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Differential expression of multidrug-resistance genes in *Trichophyton rubrum*

Maíra Pompeu Martins, Antonio Rossi*, Pablo Rodrigo Sanches, Nilce Maria Martinez-Rossi

Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, 14049-900, Ribeirão Preto, SP, Brazil

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ABSTRACT

Treatment of dermatophytosis is generally a long and challenging process, deeply affected by drug resistance owing to efflux-mediated activity. These drug-pumping mechanisms involve overexpression of transporter proteins with the ability to extrude a wide variety of structurally and functionally unrelated compounds. The ATP-binding cassette transporter and the major facilitator are the two largest superfamilies of transporters, expressed ubiquitously in all living organisms. Here, we examined the transcription modulation of both families of transporter genes in the dermatophyte *Trichophyton rubrum* upon challenge with sub-lethal doses of undecanoic acid or acriflavine. Data derived from RNA sequencing revealed transporters functioning in specific patterns according to the stressing condition, suggesting that each drug recruits specific physiological pathways. Synergistic transport activity may be acting to overcome drug toxicity, demonstrating that multidrug resistance transporters cooperate to induce drug resistance and fungal survival in an unpredictable manner.

Keywords: RNA-seq; ATP-binding cassette superfamily; Major facilitator superfamily; Dermatophyte; Antifungal efflux

1. Introduction

Dermatophytes are a specialized group of filamentous fungi that colonize keratinized tissues. They are the most commonly diagnosed pathogens in superficial infections, with *Trichophyton rubrum* being responsible for the majority of infective cases [1, 2]. Treatment of dermatophytosis is generally a long and challenging process, deeply affected by the small number of available antifungal drugs, the limited number of cellular targets, and the occurrence of drug resistance [3, 4].

Among mechanisms that render the fungus resistant or tolerant to toxic compounds, overexpression of drug efflux pumps belonging to the ATP-binding cassette (ABC) superfamily or to the major facilitator superfamily (MFS) comprise a major challenge [5, 6]. Both superfamilies consist of integral membrane proteins, with a conserved domain architecture [5]. These multidrug resistance (MDR) transporter genes are active against diverse unrelated chemical compounds and extrude them from the cell [7, 8].

The large number of genes encoding these transporters and the clinical relevance of efflux-mediated drug resistance supports the need to elucidate the molecular features involved in transporter interactions and pumping activity [5]. Further, because of their association with the prominent efflux-mediated pleiotropic resistance, the relevance of these transporters in fungal pathogenicity that acts as a virulence factor is thus becoming evident [6, 9].

In dermatophytes, evaluation of transcription profiles of ABC transporter genes *pdr1, mdr2,* and *mdr4* showed a synergistic activity among them in response to antifungal drug exposure. Further, among the four dermatophytes evaluated, each presented a gene-specific transcriptional profile [10]. The Δ*mdr2* mutant strain showed reduced infectivity on human nails and an enhanced sensitivity to drugs including terbinafine [6, 11]. The transcript levels of the *mdr1* gene were observed to be induced in response to drugs such as griseofulvin and itraconazole, suggesting its participation in antifungal resistance [12]. These results indicate a particular efflux activity, dependent on the chosen

***Corresponding author:** Antonio Rossi, anrossi@usp.br

drug, the gene evaluated, and the organism analyzed.

Global gene-expression analysis is a prominent approach to evaluate modulatory changes during environmental challenge. Thus, through RNA sequencing (RNA-seq) interpretation of a myriad molecular functions in diverse organisms has been favored [13, 14]. In *Trichophyton rubrum*, two RNA-seq data revealed transcriptional modulation in response to antifungal-active compounds, undecanoic acid and acriflavine [15, 16]. Among the identified differentially expressed genes, we selected those coding for ABC and MFS transporters and evaluated their transcriptional profile in response to stressing conditions.

Here we aimed to evaluate the drug efflux-related gene expression, attempting to examine the role of these drugs in modulating gene expression.

2. Materials and Methods

2.1. Data analysis and gene selection

The prediction of ABC and MFS domain-containing proteins was performed using the HMMER v3.1b2 pipeline [17] in the *T. rubrum* CBS 118892 (Centraalbureau voor Schimmelcultures, The Netherlands) genome sequence, available at ftp://ftp.broadinstitute.org/pub/annotation/fungi . The hidden Markov model (HMM) was built utilizing a Pfam multiple alignment based-search of 55 sequences corresponding to the ABC domain-containing proteins and other Pfam multiple alignment based-search of 192 sequences corresponding to the MFS domain-containing proteins, both from different organisms (https://pfam.xfam.org/family/PF00005 and https://pfam.xfam.org/family/PF07690 respectively). The resulting models with epitopes of consensus sequences of ABC and MFS domain-containing proteins were used to search for homologs in *T. rubrum* protein sequences. The genes, whose codes for the hereinafter identified proteins were confronted with the differentially expressed genes (DEG) identified in the *T. rubrum* undecanoic acid and acriflavine RNA-seq libraries, are available at the GEO database under accession nos. GSE102872 and GSE40425. The selected genes are presented in Table 1.

