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# The *pmr* Gene, Encoding a $\text{Ca}^{2+}$ -ATPase, Is Required for Calcium and Manganese Homeostasis and Normal Development of Hyphae and Conidia in *Neurospora crassa*

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The *pmr* gene is predicted to encode a  $\text{Ca}^{2+}$ -ATPase in the secretory pathway. We examined two strains of *Neurospora crassa* that lacked PMR: the  $\Delta pmr$  strain, in which *pmr* was completely deleted, and *pmr*<sup>RIP</sup>, in which the gene was extensively mutated. Both strains had identical, complex phenotypes. Compared to the wild type, these strains required high concentrations of calcium or manganese for optimal growth and had highly branched, slow-growing hyphae. They conidiated poorly, and the shape and size of the conidia were abnormal. Calcium accumulated in the  $\Delta pmr$  strains to only 20% of the wild-type level. High concentrations of  $\text{MnCl}_2$  (1 to 5 mM) in growth medium partially suppressed the morphological defects but did not alter the defect in calcium accumulation. The  $\Delta pmr \Delta nca-2$  double mutant (*nca-2* encodes a  $\text{Ca}^{2+}$ -ATPase in the plasma membrane) accumulated 8-fold more calcium than the wild type, and the morphology of the hyphae was more similar to that of wild-type hyphae. Previous experiments failed to show a function for *nca-1*, which encodes a SERCA-type  $\text{Ca}^{2+}$ -ATPase in the endoplasmic reticulum (B. J. Bowman, S. Abreu, E. Margolles-Clark, M. Draskovic, and E. J. Bowman, *Eukaryot. Cell* 10:654-661, 2011). The *pmr*<sup>RIP</sup>  $\Delta nca-1$  double mutant accumulated small amounts of calcium, like the  $\Delta pmr$  strain, but exhibited even more extreme morphological defects. Thus, PMR can apparently replace NCA-1 in the endoplasmic reticulum, but NCA-1 cannot replace PMR. The morphological defects in the  $\Delta pmr$  strain are likely caused, in part, by insufficient concentrations of calcium and manganese in the Golgi compartment; however, PMR is also needed to accumulate normal levels of calcium in the whole cell.

Calcium is an important signaling molecule in all cells, essential for growth yet toxic if cytosolic concentrations increase above micromolar concentrations. The “filamentous” morphology of the nonyeast fungi has been postulated to be controlled by intracellular calcium gradients (25, 28, 40, 46). However, the transporters that regulate calcium homeostasis in filamentous fungi have not been investigated to nearly the extent that they have been in some yeasts.

*Saccharomyces cerevisiae* has been the primary model organism for the study of proteins that move calcium across biological membranes (9, 11, 54). In *S. cerevisiae*, three proteins have been shown to be particularly important. Pmc1p, a  $\text{Ca}^{2+}$ -ATPase, and Vcx1p, a  $\text{Ca}^{2+}/\text{H}^+$  exchange protein, reside in the vacuolar membrane. They transport calcium from the cytosol into the vacuole, thus sequestering more than 95% of the cellular calcium (12, 15, 17, 45).  $\Delta pmc1$  strains accumulate only 20% of the calcium seen in the wild type.  $\Delta vcx1$  strains accumulate calcium to the same levels observed for the wild type, but  $\Delta pmc1 \Delta vcx1$  double mutant strains accumulate only 10% of the wild-type levels and do not grow in media with elevated levels of calcium (12). The SPCA-type  $\text{Ca}^{2+}$ -ATPase Pmr1p is seen primarily in the Golgi compartment. It delivers calcium and manganese to organelles in the secretory pathway (2, 16, 41, 48, 55, 56). In addition, *S. cerevisiae* has calcium channels in the vacuolar membrane (Yvc1p) (14, 19, 27, 42) and in the plasma membrane (the Cch1p/Mid1p complex) (19, 27, 42).

Sequencing of the genomes of many filamentous fungi has revealed larger numbers of calcium transporters in these organisms (3, 26, 58). In addition to homologs of *Vcx1* and *Pmc1*, *Neurospora crassa* and *Aspergillus* species, for example, have genes encoding 2 to 5 PMCA-type transporters (the family to which yeast

*PMC1* belongs). Unlike yeasts, the filamentous fungi also have the SERCA-type  $\text{Ca}^{2+}$ -ATPase found in the endoplasmic reticulum (ER) of plants and animal cells (6, 31). The PMR  $\text{Ca}^{2+}$ -ATPase has been investigated in *Aspergillus niger* and *Aspergillus fumigatus*. The deletion of this enzyme in *A. fumigatus* was reported previously to reduce radial growth by 60% and to cause defects in the synthesis of the cell wall (43). In contrast, the loss of PMR in *A. niger* did not cause a significant change in the growth rate or hyphal morphology (3, 57). In both species of *Aspergillus*, the PMR deletion strains grew poorly in media with low concentrations of calcium.

Using *N. crassa* as our model organism, we have investigated the cellular distribution and function of calcium transporters (4, 5). The *cax* gene encodes the homolog of *Vcx1*. As in *S. cerevisiae*, this  $\text{Ca}^{2+}/\text{H}^+$  exchanger is important for sequestering calcium in vacuoles. The *nca-2* and *nca-3* genes encode PMCA-type  $\text{Ca}^{2+}$ -ATPases. Different from *S. cerevisiae* but similar to animal cells, they appear to function primarily in the plasma membrane, pumping calcium out of the cell. The deletion of *nca-2* in *N. crassa* causes a 4- to 10-fold elevation of the cell calcium concentration. The *nca-1* gene encodes a SERCA-type  $\text{Ca}^{2+}$ -ATPase, which, when tagged with green fluorescent protein (GFP), is observed in the nuclear envelope and ER, again as in animal cells. The  $\Delta nca-1$

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strains of *N. crassa* were indistinguishable from the wild-type strain. Indeed, we were surprised that deletions of the genes *cax*, *nca-1*, *nca-2*, and *nca-3* or double and triple combinations of these deletions failed to cause significant changes in cell morphology: the sizes and branching patterns of hyphae were much like those of the wild type.

