

# The sterol-binding activity of PATHOGENESIS-RELATED PROTEIN 1 reveals the mode of action of an antimicrobial protein

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## SUMMARY

Pathogenesis-related proteins played a pioneering role 50 years ago in the discovery of plant innate immunity as a set of proteins that accumulated upon pathogen challenge. The most abundant of these proteins, PATHOGENESIS-RELATED 1 (PR-1) encodes a small antimicrobial protein that has become, as a marker of plant immune signaling, one of the most referred to plant proteins. The biochemical activity and mode of action of PR-1 proteins has remained elusive, however. Here, we provide genetic and biochemical evidence for the capacity of PR-1 proteins to bind sterols, and demonstrate that the inhibitory effect on pathogen growth is caused by the sequestration of sterol from pathogens. In support of our findings, sterol-auxotroph pathogens such as the oomycete *Phytophthora* are particularly sensitive to PR-1, whereas sterol-prototroph fungal pathogens become highly sensitive only when sterol biosynthesis is compromised. Our results are in line with previous findings showing that plants with enhanced PR-1 expression are particularly well protected against oomycete pathogens.

**Keywords:** PR-1, CAP protein, antimicrobial protein, *Phytophthora*, sterol binding, plant immunity.

## INTRODUCTION

PATHOGENESIS-RELATED PROTEIN 1 (PR-1) was discovered as the most abundant of several extracellular proteins expressed in pathogen-challenged *Nicotiana tabacum* (tobacco) plants (Gianinazzi *et al.*, 1970; Van Loon and Van Kammen, 1970). PR-1 accumulated locally and systemically, suggesting an involvement in systemic acquired resistance that represents a form of plant immunization (Van Loon *et al.*, 2006). Whereas functions could be assigned to other members of the original PR protein families (Kauffmann *et al.*, 1987; Legrand *et al.*, 1987; Van Loon and Van Strien, 1999), the mode of action of PR-1 remained elusive. In line with an immunity-related function, PR-1 orthologs of different plant species exhibited *in vitro* antimicrobial activity (Niderman *et al.*, 1995; Rauscher *et al.*, 1999; Kiba *et al.*, 2007; Li *et al.*, 2009; Zhu *et al.*, 2012) and transgenic tobacco plants with enhanced PR-1 expression were more resistant to disease (Alexander *et al.*, 1993; Kiba *et al.*, 2007). PR-1 is a member of the CAP protein family [cysteine-rich secretory protein (CRISP),

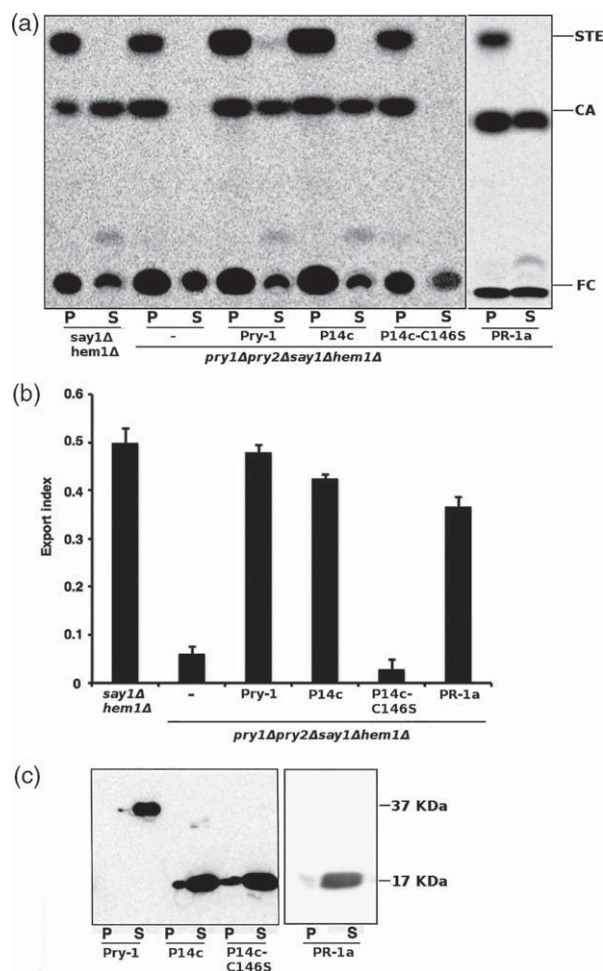
antigen 5, and pathogenesis-related 1], also known as the SCP family (sperm coating protein family). CAP proteins share a domain of approximately 150 amino acids (Gibbs *et al.*, 2008; Cantacessi *et al.*, 2009; Figure S1) that adopts a unique  $\alpha$ - $\beta$ - $\alpha$  sandwich fold to create a large central cavity (Fernández *et al.*, 1997; Szyperski *et al.*, 1998; Serrano *et al.*, 2004; Asojo *et al.*, 2005). The biochemical activity of CAP proteins has remained a mystery until the recent finding that yeast CAP proteins bind sterols (Choudhary and Schneider, 2012). Here, we reinvestigate the biochemical activity of PR-1 proteins and link this activity to the mode of action as an antimicrobial protein.

