

## A comparative genomic analysis of the calcium signaling machinery in *Neurospora crassa*, *Magnaporthe grisea*, and *Saccharomyces cerevisiae*

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### Abstract

A large number of Ca<sup>2+</sup>-signaling proteins have been previously identified and characterized in *Saccharomyces cerevisiae* but relatively few have been discovered in filamentous fungi. In this study, a detailed, comparative genomic analysis of Ca<sup>2+</sup>-signaling proteins in *Neurospora crassa*, *Magnaporthe grisea*, and *S. cerevisiae* has been made. Our BLAST analysis identified 48, 42, and 40 Ca<sup>2+</sup>-signaling proteins in *N. crassa*, *M. grisea*, and *S. cerevisiae*, respectively. In *N. crassa*, *M. grisea*, and *S. cerevisiae*, 79, 100, and 13% of these proteins, respectively, were previously unknown. For *N. crassa*, *M. grisea*, and *S. cerevisiae*, respectively, we have identified: three Ca<sup>2+</sup>-permeable channels in each species; 9, 12, and 5 Ca<sup>2+</sup>/cation-ATPases; eight, six, and four Ca<sup>2+</sup>-exchangers; four, four, and two phospholipase C's; one calmodulin in each species; and 23, 21, and 29 Ca<sup>2+</sup>/calmodulin-regulated proteins. Homologs of a number of key proteins involved in the release of Ca<sup>2+</sup> from intracellular stores, and in the sensing of extracellular Ca<sup>2+</sup>, in animal and plant cells, were not identified. The greater complexity of the Ca<sup>2+</sup>-signaling machinery in *N. crassa* and *M. grisea* over that in *S. cerevisiae* probably reflects their more complex cellular organization and behavior, and the greater range of external signals which filamentous fungi have to respond to in their natural habitats. To complement the data presented in this paper, a comprehensive web-based database resource (<http://www.fungalcell.org/fdf/>) of all Ca<sup>2+</sup>-signaling proteins identified in *N. crassa*, *M. grisea*, and *S. cerevisiae* has been provided.

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### 1. Introduction

Calcium plays a major role as an intracellular signal molecule, and eukaryotic cells possess a complex array of Ca<sup>2+</sup>-permeable channels, -pumps, -transporters, and other Ca<sup>2+</sup>-signaling proteins. These components of the Ca<sup>2+</sup>-signaling machinery are involved in the transduction of a wide variety of external signals through numerous stimulus-response pathways within the cellular

signal transduction network (Berridge et al., 2003; Sanders et al., 2002). The resting level of cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>c</sub>) is kept very low (typically 50–100 nM) and this is maintained by active Ca<sup>2+</sup>-pumps and -transporters, and the Ca<sup>2+</sup>-buffering capacity of the cytoplasm. However, [Ca<sup>2+</sup>]<sub>c</sub> becomes an intracellular signal when its concentration is transiently increased as a result of the activation of Ca<sup>2+</sup>-permeable channels allowing Ca<sup>2+</sup> to flow down a concentration gradient into the cytoplasm from Ca<sup>2+</sup>-storage organelles or from the external medium. Following the [Ca<sup>2+</sup>]<sub>c</sub> increase, the [Ca<sup>2+</sup>]<sub>c</sub> recovers to its original resting level by

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the increased activity of  $\text{Ca}^{2+}$ -pumps and -transporters. Different combinations of  $\text{Ca}^{2+}$ -channels, -pumps, -transporters and other  $\text{Ca}^{2+}$ -signaling proteins are used by cells to produce  $\text{Ca}^{2+}$  signals with different dynamic spatial and temporal characteristics (Berridge et al., 2003).  $[\text{Ca}^{2+}]_c$  dynamics are now being routinely measured in living fungal cells (Nakajima-Shimada et al., 1991; Nelson et al., 2004).

Animal and plant cells possess very complex  $\text{Ca}^{2+}$ -signaling machineries (Berridge et al., 2003; Sanders et al., 2002). In contrast, the budding yeast, *Saccharomyces cerevisiae*, has a much simpler  $\text{Ca}^{2+}$ -signaling apparatus which has been reported to regulate the cell cycle, mating, sensing of glucose and glucose starvation, resistance to salt stress and cell survival (Cyert and Thorner, 1992; Fischer et al., 1997; Matheos et al., 1997; Nakajima-Shimada et al., 1991; Sun et al., 1992; Tisi et al., 2004). In filamentous fungi, in which growth patterns and development are more complex, there is evidence for the involvement of  $\text{Ca}^{2+}$  in far more physiological processes, including the cell cycle, sporulation, spore germination, hyphal tip growth, hyphal orientation, hyphal branching, and circadian rhythms (Gadd, 1994; Shaw and Hoch, 2001). The sequencing of the *Neurospora crassa* genome has indicated that this fungus possesses a substantive 'toolkit' of  $\text{Ca}^{2+}$ -signaling proteins. However, this toolkit showed significant differences with those in animal and plant cells, especially in relation to proteins associated with  $\text{Ca}^{2+}$ -release from intracellular  $\text{Ca}^{2+}$  stores (Borkovich et al., 2004; Galaghan et al., 2003). Now that several fungi have had their genomes sequenced, it has become possible to make a detailed comparative genomic analysis of the  $\text{Ca}^{2+}$ -signaling machinery in filamentous fungi and yeasts.

The genome sequences of the non-filamentous fungal model organisms *Saccharomyces cerevisiae* (<http://genome-www.stanford.edu/Saccharomyces/>) and *Schizosaccharomyces pombe* ([http://www.sanger.ac.uk/Projects/S\\_pombe/](http://www.sanger.ac.uk/Projects/S_pombe/)) were published in 1996 and 2002, respectively (Goffeau et al., 1996; Wood et al., 2002). The genome sequence of *N. crassa*, the first filamentous fungus to be completely sequenced in the public sector, has recently been published (Borkovich et al., 2004; Galaghan et al., 2003; <http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>). The genome sequences of various other filamentous fungi are also now available, including *Magnaporthe grisea* (<http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>), *Aspergillus fumigatus* (<http://www.tigr.org/tdb/e2k1/afu1/>), *Aspergillus nidulans* (<http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/>), *Coprinus cinereus* ([http://www-genome.wi.mit.edu/annotation/fungi/coprinus\\_cinereus/](http://www-genome.wi.mit.edu/annotation/fungi/coprinus_cinereus/)), *Fusarium graminearum* (<http://www-genome.wi.mit.edu/annotation/fungi/fusarium/>), *Phanaerochaete chrysosporium* (<http://genome.jgi-psf.org/whiterot/>), *Candida. albi-*

*cans* (<http://genome-www.stanford.edu/fungi/Candida>), *Cryptococcus neoformans* (<http://www.tigr.org/tdb/e2k1/cna1>), *Pneumocystis. carinii* (<http://www.uky.edu/Projects/Pneumocystis>), and *Ustilago maydis* ([http://www-genome.wi.mit.edu/annotation/fungi/ustilago\\_maydis/index.html](http://www-genome.wi.mit.edu/annotation/fungi/ustilago_maydis/index.html)).

The availability of such large filamentous fungal genome databases has made it possible, for the first time, to gain detailed insights into the molecular machinery of filamentous fungi through genomic analysis. The aims of this study were: (a) to identify the  $\text{Ca}^{2+}$ -signaling proteins encoded in the genomes of the model filamentous fungus *N. crassa* and model plant pathogen *M. grisea* based on an analysis of their entire genomes; (2) to analyse in detail the  $\text{Ca}^{2+}$ -permeable channels,  $\text{Ca}^{2+}$ -pumps and  $\text{Ca}^{2+}$ -transporters in *N. crassa*; (3) to compare these  $\text{Ca}^{2+}$ -signaling proteins with those in *M. grisea* and those in the yeast model *S. cerevisiae* (the fungus in which most is known about  $\text{Ca}^{2+}$ -signaling); and (4) to provide a comprehensive web-based database resource on all  $\text{Ca}^{2+}$ -signaling proteins in *N. crassa*, *M. grisea*, and *S. cerevisiae*.

## 2. Materials and methods

A range of  $\text{Ca}^{2+}$ -signaling protein and gene sequences from plants, animals, and fungi were used to probe the (a) *N. crassa* genome database (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>); (b) *M. grisea* genome database (<http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>); and (c) *S. cerevisiae* genome database (<http://genome-www.stanford.edu/Saccharomyces/>) using the BLASTN, TBLASTN, and BLASTP algorithms. Probe sequences included several representatives from every family of  $\text{Ca}^{2+}$ -signaling proteins for which gene sequence could be retrieved. Potential hypothetical protein homologs from *N. crassa*, *M. grisea*, and *S. cerevisiae* were identified based on: (a) *E* values, (b) % identities and gaps, and (c) conserved domains present (e.g., whether the homologous regions matched conserved domains or regions in other proteins thought to be involved in  $\text{Ca}^{2+}$  related processes according to the literature or the tools made available by NCBI's Conserved Domain Database and Search Service (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>)). Hypothetical proteins and DNA coding sequences obtained in this way were entered into our own database (<http://www.fungalcell.org/dfd/>) of putative  $\text{Ca}^{2+}$ -signaling proteins. These data were stored under their locus numbers, as defined by the web page of each organism's genome project. Potential  $\text{Ca}^{2+}$ -signaling proteins and regions of DNA from our database were used to reprobe the GenBank, EMBL, DDBJ, and PDB databases through NCBI using the TBLASTN algorithm to check for similar protein or DNA sequences in other organisms. This information was also entered into our database.