In contrast to the results seen with other calcium transporters, preliminary experiments with *pmr* mutant strains of *N. crassa* showed that cells lacking this SPCA-type Ca<sup>2+</sup>-ATPase were dramatically affected both in hyphal morphology and in the ability to grow in standard media (1). Experiments with *Aspergillus* species showed that the deletion of the *pmr* gene causes defects in cell wall synthesis and slower growth, especially in media with lower concentrations of calcium (43, 51, 57). In *S. cerevisiae*, strains lacking Pmr1p accumulate higher levels of calcium and manganese and are defective in secretion and glycosylation (2, 24, 29, 30, 48). Changes in the size or shape of haploid cells have not been reported for *S. cerevisiae*; however, diploid  $\Delta pmr$  cells fail to sporulate and appear abnormal (48). In *Schizosaccharomyces pombe*, the typical cylindrical shape of cells became more rounded when the *pmr* gene was deleted (35). In yeasts, there is evidence that Pmr1p may function in the ER as well as the Golgi compartment (52). *N. crassa* has a SERCA-type Ca<sup>2+</sup>-ATPase, encoded by *nca-1*, localized to the ER (5). In this report, we constructed a *pmr nca-1* double mutant. The phenotype of this strain suggests that both the SERCA-type Ca<sup>2+</sup>-ATPase and the SPCA-type Ca<sup>2+</sup>-ATPase are needed for the normal growth of *N. crassa* and probably other filamentous fungi. The major goals of this paper are to determine the role of the PMR transporter in *N. crassa* and to ask if PMR and NCA-1 have important roles in calcium homeostasis and in morphological development.

## MATERIALS AND METHODS

**Deletion strains.** Except for the *pmr*<sup>RIP</sup> strain described below, all mutant strains were generated by the *Neurospora* Genome Project (7) and were obtained from the Fungal Genetics Stock Center (39). In these strains, the protein-encoding region was replaced by the *hph*<sup>+</sup> gene, which confers resistance to the drug hygromycin. The procedure used to generate deletion strains made use of multinucleated conidia, and viable hygromycin-resistant homokaryons were not obtained for a small percentage of the strains. For these deletions, the transformed conidia were saved as heterokaryons. The *Neurospora* Genome Project produced heterokaryon strain FGSC 11616, in which *pmr* (locus NCU03292.2) was replaced with *hph*<sup>+</sup>. We streaked conidia from FGSC 11616 and observed a small number of colonies that were slow growing and morphologically abnormal (described more fully in Results). They had the same phenotype as that of the *pmr*<sup>RIP</sup> strain described below. When crossed to wild-type strain 74A, the slow-growing, abnormal colonies represented approximately 50% of the progeny. Analysis by PCR showed that the *pmr* gene had been replaced by *hph*<sup>+</sup>. We selected one of these with the *pmr::hph*<sup>+</sup> A genotype and named it the  $\Delta pmr$  strain. The other deletion strains used,  $\Delta cax$ ,  $\Delta nca-1$ ,  $\Delta nca-2$ , and  $\Delta nca-3$ , were described previously (4).

**Isolation of strains with two mutations.** The  $\Delta pmr$  strain was fertile as either a male or a female parent. It was mated with the  $\Delta cax$ ,  $\Delta nca-2$ , or  $\Delta nca-3$  strain, and progeny were isolated by using standard genetic procedures for *N. crassa* (13). The progeny were analyzed by PCR to identify double mutants and to verify that no endogenous copy of either gene was present.

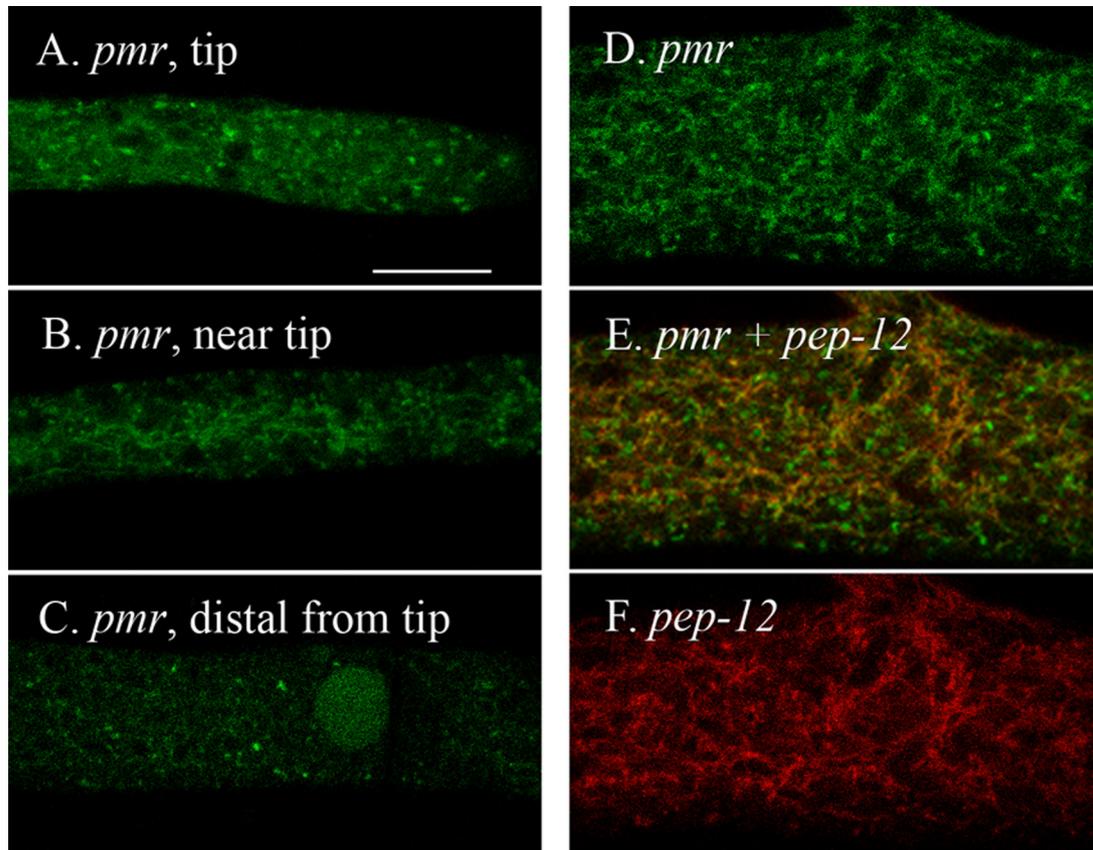
Multiple attempts to isolate the  $\Delta pmr \Delta nca-1$  double mutant strain were unsuccessful because *pmr* is closely linked to *nca-1*, separated by only 57 kb (approximately 1 map unit), and because the double mutant had a low level of viability. Therefore, we developed a method by which we

could enrich for the presence of *pmr*-null  $\Delta nca-1$  progeny. First, we generated a *pmr*-null strain with the RIPing (repeat-induced point mutations) procedure (49). A 3,211-bp fragment of DNA containing the *pmr* gene was amplified by PCR using primers 5'-ACGATGTTCTTCCCTTC TACC-3' (forward) and 5'-CCGTA C TCCACCATT T TCTC-3' (reverse). The *pmr* gene was inserted into pBM61 (38). The resulting plasmid was used to transform the *his-3* strain (FGSC 6103) of *N. crassa*, producing a strain that had both endogenous *pmr*<sup>+</sup> and a second copy of *pmr*<sup>+</sup> targeted to the *his-3* locus. The transformed strain was crossed with wild-type strain 74A. Among the progeny from this cross were isolates that had an abnormal hyphal morphology and failed to conidiate, a phenotype that we now know is indistinguishable from the phenotype of  $\Delta pmr$  strains. An isolate with abnormal morphology was crossed again to wild-type strain 74A. We selected progeny that were *his-3*<sup>+</sup> and had the abnormal-morphology phenotype. The *pmr* gene from one such isolate, named *pmr*<sup>RIP</sup>, was amplified by PCR and sequenced. The gene had 120 nucleotide changes, resulting in 80 amino acid changes, with stop codons introduced at amino acids 490 and 856 (1,025 amino acids total).

