F	CGGCAACACACGAATCTATGGT	CCGCATGTAATGTCCTCCAA
O-methyltransferase	F	AGAGATACATCCAGGCTTCGAGAT	GCGGCTATGTTACTACAATGC
Avirulence gene Avr4	F	CTTCTCGTGTCCACCATTG	AGACCCGGTATCGC GTATGAAA
MFS transporter	F	AGCCCGCAATGTCACACTTAC	TCAAGCCGCAGTATGGATCTT
Oxidoreductase	F	CAACCGAAGCA CACTTGGAA	ACTCCGCAGCTCGTCGAT
Alcohol dehydrogenase	F	TGACCAGCTCGGCAAACTCT	CGGCATCATCTCCCAATACG
ABC transporter	F	GTCGCTTGACCGTCTACTG	CATGGTAGCCTTCTGCTT CTC
135sF	F	GCTCGAGGGTTCTTGAGGAAA	CTGCCATTGGCAAGCTATAACC
11cF	F	TTCTAGAGGAAGCCACGTTGCT	GGCCATGTTGTGTGTGGTGAT
12cF	F	AGCTTGTGTTGCTGCTCCATGA	ACGATGAGAGACGACGAACGA
13cF	F	CGAGGCGCTGAGTGATATTCA	GCGGTGATGGCAATGATGTAG
17cF	F	GTGAACAGCAATGATCGTCGAA	TGGTCGATGGATCTCATGCA
23cF	F	CGAGAAGCGCCAAGAACAAT	TGAGCTAATGGCGGAAGTCTG
24cF	F	GCCGAGGTACGTTCAAATCCTT	CCAAGACGTCGTGAAGCAGAA
207sF	F	TTCGAATGGCACTTCCTGACA	CGCACGTGGAGTTGATAATCG
7cF	F	TTCCCCAAATCCGGTCATAAG	TCTCCGAAGAGACCGCATAT
20sF	F	ACGTTTGGTGCAGAAAGTT	GACATGAGTGGCGATTGTGATC
63cF	F	TCCCATCTGCACTGCCATGAA	TTACCTGCCCTTTGGGAACIT
200sF	F	TGTCGACCAGTTCTCGGATCTT	GGCAACACGACTACAACGAACA
62c	F and R	CTCCCTTGATGGCAGAGTAA	CCAACCTCAGCCACCTGTCTACA
Vacuolar ATPase	R	GGGCGCGGCATATGAC	GGCCTGAAAACACCAACCGTT
Glucosamine aminotransferase	R	CAATGCCATACCAGCAAGTCATC	GCCGCCAGTGTTCGAAA
Superoxide dismutase	R	GCTCTCTGGGCGGATCAA	GCCAGGGCAACGCAAA
Uracil transporter	R	GGAGCCAAATATCACCTGAGCTT	CGCCCCAAATCCCAAAAG
Glutathione-S-transferase	R	GCATTCCTGGTCTCATTGA	GGATAGCAAGCACCTCCATAA
Dihydroorotate reductase	R	GTTGTGCGAGCCAGCAAGT	TGGCGACATCTTGACCATGA
Phosphatase	R	GCACCGGCACTGTTTCATG	GGCTGACAAGAGCGCATTC
Quinone reductase	R	AGTCCCAGCTTCCCATAACA	TCCCGGTGGACACAACCTGT
Disulfide-isomerase	R	CTGCTTGACCAGCTCGTTGT	TCCCTCGACTCTACTGTCTGCTA
ABC transporter	R	TGTCATTACCGGCTCCATGTT	TGCTGCTGCACATAGCCTGT
35cR	R	TGCTCTCTGCGGTGGTGATC	CCTTGACAAGCCAAAGAGAAGT
36cR	R	TATTGCGTCGACCCTCGAA	CACAAGTCGGTGCTGAGATTGA
41cR	R	CCATGGCAACTTGCTGTTTT	CTCGTTATGTTCTGTTTCGTGTCAT
92cR	R	GAAAACCTGGGAGTTGTGGTTTCCAG	CCCCTTGTAATAATCCAATGC
33cR	R	CCATCTTGACGGGCATGTTA	GGAAGTGACCGTTCAAAGCA
103sR	R	CACTGATCCGGAACATTAACCA	TCTGTCCGAGCATAATTGGAA

30 min and 95 °C for 5 min. The resulting first-strand cDNA was used as a template for q-RTPCR, which was performed in a DNA Engine Opticon2 (Biorad, Hercules, CA). A single 25 µL PCR reaction included 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µL of the first strand cDNA, and 22.5 µM each forward and reverse primers. The cycling parameters were set at 95 °C for 10 s, 40 repetitions at 95 °C for 15 s, 60 °C for 1 s, and fluorescence acquisition at 77 °C for 1 s. After cycling completion, amplicons were identified using a melting point analysis protocol (60–90 °C every 0.5 °C for 1 s). The q-RTPCR reactions were performed in triplicate, and negative controls included total RNA extracted from either WT or *crg1* to check for DNA contamination in samples, and a

no-DNA control. Each sample was normalized against the 18S rRNA gene control, and fold-change relative to WT was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Toxin sensitivity assays

The WT and *crg1* mutant strains of *C. nicotianae* described previously were evaluated for resistance to cercosporin under different media and pH growth regimes. Solid complete medium (CM), malt medium (Malt), and potato dextrose agar (PDA) were prepared as described previously (Jenns *et al.*, 1989) and their pH was adjusted to either 4, 5, 6, 7 or 8 with NaOH or HCl. Plates containing different

media/pH combinations with or without 10 μ M cercosporin were inoculated using a toothpick stab technique. Two plates, each containing two stabs, were inoculated for each strain/media/pH/toxin combination and incubated for 1 week at 28 °C under continuous light. Radial growth measurements were taken at the end of the experiment. Cercosporin resistance is calculated as growth on cercosporin-containing medium as a % of growth in the absence of the toxin.

Results

Subtraction efficiency and sequence analysis

To ensure selection of differentially expressed ESTs, a subtraction efficiency test was performed to confirm a significant reduction in transcript accumulation of housekeeping genes that occurs in efficient subtractions. The vitamin B6 biosynthetic gene *PDX1*, constitutively expressed in both WT and *crg1*, was chosen to evaluate subtraction efficiency by monitoring accumulation over time. Whereas *PDX1* can be detected by cycle 20 in unsubtracted controls, it was not detected until cycle 30 in both the forward and reverse subtracted libraries (Fig. 1), indicating efficient subtraction of transcripts differentially expressed between the two strains.

