INTRODUCTION

The Ca\(^{2+}\) signalling network comprises a number of channels, transporters, sensors and effectors, and it regulates a wide spectrum of cellular processes (Cunningham, 2011; Kellermayer et al., 2003). In the unstimulated cell, the cytosolic Ca\(^{2+}\) concentration is maintained at the resting level between 50 and 200 nM, but its concentrations in the extracellular environment or the intracellular storage organelles are thousands of times higher than that in the extracellular environment or the intracellular storage organelles. The Ca\(^{2+}\) ions will be finally pumped out of the cytoplasm to the extracellular environment or the intracellular storage organelles through the Ca\(^{2+}\)-extruding ATPases.

Putative PmrA and PmcA are important for normal growth, morphogenesis and cell wall integrity, but not for viability in *Aspergillus nidulans*

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P-type Ca\(^{2+}\)-transporting ATPases are Ca\(^{2+}\) pumps, extruding cytosolic Ca\(^{2+}\) to the extracellular environment or the intracellular Ca\(^{2+}\) store lumens. In budding yeast, Pmr1 (plasma membrane ATPase related), and Pmc1 (plasma membrane calcium-ATPase) cannot be deleted simultaneously for it to survive in standard medium. Here, we deleted two putative Ca\(^{2+}\) pumps, designated AnPmrA and AnPmcA, from *Aspergillus nidulans*, and obtained the mutants ΔanpmrA and ΔanpmcA, respectively. Then, using ΔanpmrA as the starting strain, the promoter of its anpmcA was replaced with the alcA promoter to secure the mutant ΔanpmrAalcApmcA or its anpmcA was deleted completely to produce the mutant ΔanpmrAΔpmcA. Different from the case in *Saccharomyces cerevisiae*, double deletion of anpmrA and anpmcA was not lethal in *A. nidulans*. In addition, deletion of anpmrA and/or anpmcA had produced growth defects, although overexpression of AnPmc1 in ΔanpmrAalcApmcA could not restore the growth defects that resulted from the loss of AnPmrA. Moreover, we found AnPmrA was indispensable for maintenance of normal morphogenesis, especially in low-Ca\(^{2+}\)/Mn\(^{2+}\) environments. Thus, our findings suggest AnPmrA and AnPmcA might play important roles in growth, morphogenesis and cell wall integrity in *A. nidulans* in a different way from that in yeasts.

Abbreviations: PMCA, plasma membrane Ca\(^{2+}\)-ATPase; q, quantitative; RT, real-time; SERCA, sacro/endoplasmic reticulum Ca\(^{2+}\)-ATPase; SPCA, secretory pathway Ca\(^{2+}\)-ATPase.

One supplementary table is available with the online Supplementary Material.
(Bates et al., 2005). In S. cerevisiae, Pmr1 and Pmc1 cannot be deleted simultaneously for it to survive in standard medium (Cunningham & Fink, 1994). Here, we deleted the putative PmrA and/or PmcA (AnPmrA and/or AnPmcA) in Aspergillus nidulans and analysed the corresponding phenotypes. It was found that deletion of *anpmrA* and/or *anpmcA* produced defects in growth, morphogenesis and cell wall integrity. However, different from the case in *S. cerevisiae*, double deletion of *anpmrA* and *anpmcA* was not lethal in *A. nidulans*.

**METHODS**

**Strains and media.** The parental *A. nidulans* TN02A7 strain was a gift from B. R. Oakley (Ohio State University, Columbus, OH). Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting. Conidia were collected with physiological saline supplemented with 0.01% Tween 80 and filtered through eight layers of miracloth. Spore density was determined by haemocytometry and viable counting.

**Creation of *A. nidulans* mutants.** To delete the target genes, the fragments containing the selectable marker sandwiched by their respective upstream and downstream segments were created via fusion PCR as described previously (Zarrin et al., 2005). The *pyrG* selectable marker was first amplified from plasmid pXDRFP4 (Fungal Genetics Stock Center) with the primers 7/8; the upstream and downstream fragments of *anpmrA* (*anpmcA*) were amplified from the genomic DNA with the primers: R1/R3 (C1/C3) or R4/R6 (C4/C6). Then, the above three fragments were fused together with the primers R2/R5 (or C2/C5). To perform promoter replacement, partial *anpmcA* (−14 to +1081) was amplified with the primers 9/10 and cloned to plasmid pQa (gift from Professor H. M. Park, Chungnam National University, Daejeon, South Korea) prior to being transformed to Δ*anpmrA* protoplasts. The complete deletion of *anpmcA* in Δ*anpmrA* was carried out as above, except for using *A. fumigatus* *pyroA* rather than the *pyrG* selectable marker. The primers were: 11/12 for the *pyroA* expression cassette, D1/D3 for the upstream segment of *anpmcA*, D4/D6 for the downstream segment of *anpmcA* and D2/D5 for fusion PCR. All the fragments were verified by sequencing.

