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Analysis of a suppressive subtractive hybridization library of *Alternaria alternata* resistant to 2-propenyl isothiocyanate

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ABSTRACT

Background: Isothiocyanates (ITCs) are natural products obtained from plants of the Brassicas family. They represent an environmentally friendly alternative for the control of phytopathogenic fungi. However, as it has been observed with synthetic fungicides, the possibility of inducing ITC-resistant strains is a major concern. It is, therefore, essential to understanding the molecular mechanisms of fungal resistance to ITCs. We analyzed a subtractive library containing 180 clones of an *Alternaria alternata* strain resistant to 2-propenyl ITC (2-pITC). After their sequencing, 141 expressed sequence tags (ESTs) were identified using the BlastX algorithm. The sequence assembly was carried out using CAP3 software; the functional annotation and metabolic pathways identification were performed using the Blast2GO program.
Results: The bioinformatics analysis revealed 124 reads with similarities to proteins involved in transcriptional control, defense and stress pathways, cell wall integrity maintenance, detoxification, organization and cytoskeleton destabilization; exocytosis, transport, DNA damage control, ribosome maintenance, and RNA processing. In addition, transcripts corresponding to enzymes as oxidoreductases, transferases, hydrolases, lyases, and ligases, were detected. Degradation pathways for styrene, aminobenzoate, and toluene were induced, as well as the biosynthesis of phenylpropanoid and several types of N-glycan.
Conclusions: The fungal response showed that natural compounds could induce tolerance/resistance mechanisms in organisms in the same manner as synthetic chemical products. The response of *A. alternata* to the toxicity of 2-pITC is a sophisticated phenomenon including the induction of signaling cascades targeting a broad set of cellular processes. Whole-transcriptome approaches are needed to elucidate completely the fungal response to 2-pITC.

1. Introduction

The use of synthetic fungicides in agriculture caused the development of drug-resistant fungal strains [1]. The presence of toxic residues in agricultural products may have potentially adverse effects on human health [2], the environment [3], and biodiversity [4,5]. It is, therefore, important to reduce the dependency on synthetic fungicides to control phytopathogenic fungi. For this purpose, natural fungicides of plant origin are being explored. Among them, isothiocyanates (ITCs) represent promising alternatives to synthetic fungicides for the control of fungi causing postharvest fruit losses [6,7]. In addition, ITCs have attracted attention in cancer research because of their ability to inhibit carcinogenesis and cancer growth in both *in vitro* and *in vivo* models [8].

ITCs are compounds synthesized by plants from the *Brassicaceae* family, such as radish, cauliflower, and mustard. They have a potent fungicidal activity against a number of fungi, including *Alternaria alternata* [6]. *A. alternata* is a fungus that causes spot lesions on the leaves [9] and fruits [10] of a broad variety of hosts [11,12], and is considered an important generalist phytopathogen in the field and during postharvest. Another *Alternaria* species, *Alternaria brassicicola* is a specialist, which is infective to the plants of the genus *Brassicas* [13]. Because *Brassicas* plants produce ITCs as a defense mechanism against infectious microorganisms or predators, *A. brassicicola* developed a particular resistance mechanism against this strong selective pressure during their coevolution [14]. Thus, *A. brassicicola* acquired special mechanisms to resist ITCs, but such mechanisms are not present in the generalist fungus *A. alternata*. Because *A. alternata* is phylogenetically close to *A. brassicicola*, we hypothesized that *A. alternata* would respond and survive to the toxic effects of ITCs. Thus, *A. alternata* may be a useful model to study the molecular mechanisms activated in response to ITCs.

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In the previous work [15], *A. alternata*, a fungus naturally sensitive to ITCs, was found to acquire tolerance or resistance to the ITCs toxicity after a constant and prolonged exposure. That work suggested that the prolonged use of natural fungicides could induce the emergence of resistant strains, as it has been observed with synthetic chemical products.

