Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Microbiological Research 166 (2011) 566–577

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

Microbiological Research

www.elsevier.de/micres

# Differentially expressed cDNAs in *Alternaria alternata* treated with 2-propenyl isothiocyanate

María Elena Báez-Flores[a,1], Rosalba Troncoso-Rojas[a], María A. Islas Osuna[a], Marisela Rivera Domínguez[b], Barry Pryor[c], Martín Ernesto Tiznado-Hernández[a,*]

[a]*Coordinación de Tecnología de Alimentos de Orígen Vegetal, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, 83000, Mexico*
[b]*Ciencia de los Alimentos, Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo, Sonora, 83000, Mexico*
[c]*School of Plant Sciences, Division of Plant Pathology and Microbiology. The University of Arizona, P.O. Box 210036 Tucson, AZ, USA*

Received 15 October 2010 ; received in revised form 16 November 2010; accepted 20 November 2010

KEYWORDS
*Alternaria alternata*;
2-Propenyl isothiocyanate;
Suppressive substractive hybridization;
Expressed sequence tags

## Abstract

The molecular mechanism of the fungal tolerance phenotype to fungicides is not completely understood. This knowledge would allow for the development of environmentally friendly strategies for the control of fungal infection. With the goal of determining genes induced by 2p-ITC, a forward suppressive subtractive hybridization was performed using cDNAs from ITC-treated *Alternaria alternata* as a ''tester'' and from untreated fungus as a ''driver.'' Using this approach, a library containing 102 ESTs was generated that resulted in 50 sequences after sequence assembly (17 contigs and 33 singletons). Blastx analysis revealed that 38% and 40% of the sequences showed significant similarity with known and hypothetical proteins, respectively, whereas 18% were not similar to known genes. These last sequences could represent novel genes that play an unknown role in the molecular responses of fungi during adaptation to 2p-ITC. Clones similar to opsins, ABC transporters, calmodulin, ATPases and SNOG proteins were identified. Using real-time RT-PCR analysis, significant inductions of an ABC transporter and a $Ca^{++}$ ATPase during 2p-ITC treatment were discovered. These results suggest that the fungal resistance phenotype to 2p-ITC involves calcium ions and 2p-ITC efflux via an ABC transporter.
© 2010 Elsevier GmbH. All rights reserved.

*Corresponding author.
*E-mail address:* tiznado@ciad.mx (M.E. Tiznado-Hernández).

[1] Present address: Universidad Autónoma de Sinaloa, Facultad de Ciencias Químico Biológicas. Ciudad Universitaria, Culiacán Sinaloa, México.

0944-5013/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.micres.2010.11.004

Author's personal copy



## Introduction

The genus *Alternaria* is widespread and of great economic importance because it causes destructive leaf spots, foliar and blossom blight, blemishes and damage to a great variety of fruits and seeds from numerous hosts. It belongs to the group of necrotrophic fungi, which represent about 4% of the fungal diversity but cause ~80% of foliar losses in some parts of the world (Rotem, 1994). As a foliar pathogen, *Alternaria* spp. destroy host tissues through reduction of the photosynthetic potential (Agarwal et al., 1997), whereas in stored products, this fungus causes quiescent infections that penetrate the tissue, where it remains dormant until fruit conditions favor infection (Rotem, 1994). Thus, *Alternaria* spp. represent one of the most important post-harvest pathogens (Simmons, 1992).

The indiscriminate use of synthetic agrochemicals to control agricultural fungal infections has led to the development of resistance in phytopathogenic fungal populations, which is one of the most important problems in agriculture (Brent, 1995). A good alternative to chemical compound utilization in fungal disease control is natural compounds such as isothiocyanates. These compounds are part of the defense system of *Brassicas* plants and exhibit inhibitory activity against bacteria (Tajima et al., 1998), fungi (Troncoso-Rojas et al., 2005), nematodes (Kermanshai et al., 2001) and insects (Ratzka et al., 2002). In the case of fungi, isothiocyanates have demonstrated fungicidal activity against different species under *in vitro*, *in vivo* and *in solum* conditions (Tiznado-Hernández and Troncoso-Rojas, 2006).

Although isothiocyanates display strong inhibitory activity against fungal growth (Mari et al., 1993; Manici et al., 1997; Smolinska et al., 2003; Troncoso-Rojas et al., 2005; Troncoso et al., 2005), including *Alternaria alternata*, preliminary experiments in our laboratory have demonstrated that this fungus can grow in the presence of 2-propenyl isothiocyanate (2p-ITC) following a chronic exposure to sublethal doses (unpublished results).

A suitable approach to gain insight into the molecular basis of the 2p-ITC treatment response of *A. alternata* is to study the transcriptome of the ITC-tolerant fungus. Genetic analyses of plant pathogens are important in understanding epidemiology, host-pathogen co-evolution, resistance management and control methods. Comparisons of the genetic profiles of fungicide-sensitive and -resistant subpopulations within the same species are expected to elucidate the evolution of fungicide resistance (McDonald and McDermott, 1993; Báez-Flores et al., 2008). Understanding the mechanisms that underlie the development of fungal adaptation at the genetic level will allow the development of strategies to control fungal infections in a more effective and environmentally friendly manner.

Recently, the effect of 2p-ITC and benzylisothiocyanate (BITC) on *Alternaria brassicicola*, a specialized *Brassicas* pathogen, was evaluated. Differential display analysis led to the detection of the first glutathione S-transferase (GST) gene in this fungus (Sellam et al., 2006), designated *AbGSt1*, which was induced in the presence of 2p-ITC and BITC. Furthermore, the authors observed that *AbGst1* was upregulated during *A. brassicicola* infection in *Arabidopsis thaliana*, which shows a natural resistance against this pathogen.

