

Chitosan inhibits septin-mediated plant infection by the rice blast fungus *Magnaporthe oryzae* in a protein kinase C and Nox1 NADPH oxidase-dependent manner

Federico Lopez-Moya¹ , Magdalena Martin-Urdiroz² , Miriam Osés-Ruiz^{2,3} , Vincent M. Were^{2,3} , Mark D. Fricker⁴ , George Littlejohn^{2,5} , Luis V. Lopez-Llorca¹  and Nicholas J. Talbot^{2,3} 

¹Laboratory of Plant Pathology, Department of Marine Sciences and Applied Biology, University of Alicante, Alicante 03690, Spain; ²School of Biosciences, University of Exeter, Exeter, EX4 4QD, UK; ³The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK; ⁴Department of Plant Science, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK; ⁵School of Biological and Marine Sciences, Plymouth University, Portland Square Building Room A404, Drake Circus, Plymouth, PL4 8AA, UK

Summary

Author for correspondence:
Nicholas J. Talbot
Email: nick.talbot@tsl.ac.uk

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- Chitosan is a partially deacetylated linear polysaccharide composed of β -1,4-linked units of D-glucosamine and N-acetyl glucosamine. As well as a structural component of fungal cell walls, chitosan is a potent antifungal agent. However, the mode of action of chitosan is poorly understood.
- Here, we report that chitosan is effective for control of rice blast disease. Chitosan application impairs growth of the blast fungus *Magnaporthe oryzae* and has a pronounced effect on appressorium-mediated plant infection. Chitosan inhibits septin-mediated F-actin remodelling at the appressorium pore, thereby preventing repolarization of the infection cell.
- Chitosan causes plasma membrane permeabilization of *M. oryzae* and affects NADPH oxidase-dependent synthesis of reactive oxygen species, essential for septin ring formation and fungal pathogenicity. We further show that toxicity of chitosan to *M. oryzae* requires the protein kinase C-dependent cell wall integrity pathway, the Mps1 mitogen-activated protein kinase and the Nox1 NADPH oxidase. A conditionally lethal, analogue (PP1)-sensitive mutant of Pkc1 is partially remediated for growth in the presence of chitosan, while $\Delta nox1$ mutants increase their glucan : chitin cell wall ratio, rendering them resistant to chitosan.
- Taken together, our data show that chitosan is a potent fungicide which requires the cell integrity pathway, disrupts plasma membrane function and inhibits septin-mediated plant infection.

Introduction

Plant pathogenic fungi are responsible for many of the world's most serious crop diseases and yet remain very challenging to control (Fisher *et al.*, 2012, 2018). Fungicide application is often not completely effective, there are environmental consequences and resistance to fungicides is a frequent occurrence (Lucas *et al.*, 2015). Chitosan is a biopolymer obtained by partial N-deacetylation of the β -1,4-D-linked polymer of N-acetyl glucosamine, chitin (Kumar, 2000). As well as being a structural component of fungal cell walls, chitosan also displays antimicrobial activity (Allan & Hadwiger, 1979). Chitosan has a pK_a value of *c.* 6.3 and it is cationic at lower pH values, as a result of protonation of its amino groups. Chitosan inhibits the growth of filamentous fungal plant pathogens, such as *Botrytis cinerea* (Muñoz & Moret, 2010) and *Fusarium oxysporum* (Palma-Guerrero *et al.*, 2008; Al-Hetar *et al.*, 2010). Chitosan therefore shows considerable potential as a naturally occurring, novel antifungal agent, but its precise mode of action in plant pathogenic fungi remains unclear.