For the adaptation to fungicides, fungi use various mechanisms [16]. With regard to ITCs, Sellam and coworkers [17] reported that the response of *A. brassicicola* to 2p-ITC was similar to that observed under oxidative stress conditions since 35% of the transcriptionally induced genes corresponded to glutathione S-transferase (GST), glutathione peroxidase, glutamyl cysteine synthetases, thioredoxins, thioredoxin-reductases, oxidoreductases, and cytochrome P450. In addition, the mechanisms for reducing the intracellular accumulation of 2p-ITC were induced. In total, 16% of the induced genes were identified as encoding mainly ATP-binding cassettes (ABCs) and major facilitator superfamily transporter proteins [17]. In our previous work, we constructed and analyzed a suppressive subtracted hybridization (SSH) library from the mycelia of *A. alternata* treated with 2-pITC, and found expressed sequence tags (ESTs) coding RNA-binding domains and integral membrane proteins, such as ABC CDR4 transporters, opsins, ATPases, and fumarate reductases [18]. In addition, we detected the sequences coding modulating proteins of the calmodulin family (EF-hand Ca^{++}) and hypothetical S-nitrosoglutathione [18]. Although these results suggest possible molecular mechanisms of *A. alternata* adaptation to 2-pITC, additional adaptation mechanisms, including numerous metabolic pathways, exist because there are many more unexplored differentially expressed clones in the SSH library. In our previous work, a forward library was constructed, which allowed us to identify the genes that were being expressed in the treated organism but not in the control organism. Thus, the differentially expressed genes could be directly involved in the resistance process.

In this study, we analyzed 180 such unexplored clones from the forward SSH library constructed from *A. alternata* tolerant to 2-pITC. Because of their origin and nature, these unstudied clones represent a valuable genetic resource to provide additional scientific information on the molecular adaptation mechanisms of *A. alternata* to ITCs. Since the number of clones in this study was higher than in the first round of analysis, we expected to find different transcripts that were involved in previously unidentified adaptation processes in *A. alternata* 2-pITC tolerance, or were involved in known adaptation pathways to toxic compounds in other organisms. Indeed, we found a very diverse number of transcripts encoding for proteins or enzymes not detected in the first screening of the library. Further, they were not reported in previous studies focusing on the *Alternaria* tolerance to natural or synthetic compounds. These results are significant and complement previous works including ours because they reveal transcripts regulating the expression of genes and, allowed us to visualize genetic networks that are activating metabolic pathways to alleviate the toxic effect of 2-pITC on *A. alternata*.

2. Materials and methods

2.1. Library construction

A forward SSH library was constructed following the protocol of the provider company (Clontech, Palo Alto, CA, USA). The details regarding the *A. alternata*, SSH library construction can be reviewed in Baez-Flores et al. [18]. Briefly, the mRNA was isolated from *A. alternata* strain adapted to lethal levels of 2-propenyl-isothiocyanate according to the protocol published by Islas-Flores et al. [19]. cDNAs were prepared using the SMART PCR cDNA synthesis kit and subtracted with the PCR-Select DNA Subtraction Procedure (Clontech, Palo Alto CA). The differentially expressed cDNAs were cloned into p-GEM-T Easy vector

and cells of *E. coli* JM109 were transformed with them (Promega, Madison, WI).

2.2. Plasmid DNA extraction and sequencing of differentially expressed ESTs

Clones harboring ESTs in the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA), from the 2-pITC-treated *A. alternata* SSH library, were reactivated in LB agar. Then, the clones were cultivated in an LB-ampicillin broth for 24 h. Plasmid DNA was extracted using the alkaline lysis method [20] and digested with the *Rsa*I restriction enzyme (New England Biolabs® Inc. Ipswich, MA, USA). The restriction products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized in a transilluminator set at 312 nm (LA-20E; VWR Scientific, Buffalo Grove, IL, USA). The size of the insert in each clone was confirmed. The plasmid DNA was sent for sequencing (Genomic Analysis and Technology Core Facility, University of Arizona, AZ, USA) using the Sanger dideoxy sequencing technique and the M13 forward oligonucleotide.

2.3. Assembly and BLAST analysis of the sequences

To eliminate the DNA of vector origin and the adaptors used for the differentially expressed fragments' amplification, the obtained sequences were analyzed using the VecScreen program available at the National Center for Biotechnology Information (NCBI) webpage. Then, sequences corresponding to the same genes were assembled using the CAP3 Sequence Assembly Software [21]. The contigs and singletons generated were analyzed by the BLAST program using the algorithms BlastN [22] and BlastX [23].

2.4. Submission of genetic sequences to DNA databases

The sequences collected in this work were deposited in DDBJ/EMBL/GenBank using the Sequin software available at the NCBI web page. The ESTs were deposited under the accession numbers JK036089–JK036229. The contigs were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under Bioproject PRJNA260095. The *A. alternata* Transcriptome Shotgun Assembly Project (TSA) has the accession number GBZG00000000. The version described in this paper is the first version (GBZG01000000) and consists of sequences GBZG01000001–GBZG01000030.