## RESULTS AND DISCUSSION

Tobacco PR-1a (*Nt*PR1a) that belongs to the originally described group of PR-1 proteins (Gianinazzi *et al.*, 1970; Van Loon and Van Kammen, 1970) and the closely related *Solanum lycopersicum* (tomato) P14c protein (*Sl*P14c) were chosen as candidates for biochemical analysis. PR-1a

encodes an acidic secreted protein and P14c encodes a basic PR-1 protein that is targeted to the vacuole. The two proteins share 67% sequence identity (Figure S1). The sterol-binding capacity of PR-1 proteins was analyzed in two independent ways. First, P14c and PR-1a were tested for genetic complementation of the yeast sterol export-deficient mutant *pry1Δpry2Δ*. Second, the two PR-1 proteins were tested for direct *in vitro* sterol-binding activity. The yeast CAP proteins Pry1 and Pry2 are required for the secretion of cholesteryl acetate that accumulates intracellularly in heme-deficient cells lacking the sterol deacetylase Say (Choudhary and Schneider, 2012). Yeast cells were fed with [<sup>14</sup>C]cholesterol and the export of labeled cholesteryl acetate into the medium was quantified. Expression of P14c or PR-1a both rescued the defect in export of cholesteryl acetate of the *pry1Δpry2Δ* double mutant (Figure 1a). Quantification of export rates calculated as ratios between the extracellular and the sum of intra- and extracellular cholesteryl acetate revealed that expression of PR-1 proteins resulted in an export index similar to Pry1 complemented yeast cells, whereas the *pry1Δpry2Δ* mutant had a more than eightfold lower export index (Figure 1b). Hence, both PR-1 proteins rescued the deficiency of the *pry1Δpry2Δ* mutant in exporting cholesteryl acetate, suggesting that PR-1 proteins bind to cholesteryl acetate in the secretory pathway and mediate its export. In line with a specific protein–sterol interaction, a mutant version with a single amino acid substitution, P14c-C146S, failed to complement the deficiency in the export of cholesteryl acetate (Figure 1), although it was expressed and secreted at a similar level as P14c, PR-1a and Pry1 (Figure 1c). An analogous mutation in the Pry1 protein (C279S) was previously shown to negatively affect the binding of sterols without causing major changes in the secondary structure, compared with the wild-type protein (Choudhary and Schneider, 2012).

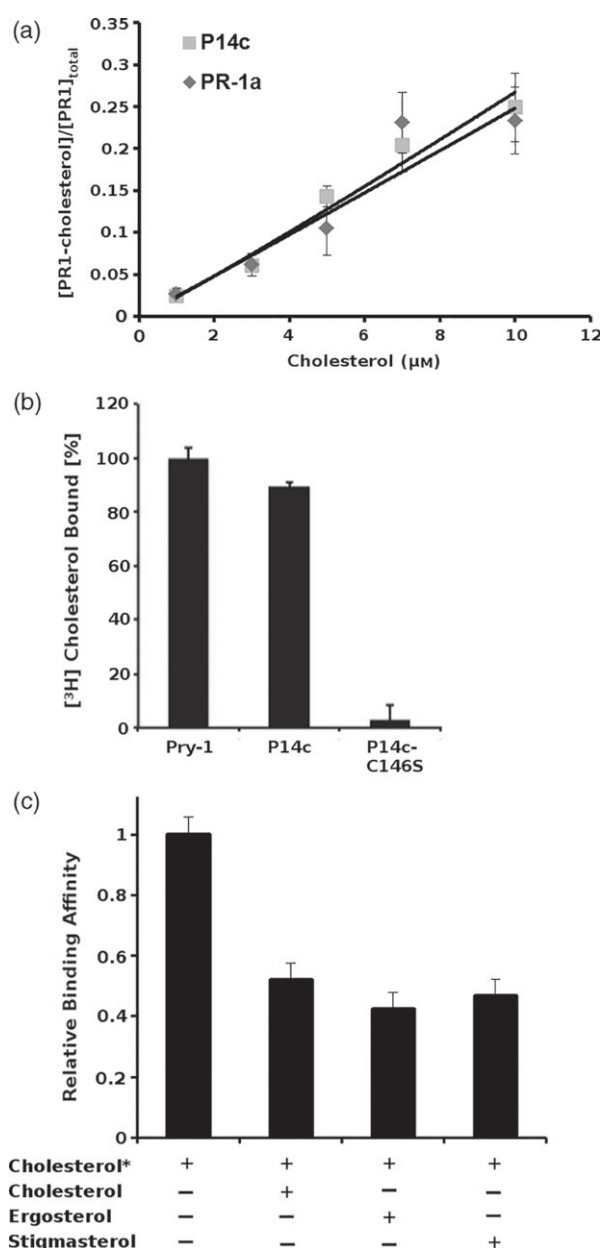
To produce recombinant PR-1 proteins, cDNAs lacking a signal peptide sequence were expressed in *Escherichia coli* as C-terminal His<sub>6</sub>-tagged fusion proteins. PR-1 proteins were purified from bacterial extracts via nickel affinity chromatography (Figure S2). To directly assess the binding of sterols *in vitro*, purified P14c or PR-1a were incubated together with [<sup>3</sup>H]cholesterol, and bound radio ligand was quantified by scintillation counting after the protein was separated from the unbound ligand by adsorption to anion exchange beads. Binding analysis with increasing concentrations of [<sup>3</sup>H]cholesterol showed nearly identical cholesterol-binding kinetics for both P14c and PR-1a with an equilibrium dissociation constant  $K_d$  of 28  $\mu$ M (Figure 2a). P14c and the yeast Pry1 protein had similar cholesterol-binding capacity, whereas the mutant version P14c-C146S completely lost the cholesterol-binding activity (Figure 2b). The binding specificity of P14c was analyzed in competition assays with other sterols (Figure 2c). Both the fungal-specific ergosterol and the natural phytosterol,