To verify the presence of inserted ESTs, a total of 62 randomly selected bacterial clones were tested by PCR using forward and reverse M13 primers (Fig. 2). The majority of clones tested harbored a single insertion, ranging from 250 to 1000 bp. From a total of 426 clones recovered, inserts from 338 (176 FLlib and 162 RLlib) were successfully sequenced. Sequence analysis revealed that some ESTs were represented in multiple clones. The number of

unique clones obtained was 80 for FLlib and 105 for RLlib, with average insert sizes of 357 and 331 bp, respectively. The results are similar to other studies showing that 77% of SSH-recovered clones having inserts (Cramer & Lawrence, 2001) and insert sizes of 0.15–0.75 kb (Shim & Dunkle, 2002).

Functional classification of ESTs

All sequenced ESTs were compared with the sequences publicly available in the NCBI nonredundant and COGEME databases using BLASTX or TBLASTX algorithms, respectively. Only six ESTs were found that were common to both libraries. Two of these clones do not show significant homology to other protein sequences available publicly, while the remaining four are similar to an ABC-type multidrug transport protein from *Aspergillus nidulans* ($E = 8 \times 10^{-28}$), a phenylacetyl-CoA ligase from *Aspergillus fumigatus* ($E = 9 \times 10^{-54}$), and two hypothetical proteins

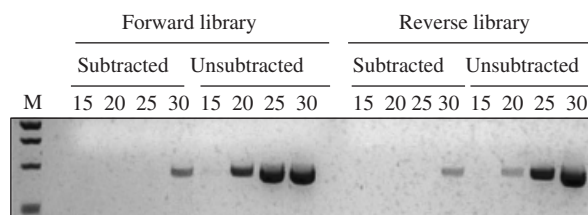


Fig. 1. Assay to test the subtraction efficiency of the SSH protocol. PCR amplification of *PDX1*, a vitamin B6 biosynthetic gene expressed constitutively in *Cercospora nicotianae*, was used to test for subtraction efficiency. Subtracted and unsubtracted samples from both forward and reverse libraries were used as templates. Numbers indicate the PCR cycle at which a 5 μ L sample was collected for subsequent electrophoresis. M refers to the 1 kb molecular marker.

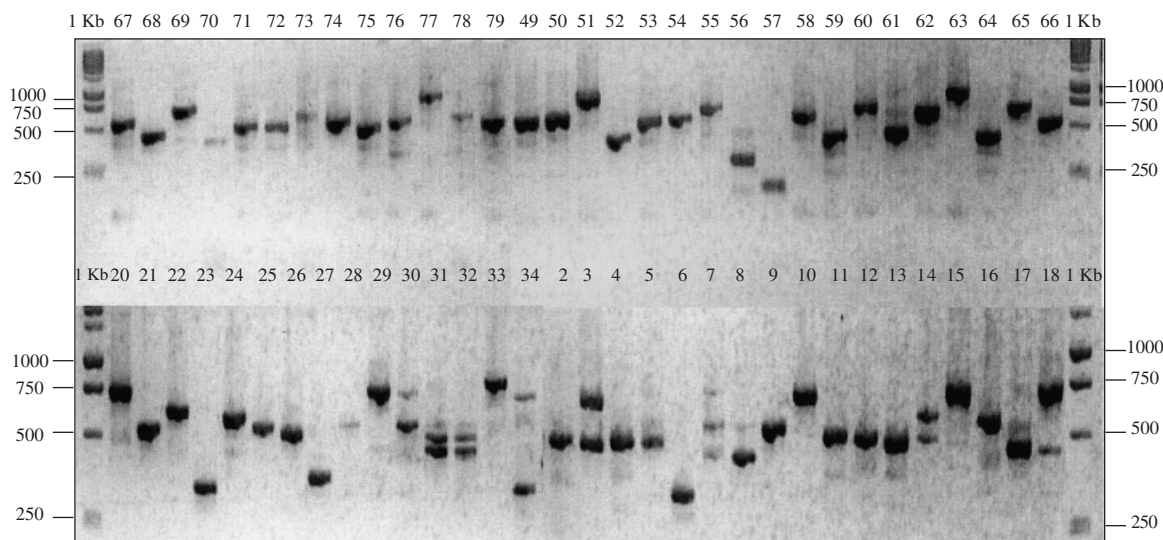


Fig. 2. PCR amplification of selected clones harboring ESTs. ESTs were amplified using primers specific to vector sequences flanking the insert, and the resulting products were electrophoresed in a 1% agarose gel. Most clones contained a single insert.

from *Magnaporthe grisea* ($E = 2 \times 10^{-23}$) and *Cladosporium fulvum* ($E = 2 \times 10^{-13}$).

For sequences unique to the FLib, 47 ESTs had significant homology ($E \leq 1 \times 10^{-5}$) to proteins of known function (Table 2). In addition, seven ESTs showed significant homology to hypothetical proteins. For sequences unique to the RLib, a total of 48 ESTs showed significant homology ($E \leq 1 \times 10^{-5}$) to known proteins, and 12 showed significant homology to hypothetical proteins (Table 3). In addition, the FLib and RLib included 26 and 45 sequenced ESTs, respectively, without significant homology to genes found in any of the databases (data not shown), suggesting that these sequences may be unique to *Cercospora* spp. or closely related fungi whose genomes have not yet been sequenced.

Sequenced ESTs from the FLib and RLib that were assigned a putative function based on homology were grouped into functional categories according to the KEGG classification (<http://www.genome.jp/kegg/>). For most categories, only slight differences were found between the number of ESTs identified either in the FLib or the RLib and assigned to a specific category (Fig. 3). The greatest difference in the numbers of sequences in functional categories was in the 'energy metabolism' category. This category contained ESTs showing significant homology to genes involved in ATP synthesis and transport such as vacuolar ATPase (DW985971), two ATP synthases (DW986064, DW986046), a cytochrome *b* (DW986052) and a mitochondrial ADP/ATP translocase (DW985966). Other categories that showed larger than a 3% difference in ESTs between the two strains were 'amino acid metabolism' and 'genetic information processing,' where the number of assigned genes was higher in the FLib and the RLib, respectively.