Chitosan inhibits the growth of sensitive fungi, causing massive membrane permeabilization (Palma-Guerrero *et al.*, 2009; Palma-Guerrero *et al.*, 2010). In *Neurospora crassa*, chitosan exposure causes generation of an oxidative response, which leads to membrane permeabilization and cell death (Lopez-Moya *et al.*, 2015). Recent studies, however, have also demonstrated that cell wall composition plays a key role in fungal sensitivity to chitosan (Aranda-Martinez *et al.*, 2016) as well as mitochondrial activity (Jaime *et al.*, 2012). Transcriptional profiling studies, meanwhile, have revealed that cytoskeletal functions and cell integrity pathway genes (such as *Slr2*) are also compromised by chitosan exposure in the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (Zakrzewska *et al.*, 2005, 2007; Shih *et al.*, 2019). Genes related to actin polymerization were also repressed by chitosan treatment in the filamentous fungus *N. crassa*, (Lopez-Moya *et al.*, 2016), suggesting that cell wall- and actin-dependent functions, such as cell polarity, exocytosis, endocytosis, cytokinesis and organelle movement, may be affected by chitosan exposure (Barja *et al.*, 1993; Berepiki *et al.*, 2010). In all of these reports, however, it is

unclear whether the observations reveal the mode of action of chitosan, or rather the pleiotropic effects it has on cell viability. It is therefore vital to carry out more comprehensive investigations of the manner in which chitosan affects fungal viability, in order to determine its likely efficacy as a novel fungicide.

In this report, we describe a series of experiments designed to determine the mode of action of chitosan in the control of a major crop disease-causing fungus, *Magnaporthe oryzae*, which has a global impact on food security (Zhang *et al.*, 2009; Wilson & Talbot, 2009; Castroagudin *et al.*, 2015). *Magnaporthe oryzae* is the causal agent of blast disease, responsible for up to 30% losses to the annual rice harvest (Talbot, 2003). *Magnaporthe oryzae* infects rice cells using specialized infection structures called appressoria. These structures generate enormous turgor, applied as physical force to penetrate epidermal cells and infect rice tissues (Wilson & Talbot, 2009). The dome-shaped appressorium possesses a thick melanin layer in its cell wall, critical for plant infection (Chumley & Valent, 1990), and can generate up to 8.0 MPa of pressure (Howard *et al.*, 1991; Howard & Valent, 1996), by accumulating molar concentrations of glycerol and other polyols as compatible solutes (de Jong *et al.*, 1997). Appressorium turgor is sensed by the Sln1 histidine-aspartate kinase, which acts via the protein kinase C-dependent cell integrity pathway and the cAMP-dependent protein kinase A pathway in order to modulate glycerol accumulation and melanin biosynthesis, once a critical threshold of pressure has been reached. An NADPH oxidase-dependent regulated burst of reactive oxygen species (ROS) then occurs, leading to septin-dependent re-polarization of the appressorium and plant infection (Ryder *et al.*, 2019). Generation of ROS requires NADPH oxidases encoded by the *NOX1*, *NOX2* and *NOXR* genes, which are essential for *M. oryzae* infection (Egan *et al.*, 2007; Ryder *et al.*, 2013). In other fungi, NADPH oxidase-dependent ROS generation also plays roles in cell polarity and invasive growth. In *Sordaria macrospora*, for instance, Nox1 regulates gene expression involved in cytoskeleton remodelling, hyphal fusion and mitochondrial respiration (Dirschnabel *et al.*, 2014), while in the endophyte *Epichloë festucae*, *NOXA* is essential for polarized growth and hyphal fusion (Eaton *et al.*, 2011). In *M. oryzae*, *NOX1* is also involved in cell wall organization, and $\Delta nox1$ mutants are resistant to cell wall-perturbing agents, such as calcofluor white (Egan *et al.*, 2007). This role may be conserved in fungi, because in the mycoparasitic fungus *Coniothyrium minitans*, for example, *CmNOX1* and *CmSLT2* (an orthologue of *M. oryzae* *MPS1*), regulate localization of the cell wall integrity-associated mitogen-activated protein kinase (MAPK) and mediate changes in gene expression associated with cell integrity (Wei *et al.*, 2016).