**Figure 1.** *Solanum lycopersicum* (tomato) P14c and *Nicotiana tabacum* (tobacco) PR-1a rescue the defect in cholesterol acetate export of the yeast *pry1Δ pry2Δ* mutant. (a) Heme- (*hem1Δ*) and say1-deficient (*say1Δ*) yeast strains of the indicated genotypes were radiolabeled with [<sup>14</sup>C]cholesterol. Lipids extracted from the cell pellet (P) and the culture supernatant (S) were separated by thin-layer chromatography. The position of free cholesterol (FC), cholesteryl acetate (CA) and sterol esters (STE) is indicated on the right. (b) The ratio between exported to total cholesteryl acetate (CA) is plotted as the export index. Data represent means  $\pm$  SDs of four independent experiments. (c) Immunoblot analysis of yeast genotypes for expression and secretion of yeast Pry1-His<sub>6</sub>, P14c-His<sub>6</sub>, P14c-C146S-His<sub>6</sub> and PR-1a-His<sub>6</sub>. P, proteins in the cell pellet; S, proteins in the culture supernatant.

stigmasterol, competed as efficiently with the radiolabeled cholesterol for binding as the unlabeled cholesterol. Thus, P14c did not show a binding preference for a specific sterol.

The antimicrobial activity of purified PR-1 proteins was tested with *Phytophthora brassicae* that belongs to the stramenopila class of oomycetes, which includes many important plant pathogens (Roetschi *et al.*, 2001). Five hundred zoospores were incubated with increasing quantities of either purified P14c or PR-1a (Figure 3a). The growth of *P. brassicae* was strongly inhibited by the addition of PR-1 proteins. Quantification of growth based on total fatty acid



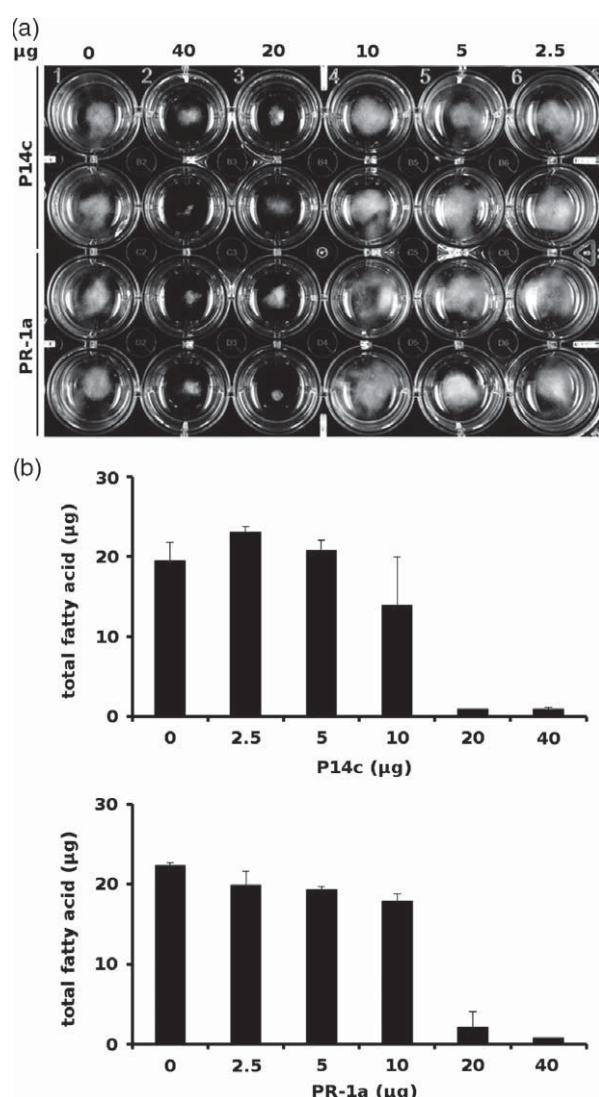
**Figure 2.** *In vitro* binding of cholesterol by PR-1 proteins. (a) Purified P14c or PR-1a proteins (100 pmol) were incubated with increasing concentrations of [<sup>3</sup>H]cholesterol and the protein-bound radio ligand was quantified by scintillation counting. Data represent means  $\pm$  SDs of two independent experiments. (b) Comparison of cholesterol-binding activity of yeast Pry1, P14c and P14c-C146S: 100 pmol of purified protein was incubated with 50 pmol of free radiolabeled cholesterol. Results are plotted relative to the ligand binding of Pry1. Data represent means  $\pm$  SDs of three independent experiments. (c) Sterol-binding specificity of P14c: 100 pmol of purified P14c was incubated with 50 pmol of [<sup>3</sup>H]cholesterol, and binding of the radio ligand competed with 50 pmol of unlabeled sterols. Data represent means  $\pm$  SDs of two independent experiments.