2.5. Sequence annotation

To assign biological functions to the transcripts encoded by the differentially expressed genes, a functional annotation of contigs and singletons was performed using the BLAST2GO (B2GO) software version 2.7.0 [24]. The analysis was carried out against the non-redundant nucleotide collection of GenBank with a minimum *E*-value of 1×10^{-6} and a high-scoring segment pair cut-off of 33. The annotation step was carried out using the program default parameters and expanded using ANNEX (Annotation Expander). A B2GO InterPro Scan [25] was performed to search for additional GO terms corresponding to functional domains. The ESTs with GO annotations received enzyme codes (EC), and the B2GO KEGG module retrieved the maps of metabolic pathways in which the tracked EC numbers participated.

3. Results

3.1. Assembly and Blast analysis

Of 180 sequenced clones, 141 ESTs met the quality requirements after sequence cleaning. The CAP3 Assembly resulted in 124 reads, consisting of 40 contigs and 84 singletons. The Blast search returned 58% of the reads with similarities to characterized proteins, 25.8%

matched hypothetical proteins, and 12% had no similarity to known sequences. Some of the reads showing significant similarities with known proteins are listed in Table 1, whereas the ESTs contained in each resultant contig are listed in Table 2. Of the sequences with similarities to hypothetical proteins, 8% were similar to *Pyrenophora teres* f. terms, 5.6% to *Pyrenophora tritici-repentis*, and 4% to *Setosphaeria turcica* proteins.

Among the sequences similar to known proteins were those corresponding to regulatory checkpoint 1 (CHK1) and WSC domain-containing proteins, LemA and major Woronin body proteins, SLA1- and CFEM-domain GPI-anchored proteins, and GTP-binding, zinc finger and actin-bundling proteins. Also, we found ESTs similar to oxidoreductases enzymes (EC 1.2.1.23, EC 1.3.11.60, EC 1.3.8.8, and EC 1.2.1.65); transferases (EC 2.1.1, and EC 2.7.8); hydrolases

Table 1
Sequences from a suppressive subtracted hybridization library of *A. alternata* resistant to 2-propenyl isothiocyanate that are similar to known proteins. The results were obtained using the BlastX algorithm against the non-redundant database from GenBank (*E*-value < 1e − 05).