The Nox2-regulated synthesis of ROS is necessary for recruitment and organization of four septins to the appressorium pore (Ryder *et al.*, 2013), where they form a hetero-oligomeric ring complex, essential for repolarization of the appressorium (Dagdas *et al.* 2012). The septin ring rigidifies the cortex of the appressorium, acting as a scaffold for F-actin organization. Septins also act as a lateral diffusion barrier, required for localization of polarity determinants such as Chm1 (Li *et al.*, 2004), Tea1 and Las17 (Dagdas *et al.*, 2012).

In this study, we set out to determine the effect of exposure to chitosan on infection-related development by *M. oryzae*. Chitosan is a cell wall component in *M. oryzae*, important in adhesion and appressorium formation (Geoghegan & Gurr, 2016). We were interested in whether exposure to exogenous chitosan was, however, toxic to the fungus, as shown for other fungi and, if so, how it acts on the cellular events necessary for plant infection. Here, we provide evidence that chitosan exposure is fungicidal to *M. oryzae*, causing membrane permeabilization and preventing appressorium-mediated plant infection. Importantly, we show that the cell integrity pathway and Nox1 NADPH oxidase activity are essential for toxicity of chitosan, providing insights into its mode of action.

Materials and Methods

Fungal strains and growth conditions

The wild-type strain of *Magnaporthe oryzae*, Guy11 (Leung *et al.*, 1988) and transgenic rice blast isolates expressing Sep4-GFP::H1-RFP, Sep5-GFP, gelsolin-GFP, Chm1-GFP, Tea1-GFP and Grx1-roGFP2 strains were stored in the laboratory of one of the authors (NJT). Gene replacement mutants $\Delta nox1$, $\Delta nox2$, $\Delta nox1nox2$ and $\Delta mps1$ and the *pkc1^{AS}* mutant were generated as described previously (Xu *et al.*, 1998; Ryder *et al.*, 2013; Penn *et al.*, 2015). All fungal strains were grown on complete medium (CM) at 24°C under a 12 h : 12 h, light : dark photoperiod (Talbot *et al.*, 1993). Conidial suspensions were obtained in sterile distilled water (SDW) by scraping the surface of 12-d-old plate cultures with a spreader, before being filtered through Miracloth (Calbiochem, San Diego, CA, USA) and concentrated by centrifugation (16 873 g for 1 min).

Preparation of chitosan

Medium-molecular-weight chitosan (70 kDa) with an 85% deacetylation degree (T8; Supporting Information Table S1) was provided by Marine BioProducts GmbH (Bremerhaven, Germany). Chitosan solutions were prepared as described by Palma-Guerrero *et al.* (2008). The resulting solution was dialysed against distilled water and autoclaved. Chitosan solutions were stored at 4°C until used, and never stored for > 30 d.

Exposure of *M. oryzae* to chitosan during plant infection

To evaluate the effect of chitosan on the pathogenicity of *M. oryzae*, leaf spot and spray infection assays were performed. Conidia of Guy11 were collected and suspensions adjusted to 10^5 conidia ml⁻¹. Leaf spot bioassays were performed by inoculating 20 µl droplets of 1×10^5 conidia ml⁻¹ onto the adaxial surfaces of detached rice leaves of the blast-susceptible dwarf Indica variety CO-39 (Talbot *et al.*, 1993) using four replicate leaves per treatment. Inoculated rice leaves were incubated in moist chambers and after 5 d, the size of the resulting rice blast disease lesion was recorded. Treatments included a control experiment (no chitosan) and conidial suspensions containing either 1

or 5 mg ml⁻¹ chitosan. To evaluate the effect of chitosan on invasive growth, wounded infection assays were carried. A hypodermic needle was used to gently scrape the surface of detached CO-39 leaves. Guy11 conidia were diluted to a final concentration of 1 × 10⁵ conidia ml⁻¹ in SDW in the presence or absence of 1 or 5 mg ml⁻¹ chitosan. Wounded leaves were placed on 2% water agar and inoculated with 20 µl of conidial suspension, with four leaves per treatment and 3 biological replicates. Lesions were scored 5 d post-infection. For leaf spray bioassays, CO-39 rice plants at the three-leaf stage (normally, 21 d old) were sprayed with 5 ml of a suspension of 5 × 10⁴ conidia ml⁻¹ using an artist's airbrush (Badger Air-Brush Co, Franklin, IL, USA). Plants were also sprayed with chitosan only (no conidia) and all experiments were repeated three times. Inoculated plants were placed in plastic bags for 2 d to achieve high humidity and symptoms scored after 5 d. Leaves were collected and rice blast lesions quantified.