content per well revealed that 20  $\mu$ g of PR-1 protein caused a 96% (P14c) and 90% (PR-1a) reduction in growth of *P. brassicae* (Figure 3b); 20  $\mu$ g of PR-1 protein corresponds

to a concentration of 1.1  $\mu$ M. Hence, 2.2 nmol of PR-1 protein per *P. brassicae* germling was sufficient to inhibit growth. As both tomato P14c and tobacco PR-1a had very similar biochemical properties, we focused on P14c for testing the relationship between sterol-binding and antimicrobial activity. The growth inhibitory activity of purified P14c towards *P. brassicae* was analyzed in the presence of increasing concentrations of free cholesterol (Figure 4a). The basic sterol concentration in the standard cleared V8 medium, as determined by GC-MS, was 145 nM. Growth inhibition by P14c was significantly reduced at a concentration of free cholesterol of 2.5  $\mu$ M, and completely abolished at 10  $\mu$ M. Hence, the addition of free cholesterol titrated P14c antimicrobial activity. No growth-promoting effect of cholesterol was observed in the absence of P14c (Figure S3). The loss of sterol-binding activity of the mutant P14c-C146S protein (Figures 1b and 2b) correlated with a loss of antimicrobial activity (Figure 4b). These results clearly show that the antimicrobial activity is based on the sterol-binding activity of P14c. Microscopical analysis of *P. brassicae* treated with P14c revealed severely stunted hyphal growth compared with the untreated control (Figure 4c). The genus *Phytophthora* and other members of the oomycete order Peronosporales are sterol auxotrophs (Nes, 1987). They lack sterol biosynthetic activity and accumulate sterols available from their environment in their membranes (Gonzales and Parks, 1981). The nutritional dependence on external sterol supplies is expected to make *Phytophthora* especially vulnerable to perturbation by sterol-binding proteins. Indeed, P14c was much less effective in inhibiting the growth of sterol prototrophic fungi. The growth of the fungal pathogens *Aspergillus niger* and *Botrytis cinerea* was unaffected by concentrations of P14c that effectively blocked the growth of *P. brassicae* (Figure 5a,b). Both fungi became sensitive to P14c, however, when fungal sterol biosynthesis was reduced by the addition of a sublethal dose of the fungicidal sterol biosynthesis inhibitor difenoconazole. The addition of 20  $\mu$ g of P14c to media containing 10 pmol difenoconazole enhanced growth inhibition of *B. cinerea* from 13 to 84%, and that of *A. niger* from 27 to 86% (Figure 5b). Hence, the susceptibility of filamentous pathogens to PR-1 proteins appears to depend on their sterol biosynthetic capacities. A similar argument may explain why plant cells are not sensitive to PR-1. Much higher effective PR-1 concentrations were reported for the inhibition of fungal pathogens (Zhu *et al.*, 2012). The enhanced P14c sensitivity of sterol-compromised fungi further strengthens the conclusion that P14c antimicrobial activity is linked to sterols.