We next crossed the  $\Delta nca-1$  strain obtained from the *Neurospora* Genome Project to the *his-3* strain (*his-3* lies 10 map units from *pmr*), generating the *his-3 \Delta nca-1* strain, which is a histidine auxotroph that is resistant to hygromycin. The *his-3 \Delta nca-1* strain was crossed with the *pmr*<sup>RIP</sup> strain, and ascospores were germinated on agar plates with Vogel's minimal medium containing 2% sucrose and hygromycin (400  $\mu$ g/ml). The order of the genes is *his-3*, *pmr*, and *nca-1*. The only progeny that could grow were *his*<sup>+</sup> and hygromycin-resistant progeny, produced by recombination between the *his-3* and  $\Delta nca-1$  genes. Ten percent of the recombinants were predicted to be *pmr*<sup>RIP</sup>  $\Delta nca-1$  recombinants. More than 99% of the viable progeny had a normal morphology, as expected for *his-3*<sup>+</sup>  $\Delta nca-1$  strains. A few of the viable progeny grew very slowly with an abnormal morphology (described in Results). Analysis by PCR (for the  $\Delta nca-1$  mutation) and sequencing (for the *pmr*<sup>RIP</sup> mutation) showed that these progeny were *pmr*<sup>RIP</sup>  $\Delta nca-1$  strains.

**Generation and analysis of strains with GFP- and red fluorescent protein (RFP)-tagged proteins.** We identified *N. crassa* homologs of the VPS52 (NCU05273) and VRG4 (NCU06198) genes of *S. cerevisiae*. Vps52p is a component of the Golgi-associated retrograde protein complex, required for the recycling of proteins from endosomes to the late Golgi compartment (8). Vrg4p is a GDP-mannose transporter (44). Primers 5'-cacatctagaCACCATGGCCAACAAGAGAAAC-3' (forward) and 5'-gggtggatccAAGCATTGCGGCATCCCTG-3' (reverse) were used for pvrg-4-GFP (the region in uppercase letters is the sequence in the gene, and the region in lowercase letters contains the restriction site used for construction of the plasmid). The primers used to construct pvps-52-GFP, pRFP-vps-52, ppmr-GFP, and pRFP-pmr were described previously (5). The product of the NCU06777 gene served as a marker of tubular vacuolar membranes. We previously reported that this gene encodes the homolog of VAM3 from *S. cerevisiae* (5). As pointed out by an anonymous reviewer, this is almost certainly incorrect. *N. crassa* and other filamentous fungi lack VAM3, and NCU06777 appears to encode the homolog of PEP12, which has a similar sequence (22). Pep12p is a multifunctional syntaxin involved in trafficking to the prevacuolar compartment in *S. cerevisiae* (20); a high level of expression of PEP12 can suppress defects in VAM3 mutants (21). We have shown that the *N. crassa* protein encoded by NCU06777 colocalizes with the vacuolar H<sup>+</sup>-ATPase (VMA-1 and VMA-5) and the vacuolar calcium/H<sup>+</sup> transporter (CAX) (5). The construction of plasmids, the transformation of *N. crassa* strain 74A (FGSC 987), and analysis by confocal microscopy were carried out as described previously (5).

**Analysis of growth and morphology.** The growth yields of wild-type and mutant strains were measured by using Vogel's medium with 2% sucrose (13) in which CaCl<sub>2</sub> and MnCl<sub>2</sub> had been omitted. CaCl<sub>2</sub> and MnCl<sub>2</sub> were added as indicated in the figure legends. Six milliliters of medium was put into 20-ml vials; each vial was then inoculated with 100,000 conidia, and conidia were grown at 30°C for 2 days. Mycelia were



**FIG 1** Intracellular location of PMR. The scale bar in panel A is 10  $\mu\text{m}$ . All panels are shown at the same magnification. (A to C) Cells have been transformed with *pmr*<sup>+</sup>::*sgfp*. The regions shown are the hyphal tip (A) and the regions 150  $\mu\text{m}$  from the tip (B) and 2 mm from the tip (C). (D to F) Shown is the region 150  $\mu\text{m}$  from the tip in a heterokaryon formed by coinoculating strains transformed with *pmr*<sup>+</sup>::*sgfp* and *rfp*::*pep-12*<sup>+</sup>. Panels D and F were merged in panel E.

collected by filtration, rinsed with water, and dried. All growth experiments were done at least twice, with triplicate samples in each experiment.

To observe growth on solid medium (2% agar), we used Vogel's medium containing normal concentrations of  $\text{MnCl}_2$  (0.3  $\mu\text{M}$ ) and  $\text{CaCl}_2$  (0.68 mM) and 2% sucrose. Some plates were supplemented with 3 mM  $\text{MnCl}_2$  or 10 mM  $\text{CaCl}_2$ , as indicated. Plates were inoculated with a 3-mm plug of mycelium from another plate and incubated at 30°C for the times indicated. The morphology of hyphae at the edge of the colonies was photographed after 24 h. In other experiments, we used the same medium and growth conditions but spread conidia onto the agar plates at a low density. Colonies growing from single conidia were photographed after 24 h of growth.

To obtain conidia from wild-type strain 74A and the  $\Delta pmr$  strain, we inoculated agar plates with Vogel's medium (2% agar and 2% sucrose) in the center of the plates and grew the conidia for 3 days at 30°C and for an additional 7 to 10 days on the benchtop at room temperature. As described in Results, the  $\Delta pmr$  strain produced conidia only after 10 days postinoculation and only in a narrow region around the rim of the plate. Strain 74A produced abundant conidia starting 3 to 4 days after inoculation. Conidia were suspended in water and photographed to measure the conidial diameter. For the rod-shaped conidia produced by the  $\Delta pmr$  strain, the average of the width and length was used in place of the diameter.