Conserved protein domains were analysed using the NIH tools CDD and CDART (<http://www.ncbi.nlm.nih.gov>). Hydrophilicity plots were performed using the Kyte–Doolittle method using a web-based program provided by the Weizmann Institute of Science ([http://bioinformatics.weizmann.ac.il/hyd-bin/plot\\_hydroph.pl](http://bioinformatics.weizmann.ac.il/hyd-bin/plot_hydroph.pl)). Prediction of putative transmembrane segments was done using PredictProtein, from the server at EMBL ([http://www.embl-heidelberg.de/predictprotein/submit\\_def.html](http://www.embl-heidelberg.de/predictprotein/submit_def.html)). Multiple sequence alignment and generation of maximal parsimony dendrograms was done using the Clustal X program (Thompson et al., 1997). Maximal parsimony dendrograms were drawn using njplot (<http://pbil.univ-lyon1.fr/software/njplot.html>).

A MySQL database was used for data storage (<http://www.mysql.com>). The web interface and underlying software to our database was written in perl (<http://www.perl.com>) and html (<http://www.w3.org>). It was written and run on a standard PC running SuSE Linux version 7.3 (<http://www.suse.com> and <http://www.linux.org>).

### 3. Results

#### 3.1. Interactive Web-based database of $Ca^{2+}$ -signaling proteins

An interactive database with a web interface was prepared (<http://www.fungalcell.org/dfd/>) and used as a

repository for detailed information regarding all the proteins described in this paper. This website should be used to supplement the information presented below. The advantages of this approach for data storage and access are the provision of: (1) a dynamic and convenient, interactive interface for the public to access these data; (2) numerous ways for the user to filter the data to view or search for what one wants; (3) access to sequence data for all the proteins and genes described in the database; (4) hyperlinks to other sources of information specific to the data within the database (e.g., automatic searches for conserved domains within a protein and other functions); and (5) the flexibility to add unlimited additional functions in the future. A local BLAST facility was also set up (<http://www.fungalcell.org/blast/>) to enable the sequence data deposited in our database to be searched directly using the NCBI BLAST software package (software provided courtesy of NCBI at <http://www.ncbi.nlm.nih.gov/Ftp/>).

#### 3.2. Calcium-signaling proteins previously identified in filamentous fungi and budding yeast

A search of the literature and the NCBI Entrez–Protein database revealed a number of previously identified filamentous fungal  $Ca^{2+}$ -signaling proteins (Table 1). In all filamentous fungi only one  $Ca^{2+}$ -permeable channel, Cch1, has been previously identified (NCBI Accession No. AF393474). Six  $Ca^{2+}$ -ATPases and one  $Ca^{2+}/H^+$  exchanger comprise the total number of these types of

Table 1  
 $Ca^{2+}$ -signaling proteins previously identified in filamentous fungi

Protein class	Protein name/locus	Organism	References
$Ca^{2+}$ -permeable channel	CCH1	<i>Aspergillus nidulans</i>	NCBI #AF393474
$Ca^{2+}$ -ATPase	pmrA	<i>A. niger</i>	Yang et al. (2001a)
$Ca^{2+}$ -ATPase	NCA-1, NCA-2, NCA-3, PMR-1, PH-7	<i>Neurospora crassa</i>	Benito et al. (2000)
$Ca^{2+}/H^+$ -exchanger	CAX	<i>N. crassa</i>	Margolles-Clark et al. (1999)
Calmodulin	CMD	<i>N. crassa</i>	Capelli et al. (1993); Melnick et al. (1993)
Calmodulin	CMDA	<i>A. nidulans</i>	Rasmussen et al. (1990)
Calmodulin	AAK69619	<i>Fusarium proliferatum</i>	Kwon et al. (2001)
Calcineurin A (catalytic subunit)	CNA	<i>N. crassa</i>	Higuchi et al. (1991)
Calcineurin A (catalytic subunit)	CNAA	<i>A. nidulans</i>	Rasmussen et al. (1994)
Calcineurin A (catalytic subunit)	CNA1	<i>Filobasidiella neoformans</i>	Odom et al. (1997)
Calcineurin A (catalytic subunit)	CNAA	<i>A. oryzae</i>	Juvvadi et al. (2001)
Calcineurin A (catalytic subunit)	CNA/AAL47191	<i>Exophiala dermatitidis</i>	NCBI #AAL47191
Calcineurin B (regulatory subunit)	CNB	<i>N. crassa</i>	Kothe and Free (1998)
Calcineurin B (regulatory subunit)	CNB1	<i>F. neoformans</i>	Fox et al. (2001)
$Ca^{2+}$ /calmodulin-dependent protein kinase	FCaMK	<i>Arthrobotrys dactyloides</i>	Tsai et al. (2002)
$Ca^{2+}/CaM$ dependent protein kinase B	CaMK I/IV homolog cmkB	<i>A. nidulans</i>	Joseph and Means (2000)
$Ca^{2+}/CaM$ dependent protein kinase C	CaMKK a/b homolog cmpk	<i>A. nidulans</i>	Joseph and Means (2000)
$Ca^{2+}/CaM$ dependent protein kinase	CMKA	<i>A. nidulans</i>	Kornstein et al. (1992)
$Ca^{2+}/CaM$ -dependent kinase-1	CAMK-1	<i>N. crassa</i>	Yang et al. (2001b)
Calmodulin-dependent protein kinase	CgCMK	<i>Colletotrichum gloeosporioides</i>	Kim et al. (1998)
Phospholipase C	NCPLC-1, NCPLC-2, NCPLC-3	<i>N. crassa</i>	Jung et al. (1997)
Phospholipase C	ANPLC1	<i>A. nidulans</i>	Jung et al. (1997)
Phospholipase C	BCPLC1	<i>Botryotinia fuckeliana</i>	Jung et al. (1997)
Phospholipase C	MPLC1	<i>Magnaporthe grisea</i>	NCBI #AAC72385

proteins previously described in filamentous fungi. Calcineurin has been identified in five species of filamentous fungi, and calmodulin (CaM) in three. Several putative isozymes of phospholipase C (PLC) have been identified in *N. crassa*, *A. nidulans*, *M. grisea*, and *Botryotinia fuckeliana*, although their actual cellular functions have not been analysed experimentally.

In budding yeast, three Ca<sup>2+</sup>-permeable channels, four Ca<sup>2+</sup>-ATPases, one Ca<sup>2+</sup>/H<sup>+</sup>-exchanger, one Ca<sup>2+</sup>/Na<sup>+</sup>-exchanger, one PLC, and several other proteins involved in Ca<sup>2+</sup>-transport and homeostasis have all been previously identified (Table 2). In contrast to the case with filamentous fungi, all of these proteins have been investigated experimentally, and have had their cellular functions analysed.

The number of reports on Ca<sup>2+</sup>-signaling proteins in filamentous fungi is currently very low (Table 1). To create an appropriate database of Ca<sup>2+</sup>-signaling proteins in these organisms, we performed a detailed BLAST analysis of the genomes of two filamentous fungi (*N. crassa* and *M. grisea*) and budding yeast (*Saccharomyces cerevisiae*).

### 3.3. Calcium-signaling proteins present in filamentous fungi and budding yeast

Our BLAST analysis identified 48, 42, and 40 Ca<sup>2+</sup>-signaling proteins in *N. crassa*, *M. grisea*, and *S. cerevisiae*, respectively (<http://www.fungalcell.org/fdf/> and Tables 3 and 4). In *N. crassa*, and *M. grisea*, 79 and 100% of these proteins, respectively, were previously unknown. In *S. cerevisiae* only 13% were previously unknown. With the exception of Ecm27p, which has already been named, these previously unknown hypothetical Ca<sup>2+</sup>-signaling proteins are YOR291W, YNL321W, YDL206W, YGR058W, and YDR287W (<http://www.fungalcell.org/fdf/>). The number of Ca<sup>2+</sup>-signaling proteins discovered in *N. crassa* and *M. grisea* represents almost 0.5% of the estimated ~10,000 and ~11,000 proteins encoded by their respective genomes

(Borkovich et al., 2004; Galaghan et al., 2003; <http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>). Clearly then, genes encoding Ca<sup>2+</sup>-signaling proteins are an important component of fungal genomes. The proteins discovered were divided into several categories for further analysis. These were: (1) Ca<sup>2+</sup>-permeable channels; (2) Ca<sup>2+</sup>-pumps; (3) Ca<sup>2+</sup>-transporters; and (4) other proteins important for Ca<sup>2+</sup>-signaling. Most of the proteins in the latter category will not be discussed here but are included on the website provided (<http://www.fungalcell.org/fdf/>).

### 3.4. Calcium-permeable channels

Ca<sup>2+</sup>-permeable channels regulate the passive flow of Ca<sup>2+</sup> across cell membranes into the cytoplasm. There are several different types of Ca<sup>2+</sup>-permeable channel and they are usually grouped according to their method of activation (Bootman et al., 2001). Voltage-gated Ca<sup>2+</sup>-permeable channels are comprised of four subunits, each consisting of six transmembrane (TM) spans, within which a putative ion-conducting pore region is present (Kreusch et al., 1998). Our BLAST analysis revealed three Ca<sup>2+</sup>-permeable channels falling into three separate groups (I–III) in each of the three fungi investigated (Fig. 1; Table 3). All of these proteins were previously unknown in *N. crassa* and *M. grisea*.

