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 Ca$^{2+}$ reservoirs to set the stage for the next cycle (Wuytack et al., 2003; Yu et al., 2012). P-type Ca$^{2+}$-transporting ATPases are responsible for this process, i.e. pumping Ca$^{2+}$ against the ion gradients at the expense of energy derived from ATP (Bowman et al., 2011; Catty et al., 1997). In humans, to date, nine different Ca$^{2+}$ pumps corresponding to at least 30 splice variants have been identified and classified into three groups: PMCA (plasma membrane Ca$^{2+}$-ATPase), SERCA (sacro/endoplasmic reticulum Ca$^{2+}$-ATPase) and SPCA (secretory pathway Ca$^{2+}$-ATPase), according to their tissue distribution and function (Micaroni et al., 2010; Shull, 2000).

In Saccharomyces cerevisiae, three Ca$^{2+}$ pumps have so far been identified and characterized. Pmr1 (plasma membrane ATPase related) is the Ca$^{2+}$/Mn$^{2+}$ pump localized in the Golgi apparatus, whilst Pmc1 (plasma membrane calcium-ATPase) and Cod1 (control of 3-hydroxy-3-methylglutaryl-CoA reductase degradation) are the Ca$^{2+}$ pumps located in the vacuole and the endoplasmic reticulum, respectively (Cronin et al., 2000; Cunningham & Fink, 1994). Mutation of Pmr1 leads to retention of Ca$^{2+}$ and Mn$^{2+}$ in the cytoplasm. The elevated Ca$^{2+}$ causes continuous activation of the Ca$^{2+}$ signalling pathway, whilst Mn$^{2+}$ is toxic to some cytosolic enzymes (Lauer Júnior et al., 2008; Park et al., 2001; Wuytack et al., 2003). At the same time, insufficient supply of Mn$^{2+}$ to the Golgi apparatus impairs the glycosylation procedure, because many glycosyltransferases use it as the cofactor

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 ΔanpmcA 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.
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 \textit{anpmrA} and/or \textit{anpmcA} produced defects in growth, morphogenesis and cell wall integrity. However, different from the case in \textit{S. cerevisiae}, double deletion of \textit{anpmrA} and \textit{anpmcA} was not lethal in \textit{A. nidulans}.

**METHODS**

**Strains and media.** The parental \textit{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. Strains were maintained in 30 % glycerol and stocked at 

**Creation of \textit{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 \textit{pyrG} selectable marker was first amplified from plasmid pXDRFP4 (Fungal Genetics Stock Center) with the primers 7/8; the upstream and downstream fragments of \textit{anpmrA} (\textit{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 \textit{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 \textit{Anpmt} protoplasts. The complete deletion of \textit{anpmt} in \textit{Anpmt} was carried out as above, except for using \textit{A. fumigatus} \textit{pyrA} rather than the \textit{pyrG} selectable marker. The primers were: 11/12 for the \textit{pyrA} expression cassette, D1/D3 for the upstream segment of \textit{anpmt}, D4/D6 for the downstream segment of \textit{anpmt} and D2/D5 for fusion PCR. All the fragments were verified by sequencing. Transformation was performed as described previously (Bussink & Osmani, 1998; Jiang et al., 2014). Confirmation of the mutants was carried out through PCR. Three couples of primers, R1/R6, R1/8 and 7/8 (C1/C6, C1/8 and 7/8), were employed to identify \textit{Anpmt} (\textit{AnpmcA}). For \textit{Anpmt16Apmt} and \textit{AnpmcA}, we used primer pairs 13/14 and D1/D2, respectively. All the primers are listed in Table S1 (available in the online Supplementary Material). The amplification conditions were 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1–3 min.