In addition to differences in representation within KEGG categories, ESTs with putative functions relevant to known mechanisms involved in cercosporin resistance were also identified, such as transport proteins and proteins involved in detoxification of noxious compounds. The FLib contained several ESTs with putative diverse transport functions (Table 2). These include members of the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters involved in detoxification of drugs and xenobiotic compounds (ESTs, DW986035, DW985942 and DW986000). One of these (DW985942) has been characterized in the closely related fungus *Cercospora kikuchii* as cercosporin facilitator protein (CFP), proposed to be the cercosporin transporter also important in resistance (Callahan *et al.*, 1999). Additional ESTs with homology to proteins known to play a role in the detoxification of noxious compounds include a cytochrome P450 monooxygenase (ES408467) and a cyanide hydratase (DW985998).

The *crg1*-null mutant shows decreased cercosporin production relative to WT; consequently, and as expected, ESTs with homology to fungal toxin biosynthetic genes were also identified in the FLib (Table 2). These include two genes found in the cercosporin biosynthetic gene cluster, an oxidoreductase (DW985946) and an *O*-methyltransferase (DW986049) (Chen *et al.*, 2007), as well as a polyketide synthase involved in fumonisin biosynthesis (DW985955) and a peptide synthase from the bacterium *Ralstonia solanacearum* (DW986053).

Because the *crg1*-null mutant is sensitive to cercosporin but still produces the toxin, an up-regulation of general oxidative stress genes in the RLib was expected. Consistent with this prediction, the RLib contained homologs to genes responsive to oxidative stress, including those encoding superoxide dismutase (DW985988), glutathione-S-transferase (DW986001), a disulfide-isomerase precursor (DW986020) and a peptide methionine sulfoxide reductase (DW986108) (Table 3).

q-RTPCR of selected genes

q-RTPCR was used to confirm and measure the differential expression of selected ESTs recovered in the two libraries. A total of 39 ESTs from both FLib and RLib were selected for analysis based on the putative function of relevance to cercosporin resistance and production as well as randomly selected genes (Fig. 4). The genes selected from the FLib (Fig. 4a) included those with homology to a flavohemoprotein involved in oxidative stress responses, several putative multidrug transporters, a cyanide hydratase involved in the detoxification of xenobiotic products, a cysteine synthase requiring vitamin B6 as a cofactor, homologs to cercosporin biosynthesis genes (oxidoreductase and *O*-methyltransferase), and randomly selected genes encoding a putative alcohol dehydrogenase, the avirulence gene *AVR4*, and several hypothetical proteins. Genes recovered in the FLib should be down-regulated in the *crg1* mutant relative to WT. Of the FLib genes tested, 56.5% (13 of 23 ESTs) were down-regulated in the *crg1*-null strain, with expression ranging from one-fourth to more than 300-fold less than WT (Fig. 4a). The remaining 10 FLib ESTs were the same or slightly (< 2-fold) up-regulated in the *crg1* mutant.

A total of 16 genes were selected from the RLib (Fig. 4b). Of the 16 genes, 10 (63%) were up-regulated as expected in *crg1*, four (25%) did not have differential expression, and two (13%) were down-regulated. The three most up-regulated ESTs included those encoding glutathione-S-transferase and disulfide isomerase, likely induced as a general response to oxidative stress caused by the presence of cercosporin. In addition, a putative uracil transporter known to be located immediately downstream of *CRGI*

Table 2. Putative protein homologs (E -values $\leq 1.00 \times 10^{-5}$) recovered from the subtracted forward library (FLib)