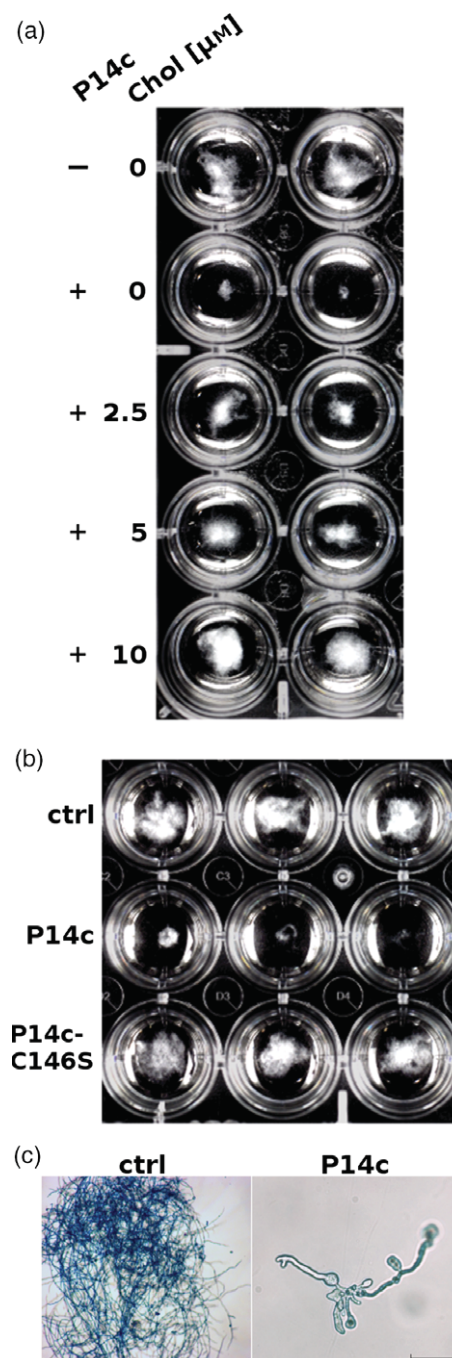
Two non-exclusive options may explain how PR-1 interferes with sterol function in microbes. Either PR-1 proteins bind sterols in the media to interfere with their uptake or they cause damage by directly acquiring sterols from pathogens. In the latter case PR-1 proteins would have to