**Chemical analysis of the cell wall.** Conidia were inoculated into 100 ml liquid MMPDRUU at a concentration of 106 ml−1 and shaken (250 r.p.m.) at 37 °C for 36 h. Three aliquots of 10 mg 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_002574746; PMCA4, NP_001675; SERCA1, NP_004311; SERCA2, XP_000253945; SERCA3, XP_000256713; SPC1A, XP_000254741; SPC2A, 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, U51272.1; 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 \textit{A. nidulans}**

By searching the National Center for Biotechnology Information website in the genomic database of \textit{A. nidulans}, we identified the homologues of \textit{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 (pfam00689), E1-E2 ATPase (pfam00122), halocid dehydrogenase-like hydrolases (cd01427) and cation ATPase C (pfam00689) domains conserved in P-type Ca2+-transporting ATPases. A phylogenetic tree recreated 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, \textit{anpmt} or \textit{anpmcA} was replaced completely with the \textit{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 anpmrA or anpmcA had been replaced successfully by the pyrG selectable marker at the correct site. They were designated DpyrG 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, respectively.

**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 (Panzizzo 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.
It was found that although ΔanpmcA could maintain normal morphogenesis similar to WT, ΔanpmrA and ΔanpmrΔpmcA demonstrated an abnormal hyphal pattern (Fig. 5e–h). A similar but more severe growth defect phenotype could be obtained after adding EGTA (which has a major affinity for Ca\(^{2+}\) and minor affinity for Mn\(^{2+}\)) to the medium. It was demonstrated an abnormal hyphal pattern could be restored to 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 ΔanpmrΔpmcA 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 ΔanpmrΔpmcA was 128, 110 and 115% or 120, 112 and 136% of WT, respectively (all \(P<0.05\)).

Alkali-soluble glucans are composed primarily of \(\alpha\)-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-\(\alpha\)-glucan was synthesized by the synthase AgsB, whilst fksA is the only gene that encodes 1,3-β-glucan synthase (Fujioka et al., 2007; Yoshimi et al., 2013). In addition, chsB and csmA genes encode chitin synthases that play crucial roles in the maintenance of cell wall integrity in A. nidulans (Ichinomiya et al., 2005). Thus, we analysed the mRNA expression levels of these genes, and found that the csmB expression levels in ΔanpmrA, ΔanpmcA and ΔanpmrΔpmcA were 70, 124 and 96% of WT, whereas the 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 Ca\(^{2+}\)-ATPases was consistent with their evolutionary relationships (Fig. 1). In *S. cerevisiae*, continuous activation of the intracellular Ca\(^{2+}\) signalling pathway could result in upregulation of P-type
**Fig. 4.** Growth characteristics of the mutants. (a–d) Conidia ($10^4$) 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 $\triangle$anpmrA and $\triangle$anpmrA$\triangle$pmcA, 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 $10^5$ 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 Mn$^{2+}$, although $\triangle$anpmcA had similar morphology to WT, the morphogenesis of $\triangle$anpmrA and $\triangle$anpmrApmcA were obviously abnormal. (i–l) When the conidia were inoculated to MMPDRUU containing 4 mM EGTA, $\triangle$anpmrA and $\triangle$anpmrApmcA 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 \textit{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 \textit{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 \textit{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.

\textbf{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 \(\Delta\Delta Ct\) 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 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 a 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 ΔanpmrA 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 ΔanpmcA 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*.

**REFERENCES**


**Table 1. Cell wall ingredient analysis**

Conidia were inoculated to MMPDRUU and cultured at 37 °C for 36 h. Three aliquots of 10 mg lyophilized mycelia were used as independent samples for analysis, as described in Methods. Values represent mean ± SD. The assay was repeated twice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell wall components [µg (10 mg dry mycelia)$^{-1}$]</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Proteins</td>
<td>Alkali-soluble glucans</td>
<td>Chitin</td>
<td>Alkali-insoluble glucans</td>
</tr>
<tr>
<td>TN02A7</td>
<td>446 ± 23</td>
<td>420 ± 19</td>
<td>644 ± 39</td>
<td>1399 ± 52</td>
</tr>
<tr>
<td>ΔanpmrA</td>
<td>412 ± 13</td>
<td>355 ± 18</td>
<td>824 ± 14</td>
<td>1683 ± 41</td>
</tr>
<tr>
<td>ΔanpmcA</td>
<td>452 ± 14</td>
<td>452 ± 32</td>
<td>709 ± 29</td>
<td>1569 ± 78</td>
</tr>
<tr>
<td>ΔanpmrA ΔanpmcA</td>
<td>440 ± 19</td>
<td>412 ± 37</td>
<td>743 ± 36</td>
<td>1906 ± 92</td>
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