2.2. T. rubrum strain and culture conditions used in the RNA -seq libraries assemblage

T. rubrum mycelia obtained from 96 h-culture, starting from approximately 10^6 conidia mL^{-1} in Sabouraud dextrose broth (SDB), challenged with 1.75 µg/mL of acriflavine (Sigma Aldrich Corp., USA), which corresponds to 70% of its MIC (minimal inhibitory concentration), or with 17.5 µg mL-1 (70% MIC) of undecanoic acid (Sigma Aldrich Corp., USA) were used for RNA extraction and sequencing as previously published [15, 16]. The strain was maintained as described previously [18, 19].

3. Results and discussion

A total of 44 DEGs coding for ABC or MFS transporters were identified in two libraries. Among them, 11 are responsive to both conditions (Table 1.A). Other 19 were modulated exclusively in response to acriflavine (Table 1.B), and 14 respond only to undecanoic acid exposure (Table 1.C). A higher number of MFS transporters were modulated in our experimental conditions, in relation to the ABC transporter genes.

Among the genes modulated in response to both tested drugs, four, identified as TERG_01443, TERG_08336, TERG_02283, and TERG_01623, were inversely modulated in response to the drugs chosen. Drug exposure repressed the other concomitantly modulated MDR transporter genes, four of them belonging to the ABC transporter family and the other three were MFS transporters. Two of the repressed MFSs were siderophore iron transporters.

In response to acriflavine, more genes were induced than repressed, belonging mainly to the MFS transporter superfamily than to the ABC transporters. Among the induced genes, we identified a phosphate permease that was highly modulated (log_2 = 9.58) presenting two MFS transporter domains.

Undecanoic acid modulated less MDR transporter genes than acriflavine, mainly inducing their expression rather than repressing it. As observed with acriflavine, more MFS transporter genes were responsive to undecanoic acid exposure. The inductive effect of MDR transporters is expressively more related to the initial exposure than to the later time period of exposure.

The smallest number of genes modulated in both conditions compared to those that are drug-specific highlights a drug-dependent activation. Under undecanoic acid challenge, these genes responded in the earliest time period. Except for the ABC transporter TERG_01443, the other three genes responded exclusively in the latest time period of exposure to acriflavine.

Only three of the DEGs were modulated at all time points, for each drug tested: TERG_06679, TERG_00955, and TERG_00402 remained repressed during the 24 h of acriflavine exposure, and TERG_08130, TERG_05055, and TERG_03719 remained downregulated in the two time points of exposure to undecanoic acid.

4.Discussion

Antifungal-active compounds challenge fungal survival. Through activation of efflux pumps such as the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters, multiple cytotoxic chemicals are actively extruded from fungal cells, thus playing an important role in the multiresistance phenomenon [20]. These hostile environments provoked by each different substance force fungi to modulate diverse and specific transporters, seeking to counter their harmful effects.

Table 1 | MFS and ABC transporter genes modulated in response to acriflavine (ACF) or undecanoic acid (UDA) exposure in *T. rubrum*. Modulatory data correspond to the control (in the absence of drug) compared at each time point of drug exposure.