Sequence ID	GenBank accession number	Size (bp)	Similar sequence in GB/organism/accession number	Coverage %	E-value	Identity %
Aaitc271	JK036093	573	Similar to 60S ribosomal protein L28 [*Leptosphaeria maculans* JN3] > emb|CBY00944.1|	47%	2e − 50	90%
Aaitc277	JK036098	382	Vacuolar ATP synthase catalytic subunit A [*Pyrenophora tritici-repentis* Pt-1C-BFP] > gb|EDU43928.1|	91%	1e − 68	94%
Aaitc293	JK036107	420	Putative chitin- domain 3 protein [*Botryotinia fuckeliana* BcDW1] EMR83340.1	36%	3e − 08	56%
Aaitc295	JK036108	699	Woronin body major protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU41317.1|	54%	2e − 17	58%
Aaitc297	JK036109	404	Similar to hydrolase [*L. maculans* JN3] > emb|CBX99475.1|	94%	7e − 71	87%
Aaitc300	JK036112	448	40S ribosomal protein S26 [*Tuber melanosporum* Mel28] > emb|CAZ81138.1|	33%	8e − 15	94%
Aaitc311	JK036120	437	ATP synthase subunit alpha, mitochondrial precursor [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU50467.1|	37%	2e − 21	82%
Aaitc314	JK036123	379	40S ribosomal protein S17 [*Aspergillus fumigatus* Af293]	69%	3e − 54	95%
Aaitc318	JK036127	457	Cystathionine gamma-lyase [*P. tritici-repentis* Pt-1C-BFP]	38%	2e − 25	88%
Aaitc323	JK036129	410	Translational activator [*Colletotrichum orbiculare* MAFF 240422] ENH88059.1	48%	4e − 13	79%
Aaitc324	JK036130	406	GPI anchored CFEM domain containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU40367.1|	62%	5e − 42	83%
Aaitc326	JK036131	571	Cytoskeleton assembly control protein SLA1 [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU50968.1|	26%	1e − 23	96%
Aaitc332	JK036134	732	GTP-binding protein 128up [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU39679.1|	59%	2e − 96	97%
Aaitc344	JK036142	575	Similar to phosphatidyl synthase [*L. maculans* JN3] > emb|CBX91093.1|	55%	7e − 54	81%
Aaitc346	JK036144	456	Zinc finger containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU49999.1|	59%	1e − 41	77%
Aaitc383	JK036152	610	40S ribosomal protein S27 [*Pyrenophora teres* f. teres 0–1] > gb|EFQ88323.1|	50%	1e − 56	92%
Aaitc388	JK036155	516	Putative actin-bundling protein [*Neofusicoccum parvum* UCRNP2] EOD46166.1	94%	2e − 95	89%
Aaitc405	JK036165	388	Glycoside hydrolase family 16 protein [*Setosphaeria turcica* Et28A] EOA83343.1	51%	9e − 21	89%
Aaitc411	JK036169	423	rRNA methyltransferase NOP1 [*Pyrenophora teres* f. teres 0–1] > gb|EFQ87989.1|	73%	2e − 66	99%
Aaitc442	JK036185	497	Carbohydrate-binding module family 18 protein [*Bipolaris maydis* C5] > gb|ENI09831.1|	85%	2e − 83	90%
Aaitc449	JK036189	607	Salicylaldehyde dehydrogenase [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU43682.1|	67%	4e − 66	86%
Aaitc456	JK036193	348	40S ribosomal protein S26 [*T. melanosporum* Mel28] > emb|CAZ81138.1|	30%	3e − 05	97%
Aaitc457	JK036194	401	Endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase [*Pseudozyma hubeiensis* SY62]	95%	1e − 32	64%
Aaitc462	JK036198	490	Similar to importin beta-4 subunit [*L. maculans* JN3] > emb|CBX96195.1|	74%	2e − 68	93%
Aaitc464	JK036200	308	Arrestin domain containing protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU48753.1|	88%	2e − 51	91%
Aaitc466	JK036201	400	Mitochondrial fusion GTPase protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU42985.1|	95%	1e − 73	94%
Aaitc473	JK036207	853	Streptomycin biosynthesis protein StrI [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU51230.1|	93%	7e − 158	78%
Aaitc474	JK036208	727	Pentatricopeptide repeat protein [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU39902.1|	97%	1e − 141	90%
Aaitc485	JK036216	323	Histone H2A [*Coniosporium apollinis* CBS 100218] EON65154.1	31%	3e − 12	97%
Aaitc490	JK036221	526	ABC transporter CDR4 [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU44273.1|	97%	4e − 50	59%
Aaitc493	JK036224	480	Iron sulfur cluster assembly protein 1, mitochondrial precursor [*P. tritici-repentis* Pt-1C-BFP] > gb|EDU44735.1|	71%	1e − 75	98%

Table 2
ESTs in contigs.

Contigs (Lab ID)	Contig accession number (TSA)	ESTs in contig (Lab ID)	EST GenBank accession number
Aaitcas18	GBZG01000001	Aaitc412	JK036170
		Aaitc273	JK036095
		Aaitc455	JK036192
Aaitcas19	GBZG01000002	Aaitc389	JK036156
		Aaitc274	JK036096
		Aaitc394	JK036158
		Aaitc482	JK036214
Aaitcas20	GBZG01000003	Aaitc448	JK036229
		Aaitc290	JK036105
		Aaitc491	JK036222
Aaitcas21	GBZG01000004	Aaitc306	JK036116
		Aaitc307	JK036117
		Aaitc434	JK036181
		Aaitc470	JK036205
Aaitcas22	GBZG01000005	Aaitc381	JK036150
		Aaitc343	JK036141
		Aaitc425	JK036172
		Aaitc268	JK036090
		Aaitc331	JK036133
		Aaitc387	JK036154
		Aaitc495	JK036226
		Aaitc496	JK036227
		Aaitc461	JK036197
		Aaitc481	JK036213
Aaitcas23	GBZG01000006	Aaitc428	JK036175
		Aaitc358	JK036149
		Aaitc341	JK036140
		Aaitc329	JK036132
		Aaitc450	JK036190
		Aaitc410	JK036168
		Aaitc467	JK036202
Aaitcas24	GBZG01000007	Aaitc349	JK036146
		Aaitc435	JK036182
		Aaitc489	JK036220
Aaitcas25	GBZG01000008	Aaitc441	JK036184
		Aaitc409	JK036167
		Aaitc477	JK036210
Aaitcas26	GBZG01000009	Aaitc278	JK036099
		Aaitc458	JK036195
Aaitcas27	GBZG01000010	Aaitc356	JK036148
		Aaitc484	JK036215
Aaitcas28	GBZG01000011	Aaitc399	JK036160
		Aaitc492	JK036223
Aaitcas29	GBZG01000012	Aaitc475	JK036209
		Aaitc401	JK036161
		Aaitc288	JK036103
		Aaitc267	JK036089
		Aaitc285	JK036102
		Aaitc404	JK036164
		Aaitc403	JK036163
		Aaitc460	JK036196
		Aaitc471	JK036206
Aaitcas30	GBZG01000013	Aaitc478	JK036211
		Aaitc426	JK036173
Aaitcas31	GBZG01000014	Aaitc486	JK036217
		Aaitc494	JK036225
Aaitcas32	GBZG01000015	Aaitc488	JK036219
		Aaitc469	JK036204
Aaitcas33	GBZG01000016	Aaitc338	JK036138
		Aaitc1A	
Aaitcas34	GBZG01000017	Aaitc348	JK036145
		Aaitc33	
Aaitcas35	GBZG01000018	Aaitc427	JK036174
		Aaitc65	
Aaitcas36	GBZG01000019	Aaitc430	JK036177
		Aaitc161	
Aaitcas37	GBZG01000020	Aaitc463	JK036199
		Aaitc133	
Aaitcas38	GBZG01000021	Aaitc143	
		Aaitc46	
Aaitcas39	GBZG01000022	Aaitc150	
		Aaitc436	JK036183
		Aaitc301	JK036113
Aaitcas40	GBZG01000023	Aaitc227	
		Aaitc315	JK036124