Leaf sheath bioassays

Leaf sheath fragments (2–3 cm long) were obtained from 21-d-old seedlings of rice cultivar CO-39. Leaf sections were inoculated with 50 µl of a conidial suspension of 1 × 10⁵ conidia ml⁻¹ and incubated in moist chambers for 30 h at 24°C. Transverse sections were cut with a razor blade and mounted in water. Micrographs were recorded using an IX-81 Olympus inverted microscope. After 30 h, the frequency of *M. oryzae*-rice cell invasion was recorded (*n* = 50 cells observed, in three replicate experiments).

Evaluation of the effect of chitosan exposure on growth and development of *M. oryzae*

Conidia of Guy11, $\Delta nox1$, $\Delta nox2$ and $\Delta nox1nox2$ mutants were incubated on hydrophobic glass coverslips in the presence of 0, 0.1, 0.5, 1, 2 and 5 mg ml⁻¹ chitosan. The frequency of conidial germination was determined after 2 h. The frequency of appressorium development was scored after 4, 6, 8, 16 and 24 h.

The effect of chitosan on mycelium growth of *M. oryzae* was tested as follows. Guy 11, $\Delta nox1$, $\Delta nox2$, $\Delta nox1nox2$, $\Delta mps1$ and $pkc1^{AS}$ were grown on CM liquid medium for 48 h on an orbital shaker (150 rpm) at 24°C with a 12 h: 12 h photoperiod. Mycelium was then transferred to CM medium in the presence/absence of chitosan (0.5 and 1 mg ml⁻¹) and incubated for a further 48 h. Mycelium was then collected and washed twice with SDW before being lyophilized and the DW was recorded. We also carried out growth kinetic analysis as follows. Ten-day-old *M. oryzae* CM cultures were homogenized in a Waring blender (Waring Commercial, McCConnellsburg, PA, USA). The resulting homogenates were adjusted to OD₄₉₀ < 0.0825 (Clinical & Laboratory Standards Institute, 2008) in an Infinite 200 Pro 96-well spectrophotometer (Tecan, Männedorf, Switzerland). Chitosan (0.05–5 mg ml⁻¹) was then added to CM medium and dispensed to 96-well microtitre plates (Sterillin Ltd, Newport, UK). Growth kinetics of *M. oryzae* mutants were established by measuring optical density at 490 nm

(OD₄₉₀) (Lopez-Moya *et al.*, 2015) in a multiwell spectrophotometer (Tecan) every hour for 140 h. Using the same methodology, we also evaluated the synergistic effect of chitosan with caspofungin (0.1 µg ml⁻¹).

Physiological and cellular response of *M. oryzae* to chitosan during appressorium differentiation

Reporter strains Sep4-GFP:H1-RFP, Sep5-GFP, gelsolin-GFP, Chm1-GFP, Tea1-GFP and Grx1-roGFP2 were used to evaluate the effect of chitosan on cytoskeletal organization, plasma membrane integrity and cellular oxidative status. A conidial suspension of each strain was incubated in the presence or absence of 5 mg ml⁻¹ chitosan for 4, 6, 8 and 24 h to visualize appressorium development. FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethyl amino) phenyl) hexatrienyl) pyridinium dibromide) was used to evaluate integrity of the plasma membrane in *M. oryzae* appressoria exposed to 5 mg ml⁻¹ chitosan. Germlings were observed 24 h after inoculation. A Grx1-roGFP2 strain (Samalova *et al.*, 2014) was used to detect changes in glutathione oxidation. This has a glutaredoxin (Grx) subunit to improve response kinetics to oxidation, in order to evaluate the oxidative response of *M. oryzae* appressoria to either 1 or 5 mg ml⁻¹ chitosan (Samalova *et al.*, 2014). Maximum fluorescence is therefore related to accumulation of oxidized glutathione, a measurement to determine ROS concentrations.