Clone ID #	EST Genbank accession #	Size (bp)	Putative EST homolog	Organism	E -value	NCBI accession # or unisequence ID*
5cF	DW985943	549	Heat shock protein 90	<i>Podospora anserina</i>	$3.00E^{-83}$	O43109
55sF [†]	DW986049	438	O-methyltransferase	<i>Cercospora nicotianae</i>	$2.00E^{-79}$	ABK64180
195sF	DW986080	584	Phosphoketolase	<i>Cryptococcus neoformans</i>	$8.00E^{-77}$	AAW46259
8cF [†]	DW985946	645	Oxidoreductase	<i>Cercospora nicotianae</i>	$8.00E^{-74}$	ABK64182
4cF [†]	DW985942	366	Cercosporin transporter	<i>Cercospora kikuchii</i>	$2.00E^{-60}$	AAC78076
13cF	DW985950	648	Hypothetical protein	<i>Magnaporthe grisea</i>	$4.00E^{-57}$	XP_361188
65sF	DW986052	461	Cytochrome <i>b</i>	<i>Cercospora kikuchii</i>	$4.00E^{-56}$	BAE53454
89cF [†]	DW985998	463	Cyanide hydratase	<i>Botryotinia fuckeliana</i>	$5.00E^{-51}$	BfCon[0544]*
21sF	DW986041	331	Pyruvate dehydrogenase β chain precursor	<i>Neurospora crassa</i>	$3.00E^{-49}$	EAA36154
15cF	DW985952	371	Integral membrane protein	<i>Aspergillus fumigatus</i>	$2.00E^{-42}$	EAL88317
60sF	ES408467	451	Cytochrome P450 monooxygenase	<i>Magnaporthe grisea</i>	$9.00E^{-42}$	MagCon[10214a]*
9sF [†]	DW986035	358	ATP-binding cassette transporter	<i>Meloidogyne graminicola</i>	$3.00E^{-41}$	AAK62340
10sF	DW986036	521	α subunit of succinyl-CoA ligase	<i>Saccharomyces cerevisiae</i>	$3.00E^{-37}$	CAA64059
1cF [†]	DW985939	301	Cysteine synthase	<i>Aspergillus fumigatus</i>	$5.00E^{-36}$	EAL84542
171sF	DW986074	347	Heat shock protein	<i>Magnaporthe grisea</i>	$8.00E^{-34}$	AA07628
207sF	DW986083	449	Hypothetical protein	<i>Aspergillus oryzae</i>	$1.00E^{-30}$	BAE62964
84sF	DW986055	224	Hypothetical protein	<i>Magnaporthe grisea</i>	$2.00E^{-30}$	MagCon[0894a]*
156sF	DW986070	337	Heat shock protein	<i>Aspergillus niger</i>	$2.00E^{-30}$	CAA72292
8sF	DW986034	243	Fructose 1,6-bisphosphate aldolase	<i>Aspergillus oryzae</i>	$4.00E^{-29}$	BAB12232
26cF	DW985963	361	Casein kinase II, α chain	<i>Neurospora crassa</i>	$3.00E^{-28}$	EAA67474
59sF	DW986050	219	O-acetylhomoserine (thiol)-lyase	<i>Neurospora crassa</i>	$5.00E^{-28}$	CAB99179
203sF	DW986082	386	Alternative oxidase	<i>Blumeria graminis</i>	$3.00E^{-27}$	AAL56983
154sF	DW986069	473	Acetolactate synthase	<i>Magnaporthe grisea</i>	$2.00E^{-25}$	XP_962652
44sF	DW986046	269	ATP synthase α chain-like protein	<i>Magnaporthe grisea</i>	$7.00E^{-25}$	AA07676
91cF	DW985999	256	Peroxisomal membrane protein (peroxiredoxin)	<i>Penicillium citrinum</i>	$2.00E^{-23}$	AAD42074
26sF [†]	DW986044	335	Alcohol dehydrogenase	<i>Aspergillus fumigatus</i>	$2.00E^{-23}$	EAL93622
53sF	DW986048	346	Vacuolar serine protease	<i>Penicillium chrysogenum</i>	$2.00E^{-21}$	AAG44693
133sF	DW986064	201	ATP synthase subunit	<i>Aspergillus nidulans</i>	$5.00E^{-20}$	XP_659919
29cF	DW985966	373	ADP/ATP translocase	<i>Neurospora crassa</i>	$6.00E^{-20}$	CAE75740
113sF	DW986061	378	1-Aminocyclopropane-1-carboxylate synthase	<i>Penicillium citrinum</i>	$4.00E^{-19}$	BAA92149
121sF	DW986062	297	Microtubule-interacting protein	<i>Aspergillus fumigatus</i>	$7.00E^{-19}$	EAL90024
17sF	DW986039	269	C2H2 finger domain protein	<i>Aspergillus fumigatus</i>	$1.00E^{-17}$	EAL87913
18cF	DW985955	255	Polyketide synthase	<i>Gibberella moniliformis</i>	$1.00E^{-16}$	AA074815
103cF [†]	DW986000	456	Multidrug resistance dihydroxy MFS transporter	<i>Saccharomyces cerevisiae</i>	$2.00E^{-16}$	NP_009739
166sF	DW986073	161	C-8 sterol isomerase	<i>Neurospora crassa</i>	$4.00E^{-16}$	CAC28749
60cF	DW985994	104	40S ribosomal protein S18	<i>Meloidogyne graminicola</i>	$2.00E^{-14}$	Mgc10e01f*
81sF	DW986054	561	Hydroxyphenyl propionic acid monooxygenase	<i>Rhodococcus</i> spp.	$2.00E^{-14}$	AAF81824
20cF	DW985957	367	Autophagic vesicle breakdown protein	<i>Candida albicans</i>	$1.00E^{-13}$	XP_717509
173sF	DW986075	440	SH3 domain-containing protein	<i>Gibberella zeae</i>	$1.00E^{-13}$	GzCon[1137]*
24cF	DW985961	285	Hypothetical protein	<i>Magnaporthe grisea</i>	$2.00E^{-12}$	XP_361188
187sF	DW986077	232	Choline dehydrogenase	<i>Aspergillus fumigatus</i>	$5.00E^{-12}$	EAL86238
78sF	DW986053	406	Peptide synthase protein	<i>Ralstonia solanacearum</i>	$1.00E^{-11}$	NP_522978
22cF	DW985959	266	Oxidoreductase (Zn-binding dehydrogenase)	<i>Aspergillus fumigatus</i>	$4.00E^{-11}$	EAL89621
16cF	DW985953	431	Cell adhesion protein	<i>Gibberella zeae</i>	$1.00E^{-09}$	Gz31372987*
2cF [†]	DW985940	135	Flavohemoprotein	<i>Aspergillus oryzae</i>	$2.00E^{-09}$	CAF32307
97sF3	DW986058	280	Sterol desaturase family protein	<i>Aspergillus fumigatus</i>	$9.00E^{-09}$	EAL84630
25sF	DW986043	342	60S ribosomal protein	<i>Meloidogyne graminicola</i>	$2.00E^{-08}$	Mg[1171]*
97sF5	DW986057	244	Pol polyprotein homolog	<i>Cladosporium fulvum</i>	$2.00E^{-08}$	AAF21678
21cF	DW985958	124	Hypothetical protein	<i>Gibberella zeae</i>	$1.00E^{-07}$	GzCon[4039]*
108sF	DW986060	183	U-box domain protein	<i>Aspergillus fumigatus</i>	$1.00E^{-07}$	EAL93399
25cF [†]	DW985962	276	Avr4_Clafu race-specific elicitor	<i>Cladosporium fulvum</i>	$6.00E^{-07}$	CAA55403
11sF	DW986037	318	Hypothetical protein	<i>Meloidogyne graminicola</i>	$3.00E^{-06}$	Mga0262f*
61sF	DW986051	238	Monophenol monooxygenase	<i>Meloidogyne graminicola</i>	$1.00E^{-05}$	Mg[0247]*
200sF	DW986081	319	Hypothetical protein	<i>Neurospora crassa</i>	$1.00E^{-05}$	EAA30338

*Unisequence ID from COGEME.

[†]Clones were selected for q-RTPCR analysis.

Table 3. Putative protein homologs (E -values $\leq 1.00 \times 10^{-5}$) recovered from the subtracted reverse library (RLiB)

