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Plant pathogenesis-related proteins of the cacao fungal pathogen *Moniliophthora perniciosa* differ in their lipid-binding specificities

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ABSTRACT

Moniliophthora perniciosa is the causative agent of witches' broom disease, which devastates cacao cultures in South America. This pathogenic fungus infects meristematic tissues and derives nutrients from the plant apoplast during an unusually long-lasting biotrophic stage. In order to survive, the fungus produces proteins to suppress the plant immune response. Proteins of the Pathogenesis Related 1 (PR-1)/CAP superfamily have been implicated in fungal virulence and immune suppression. The genome of *M. perniciosa* encodes eleven homologues of plant PR-1 proteins, designated MpPR-1 proteins, but their precise mode of action is poorly understood. In this study, we expressed MpPR-1 proteins in a yeast model lacking endogenous CAP proteins. We show that some members of the MpPR-1 family bind and promote secretion of sterols whereas others bind and promote secretion of fatty acids. Lipid-binding by purified MpPR-1

occurs with micromolar affinity and is saturable *in vitro*. Sterol binding by MpPR-1 requires the presence of a flexible loop region containing aromatic amino acids, the caveolin-binding motif. Remarkably, MpPR-1 family members that do not bind sterols can be converted to sterol binders by a single point mutation in the caveolin-binding motif. We discuss the possible implications of the lipid-binding activity of MpPR-1 family members with regard to the mode of action of these proteins during *M. perniciosa* infections.

Witches' Broom Disease of cacao (WBD) is one of the most devastating plant diseases in Southern America, and a major threat to cacao production, resulting in extensive economic losses in affected countries (1, 2). WBD is caused by the basidiomycete fungus *Moniliophthora perniciosa* (3). This hemibiotrophic fungus has an atypical

prolonged biotrophic stage of up to 90 days, during which it slowly grows in the apoplast of infected plants, inducing conspicuous morphological alterations that culminate with the formation of anomalous structures called “green brooms”. These chlorotic and swollen shoots are formed as a consequence of a hormonal imbalance and intense reprogramming of the plant metabolism induced by the fungal infection (4, 5). The necrotrophic phase of the disease begins upon death of plant tissues, which are invaded by the proliferative mycelia. *M. pernicioso* also infects cacao flowers and fruits, causing swelling and abnormal ripening of the fruits and ultimately death of the infected tissues (1).

A genome draft of *M. pernicioso* (6), a comprehensive transcriptomic analysis of the biotrophic stage of the interaction (4), and a series of functional analyses of *M. pernicioso* proteins provided insights into this intriguing disease (7–10).

Members of the CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1) superfamily of proteins, also known as SCP/TAPS (Sperm-Coating Protein/Tpx-1/Ag5/PR-1/Sc7) proteins, are present throughout the eukaryotic kingdom (11, 12). In plants, pathogenesis-related 1 (PR-1) superfamily members serve markers of an induced defense against pathogens (13). Interestingly, CAP proteins are abundantly expressed by human pathogenic nematodes such as *Schistosoma mansoni* and *Necator americanus* during infection, and were speculated to function as immunomodulators to inhibit host defense (14). A CAP protein from a single isolate of *Fusarium oxysporum* f. sp. *lycopersici*, which can cause disease on both tomato plants and immunodepressed mice, is important in the infection of the animal model. However, this protein is not essential for virulence on tomato (15). Gr-Vap1, a CAP protein from the potato cyst nematode *Globodera rostochiensis*, on the other hand, induces the loss of basal immunity to unrelated pathogens when heterologously expressed in plants (16). In addition, the knock-down of Gr-Vap1 expression reduces the infectivity of nematode, indicating that this protein is important for pathogen-induced suppression of

plant defense. An immunomodulatory function was also suggested for tablysin-15, a CAP protein from the horsefly *Tabanus yao*. Tablysin-15 binds leukotrienes and free fatty acids and thereby might inhibit the proinflammatory effects of leukotrienes that are released from mast cells in the vicinity of the bite (17).

The *Saccharomyces cerevisiae* CAP proteins, the Pry proteins (pathogen related in yeast) function to bind and export sterols and fatty acids out of the cells (18, 19). The sterol binding activity is conserved among many CAP superfamily proteins, since expression of human CRISP2, parasitic SmVAL4, or plant PR-1 rescues the lack of Pry function in yeast, and these proteins bind cholesterol *in vitro* (18, 20, 21). Expression of tablysin-15, however, does not rescue the sterol export phenotype but rescues export of fatty acids (19). The sterol and fatty acid binding and export function of the yeast CAP proteins is confined to the CAP domain, because expression of the CAP domain alone is sufficient to rescue the sterol and fatty acid export phenotype of a *pry1Δ pry2Δ* double mutant, and the CAP domain of Pry1 alone binds sterols and fatty acids at distinct sites *in vitro* (19, 22, 23). Sterol binding by Pry proteins requires the displacement of a flexible loop containing aromatic amino acids, termed the caveolin-binding motif (CBM) within the CAP domain, since point mutations within this motif abolish sterol export and binding, whereas fatty acid binding takes place in the groove between two parallel running helices, $\alpha 1$ and $\alpha 3$ (19, 24). Taken together, these data indicate that CAP proteins may exert an immunomodulatory function through binding hydrophobic ligands, such as sterols or related small hydrophobic compounds and fatty acids and their derivatives such as leukotrienes (19, 23, 25).

The genome sequence of *M. pernicioso* indicates the presence of 11 CAP family members in this phytopathogen (6, 26). Furthermore, gene expression analyses revealed that some of *M. pernicioso* CAP genes, termed *MpPR-1* genes, are highly and specifically expressed during the green broom stage of WBD, and in germinating basidiospores (4, 26). These results suggest that

some MpPR-1 proteins function during the *M. pernicioso* infective process.

In this work, we examine whether MpPR-1 proteins have lipid-binding activity *in vivo*, by complementing yeast Pry mutants, and *in vitro*, through sterol and fatty acid binding assays. Five of the ten MpPR-1 family members tested, MpPR-1c, MpPR-1d, MpPR-1g, MpPR-1j and MpPR-1k complemented the defect in sterol export of yeast mutants lacking Pry function. Consistent with their activity *in vivo*, MpPR-1d and MpPR-1k bound cholesterol *in vitro*. Interestingly, two the MpPR-1 family members that did not bind sterols, MpPR-1e and MpPR-1i, bound fatty acids *in vitro* and they also complemented the block in fatty acid export of mutant cells lacking their endogenous CAP function. Closer inspection of the sequence of the CBM of these four MpPR-1 proteins revealed that proteins that bound sterols have a conserved CBM whereas those that did not bind harbored substitutions of an aromatic residue at position 3 of the CBM. Remarkably, single mutations within the CBM converted a sterol binder into a non-binder and *vice versa*. These data thus substantiate the importance of the CBM for sterol binding and they suggest that the number of CAP proteins present in *M. pernicioso* and their sequence diversity might serve to bind a variety of small hydrophobic ligands to suppress the immune defense of its host.

RESULTS

MpPR-1 proteins export sterols *in vivo*

To determine whether the CAP proteins from the cacao pathogen *M. pernicioso* could bind sterols, we first tested whether they would complement a block in sterol export of yeast cells lacking their endogenous CAP family members Pry1 and Pry2. The yeast Pry proteins bind cholesterol *in vitro* and are required for the export of cholesteryl acetate into the culture supernatant *in vivo* (18). To test whether the expression of the MpPR-1s in *pry1Δ pry2Δ* double mutant cells rescued the defect in cholesterol export, *hem1* and *say1* (*hem1Δ say1Δ*) deficient cells of the indicated genotype were cultivated in the presence of radiolabeled [¹⁴C]-cholesterol overnight. In these experiments, heme deficient cells are being

used because unlike wild-type cells, heme mutants can take up sterols from the media. In addition, these cells are deficient for the sterol deacetylase, *Say1*, and thus secrete cholesteryl acetate into the culture medium (27). Cells were then washed and diluted in fresh medium to allow the export of cholesterol and cholesteryl acetate. Lipids were extracted from the cell pellet (P) and the culture supernatant (S), separated by thin-layer chromatography (TLC), visualized, and quantified by phosphorimaging. The relative percentages of cholesteryl acetate (CA) that was exported by the cells was plotted as an export index (ratio between extracellular CA and the sum of intracellular and extracellular CA). Mutant yeasts expressing *MpPR-1c*, *MpPR-1d*, *MpPR-1g*, *MpPR-1j*, and *MpPR-1k* exported high levels of CA into the culture supernatant, indicating that these *MpPR-1* genes functionally complement the absence of yeast CAP proteins (*pryΔ*) *in vivo* (Fig. 1). The expression of *MpPR-1a*, *MpPR-1b*, *MpPR-1e*, *MpPR-1f*, or *MpPR-1i*, on the other hand, failed to rescue the block in sterol export. *MpPR-1h* could not be tested because the transformants died after initial growth, suggesting that this gene is toxic to yeast growth (data not shown). These results thus indicate that some but not all of the CAP proteins from *M. pernicioso* could bind sterols *in vivo*.