Group I Ca<sup>2+</sup>-permeable channels included the budding yeast Cch1p protein. Cch1p bears sequence similarity to the  $\alpha 1$ , catalytic subunit of voltage-gated Ca<sup>2+</sup>-permeable channels (e.g., *Rattus norvegicus*, Fig. 2) and was localized in the yeast plasma membrane by Locke et al. (2000). The filamentous fungal homologs of Cch1p (*N. crassa* NCU02762.1 and *M. grisea* MG5643.4) were very similar to the yeast protein ( $E = 0$  for both). Like the  $\alpha 1$  subunit, the fungal proteins contain four hydrophobic repeat units (I–IV), each consisting of six TM domains (Fig. 2) that tetramerize to form the core of the Ca<sup>2+</sup>-channel (an aqueous pore). Most of the sequence identity between the fungal and

Table 2  
Ca<sup>2+</sup>-signaling proteins previously identified in *Saccharomyces cerevisiae*

Protein class	Protein name/locus	References
Ca <sup>2+</sup> -permeable channel	Cch1p, Mid1p, Yvc1p	Paidhungat and Garrett (1997); Iida et al. (1994); Kanzaki et al. (1999); Palmer et al. (2001)
Non-specific cation channel	Pmp3p	Navarre and Goffeau (2000)
Ca <sup>2+</sup> -ATPase	Pmc1p, Pmr1p, Spf1p, Neo1p	Degand et al. (1999); Park et al. (2001); Cronin et al. (2002); Catty and Goffeau (1996)
Ca <sup>2+</sup> -transporter	Ccc1p	Lapinskas et al. (1996)
Ca <sup>2+</sup> /H <sup>+</sup> -exchanger	Vcx1p	Miseta et al. (1999)
Calmodulin	Cmd1p	Davis et al. (1986)
Calcineurin A (catalytic subunit)	Cna1p, Cna2p	Cyert et al. (1991)
Calcineurin B (regulatory subunit)	Cnb1p	Cyert and Thorner (1992)
CaM-dependent protein kinase	Cmk1p, Cmk2p	Cyert (2001)
Calnexin	Cne1p	de Virgilio et al. (1993)
Phospholipase C	Plc1p	Yoko-o et al. (1993)

Table 3  
Ca<sup>2+</sup>-permeable channels, Ca<sup>2+</sup>-pumps and Ca<sup>2+</sup>-transporters in *N. crassa*, *M. grisea*, and *S. cerevisiae*

Class of protein	Proteins in <i>N. crassa</i>		Closest homologs in:			
			<i>M. grisea</i>		<i>S. cerevisiae</i>	
	Name	No.	Name	No.	Name	No.
Ca <sup>2+</sup> -permeable channel	NCU02762.1	3	MG05643.4	3	Cch1p	3
	NCU06703.1		MG04001.4		Mid1p	
	NCU07605.1		MG09828.4		Yvc1p	
Cation pump (Ca <sup>2+</sup> unless otherwise indicated)	NCA-1	9	MG04550.4, 2.852, 18780–19520	12	Pmr1p	5
	NCA-2		MG02487.4,		Pmc1p	
	NCA-3		MG04890.4		Pmc1p	
	PMR1		MG09892.4, MG10730.4 <sup>a</sup> ,		Pmr1p	
	PH-7, ENA-1		MG02074.1F		Ena2p <sup>b</sup>	
	NCU07966.1		2.1107, 34917–37626			
	NCU04898.1		2.1792, 25841–29738 <sup>a</sup>		Spf1p	
	NCU03818.1		MG04066.4, MG05078.4 <sup>b</sup>		Neo1p, Ena2p <sup>b</sup>	
	NCU01437.1 <sup>a</sup>		MG06925.4 <sup>a</sup> , MG07971.4		YOR291W <sup>a</sup> , Ena5p <sup>b</sup>	
Ca <sup>2+</sup> -transporters (Ca <sup>2+</sup> /H <sup>+</sup> unless otherwise indicated)	CAX	8	2.175, 3011–3697	6	Vcx1p	4
	NCU00916.1		MG01193.4		Vcx1p	
	NCU00795.1		MG08710.4		Vcx1p	
	NCU06366.1		None		none	
	NCU07711.1		MG04159.4		Vcx1p	
	NCU05360.1		MG01381.4		YNL321W	
	NCU02826.1 <sup>c</sup>		MG01638.4 <sup>c</sup>		YDL206W <sup>c</sup> , Ecm27p <sup>c</sup>	
	NCU08490.1 <sup>c</sup>		None		none	

<sup>a</sup> Undefined cation-ATPase.

<sup>b</sup> Na<sup>+</sup>-ATPase.

<sup>c</sup> Ca<sup>2+</sup>/Na<sup>+</sup>-exchanger.

Table 4  
Phospholipase C's and other important Ca<sup>2+</sup> and/or CaM binding proteins in *N. crassa*, *M. grisea*, and *S. cerevisiae*

Protein class	Proteins in <i>N. crassa</i>		Closest homologs in:			
			<i>M. grisea</i>		<i>S. cerevisiae</i>	
	Name	No.	Name	No.	Name	No.
Phospholipase C- $\delta$	NCU01266.1	4	MG02444.4	4	Plc1p	1
	NCU06245.1		MG05332.4		Plc1p	
	NCU09655.1		MG05905.4		Plc1p	
	NCU02175.1		MG02682.4		Plc1p	
Calmodulin	CMD 1	1	MG06884.2	1	Cmd1p	1
Calcineurin (catalytic)	CNA-1	1	MG07456.2	1	Cna1p, Cna2p	2
Calcineurin (regulatory)	CNB-1	1	MG06933.4	1	Cnb1p	1
Ca <sup>2+</sup> and/or CaM binding proteins	NCU02283.1	9	MG00925.4	9	Cmk1p, Cmk2p	11
	NCU09123.1		MG09912.4		Cmk1p, Cmk2p	
	NCU06177.1		MG06421.4		Pak1p	
	NCU09212.1		MG08547.4		Rck2p, Rck1p	
	NCU00914.1		MG01196.4		Kin4p, Arp8p	
	NCU02814.1		MG01596.4		Dun1p, Rad53p	
	NCU06347.1		MG06180.4		End3p	
Calnexin	NCU09265.1		MG01607.4		Cne1p	
Calpactin I heavy chain	NCU04421.1		MG06847.4		no hit	
Calreticulin	NCU09265.1		MG01607.4		Cne1p	

mammalian Ca<sup>2+</sup>-channel subunits is present within regions thought to play key roles in defining channel specificity (domain P) (Fig. S2 at <http://www.fungalcell.org/fdf/>) and voltage dependence (TM domain S4) (Paidhungat and Garrett, 1997). All four hydrophobic domains (I–IV) contain amino acid residues indicative

of the Ca<sup>2+</sup>-selective P segment, and three (II, III, and IV) of the four (Fig. 2B) contain a highly conserved glutamate residue that is thought to play a critical role in Ca<sup>2+</sup> coordination (Fig. S2a at <http://www.fungalcell.org/fdf/>; Paidhungat and Garrett, 1997). Each of the S4 segments of domains I, II, and III contain repeated

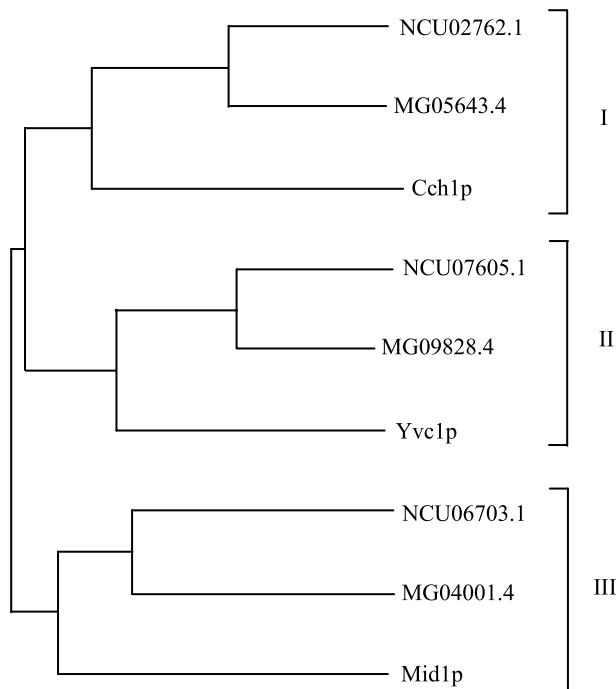


Fig. 1. Maximal parsimony dendrogram of  $\text{Ca}^{2+}$ -permeable channels identified in *N. crassa*, *M. grisea*, and *S. cerevisiae*. Neither rigorous calculation of evolutionary distances nor phylogenetic relationship can be inferred with confidence from this tree.

motifs of a positively charged residue followed by two hydrophobic residues (Fig. S2b at <http://www.fungalcell.org/fdf/>). Similar segments have been shown to act as voltage sensors in ion channels of higher eukaryotes (Paidhungat and Garrett, 1997). The hydrophobic domains (I, II, III, and IV) matched the PFAM00520

domain, found in  $\text{Na}^{+}$ -,  $\text{K}^{+}$ -, and  $\text{Ca}^{2+}$ -permeable channels and consist of six TM helices in which the last two helices flank a loop which determines ion selectivity (<http://www.sanger.ac.uk/Software/Pfam>).