**Measurement of calcium uptake.** Strains were grown in 20-ml vials containing 6 ml of Vogel's medium with 2% sucrose and 0.3  $\mu\text{Ci}$   $^{45}\text{Ca}$ . Vials were inoculated with conidia (300,000 conidia per ml) from 74A and the  $\Delta pmr$ ,  $\Delta pmr \Delta cax$ , and  $\Delta pmr \Delta nca-3$  strains. For the aconidial *pmr*<sup>RIP</sup>  $\Delta nca-1$  and  $\Delta pmr \Delta nca-2$  strains, a 200- $\mu\text{l}$  aliquot of mycelium from a

liquid culture (approximately 0.1 mg [dry weight]) was used as an inoculum. Experiments with the  $\Delta pmr$  strain showed that the amounts of calcium accumulated per mg dry weight were the same in cultures inoculated with conidia or mycelia. The strains were grown for 2 to 6 days until they reached a dry weight of approximately 2 mg/ml, which is in the mid-log phase. Mycelia were collected by filtration and rinsed four times with 6-ml aliquots of Vogel's medium supplemented with 20 mM  $\text{CaCl}_2$ . The dry weight was determined, and the uptake of  $^{45}\text{Ca}$  was measured with a liquid scintillation counter. The experiments were performed at least twice with triplicates for each strain.

## RESULTS

**Intracellular location of PMR.** To visualize the location of PMR, we constructed recombinant genes to produce proteins with GFP fused to the C terminus or RFP fused to the N terminus. These recombinant genes were introduced into wild-type strain 74A and the  $\Delta pmr$  strain. With the RFP-PMR protein, the morphology and growth defects observed for the  $\Delta pmr$  strain were unchanged, while the PMR-GFP protein restored nearly normal growth and morphology (data not shown). We observed PMR-GFP in irregularly shaped particles roughly 0.5 to 1.0  $\mu\text{m}$  in diameter. These particles occurred in all regions of the hypha (Fig. 1A to C) but were most abundant near the tip (Fig. 1A). In the region where the tubular vacuolar network was observed (50 to 200  $\mu\text{m}$  behind the tips), we also saw PMR-GFP located in thin tubules (Fig. 1B). The tubules were part of the tubular vacuolar network

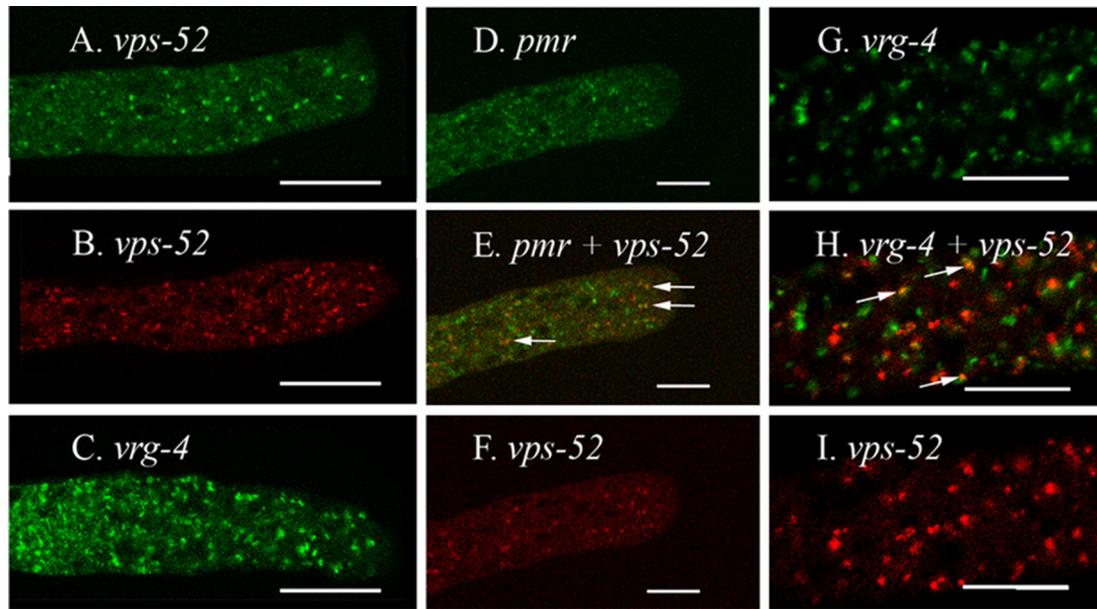


FIG 2 Intracellular location of the PMR, VPS-52, and VRG-4 proteins. The scale bars are 10  $\mu\text{m}$  in panels A to F and 5  $\mu\text{m}$  in panels G to I. (A to C) Cells were transformed with *vps-52*<sup>+</sup>::*sgfp* (A), *rfp*::*vps-52*<sup>+</sup> (B), and *vrg-4*<sup>+</sup>::*sgfp* (C). (D to F) Shown is a region at the hyphal tip in a heterokaryon formed by the coinoculation of strains transformed with *pmr*<sup>+</sup>::*sgfp* (D) and *rfp*::*vps-52*<sup>+</sup> (F). (Panels D and F were merged in panel E. (G to I) Shown is a heterokaryon formed by coinoculating strains transformed with *vrg-4*<sup>+</sup>::*sgfp* (G) and *rfp*::*vps-52*<sup>+</sup> (I). Panels G and I were merged in panel H. Arrows indicate particles with both GFP and RFP. The region shown is approximately 100  $\mu\text{m}$  from the hyphal tip.

because they colabeled with RFP-PEP-12 (discussed in Materials and Methods) (Fig. 1D to F). The small green particles in this region did not overlap RFP-PEP-12. To see if the particles visualized with PMR-GFP were components of the Golgi compartment, we tagged two putative Golgi-localized proteins with GFP and RFP. The proteins which we used were encoded by *N. crassa* homologs of the *S. cerevisiae* genes *VPS52* and *VRG4*. We observed VPS-52-GFP and RFP-VPS-52 in small particles similar in size to those seen with PMR-GFP (Fig. 2A and B). In heterokaryons made by fusing VPS-52-GFP and RFP-VPS-52, we saw the two tagged proteins in the same compartments (data not shown). VRG-4-GFP gave the strongest signal of any of the Golgi markers, appearing in organelles of similar sizes but in a greater abundance than PMR-GFP, VPS-52-GFP, or RFP-VPS-52 (Fig. 2C). In a heterokaryon expressing both PMR-GFP and RFP-VPS-52, a small proportion of the tagged particles had both GFP and RFP (Fig. 2D to F). Similarly, a heterokaryon expressing both VRG-4-GFP and RFP-VPS-52 had a small proportion of particles with both GFP and RFP (Fig. 2G to I). Despite several attempts, we did not succeed in making a red-tagged version of PMR or VRG-4 that we could use to see if PMR and VRG-4 colocalized. The localization data are discussed further below.