Sequence analysis of the caveolin-binding motif of *MpPR-1* proteins

To understand why some of the CAP proteins from *M. pernicioso* could bind sterols whereas other could not, we analyzed their sequence at the caveolin-binding motif (CBM). Our previous studies had revealed that the CBM is important for sterol binding of the yeast Pry1 protein (24). The hallmark of the CBM is the presence of aromatic amino acids in positions 1, 3, and 8 of the motif (ØxØxxxxØ) (24, 28, 29). While the first aromatic residue is conserved in all MpPR-1 proteins, the aromatic residues at positions 3 and 8 of the motif are only conserved in MpPR-1c, MpPR-1d, MpPR-1g, and MpPR-1j, as revealed by the sequence alignment shown in Fig. 2. MpPR-1k, has an alanine in position 3 of the CBM, which appears to be functional for sterol binding.

Thus, MpPR-1 family members that bound sterols *in vivo* also had a conserved CBM. Conversely, in MpPR-1a, MpPR-1b, MpPR-1e, MpPR-1f and MpPR-1i, which failed to complement the deficiency in the export of cholesteryl acetate, either only position 3 (MpPR-1a, MpPR-1e) or both positions (MpPR-1b, MpPR-1f, MpPR-1i) within the CBM contain non-aromatic residues (Fig. 2). MpPR-1i is somewhat atypical as the loop region containing the CBM appears to be shortened, essentially resulting in a deletion of the residue in position 8 of the sequence motif (Fig. 2).

Role of the CBM in sterol binding by M. perniciosus CAP proteins

To evaluate the functional importance of the aromatic residues at position 3 of the CBM for sterol binding, we generated mutations in MpPR-d, MpPR-1e, MpPR-1i and MpPR-1k. The rationale was to convert non-binding MpPR-1 family members into sterol binding ones, and *vice-versa*. To convert MpPR-1d into MpPR-1e, tyrosine at position 155 of MpPR-1d was mutated to proline (MpPR-1d→e; MpPR-1d^{Y155P}). To convert MpPR-1e to MpPR-1d, proline at position 103 of MpPR-1e was mutated to tyrosine (MpPR-1e→d; MpPR-1e^{P103Y}). To convert MpPR-1k to MpPR-1i, alanine at position 107 of MpPR-1k was mutated to aspartic acid (MpPR-1k→i; MpPR-1k^{A107D}), and to convert MpPR-1i to MpPR-1k aspartic acid at position 112 was mutated to alanine (MpPR-1i→k; MpPR-1i^{D112A}; see Fig. 2, amino acids boxed in red). The mutants were analyzed by the yeast complementation assay and results are depicted in Fig. 3. The conversion of MpPR-1d→e and that of MpPR-1k→i by exchanging the aromatic residues in position 3 of the CBM to either proline (MpPR-1d→e) or aspartic acid (MpPR-1k→i) resulted in a loss of sterol export. Conversely, the introduction of an aromatic residue at position 3 of the CBM of the non-binding MpPR-1e (MpPR-1e→d) and the removal of a negatively charged aspartic acid in MpPR-1i (MpPR-1i→k), resulted in a gain of sterol export phenotype. Thus, non-binding MpPR-1s can be converted to sterol binders by the introduction of an aromatic residue or alanine at position 3

of the CBM, whereas a sterol-binding MpPR-1 can be converted to a non-binder by substitution of the aromatic residues at position 3 of the CBM.

MpPR-1 proteins bind cholesterol in vitro

To test more directly whether the MpPR-1 proteins bind sterols *in vitro*, MpPR-1d and MpPR-1k were expressed as C-terminally polyhistidine tagged versions in *E. coli* and they were affinity purified on nickel agarose beads. Sterol binding was then assessed using increasing concentrations of the radioligand [³H]-cholesterol (0-400 pmol) and a constant concentration of the purified proteins (100 pmol). These experiments revealed a concentration-dependent increase in radioligand binding, indicating that MpPR-1d and MpPR-1k bind cholesterol *in vitro* with both proteins displaying saturable binding (Fig. 4A, B). In accordance with the observation that MpPR-1d→e and MpPR-1k→i mutant versions lost the capacity to export sterols *in vivo*, these mutant versions displayed poor cholesterol binding *in vitro* (Fig. 4A, B). MpPR-1e and MpPR-1i, on the other hand, which did not complement the sterol export phenotype *in vivo*, also displayed poor binding when tested *in vitro*, thus confirming the results of the *in vivo* experiments (Fig. 4C, D). Conversely, the corresponding gain of function mutants, MpPR-1e→d and MpPR-1i→k displayed an increased affinity for sterols also in the *in vitro* assay. Taken together, these data indicate that the sterol export phenotype observed in the *in vivo* assay correlates well with the relative affinity of the respective MpPR-1 family member for sterols as determined *in vitro* and thus reinforce the importance of the aromatic residue in position 3 of the CBM for sterol binding.

Sterol binding specificity of MpPR-1d

To examine the ligand-specificity of MpPR-1d, the ability of various steroids to compete with radiolabeled cholesterol for binding to MpPR-1d, the *M. perniciosus* CAP family member with the highest affinity for cholesterol, was determined using a competitive binding assays (Fig. 5). Addition of unlabeled cholesterol or that of cholesteryl acetate (50 and 500 pmol)

competed with the radiolabeled cholesterol for binding to MpPR-1d, indicating that sterol binding is specific. Additionally, both, the plant sterol sitosterol and the fungal sterol ergosterol efficiently competed with the radiolabeled cholesterol for binding to MpPR-1d (Fig. 5). These results thus indicate that these MpPR-1 family members can bind different types of sterols including those from plants and fungi.

MpPR-1 proteins bind fatty acids in vivo and in vitro

Given that the horsefly CAP protein, tablysin-15 and the yeast Pry1 bind fatty acids, we finally tested whether the four *M. perniciosus* CAP family members, which we analyzed in more detail for sterol binding, MpPR-1d, MpPR-1e, MpPR-1i, and MpPR-1k would bind fatty acids as well (17, 19). Therefore, we first tested whether expression of these MpPR-1 proteins in yeast mutants lacking the two major acyl-CoA synthetases, Faa1 and Faa4, in addition to Pry1 and Pry2, would result in export of fatty acids. *faa1Δ faa4Δ* double mutant cells have previously been shown to export fatty acids into the culture media and this fatty acid export requires the yeast CAP family members, the Pry proteins (19, 30). Quantification of fatty acids exported from *pry1Δ pry2Δ faa1Δ faa4Δ* mutant cells revealed that the expression of either MpPR-1e or MpPR-1i promoted fatty acid export whereas export was reduced to basal levels in cells expressing either MpPR-1d or MpPR-1k (Fig. 6A). These data thus indicate that the MpPR-1 proteins that did not bind or export cholesterol, MpPR-1e and MpPR-1i, promoted fatty acid export, whereas those MpPR-1 proteins that bound and exported cholesterol did not export fatty acids, i.e., MpPR-1d and MpPR-1k.