Clone ID #	EST Genbank accession #	Size (bp)	Putative EST homolog	Organism	E -value	NCBI accession # or unisequence ID*
46cR3	DW985982	953	Phosphoenolpyruvate carboxykinase	<i>Emericella nidulans</i>	$1.00E^{-66}$	AAL10705
46sR	DW986092	478	HAD-superfamily hydrolase	<i>Sphingopyxis alaskensis</i>	$3.00E^{-60}$	EAN48415
51cR	DW985987	327	Cytosolic asparaginyl-tRNA synthetase	<i>Aspergillus fumigatus</i>	$2.00E^{-48}$	EAL87656
48cR	DW985984	301	Aldehyde dehydrogenase	<i>Cladosporium fulvum</i>	$4.00E^{-45}$	AAF82789
206sR	DW986125	409	Glycosylphosphatidylinositol transamidase	<i>Aspergillus fumigatus</i>	$1.00E^{-40}$	EAL89968
80cR	DW986015	290	Succinate-semialdehyde dehydrogenase	<i>Aspergillus fumigatus</i>	$2.00E^{-39}$	EAL92853
68cR	DW986129	355	Putative 50s ribosomal protein	<i>Aspergillus fumigatus</i>	$2.00E^{-39}$	EAL93148
34cR†	DW985971	439	Vacuolar ATPase proteolipid subunit	<i>Neurospora crassa</i>	$8.00E^{-39}$	AAK13465
53cR†	DW985989	569	Uracil transporter	<i>Cercospora nicotianae</i>	$5.00E^{-38}$	AAG38103
184sR5	DW986119	411	ZIP zinc transport protein	<i>Aspergillus fumigatus</i>	$9.00E^{-38}$	XP_659437
146sR5	DW986113	467	Steroid monooxygenase	<i>Aspergillus fumigatus</i>	$2.00E^{-36}$	EAL91847
197sR3	DW986123	378	Aldo-keto reductase	<i>Aspergillus fumigatus</i>	$4.00E^{-36}$	EAL86987
94cR†	DW986026	411	Phosphatase 2A regulatory B subunit	<i>Neurospora crassa</i>	$1.00E^{-35}$	AAD15987
95cR†	DW986130	444	Quinone reductase	<i>Aspergillus fumigatus</i>	$1.00E^{-35}$	EAL91468
77sR3	DW986096	427	Hypothetical protein	<i>Aspergillus fumigatus</i>	$2.00E^{-35}$	EAL88279
47cR	DW985983	386	Signal sequence receptor α subunit	<i>Aspergillus oryzae</i>	$6.00E^{-34}$	BAE61907
43cR†	DW985980	388	Glucosamine-fructose-6-P aminotransferase	<i>Emericella nidulans</i>	$1.00E^{-33}$	AAW49003
95sR3	DW986105	276	Glycosyl transferase	<i>Aspergillus fumigatus</i>	$1.00E^{-31}$	EAL84995
37sR	DW986091	503	Coatomer β subunit	<i>Aspergillus fumigatus</i>	$2.00E^{-30}$	EAL90424
69cR	DW986004	285	Glucose-regulated precursor	<i>Neurospora crassa</i>	$5.00E^{-30}$	EAA27331
71cR	DW986006	374	Hypothetical protein	<i>Aspergillus oryzae</i>	$1.00E^{-29}$	BAE62739
31cR	DW985968	308	β (1-3) glucanosyltransferase	<i>Aspergillus fumigatus</i>	$2.00E^{-27}$	EAL93577
9sR†	DW986086	255	Dihydroorotate reductase	<i>Aspergillus fumigatus</i>	$7.00E^{-27}$	EAL93396
86cR†	DW986021	300	ABC multidrug transporter	<i>Aspergillus fumigatus</i>	$3.00E^{-25}$	EAL92840
50cR	DW985986	215	Fimbrin (actin-filament bundling protein)	<i>Gibberella zeae</i>	$9.00E^{-25}$	EAA67746
72cR	DW986007	178	Transketolase	<i>Aspergillus niger</i>	$2.00E^{-24}$	BAE60691
108sR	DW986108	249	Peptide methionine sulfoxide reductase	<i>Aspergillus fumigatus</i>	$5.00E^{-23}$	EAL87411
214sR3	DW986128	172	Hypothetical protein	<i>Aspergillus nidulans</i>	$2.00E^{-21}$	EAA61671
42cR	DW985979	341	Oxidoreductase	<i>Cryptococcus neoformans</i>	$5.00E^{-19}$	AAW45419
84cR	DW986019	212	Hypothetical protein	<i>Aspergillus nidulans</i>	$5.00E^{-19}$	EAA60899
55cR	DW985991	281	Hypothetical protein	<i>Magnaporthe grisea</i>	$5.00E^{-17}$	XP_366565
88cR	DW986023	294	Soluble fumarate reductase	<i>Aspergillus fumigatus</i>	$5.00E^{-17}$	EAL85317
77cR	DW986012	259	3-ketoacyl-CoA thiolase peroxisomal A	<i>Aspergillus fumigatus</i>	$9.00E^{-17}$	EAL89695
90cR	DW986024	335	SREBP cleavage activating protein	<i>Meloidogyne graminicola</i>	$1.00E^{-16}$	Mga0275f*
51sR	DW986093	481	Proteasome regulatory subunit 12	<i>Aspergillus fumigatus</i>	$6.00E^{-16}$	EAL88462
96cR	DW986027	266	Amino acid transporter/permease	<i>Amanita muscaria</i>	$2.00E^{-15}$	CAB38005
165sR5	DW986116	227	Hypothetical protein	<i>Magnaporthe grisea</i>	$3.00E^{-14}$	MagCon[2205a]*
195sR	DW986122	279	Protein transport protein	<i>Aspergillus fumigatus</i>	$5.00E^{-14}$	EAL85624
65cR†	DW986001	337	Glutathione S-transferase	<i>Azotobacter vinelandii</i>	$1.00E^{-13}$	EAM04839
84sR5	DW986099	196	Hydroxymethylglutaryl-CoA lyase	<i>Neurospora crassa</i>	$2.00E^{-13}$	EAA34006
67cR	DW986003	122	Putative Cis-prenyltransferase	<i>Aspergillus fumigatus</i>	$3.00E^{-12}$	EAL89656
78cR	DW986013	286	ER membrane domain protein	<i>Aspergillus fumigatus</i>	$9.00E^{-12}$	EAL93330
74cR	DW986009	213	Vacuolar protein sorting factor	<i>Aspergillus fumigatus</i>	$1.00E^{-11}$	EAL89587
40cR	DW985977	290	Hypothetical protein	<i>Meloidogyne graminicola</i>	$9.00E^{-11}$	Mg[0147]*
52cR†	DW985988	115	Copper-zinc superoxide dismutase	<i>C. militaris</i>	$2.00E^{-10}$	XP_366549
94sR3	DW986104	162	Hypothetical protein	<i>Gibberella zeae</i>	$7.00E^{-10}$	XM_384502
138sR	DW986111	294	Dihydroorotase	<i>Neurospora crassa</i>	$2.00E^{-09}$	CAD71051
45cR	DW985981	293	Pathogenesis related protein	<i>Botryotinia fuckeliana</i>	$5.00E^{-09}$	BfCon[0410]*
88sR	DW986101	384	Hypothetical protein	<i>Gibberella zeae</i>	$1.00E^{-08}$	EAA76778
37cR	DW985974	450	Signal peptidase complex subunit 1	<i>X. tropicalis</i>	$1.00E^{-07}$	NP_001016638
32sR	DW986089	489	Hypothetical protein	<i>Cladosporium fulvum</i>	$1.00E^{-07}$	Cf8667490*
210sR5	DW986127	298	Protein translocation complex component	<i>Aspergillus fumigatus</i>	$1.00E^{-07}$	EAL90825
85cR†	DW986020	206	Disulfide-isomerase precursor	<i>Neurospora crassa</i>	$2.00E^{-07}$	EAA32279
79sR	DW986098	174	Glutamate receptor	<i>Magnaporthe grisea</i>	$3.00E^{-07}$	MagCon[0061a]*
79cR	DW986014	412	Hex2 protein phosphatase	<i>Aspergillus fumigatus</i>	$6.00E^{-07}$	EAL84897
140sR5	DW986112	247	Hypothetical protein	<i>Magnaporthe grisea</i>	$1.00E^{-06}$	Mag14180285*