Table 2 (*continued*)

Contigs (Lab ID)	Contig accession number (TSA)	ESTs in contig (Lab ID)	EST GenBank accession number
Aaitcas41	GBZG01000024	Aaitc289	JK036104
		Aaitc276	JK036097
Aaitcas42	GBZG01000025	Aaitc299	JK036111
		Aaitc279	JK036100
Aaitcas43	GBZG01000026	Aaitc322	JK036128
		Aaitc305	JK036115
Aaitcas44	GBZG01000027	Aaitc345	JK036143
		Aaitc316	JK036125
Aaitcas45	GBZG01000028	Aaitc335	JK036136
		Aaitc269	JK036091
		Aaitc304	JK036114
		Aaitc308	JK036118
		Aaitc310	JK036119
		Aaitc317	JK036126
Aaitcas46	GBZG01000029	Aaitc443	JK036186
		Aaitc337	JK036137
		Aaitc429	JK036176
Aaitcas47	GBZG01000030	Aaitc447	JK036188
		Aaitc451	JK036191
		Aaitc433	JK036180

(EC 3.6.5.1, EC 3.2.1, EC 3.1.1.2, EC 3.6.4.13, and EC 3.6.3.14); a lyase (EC 4.4.1.1); and a ligase (EC 6.2.1.17).

3.2. Functional annotation and metabolic pathways

In the functional annotation procedure, the B2GO software assigned GO terms to 54% of the reads set, whereas no results were obtained for 13%, 22%, and 9.6% of the sequences during the blasting, mapping, or annotation processes, respectively. A total of 265 annotations and 17 EC numbers (for 16 sequences) were retrieved. The annotation distribution, according to GO, showed that the best-represented categories at level two were molecular function and biological process. In the molecular function category (Fig. 1a), more transcripts were assigned to binding (38) and catalytic activity (33) followed by structural molecule activity (13). In the biological process category (Fig. 1b), the main clusters were cellular and metabolic process (both with 44 GO terms) followed by single organisms and localization (22 and 17 GO terms, respectively).

Based on the EC codes assigned to sequences and the KEGG maps retrieved by B2GO, we obtained transcripts having enzymatic functions involved in the metabolic pathways. The exposure of *A. alternata* to 2-pITC, differentially induced enzymes participating in the metabolism of nitrogen, pyruvate, cysteine and methionine, selenium compounds, propanoate, phenylalanine, butanoate, methane, glycine, serine, and threonine. Also, the enzymes participating in styrene, aminobenzoate, and toluene degradation were induced. Furthermore, the enzymes involved in catalyzing steps in glycolysis, gluconeogenesis, citrate cycle, oxidative phosphorylation, and carbon fixation pathways, were detected. Finally, the enzymes involved in the phenylpropanoid pathway and several types of N-glycan biosynthesis were also differentially induced.

