Poster Category 9: The Fungal Cell Wall

PR9.1

***Magnaporthe oryzae* evades MAMP (microbe-associated molecular pattern)-triggered immunity of the host rice with surface α-1,3-glucan on the cell wall**

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Plants evoke innate immune defenses against fungal challenges upon recognition of MAMPs such as chitin, a major cell wall component of fungi. Nevertheless, fungal pathogens somehow circumvent the innate immunity of host plants. We previously reported that the rice blast fungus *Magnaporthe oryzae* masks cell wall surface with α-1,3-glucan, an undegradable polysaccharides for many plants, in response to a plant wax component via activation of Mps1 MAPK signaling (Fujikawa et al., 2009). We further studied role of α-1,3-glucan in *M. oryzae*-rice interactions. A *M. oryzae* mutant lacking α-1,3-glucan normally produced infectious structures. However, the inoculation of the mutant rapidly induced defense responses of susceptible rice plants and, as a result, the fungal infection was completely blocked. Moreover, a transgenic rice expressing a bacterial α-1,3-glucanase rapidly responded to the *M. oryzae* and showed strong resistance to the fungal infection. Overall, our results suggest that the surface α-1,3-glucan plays indispensable roles in escaping the host innate immunity and consequently in establishing the infection in *M. oryzae*.

PR9.2

Role of hydrolases produced by yeast antagonists in the biocontrol of postharvest pathogens of apples and peaches

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Biocontrol of postharvest pathogens by antagonistic yeasts involves several modes of action. Different yeast – including strains of Pichia guilliermondii, Metschnikowia pulcherrima, M. fructicola and Aureobasidium pullulans – have been studied for their biocontrol mechanism. Production of hydrolases is one of the components of the mechanism of action: enzymatic assays permitted to evidence exo-1,3-beta-glucanase, chitinase and alkaline protease activities. An exo-1,3-beta-glucanase gene of 1,224 bp without introns (PgExg1 gene) was amplified from the genomic DNA of P. guilliermondii M8. The gene belongs to the cellulose superfamily. Similarly, two chitinase genes (MpChi1 and MfChi1 genes) were amplified from the genomic DNA of M. pulcherrima strain MACH1 and M. fructicola strain AP47. Both genes lack introns and belong to GH18-chitinase-like superfamily. An alkaline protease gene of 1,351 bp (ALP5) was amplified respectively from the genomic DNA of A. pullulans PL5. The cDNAALP5 gene had a 18-amino acid signal peptide, two introns, one N-gylcosylation, one histidine active site, one serine active site. The protein encoded had 100% homology with the protease enzyme (ALP2) of the sea yeast A. pullulans. Expression in Escherichia coli, followed by identification with Western-blotting, purification with Ni-NTA and analysis with enzyme assay, yielded a homogeneous recombinant cDNAALP5 which hydrolyzed the substrate casein and inhibited pathogen mycelia growth. Production of hydrolases may can greatly contribute to the biocontrol effectiveness.

PR9.3

Vacuolar H+-ATPase plays a key role in cell wall biosynthesis of Aspergillus niger

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The identification of suitable targets is crucial for the discovery and development of new antifungals. Since the fungal cell wall is an essential organelle, the identification of genes involved in cell wall biosynthesis is expected to help discover new antifungal targets. From our collection of cell wall mutants, we selected a thermosensitive, osmotic-remediable mutant with decreased resistance to SDS for complementation analysis. The phenotypes of this mutant were complemented by a gene encoding a protein with high sequence similarity to subunit D of the eukaryotic Vacuolar-H+-ATPase (VmaD). Genetic analysis revealed that the mutant allele encodes a protein that lacks 12 amino acids at the C-terminus. Deletion of the entire gene resulted in very poor growth. The conditional mutant displayed several phenotypes that are typical to V-ATPase mutants, including increased sensitivity to zinc ions and reduced acidification of the vacuole as observed by quinacrine staining. Treatment of A. niger germlings with the V-ATPase inhibitor bafilomycinB1 also induced the expression of the agsA gene. Furthermore genes involved in cell wall reassembly like fksA, agsA and phiA are clearly up-regulated in the conditional mutant. Moreover, expression of cell wall related genes can be induced by treatment with the V-ATPase inhibitor bafilomycinB1. Our results indicate that the ATP-driven transport of protons and acidification of the vacuole is crucial for the strength of the fungal cell wall and that reduced activity of the V-ATPase induces the cell wall stress response pathway.