ID	ACF vs. control - log_2 (RNA-seq fold change)			UDA vs. control - log_2 (RNA-seq fold change)		Number of MFS or ABC Transporter* domains	HMM (E-value)	Gene Product Name
	3 h	12 h	24 h	3 h	12 h			
Table 1.A – modulated in response to exposure of ACF and UDA								
TERG_01443	1.80		1.52	-1.80		2*	4.10E-49	ABC multidrug transporter (*T. tonsurans*)
TERG_08336			-1.59	2.51		2	1.20E-45	MFS multidrug transporter, putative (*A. benhamiae*)
TERG_02283			-1.61	2.11		1	1.70E-32	MFS transporter, putative (*T. verrucosum*)
TERG_01623			1.72	-2.02		1	2.30E-39	MFS transporter (T. equinum)
TERG_08613		-1.70	-2.14		-2.04	2*	4.40E-72	ABC multidrug transporter mdr2, putative (*A. benhamiae*)
TERG_08130		-1.70	-1.77	-2.03	-2.28	2*	6.00E-36	ABC ATPase (*T. equinum*)
TERG_04323		-1.60	-1.56	-1.61		2*	1.00E-57	ATP-dependent bile acid permease (*T. equinum*)
TERG_05617	-2.04				-1.89	1*	3.00E-02	Hypothetical protein
TERG_06679	-2.67	-3.26	-3.82		-2.14	2	4.40E-18	MFS transporter, putative (*A. benhamiae*)
TERG_08619	-2.76	.	-1.72	-3.56		2	3.20E-17	Siderophore iron transporter mirB (*T. equinum*)
TERG_08620	-2.25	.	-2.61	-2.35		2	3.30E-18	Siderophore iron transporter (*T. equinum*)
Table 1.B – modulated only in response to exposure of ACF								
TERG_04952	2.14					1*	1.20E-35	Multidrug resistance protein (*T. equinum*)
TERG_07801		2.60	3.13			1*	6.64E-12	ABC multidrug transporter *mdr4*
TERG_00762	1.55		1.58			2*	9.60E-04	Vesicular-fusion protein SEC18 (*M. gypseum*)
TERG_07921	1.86					1*	3.90E-02	Denylylsulfate kinase
TERG_02583			9.58			2	1.30E-22	Phosphate permease (*A. benhamiae*)
TERG_04400	1.56					2	2.00E-21	MFS monosaccharide transporter, putative (*T. verrucosum*)
TERG_03174		3.69	4.97			2	7.50E-11	MFS siderochrome iron transporter MirB (*T. verrucosum*)
TERG_02369		1.81				1	5.50E-36	MFS transporter (*T. tonsurans*)
TERG_08059		1.70					1.40E-18	Sugar transporter (*T. equinum*)
TERG_01623			1.72			1	4.16E-12	MFS transporter
TERG_03933			-1.73			2*	2.00E-53	ABC metal ion transporter
TERG_00955	-1.66	-1.79	-2.29			2*	1.30E-52	ABC drug exporter AtrF (*T. verrucosum*)
TERG_00402	-1.60	-2.10	-2.25			1*	2.30E-19	ABC multidrug transporter, putative (*T. verrucosum*)
TERG_07216	-1.50					1*	1.10E-01	Hypothetical protein
TERG_00008			-1.68			2	6.70E-19	MFS phospholipid transporter (*T. tonsurans*)
TERG_00820			-2.30			2	7.90E-47	MFS multidrug transporter, putative (*A. benhamiae*)
TERG_05153		-1.89	-2.37			2	3.50E-26	MFS transporter, putative (*A. benhamiae*)
TERG_07539	-1.64		-3.49			1	6.20E-48	Multidrug resistance protein (*T. tonsurans*)
TERG_06650	-1.65					2	2.70E-30	MFS monocarboxylate transporter, putative (*A. benhamiae*)
Table 1.C – modulated only in response to exposure of UDA								
TERG_04224				2.41		2*	7.70E-55	ABC transporter
TERG_02508				1.91		2*	1.40E-44	ABC multidrug transporter, putative (*A. benhamiae*)
TERG_06361				2.13		1*	1.60E-02	ATP-dependent protease La
TERG_00162				3.88		1	1.80E-47	MFS multidrug transporter, putative (*A. benhamiae*)
TERG_00163				2.12		2	7.40E-19	Siderochrome-iron transporter, putative (*A. benhamiae*)
TERG_05575				2.65		2	7.70E-42	MFS multidrug transporter (*T. tonsurans*)
TERG_05199				2.06		2	1.30E-40	MFS gliotoxin efflux transporter GliA (*T. verrucosum*)
TERG_05466					1.80	2	6.80E-25	MFS transporter, putative (*T. verrucosum*)
TERG_04227				-2.60		2*	4.40E-38	ABC transporter (*T. tonsurans*)
TERG_04514				-1.86		1*	8.30E-02	Cell division control protein 12 (*T. tonsurans*)
TERG_04308				-2.54		2	1.40E-30	MFS sugar transporter (*T. tonsurans*)
TERG_03984					-1.86	1	3.90E-37	Major facilitator superfamily transporter MFS-1 (*M. canis*)
TERG_05055				-2.02	-1.64	1	4.80E-44	MFS multidrug transporter (*T. tonsurans*)
TERG_03719				-2.84	-2.87	2	4.60E-11	MFS sugar transporter (*T. tonsurans*)

We examined the transcription modulation of ABC and MFS transporter genes in the dermatophyte *T. rubrum* challenged with sub-lethal doses of undecanoic acid or acriflavine. The differentially expressed genes were subdivided in three groups: those responsive to both drugs, and genes transcribed exclusively in response to each of the chosen drugs.