4. Discussion

Among the differentially expressed ESTs of *A. alternata* tolerant to 2-pITC, Aaitc346 (GenBank accession number JK036144) showed significant similarity to a Zn-finger transcription factor. This protein is induced, along with ABC transporters, in *Aspergillus fumigatus* after exposure to voriconazole [26] and in *Fusarium graminearum* after exposure to tebuconazole [27]. In plants, these proteins are involved in defense pathways and are induced in response to several types of stress [28]. In *F. graminearum*, the Zn-finger transcription factor tac1p, which is a transcriptional activator of *CDR* genes, regulates the expression of *CDR1* and *CDR2*, which encode ABC transporters in

azole-resistant clinical isolates of *Candida albicans* [29]. Moreover, *Botrytis cinerea* that are resistant to several fungicides overexpress an ABC transporter, which is induced by a mutation in the putative Zn-finger transcription factor Mrr1 [30]. In our previous work, using real-time RT-PCR, we confirmed the overexpression of an ABC transporter in *A. alternata* in response to 2-pITC [18]. Thus, the Zn-finger transcription factor is induced by 2-pITC, which activates the expression of the ABC multidrug CDR4 transporters in *A. alternata* to provide tolerance to 2p-ITC.

In our current work, we found a sequence similar to a protein containing a WSC domain [31]. The WSC1 gene encodes an integral membrane protein (Wsc1p) that functions as a stress sensor. In yeast, this protein participates in the monitoring of cell wall integrity and the activation of the protein kinase C (PKC) pathway in response to external stress signals, such as cell wall perturbations. Wscp1 also regulates 1,3-β-glycan synthesis [31,32], a metabolic pathway identified in the B2GO analysis.

Among the 2p-ITC induced-transcripts of *A. alternata*, we also found a sequence encoding a karyopherin, Kap123. This molecule has an important role in cell integrity, which also depends on PKC pathway activity that regulates the secretion and vesicular transport pathway. The cellular integrity pathway is activated after cell wall damage [33]. Other sequences involved in cellular integrity are those encoding the major Woronin body protein, the GPI-anchored CFEM domain-containing protein, and the oxidoreductase glyoxal oxidase. The Woronin bodies are proteinic corpuscles that move to the septal pore of filamentous fungi in response to cellular

damage [34], whereas the GPI-anchored CFEM domain-containing proteins are involved in cell wall stabilization [35]. Glyoxal oxidase may have a role in fungal detoxification [36]. The induction of this enzyme in 2p-ITC-treated *A. alternata* suggests it has a role in the detoxification of 2p-ITC.

Other genes induced in *A. alternata* after 2p-ITC treatments are GTPases. Some of these molecules are activated by stress, and in turn, they activate the Pkc1 kinase in the PKC pathway [32]. The interruption of CgBem2, which encodes a GTPases activator protein in *Candida glabrata*, resulted in azole susceptibility, suggesting a function for GTPases in the survival to stress caused by antimycotics [37]. Thus, the induction of GTPases in *A. alternata* after 2p-ITC treatments suggests its participation in the resistance to 2p-ITC.