Group II  $\text{Ca}^{2+}$ -permeable channels included the yeast Mid1p protein. Mid1p is a stretch-activated, plasma membrane located,  $\text{Ca}^{2+}$ -permeable channel (Iida et al., 1994; Kanzaki et al., 1999). Recent data suggest that the Mid1p protein may also be present in the endoplasmic reticulum (Yoshimura et al., 2004). The filamentous fungal homologs of Mid1p (*N. crassa* NCU06703.1 and *M. grisea* MG04001.4) were quite similar to the yeast protein ( $E = 4e-28$  and  $E = 1e-32$ , respectively) although the filamentous fungal proteins were larger (NCU06703.1 by 22% and MG04001.4 by 38%). None of the  $\text{Ca}^{2+}$ -permeable channels in this class had overall sequence similarity with known plant or animal ion channels.

Several features are thought to be important in Mid1p function (Maruoka et al., 2002; Tada et al., 2003) (Fig. 3). These include a carboxy-terminal region containing three possible functional motifs, and a cysteine-rich region at the carboxy end of the protein. The hydrophobic regions of Mid1p, which were partially similar to those in known ion channels (Tada et al., 2003), were found to be partially conserved in the *N. crassa* and *M. grisea* homologs. The carboxy-terminal region of the protein has previously been postulated to be a regulatory region for the Mid1p channel (Iida et al., 1994). Every cysteine residue within the cysteine-rich region previously defined in Mid1p (Maruoka et al., 2002) was conserved in all three fungal homologs. This cysteine-rich region is essential (Maruoka et al., 2002)

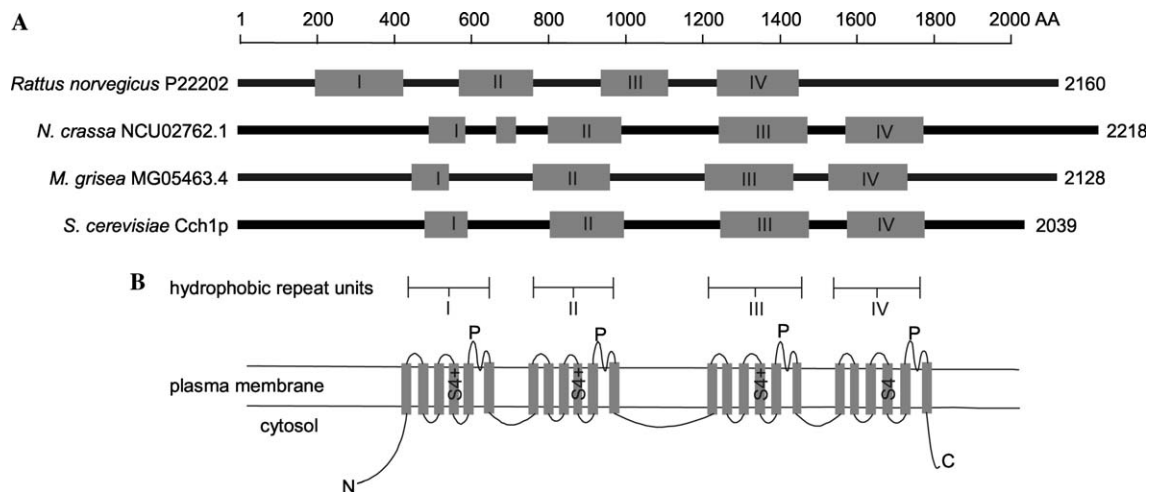


Fig. 2. Protein alignments and characteristics of the group I  $\text{Ca}^{2+}$ -permeable channels, *S. cerevisiae* Cch1p and its homologs in *N. crassa* and *M. grisea* (the  $\alpha 1$ , catalytic subunit of the voltage-gated  $\text{Ca}^{2+}$ -permeable channel of *Rattus norvegicus* is shown for reference). (A) Protein alignments: grey boxes indicate regions homologous to the PFAM00520 domain which is involved in ion transport. (B) Schematic representation of transmembrane regions in L-type  $\text{Ca}^{2+}$ -permeable channels (adapted from Paidhungat and Garrett, 1997): grey boxes indicate transmembrane regions. The four hydrophobic repeats are marked I to IV. Domains P and S4 are thought to play key roles in defining channel specificity and voltage dependence, respectively (Paidhungat and Garrett, 1997). Plus signs on the S4 domains of repeats I, II, and III indicate the positively charged amino acids.

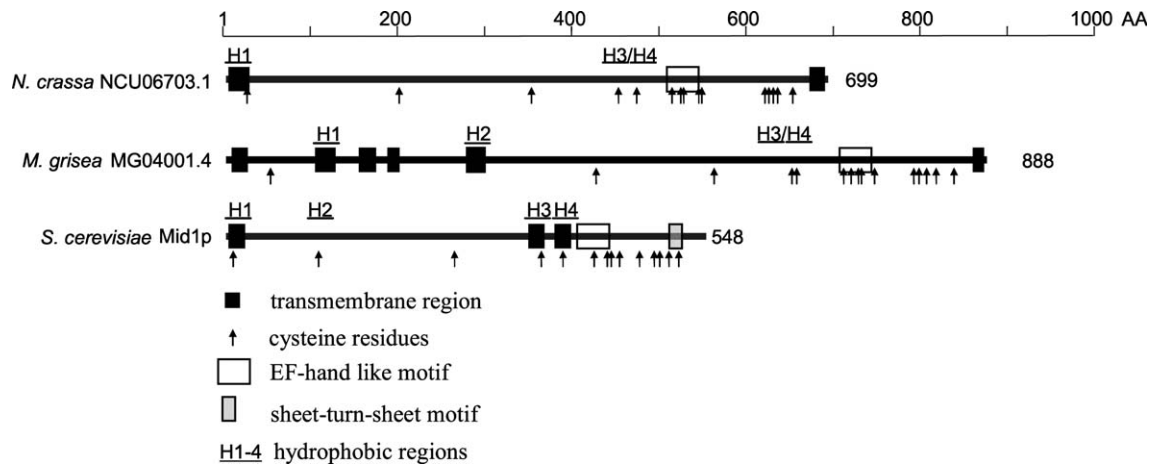


Fig. 3. Protein alignments and characteristics of the group II  $\text{Ca}^{2+}$ -permeable channels, *S. cerevisiae* Mid1p, and its homologs in *N. crassa* and *M. grisea*.

and contains a putative casein kinase 2 phosphorylation motif which is absent from the filamentous fungal homologs examined and was found to be non-essential for Mid1p function (Maruoka et al., 2002). An EF-hand like structure, also in this region, has been shown to be essential for Mid1p function (Maruoka et al., 2002) and was well conserved between the three fungi (Fig. 3; Fig. S3a at <http://www.fungalcell.org/fdf/>). There is conflicting data on the importance of a sheet-turn-sheet motif in the carboxy end of *S. cerevisiae* Mid1p (Maruoka et al., 2002). However, our sequence analysis suggests that it is not important in *N. crassa* and *M. grisea* as it was not conserved between budding yeast and filamentous fungi (Fig. S3b at <http://www.fungalcell.org/fdf/>).

Group III  $\text{Ca}^{2+}$ -permeable channels included the yeast Yvc1p protein. Yvc1p is a voltage-dependent  $\text{Ca}^{2+}$ -activated  $\text{Ca}^{2+}$ -permeable channel located in the

budding yeast vacuolar membrane. The filamentous fungal homologs of Yvc1p (*N. crassa* NCU07605.1 and *M. grisea* MG09828.4) were very similar to the yeast protein ( $E = 9\text{e}-90$  and  $E = 1\text{e}-114$ , respectively) (Fig. 4). NCU07605.1 was  $\sim 50\%$  larger than MG09828.4 and Yvc1p. However, the first 50% (650 residues) of NCU07605.1 has no homology to known proteins and possibly represents a sequencing or annotation error but this will have to be verified. Yvc1p, and its filamentous fungal homologs, have significant homology to the transient receptor potential (TRP) family of ion channels (Vennekens et al., 2002). Hydrophilicity and domain prediction indicate that Yvc1p (Palmer et al., 2001), MG09828.4, and the last 50% of NCU07605.1 contain between six and eight TM domains, six of which match the PFAM00520 ion transport protein domain (see Figs. 4A and B). This domain family contains  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  ion channels. The most significant