In a more recent study, the suppressive subtractive hybridization technique (Diatchenko et al., 1996; Chenchick et al., 1998) was used to generate a cDNA library from germinated conidia of *A. brassicicola* treated with 2p-ITC (Sellam et al., 2007). Among the genes that were transcriptionally induced by 2p-ITC, 35% were found to be involved in the oxidative stress response: GSTs, glutathione peroxidases, glutamylcysteine synthetases, thioredoxins, thioredoxin reductases, oxidoreductases and cytochrome P450s. The response also included mechanisms involved in limiting the intracellular accumulation of the compound, as suggested by the finding that 16% of the cDNAs induced by ITC treatment encoded transporter proteins, mostly pleiotropic drug resistance types, such as ATP binding cassettes and the major facilitator superfamily (MFS).

The fungus *A. brassicicola* is a specialized *Brassicas* pathogen. In contrast, *A. alternata* is a generalist pathogen, and consequently, it is not expected to be tolerant to ITCs. In this regard, this organism represents a good model to study the effects of ITCs in fungi that are not exposed naturally to these compounds. An approach to gain insight into the mechanism by which *A. alternata* develops ITC tolerance involves the identification of differentially expressed genes in ITC-treated *A. alternata* using suppressive subtractive hybridization (SSH), which allows the detection of genes that are expressed in response to a specific stress or treatment. The goal of the present study was to understand the molecular mechanism that permits *A. alternata* growth in the presence of isothiocyanates.

Author's personal copy



## Materials and methods

### Fungal isolation and exposure to 2p-ITC

*A. alternata* was originally recovered from field fruit of tomato (*Lycopersicum esculentum*), purified by monospore isolation and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 4 °C. Fresh subcultures were generated by transferring a mycelia disk to a Petri dish containing 10 ml of PDA media covered with a cellophane membrane. A filter paper disk soaked with 9.7 μM 2p-ITC (Sigma—Aldrich Chemical Co., Milwaukee, Wisconsin) was collocated in the lid of each dish. The dishes were then sealed with parafilm and incubated at 28 °C for 5 days. The control fungus was inoculated and incubated using the same conditions except that the filter paper disk was soaked with distilled water. The mycelia of both fungi were harvested and maintained at −80 °C until use.

### RNA extraction

Whole-cell RNA from *A. alternata* ("tester" and "driver") was isolated according to the RNA extraction protocol published by Islas-Flores et al. (2006), which combines the TRIzol (Invitrogen, Carlsbad, California) and RNeasy methods (Qiagen Biosciences, Maryland) to avoid the co-precipitation of melanin with RNA (Islas-Flores et al., 2006).

### Suppression subtractive hybridization (SSH)

The cDNAs were synthesized from 1 μg of total RNA isolated from 2p-ITC treated *A. alternata* and control using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, California) according to the manufacturer's recommendations. The cDNA populations were then subtracted with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, California) using the cDNAs from the treated fungus as a "tester" and those from the control fungus as a "driver." The efficiency of subtraction was evaluated by amplifying a fragment of the *A. alternata* β-tubulin gene (before and after the subtractive procedure) using primers that were designed using the Primer3 program (Rozen and Skaletsky, 2000). Specific primers are required because the internal control of the PCR-Select cDNA Subtraction Kit is designed to amplify a fragment of the glyceraldehyde-3-phosphate dehydrogenase enzyme, and the sequence of this gene displays a low similarity to that of *A. alternata* according to the multiple sequence analysis conducted using the software ClustalW (Thompson et al., 1994).

The differentially expressed fragments obtained using the SSH procedure were ligated into the pGEM-T Easy vector (Promega, Madison, Wisconsin), and the recombinant plasmids were transformed into *E. coli* JM109 competent cells to create libraries containing the generated fragments. White/blue screening was performed to identify the recombinant clones. Clones carrying DNA fragments were grown overnight in LB-ampicillin to amplify the inserted clones. Plasmid DNA extraction was performed using the alkaline lysis method (Sambrook et al., 1989). The presence and the size of fragments in each putative recombinant clone were verified by digestion with *Rsa* I, and the fragments were separated by electrophoresis in a 1% agarose gel together with a 1 kb plus DNA ladder (Invitrogen, Carlsbad, California). The DNA fragments were stained with ethidium bromide and visualized using a transilluminator set at 312 nm (LA-20E, VWR Scientific, Buffalo Grove, Illinois). The gels were digitalized using a KODAK 4000MM Digital Imaging System (Kodak's Graphic Communications, Rochester, New York).

### Clone sequencing, assembly and analysis

Selected positive clones were sequenced using the M13 forward oligonucleotide primer at the Genomic Analysis and Technology Core Facility, University of Arizona, Tucson, Arizona, U.S.A. The obtained sequences were analyzed to identify vector fragments and adaptors, using the VecScreen program available at the NCBI website. Foreign DNA fragments were removed from the sequences, which were then assembled to eliminate redundancy using the CAP3 Sequence Assembler Program (Huang and Maddan, 1999). The resultant contigs and singletons were analyzed using the BLAST program with the Blastn (Zheng Zhang et al., 2000) and Blastx (Altschul et al., 1990) algorithms.