Fatty acid export by Pry in *faa1Δ faa4Δ* mutant cells is essential as a quintuple mutant lacking both the acyl-CoA synthetases and all three of the Pry proteins is lethal (*pry1Δ pry2Δ pry3Δ faa1Δ faa4Δ*) (19). To examine whether expression of either one of these MpPR-1 proteins would rescue the lethality of this quintuple mutant strain we tested for loss of a *URA3* marked plasmid containing *FAA1*. When cultivated on media containing (5-FOA, 5-fluoroorotic acid), which counterselects for the

presence of *URA3*, cells expressing *MpPR-1e* or *MpPR-1i* were able to grow but not those expressing *MpPR-1d* or *MpPR-1k*. This *in vivo* test thus confirms the results of the fatty acid export assay and indicates that expression of either *MpPR-1e* or *MpPR-1i* but not that of *MpPR-1d* or *MpPR-1k* complements the fatty acid export block imposed by the absence of the endogenous CAP proteins in yeast (Fig. 6B).

To test whether these MpPR-1 proteins would also bind fatty acid *in vitro*, polyhistidine-tagged versions were again expressed in *E. coli* and purified by affinity chromatography. They were then incubated with increasing concentration of [³H]-palmitic acid and binding of the radioligand was quantified. The results of this analysis are consistent with the *in vivo* data and indicate that both MpPR-1e and MpPR-1i bound fatty acids whereas MpPR-1d and MpPR-1k displayed background binding (Fig. 7A). Thus, the MpPR-1 family members that export fatty acids in the *in vivo* assay also bound fatty acids when tested *in vitro*.

To test whether the mutant versions of these proteins could now bind both ligands simultaneously, we performed *in vitro* binding assays with [³H]-cholesterol (50 pmol) alone, [³H]-palmitic acid (50 pmol) alone, and with both radioligands being present simultaneously (each at 50 pmol). The presence of both radioligands in the binding assay resulted in a duplication of the radioactivity associated with the proteins, indicating that all three proteins tested in this assay, Pry1, MpPR-1e→d, and MpPR-1i→k can bind both fatty acids and sterols simultaneously (Fig. 7B).

To assess the fatty acid chain length specificity of MpPR-1i and MpPR-1e, we performed competition binding experiments with unlabeled fatty acids of various chain length. The results of these experiments indicate that long chain fatty acids (C₁₆, C₁₈), but not C₂₀ competed with [³H]-palmitic acid for binding to these proteins (Fig. 8A). Short- (C₁₀), medium- (C₁₂, C₁₄) and very long-chain (C₂₂) fatty acids, on the other hand, failed to compete with the radioligand. The polyunsaturated arachidonic acid, the precursor to eicosanoids, however, bound to both MpPR-1i and MpPR-1e. The acyl chain substrate preference of MpPR-1i and MpPR-1e is thus comparable that that

previously observed for tablysin-15 and Pry1 (17, 19).

DISCUSSION

Witches' broom disease of cacao is caused by *M. perniciosa*, a hemibiotrophic basidiomycete phytopathogen that contains 11 genes from CAP superfamily (26). Based on gene expression analysis, *MpPR-1* genes could have roles in *M. perniciosa* pathogenicity as *MpPR-1c*, *MpPR-1f*, *MpPR-1g*, *MpPR-1h*, *MpPR-1i* and *MpPR-1k* had highest expression in green broom stage, the hallmark of WBD progression (26). CAP homologues of *S. cerevisiae* are required for sterol and fatty acid export in yeast and they bind cholesterol and fatty acids *in vitro* (18, 19). In the present study, we examined whether the MpPR-1 proteins could act as sterol and fatty acid binding and export proteins. Such studies are not possible in *M. perniciosa*, because transformation of this fungus is very inefficient and they are unstable. In addition, *M. perniciosa* cannot be cultivated *in vitro* in its monokaryotic, biotrophic form as it rapidly converts to the dikaryotic, necrotrophic form, which is not of interest to understand its pathogenicity. We thus turned to yeast a model lacking endogenous CAP proteins for the expression and functional characterization of MpPR-1 family members. From the yeast complementation assay, two classes of *MpPR-1* genes could be distinguished: the expression of *MpPR-1c*, *MpPR-1d*, *MpPR-1g*, *MpPR-1j* and *MpPR-1k* cDNAs rescued the sterol export phenotype of yeast mutants lacking their endogenous CAP proteins. On the other hand, *MpPR-1a*, *MpPR-1b*, *MpPR-1e*, *MpPR-1f* and *MpPR-1i* failed to complement the sterol export phenotype. However, expression of the sterol non-binding *MpPR-1e* and *MpPR-1i* complemented the fatty acid export defect of mutant cells lacking their endogenous CAP proteins and they bound fatty acids *in vitro*. These data thus indicate that CAP proteins can bind various types of small hydrophobic compounds such as sterols or fatty acids. Whereas the yeast Pry1 protein binds both of these ligands by two discrete, non-overlapping lipid-binding sites (19). The *M. perniciosa* PR-1 proteins, on the other hand,

bind only one of the two ligands tested here. These MpPR-1 proteins, however, might bind as yet unidentified ligands and this ligand binding and sequestration is likely important for the pathogenicity of the fungus under specific growth conditions.

However, we find no strict correlation between expression of a particular *MpPR-1* family member during the fungal life cycle and its lipid ligand specificity. From the six *MpPR-1* genes up-regulated in green broom stage (*MpPR-1c*, *MpPR-1f*, *MpPR-1g*, *MpPR-1h*, *MpPR-1i* and *MpPR-1k*), three encode PR-1 proteins that bind sterols (*MpPR-1c*, *MpPR-1g*, *MpPR-1k*) (4). Of the remaining, two encode PR-1 proteins that bind fatty acids (*MpPR-1f*, *MpPR-1i*) and one could not be tested due to its toxicity in yeast (*MpPR-1h*). From these data, it thus seems likely that binding of both sterols and fatty acids is important in WBD progression.

The data presented here are consistent with the proposition that CAP proteins constitute a class of secreted lipid binding proteins and that they serve to sequester hydrophobic compounds that are secreted by the respective host to modulate its immune response. Such a possible mode of action of CAP proteins is supported by the observation that yeast mutants lacking Pry function are hypersensitive to eugenol, a member of the alkylbenzene class of compounds present in clove oil, nutmeg, cinnamon, and bay leaf, used as local antiseptic and anesthetic. Importantly, this eugenol sensitivity is proportional to expression levels of Pry proteins and eugenol competes with cholesterol for binding to Pry *in vitro*, indicating that the flexible loop harboring the CBM can not only bind sterols but also other small hydrophobic compounds such as for example eugenol, thereby protecting cells from its toxicity (18, 23). A possible sequestration-based mode of action of CAP family members in plant innate immunity is supported by the fact that plant PR-1 binds sterols *in vitro* and addition of the purified PR-1 protein inhibits growth of the sterol-auxotrophic plant pathogenic oomycete *Phytophthora* (21). Thus, CAP family members secreted by both the host and the pathogen may serve to sequester small

hydrophobic compounds required for the survival of either one (31, 32).

M. perniciosus PR-1 proteins harbor a fold that is characteristic of all CAP proteins, an alpha-beta-alpha sandwich fold (26, 33). The sterol-binding domain within the CAP domain is composed of a flexible loop containing aromatic amino acids (24). This caveolin-binding motif is conserved in the MpPR-1 members that binds sterols, but in members that do not bind cholesterol, the CBM contains substitutions of the important residues at position 3 and 8 of the motif. Remarkably, MpPR-1 proteins that failed to bind sterols could be converted to sterol binders by the exchange of the amino acid in position 3 of the CBM and *vice-versa*.

In contrast to the sterol-binding CBM, the structural requirements for the fatty acid binding pocket are less well defined. The fatty acid-binding pocket of tablysin-15 is composed of two helices ($\alpha 1$ and $\alpha 3$) that run parallel to each other and a shorter third helix ($\alpha 4$) running perpendicular and forming the bottom closure of the fatty acid-binding pocket (17). Our analysis of fatty acid binding by Pry1 revealed that a conserved valine (position 254 of Pry1) at the bottom of the binding groove is important for binding fatty acids (19). This valine is conserved in MpPR-1e (position 114) and substituted by an isoleucine in MpPR-1i (position 121), which both bind fatty acids. However, this valine is also conserved in MpPR-1d (position 170) and substituted by alanine in MpPR-1k (position 121), which both do not bind fatty acids (Fig. 8B). Thus, to define the sequence requirements of this second lipid binding pocket, more structural information of different MpPR-1 family members will be required.