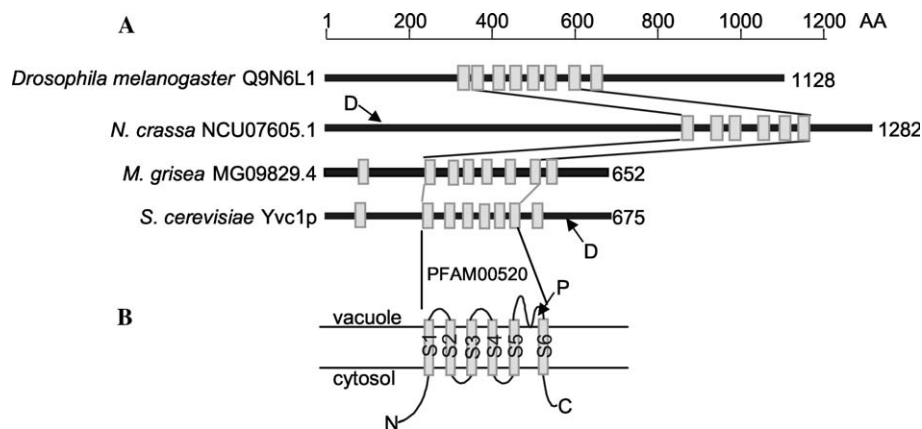


Fig. 4. Protein alignments and characteristics of the Class III  $\text{Ca}^{2+}$ -permeable channels in *N. crassa*, *M. grisea* and *S. cerevisiae* (the *Drosophila melanogaster* TRP  $\text{Ca}^{2+}$ -channel, Q9N6L1, is shown for reference). (A) Protein alignments: The PFAM00520 domain (indicated) contains ion channels that have 6 transmembrane helices (grey boxes) where the last two helices flank a loop which determines ion selectivity. (B) Schematic representation of transmembrane regions in a L-type  $\text{Ca}^{2+}$ -channel (Palmer et al., 2001). S1 to S6 are the PFAM00520 transmembrane regions (Xu et al., 2000) including a putative pore region (P). putative  $\text{Ca}^{2+}$ -binding DDDD motifs (D) are also shown (Palmer et al., 2001).

homology to other TRP channels was found in the predicted sixth TM domain, S6 (Fig. S4a at <http://www.fungalcell.org/fdf/>), which forms part of the ion conduction pathway and is intimately associated with deactivation gating in cation channels (Palmer et al., 2001). Within the region matching PFAM00529, all six TM domains were highly conserved in the proteins examined, although NCU07605.1 was missing large parts of TM domain 4 and 5 (Fig. S4 a at <http://www.fungalcell.org/fdf/>). The C-terminal portion of Yvc1p contains a DDDD motif (Fig. 4) that may be Ca<sup>2+</sup>-regulated similar to the Ca<sup>2+</sup>-binding bowl in Ca<sup>2+</sup>-activated K<sup>+</sup>-channels (Palmer et al., 2001; Schreiber and Salkoff, 1997). This motif is present in the N-terminal region of NCU07605.1 but is absent from MG09828.4. The significance of this difference needs to be analysed experimentally.

*Saccharomyces cerevisiae* Cch1p, Mid1p, and Pmc1p (a Ca<sup>2+</sup>-ATPase—see below) all appear to function within a single pathway that promotes the acquisition and concentration of Ca<sup>2+</sup> into secretory organelles. This pathway closely parallels the capacitative Ca<sup>2+</sup> entry pathway of animal cells (Locke et al., 2000). Mid1p has also been shown to be necessary for mating (Iida et al., 1994), and based on electrophysiological and biophysical studies, it has been suggested that Mid1p might function in sensing membrane stretch and generating Ca<sup>2+</sup> signals during mating (Kanzaki et al., 1999).

*Saccharomyces cerevisiae* Yvc1p mediates Ca<sup>2+</sup>-release in response to hyperosmotic shock and thus seems to be part of the sensory mechanism allowing yeast cells to adapt to hyperosmotic stress in the environment (Denis and Cyert, 2002).

### 3.5. Ca<sup>2+</sup>-pumps

Calcium-ATPases hydrolyze ATP to drive the active transport of Ca<sup>2+</sup> across biological membranes. They reduce [Ca<sup>2+</sup>]<sub>c</sub> by pumping Ca<sup>2+</sup> into internal stores, or across the plasma membrane and out of the cell (Moller et al., 1996). Ca<sup>2+</sup>-ATPases fall into the superfamily of P-type (or E1–E2 type) ATPases. Although there are large differences in primary structure and low overall similarity within the P-type ATPase family, eight conserved regions (A–H) have been identified (Axelsen and Palmgren, 1998, 2001). These enzymes typically encode a protein of approximately 100 kDa with 10 transmembrane helices. A large hydrophilic region following the fourth helix contains the site of ATP hydrolysis.

In animal cells the SERCA type Ca<sup>2+</sup>-ATPase, so named because it is found in the sarcoplasmic/endoplasmic reticulum, plays a major role in regulating [Ca<sup>2+</sup>]<sub>c</sub> levels. *Saccharomyces cerevisiae* does not have a SERCA Ca<sup>2+</sup>-ATPase, and several investigators have generalized this observation to report that all fungi lack the enzyme (Axelsen and Palmgren, 1998; Cronin et al.,

2002). As shown in Fig. 6A and Table 3 the Pmc1p enzyme of *S. cerevisiae* has high sequence similarity to the SERCA Ca<sup>2+</sup>-ATPases, but it belongs to a different subfamily of proteins with homologs in both *N. crassa* and *M. grisea* (described below). The recent sequencing projects, however, have shown that filamentous fungi have Ca<sup>2+</sup>-ATPases that are significantly more similar to the SERCA type enzyme: NCA-1 in *N. crassa* and MG045504 in *M. grisea*. All the amino acids involved in Ca<sup>2+</sup>-binding in SERCA were conserved in *N. crassa* NCA-1, which also showed an ER retention signal (KKKDL) in the carboxy terminus (Fig. 6A; Benito et al., 2000). This motif was not present in the *M. grisea* P-type ATPases analysed. The function of the SERCA-type enzyme has not been explored in filamentous fungi.

In *S. cerevisiae* the only P-type ATPase that appears to have a major role in regulation of [Ca<sup>2+</sup>]<sub>c</sub> is the Pmc1p enzyme. This Ca<sup>2+</sup>-ATPase is found in the vacuolar membrane. It works in concert with another vacuolar membrane protein Vcx1p, a Ca<sup>2+</sup>/H<sup>+</sup> antiporter, to remove excess Ca<sup>2+</sup> from the cytosol (Denis and Cyert, 2002). Both *N. crassa* and *M. grisea* have a pair of highly similar proteins (NCA-2 and NCA-3; MG02487.4 and MG04890.4) that appear to be homologs of Pmc1p (Fig. 5; Table 3). Preliminary data from *N. crassa* indicates that NCA-2 has the same function as Pmc1p in *S. cerevisiae*. *nca-3* is expressed and its expression is elevated in media with high levels of calcium, but mutants lacking *nca-3* have no observable phenotype (Abreu et al., 2003).

The *PMR1* gene of *S. cerevisiae* encodes an enzyme that can transport Ca<sup>2+</sup> or Mn<sup>2+</sup> (Mandal et al., 2003). It is not yet clear which ion is the major substrate in vivo. The primary function of the enzyme appears to be maintenance of the correct ion composition within the Golgi. Mutant strains that lack the enzyme are defective in the processing and maturation of enzymes that traverse the endomembrane network (Vashist et al., 2002). Filamentous fungi have one homolog of *PMR1*, also named *pmr1* in *N. crassa*, and MG09892 in *M. grisea*. Preliminary experiments in *N. crassa* indicate that *pmr1* has the same function as in *S. cerevisiae* (Abreu et al., 2003; Bowman and Abreu, 2004).

Although they have a high degree of sequence similarity to Ca<sup>2+</sup>-ATPases the proteins in P<sub>2D</sub>, P<sub>4</sub>, and P<sub>5</sub> families (Fig. 5) probably do not transport Ca<sup>2+</sup>. The *SPF1* gene of *S. cerevisiae* has a function similar to that of *PMR1* and works in concert with it. Spf1p is needed for proper protein glycosylation and protein folding in the endoplasmic reticulum (Vashist et al., 2002). We identified homologs of Spf1p in *N. crassa* (NCU04898.1) and *M. grisea* (MG06925.4), but no functional studies have been done. The filamentous fungi have several homologs to the *ENA2* and *ENA5* genes of *S. cerevisiae*, that appear to encode Na<sup>+</sup>-pumps (Benito et al., 2000; Rodriguez-Navarro et al., 1994) (Table 3, Fig. 5). The

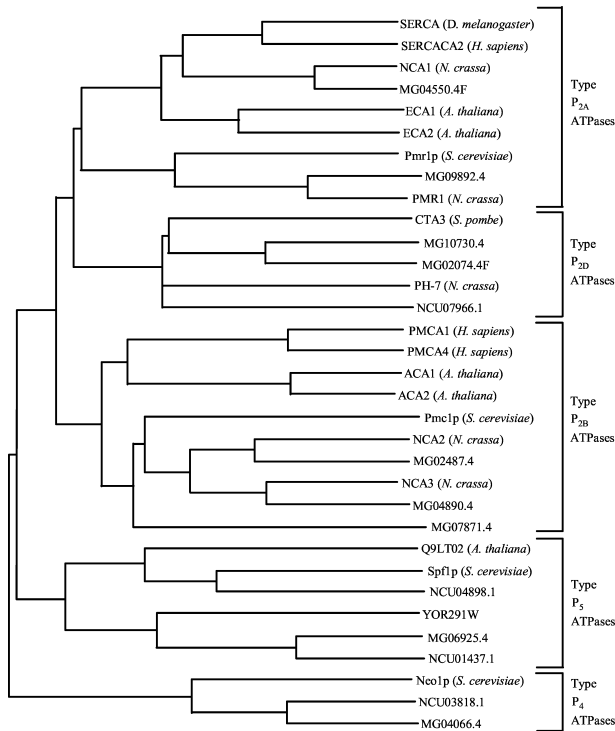


Fig. 5. Maximal parsimony dendrogram of P-type ATPases identified in *N. crassa*, *M. grisea*, and *S. cerevisiae*. Only the type-P<sub>2A</sub> and type-P<sub>2B</sub> ATPases are likely to be Ca<sup>2+</sup>-ATPases. Examples of P-type-ATPases identified from other organisms are: SERCA (type P<sub>2A</sub>) sequences *D. melanogaster* SERCA (NCBI #A36691), *H. sapiens* SERCA2 (NCBI #P16615) and *A. thaliana* ECA1 (NCBI #AAF36087) and ECA2 (NCBI #CAA10659); PMCA (type P<sub>2B</sub>) *H. sapiens* PMCA1 (NCBI #P20020) and PMCA4 (NCBI #P23634), and *A. thaliana* ACA1 (NCBI #CAA49559) and ACA2 (NCBI #T04721); type P<sub>2D</sub> sequences *S. pombe* CTA3 (NCBI #P22189); type 5 sequences *A. thaliana* (NCBI #Q9LT02). Neither rigorous calculation of evolutionary distances nor phylogenetic relationship can be inferred with confidence from this tree.