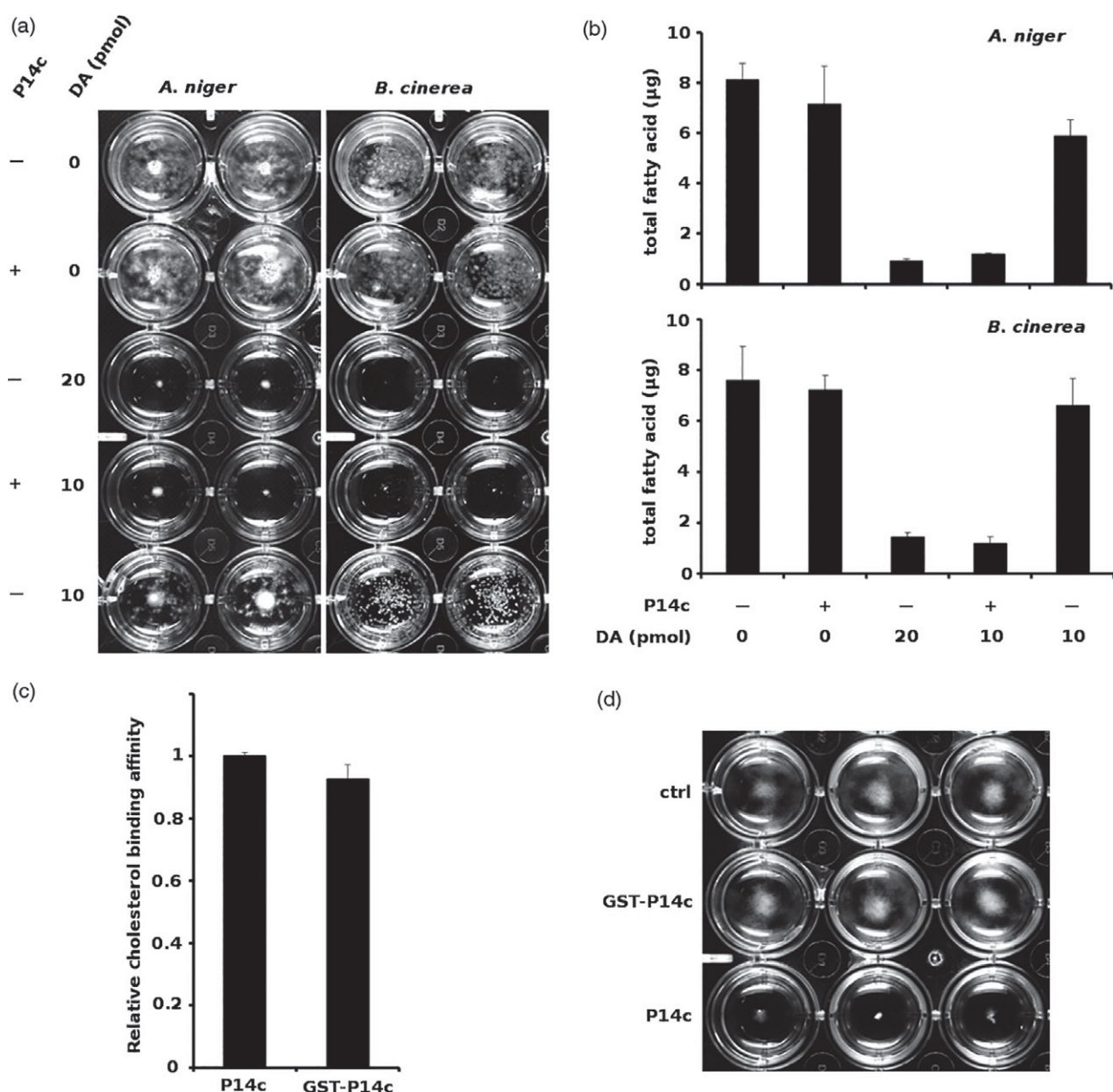


**Figure 3.** Test of PR-1 proteins for antimicrobial activity. (a) Five hundred zoospores of *Phytophthora brassicae* were incubated with increasing quantities of either P14c or PR-1a protein. The experiment was repeated four times with similar results. Pictures were taken 5 days after germination. (b) Quantification of growth inhibition based on total fatty acid content per well.

pass the cell wall to gain access to sterols in the microbial plasma membrane. Based on their low molecular weight of below 20 kDa and their compact structure, secreted PR-1 proteins can diffuse through the cell walls of plants to accumulate in the apoplastic space, and have also been detected within fungal cell walls (Cordier *et al.*, 1998; Van Loon *et al.*, 2006). To interfere with access to the pathogen plasma membrane, an N-terminal GST-tag was added to P14c-His<sub>6</sub>, resulting in a much larger 46.6-kDa fusion protein. GST-tagged P14c was expressed in *E. coli*, and the purified protein (Figure S4) was analyzed for sterol-binding and antimicrobial activity. Comparative *in vitro* sterol-



**Figure 4.** Sterol-binding activity of PR-1 proteins is essential for antimicrobial activity. (a) Antimicrobial activity of P14c towards *Phytophthora brassicae* in the presence of increasing quantities of free cholesterol: 20 µg of purified P14c was incubated for 1 h in media containing different quantities of free cholesterol before the addition of 500 zoospores. The experiment was repeated twice with similar results. (b) Comparison of antimicrobial activity of P14c with the mutated P14c-C146S with compromised sterol-binding activity: 20 µg of purified protein was incubated with 500 zoospores of *P. brassicae*. The experiment was repeated once with similar results. Pictures were taken 5 days after germination (a and b). (c) Microscopical analysis of growth phenotype: *P. brassicae* was incubated in standard cleared V8 medium in the absence or presence of 20 µg of purified P14c. Pictures were taken 48 h after germination. Scale bars: 200 µm (ctrl) and 50 µm (P14c). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**Figure 5.** Mode of action of *Solanum lycopersicum* (tomato) P14c. (a) Effect of inhibition of sterol biosynthesis on the potential of P14c to inhibit fungal growth. Five hundred spores of either *Aspergillus niger* or *Botrytis cinerea* were incubated with different combinations of 20 μg of purified P14c and the sterol biosynthesis inhibitor difenoconazole (DA). The experiment was repeated four times with similar results. (b) Quantification of inhibition of fungal growth based on total fatty acid content per well. (c) Comparison of specific cholesterol-binding activity between P14c and GST-P14c: 100 pmol of purified protein was incubated with 50 pmol of radiolabeled cholesterol and the protein-bound radiolabel was quantified by scintillation counting. Results are expressed relative to ligand binding of P14c. Data represent means ± SDs of two independent experiments. (d) Purified P14c (20 μg) or GST-P14c (50 μg) were incubated with 500 zoospores of *Phytophthora brassicae brassicae*. The picture was taken 5 days post germination. The experiment was repeated once with similar results.

binding assays revealed no significant difference in the specific sterol-binding capacity between GST-P14c and P14c (Figure 5c); however, the antimicrobial potential of the larger protein towards *P. brassicae* was strongly compromised in comparison with equimolar quantities of P14c (Figure 5d). Hence, the binding of sterols in the medium could not explain the observed growth inhibition. The

results support the conclusion that P14c inhibits growth by direct sterol acquisition from pathogens.

## CONCLUSION

Our results reveal a new mode of action for a family of antimicrobial plant proteins by showing that the antimicrobial activity of PR-1 proteins depends on sterol

sequestration. Hence plant defense targets pathogen sterols, as do bacteria, with the production of antimycotic polyene antibiotics that bind to ergosterols in fungal membranes to cause cellular leakage, and azole fungicides that act as sterol biosynthesis inhibitors. PR-1 does not discriminate between different sterols. The potential of PR-1 to cause cellular damage appears to depend on the sterol biosynthetic capacity of an organism: sterol-auxotroph organisms such as *Phytophthora* are more sensitive to PR-1 than sterol-prototroph organisms such as fungi and plants. Interestingly, it was shown before that tobacco plants with enhanced PR-1 expression are particularly well protected against the oomycete pathogens *Phytophthora parasitica* and *Peronospora tabacina*, whereas resistance towards viral, bacterial and fungal pathogens was not enhanced (Alexander *et al.*, 1993). Enhanced disease resistance towards the fungal pathogen *B. cinerea* was reported for tobacco plants expressing a PR-1 protein from *Wasabia japonica* (Kiba *et al.*, 2007).