A more elevated number of DEGs belonging to the MFS superfamily, comparing to the ABC transporter genes, were identified in both libraries, including three genes commonly expressed and inversely modulated in the presence of the evaluated drugs. The MFS transporters correspond to the largest class of secondary active pumps in all branches of life, and the high number of gene copies indicates a highly conserved defense potential [20, 21]. MFS are capable of transporting a huge variety of substances, ranging from small solutes, in response to chemiosmotic ion gradients, to drugs presumably acting as Drug:H+ Antiporters (DHA) [20, 22].

Here, important physiological functions are affected by the drugs including the transport of siderophore-iron chelates (TERG_08619, TERG_08620, and others). As previously proposed, *T. rubrum* requires iron to overcome toxicity triggered by acriflavine exposure [15]. Since undecanoic acid also induced siderophore-related genes we supposed that an essential modulation profile of iron-related genes plays a role in stress resistance. Also affected, one sugar transporter is induced in response to undecanoic acid (TERG_08059), and the other two are repressed in the presence of acriflavine (TERG_04308, and TERG_03719). Sugar transport occurs along a concentration gradient, or operates when the availability of sugars presents relatively low concentrations [23]. Resistance to acriflavine and ethidium bromide was attributed to the *qacA* transporter from *Staphylococcus aureus*, a sugar uptake-related protein [24]. The evaluated drugs seem to oppositely affect the sugar availability in cells, recruiting MFS transporters in a particular way.

Among the induced MFS transporters, one phosphate permease is expressively upmodulated in response to acriflavine. In the presence of inorganic phosphate, Escherichia coli strains become more sensitive to acriflavine, despite the resistance they present in its absence [25]. Also, the yeast *Hansenula jadinii*, when challenged with increasing amounts of acriflavine, augments phosphorylation activity, relating the toxic effects of the drug to the phosphate availability on cells [26].

The DEGs identified as modulated in both drug conditions, excluding the inversely transcribed ones, are all repressed (Table 1.A). These genes are supposed to be more directly related to the drug extrusion activity. As they are modulated in all time points, their active recruitment appears to be time- and drug-dependent. Among those genes, the ABC ATPase (TERG_08130) is repressed in response to the two time periods of exposure to undecanoic acid. It is related to the molybdenum cofactor biosynthesis protein of *Talaromyces marneffei* thus, being related to cofactors or prosthetic group transport. Undecanoic acid also represses other cofactor-related exchangers including iron and copper transporters, suggesting an interconnection between the harmful effect of the drug to essential cycles such as carbon, sulfur, and nitrogen [27]. The putative MFS transporter (TERG_06679), repressed in response to acriflavine at all time points, is correlated to the protein phosphatase 2C from *Aspergillus oryzae* and may be repressed in an attempt to counterbalance the activity of other active phosphatases.

The transcription of the *mdr2* gene (TERG_08613), repressed in response to both drugs, is also presumably related to drug resistance. This ABC transporter and the *mdr4* gene (TERG_07801), responsive to acriflavine exposure, were previously evaluated in four dermatophyte fungi, including the herein evaluated *T. rubrum* [10]. Disruption of the *mdr2* gene induced high transcription levels of *mdr4* in the presence of griseofulvin, suggesting a counter activity of the *mdr4* gene overlapping the *mdr2* inactivation, thus providing resistance to this antifungal. With acriflavine, we observed an inverse pattern of *mdr2* and *mdr4* modulation indicating activation of *mdr4* in response to the repression of *mdr2* [15].

In different situations, it is possible to identify a synergism of activity among transporters that are apparently redundant in number and potential activity and are active in improving stress tolerance and surpassing physiological challenges in a drug-specific manner. The concomitant modulation of several MDR transporters highlights their biological importance and suggests an active bias to stimulate drug resistance, concurring somehow with fungal defense.

5.Concluding Remarks:

Our results suggest a drug-specific activation of efflux pumps, resulting in a particular pattern of transcriptional regulation, possibly, resulting in a drug-specific profiling of antifungal drug resistance. We also suggest a synergistic activity of these transporters, with a compensatory activity against stressing conditions. These observations point to a singular fungal-response that supports how antifungal drug resistance varies drastically among organisms and drug classes.

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