### Gene ontology annotation

The annotation for the contigs and singletons was carried out using the Blast2GO v.2 program available at the Bioinformatics Department, Centro de Investigación Príncipe Felipe, Valencia, Spain. Blast2GO is a bioinformatics tool that is used mainly for the automatic annotation of DNA or protein sequence data based on the gene ontology (GO) vocabulary; however, it is also used for non-model species (Götz et al., 2008). The primary goal of Blast2GO is to assign GO terms to nucleotide or pro-

Author's personal copy



**Table 1.** Sequence of the primers designed for quantitative Real-Time PCR experiments based in the known sequence of either the EST or the β-tubulin gene.

| Gene name | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| β- tubulin | ACGCTTCTCATCTCCAAGATCCGT | AGAGAGCCTCGTTGTCAATGCAGA |
| Aaitc1A | TGGGCGTCCAATCCCGTAACTATT | TACGCGGCATGTGTCTGTTTCCTA |
| Aaitc65 | ATTACCTTCATTGGGCTGGTGGGA | TAGGTAACCGTCGAACAATGGCGT |
| Aaitc128 | TTAGCCTCGAGGAACTCAGCAACA | AACTACCACTACGTCAAGCCCACA |
| Aaitc 141 | TGCGGCTTATCAGCTCTTCACTCA | TCCGACATCTTTGCCCTGACCTTT |
| Aaitc168 | AATGGCCGATGTGAAGTTCATGCC | TCTCCAAGTTCGCCAACGATGTCA |
| Aaitcas6 | AACCGACGATGAGAGCGATGACAA | AATACACGACGAGCATACCAGCGT |

tein sequences and to group them in three basic categories: biological processes, molecular functions and cellular components (Conesa and Götz, 2008).

In the present work, the initial blastx search was performed against the non-redundant NCBI database using a minimum expectation value of $1 \times 10^{-3}$ and a high scoring segment cut-off of 33. The annotation step was carried out with default parameters: the pre-evalue hit filter was $1 \times 10^{-6}$, the annotation cut-off was set at 55, and the GO weight was 5. The annotation was expanded using ANNEX (Annotation Expander), and the Blast2GO InterPro Scan was performed to search for additional GO terms that were associated with functional domains.

## Gene expression analysis by quantitative RT—PCR

From the cDNAs that were differentially expressed in the *A. alternata* 2p-ITC SSH library, six sequences that were found to play a putative role in fungal tolerance to ITC were selected for expression analyses at different times following 2p-ITC exposure. RNA was isolated at 0.5, 1, 2 or 12 h after treatment. The experiment was conducted using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, California) and the Full Velocity SYBR Green QRT-PCR MasterMix 1 Step, according to the instructions of the manufacturers. The RT-PCR cycling program was as follows: 50 °C for 30 min and 95 °C for 10 min (1 cycle); 95 °C for 10 s and 60 °C for 30 s (40 cycles); 95 °C for 15 s and 60 °C for 1 min (1 cycle). A melting point analysis was carried out (cooling the samples to 60 °C and heating 0.3 °C $s^{-1}$) to assure that only specific product was amplified. All of the samples were tested in triplicate. The β-tubulin gene was amplified simultaneously in the same plate and used as an endogenous control for normalization of the genetic expression level. A relative comparison analysis was performed using the comparative $C_T$ method. Gene primers were designed using the Primer3 software (Rozen and Skaletsky, 2000) and are shown in Table 1.

## Statistical analysis

The results of the expression experiments were analyzed using Rest© software, which performs the pairwise fixed reallocation randomization test and is designed specifically for statistical analysis of the relative expression data obtained in RT-PCR real-time experiments (Pfaffl et al., 2002).

# Results

The analysis of the subtracted cDNA library revealed that the differentially expressed fragments ranged in size from 500—900 bp. After cloning into the pGEM-T vector, a total of 102 recombinant clones were obtained that ranged in size from 250—824 bp, with an average length of 477 bp. After DNA sequencing, assembly of the different clones provided 50 fragments (17 contigs and 33 singletons). The Table 2 includes the results of the BLAST analysis performed against the non-redundant GenBank CDS database (Blastx algorithm). These analysis showed that 38% of the contigs were found to represent ESTs with significant similarity ($\leq 1e^{-06}$) to diverse, known proteins that were mostly from *Pyrenophora tritici repentis*; 40% were very similar to a hypothetical S-nitrosoglutathione (SNOG) from *Phaeosphaeria nodorum* and to PTRG proteins from *P. tritici repentis*. Furthermore, 4% of the sequences demonstrated similarity to proteins that were not reported in fungi, and 18% corresponded to unknown genes (Fig. 1).

In a BLAST search performed against the *A. brassicicola* EST collection (Blastn algorithm), 20% of the ESTs detected herein showed similarity to genes that were induced under nitrogen starvation,

Author's personal copy



**Table 2.** Results of the sequence analysis of the SSH cDNA from *A. alternata* 2p-ITC treated library by BLAST in the non redundant GenBank database (*E*-value < 1e-05).

| Contig/singlet* | Size (bp) | *E* value | GenBank Accession Number | Homologous Sequence in GenBank /Organism/ Accesion number |
|---|---|---|---|---|
| Aaitcas1 | 496 | 3e−35 | EZ000322 | Hypotetical protein PTRG 03541 *Pyrenophora tritici-repentis* XP_001933874.1 |
| Aaitcas2 | 636 | 6e−83 | EZ000323 | RNA binding domain *P. tritici-repentis* XP_001938447.1 |
| Aaitcas3 | 475 | 8e−66 | EZ000324 | Hypothetical protein SNOG_00220 *Phaeosphaeria nodorum* XP_001790911.1 |
| Aaitcas4 | 453 | 9e−76 | EZ000325 | Amino-acid permease inda1 *P. tritici-repentis* XP_001931109.1 |
| Aaitcas5 | 732 | 9e−23 | EZ000326 | Hypothetical protein *Vitis vinifera* CAN70790.1\| |
| Aaitcas6 | 512 | 4e−69 | EZ000327 | ABC multidrug CDR4 *P. tritici-repentis* XP_001941554.1 |
| Aaitcas7 | 465 | 1e−22 | EZ000328 | hypothetical protein PTRG_03541 *P. tritici-repentis* XP_001933874.1 |
| Aaitcas8 | 351 | 9e−49 | EZ000329 | Coatomer subunit alpha *P. tritici-repentis* XP_001932333.1 |
| Aaitcas9 | 585 | 1e−41 | EZ000330 | Hypothetical protein SNOG_00983 *P. nodorum* XP_001791644.1 |
| Aaitcas11 | 824 | 8e−32 | EZ000332 | Conserved hypothetical protein *P. tritici-repentis* XP_001934661.1 |
| Aaitcas12 | 656 | 1e−68 | EZ000333 | Conserved hypothetical protein *P. tritici-repentis* XP_001935771.1 |
| Aaitcas13 | 545 | 9e−47 | EZ000334 | hypothetical protein SNOG_02797 *P. nodorum* XP_001793392.1 |
| Aaitcas15 | 721 | 8e−34 | EZ000336 | Conserved hypothetical protein *P. tritici-repentis* XP_001941363.1 |
| Aaitcas16 | 536 | 1e−38 | EZ000337 | Hypothetical protein PTRG_00335 *P. tritici-repentis* XP_001930668.1 |