Table 3. Continued.

Clone ID #	EST Genbank accession #	Size (bp)	Putative EST homolog	Organism	E-value	NCBI accession # or unisequence ID*
56cR	DW985992	270	Hypothetical protein	<i>Blumeria graminis</i>	2.00E ⁻⁰⁶	Bg13904088*
107cR	DW986031	165	Aldo-keto reductase	<i>Bacillus licheniformis</i>	2.00E ⁻⁰⁶	AAU24983
81sR	DW986131	289	Thiamine biosynthesis protein	<i>Fusarium sporotrichioides</i>	3.00E ⁻⁰⁶	FsCon[0686]*
49cR	DW985985	352	Hypothetical protein	<i>Gibberella zeae</i>	7.00E ⁻⁰⁶	EAA72198

*Unisequence ID from COGEME.

†Clones were selected for q-RT-PCR analysis.

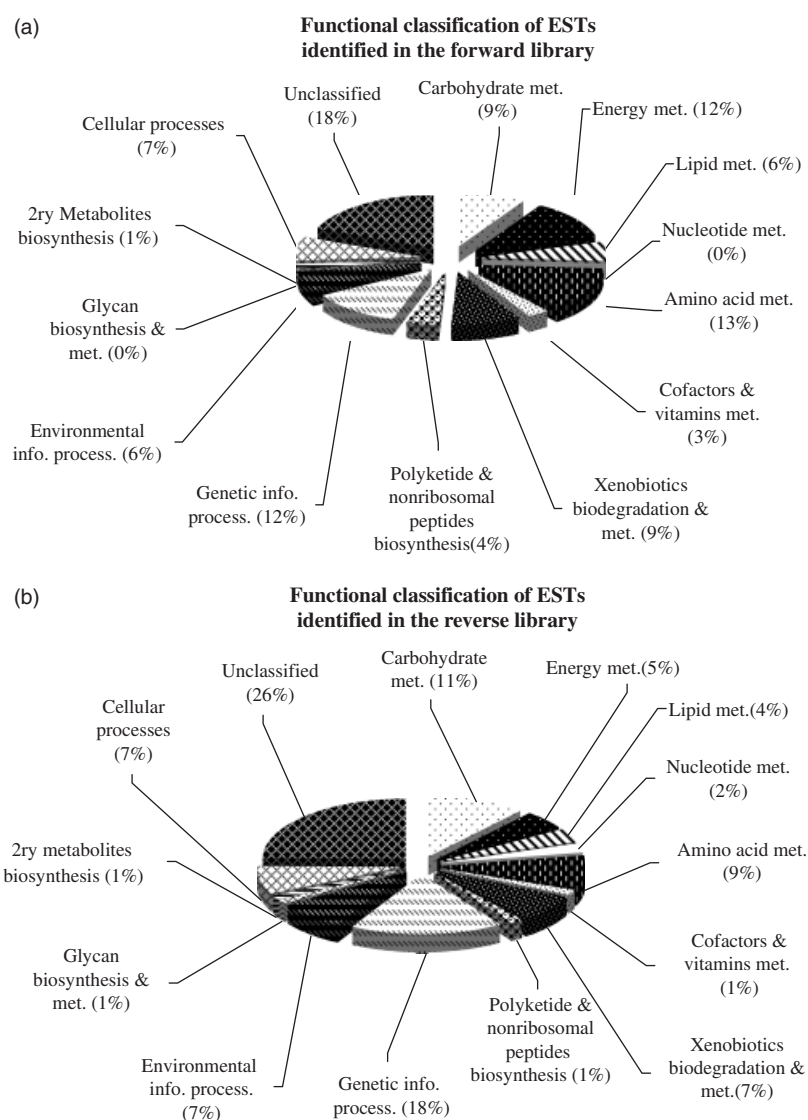


Fig. 3. Functional classification of ESTs. Percentage of ESTs from forward (a) and reverse (b) subtracted libraries with homology ($E < 1 \times 10^{-5}$) to publicly available protein sequences. Functional classifications are according to the KEGG (<http://www.genome.jp/kegg/>). Met, metabolism; 2ry, secondary; Info. Process, Information Processing.

(Chung *et al.*, 2003a) was up-regulated more than 10-fold in the *crg1*-null mutant, suggesting that CRG1 may be a repressor of this transporter. Taken together, the observations show that c. 60% of the recovered ESTs are differen-

tially regulated as predicted, and 38.5% differed by at least two-fold. These results indicate that the use of SSH is an efficient approach to identify differentially expressed genes between cercosporin-sensitive and -resistant strains.