Taken together, the data presented here show that CAP family members from the cacao pathogenic fungus, *M. perniciosus* display different lipid-binding properties. Five of the ten tested MpPR-1 family members (c, d, g, j, k) exported cholesteryl acetate *in vivo* and two of them (d and k) were tested and confirmed to bind cholesterol *in vitro*. In contrast, MpPR-1e and MpPR-1i, which did not export sterols, bound and exported fatty acids. Remarkably, both MpPR-1e and MpPR-1i could be

converted to sterol binders by a single point mutation in their CBM and the respective mutant proteins MpPR-1e \rightarrow d and MpPR-1i \rightarrow k could simultaneously bind both sterols and fatty acids *in vitro*. Thus, the diversification of the CAP family members in *M. perniciosus* may serve to bind a structurally diverse set of hydrophobic ligands at defined stages of the fungal life cycle.

EXPERIMENTAL PROCEDURES

Gene cloning and site-directed mutagenesis
cDNA was synthesized from 1 μ g total RNA using the SuperScript II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. Primers specifically designed for the amplification of each MpPR-1 gene, without the peptide signal sequence, were used in PCR reactions containing cDNA from the basidiomata (*MpPR-1a*, *MpPR-1b*, *MpPR-1d*, *MpPR-1e* and *MpPR-1j*) or green brooms stage of development (*MpPR-1c*, *MpPR-1f*, *MpPR-1g*, *MpPR-1h*, *MpPR-1i* and *MpPR-1k*). Amplicons were subcloned into pGEM T-easy plasmid (Promega, Madison, WI, USA) which was digested with appropriate restriction enzymes. Site-directed mutagenesis was performed by PCR using mismatch primers.

Yeast strains and growth conditions

Yeast strains were cultivated either in rich media (YPD; 1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose; US Biological, Swampscott, MA) or in minimal media (containing 0.67% yeast nitrogen base without amino acids (US Biological), 0.73 g/l amino acids, and 2% glucose). Media supplemented with sterols contained 0.05 mg/ml Tween 80 and 20 μ g/ml cholesterol (Sigma Chemical Co., St. Louis, MO). To bypass heme deficiency, cells were grown in media supplemented with 10 μ g/ml delta-aminolevulinic acid.

Expression and purification of MpPR-1 proteins

MpPR-1 genes and their mutant versions were cloned into the NcoI and XhoI sites of pET22b vector (Novagen, Merck, Darmstadt, Germany), which contains a PelB signal

sequence to direct the secretion of the expressed protein into the periplasmic space. The vector was transformed into *Escherichia coli* BL21 and expression of the polyhistidine-tagged protein was induced by lactose at 24°C overnight. Cells were harvested, lysed, and incubated with Ni-NTA beads (Qiagen, Hilden, Germany). Beads were washed and proteins were eluted with imidazole and quantified. Protein concentration was determined by Lowry assay using Folin reagent and BSA as standard.

In vivo sterol export assay

The yeast sterol export assay is based on the secretion of cholesterol acetate (CA) into the culture supernatant and was performed as previously described (27). Yeast mutants deficient in heme biosynthesis (*hem1Δ*) and lacking the sterol deacetylase *Say1* (*say1Δ*) were cultivated overnight in the presence of 20 μg/ml cold cholesterol and 0.5% Tween 80. Cells were harvested by centrifugation, washed twice with synthetic complete (SC) media, diluted to an OD₆₀₀ of 1.0 into fresh media containing 0.025 μCi/mL [¹⁴C]-cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO) and grown overnight. Cells were washed with SC media and cultivated for another day with non-radiolabeled cholesterol. Cells were centrifuged and lipids were extracted from the cell pellet and the culture supernatant using chloroform/methanol [1:1, (v/v)]. Samples were dried and separated by thin-layer chromatography on silica gel 60 plates (TLC; Merck, Darmstadt, Germany) using the solvent system petroleum ether/diethyl ether/acetic acid [70:30:2, per volume]. TLCs were exposed to phosphorimager screens and radiolabeled lipids were visualized and quantified using a phosphorimager (GE Healthcare, Little Chalfont, UK). The export index was calculated as the ratio of extracellular CA to the sum of intracellular and extracellular CA. Export experiments were performed in triplicate and the export index is given as the mean ± standard deviation of three independent experiments.

Fatty acid export and quantification

Wild-type and mutant cells were grown in SC medium at 30°C. Cells were washed once in cold water, and a fatty acid standard (C17:0; 50 μg; Sigma) was added. Cells were disrupted using glass beads, and lipids were extracted using chloroform/methanol (1:1; per volume) for cellular lipids and with chloroform/methanol/concentrated HCl (1:2:0.03; per volume) for extracellular fatty acids. Fatty acid methyl esters (FAMES) were prepared from 5 A_{600nm} units of yeast cells using boron trifluoride at 100 °C for 45 min and were recovered by extraction with petrol ether. After evaporation, extracts were resuspended in hexane. FAMES were separated with an Agilent 7890A gas chromatograph (GC) equipped with a DB-23 capillary column (30 m 0.25 mm 0.25 m) (Agilent Technologies, Santa Clara, CA). The temperature of the injection port was set to 250°C, its pressure to 26.24 p.s.i. (average velocity, 48.17 cm/s), and the septum purge flow to 3 ml/min. Split injections occurred through an Agilent 7693A automated liquid sampler. The initial oven temperature (100°C; held for 2 min) was increased to 160°C at a rate of 25°C/min and was then increased again to 250°C at 8°C/min. The final oven temperature was held for an additional 4 min. FAMES were detected with a flame ionization detector (Agilent Technologies) set at 270°C with H₂, air, and helium flows set at 30, 400, and 27.7 ml/min, respectively. FAMES were quantified relative to the internal standard, and the relative response factor for each FAME was determined from a four-level calibration curve (r^2 , 0.999).

In vitro lipid binding and competition assay

To determine lipid binding *in vitro*, a radioligand-binding assay was performed as described previously (18, 34, 35). Purified protein (100 pmol) in binding buffer (20 mM Tris, pH 7.5, 30 mM NaCl, 0.05% Triton X-100) was incubated with [³H]-cholesterol or [³H]-palmitic acid (0–400 pmol) for 1 h at 30°C. The protein was then separated from the unbound ligand by adsorption to Q-sepharose beads (GE Healthcare); beads were washed with washing buffer (20 mM Tris pH 7.5), proteins were eluted (20 mM Tris, pH 7.5, 1 M NaCl), and the radioligand was quantified by scintillation counting. To determine non-

specific binding, the binding reaction was performed without the addition of protein into the binding assay. For competition binding assays, unlabeled sterols or fatty acids (Sigma Chemical Co., St Louis, MO) were included in the binding reaction together with an equal concentration to the respective radiolabeled ligand (50 pmol).

At least two independent experiments were performed under each experimental condition and data is reported as the mean \pm standard deviation. Statistical significance of data was analyzed by a multiple Student's t test (Prism, GraphPad Software, La Jolla, CA).

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Author contributions: R. D. conducted most of the experiments, analyzed the results, and generated the figures. O. E. A. helped in fatty acid binding and export experiments. R. M. B. extracted fungal RNA and generated the cDNA clones. P. J. P. L. T., J. M. C. M., R. M. B., R. D., G. A. G. P., and R. S. conceived the idea for the project and analyzed the data. R. M. B., R. D., J. M. C. M., G. A. G. P., and R. S. wrote and edited the manuscript.