PR9.4

Identification Of Chitin Synthase Genes (*CHS*) In The Postharvest Pathogen *Pencillium digitatum*. Changes Of Expression During Growth And Citrus Fruit Infection.

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The main citrus postharvest pathogen is *Penicillium digitatum*, a necrotrophic fungus with a narrow host range that penetrates citrus fruit through injured peel. Fungal cell wall (CW) is composed of chitin, glucans, mannans and glycoproteins, and is considered an excellent potential target for the development of novel antifungals. Chitin is synthesized by a complex set of chitin synthase genes (*Chs*) that belong up to seven distinct gene families in filamentous fungi. We have carried out the isolation and characterization of chitin synthase genes (*Chs*) of *P. digitatum*. Using distinct sets of degenerate primers designed from conserved regions of *Chs* genes of yeast and filamentous fungi, PCR methods and a DNA genomic library, five complete *Chs* genes (*PdigChsI*, *PdigChsII, PdigChsIII*, *PdigChsV* and *PdigChsVII*) were identified, isolated, sequenced and characterized. A very high sequence identity and strong synteny was found with corresponding regions from the genome of *Penicillium chrysogenum.* Gene expression of *P. digitatum Chs* genes during mycelium axenic growth under different conditions and infection of citrus fruit was quantified using qRT-PCR. *PdigChsIII* had the highest expression among the five genes by one order of magnitude, while *PdigChsII* had the lowest. Results suggest that *PdigChsI*, *PdigChsV* and *PdigChsVII* could have a specific role during the interaction with citrus, since their expression was up-regulated at late times of infection. *PdigChsV* and *PdigChsVII* co-expressed in all the experiments carried out and the analysis of their genomic sequences revealed that they are separated by a 1.77 kb intergenic region that contains several conserved regulatory motifs.

PR9.5

Development of a screening method for (synthetic) anti-fungal peptides

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Drug resistant fungi are a rapidly upcoming problem and a threat to public health. Triazole agents, the most used group of antifungal drugs, become less effective to mold infections. Successful treatment of e.g. aspergillosis requires active drug therapy. The demand for new and effective anti-microbial agents is rising. Natural antimicrobial compounds known as host defense peptides or antimicrobial peptides (AMPs) are involved in the direct destruction of various microorganisms. AMPs form a promising new source for drug therapy towards fungi. Fungal cell wall biosynthesis is a potential target for drug development. The cell wall integrity pathway plays an important role in cell wall synthesis. *Aspergillus niger* alpha-1,3-glucan synthase (*AgsA*), is strongly and specifically up-regulated in response to cell wall stress (Damveld et.al., 2005b; Meyer et.al., 2007). This gene is used for the construction of a cell wall stress *A. niger* reporter strain.The goal of this research is to develop a fast and hightroughput system for anti-fungal peptide screening.

PR9.6

Fungal a-arabinofuranosidases of glycosyl hydrolase families 51 and 54 show arabinofuranosyl- and galactofuranosyl hydrolyzing activity

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The filamentous fungus *Aspergillus niger* secretes two α-L-arabinofuranosidases that are encoded by the *abfA* and *abfB* genes. Expression of these genes in *Pichia pastoris* and enzymatic characterization of the purified recombinant AbfA and AbfB proteins revealed that both enzymes do not only hydrolyze p-Nitrophenyl-α-L-arabinofuranoside (pNp-α-Ara*f*), but are also capable of hydrolyzing p-Nitrophenyl-β-D-galactofuranoside (pNp-β-Gal*f*). Both AbfA and AbfB showed a higher specific activity towards pNp-α-Ara*f* than towards pNp-β-Gal*f*. Molecular modeling of the AbfB protein with pNp-α-Ara*f* or pNp-β-Gal*f* confirmed the possibility for AbfB to interact with both substrates in a similar manner. The *P. pastoris* expressed AbfA and AbfB proteins did not show hydrolyzing activity towards galactomannan isolated from *A. niger*, despite the presence of β-linked terminal and internal galactofuranosyl moieties within this compound. These data suggest that the AbfA and AbfB proteins are not responsible for the β-galactofuranosidase activity detected in the culture medium of *A. niger*. In addition, culture medium from an AbfA knockout *A. niger* strain showed a similar β-galactofuranosidase activity compared to the medium of a similarly grown parental *A. niger* strain, which does not support a role for AbfA in Gal*f* hydrolysis. In summary our studies show that both AbfA and AbfB contain a Gal*f*-hydrolyzing activity which may have a biological function by hydrolyzing natural Gal*f*-containing substrates from other organisms, or may be a side activity of the Ara*f*-hydrolyzing capacity.