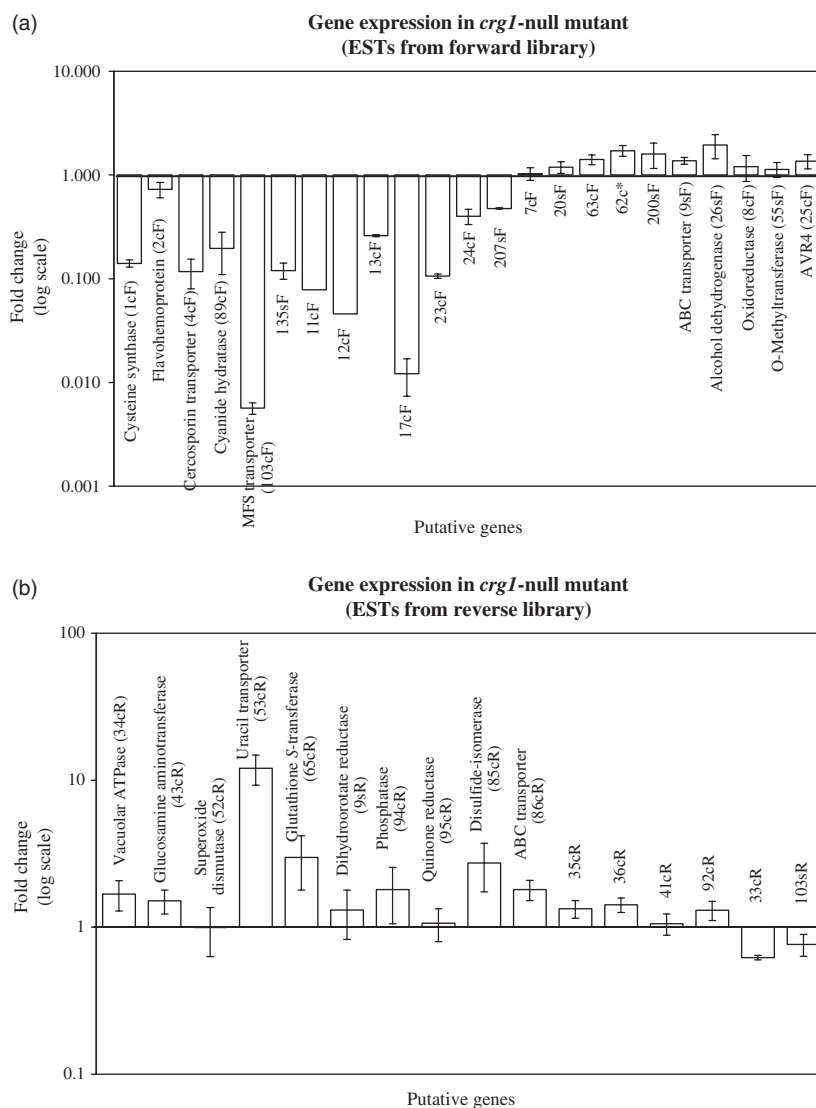


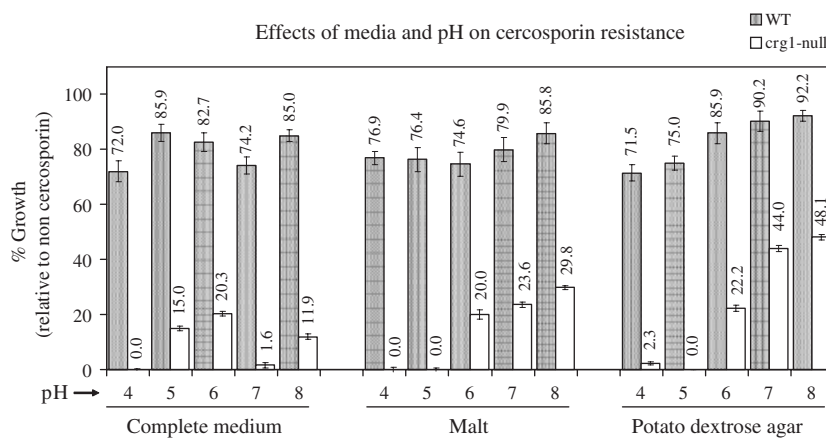
Fig. 4. q-RT-PCR analysis. Expression of selected genes in the cercosporin-sensitive *crg1*-null mutant strain were represented as fold-change relative to expression in the cercosporin-resistant WT (set to 1). (a) Expression of ESTs identified in the FLib. (b) Expression of ESTs identified in the RLib. Scale bars represent means from two different experiments with their associated SD. *Clone 62c was identified in both FLib and RLib.

Effects of pH and medium on toxin resistance

The role of CRG1 in the regulation of toxin resistance and biosynthesis is largely unknown, and its specific function as a transcription factor has not been elucidated. Sequence analysis revealed that CRG1 shows homology (score 186, E value = $4E^{-45}$) to a putative weak-acid response transcription factor from *A. fumigatus* (Genbank accession #: XP 746768.1). A homolog of this element found in *Saccharomyces cerevisiae* regulates stress adaptation through transcriptional induction of transporters involved in acid detoxification and indirectly affects pH homeostasis (Kren *et al.*, 2003). To investigate whether the *crg1* mutant is altered in pH responses, the effects of medium and pH on cercosporin toxicity to WT and *crg1* mutant strains were

tested (Fig. 5). Depending on the medium and pH, growth of the WT strain in the presence of cercosporin ranged from 71.5% to 92.2% of growth in the absence of cercosporin, with slightly less resistance at acidic pH, particularly on the PDA medium. This difference may be due to greater lability of the cercosporin molecule at high pH. By contrast, growth of the *crg1*-null strain was strongly affected by pH (Fig. 5). On two of the three media tested, growth of the *crg1* null mutant was virtually completely inhibited (0–2% growth relative to noncercosporin controls) at pH levels below pH 6. These observations indicate that the *crg1* null mutant is unable to defend itself against cercosporin toxicity under low-pH conditions, suggesting that this mutant is altered in the maintenance of normal pH that may be needed for cercosporin resistance.

Fig. 5. Effects of pH and medium on resistance to cercosporin. The WT and *crg1* mutant strains were grown for 1 week under continuous light on three media (CM, Malt and PDA) adjusted to the pH values shown and amended with 10 μ M cercosporin or without cercosporin. Scale bars represent growth of WT or the *crg1*-null strains in the presence of cercosporin expressed as a percentage of their growth in the absence of the toxin. Means and associated SD were calculated by averaging four fungal colonies.