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FOOTNOTES

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The abbreviations used are: FAMES, fatty acid methyl esters; TLC, thin-layer chromatography;

FIGURE LEGENDS

FIGURE 1. Some *MpPR-1* family members complement the sterol export defect of yeast mutants lacking *Pry* function. (A) Heme and *Say1* deficient double mutant cells (*hem1Δ say1Δ*) containing either no plasmid (-), an empty plasmid (Empty), a plasmid-borne copy of *Pry1* or of the indicated *MpPR-1* family member were cultivated in the presence of radiolabeled [¹⁴C]-cholesterol. Lipids were extracted from the cell pellet (P) and the culture supernatant (S), separated by thin layer chromatography (TLC) and visualized by phosphorimaging. The position of free cholesterol, FC; cholesteryl acetate, CA; sterol esters, STE; and that of unidentified lipids * are indicated to the left. The experiment was repeated 5-times with similar results. (B) Quantification of the export of cholesteryl acetate (CA). The export index indicates the relative levels of CA exported by the cells (the ratio between extracellular CA and the sum of intracellular and extracellular CA). Data represent mean ± SD of 5 independent experiments. *Asterisks* denote statistical significance of the export phenotype relative to *hem1Δ say1Δ* double mutant cells (***, *p* < 0.0001). n.s.; not significant.

FIGURE 2. Comparison of the caveolin-binding motif (CBM) of the eleven *MpPR-1* family members from *M. perniciosa*. The position of aromatic amino acids (ø) within the CBM is indicated at the top. Residues in red squares at position 3 of the motif mark the amino acids that were mutated. The aromatic amino acid in position 8 of the CBM is conserved only in few family members. Proteins selected for further analysis are highlighted in yellow, *MpPR-1d*, *MpPR-1e*, *MpPR-1i*, and *MpPR-1k*.

FIGURE 3. Single point mutations in the CBM convert MpPR-1s that do not export sterols into sterol exporters. (A) Substitutions of aromatic amino acids in the third position of the CBM convert the sterol exporting MpPR-1d and MpPR-1k into non-exporting mutant versions (MpPR-1d→e and MpPR-1k→i). Conversely, introduction of an aromatic residue at position 3 of the CBM results in the conversion of the non-sterol exporting MpPR-1e and MpPR-1i into gain of function versions (MpPR-1e→d and MpPR-1i→k). *hem1Δ say1Δ* double mutant cells expressing the indicated wild-type (wt) or mutant version of MpPR-1 proteins were radiolabeled with [¹⁴C]-cholesterol. Lipids were extracted from the cell pellet (P) and the culture supernatant (S) and separated by TLC. The position of free cholesterol, FC; cholesteryl acetate, CA; steryl esters, STE; and that of an unidentified lipid * are indicated to the left. The experiment was repeated 3-times with similar results. (B) The export index was plotted as the ration between exported to total CA. Data represent mean ± SD of 3 independent experiments. Asterisks denote statistical significance of the export phenotype (***, $p < 0.0001$).

FIGURE 4. MpPR-1 bind cholesterol *in vitro*. (A-D) MpPR-1 family members that export sterols *in vivo* also bind sterols *in vitro*. Sterol binding by the indicated MpPR-1 wild-type (wt) protein and the respective mutant versions was assessed using 100 pmol of purified protein and an increasing concentration of [³H]-cholesterol (0-400 pmol). The protein was separated from unbound ligand by adsorption to an anion-exchange matrix and bound radioligand was quantified by scintillation counting. Background binding was recorded by performing the binding assay in the absence of protein. Data represent mean ± SD of 3 independent experiments. n.d.; not determined.

FIGURE 5. MpPR-1d binds cholesteryl acetate, sitosterol and ergosterol *in vitro*. Binding specificity of MpPR-1d was assessed in a competition binding assay in which an unlabeled sterol competes with [³H]-cholesterol for binding to the protein. Purified MpPR-1d protein (100 pmol) was incubated with 50 pmol of [³H]-cholesterol and either an equal concentration (50 pmol) or an excess (500 pmol) of the indicated sterol. Data represent mean ± SD of 2 independent experiments. Asterisks denote statistical significance relative to the control containing only the radiolabeled cholesterol and purified MpPR-1d (**, $p < 0.001$; *, $p < 0.01$).

FIGURE 6. *MpPR-1* family members that do not export sterols export fatty acids. (A) Wild-type (wt), *faa1Δ faa4Δ* double mutant and *pry1Δ pry2Δ faa1Δ faa4Δ* quadruple mutant cells expressing the indicated *MpPR-1* family members were cultivated in minimal medium overnight at 30°C. Lipids were then extracted from the cell pellet and the culture supernatant and fatty acids were quantified by GC-MS. Values are plotted as export index representing the amount of exported fatty acids relative to total fatty acids present in the cell pellet and the supernatant. Data represent mean ± SD of 3 independent experiments. Asterisks denote statistical significance relative to the *faa1Δ faa4Δ* double mutant (**, $p < 0.001$). n.s.; not significant. (B) MpPR-1 family members that do not export fatty acids do not provide the essential function of CAP proteins in cells that export fatty acids. Wild-type and quintuple mutant cells lacking Pry function and the acyl-CoA synthetases Faa1 and Faa4 expressing a plasmid borne copy of either Pry1 or the indicated MpPR-1 family member were serially diluted 10-fold and spotted on plates with the indicated media. The *URA3*-marked plasmid-borne copy of *FAA1* present in these cells cannot be lost on 5-FOA media in cells expressing *MpPR-1d* or *MPPR-1k*, the two MpPR-1 family members that do not export or bind fatty acids.

FIGURE 7. MpPR-1 family members that export fatty acids *in vivo* also bind fatty acids *in vitro*. (A) Fatty acid binding to the indicated MpPR-1 family members was assessed by an *in vitro* binding assay with purified protein (100 pmol) and an increasing concentration of [³H]-palmitic acid (0-

400 pmol). The protein was separated from unbound ligand by adsorption to an anion-exchange matrix and bound radioligand was quantified by scintillation counting. Background binding was recorded by performing the binding assay in the absence of protein. (B) CAP family members can bind both sterols and fatty acids simultaneously. Radioligand binding by yeast Pry1 and the respective gain of function versions of MpPR-1, MpPR-1e→d, and MpPR-1i→k, was assessed using 100 pmol of purified protein and either [³H]-cholesterol (50 pmol), [³H]-palmitic acid (50 pmol), or both [³H]-cholesterol and [³H]-palmitic acid (each at 50 pmol) in the binding reaction. Data represent mean ± SD of 3 independent experiments.

FIGURE 8. MpPR-1 family members bind saturated long chain and polyunsaturated fatty acids. (A) Fatty acid binding specificity was assessed by a competition binding assay in which an equal concentration (50 pmol) of the unlabeled fatty acid of the indicated chain length and degree of unsaturation competes with the radiolabeled [³H]-palmitic acid (50 pmol) for binding to the protein (100 pmol). Data represent mean ± SD of 3 independent experiments. *Asterisks* denote statistical significance relative to the control containing only the radiolabeled palmitic acid and purified MpPR-1i or MpPR-1e, respectively ((***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.01$). n.s.; not significant. (B) Sequence alignment around the fatty acid-binding pocket. The sequence of tablysin-15, yeast Pry1, and the *M. perniciosus* MpPR-1 family members analyzed in more detail are shown. Numbering on the top corresponds to the amino acid positions in Pry1. Valine at position 254 of Pry1 (boxed in green) is important for fatty acid binding (19). Key residues shown in colors are those forming the fatty acid binding pocket in tablysin-15 (17). Panel B adapted from (19).