In our library, we also found a sequence similar to an SLA1 protein, an actin-cytoskeleton regulatory complex component (PAN1), and an ubiquitin-binding protein [38]. Moreover, actin-binding proteins that direct the response to external stimuli [39] were detected. Additionally, sequences similar to proteins containing Lem domains were identified. In yeast, these proteins participate in the genome and nuclear structure organization [40] while, in *Caenorhabditis elegans*, they have a role in the response to DNA damage [41]. Another transcript induced by 2p-ITC codes for the regulatory protein CHK1, which is also involved in responses to DNA damage and cell survival [42]. In *Clonostachys rosea*, the CHK1 protein was induced in response to oxidative stress by toxins [43]. Because 2p-ITC is a compound causing oxidative stress, it is feasible that CHK1 was induced in *A. alternata* after the 2p-ITC treatment.
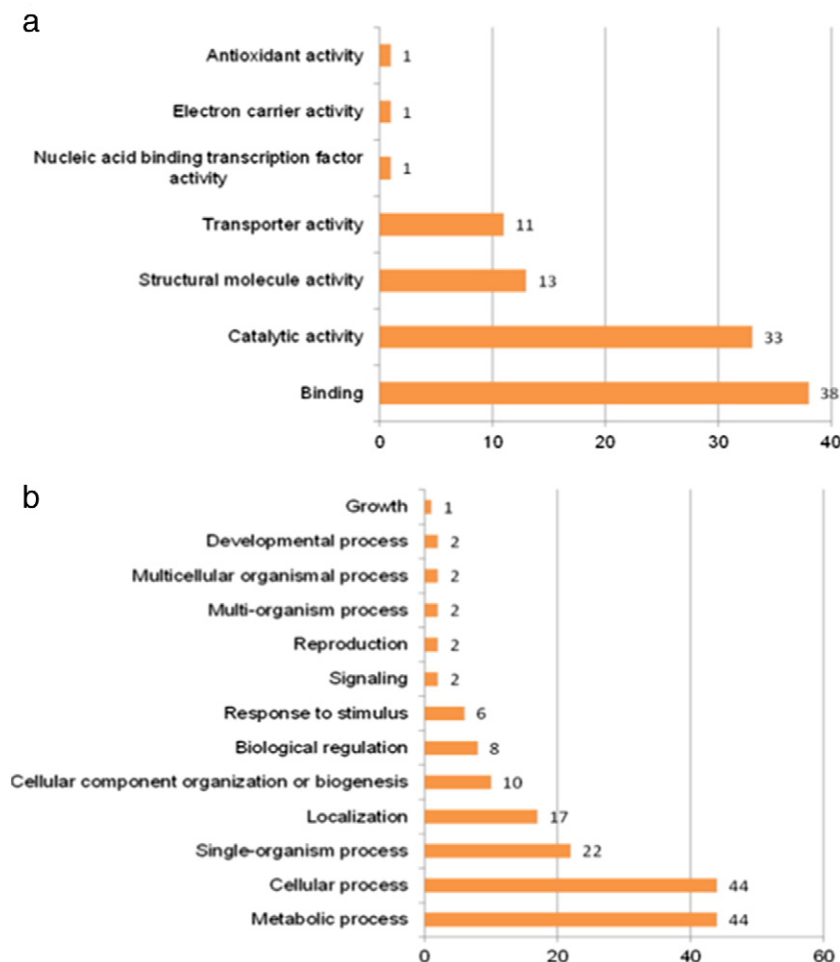

Fig. 1. Results of the annotation of sequences from a suppressive subtracted hybridization library of *A. alternata* resistant to 2-propenyl isothiocyanate. The distribution of gene ontology (GO) terms is shown at a GO level 2. The number in each category shows the frequency for each GO term. a) Molecular function; b) Biological process.

Several sequences detected in the library suggest that 2p-ITC induces a large variety of proteins and enzymes involved in the activation of signal cascades that promote general cellular responses oriented to cell reparation and maintenance. Such sequences encode 40S (S0, S17, S18, S26, and S27) and 60S (P0, L20, L21, and L28) ribosomal subunits; 1-α-elongation factor; methyltransferases; transcripts involved in amino acid biosynthesis (aminotransferase and cystathionine gamma-lyase); sequences participating in fungal pH adaptation (arrestin domain-containing proteins) [44]; proteins implicated in RNA metabolism (pentatricopeptide repeat motif) [45] or, functioning as chaperones (DEAD box proteins) [46]. Additionally, were identified the transcripts involved in the regulation of genetic expression (Fe–S clusters) [47] and cell cycle progression, as well as in ribosome biogenesis (GTP-binding proteins) [48]. According to their reported functions, none of these proteins can directly confer resistance to the fungus against fungicides. However, the overexpression of these molecules provides protection against the effects of 2p-ITC on the structure and function of the fungal cell. Thus, the cell uses all possible genetic, biochemical, and structural strategies to survive the toxicity of 2p-ITC.

In our previous study, the tolerance of *A. alternata* to 2p-ITC required mainly calcium ions and the efflux of the compound by an ABC transporter. There is evidence that the genes induced in *A. alternata* by 2p-ITC, are also expressed in response to synthetic fungicides [26]. When *A. alternata* was treated with the synthetic compound carbonyl sulfide, 510 cDNAs differentially expressed were found. These genes are related to general metabolism, growth, cellular division, defense, cellular transport, and signal transduction [49], similar to our work results. Therefore, it should be emphasized that even natural compounds could induce tolerance/resistance mechanisms in organisms in the same manner as synthetic chemical products if they were not used properly. In a study with fungal toxins Kosawang et al. [43] using a SSH experimental approach reported 443 and 446 differentially expressed clones induced in *Clonostachys rosea* by deoxynivalenol (DON) and zearalenone (ZEA) toxins, respectively. DON induced proteins involved in the stress response as well as metabolic enzymes (cytochromes c oxidase and P450) while ZEA induced the detoxifying enzyme HD101 and ABC pleiotropic drug transporters. These authors concluded that the tolerance to fungal toxins in *C. rosea* was provided by a broad range of genes playing a role in metabolism and transport. Based on the increase of transcripts encoding the metabolic enzymes CYP450 and COX, the sugar transporters HXT2 and H+-ATPase, as well as the Hsp70 and Hsp90 proteins, they stated that the cellular energy was used to synthesize proteins that were inactivated by the toxins.