Discussion

Work in the authors' laboratory focuses on understanding how *Cercospora* spp. protect themselves from the toxic effects of cercosporin, a secondary metabolite that generates ROS and is used by these fungi as a virulence factor during plant pathogenesis. As mentioned previously, several protection mechanisms have been proposed; however, no single mechanism has been found to be solely responsible for providing cercosporin protection in *Cercospora* spp., suggesting that resistance is complex and most likely multi-genic. In addition, efforts to understand toxin resistance in *Cercospora* spp. have been limited by the lack of sequence information and implementation of a high-throughput approach to study this system. The isolation and characterization of a transcription factor (CRG1) that is partially responsible for conferring self-resistance to cercosporin in the tobacco pathogen *C. nicotianae* (Chung *et al.*, 2003b) allowed the authors to pursue the identification of genes regulated by CRG1. To this end, a reverse genetics approach was adopted to identify genes differentially expressed between the cercosporin-resistant WT *C. nicotianae* and a cercosporin-sensitive *crg1* mutant lacking the transcription factor CRG1.

Reverse- and forward-subtracted cDNA libraries were constructed using SSH, and a collection of 185 ESTs was obtained. This number compares well with that recovered in similar studies (Cramer & Lawrence, 2001). The usefulness of SSH for recovering relevant ESTs was confirmed by the subtraction efficiency assay as well as by the fact that most sequences recovered were unique to either the FLlib or RLlib. Further, relative to the WT, genes in the *crg1*-null mutant behaved as expected when tested by q-RTPCR: those identified in the FLlib were mostly down-regulated, while those identified in the RLlib were mostly up-regulated. The authors' collection represents the largest number of sequences for the tobacco pathogen *C. nicotianae* deposited in the GenBank for public use (<http://www.ncbi.nlm.nih.gov/>).

The FLlib contained ESTs of potential significance to the authors' interest in cercosporin resistance. For example, several ESTs with homology to ABC and MFS transporters were identified. The most prominent function of these transporters is to provide protection against natural toxic compounds, and in plant pathogenic fungi they can mediate secretion of fungal toxins (Stergiopoulos *et al.*, 2002). Two different MFS transporters from *C. kikuchii* (CFP) and *C. nicotianae* (CBT4) have been characterized (Callahan *et al.*, 1999; Choquer *et al.*, 2007). CBT4 is part of the cercosporin biosynthesis cluster, and CFP was identified as encoded by a light-induced gene. In both instances, deletion of these transporters results in a reduction in cercosporin production and fungal virulence. Furthermore, *cfp* deletion mutants are partially sensitive to exogenous cercosporin. The authors proposed that CFP actively exports cercosporin and contributes to toxin auto-resistance in *C. kikuchii*. An EST homologous to CFP (DW985942) has been recovered, and it was found that this gene is down-regulated in the cercosporin-sensitive *crg1* mutant strain. However, *crg1*-null strains overexpressing a CFP homolog isolated from *C. nicotianae* show only a slight increase in resistance to exogenous cercosporin and grow less than 25% of WT (unpublished results), suggesting that additional mechanisms are responsible for cercosporin self-resistance in *C. nicotianae*. The role of the other putative ABC and MFS transporters identified through the subtractive library is presently being investigated. Sequence analysis indicates that these transporters are different from those reported earlier to play a role in cercosporin detoxification, namely Snq2p from *S. cerevisiae* (Ververidis *et al.*, 2001) and *Bcmfs1* from *Botrytis cinerea* (Hayashi *et al.*, 2002) (unpublished results), thus confirming the novelty of the transporters identified in the library.

Because WT and *crg1* differ in their ability to produce cercosporin, it was not surprising to recover ESTs with homology to polyketide biosynthetic genes, including two that are part of the cercosporin biosynthesis cluster:

DW985946, encoding an oxidoreductase and DW986049, encoding an *O*-methyltransferase (Chen *et al.*, 2007). The pleiotropic relationship between cercosporin biosynthesis and resistance is not clear. Toxin-deficient strains unable to produce cercosporin (Sollod *et al.*, 1992) and deletion mutants in the cercosporin biosynthetic pathway remain resistant to the toxin (K.R. Chung, personal communication), suggesting that production and resistance are under different genetic controls. In this study, nonbiosynthetic genes whose expression differed by at least two-fold between the cercosporin-resistant WT and susceptible *crg1* strains have been identified. These include a putative cysteine synthase, a cyanide hydratase, two MSF transporters and several unclassified ESTs from the FLib as well as a putative uracil transporter from the RLib. Their role in cercosporin resistance is presently under investigation. Interestingly, ESTs were recovered in the RLib with homology to genes known to be induced by oxidative stress such as those encoding glutathione-*S*-transferase and disulfide isomerase. These results indicate that oxidate-stress-related genes are up-regulated in the *crg1* mutant, a result consistent with the prediction that the lack of normal cercosporin-resistance mechanisms in this mutant places it under oxidative stress and requires the induction of other antioxidant defenses.

The functional classification of ESTs suggests that cellular energetics may be compromised in the *crg1* mutant strain. Changes in cellular respiration and therefore in energy metabolism in *crg1* may be the result of the oxidative-stress conditions encountered by this strain (Joseph-Horne *et al.*, 2001). In addition, one of the factors directly linked to energy metabolism is intracellular pH and pH gradients, which are regulated by plasma membrane, vacuolar, and mitochondrial ATPases (Hesse *et al.*, 2002). In this work, a vacuolar ATPase (DW985971) from the RLib up-regulated in the *crg1* mutant was identified. The recovery of this gene, along with the homology of CRG1 to an acid-response transcriptional regulator, led the authors to test the impact of pH on cercosporin toxicity to the *crg1* null mutant. Cercosporin toxicity assays revealed that the sensitivity of the *crg1* null mutant to cercosporin is strongly impacted by pH, with an almost complete lack of growth in the presence of cercosporin on two media at pH levels below 6. These results suggest that this strain may be compromised in its ability to detect an acidic environment and adjust its intracellular pH accordingly, and that maintenance of normal cellular pH is required for normal cercosporin resistance. Studies to address the role of CRG1 in intracellular pH regulation and its relationship with cercosporin resistance are presently being initiated.

In summary, a collection of ESTs has been obtained, sequenced and categorized from the tobacco pathogen *C. nicotianae* that are likely directly or indirectly regulated by the transcription factor CRG1 and may be involved

in auto-resistance to cercosporin, a toxin produced by plant pathogens of the genus *Cercospora*. The information generated in this study is presently being used to isolate full-length genes and further characterize their role in cercosporin protection. The studies will not only contribute to a better understanding of cellular resistance to cercosporin and oxidative stress but also to the development of novel strategies to control this group of economically important plant pathogens.

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