For epifluorescence microscopy, an IX-81 Olympus inverted microscope connected to a CoolSNAP HQ2 camera (Teledyne Photometrics, Tucson, AZ, USA) was used. Three-dimensional projections were captured using a Leica (Wetzlar, Germany) SP8 LSCM laser confocal scanning microscope, with HyD detectors HC PL APO CS2. Lasers (488 and 561 nm) were used for excitation of GFP and RFP, respectively. METAMORPH 7.5 (Molecular Devices, San Jose, CA, USA) and IMAGEJ software (National Institutes of Health, Bethesda, MD, USA) were used for image analysis. Three-dimensional reconstructions were performed with Leica LAS software.

Protoplast release assay

To evaluate the effect of chitosan on the cell wall composition of *M. oryzae*, we scored the frequency of protoplast release using cell wall-degrading enzymes on chitosan-grown mycelia. Guy11 was first grown in CM in the presence or absence of 0.5 and 1 mg ml⁻¹ chitosan for 48 h. Mycelium was then collected using Mira cloth (MilliporeSigma, Burlington, MA, USA) and washed twice with SDW, before being transferred to a conical tube with 40 ml of Osmotically Stabilized Medium (OM) buffer (1.2 M MgSO₄, 10 mM NaPO₄, pH 5.8) in the presence of 500 mg Glucanex (Sigma) at pH 5.8. Tubes were then shaken gently to disperse hyphal clumps and then incubated for 3 h at 30°C with gentle (75 rpm) shaking. Isolated protoplasts were transferred to sterile polycarbonate or polysulfonate Oakridge tubes (Nalgene Oakridge Tubes, Thermo Fisher Waltham, MA, USA) and overlaid with an equal volume of cold ST buffer (0.6 M sorbitol and 0.1 M Tris-HCl; pH 7.0), before centrifugation at 5000 g at 4°C

for 15 min. Protoplasts were recovered from the OM/ST interface and transferred to a clean Oakridge tube. The tube was filled with STC (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5 and 10 mM CaCl_2). Protoplasts were concentrated by centrifugation at 3000 g for 10 min at 4°C, in a swinging bucket rotor, before being washed twice more with 10 ml STC. Protoplasts were resuspended in 100 μl STC buffer and counted.

Quantification of β -1,3 glucan and chitin in cell walls of *M. oryzae*

Chitin content of mycelium was estimated by determining the amount of N-acetyl-D-glucosamine according to the method of Bowman *et al.*, (2006) with some modifications as Aranda-Martinez *et al.* (2016). Guy11 and Δnox1 strains were first grown in CM for 48 h, mycelium was collected, washed twice with SDW and transferred to CM for 48 h in the presence or absence of 1 mg ml^{-1} chitosan. Mycelium was then collected by centrifugation, washed twice in SDW and lyophilized. Mycelium was ground in liquid nitrogen, and 30 mg per sample hydrolysed in 1 ml 6 M HCl at 110°C for 6 h. The HCl was removed by aeration and samples were resuspended in 1 ml of SDW before being centrifuged twice at 16 873 g for 20 min in a microfuge. A 0.5 ml aliquot of the supernatant from each sample was mixed with 0.1 ml of 0.16 M sodium tetraborate pH 9.1 and then heated at 100°C for 3 min. After cooling, 3 ml of p-dimethylamine benzaldehyde (DMAB) solution (10% DMAB in glacial acetic acid containing 12.5% 10 N HCl, diluted with 9 vol. of glacial acetic acid) was added. The mixture was then incubated for 20 min at 37°C and absorbance at 595 nm was measured in a Genios™ Multiwell Spectrophotometer (Tecan). A standard curve was generated using 0–20 mg ml^{-1} N-acetyl-D-glucosamine (Sigma) treated as described previously.