**Chemical analysis of the cell wall.** Conidia were inoculated into 100 ml liquid MMPDRU at a concentration of 10⁶ ml⁻¹ and shaken (250 r.p.m.) at 37 °C for 36 h. Three aliquots of 10 μl lyophilized mycelia were used as independent samples for analysis. Each sample was boiled for 5 min in 2 ml 50 mM Tris/HCl buffer containing 2% SDS, 100 mM Na-EDTA, 40 mM β-mercaptoethanol and 1 mM PMSF (Elorza et al., 1985; Hearn & Sietsma, 1994) to remove unbound cell wall proteins. After treatment with 3% NaOH at 75 °C for 1 h, proteins and alkali-soluble glucans were released. Alkali-insoluble glucans and chitin were digested in 2 ml 96% formic acid at 100 °C for 4 h, which was then evaporated by lyophilization. The residues were dissolved in 2 ml distilled water. Proteins were quantified by using the Lowry protein assay (Lowry et al., 1951). Glucans and chitin were estimated by determining the amount of glucose and N-acetylglucosamine released after digestion, respectively. Glucose was measured by using the phenol sulfuric acid method (Dubois et al., 1951). N-acetylglucosamine was measured by using the method described by Lee et al. (2005). The experiment was repeated twice.

**Quantitative real-time (qRT)-PCR.** Total RNAs were isolated using TRIzol (Invitrogen). cDNAs were synthesized utilizing a HiScript Q Select RT SuperMix for qPCR kit (Vazyme) according to the manufacturer’s instructions. qRT-PCR was carried out in triplicates and repeated on two independent replicates in a StepOne Real-Time PCR System (Life Technologies) with SYBR Green Master Mix (Vazyme) according to the manufacturer’s instructions. The relative expression was normalized to the tubulin gene (Colabardini et al., 2010; Trevisan et al., 2011) and calculated using the ΔΔCt method (Alam et al., 2012). The primers used are listed in Table S1.

**GenBank accession numbers.** PMCA1, XP_005268976; PMCA2, NP_001001331; PMCA3, XP_005274746; PMCA4, NP_001675; SERCA1, NP_004311; SERCA2, XP_005253945; SERCA3, XP_005256713; SPCA1, XP_005247411; SPCA2, NP_001273456; S. cerevisiae Pmr1, NP_011348; S. cerevisiae Pmc1, NP_011509; AnPmrA, XP_680733; AnPmcA, XP_658793; chsB, D21269.1; csmA, AB000125.3; fksA, U31227.1; ageB, BN001306.1; tubulin, M17520.1.

**Statistics.** Data were given as mean ± sd. Statistical significance was estimated with SPSS 13.0 using either Student’s *t*-test or one-way ANOVA. *P* values < 0.05 were considered statistically significant.

**RESULTS**

**In silico analysis of AnPmrA and AnPmcA from *A. nidulans***

By searching the National Center for Biotechnology Information website in the genomic database of *A. nidulans*, we identified the homologues of *S. cerevisiae* Pmr1 (GenBank accession number NP_011348) and Pmc1 (GenBank accession number NP_011509), designated AnPmrA (GenBank accession number XP_680733) and AnPmcA (GenBank accession number XP_658793), respectively. Both of them have the cation ATPase N-terminal domain (pfam00689) domains conserved in P-type Ca²⁺-translocating ATPases. A phylogenetic tree revealed on the basis of full-length sequences split into two primary clades, corresponding to the PMCA orthologues and the Golgi apparatus/endoplasmic reticulum Ca²⁺-ATPase homologues, including SERCA and SPCA. As expected, AnPmrA was on the branch related to human SPCA ATPases, whereas AnPmcA had a relatively closer relationship with human PMCA ATPases (Fig. 1).