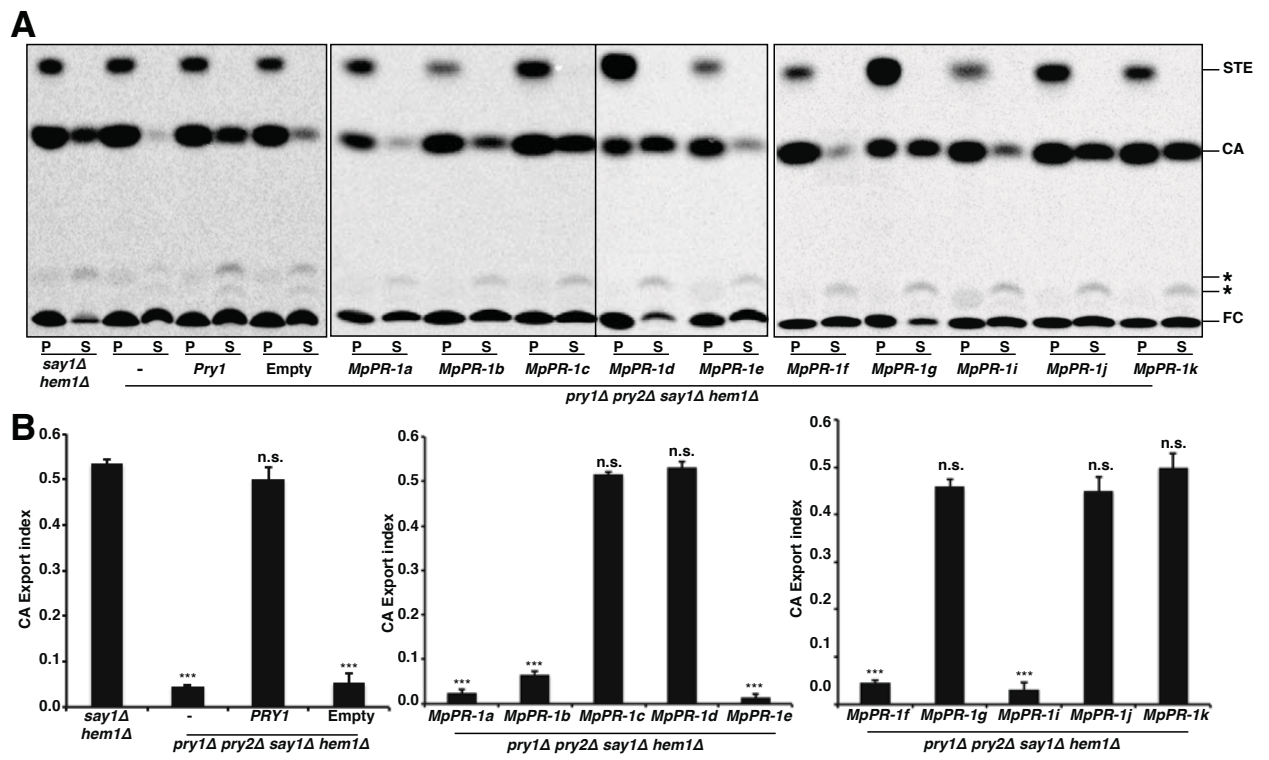


FIGURE 1

Position CBM	1	3	3	3	3	3	8	15							
	Ø	X	Ø	X	X	X	Ø								
<i>MpPR-1a/111-121</i>	Y	N	S	A	N	P	A	Y	N	-	-	-	-	H	W
<i>MpPR-1b/322-332</i>	Y	D	S	N	N	P	K	A	S	-	-	-	-	H	F
<i>MpPR-1c/133-147</i>	Y	D	Y	Q	H	P	G	F	S	M	S	T	G	H	F
<i>MpPR-1d/153-167</i>	Y	D	Y	N	K	P	G	F	S	T	A	T	G	H	F
<i>MpPR-1e/101-111</i>	Y	D	P	N	N	P	Q	Y	S	-	-	-	-	H	F
<i>MpPR-1f/100-110</i>	Y	N	P	Q	N	P	E	S	S	-	-	-	-	S	W
<i>MpPR-1g/96-107</i>	Y	N	Y	-	-	P	H	V	S	E	A	T	-	P	W
<i>MpPR-1h/184-194</i>	Y	G	G	D	D	G	L	L	D	-	-	-	-	H	L
<i>MpPR-1i/110-118</i>	F	P	D	D	R	S	I	-	-	-	-	-	-	P	W
<i>MpPR-1j/144-158</i>	Y	D	Y	D	D	P	H	F	S	N	S	T	G	H	F
<i>MpPR-1k/105-118</i>	F	N	A	M	I	K	A	W	I	P	D	D	-	P	Y