*CTA3* gene of *S. pombe* was originally thought to encode a Ca<sup>2+</sup>-ATPase, but phylogenetically it groups with Na<sup>+</sup>-ATPases and a recent report suggests it may be a K<sup>+</sup>-ATPase (Benito et al., 2002). Both *N. crassa* and *M. grisea* appear to have homologs of *CTA3* so it will be important to determine if these proteins have a role in K<sup>+</sup> transport. The *NEO1* gene of *S. cerevisiae* may encode a “flippase,” an enzyme that moves phospholipid molecules from one half of the lipid bilayer to the other (Hua and Graham, 2003). A single homolog is present in both *N. crassa* (NCU03818.1) and *M. grisea* (MG04066.4).

Thus our analysis revealed nine Ca<sup>2+</sup>- or cation-ATPases in *N. crassa* (four of which were novel) and 12 in *M. grisea* (all novel) (Table 3). Three of the *M. grisea* Ca<sup>2+</sup>/cation-ATPases did not correspond to hypothetical proteins in the *M. grisea* database and were therefore not analysed in detail during this study. In budding yeast, our analysis identified only five Ca<sup>2+</sup>/cation-ATPases of which one, YOR291W, was of unknown

function but had been previously described (Catty et al., 1997). Eleven other ATPases have been identified in *S. cerevisiae*, although none of these have been classified as Ca<sup>2+</sup>-ATPases (Catty et al., 1997). Five of the *N. crassa* P-type ATPases had been previously discovered (Benito et al., 2000) and found to be distributed in all branches of type P<sub>2</sub> ATPases except the branch of animal Na<sup>+</sup>/K<sup>+</sup>-ATPases (P<sub>2C</sub>) (Benito et al., 2000). The novel Ca<sup>2+</sup>- and cation-ATPases discovered, were classified according to their alignment with known ATPases (Fig. 5 and Fig. S6 at <http://www.fungalcell.org/fdf/>).

In the *M. grisea* databases two proteins, MG04550.4 and MG02074.4, had only four TM regions and showed a complete absence of TM 4 and TM 4-6, respectively. These proteins were also very short, having only 588 and 221 residues, respectively, while the other P-type ATPases analysed ranged between 1094 and 2005 amino acids in length. Further analysis revealed that these proteins had been predicted incorrectly. The correct proteins were identified and entered into our database under the names MG04550.4F and MG02074.4F (F for full). As described above MG04550.4F is a SERCA-type ATPase which has no close homolog in *S. cerevisiae*. MG02074.4F is probably a Na<sup>+</sup>-ATPase, homologous to *Ena5p* in *S. cerevisiae* (<http://www.fungalcell.org/fdf/>).

### 3.6. Ca<sup>2+</sup>-exchangers

Like P-type ATPases, Ca<sup>2+</sup>-exchangers serve to reduce the concentration of [Ca<sup>2+</sup>]<sub>c</sub> to resting level and to transport Ca<sup>2+</sup> into Ca<sup>2+</sup>-storage organelles. This is achieved by the exchange of positive ions across membranes. In plants, Ca<sup>2+</sup>/H<sup>+</sup>-antiporters are the most common form of Ca<sup>2+</sup>-exchanger and usually require a Ca<sup>2+</sup>/H<sup>+</sup> stoichiometry of at least three (Blackford et al., 1990). Several of the 11 putative Ca<sup>2+</sup>-exchangers (CAXs) in *A. thaliana* (Mäser et al., 2001) have been localised in the vacuolar membrane (Mäser et al., 2001; Sanders et al., 2002). In animals Ca<sup>2+</sup>/Na<sup>+</sup>-antiporters are the primary Ca<sup>2+</sup>-exchangers present. *Saccharomyces cerevisiae* has only one previously identified Ca<sup>2+</sup>/H<sup>+</sup>-exchanger (Vcx1p/Hum1p) and it is localised in the vacuolar membrane (Cunningham and Fink, 1994; Miseta et al., 1999; Pozos et al., 1996). The *A. thaliana* Ca<sup>2+</sup>/H<sup>+</sup>-antiporter CAX1 is (and CAX3 and VCAX1 are probably) regulated at the posttranslational level by a mechanism of N-terminal auto-inhibition (Pittman and Hirschi, 2001; Pittman et al., 2002a,b). However, apart from CAX1, very little is known about the post-translational regulation mechanisms of Ca<sup>2+</sup>/H<sup>+</sup>-antiporters from any species.

Our analysis identified six Ca<sup>2+</sup>/H<sup>+</sup>-exchangers and two Ca<sup>2+</sup>/Na<sup>+</sup>-exchangers in *N. crassa*, and five Ca<sup>2+</sup>/H<sup>+</sup>-exchangers and one Ca<sup>2+</sup>/Na<sup>+</sup>-exchanger in *M. grisea* (Table 3; Figs. 7–10). All of the *M. grisea* genes appeared to have a corresponding homolog in *N. crassa*.

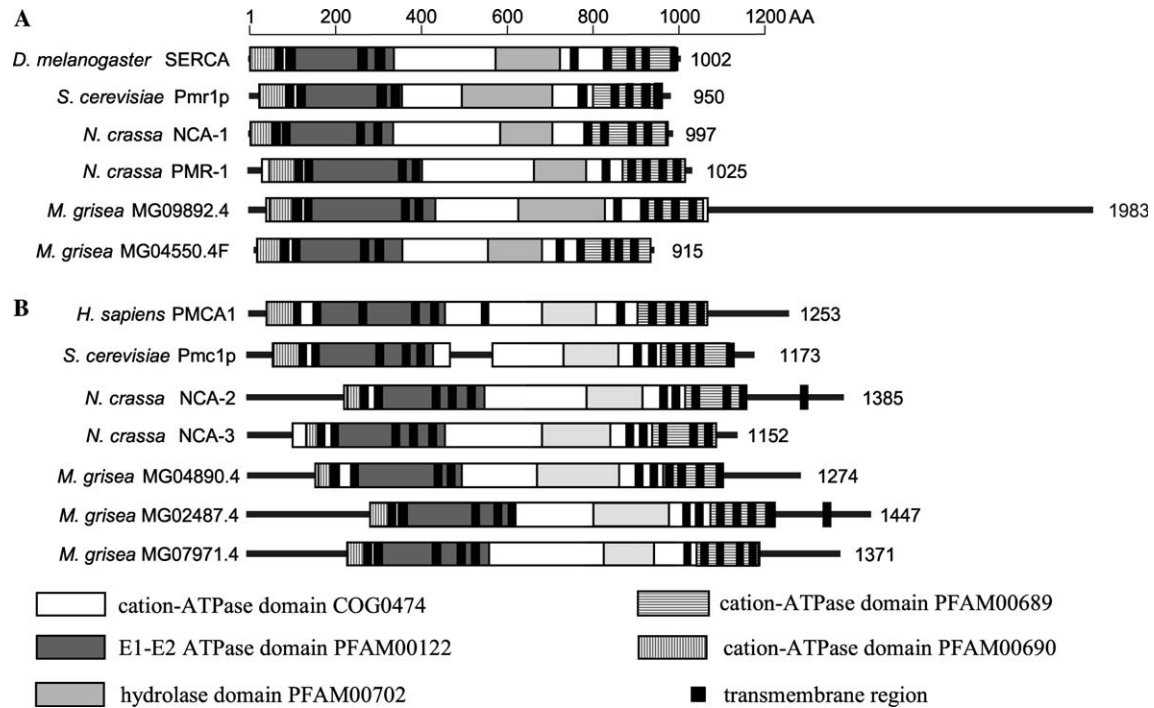


Fig. 6. Protein alignments and characteristics of  $\text{Ca}^{2+}$ -ATPases in *S. cerevisiae*, *N. crassa*, and *M. grisea*. (A) Type  $\text{P}_{2A}$  ATPases (of *S. cerevisiae*, *N. crassa*, and *M. grisea*. The  $\text{Ca}^{2+}$ -ATPase SERCA [NCBI #A36691] of *Drosophila melanogaster* is shown for reference. (B) Type  $\text{P}_{2B}$  ATPases from *N. crassa*, *M. grisea*, and *S. cerevisiae*. *H. sapiens* type  $\text{P}_{2B}$   $\text{Ca}^{2+}$ -ATPase PMCA1 (NCBI #P20020) is shown for reference.