Several publications using a differential expression approach to studying the response to natural and synthetic compounds have reported different numbers of genes induced. However, the reported number of metabolic pathways by using more robust protocols is modest, considering the robustness of the techniques used. The number of genes reported as induced by synthetic compounds in the literature is higher than in this work. This fact is not related to the treatment, but rather to the methodology. For instance, using microarrays, Ferreira et al. [26] found 2271 genes differentially expressed in *A. fumigatus* exposed to voriconazole. These authors reported increased transcripts levels of genes involved in a variety of cellular functions: *e.g.* transporters, transcription factors as well as proteins involved in cell metabolism. In agreement with our results, these authors found that the induction of two C2H2 zinc finger domains, ATPase and calmodulin transcription factors putatively involved in the response to stress conditions imposed by the voriconazole treatment. These authors hypothesized that these proteins could have as target genes participating in detoxification (transporters).

Another study using microarrays in *F. graminearum* (Becher et al. [27]), reported 1058 differentially expressed genes in response to azole

treatment and of them, 596 showed significantly increased transcript levels. The functional annotation of these genes found the ergosterol biosynthesis as an important pathway in the fungal response to azole. Also, the authors found transcripts differentially expressed encoding ABC transporters and transcription factors, presumably involved in mechanisms to decrease the toxic effect of the fungicide. They studied the expression level of 31 genes, out of which, they found five genes with increased transcript levels. These genes were involved in sterol metabolic processes. In contrast to our results, these authors reported a decreased expression of genes related to amino acid transport and metabolism. Even though this article reports a rather large study, the analysis of the genes with increased transcripts levels (GO annotation) identified only a few functional categories significantly represented; among them, the sterol biosynthetic process and tetracyclic and pentacyclic triterpene metabolism were found as the most represented functional categories. Additionally, isoprenoid metabolism and heme-binding proteins were important processes and functional category, respectively. The mentioned work found the induction of proteins with hexosyltransferase activity as well as proteins involved in membrane processes, mycelium development, respiratory chain, and energy generation. These authors assume that many of the induced genes exhibit a non-specific stress response caused by the azole membrane perturbations. However, they observed that the majority of the overexpressing genes responded specifically to the fungicide treatment. All of the pathways reported in that work were identified in our study. As compared with the mentioned published works, this is rather small due to the experimental approach utilized. However, we have a great variety of transcripts induced.

5. Concluding remarks

The results herein presented revealed a broader set of transcripts and cellular activities putatively implicated in the survival of *A. alternata* exposed to 2p-ITC. The induction of signaling cascades targeting diverse cellular processes is evident. These involve mainly the pathways of defense and stress response, cell wall integrity, cytoskeleton organization, and destabilization, as well as exocytosis and transport. Additionally, genome and nuclear structure organization, protein and ribosome synthesis, cell cycle progression, and DNA damage response activities were induced. Furthermore, some metabolic pathways underlying the fungal genetic responses against toxic compounds were identified. Interestingly, the proteins and enzymes that were induced in response to 2p-ITC can also be induced by the fungal resistance to different synthetic compounds. By performing a complete analysis of the *A. alternata* SSH library, a larger number of biological processes and molecular functions induced by 2p-ITC were identified. These findings show that the response of *A. alternata* to the toxic effects of 2-PITC is a complex and sophisticated defense mechanism. However, it is possible that the expression of some genes is elicited by a primary response of other genes to the toxicity of 2p-ITC, as suggested by the expression of genes related to the promotion of general cellular responses linked to growth and maintenance processes. To better understand the role for each transcript, RNA-seq and microarray studies should be performed. Thus, the complete knowledge of the tolerance mechanisms of *A. alternata* to 2-pITC will require the use of whole transcriptomic sequencing, genetic-level expression analyses, and functional analyses, which will allow us to study all of the genes involved and their functions in the resistance process.

Conflict of interest

The authors declare that they have no conflict of interest.

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