FIGURE 2

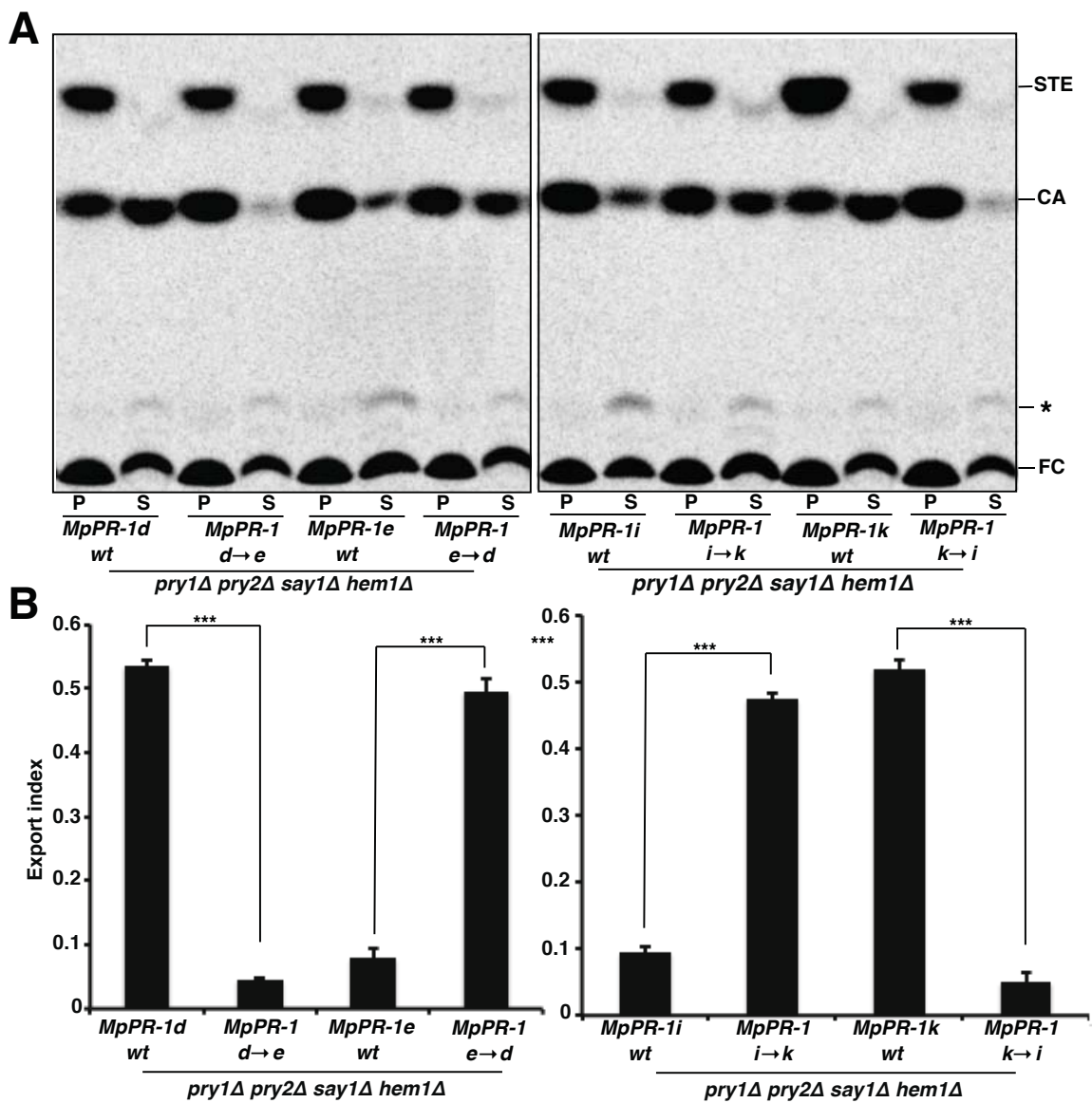


FIGURE 3

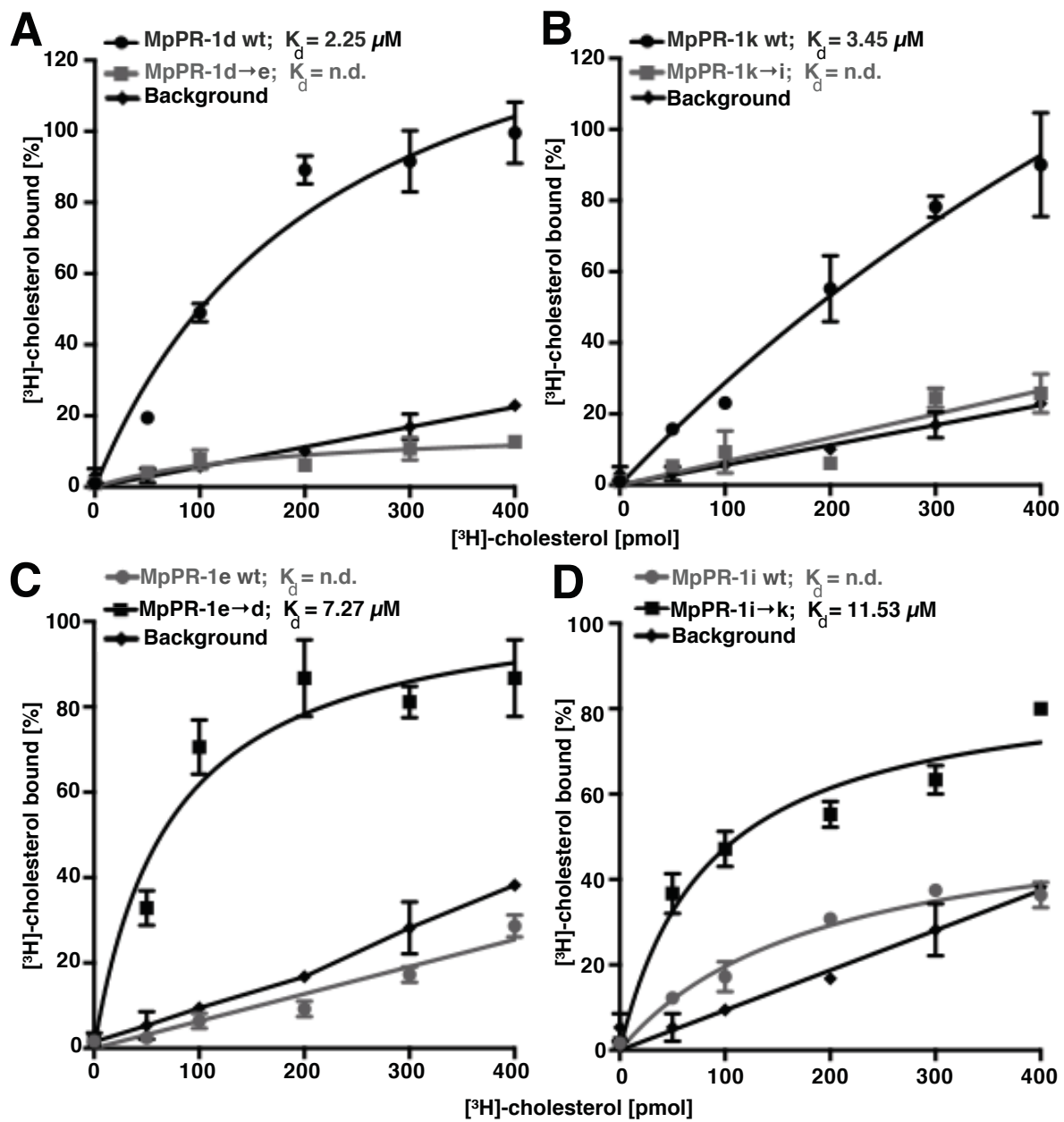


FIGURE 4

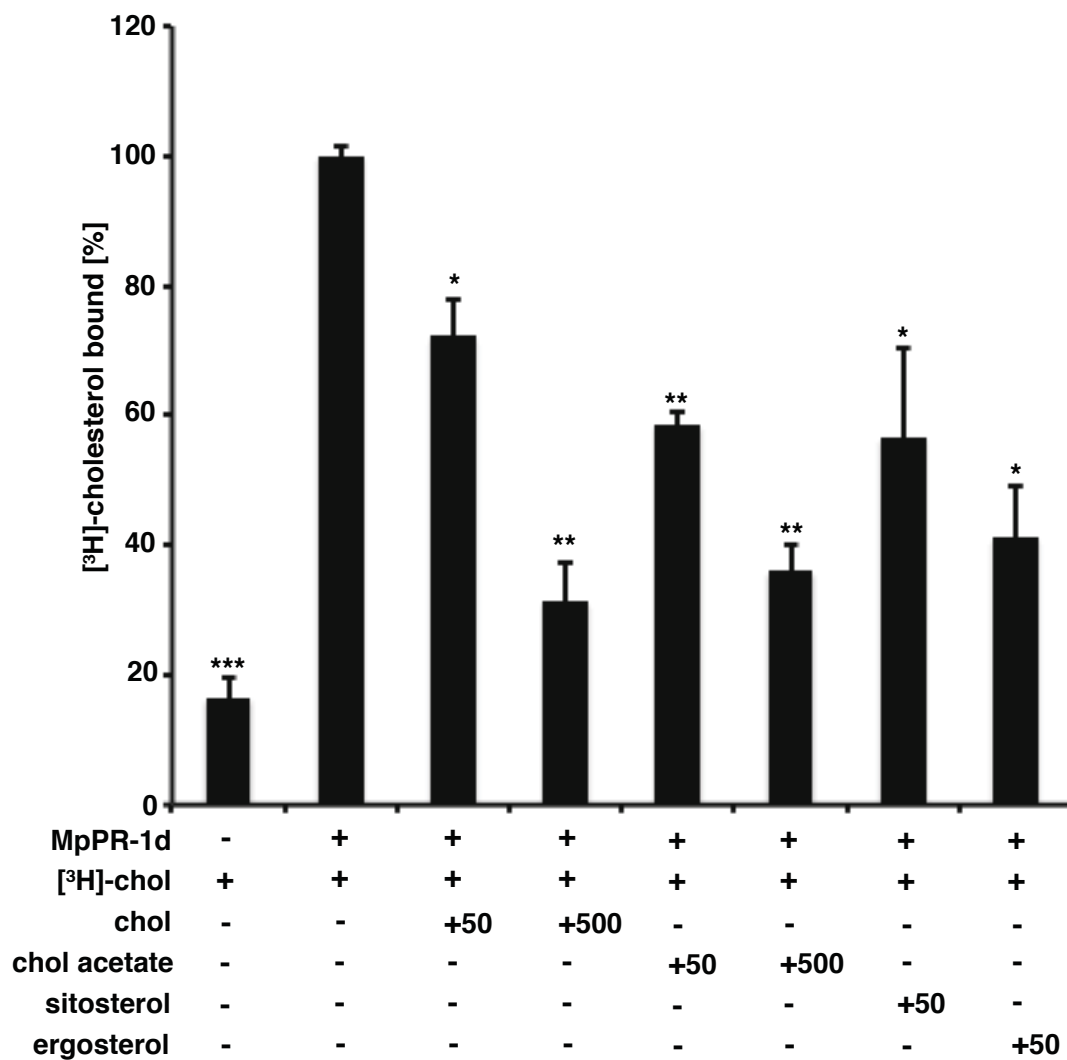