PR9.7

Lectin-like proteins in *Piriformospora indica*

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The identification of microbial cell wall components by the plant host is an effective strategy to seek for potential threads. In order to avoid identification by the host, which may lead to oligosaccharide-triggered immunity, microbes have evolved different strategies, like cell wall masking. Recent findings show that lectin-like proteins play an important role in the suppression of oligosaccharide-triggered immunity. The symbiotic root endohyte *Piriformospora indica* can evade detection and suppresses immunity triggered by various microbe-associated molecular patterns, but the underlying mechanisms remain unclear. Our hypothesis is that the ability of *P. indica* to colonize roots from a wide range of unrelated plants depends on the evolution of strategies for broad immune system evasion and suppression. Comparative genomics revealed a significant expansion of lectin-like proteins containing either one or a combination of the carbohydrate binding domains LysM (chitin-binding); WSC (glucan-binding) and CBM1 (cellulose-binding) in the genome of *P. indica*. These putatively secreted proteins are also induced at the pre-penetration stage and early biotrophic phase and we therefore speculate that they are involved in modulating recognition by masking microbe-associated molecular patterns (MAMPs) and thus avoiding recognition by the host plant. Based on expression profiles data *in planta* we are functionally characterizing selected candidate genes within these 3 different categories.

PR9.8

A Gly579Arg mutation in the *Aspergillus fumigatus pkcA* encoding gene leads to defects in the cell wall integrity maintenance

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Aspergillus fumigatus is a ubiquitous mold that causes a number of clinical diseases in humans including invasive pulmonary aspergillosis, the life-threatening form of infection. The CWIP (cell wall integrity pathway) signaling cascade is activated in fungal cells under stressing conditions and plays a role in the adaptation of several fungal pathogen to the human host. In many fungi, CWIP is launched via the activation of protein kinase C which is ultimately associated to the transcription of a number of genes related to cell wall reinforcement and remodeling. We have recently observed that *pkcA* is an essential gene in *A. fumigatus*. Here we constructed a Gly579Arg mutant through DNA-mediated transformation of a engineered cassette which comprises a G2044C transversion located in the cysteine-rich C1B regulatory domain. To test the involvement of $pkcA^{G579R}$ in the CWIP, different concentrations of conidia from the wild type and $pkcA^{G579R}$ strains were spotted on complete and minimal agar plates supplemented with different concentrations of substances disturbing/interfering CWI (congo red, calcofluor white, caffeine, glucanex, anidulafungin, SDS). The sensitivity of the $pkcA^{G579R}$ to these drugs were increased and could be partially restored by D-sorbitol. Polar and vegetative growth of the $pkcA^{G579R}$ mutant strain were also considerably affected mainly at 30°C and 45°C. No defects were observed in the hyphal morphology or in the asexual reproductive structures. These data reinforces the role of *pkcA* signaling cascade in cell wall maintenance in *A. fumigatus*. Virulence assays for the $pkcA^{G579R}$ mutant are in progress.