**Effect of calcium and manganese on the growth of strains lacking PMR.** Previous reports have shown that the PMR protein has a role in the transport of both calcium and manganese in fungi (2, 24, 30, 36, 48, 57). We compared the growths of the  $\Delta pmr$  strain and wild-type strain 74A in Vogel's minimal medium, which is widely used for *N. crassa*. This medium contains 8.9 mM citrate, a weak chelator of divalent cations. In preliminary experiments, we found that the omission of citrate allowed the  $\Delta pmr$  strain to grow in a mass nearly as well as the wild type, presumably using trace levels of divalent cations. In the experiments shown in

Fig. 3, the medium contained citrate. The growth of the wild-type strain was barely affected by the omission of both calcium and manganese from Vogel's medium, while the  $\Delta pmr$  strain grew poorly in this medium (Fig. 3A and B). The addition of calcium up

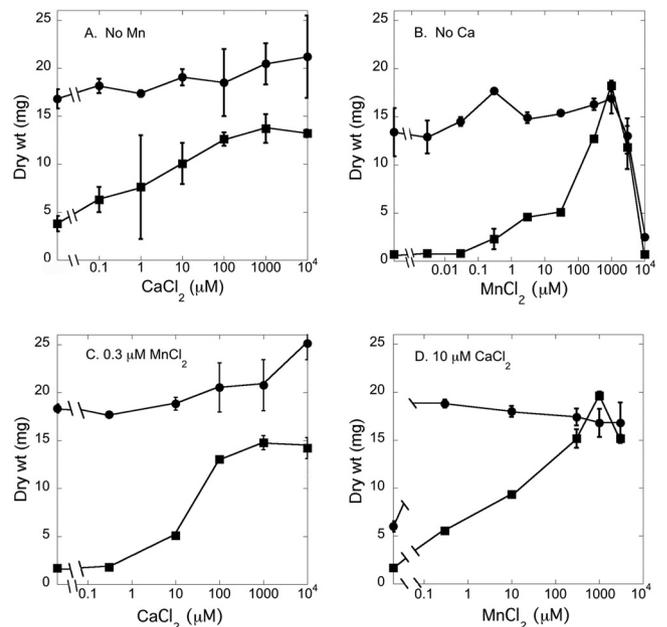


FIG 3 Growth of wild-type and  $\Delta pmr$  strains with various concentrations of calcium and manganese. Strains were grown in Vogel's medium with the indicated concentrations of CaCl<sub>2</sub> and MnCl<sub>2</sub>, as described in Materials and Methods. Circles represent wild-type strain 74A, and squares represent the  $\Delta pmr$  strain.

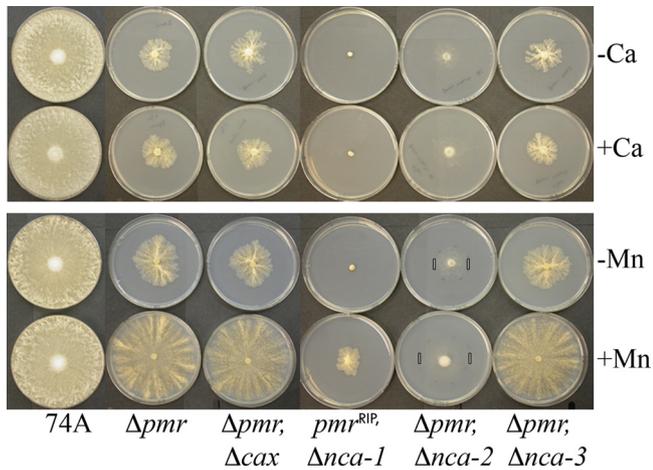


FIG 4 Growth of wild-type and mutant strains on agar plates. Strains were grown for 2 days at 30°C, as described in Materials and Methods. As indicated, either 1 mM  $\text{MnCl}_2$  or 10 mM  $\text{CaCl}_2$  was added to some plates. The black bars on the plates with the  $\Delta pmr \Delta nca-2$  strain show the position of the edge of the colony, which is barely visible in the photograph.

to 1 mM increased the growth yield for the  $\Delta pmr$  strain, although maximal growth was still less than that for the wild type. The addition of manganese at a concentration of 1 mM produced the same growth yield for the  $\Delta pmr$  strain as that for the wild type. Higher concentrations of manganese were toxic to both the  $\Delta pmr$  and wild-type strains.

Similar results were obtained if we omitted only one of the divalent cations. The growth of the  $\Delta pmr$  strain approached that of the wild type in medium with 0.1 to 10.0 mM calcium (Fig. 3C). At manganese concentrations of 0.3 to 3.0 mM, the growth of the  $\Delta pmr$  strain was equivalent to that of the wild type (Fig. 3D). Note that the wild type grew poorly in medium with 10  $\mu\text{M}$  calcium and no added manganese (Fig. 3D), perhaps indicating that calcium can block the uptake of trace amounts of manganese. The addition of just 0.3  $\mu\text{M}$  manganese was sufficient for the maximal growth of the wild type. These results show that PMR plays an important role in providing the calcium and manganese needed for growth.

**Morphological defects in the  $\Delta pmr$  strain.** When grown on agar plates with Vogel's minimal medium, the  $\Delta pmr$  strain exhibited an unusual colony morphology. The linear growth rate was much lower, 0.23 mm/h, than the growth rate for the wild type, 4.6 mm/h. The edge of the colony was highly branched, the production of aerial hyphae was suppressed, and the general appearance of the mycelial mat was different (Fig. 4). The addition of a high concentration of calcium (10 mM) did not affect the growth rate or morphology. The addition of manganese (1 mM) stimulated the radial growth of the  $\Delta pmr$  strain 4-fold and partially corrected the morphological defects. Higher concentrations of calcium or manganese had no additional effects or were toxic.

The  $\Delta pmr$  strain did not produce conidia (asexual spores) when cultured in ways that result in abundant conidial production from the wild type, for example, by growth in agar slants or in Erlenmeyer flasks. The only reproducible way that we found to produce conidia was to grow  $\Delta pmr$  cells on agar plates for at least 10 days. Conidia formed in the narrow space where the plate contacted the lid (see data for the  $\Delta pmr$  strain in Fig. 8). The conidia that were produced were highly variable in size and shape. The