Interestingly, individual plant species contain multiple PR-1-like proteins, such as Arabidopsis, with 22 family members. Only the expression of PR-1 (At2G14610) is strongly induced upon pathogen challenge, suggesting that PR-1-like proteins function beyond plant disease protection. PR-1 proteins are either secreted or accumulate in the vacuole. PR-1a secreted into the intercellular space can make immediate contact with an invading pathogen. In contrast, P14c is predicted to accumulate in the vacuole, based on its C-terminal vacuolar targeting sequence. Hence, P14c will only come into contact with an invading pathogen upon lysis of the host cell. Similar to the situation with antimicrobial hydrolases (Mauch and Staehelin, 1989), we speculate that the vacuolar storage enhances the antimicrobial efficiency of PR-1 proteins, as pathogens might not be able to counteract the sudden contact with high concentrations of PR-1 with a fast enough adjustment of sterol biosynthesis.

Mature PR-1a and P14c consist of an isolated CAP domain with no additional flanking sequences. The demonstration of a sterol-binding activity of PR-1 proteins lends further support to the hypothesis that the CAP domain functions as a bona fide sterol-binding domain (Choudhary and Schneider, 2012). The biochemical activity and mode of action of PR-1 proteins may be relevant beyond plant immunity, as a number of animal CAP proteins have been suggested to have immunity-related activities (Gibbs *et al.*, 2008).

## EXPERIMENTAL PROCEDURES

### Heterologous expression of PR-1 proteins in *E. coli*

SP14c and NtPR-1a cDNAs without signal peptide sequence and including a C-terminal His<sub>6</sub>-tag were synthesized (GenScript) and cloned into the bacterial expression vector pET24d (Novagen, now

Merck Millipore, <http://www.merckmillipore.com>). Transformed BL21 bacteria were cultured in Luria Bertani (LB) broth at 37°C to an optical density of OD<sub>600</sub> 0.5. The bacteria were further incubated at 16°C to OD<sub>600</sub> 0.7 before the induction of PR-1 production by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Bacterial cells were harvested 12 h post-induction by centrifugation, resuspended in 10 mM Tris-HCl, pH 8, and lysed with a microfluidizer.

### PR-1 protein purification

His<sub>6</sub>-tagged proteins were purified from bacterial extracts using Ni-NTA affinity resin following the manufacturer's instructions (Qiagen, <http://www.qiagen.com>). Bound proteins were eluted with a solution of 0.5 M imidazole, 0.5 M NaCl and 10 mM Tris-HCl, pH 8, and the eluted fraction was desalted with Sephadex G-25 equilibrated with 10 mM Tris-HCl, pH 8. Protein concentration was determined by the Lowry method using BSA as a standard.

### SDS-PAGE and immunoblotting

SDS-PAGE analysis and immunoblotting were performed following standard procedures. Anti-His-tag antibodies (Qiagen) were used for immunodetection. Blots were developed using peroxidase-conjugated anti-mouse secondary antibody and SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, <http://www.thermofisher.com>).

### Antimicrobial assays

*Phytophthora brassicae* was cultivated on solid V8 media (Erwin and Ribeiro, 1996), and *A. niger* and *B. cinerea* were cultivated on potato dextrose agar (Sigma-Aldrich, <http://www.sigmaaldrich.com>) at 18°C. Production of zoospores of *P. brassicae* was performed as described by Roetschi *et al.* (2001). Antimicrobial tests were performed in 24-well microtiter plates in a total volume of 1 ml, containing a final concentration of 3% of cleared V8 vegetable juice and 10 mM Tris-Cl, pH 8. After the addition of a defined number of spores the microtiter plates were incubated on a shaking platform (75 rpm; 18°C) and hyphal growth was analyzed 5 days later.

### Quantification of microbial biomass

The mycelium was harvested via filtration using a 0.45 μm polyvinylidene difluoride (PVDF) membrane. The quantification of fatty acids was carried out according to Lu *et al.* (2015), with some modifications. Briefly, free and esterified fatty acids were (trans-) methylated by incubating the mycelium in 1.5 ml 5% (v/v) sulfuric acid in methanol at 85°C for 45 min in the presence of 50 μg of butylated hydroxytoluene as the antioxidant and 5 μg of triheptadecanoylglycerol as the internal standard. After cooling, 2.25 ml 0.9% (w/v) of sodium chloride was added and fatty acid methyl esters (FAMES) were extracted three times with 2 ml of hexane. The combined hexane phases were evaporated under a stream of nitrogen. The residue was suspended in 150 μl of heptane and analyzed using a gas chromatography flame ionization detector (GC-FID), equipped with a DB-23 capillary column (30 m × 250 μm, 0.25 μm film thickness; Agilent Technologies, <http://www.agilent.com>). The injection was performed at 250°C with a split of 20:1. The initial oven temperature of 100°C was held for 2 min, followed by an increase to 160°C with a rate of 25°C min<sup>-1</sup>. Afterwards the temperature was increased to 250°C with a rate of 8°C min<sup>-1</sup>, and held for 4 min. FAMES were detected with an FID set at 270°C and quantified relative to the internal standard using a four-level calibration curve.