**Creation of mutants**

Using TN02A7 as the recipient strain, *anpmrA* or *anpmcA* was replaced completely with the *pyrG* selectable marker to
obtain the single-deletion mutants. After transformation, the genomic DNA from the colonies that emerged on the selective media was isolated as the template to perform PCR and a representative result is demonstrated in Fig. 2(a, b). As expected, the pyrG selectable marker and the upstream pyrG fragment were amplified from these colonies, but not from the TN02A7 control, meaning that the upstream pyrG mutants were obtained successfully just as expected, and deleted to yield the mutants. As shown in Fig. 2(c, d), the fragment were amplified from these colonies. In addition, AnPmcA overexpression could not make up for the loss of AnPmrA in A. nidulans.

### Double deletion of AnPmrA and AnPmcA is viable

In _S. cerevisiae_, _pmr1_ and _pmc1_ cannot be deleted simultaneously for it to survive in standard medium (Cunningham & Fink, 1994). We assumed that this might also be the case in _A. nidulans_. Therefore, we constructed the conditional mutant _ΔanpmrAΔanpmcA_, in which _anpmrA_ was deleted completely, but the expression of _anpmcA_ could be affected by carbon sources via the _alcA_ promoter. It has been proven that the gene under the regulation of _alcA_ will be overexpressed in the presence of an inducer such as threonine or ethanol, or will be repressed in the presence of glucose (Panozzo _et al._, 1998). As shown in Fig. 3, _ΔanpmrAΔanpmcA_ could grow both on the inducible medium and on the repressive medium, indicating double deletion of _anpmrA_ and _anpmcA_ in _A. nidulans_ was not lethal. Consistent with this, using _ΔanpmrAΔanpmcA_, in which both _anpmrA_ and _anpmcA_ were deleted completely, further confirmed that simultaneous deletion of _anpmrA_ and _anpmcA_ was viable in _A. nidulans_.

### AnPmrA and AnPmcA are involved in normal growth and morphogenesis maintenance

Conidia (10⁴) from these strains were dot inoculated on the surface of solid YUU media to compare their mean growth rates. The mutants demonstrated obviously reduced growth rates compared with TN02A7, corresponding to 15.42 ± 0.42, 13 ± 0.27, 14.42 ± 0.32 and 11.92 ± 0.17 mm day⁻¹ in TN02A7, _ΔanpmrA_, _ΔanpmcA_ and _ΔanpmrAΔanpmcA_, respectively (P < 0.05) (Fig. 4a–d). To test if overexpression of AnPmcA could restore the growth defects that stemmed from the deletion of AnPmrA, we inoculated conidia from _ΔanpmrAΔanpmcA_ and TN02A7 to the induction media, and found that the growth of _ΔanpmrAΔanpmcA_ was not recovered to WT (Fig. 4e–h). The results demonstrated that _anpmrA_ and _anpmcA_ were indispensable for normal vegetative growth. In addition, AnPmcA overexpression could not make up for the loss of AnPmrA in _A. nidulans_.

We observed the morphogenesis of the mutants cultured in liquid YUU or MMPDRUU media, but did not find any obviously detectable difference between these mutants and WT (Fig. 5a–d). MMPDRUU is the minimal medium containing ~25 μM Mn²⁺ but no Ca²⁺, theoretically. Here, we prepared a medium containing all of the nutritional components of MMPDRUU except for Mn²⁺, and used this medium to culture TN02A7 and the mutants.

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**Fig. 1. In silico analysis of AnPmrA and AnPmcA in _A. nidulans_.** (a) Protein alignment was performed with CLUSTAL X 2, and the phylogenetic tree was recreated using MEGA 5.2 to examine the evolutionary relationships among homologues from human, _S. cerevisiae_ and _A. nidulans_. (b) The domain analysis was done at the website http://pfam.sanger.ac.uk/search/sequence.
(a) R1/R6 R1/8 pyrG R1/R6 R1/8 pyrG