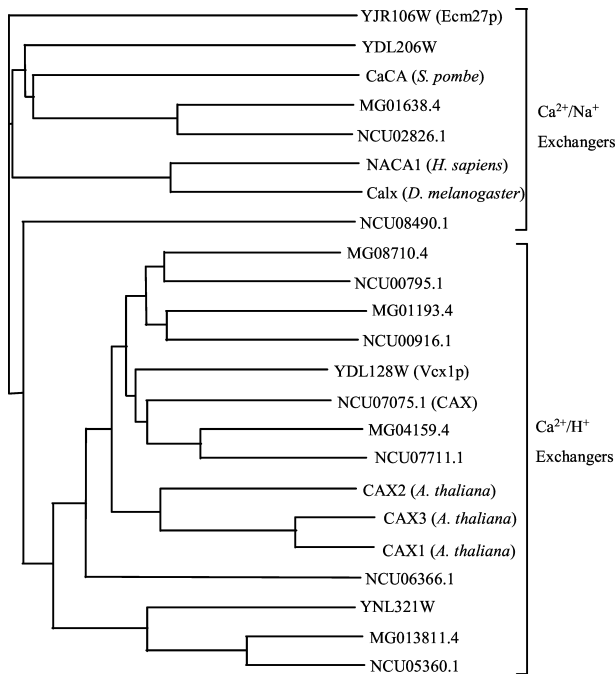


Fig. 7. A maximal parsimony dendrogram of  $\text{Ca}^{2+}$ -transporters identified in *N. crassa*, *M. grisea*, and *S. cerevisiae*. Examples of  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchanger sequences from other organisms are: *S. pombe* CaCa (NCBI #NP\_593332), *H. sapiens* NACA1 (NCBI #P32418) and *D. melanogaster* Calx (NCBI #NP\_732577); Examples of  $\text{Ca}^{2+}/\text{H}^{+}$ -exchanger sequences from other organisms are: *A. thaliana* CAX2 (NCBI #AAM19859), CAX3 (NCBI #At3g51860), and CAX1 (NCBI #AAL66749). Neither rigorous calculation of evolutionary distances nor phylogenetic relationship can be inferred with confidence from this tree.

But *N. crassa* had two additional genes (NCU06366.1 and NCU08490.1) that were significantly different from all the others (Fig. 7). Of all these proteins, only one, *N. crassa* CAX, has been characterized. Like the *Vcx1* gene of *S. cerevisiae* it encodes a  $\text{Ca}^{2+}/\text{H}^{+}$ -exchanger in the vacuolar membrane that plays a major role in regulating the concentration of  $[\text{Ca}^{2+}]_c$  (Margolles-Clark et al., 1999). The gene that we found in *M. grisea* most similar to *cax* was not predicted by the automated analysis done by the Whitehead Institute. This protein was therefore entered into our database as 2.175\_3011-3697 where the numbers indicate contig\_start-stop. Further investigation showed that this sequence is not complete and that only the last part of the protein (from residues 221 to 432) is present. The beginning of the protein (residues 1–137 as determined by identity to its *N. crassa* homolog NCU07075.1) appears to reside on contig 2.176. The middle of this protein (residues 137–221) could not be found and does not appear to be present in the current *M. grisea* genome database, although it is likely to exist in the genome itself. None of the fungal  $\text{Ca}^{2+}$ -exchangers identified contained regions homologs to the N-terminal regulatory domain found in *A. thaliana* CAX1 (Pittman et al., 2002b). All the proteins analysed had between nine and 14 predicted TM domains, in good agreement with known  $\text{Ca}^{2+}$ -transporters (Pittman et al., 2002a,b). In yeast two  $\text{Ca}^{2+}/\text{H}^{+}$ -exchangers (one novel) and two  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers (both novel) were identified.

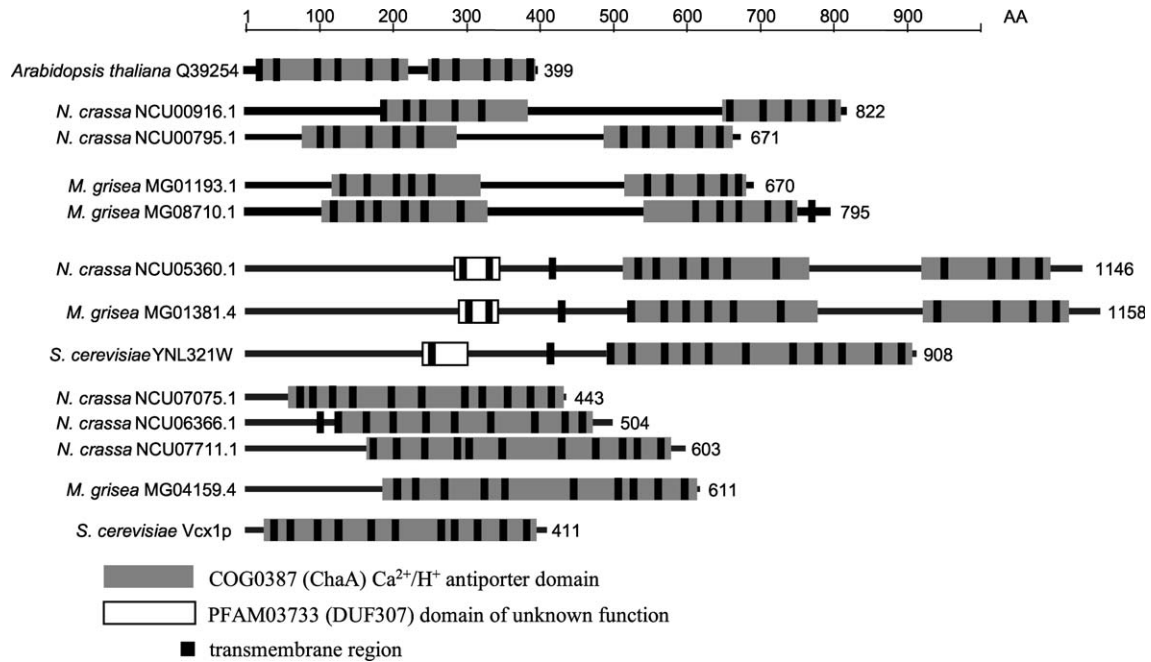


Fig. 8. Protein alignments and characteristics of  $\text{Ca}^{2+}/\text{H}^{+}$ -exchangers in *N. crassa*, *M. grisea*, and *S. cerevisiae*. Domain COG038 (ChaA) is characteristic of  $\text{Ca}^{2+}/\text{H}^{+}$ -exchanger membrane proteins. Domain PFAM03733 (DUF307) is a domain of unknown function occurring in some putative membrane proteins.

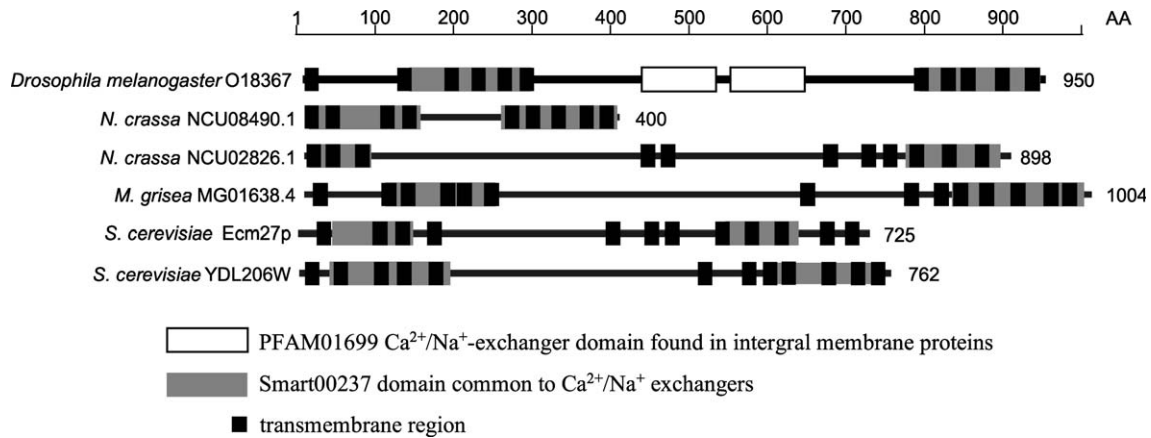


Fig. 9. Protein alignments and characteristics  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers in *N. crassa*, *M. grisea*, and *S. cerevisiae*. Domain PFAM01699 is characteristic of a family of  $\text{Ca}^{2+}/\text{Na}^{+}$  membrane proteins. Domain smart00237 is the Calx  $\beta$  domain found in  $\text{Na}^{+}/\text{Ca}^{2+}$ -exchangers and integrin- $\beta$ 4.

A maximal parsimony dendrogram showing  $\text{Ca}^{2+}$ -transporter grouping constructed from the proteins identified, along with examples of  $\text{Ca}^{2+}/\text{H}^{+}$  and  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers from other organisms had two main branches (Fig. 7). With the exception of NCU08490.1,  $\text{Ca}^{2+}/\text{H}^{+}$ -exchangers (predicted on the basis of conserved domains and homology to known proteins (Figs. S7–S9 at <http://www.fungalcell.org/fdf/>) were found in one branch and  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers in the other). Although NCU08490.1 was found in the  $\text{Ca}^{2+}/\text{H}^{+}$ -exchanger branch of the tree, it was thought to be a  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchanger as it shows homology to the ECM27  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchanger domain (CDD #10401) (Fig. S8 at

<http://www.fungalcell.org/fdf/>). Homology between predicted  $\text{Ca}^{2+}/\text{H}^{+}$ -exchanger proteins and the ChaA (CDD #COG0387) consensus  $\text{Ca}^{2+}/\text{H}^{+}$ -antiporter domain was greater (32–42% identities) than between predicted  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers and the ECM27 (CDD #10401) consensus  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchanger domain (26–31% identities) (Figs. S8 and S9 at <http://www.fungalcell.org/fdf/>).