## Determination of sterols in cleared V8 medium

One milliliter of 100% cleared V8 media was mixed with 3 ml of MeOH, 2 ml 0.5% pyrogallol, 2 ml 60% KOH and 50 µg of cholesterol (as the internal standard). The samples were incubated for 2 h at 90°C for saponification and extracted with heptane. After heptane evaporation, the samples were dissolved in 50 µl pyridine and 50 µl N,O'-bis-(trimethylsilyl)-trifluoroacetamide. Sample separation was carried out with an Agilent HP-5 ms column mounted in an HP 6890 series gas chromatograph coupled to an MS detector, with helium as the carrier gas. Standards of individual sterols were used for quantification. One milliliter of 3% cleared V8 medium used for the antimicrobial tests contained 60 pmol of stigmasterol, 60 pmol of campesterol and 25 pmol of  $\beta$ -sitosterol.

## Yeast expression constructs and transformation

*SP14c-His<sub>6</sub>*, *SP14c-C146S-His<sub>6</sub>* and *NtPR-1a-His<sub>6</sub>* cDNAs without signal peptide sequence were PCR-amplified with primers containing alcohol dehydrogenase (ADH) sequences for homologous recombination into the yeast vector pRS416 containing the pre-pro alpha factor signal sequence (Agilent Technologies). Integration was controlled using a forward primer in the ADH1 promoter and a reverse primer specific for each individual cDNA. The final constructs allow the expression of PR-1 proteins with a pre-pro alpha factor signal sequence for secretion under the control of the yeast ADH promoter.

## Yeast sterol export assay

The yeast sterol export assay based on the secretion of cholesterol acetate was performed as described by Choudhary and Schneider (2012) using heme-deficient yeast cells lacking the sterol deacetylase Say1. Heme deficiency allows the efficient uptake of [<sup>14</sup>C]-cholesterol, and a lack of Say1 leads to an accumulation of cholesterol acetate that is exported with the help of Pry proteins. Yeast cells (*heme1Δsay1Δ*) were first cultivated overnight in SC-Leu medium containing 20 µg ml<sup>-1</sup> cold cholesterol and 0.5% Tween 80. Ten optical density units of cells were washed with SC-Leu media and further cultivated overnight in the presence of [<sup>14</sup>C]-cholesterol (0.025 µCi ml<sup>-1</sup>). The cells were washed with SC-Leu media and cultivated for another day with cold cholesterol. After centrifugation the lipid fraction was extracted from pellet and supernatant, respectively, with chloroform/methanol (1:1, v/v), 250 cps of each fraction was dried, resuspended in 25 µl of chloroform/methanol (1:1, v/v), spotted on a thin layer chromatography plate (silica gel 60; Merck) and separated with petroleum ether/diethyl ether/acetic acid (70:30:2, v/v/v). The TLC-separated radiolabeled lipids were visualized with a phosphorimager (Bio-Rad, <http://www.bio-rad.com>) and quantified by radio scanning.

## In vitro cholesterol-binding assays

The sterol-binding assay was performed essentially as described by Im *et al.* (2005). In a total volume of 100 µl of binding buffer (final concentration of 20 mM Tris, pH 6.5, 30 mM NaCl, 0.05% Triton X-100), the assay contained [<sup>3</sup>H]-cholesterol (50–700 pmol; 50 Ci mmol<sup>-1</sup>) and purified protein (50–100 pmol). After incubation for 90 min at 30°C on a shaking platform, PR-1 proteins were separated from the unbound ligand by adsorption to Q Sepharose ion-exchange beads (GE Healthcare Life Sciences, <http://www.gelifesciences.com>). The beads were washed three times with 1 ml washing buffer (20 mM Tris, pH 7.5), proteins were eluted by the addition of 0.5 ml of elution buffer (20 mM Tris, pH 7.5, 1 M NaCl) and the bound radio ligand was quantified by

scintillation counting. For competition assays, 50 pmol of unlabeled cholesterol, stigmasterol or ergosterol was included in the binding reaction, together with 50 pmol of [<sup>3</sup>H]-cholesterol. The reported values were corrected for nonspecific binding by the ion-exchange beads alone.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Alignment of PR-1 proteins with the yeast CAP protein Pry1.

**Figure S2.** SDS-PAGE analysis of purified PR-1 proteins.

**Figure S3.** Effect of cholesterol on growth of *Phytophthora brassicae*.

**Figure S4.** SDS-PAGE analysis of purified GST-P14c protein.

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