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PR9.9

Genes Involved in Protein Glycosylation Determine the Sensitivity of Saccharomyces cerevisiae to the Cell-Penetrating Antifungal Peptide PAF26

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Protein glycosylation is a complex process that occurs through the N- or O-glycosylation pathways by the coordinated regulation of numerous genes and enzymes. Several glycosylation genes have been previously involved in the sensitivity of fungi to antifungal peptides and proteins. We have characterized the synthetic hexapeptide PAF26 as a cell-penetrating and non-lytic antifungal peptide that inhibits Saccharomyces cerevisiae and filamentous fungi. We searched the S. cerevisiae public collection of deletion mutants for glycosylation genes whose deletion altered sensitivity to PAF26. We observed that deletion of genes coding for the conserved protein O-mannosyltransferases (PMT) responsible for the addition of the first mannosyl residue of O-linked carbohydrates and for the EOS1 enzyme involved in N-glycosylation of cellular proteins, among other genes, resulted in specific increased resistance to diverse antifungal peptides including PAF26. Many cell wall (CW) proteins in fungi are glycosylated and/or anchored to the CW by diverse glycan structures. Microscopic visualization of S. cerevisiae cells exposed to fluorescently labelled peptide has shown that PAF26 firstly interacts with the cell envelope, prior to cell internalization, and subsequent causes intracellular cell death. Protoplasts lacking CWs interacted poorly with the peptide, and were more resistant to peptide killing than cells possessing CWs. Microscopic studies on the S. cerevisiae Δeos1 deletion mutant demonstrated a blockage of peptide internalization into cells. Interestingly, protoplasts obtained from this mutant behaved similarly to the parental strain. Collectively, these observations indicate that EOS1p exerts its activity through the glycosylation of CW protein(s) involved in the internalization of antifungal peptides.

PR9.10

Galactofuranose biosynthesis in *Aspergillus niger* provides new opportunities for industrial applications in the field of red and white biotechnology

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Galactofuranose-containing glycoconjugates are present in numerous microbes, many of which are pathogenic for humans. Metabolic aspects of the monosaccharide have proven difficult to elucidate, because galactofuranose metabolites and glycoconjugates are relatively unstable during analyses. Recent advances with genetic approaches have facilitated a better understanding of galactofuranose metabolism. Galactofuranose (Gal*f*) the five-ring isomer of galactopyranose (Gal*p*), is an essential component of the cell wall and required for a structural integrity [1-2]. Recently it has been postulated that Gal*p* bound to UDP, is converted to Gal*f* by a UDP-galactopyranose mutase (UGMA) and subsequently transported into the Golgi by a putative gal*f*-transporter namely GlfB [3] for the further biosynthesis of e.g. galactomannan, galactoaminogalactan and cell wall glycoproteins(galactomanno-proteins) [4-6]. The sugar units can be cross-linked with each other via covalent or by hydrogen bonds but the actual composition is changing depending on the respective fungal species and environmental factors [5, 6, 8].

Based on homology search we have identified two putative Gal*f*-transporters in *A. niger* (GlfB homologue) UGTA/UGTB and investigated their role in the biosynthesis of Galf-containing glycoconjugates and their role in Galf containing cell wall compartments. Moreover, we evaluated growth and morphological effects of the deletion of the corresponding genes in relation to fungal biology.

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PR9.11

The product of NRPS 4 from *Fusarium graminearum* - A fungal raincoat?

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Nonribosomal peptide synthetases (NRPSs) are large multi-modular enzyme-complexes that produce small peptides independently of the ribosomal machinery. *Fusarium graminearum* has 19 putative NRPS genes for which the products of three are known. These are all iron chelating siderophores, whereas the products of the remaining predicted NRPSs are unknown.

The *NRPS4* of *F. graminearum* encodes an 844 kDa enzyme consisting of five modules and is conserved throughout all sequenced *Fusarium* species and across a range of other plant pathogenic filamentous fungi. Deletion of *NRPS4* in *Cochliobolus heterostrophus* and *NRPS 2* in *Alternaria brassicicola* (48% and 49% consensus identity to *FgNRPS4,* respectively) resulted in phenotypes where surface hydrophobicity was significantly reduced. In the present study we have generated deletion and over expression mutants of *NRPS4* in *F. graminearum.* Both mutants show a clear phenotype as the deletion mutant displays a reduced surface hydrophobicity whereas the over expression mutant is completely water resistant.

PR9.12

Characterisation of *Aspergillus niger* chitinases involved in aging identifies a novel activity in fungal GH18 chitinases

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The filamentous fungus *Aspergillus niger* has significant industrial importance as a production host for metabolites and extracellular enzymes. Fungal aging and stress conditions during industrial fermentations may lead to the initiation of sporulation and autolysis, during which glycoside hydrolases (GH) can modify the cell wall structure. A full understanding of processes taking place during aging and nutrient starvation contributes to the identification of strategies to increase fermentation efficiency.