The β -1,3-glucan content of the fungal cell wall was determined according to Shedletzky *et al.*, (1997) with some modifications (Aranda-Martinez *et al.*, 2016). Mycelium of Guy11 and Δnox1 was transferred to CM in the presence or absence of 1 mg ml^{-1} chitosan for 48 h. Mycelium were collected by centrifugation, washed twice in SDW and then hydrolysed with 0.1 M NaOH before being lyophilized. Mycelium was ground in liquid nitrogen, and resuspended in 0.5 ml 1 M NaOH. Samples were incubated at 80°C for 30 min with 0.5 mm zirconia/silica beads (Biospec, Bartlesville, OK, USA) and vortexed several times at full speed for 10 min each for tissue disruption. Aliquots (100 μl) from each sample were then mixed with 400 μl of 1 M NaOH. Aniline blue mix (2.1 ml; 40 vol. of 0.1% aniline blue, 21 vol. of 1 N HCl and 59 vol. of 1 M glycine/NaOH buffer, pH 9.5) was then added to each sample. These were vortexed and incubated at 50°C for 30 min, and then incubated for 30 min at room temperature. Fluorescence was quantified in a Jasco Model FP-6500 spectrofluorometer (Oklahoma City, OK, USA) using 400 nm excitation and 460 nm emission wavelengths. A standard curve for β -1,3-glucan was constructed using 0–50 mg ml^{-1} curdlan (Megazyme, Irishtown, Bray, Co. Wicklow, Ireland) dissolved in 0.1 M NaOH and also heating for 30 min at 50 °C in 1 N NaOH.

Quantification of gene expression by qRT-PCR

Total RNA was obtained from Guy11 and Δnox1 cultures prepared after 48 h growth in CM and further 4, 8 and 24 h growth in the presence or absence of 1 mg ml^{-1} chitosan. Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Total RNA was treated with TurboDNA free (Ambion) to remove DNA remains. First-strand cDNA was then synthesized using retro-transcriptase RevertAid (Thermo Fisher Scientific) primed with oligo dT (Thermo Fisher Scientific). Gene expression was quantified using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) with SYBR Green and ROX (Roche). Gene quantification was performed in a Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was estimated by the $\Delta\Delta\text{Ct}$ methodology (Livak & Schmittgen, 2001) with three technical replicates per condition. Primers used to quantify the expression of *M. oryzae* genes in response to chitosan are shown in Table S2. Ubiquitin-conjugate enzyme (MGG_04081) and glyceraldehyde-3-phosphate dehydrogenase (MGG_01081) genes were used as endogenous controls for all experiments (Che Omar *et al.*, 2016), as their expression showed Ct stability for all conditions tested. We did not use β -tubulin and actin, commonly used as fungal housekeeping genes, because chitosan modifies their expression (Lopez-Moya *et al.*, 2016). All experiments were repeated three times.

Results

Exposure to chitosan reduces the ability of *M. oryzae* to cause rice blast disease

In order to determine the effect of chitosan on the growth and development of the rice blast fungus *M. oryzae*, conidia were exposed to chitosan and then used to inoculate the susceptible rice cv CO-39. In leaf drop experiments, *M. oryzae* normally generates a large necrotic, sporulating disease lesion, as shown in Fig. 1(a). However, when leaves were inoculated with *M. oryzae* in the presence of chitosan, mildly necrotic, nonsporulating lesions resulted (Fig. 1a). To determine if reduced disease symptoms were solely associated with reduced frequency of appressorium-mediated infection, rice leaves were wounded before inoculation with *M. oryzae* in the presence or absence of chitosan (Fig. 1a). Wounding rice leaves before infection by *M. oryzae* results in large necrotic, sporulating disease lesions. In the presence of chitosan we observed reduced blast disease symptoms, with little sporulation, suggesting that the ability of the fungus to invade plant tissue was also affected (Fig 1a). Conidial suspensions were then used to spray-inoculate rice seedlings. Exposure to chitosan (5 mg ml^{-1}) significantly ($P < 0.05$) reduced the generation of rice blast disease lesions (Fig. 1b,c). By contrast, spraying chitosan in a mock inoculation did not cause any damage or response in rice leaves (Fig. 1b). Microscopy of rice leaf sheath preparations inoculated with *M. oryzae* conidia exposed to chitosan (5 mg ml^{-1}) showed 75% reduction in the number of cells