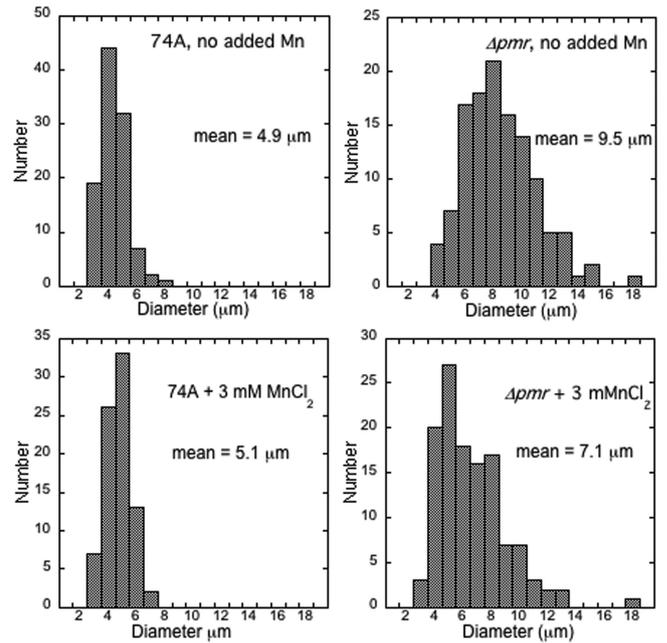


FIG 5 Sizes of conidia from wild-type and  $\Delta pmr$  strains. Conidia were harvested from cultures grown on agar plates containing Vogel's medium with no supplements (top) or with 3 mM  $\text{MnCl}_2$  (bottom).

conidia from the wild type had an average diameter of 4.9  $\mu\text{m}$ , while the average diameter for conidia of the  $\Delta pmr$  strain was nearly twice as large, 9.5  $\mu\text{m}$  (Fig. 5, top). The addition of  $\text{MnCl}_2$  to the medium did not significantly change the size of conidia compared to the size of the wild-type conidia, but it did affect the size of conidia of the  $\Delta pmr$  strain, resulting in the production of conidia that were a bit more like those of the wild type (Fig. 5, bottom). The shape of conidia is shown in Fig. 6. For the  $\Delta pmr$  strain, conidia were often highly vacuolated or shaped like rods. The addition of manganese only partially reduced the proportion of abnormal conidia.

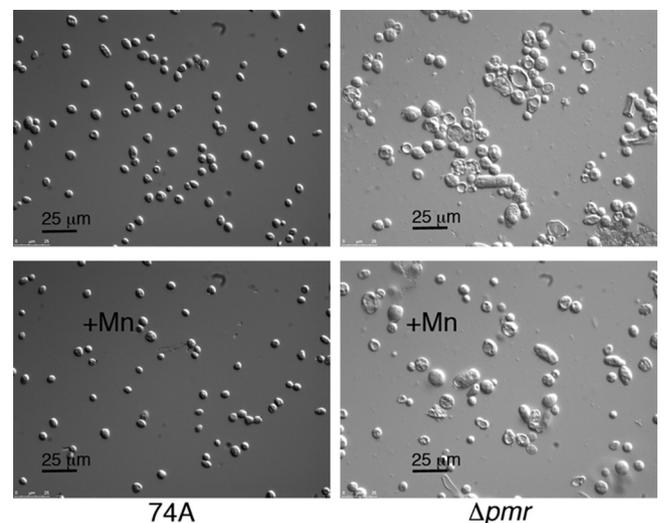
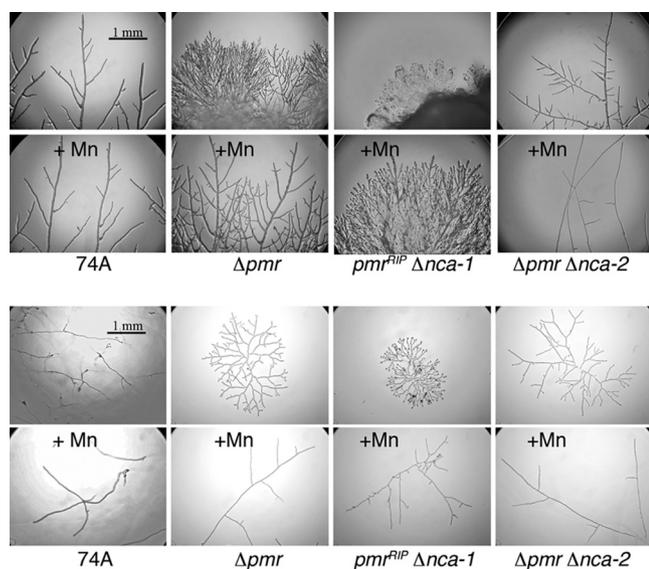


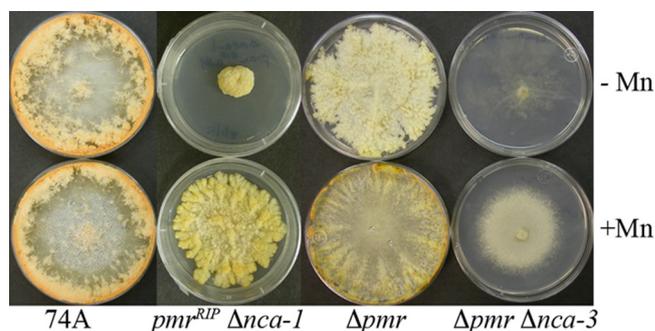
FIG 6 Effect of high concentrations of  $\text{MnCl}_2$  on the size and shape of conidia. The conidia measured in Fig. 5 are shown.



**FIG 7** Hyphal morphology of wild-type and mutant strains. The top eight photographs show the edges of colonies inoculated with 3-mm plugs of mycelia and grown for 24 h at 30°C. The bottom eight photographs show colonies growing from single conidia after 24 h at 30°C. All photographs were taken at the same magnification. The strains were grown on Vogel's medium, with and without added MnCl<sub>2</sub>, as described in Materials and Methods.

A microscopic examination of the leading edge of colonies showed that  $\Delta pmr$  hyphae were more highly branched than those of the wild type and were less likely to develop the pattern seen in the wild type, a few large hyphae with subsidiary branches (Fig. 7, top). To obtain quantitative data, we took time-lapse photographs during 8 h of growth at room temperature, observing hyphae at the leading edge of the colony. The wild-type strain formed a branch at an average time interval of 4.9 min, and the average distance between branches was 0.35 mm. The  $\Delta pmr$  strain formed a branch at an average time interval of 10.8 min, and the average distance between branches was 0.07 mm. The differences were even more pronounced when early growth from a single conidium was observed (Fig. 7, bottom). The wild type produced long hyphae with few branches, often as much as 1 mm apart. Many hyphae grew into the agar (out of the focal plane in Fig. 7). Hyphae from the  $\Delta pmr$  strain grew almost exclusively on the agar surface, with frequent branches. The addition of manganese did not significantly affect the wild type. For the  $\Delta pmr$  strain, hyphal growth with a high manganese concentration was still largely on the surface, but the branching pattern was more similar to that of the wild type.