Using transcriptome analysis, we identified glycoside hydrolases of *A. niger* produced by the aging and nutrient starved mycelium. Two enzymes were heterologously expressed, purified and characterised to gain more insight in their physiological and metabolic function. Both CfcA and CfcI belong to GH family 18, which fungal members consist mainly of (putative) chitinases. CfcA releases mainly chitobiose from the non-reducing end of chitin oligosaccharides and from chitin present in the fungal cell wall.

CfcI is capable of hydrolysing chitotriose and longer chitin oligosaccharides. CfcI by itself is not capable of releasing products from the fungal cell wall. However, *cfcI* is expressed together with *ctcB,* which encodes a putative endochitinase. The oligosaccharides generated by CtcB activity on cell walls may act as substrates for CfcI. CfcI functions by cleaving off monomers, possibly in a processive mode, acting on the reducing end of the oligosaccharide substrates. To the best of our knowledge, this activity has not been reported before for fungal chitinases of glycoside hydrolase family 18.

PR9.13

Structural and functional characterization of *Candida glabrata* epithelial adhesins

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Candida glabrata is an emerging human pathogen that is responsible for about 15 % of mucosal and systemic fungal infections. The genome of *C. glabrata* encodes a set of more than twenty surface-exposed, epithelial adhesins (Epa) for host cell adhesion and colonization. Host glycan recognition by the Epa protein family is governed by their adhesin (A) domains and hence crucial for discrimination of various target tissues. In this study, we focus on the structural and biochemical characterization of different Epa A domains to obtain detailed insights into ligand specificity. The crystal structure of the Epa1A domain reveals how core1 and core2 mucin-type O-glycans are recognized by the major Epa1 subtype. Structural and functional characterization of subtype-switched Epa variants shows that specificity is governed by two loops, CBL1 and CBL2, involved in calcium binding. Together with a conserved tryptophan, these loops organize the recognition site into an inner subsite for general, calcium-dependent galactose binding and an outer subsite for specific interactions with different types of host glycans. These structural insights show that the Epa family consists of at least four different subtypes that are discriminated from each other by conserved sequences within the CBL2 region. Overall, our study demonstrates how *C. glabrata* colonizes host tissues and provides a promising structural basis for the development of tailored antimycotics.

PR9.14

Structural and functional analysis of *Saccharomyces cerevisiae* cell surface adhesins

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Saccharomyces cerevisiae contains a set of cell wall-associated proteins, the flocculins, which confer adhesion. The highly related Flo1, Flo5 and Flo9 proteins confer cell-cell adhesion by lectin-like and calcium-dependent binding of mannoproteins on neighboring cells. This type of adhesion is known as flocculation and allows *S. cerevisiae* to form protective multicellular flocs. A further structurally unrelated flocculin, Flo11, enables yeast cells to adhere to abiotic surfaces such as agar and plastic. Finally, Flo10 represents a flocculin, whose function is discussed controversially, because it has been described to confer both flocculation and agar adhesion when overproduced. In this project, we have initiated a structural and functional analysis of the Flo10 adhesion domain (= A domain). Modeling of Flo10A reveals a high structural similarity to Flo5A and shows that Flo10A is a PA14-related protein. However, Flo10A differs from Flo5A by its subdomain, which in case of Flo5A has been suspected to confer ligand binding specificity. By using a *FLO11*-based expression system we find that Flo10A, in contrast to Flo5A, does not confer flocculation. Furthermore, deletion of the Flo5A subdomain results in a loss of flocculation, while mutual exchange of the Flo10 and Flo5 subdomains is sufficient to swap functionality. Our results highlight the crucial role of adhesin subdomains in conferring ligand binding specificity to PA14-related proteins.

PR9.15

gcsA*, an ARF-GAP-ENCODING GENE IN *Aspergillus fumigatus.