TN02A7 ΔanpmrA

(b) C1/C6 C1/8 pyrG C1/C6 C1/8 pyrG

TN02A7 ΔanpmcA

(c) ΔanpmrA ΔanpmrA ΔalcA ΔpmcA

ΔanpmrA

(d) ΔanpmrA ΔpyroA ΔanpmcA

ΔanpmrA ΔpmcA
AnPmrA and AnPmcA simultaneously was not lethal. The conditional mutant ΔanpmrAalcApmcA could grow in both media, indicating double deletion of threonine and 10 ml glycerol ml⁻¹ could be restored into the WT status by 1 M sucrose, suggesting variations might take place in the cell wall of the mutants (Fig. 6a–f). After sequential alkali and acid treatment, hyphal cell wall proteins were separated, and the glucans and chitin were decomposed into glucose and N-acetylglucosamine for quantitative analysis. As demonstrated in Table 1, the proteins and alkali-soluble glucans in ΔanpmrA were 92% and 85% of WT, respectively. Meanwhile, the counterparts in ΔanpmcA and ΔanpmrAΔpmcA were approximately similar to WT (101 and 108% or 99 and 98% of WT, respectively). At the same time, the mass of chitin or alkali-insoluble glucans in ΔanpmrA, ΔanpmcA and ΔanpmrAΔpmcA was 128, 110 and 115% or 120, 112 and 136% of WT, respectively (all P<0.05).

Alkali-soluble glucans are composed primarily of α-glucans, whereas alkali-insoluble glucans contain 1,3-β-glucan as the main component (Futagami et al., 2011). In A. nidulans, at least 50% of 1,3-α-glucan was synthesized by the synthase AgsB, whilst fksA is the only gene that encodes 1,3-β-glucan synthase (Futagami et al., 2005). Thus, we analysed the mRNA expression levels of these genes and found that the expression levels of these genes are 126, 110 and 156% of WT, respectively. In addition, the mRNA levels of csmA were 157, 106 and 144% of WT, respectively. In TN02A7, the csmA mRNA levels were 92% and 85% of WT, respectively. In the mutants, the mRNA levels corresponding to fksA were 126, 110 and 156% of WT, respectively. In addition, the mRNA levels of chsB or csmA in these mutants were 157, 106 and 144% or 132, 103 and 126% of the control, respectively (Fig. 6g). These data demonstrated that the mutants remodelled their cell wall architectures probably through regulating the expression of genes responsible for cell wall synthesis.

DISCUSSION

There are at least seven homologues corresponding to Pmr1 and Pmc1 of S. cerevisiae, among which AnPmrA and AnPmcA show the highest homology. The position of AnPmrA and AnPmcA on the phylogenetic tree recreated together with the nine human Ca²⁺-ATPases was consistent with their evolutionary relationships (Fig. 1). In S. cerevisiae, continuous activation of the intracellular Ca²⁺ signalling pathway could result in upregulation of P-type ATPases.
Fig. 4. Growth characteristics of the mutants. (a–d) Conidia (10⁴) from TN02A7 and the mutants were dot inoculated on the surface of solid YUU media and maintained at 37 °C for 3 days. The mean growth rates of the mutants, especially Δanpma and ΔanpmaΔpmcA, were obviously less than that of the WT control (P<0.05). (e–h) Overexpression of AnPmcA induced by threonine could not recover the growth defects that stemmed from the loss of AnPmrA, both on the solid medium (e, f) and in the liquid medium cultured at 37 °C for 16 h (g, h). Bar, 20 μm.

Fig. 5. Morphogenesis of the mutants. (a–d) Conidia were inoculated to MMPDRUU at a density of 10⁵ ml⁻¹ and maintained at 37 °C for 16 h. Hyphal morphogenesis of the mutants resembled that of WT. (e–h) In MMPDRUU depleted of Mn²⁺, although Δanpma had similar morphology to WT, the morphogenesis of Δanpma and ΔanpmaPmcA were obviously abnormal. (i–l) When the conidia were inoculated to MMPDRUU containing 4 mM EGTA, Δanpma and ΔanpmaPmcA demonstrated more severe defects in hyphal morphogenesis compared with the phenotypes observed immediately above. Bar, 20 μm.
Na\(^+\)-ATPase expression and, finally, Na\(^+\) tolerance (Park et al., 2001). Here, double deletion of AnPmrA and AnPmcA was not lethal in A. nidulans (Fig. 3) and this double-deletion mutant demonstrated salt sensitivity rather than salt tolerance (data not shown), indicating that high concentrations of Ca\(^{2+}\) were not retained in the cytoplasm. This phenomenon could be explained as follows. (i) The Ca\(^{2+}\)-ATPases in the plasma membrane, which does not exist in S. cerevisiae, pumped the Ca\(^{2+}\) out of the cell. (ii) There is more than one Ca\(^{2+}\)-ATPase in the vacuole or the Golgi apparatus of A. nidulans, and the retained one(s) can compensate for the loss of AnPmrA and AnPmcA; (iii) The rest of the Ca\(^{2+}\)-ATPases could produce splice variants, which could be positioned in the vacuole and/or the Golgi apparatus to function partially as AnPmrA and AnPmcA.