**Phenotypes of double mutant strains.** We previously observed that strains lacking two calcium transporters can reveal unpredicted phenotypes. For example, the growth of the  $\Delta cax$  strain is indistinguishable from that of the wild type in media with 0.01 to 400 mM CaCl<sub>2</sub>. However, the growth of the  $\Delta cax \Delta nca-2$  strain is highly sensitive to calcium, much more so than strains lacking only NCA-2 (4). We constructed double mutant strains with the  $\Delta pmr$  mutation and either the  $\Delta cax$ ,  $\Delta nca-1$ ,  $\Delta nca-2$ , or  $\Delta nca-3$  mutation (Fig. 4). The radial growth rates and morphologies of the  $\Delta pmr \Delta cax$  and  $\Delta pmr \Delta nca-3$  strains were indistinguishable from those of the single  $\Delta pmr$  strain. The  $pmr^{RIP} \Delta nca-1$  and  $\Delta pmr \Delta nca-2$  strains had severe, but different, defects in growth and morphology.



**FIG 8** Wild-type and mutant strains grown on agar plates for 10 days. Strains were inoculated with 3-mm plugs of mycelia grown on Vogel's medium, with and without added MnCl<sub>2</sub>. Strains were grown for 3 days at 30°C, followed by 7 days at room temperature.

NCA-1 is a SERCA-type Ca<sup>2+</sup>-ATPase in the ER. The  $\Delta nca-1$  strain of *N. crassa* has no observable defect in growth, morphology, or calcium uptake, either by itself or in combination with the  $\Delta nca-2$ ,  $\Delta nca-3$ , or  $\Delta cax$  mutation (4). The  $pmr^{RIP} \Delta nca-1$  double mutant exhibited extreme defects in growth and morphology. Conidiation was never observed. On agar plates containing Vogel's minimal medium, the strain barely grew in 3 days, forming a tiny ball of mycelia (Fig. 4). After 2 weeks, the ball of mycelia enlarged but never spread across the plate. The addition of manganese moderately stimulated radial growth but not to the extent observed for the  $\Delta pmr$  strain (Fig. 8). Hyphae in the  $pmr^{RIP} \Delta nca-1$  colony remained tubular, but tiny, and even more highly branched than in the  $\Delta pmr$  strain (Fig. 7). A high frequency of split hyphal tips was observed. As with the  $\Delta pmr$  strain, the addition of manganese, but not calcium, partially suppressed the morphological defects (Fig. 4 and 8). The results show that NCA-1 is indeed functional; however, the effects of the deletion of the gene encoding *nca-1* were visible only in cells lacking a functional PMR transporter.

In a previous report, we suggested that NCA-2 resides in the plasma membrane in *N. crassa*, pumping calcium out of the cell (4). The  $\Delta pmr \Delta nca-2$  strain grew more slowly than either of the single mutants. On agar plates, the mycelial mat was much less dense, and conidiation was never observed. However, the morphology of individual hyphae was surprisingly more like that of the wild type (Fig. 7), suggesting that the deletion of *nca-2* partially suppresses the morphological defects of the  $\Delta pmr$  mutation. The hyphal morphology of the  $\Delta pmr \Delta nca-2$  strain was even more like that of the wild type if manganese was added (Fig. 7, bottom).

**Uptake of calcium by the  $\Delta pmr$  and double mutant strains.** We wanted to determine if the deletion of *pmr* affected the amount of calcium in cells. Wild-type strain 74A and mutant strains were grown in standing liquid cultures to approximately the mid-log phase. The amount of calcium in the  $\Delta pmr$  strain was only 20% of that in the wild-type strain. The  $pmr^{RIP} \Delta nca-1$ ,  $\Delta pmr \Delta nca-3$ , and  $\Delta pmr \Delta cax$  double mutants had similarly low levels of calcium (Fig. 9A). We reported previously that the  $\Delta nca-2$  strain accumulates 4- to 10-fold more calcium than the wild type (4). The  $\Delta pmr \Delta nca-2$  double mutant accumulated 8-fold more calcium than the wild type (Fig. 9A), showing that the loss of *nca-2* is epistatic to the loss of *pmr*. The accumulation of calcium was also measured in medium with 1 mM MnCl<sub>2</sub> (Fig. 9B). The results were qualitatively the same, showing that high levels of manganese

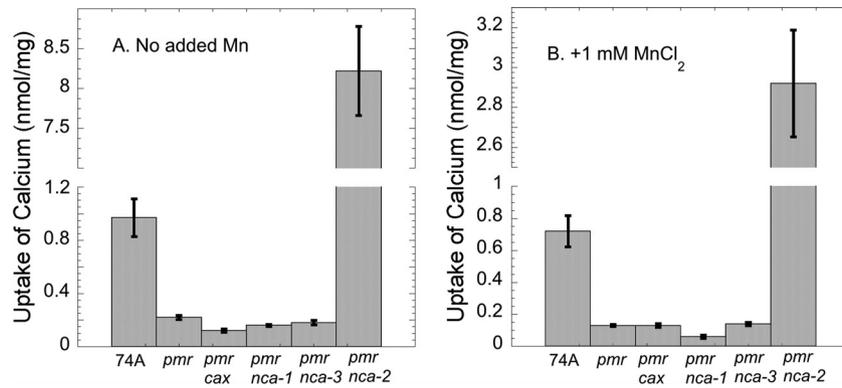


FIG 9 Uptake of calcium by wild-type and mutant strains. The strains were grown at 30°C in liquid Vogel's medium for 2 to 6 days. For panel B, the medium was supplemented with 1 mM  $\text{MnCl}_2$ . The accumulation of  $^{45}\text{Ca}$  was determined as described in Materials and Methods.

did not suppress the defect in calcium accumulation in  $\Delta pmr$  strains.

## DISCUSSION

The data from this and two previous reports (4, 5) provide a reasonably comprehensive view of the role of calcium transport proteins in *N. crassa* (Fig. 10). The locations and functions of these transporters in this filamentous fungus make it more similar to animal cells than to yeasts. Like the yeasts, *N. crassa* has a calcium/ $\text{H}^+$  antiporter, encoded by the *cax* gene, in the vacuolar membrane. Like animal cells, the PMCA-type  $\text{Ca}^{2+}$ -ATPase, NCA-2, appears to function in the plasma membrane, pumping calcium out of the cell. *N. crassa* and other filamentous fungi in fact have multiple homologs of the PMCA-type  $\text{Ca}^{2+}$ -ATPase (3, 18, 58). The function of these other PMCA homologs has not yet been discovered, although we have shown that NCA-3 from *N. crassa* is located in the plasma membrane. Yeasts and animal cells have an SPCA-type  $\text{Ca}^{2+}$ -ATPase (PMR) in the secretory pathway. In *S. cerevisiae*, Pmr1p was proposed previously to do double duty, functioning in the ER and the Golgi compartment (52), whereas animal cells and filamentous fungi have a SERCA-type  $\text{Ca}^{2+}$ -ATPase in the ER.