Author's personal copy



**Table 2.** (*Continued*)

| Contig/singlet* | Size (bp) | *E* value | GenBank Accession Number | Homologous Sequence in GenBank /Organism/ Accesion number |
|---|---|---|---|---|
| Aaitcas17 | 538 | 7e−84 | EZ000338 | Elongation factor 1-alpha *P. tritici-repentis* XP_001930631.1 |
| Aaitc1A | 508 | 9e−39 | GE467965 | Opsin-1 *P. tritici-repentis* XP_001937307.1 |
| Aaitc6A | 291 | 2e−37 | GE467968 | Benomyl/methotrexate resistance protein *P. tritici-repentis* XP_001937580.1 |
| Aaitc11A | 729 | 2e−116 | GE467971 | Aldehyde dehydrogenase *P. tritici-repentis* XP_001933942.1 |
| Aaitc15A | 345 | 1e−57 | GE467974 | Aromatic amino acid aminotransferase 1 *P. tritici-repentis* XP_001941234.1 |
| Aaitc27 | 378 | 9e−45 | GE467984 | Hypothetical protein SNOG_04093 *P. nodorum* XP_001794520.1 |
| Aaitc29 | 300 | 8e−30 | GE467986 | 60S ribosomal protein L18ae *P. tritici-repentis* XP_001935145.1 |
| Aaitc33 | 332 | 8e−30 | GE467988 | Hypothetical protein SNOG_03478 *P. nodorum* XP_001794041.1 |
| Aaitc65 | 318 | 1e−46 | GE468002 | Calcium-transporting ATPase 1 *P. tritici-repentis* XP_001942035.1 |
| Aaitc68 | 317 | 2e−11 | GE468003 | Conserved hypothetical protein *P. tritici-repentis* XP_001941608.1 |
| Aaitc101 | 611 | 6e−84 | GE468006 | Histone acetyltransferase MYST2 *P. tritici-repentis* XP_001941960.1 |
| Aaitc108 | 609 | 5e−63 | GE468013 | 60S ribosomal protein L21-A *P. tritici-repentis* XP_001937444.1 |
| Aaitc110 | 409 | 2e−37 | GE468015 | 40S ribosomal protein S18 *P. tritici-repentis* XP_001940789.1 |
| Aaitc121 | 532 | 4e−48 | GE468021 | Hypothetical protein PTRG_06153 *P. tritici-repentis* XP_001936486.1 |
| Aaitc126 | 491 | 3e−17 | GE468024 | Hypothetical protein Bm1_17870 *Brugia malayi* XP_001895031.1 |
| Aaitc127 | 486 | 8e−48 | GE468025 | Hypothetical protein SNOG_05837 XP_001796232.1 |
| Aaitc128 | 343 | 8e−54 | GE468026 | 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase *P. tritici-repentis* XP_001934603.1 |

Author's personal copy



**Table 2.** (*Continued*)

| Contig/singlet* | Size (bp) | *E* value | GenBank Accession Number | Homologous Sequence in GenBank /Organism/ Accesion number |
|---|---|---|---|---|
| Aaitc 141 | 383 | 3e−27 | GE468202 | EF-hand superfamily Ca2+-modulated protein *P. tritici-repentis* XP_001936278.1 |
| Aaitc143 | 508 | 2e−07 | GE468204 | Hypothetical protein SNOG_10318 *P. nodorum* XP_001800593.1 |
| Aaitc145 | 453 | 1e−64 | GE468205 | ATP-dependent RNA helicase eIF4A *P. tritici-repentis* XP_001938082.1 |
| Aaitc 148 | 393 | 2e−48 | GE468207 | Hypothetical protein SNOG_15237 *P. nodorum* XP_001805396.1 |
| Aaitc150 | 757 | 5e−54 | GE468208 | Hypothetical protein SNOG_02797 *P. nodorum* XP_001793392.1 |
| Aaitc152 | 629 | 8e−86 | GE468210 | Conserved hypothetical protein *P. tritici-repentis* XP_001932994.1 |
| Aaitc154 | 405 | 2e−56 | GE468211 | Aromatic amino acid aminotransferase 1 *P. tritici-repentis* XP_001941234.1 |
| Aaitc161 | 485 | 5e−53 | GE468212 | Fumarate reductase *P. tritici-repentis* XP_001937985.1 |
| Aaitc168 | 806 | 7e−111 | GE468213 | Hypothetical protein SNOG_14270 *P. nodorum* XP_001804465.1 |
| AAitc227 | 643 | 1e−60 | GE468215 | Hypothetical protein PTRG_00774 *P. tritici-repentis* XP_001931107.1 |

* An "s" at the end of the clone identification number is indicating an assembly, whereas a "c" is indicating a single EST sequence.