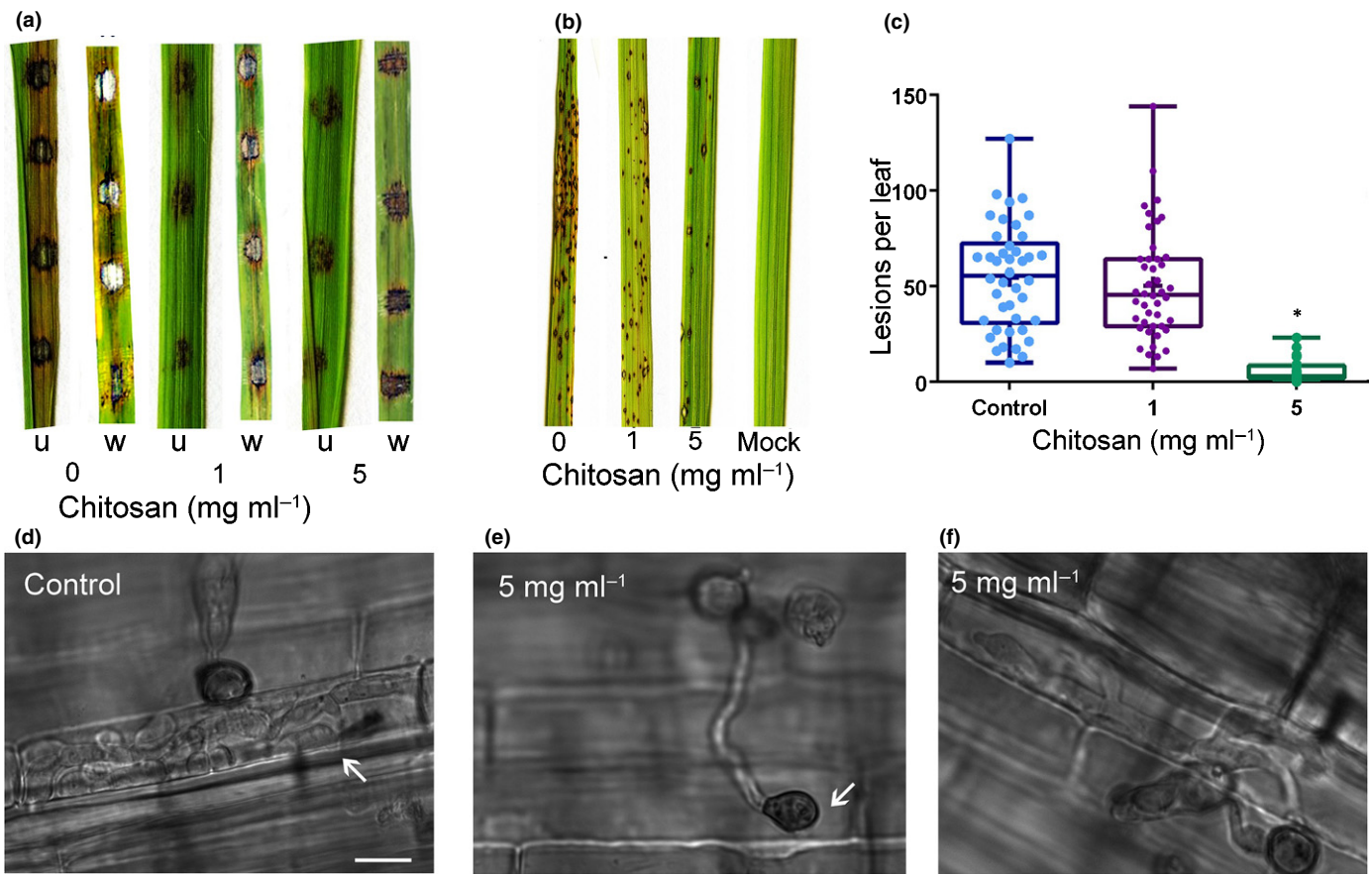


Fig. 1 Exposure to chitosan affects the ability of *Magnaporthe oryzae* to cause rice blast disease. (a) Leaves showing rice blast symptoms following leaf drop infection assays with *M. oryzae* in the presence or absence of 1 or 5 mg ml⁻¹ chitosan, after 5 d. Unwounded (u) and wounded (w) plants were used to investigate role of chitosan in inhibiting cuticle infection or invasive growth. (b) Leaves showing blast symptoms, 5 d after spray inoculation in the presence or absence of chitosan. (c) Box and whisker plots with data points shown to indicate disease lesions per leaf, following spray inoculation in the presence or absence of chitosan. Asterisk (*) indicates significant difference. Multifactorial analysis ANOVA was used to compare treatments ($P = 0.05$). Error bars are \pm SD. (d) Micrographs of leaf sheath assay of *M. oryzae* colonizing rice leaf cells after 30 h. (e) Leaf sheath assay of *M. oryzae* following chitosan exposure (5 mg ml⁻¹) shows reduced penetration peg development after 30 h. (f) Leaf sheath assay of *M. oryzae* following chitosan (5 mg ml⁻¹) exposure to show reduced invasive growth after 30 h. Bars, 10 μ m.

penetrated by the fungus, compared to nonexposed infections, as shown in Fig. 1(d). Differentiated appressoria from chitosan-treated conidia either did not penetrate epidermal rice leaf cells or displayed reduced invasive growth and colonization of adjacent epidermal cells, as shown in Fig. 1(e,f).

Exposure to chitosan impairs appressorium development and function in *M. oryzae*