Because strains lacking the SERCA-type  $\text{Ca}^{2+}$ -ATPase ( $\Delta nca-1$ ) had no observable defects, we lacked evidence that this enzyme had an important function in *N. crassa* (4). Our results support the

idea that in the absence of a  $\text{Ca}^{2+}$ -ATPase in the ER, e.g., as in the  $\Delta nca-1$  strain, the PMR transporter can function in the ER in *N. crassa*. The amount of PMR in the ER may be quite small, because we could not detect PMR-GFP in the ER, even in a  $\Delta nca-1$  background (data not shown). The deletion of both *nca-1* and *pmr* gives rise to a strain with defects far more severe than the deletion of only *pmr*. The double mutant barely grows on normal media, although it is weakly stimulated by high levels of manganese. The morphological phenotype is unusual, with the mycelia forming a spherical colony. Individual hyphae are still tubular but highly branched. Thus, our analysis of the *pmr*<sup>RIP</sup>  $\Delta nca-1$  strain strongly indicates that NCA-1 does have a functional role in *N. crassa*.

To see the location of PMR in *N. crassa*, we tagged the protein with GFP. It was predominately localized in 0.5- to 1.0- $\mu\text{m}$  particles, most abundant near the hyphal tip. In other organisms, the SPCA family of  $\text{Ca}^{2+}$ -ATPases has been observed in the Golgi compartment (41, 55). The *N. crassa* homologs of Vps52p and Vrg4p, proteins that have been observed in the Golgi compartment in *S. cerevisiae* and other organisms (8, 32, 44), appeared in particles similar in size and distribution to the particles visualized with PMR-GFP.

However, the observation of *N. crassa* hyphae expressing both PMR-GFP and RFP-VPS-52 indicated that these proteins were not all in the same compartment. In merged images, 5 to 15% of the particles contained both proteins, but most of the tagged particles appeared as different, spatially separated compartments. Other investigators reported observations of different Golgi proteins in different compartments of the Golgi compartment (33). Thus, our data are consistent with a Golgi localization for PMR in *N. crassa*. The particles that we saw may be early Golgi cisternae, some of which were transitioning to late Golgi cisternae. However, other explanations are also possible. VPS-52 was also shown previously to interact with endosomes (8, 47, 50). PMR could also be localized to vesicles that travel between the ER and the Golgi compartment. Determining the exact location of PMR, and, indeed, of other secretory pathway proteins in *N. crassa*, will be a challenging project.

The complex phenotype of the  $\Delta pmr$  strain indicates that PMR has multiple important functions. Consistent with data from previous reports, PMR appears to transport both calcium and manganese (2, 24, 30, 36, 48, 57). The wild-type strain, but not the  $\Delta pmr$  strain, can grow at nearly normal rates in Vogel's medium in

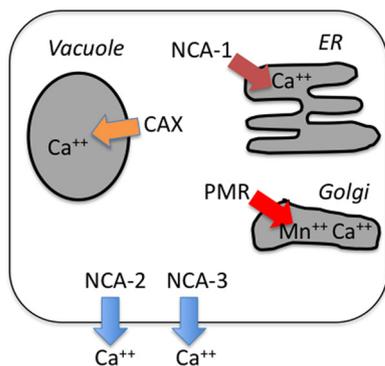


FIG 10 Model of the function of calcium transporters in *N. crassa*. Note that NCA-2 and NCA-3, when tagged with GFP or RFP, are observed in both the plasma membrane and the vacuole. However, the data do not indicate that they are functional in the vacuolar membrane (4).

which calcium and manganese have been omitted. PMR is thus required to either scavenge trace amounts of calcium and manganese from the medium or maintain sufficient levels of calcium and manganese in an intracellular compartment. Interestingly, an increase of the concentration of only one of these divalent cations is sufficient to stimulate the growth rate; i.e., calcium can substitute for manganese and vice versa. However, this is not true for the defects in hyphal morphology. A high concentration of manganese in the medium, but not calcium, partially suppressed the aberrant hyphal branching pattern and the production of atypically large, misshapen conidia. The most likely explanation is that enzymes involved in glycosylation require manganese as a cofactor and that PMR is required to pump manganese into the Golgi compartments where these enzymes reside (2, 16, 53). Support for this hypothesis comes from the recent report that the deletion of OCH1, a 1,6-mannosyltransferase, causes morphological defects in *N. crassa* that are very similar to what we observed for the  $\Delta pmr$  strain (34).

Given the observations that more than 95% of cell calcium is stored in vacuoles (4, 15, 23), we were surprised to find that the  $\Delta pmr$  strain accumulates 80% less calcium than the wild type. In *S. cerevisiae*,  $\Delta pmr1$  strains accumulate more calcium than the wild type, supporting the hypothesis that Pmr1p has a role in calcium efflux in this organism, presumably by pumping calcium into secretory vesicles which then fuse with the plasma membrane (10, 24, 29, 37). The loss of PMR in *N. crassa* may disrupt Golgi function and thereby disrupt the synthesis of compartments in the cell that normally accumulate calcium. We must also consider the possibility that a loss of function, such as an accumulation of normal levels of calcium, may simply be an indirect consequence of the poor growth of the  $\Delta pmr$  strain.

Do the phenotypes of  $\Delta pmr$  and  $pmr^{RIP} \Delta nca-1$  strains provide evidence for or against the hypothesis that calcium plays a key role in polarized growth and/or branching in filamentous fungi? The lack of morphological defects in  $\Delta nca-1$ ,  $\Delta nca-2$ ,  $\Delta nca-3$ , or  $\Delta cax$  strains led us to conclude that these calcium transporters were not essential for a calcium signal involved in polarized growth. In contrast, the  $\Delta pmr$  and  $pmr^{RIP} \Delta nca-1$  strains have severe morphological defects that are only partially suppressed by added manganese. The hyphae are still tubular, with new growth occurring at the tip, albeit at a greatly reduced rate, but the frequency and pattern of branching are highly dependent on PMR and also on NCA-1 in the absence of PMR. Also noteworthy is the phenotype of the  $\Delta pmr \Delta nca-2$  strain, which has a more nearly normal hyphal morphology. The deletion of NCA-2 causes excess calcium to accumulate in *N. crassa* (4), a phenomenon also observed for the  $\Delta pmr \Delta nca-2$  double mutant strain. Thus, there is a correlation: the  $\Delta pmr$  strain has small amounts of calcium and exhibits hyperbranching, while the  $\Delta pmr \Delta nca-2$  strain has elevated concentrations of calcium and suppressed hyperbranching. However, these experiments do not rule out the possibility that the morphological defects are a secondary effect. Insufficient calcium may disrupt secretory pathway functions that in turn cause defects in polarized growth and branching.

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