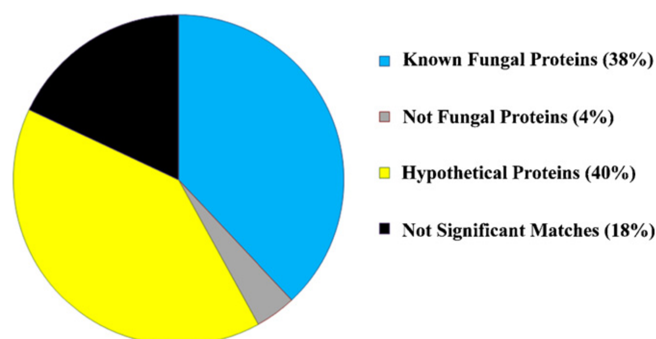


**Fig. 1.** Results of the bioinformatic analysis of the EST's induced by the treatment of *A. alternata* with 2-propenyl isothiocyanate. The data was obtained by a BLAST analysis performed against the non-redundant GenBank CDS database using the Blastx algorithm. Under hypothetical proteins are several EST's similar to the S-nitrosoglutathione (SNOG) from *Phaeosphaeria nodorum*.

Author's personal copy



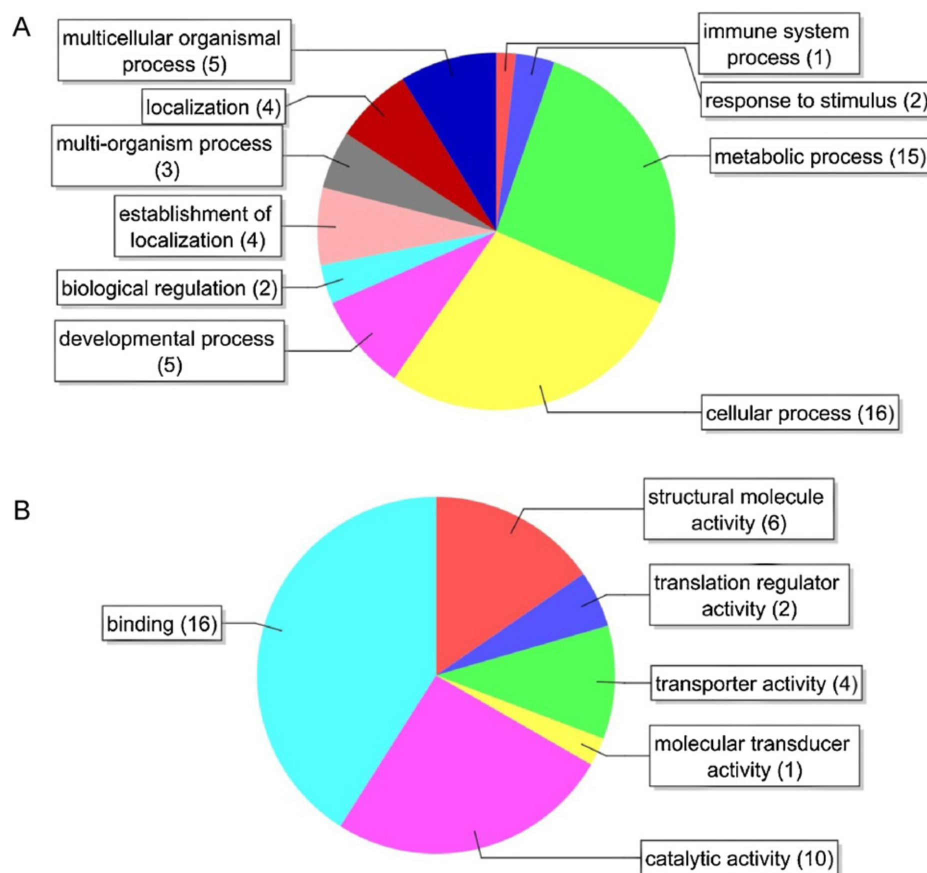


**Fig. 2.** Distribution of Gene Ontology Terms (Level 2), for transcripts obtained from *A. alternata* tolerant to 2-propenyl-Isothiocyanate. The numbers in graph categories, show the frequency for each GO term in the unigenes set. (A). Biological Process. (B). Molecular Function.

and one sequence (Aaitcas10) was similar to clone altr304xn06 (GB|DN477570.1|), which was obtained from a mycelial culture of *A. brassicicola* infecting *Brassica napus*, a plant that produces different isothiocyanates (Cramer et al., 2006). Additionally, one EST (Aaitc150) was similar to clone CmxP2E8 (GB|DY543110.1|), which was obtained from *A. brassicicola* conidia that were exposed to camalexin (Sellam et al., 2007). Another EST was similar to cDNA clone P3G9 (gb|CA405384.1|), which was obtained from spores germinating on the *Arabidopsis* leaf surface (Cramer and Lawrence, 2004).

## Annotation and GO analysis

The Blast2GO annotation provided ≥1 GO terms for 23 sequences (46%) from the total set. A total of 106 GO terms were retrieved. Among them, 42.4% could be assigned to biological processes, 40.5% to molecular functions and 17% to cellular components. The graph showing the biological processes is presented in Fig. 2A, in which the major categories at level 2 were cellular processes (GO:0009987), metabolic processes (GO:0008152), developmental processes (GO:0032502) and processes specific to multicelular organisms (GO:0032501). By increasing the ontology level up to 4, the cellular process indicated phenomena such as ribonucleoprotein complex biogenesis and assembly (GO:00226013), organelle organization and biogenesis (GO:006996), and gene expression (GO:0010467), whereas metabolic processes were integrated mostly via primary metabolic processes (GO:0044238), protein metabolic processes (GO:0019538), cellular macromolecule metabolic processes (GO:0044260), macromolecule biosynthetic processes (GO:0009059) and biopolymer metabolic processes (GO:0043883). Developmental processes were represented by mycelial development (GO:0043581).