Fig. 6. Sensitivity of the mutants to the cell wall disturbing agents Calcofluor white (CFW) and Congo red (CR). (a) Minimal medium alone, (b) minimal medium containing 50 \(\mu\)g Calcofluor white ml\(^{-1}\), (c) 100 \(\mu\)g Congo red ml\(^{-1}\), (d) 1 M sucrose, (e) 50 \(\mu\)g Calcofluor white ml\(^{-1}\) and 1 M sucrose or (f) 100 \(\mu\)g Congo red ml\(^{-1}\) and 1 M sucrose were dot inoculated with conidia from TN02A7 and the mutants, and cultured at 37 °C for 2–3 days. (g) Relative quantification analysis of the mRNA levels. Total RNA isolated from the hyphae (37 °C, 36 h) was reverse transcribed as the template, as described in Methods. The tubulin gene was used as the reference and the fold change with respect to WT was calculated using the \(\Delta\Delta C_t\) method (\(P<0.05\)).
Fungal vacuoles have a range of cellular functions, such as regulating internal hydrostatic pressure, maintaining an intracellular acid pH and digesting various materials for reuse (Moreno & Docampo, 2009; Wada & Anraku, 1994). The Golgi apparatus is another endomembrane system organelle that plays important roles in modifying, packaging and sorting different molecules to their final destinations (Jiang et al., 2011; Nilsson et al., 2009). AnPmrA and AnPmcA could pump Ca\(^{2+}\) and/or Mn\(^{2+}\) to their respective destinations to maintain the normal functions of the organelles in which they reside; therefore, it was not difficult to understand the growth defects resulting from deletion of them (Fig. 4a–d). The abnormal morphogenesis of the mutants could be explained from the aspect of the cell wall, which has the function of maintaining regular morphology (Upadhyay & Shaw, 2006). Null mutation of AnPmrA weakened the ability of the cell to supply Ca\(^{2+}/\)Mn\(^{2+}\) to the Golgi apparatus lumen. In the environment without Ca\(^{2+}/\)Mn\(^{2+}\), the activities of the enzymes dependent on these ions responsible for synthesizing, transporting and depositing the cell wall components were greatly impaired, resulting in the corresponding abnormal morphogenesis (Fig. 5).

We observed the negative effects of deleting AnPmrA or AnPmcA on the cell wall, i.e. all the mutants demonstrated hypersensitivity to the well-known cell wall defect indicators, Calcofluor white and Congo red (Fig. 6b, c). Further analysis verified the changes concerning the cell wall ingredients (Table 1) and cell wall synthesis gene expression levels (Fig. 6g). The decline of glycoproteins and alkali-soluble glucans in Δanp mrA might also result from the limited activities of the enzymes dependent on Ca\(^{2+}/\)Mn\(^{2+}\) responsible for synthesizing or sorting them, whilst a small increase of the counterparts in Δanp mcA could result from the compromised ability of the vacuole to process the obsolete molecules. As a compensation, the mass of chitin and alkali-insoluble glucans increased, which has been observed in many mutants (Kapteyn et al., 1999; Wang et al., 2012). The hypersensitivity of the mutants to Calcofluor white and Congo red might result from disruption of this compensation, because both Calcofluor white and Congo red could impede correct polymerization and deposition of chitin and 1,3-β-glucan on the cell wall (Herth, 1980; Roncero & Durán, 1985).

In conclusion, via deletion of AnPmrA and AnPmcA, we found that they were important for normal growth, morphogenesis and cell wall integrity, but not viability in *A. nidulans*.

### ACKNOWLEDGEMENTS

This work was supported financially by the National Natural Science Foundation of China [NSFC31100023 (J. H.) and NSFC81330035 (L. L.)], Natural Science Foundation of the Jiangsu Higher Education Institutions of China [11KJA180005 (L. L.) and 12KJB180006 (S. Z.)], and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions (L. L.). We thank Professor H. M. Park (Chungnam National University, Daejeon, South Korea) for the gift of plasmid pQa.

### REFERENCES


Characterization of AnPmrA and AnPmcA mutants


Edited by: V. Cid