### 3.7. Other important $\text{Ca}^{2+}$ -signaling proteins found

Many other important  $\text{Ca}^{2+}$ -signaling proteins were discovered during this analysis. Table 4 summarizes the

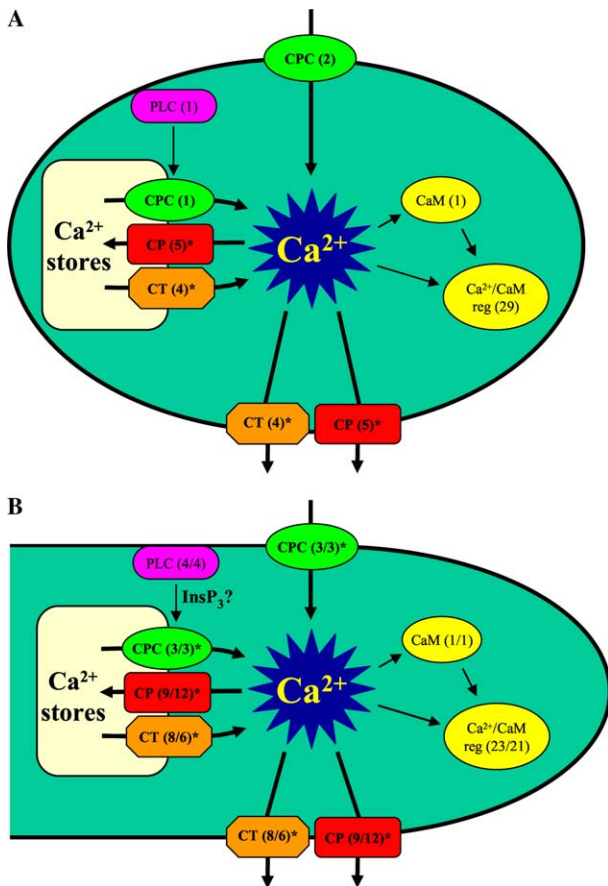


Fig. 10. Overview of major intracellular  $\text{Ca}^{2+}$ -signaling proteins in (A) *S. cerevisiae* and (B) *N. crassa* and *M. grisea*. Asterisk, location in plasma membrane and/or organelle membranes not determined; CPC,  $\text{Ca}^{2+}$ -permeable channel; CP,  $\text{Ca}^{2+}$ -ATPase; CT,  $\text{Ca}^{2+}/\text{H}^{+}$ -transporter; PLC, phospholipase C; CaM, calmodulin;  $\text{Ca}^{2+}/\text{CaM}$  reg,  $\text{Ca}^{2+}$  and/or calmodulin regulated. Numbers in brackets are number of proteins in that class. Where two numbers are given separated by a backslash, the first number refers to *N. crassa* and the second number to *M. grisea*.

phospholipase C, calmodulin, calcineurin,  $\text{Ca}^{2+}$  and/or calmodulin dependent protein kinase, calnexin, calpactin I heavy chain and calsequestrin proteins found. The remaining  $\text{Ca}^{2+}$ -signaling proteins not described here represent a diverse range of  $\text{Ca}^{2+}$  and/or calmodulin binding proteins that play important roles in transducing the  $\text{Ca}^{2+}$ -signals resulting from the activity of the proteins described in detail in this paper. These proteins are detailed in the database of  $\text{Ca}^{2+}$ -signaling proteins at <http://www.fungalcell.org/fdf/>.

### 3.8. Important $\text{Ca}^{2+}$ -signaling proteins not found

A surprising difference between  $\text{Ca}^{2+}$ -signaling in the fungi examined as compared with plants and animals was also revealed by this analysis. An important aspect of  $\text{Ca}^{2+}$ -signaling in plant and animal cells involves  $\text{Ca}^{2+}$  release from internal stores. This is commonly

mediated by the second messengers inositol 1,4,5 trisphosphate ( $\text{InsP}_3$ ) and cADP ribose, sphingolipids, NAADP or by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (Berridge et al., 2003).  $\text{InsP}_3$  is present within *N. crassa* hyphae (Lakin–Thomas, 1993) and physiological evidence, including  $\text{InsP}_3$ -activated  $\text{Ca}^{2+}$ -channel activity associated with isolated vacuolar compartments, supports a role in  $\text{Ca}^{2+}$ -signaling in filamentous fungi (Cornelius et al., 1989; Schultz et al., 1990; Silverman-Gavrilla and Lew, 2001, 2002, 2003). In *S. cerevisiae*,  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  increases have recently been shown to occur (Tisi et al., 2004). Four novel PLC- $\delta$  subtype proteins, which synthesize  $\text{InsP}_3$ , were identified in both *N. crassa* and *M. grisea* (Table 4), and one is found in *S. cerevisiae* (Yoko-o et al., 1993). In spite of this, none of the fungi analysed here possessed recognizable  $\text{InsP}_3$  receptors. In addition none of the following components of  $\text{Ca}^{2+}$  release mechanisms from plant and animal internal stores were found in the fungi analysed: (a) ADP ribosyl cyclase, which synthesises cADP ribose or NAADP; (b) ryanodine receptor proteins, which are key components of  $\text{Ca}^{2+}$ -release mechanisms in plant and animal cells; (c) sphingosine kinases, which catalyse the formation of sphingosine 1-phosphate; and (d) SCAMPER (a sphingolipid-activated protein that is a possible target for sphingolipids, Berridge et al., 2003 homologs).

Extracellular calcium-sensing receptor proteins have been identified in both animals (Brown et al., 1993) and plants (Han et al., 2003), but neither possessed homologs in *N. crassa*, *M. grisea* or *S. cerevisiae*.

## 4. Discussion

This analysis has identified many of the proteins likely to be involved in  $\text{Ca}^{2+}$ -signaling in two important filamentous fungi (*N. crassa* and *M. grisea*), as well as in the budding yeast, *S. cerevisiae* (see summary diagram in Fig. 10). These proteins include previously unknown  $\text{Ca}^{2+}$ -permeable channels,  $\text{Ca}^{2+}$ -ATPases,  $\text{Ca}^{2+}/\text{H}^{+}$ -exchangers,  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchangers, phospholipase C proteins and  $\text{Ca}^{2+}$  and/or CaM binding proteins. A web-based resource (<http://www.fungalcell.org/fdf/>), containing detailed supplementary information regarding all of the proteins described in this analysis, has been made available.

Looking at  $\text{Ca}^{2+}$ -permeable channels,  $\text{Ca}^{2+}$ -pumps and transporters, PLC's, CaM and calcineurin alone, *N. crassa* and *M. grisea* have 35 and 37% more of these  $\text{Ca}^{2+}$ -signaling proteins than *S. cerevisiae*, respectively. These results highlight both the potential importance and likely greater complexity of  $\text{Ca}^{2+}$ -signaling in filamentous fungi in comparison to budding yeast. Given the greater range of external signals that filamentous fungi have to respond to in their natural habitats, and

also their greater complexity, in comparison with budding yeast, this finding is not surprising.

More Ca<sup>2+</sup>- and/or CaM-binding proteins have been identified in *S. cerevisiae* than in *N. crassa* or *M. grisea*. One of the limitations of a sequence-based comparative analysis is the difficulty in identifying proteins with defined functions that lack sufficient sequence similarity to known counterparts in other organisms. This probably explains why we were not able to identify a greater number of Ca<sup>2+</sup> and/or CaM-regulated proteins in the filamentous fungal genomes we probed. This will require functional analyses of filamentous fungal genes, as has been performed much more extensively in budding yeast.

Notable differences between the Ca<sup>2+</sup>-signaling machinery of fungi, animals, and plants were observed (see Table 47 in Borkovich et al., 2004 and Table S1 at <http://www.fungalcell.org/fdf/>). These included: (1) good homologs of the Class I (Cch1p-like) Ca<sup>2+</sup>-permeable channels are present in animals but not plants; (2) homologs of the Class II (Mid1p-like) Ca<sup>2+</sup>-permeable channels are not found in animals or plants; (3) fungi possess both Ca<sup>2+</sup>/H<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup>-exchangers (plants only possess Ca<sup>2+</sup>/H<sup>+</sup>-exchangers, Sanders et al., 2002; animals primarily use Ca<sup>2+</sup>/Na<sup>+</sup>-exchangers and only occasionally use a Ca<sup>2+</sup>/H<sup>+</sup>-exchanger in mitochondrial Ca<sup>2+</sup>-homeostasis (Berridge et al., 2003).

The fungi lacked recognizable InsP<sub>3</sub> receptors, ADP ribosyl cyclase, ryanodine receptor proteins, sphingosine kinases or SCA<sub>MPER</sub> homologs, suggesting that fungi might possess intracellular Ca<sup>2+</sup>-release mechanisms that have yet to be identified. These findings indicate that these components of the fungal Ca<sup>2+</sup>-signaling machinery might provide novel antifungal targets for drug discovery (Borkovich et al., 2004; Nelson et al., 2004).

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