Interestingly, the EST Aaitc161 was annotated with GO:009410 and GO:0006805 terms, which are related to xenobiotic stimuli and xenobiotic metabolic processes, respectively. In contrast, EST Aaitc1A was annotated with GO terms that

Author's personal copy



**Table 3.** Time-course expression of six gens in *A. alternata* 2 p-ITC treated measured by quantitative real time reverse transcription-PCR using the comparative $C_T$ method. The gene transcription in each sample was normalized using the $C_T$ values from the β-tubulin housekeeping gene. The fold change represents the number of times that one gene is expressed compared with the reference sample (untreated control). Results are the mean of three repetitions indicating the standard deviations. The $\Delta C_T$ values statistically different ($p < 0.05$; pair wise fixed reallocation randomization test), from that obtained for the control fungus, are shown in bold numbers.

| Gene name | Fold change RQ ($2^{-\Delta\Delta C_T}$) ± SD | | | |
|---|---|---|---|---|
| | 30 min | 1 h | 2 h | 12 h |
| Opsin | **1.39** ± 0.05 | 0.64 ± 0.03 | 0.13 ± 0.12 | 0.62 ± 0.00 |
| $Ca^{++}$ATPase | **2.67** ± 0.4 | **6.24** ± 0.15 | **3.92** ± 0.23 | **1.50** ± 0.19 |
| $5MH_4$-PteGlu[a] | 0.22 ± 0.28 | 0.25 ± 0.20 | 0.76 ± 0.13 | 0.61 ± 0.04 |
| EFhand protein | 1.48 ± 0.20 | **2.29** ± 0.28 | **1.92** ± 0.26 | 0.42 ± 0.13 |
| SNOG protein | 1.54 ± 1.0 | 0.81 ± 0.90 | 1.12 ± 0.93 | 1.87 ± 0.61 |
| ABC transporter | **95.8** ± 0.00 | **25.6** ± 0.04 | **3.85** ± 0.04 | 1.38 ± 0.03 |

were related to the detection of abiotic stimuli (GO:009582).

By examining potential molecular functions, shown in Fig. 2B, the following major categories were identified: binding (GO:0005488), catalytic activity (GO:0003824) and structural molecule activity (GO:0005198). By increasing the ontology level up to 4 (data not shown), the binding activity could be related mostly to cation binding (GO:0043169), metal ion binding (GO:0046872), purine nucleotide binding (GO:0017076), RNA binding (GO:0003723) as well as ribonucleotide binding (GO:0032553), and a small proportion could be associated with pyridoxal phosphate binding (GO:0030170) and heme binding (GO:0020037). The catalytic activity in the higher ontology level 4 was represented mostly by hydrolase activity (GO:0016817, GO:0016788), transferase activity (GO:0016769, GO:0016741) and oxidoreductase activity (GO:0016627).

## RT-PCR analysis

Among the six genes evaluated following the 2p-ITC treatment, four demonstrated a statistically significant up-regulation ($p < 0.05$): opsin, $Ca^{++}$ATPase, EF-hand protein (calmodulin) and the ABC transporter (Table 3). The expression of the opsin gene was increased up to 1.39-fold at 0.5 h after the treatment, whereas the expression of $Ca^{++}$ ATPase increased 6.24-fold after 1 hour and then decreased 3.92- and 1.5-fold after 2 and 12 h, respectively.

The cDNA corresponding to calmodulin demonstrated 1.48-, 2.29- and 1.92-fold increases at 0.5, 1, and 2 h after the initiation of treatment, respectively. A marked increase in the expression of the ABC transporter was observed, with a 95.8-fold change in expression at 0.5 h followed by a decrease to 25.6- and 3.8-fold at 1 and 2 h after 2p-ITC treatment, respectively. After 12 h of treatment, the expression level of the ABC transporter showed and increase of only 1.3-fold. Changes in the expression level of ATPase were small compared to those observed for the ABC transporter. However, the increased expression level of ATPase was statistically significant ($p < 0.05$). ABC transporter expression decreased markedly after 2 h of treatment, which could be related to the half-life of these proteins. Previously, some reports have described half lives of five days for some ABC transporter (Wakabayashi et al., 2006).

No significant changes in expression were recorded for the SNOG and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase cDNAs.

## Discussion

In the *A. alternata* subtractive cDNA library, sequences that play central roles in the post-transcriptional regulation of gene expression were found (RNA binding domains), and several cDNAs encoding membrane integral proteins were detected: amino acid permeases, the ABC multidrug CDR4 transporter, methotrexate resistance protein, opsin, ATPases and fumarate reductase. These proteins play a role in several phenomena, such as amino acid transport (Hosie et al., 2002), the efflux of natural and chemical toxic compounds (Del Sorbo et al., 2000), synthetic fungicide resistance (Brôco et al., 1999), light-driven $H^+$ pumping activity (Bieszke et al., 1999) and maintenance of the intracellular calcium concentration (Vanoevelen et al., 2005).

Author's personal copy



The ITC treatment may directly impact nitrogen utilization in *A. alternata*, as suggested by the similarity (83—98% at the nucleotide level) demonstrated by 20% of our ESTs with *A. brassicicola* clones that were induced by nitrogen starvation. Reinforcing this hypothesis, we also detected differentially expressed permeases. Such phenomena have also been observed in *Saccharomyces cerevisiae*, which contains permeases that are highly expressed under nitrogen limitation conditions and are thought to scavenge amino acids for use as a nitrogen source (Magasanik and Kaiser, 2002). Perhaps the expression of permeases could be related to the need for new amino acids or glutathione synthesis, although additional experimental evidence is needed to support this hypothesis.