FIGURE 5

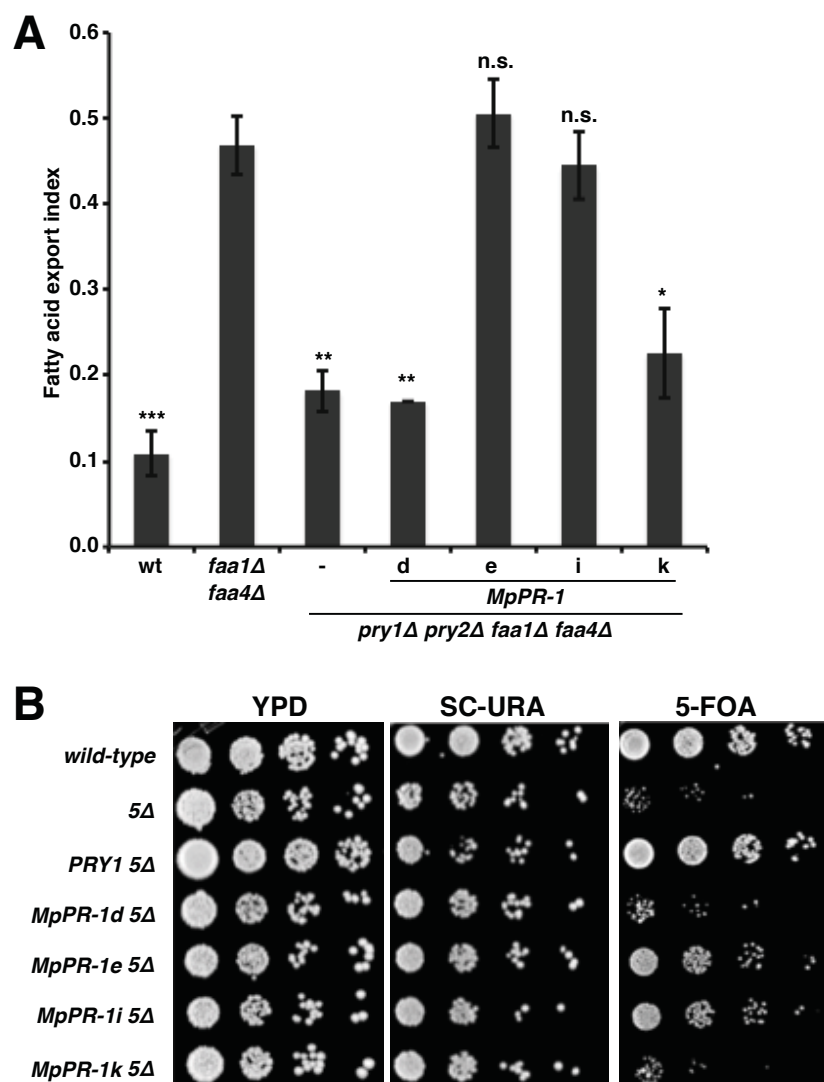


FIGURE 6

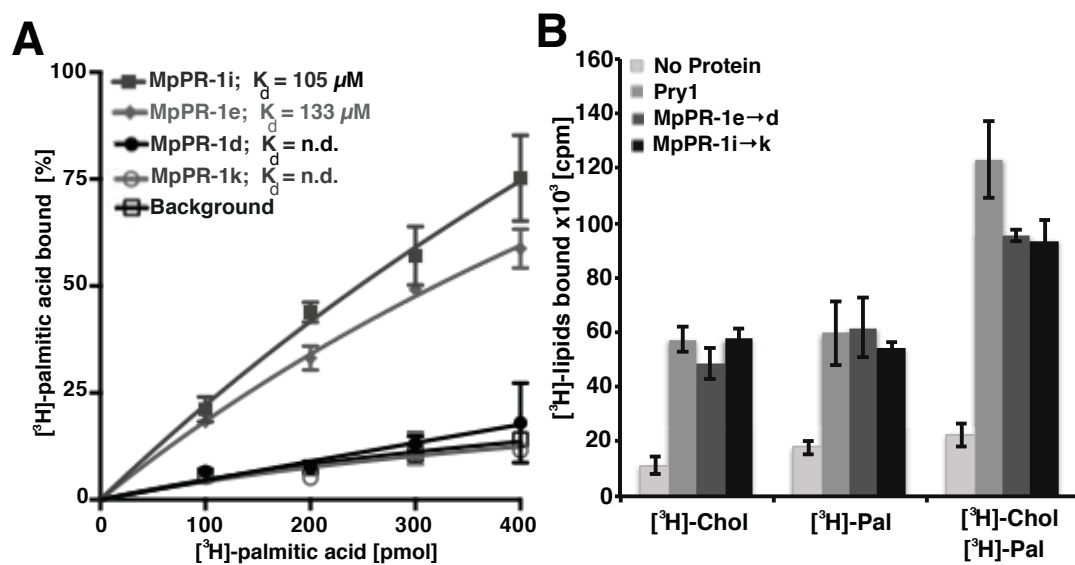


FIGURE 7

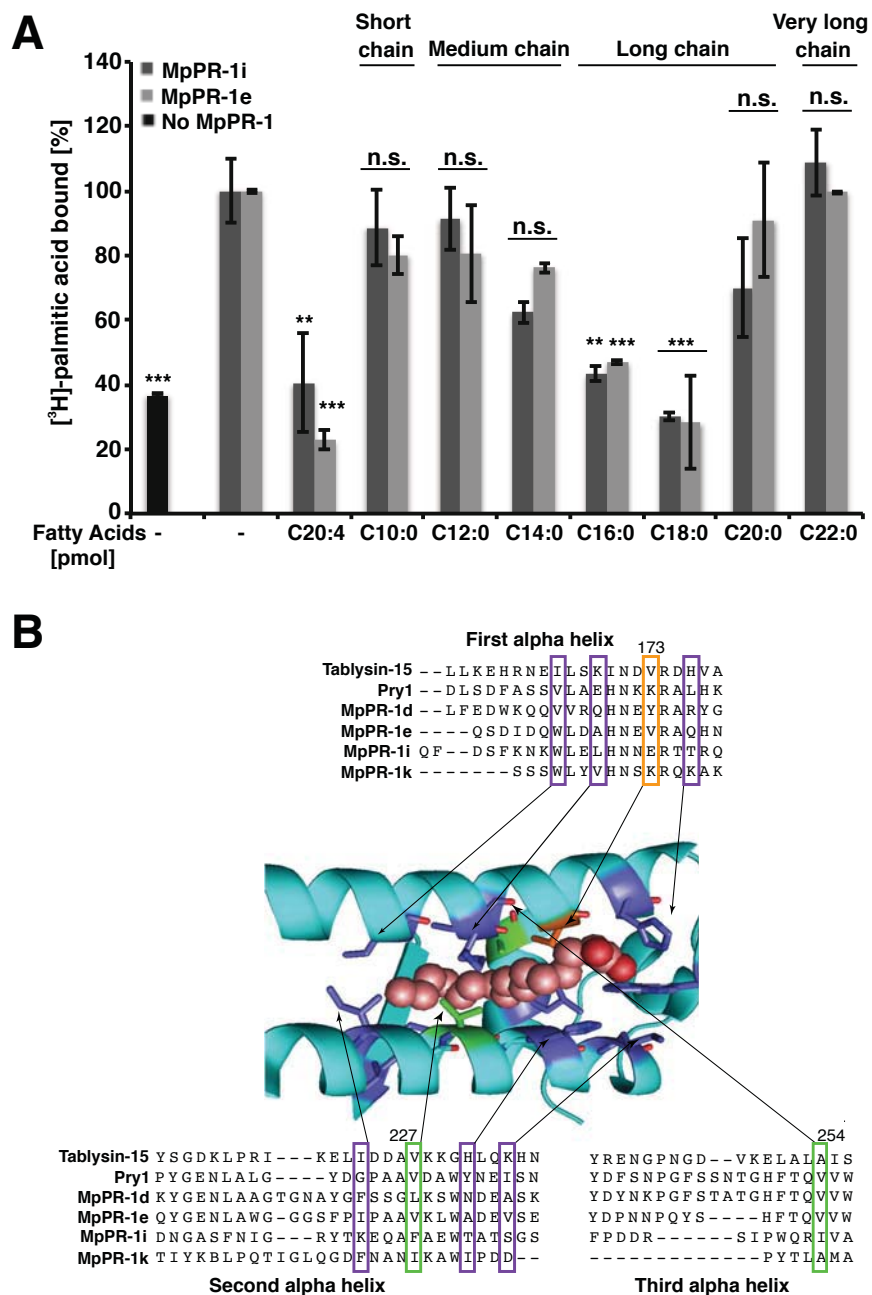


FIGURE 8