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Aspergillus fumigatus is one of the most important airborne pathogenic fungi, which has the potential to cause Invasive Pulmonary Aspergillose (IPA). Sphingolipids are the major component of the eukaryotic plasma membrane and are involved in the cell wall integrity and virulence. The ADP-ribosilation factor (ARF) family of proteins belongs to the Ras superfamily of small GTPases. The hydrolysis of ARF GTP-bound is mediated by GTPase-activating proteins (GAPs). ARF-GAPs are required for vesicular coat formation in endocytic pathway and have been related to hyphal growth, drug resistance and virulence in *C. albicans*. In this work we identified *gcsA*, the *C. albicans age3* ortholog gene in *A. fumigatus. gcsA* null mutant has normal hyphal growth, exhibit no differences in sensitivity to antifungal agents, was not able to form biofilm, and is virulent in an IPA mouse model as the wild type. Thus, despite the *gcsA* influence on the sphingolipids biosynthesis, here shown by the decreased cell polarization in the presence of Myriocin, *gcsA* null mutant has no change on antifungal sensitivity, virulence, and hyphal growth profile, suggesting a probable distinct function of this gene in the filamentous fungus *A. fumigatus*.

Acknowledgments: FAPESP

PR9.16

Functional analysis of the Mps1 MAP kinase pathway in the rice blast fungus *Magnaporthe oryzae*.

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Magnaporthe oryzae is a fungal rice pathogen responsible for large yield losses worldwide. Our project concerns the study of the Mps1 MAP kinase pathway of *M. oryzae,* involved in cell wall integrity, sporulation and pathogenicity. Mps1 is orthologue of yeast SLT2 and activates the transcription factors Rlm1, Swi4 and Swi6, while calcineurin activates Crz1. *M. oryzae* genes orthologous to yeast *CRZ1*, *MPS1*, *RLM1, SWI4,* and *SWI6* genes were identified. Swi4 and Swi6 interact with Mps1 in yeast two hybrid experiments (a) as well as Mps1 and Rlm1 (b). The main task focuses on the analysis of the Mps1 downstream elements Rlm1, Swi4 and Swi6. *Δmps1* mutants displayed an abnormal mycelial growth (no aerial hyphae), did not sporulate, and were non-pathogenic on plants as reported (c). *Δswi4* displayed phenotypes similar to *Δmps1* with milder growth and sporulation defects, while it was as non-pathogenic as *Δmps1*. *Δcrz1* and *Δswi6* mutants have a normal mycelial growth and sporulation rates, while *Δrlm1* has a reduced sporulation rate. *Δcrz1* and *Δrlm1* were non pathogenic on barley and rice, while *Δswi6* was pathogenic. These studies suggest Swi4 is the major target of Mps1 during mycelial growth, while both Rlm1 and Swi4 are the targets of Mps1 during sporulation and infection. *Δmps1, Δrlm1, Δswi4* and *Δswi6* null mutants are currently tested for their sensitivity to cell wall degrading enzymes and inhibitors of cell wall biosynthesis.

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PR9.17

Cell wall stress modulates the expression of the ram signaling network components in *Trichophyton rubrum*.

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Trichophyton rubrum is a pathogenic, cosmopolitan and anthropophilic fungi that infect keratinized tissues mainly skin and nails. The genome of several dermatophytes, including *T. rubrum*, was sequenced by the Broad Institute/NIH, enabling studies on the expression regulation of genes related to diverse cellular processes. The transcription factor (TF) Ace2 participates in a network of genes called RAM (Regulation of Ace2 activity and cellular morphogenesis), involved in the regulation of morphogenesis, cell division, and development of conidiophores. In dermatophytes this network has not yet been characterized. Therefore, the aim of this study was to identify these genes in *T. rubrum* genome, as well as their possible regulation by Ace2 through *in silico* analysis, and to evaluate the transcriptional profile of these genes in response to various cell wall disturbing agents, and the osmotic and oxidative stresses. *In silico* analysis suggested their possible regulation by this TF in *T. rubrum*. To analyze the expression of the RAM network genes, *T. rubrum* was exposed to several cell wall stressor agents. The transcriptional profile of the RAM network genes in response to disturbances in cell wall assists a better comprehension of the involvement of this pathway in regulating a variety of processes that enable cell viability during environmental stress, once the RAM signaling network components are highly conserved in eukaryotes.

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