Induction of the ABC transporter in the 2p-ITC *A. alternata* transcriptome can be explained by the finding that these transporters play a role in the secretion of specific and non-specific host toxins as a protective strategy against plant defense compounds. These processes occur in fungicide resistance (Del Sorbo et al., 2000) and in the efflux of xenobiotics via a co-transport mechanism that involves reduced levels of glutathione (Leslie et al., 2001). In agreement, ABC transporters have been found to be induced in 2p-ITC-treated *A. brassicicola* (Sellam et al., 2007). Altogether, these finding suggest a possible role for the ABC transporter in fungal resistance against the toxic effects of 2p-ITC.

Concerning the opsin gene, diverse stress conditions, such as anaerobic growth and nutrient or water stress, affect opsin gene expression in fungi (Bieszke et al., 1999). However, a clear role for opsins in fungal biology remains to be elucidated (Idnurm and Howlett, 2001) and cannot be explained based on the data obtained herein.

Fungal ATPases are all integral membrane protein $E_1$-$E_2$ (P-type) pumps that hydrolyze ATP and translocate cations across the membrane (Bowman et al., 1988). In addition, ATPases remove $Ca^{++}$ from the cytosol to decrease its cytoplasmic concentration to basal levels in response to different external stimuli (Vanoevelen et al., 2005).

In addition to the above mentioned proteins, ESTs similar to EF-hand $Ca^{++}$-modulated proteins were found, known as the calmodulin superfamily, which participate in the control of different calcium signaling pathways (Ikura and Ames, 2006).

Finally, many of the ESTs and assemblies were highly similar to hypothetical SNOG proteins. The similarity of some of the isolated cDNAs to SNOG proteins could suggest an induction of oxidative stress by the ITC treatment. SNOG proteins play an important role in diverse organisms as nitric oxide donors and are involved in protection against oxidative stress (Rosenberg et al., 1999) and apoptosis (Itoga et al., 1997). In *C. heterostrophus*, *S. nodorum*, and *A. nidulans*, orthologs of SNOG genes are related to the synthesis of transport compounds and to protection against oxidative stress (Hane et al., 2007).

In mammals, the endothelial and neuronal NO synthases are constitutively expressed enzymes, which upon stimulation, synthesize NO via a pulse-controlled $Ca^{+2}$/calmodulin mechanism (Kleinert et al., 2000). This last mechanism could help to explain the simultaneous induction of SNOGs, ATPases, and calmodulin proteins in *A. alternata* following ITC treatment. Altogether, the data generated in the present work seem to suggest an involvement of $Ca^{++}$ as a second messenger during the activation of signal transduction in response to the toxic effects of ITC.

In agreement with the presented results, the GO analysis revealed that cellular, metabolic, and developmental processes were the major categories corresponding to biological processes; in contrast, binding activity, catalytic activity and structural molecular activity were the major categories associated with molecular functions. In agreement with these findings, some of the induced gene fragments encoded integral membrane proteins and transcription factors with functions that are related to the transport of diverse molecules across membranes and to transcription initiation or elongation, respectively. Catalytic activity was represented mostly by hydrolase, transferase, and oxidoreductase activities, which might suggest a fungal response to the stress imposed by the 2p-ITC.

The data generated in this work suggest that the fungal response to the toxic effects of 2p-ITC includes the isothiocyanate efflux mediated by an ABC transporter. Calcium appears to play a role in the fungal response to the presence of 2p ITC.

The known and novel genes detected in the *A. alternata* SSH library permit a better understanding regarding the transcriptomic response of *A. alternata* to ITCs.

## Acknowledgement

We thank CONACYT (National Research Council for Science and Technology, Grant 46205, México) for providing the financial support.

Author's personal copy



## References


Agarwal A, Gark G, Devi S, Mishra D, Singh U. Ultrastructural changes in Brassica caused by *Alternaria brassicae* and destruxin B. J Plant Biochem Biotechnol 1997;6:25—8.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403—10.

Báez-Flores ME, Troncoso-Rojas R, Tiznado-Hernández ME. Biochemical and genetic responses of fungi to the toxic effect of synthetic and natural fungicides. Am J Agril & Biol Sci 2008;3(1):348—57.

Bieszke JA, Braun EL, Bean LE, Kang S, Natvig DO, Borkovich KA. The *nop-1* gene of *Neurospora crassa* encodes a seven transmembrane helix retinal-binding protein homologous to archaeal rhodopsins. Proc Natl Acad Sci USA 1999;96:8034—9.

Bowman EJ, Siebers A, Altendorf K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA 1988;85:7972—6.

Brent, KJ, 1995. Fungicide resistance in crop pathogens: how can it be managed? Brussels, GIFAP Groupement International des Associations Nationales de Fabricants de Produits Agrochimiques.

Brôco N, Tenreiro S, Viegas C, Sá-Correia I. FLR1 gene (ORF YBR008c) is required for benomyl and methotrexate resistance in *Saccharomyces cerevisiae* and its benomyl-induced expression is dependent on pdr3 transcriptional regulator. Yeast 1999;15(15):1595—608.

Conesa A, Götz S. Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics 2008 [Article ID 619832, 12 pages].

Cramer R, Lawrence C. Identification of *Alternaria brassicicola* genes expressed in planta during pathogenesis of *Arabidopsis thaliana*. Fungal Genet Biol 2004;41(2):115—28.

Cramer RA, La rota CM, Cho Y, Thon M, Craven KD, Knudson DL, et al. Bioinformatic analysis of expressed sequence tags derived from a compatible *Alternaria brassicicola*-brassica oleracea interaction. Mol Plant Pathol 2006;7(2):113—24.

Chenchick A, Zhu Y, Diatchenko L, Li R, Hill J, Siebert P.Siebert P, Larry J, editors. RT-PCR methods for gene cloning and analysis. MA: BioTechniques Books; 1998. p. 305—19.

Del Sorbo G, Schoonbeek H, De Waard M. Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol 2000;30(1):1—15.

Diatchenko L, Lau Y-FC, Campbell AP, Chenchick A, Mokadam F, Huang B, et al. Supression subtractive hybridization: a method for generating diferentially regulated or tissue specific cDNA probes and libraries. Proc Natl Acad Sci USA 1996;93:6025—30.

Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 2008;36(10):3420—35.

Hane JK, Lowe RGT, Solomon PS, Tan K-C, Conrad L, Schoch S b., et al. Dothideomycete — plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. Plant Cell 2007;19:3347—68.

Hosie AHF, Allaway D, Galloway CS, Dunsby HA, Poole PS. Rhizobium leguminosarum has a second general amino acid permease with unusually broad substrate specificity and high similarity to branched-chain amino acid transporters (Bra/LIV) of the ABC family. J Bacteriol 2002;184(15):4071—80.

Huang X, Maddan A. CAP3: a DNA sequence assembly program. Genome Res 1999;9:868—77.

Idnurm A, Howlett BJ. Characterization of an opsin gene from the ascomycete *Leptosphaeria maculans*. Genome 2001;44(2):167—71.

Ikura M, Ames JB. Genetic polymorphism and protein conformational plasticity in the calmodulin superfamily: two ways to promote multifunctionality. Proc Natl Acad Sci USA 2006;103(5):1159—64.

Islas-Flores I, Peraza-Echeverría L, Canto-Canché B, Rodríguez-García CM. Extraction of high quality, melanin free RNA from *Mycosphaerella fijiensis* for cDNA preparation. Mol Biotechnol 2006;34(1):45—50.

Itoga M, Tsuchiya M, Ishino H, Shimoyama M. Nitric oxide-induced modification of glyceraldehyde-3-phosphate dehydrogenase with NAD+ is not ADP-ribosylation. J Biochem 1997;121(6):1041—6.

Kermanshai R, McCarry BE, Rosenfeld J, Summers PS, Weretilnyk EA, Sorger GJ. Benzyl isothiocyanate is the chief or sole anthelmintic in papaya seed extracts. Phytochemistry 2001;57:427—35.

Kleinert H, Boissel J, Schwarz P, Förstermann U.Ignarro L, editor. Nitric oxide: biology and pathobiology. New York: Academic Press; 2000. p. 105—28.

Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SPC. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. Mol Pharmacol 2001;59(5):1171—80.

Magasanik B, Kaiser CA. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene 2002;290:1—18.

Manici L, Lazzeri L, Palmieri S. In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. J Agric Food Chem 1997;45(7):2768—73.

Mari M, Iori R, Leoni O, Marchi A. In vitro activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. Ann Appl Biol 1993;123(1):155—64.

McDonald B, McDermott J. Population genetics of plant pathogenic fungi. BioScience 1993;43(5):311—9.

Pfaffl M, Horgan G, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real time PCR. Nucleic Acids Res 2002;30(9):e36.

Ratzka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J. Disarming the mustard oil bomb. Proc Natl Acad Sci USA 2002;99(17):11223—8.

Rosenberg PA, Li Y, Ali S, Altiok N, Back SA, Volpe JJ. Intracellular redox state determines whether nitric oxide

Author's personal copy



is toxic or protective to rat oligodedrocytes in cultive. J Neurochem 1999;73(2):476—84.

Rotem J. The genus *Alternaria*: biology, epidemiology, and pathogenicity. St. Paul Minnesota: APS Press; 1994.

Rozen S, Skaletsky H. Bioinformatics methods and protocols. In: Misener S, Krawetz SA, editors. Methods in molecular biology., 132. Totowa, NJ: Springer; 2000. p. 365—86.

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

Sellam A, Poupard P, Simoneau P. Molecular cloning of *AbGst1* encoding a glutathione transferase differentially expressed during exposure of *Alternaria brassicicola* to isothiocyanates. FEMS Microbiol Lett 2006;258:241—9.

Sellam A, Dongo A, Guillemette T, Hudhomme P, Simoneau P. Transcriptional responses to exposure to the brassicaceous defence metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus *Alternaria brassicicola*. Mol Plant Pathol 2007;8(2):195—208.

Simmons E.Chelkowsky J, Visconti A, editors. Alternaria biology, plant disease and metabolites. Amsterdam: Elsevier Science Publishers; 1992. p. 1—35.

Smolinska U, Morra M, Knudsen G, James R. Isothiocyanates produced by *Brassicaceae* species as inhibitors of *Fusarium oxysporum*. Plant Dis 2003;87(4):407—12.

Tajima H, Kimoto H, Taketo Y, Taketo A. Effects of synthetic hydroxy isothiocyanates on microbial systems. Biosci Biotechnol Biochem 1998;62(3):491—5.

Thompson J, Higgins D, Gibson T. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22(22):4673—80.

Tiznado-Hernández ME, Troncoso-Rojas R. Control of fungal diseases with isothiocyanates. Stewart Postharvest Rev 2006;2(1):1—14.

Troncoso-Rojas R, Sánchez-Estrada A, Ruelas C, García HS, Tiznado-Hernández M. Effect of benzyl isothiocyanate on tomato fruit infection development by *Alternaria alternata*. J Sci Food Agric 2005;85(9):1427—34.

Troncoso R, Espinoza C, Sánchez-Estrada A, Tiznado ME, García HS. Analysis of the isothiocyanates present in cabbage leaves extract and their potential application to control *Alternaria* rot in bell peppers. Food Res Int 2005;38(6):701—8.

Vanoevelen J, Dode L, Baelen KV, Fairclough RJ, Missiaen L, Raeymaekers L, et al. The secretory pathway Ca/Mn-ATPase 2 is a golgi-localized pump with high affinity for Ca ions. J Biol Chem 2005;280(24):22800—8.

Wakabayashi Y, Kipp H, Arias IM. Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. J Biol Chem 2006;281(38):27669—73.

Zheng Zhang, Scott Schwartz, Lukas Wagner, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000;7(1—2): 203—14.