Chitosan exposure reduces the frequency of appressorium differentiation when applied to ungerminated *M. oryzae* conidia in a concentration-dependent manner, as shown in Fig. 2. Chitosan exposure did not affect the rate of conidial germination (Fig. S1), but after 4 h exposure to chitosan (0.5–2 mg ml⁻¹) the number of differentiated appressoria was significantly reduced ($P < 0.05$). Exposure to a concentration of 5 mg ml⁻¹ chitosan was sufficient to prevent appressorium differentiation almost entirely (Fig. 2a, b). After 6 h exposure to 5 mg ml⁻¹ chitosan, appressorium development was decreased by 85%. Exposure to low or intermediate concentrations of chitosan (between 0.5 and 2 mg ml⁻¹)

still cause significant inhibition of appressorium development. Exposure to high doses of chitosan (5 mg ml⁻¹) also affected appressorium shape, reducing the size of incipient appressoria (Figs 2c, S2) and preventing formation of the melanin layer (Fig. 2d), essential for *M. oryzae* pathogenicity (Howard & Ferrari, 1989; Howard *et al.*, 1991).

Exposure to chitosan prevents septin recruitment and organization of the appressorium pore

Appressorium repolarization requires recruitment and organization of a hetero-oligomeric ring of septin GTPases at the appressorium pore (Dagdas *et al.*, 2012). In order to determine the effect of chitosan exposure on septin-dependent plant infection, chitosan was applied to un-germinated conidia and localization of Sep4-GFP investigated by live cell imaging (Fig. 3). *M. oryzae* appressoria normally form a large septin ring, approximately 5.9 μ m in diameter (Dagdas *et al.*, 2012), as shown in Fig. 3(a). Exposure to 5 mg ml⁻¹ chitosan for 8 h led to septin accumulation as a dense body in the centre of incipient appressoria, with

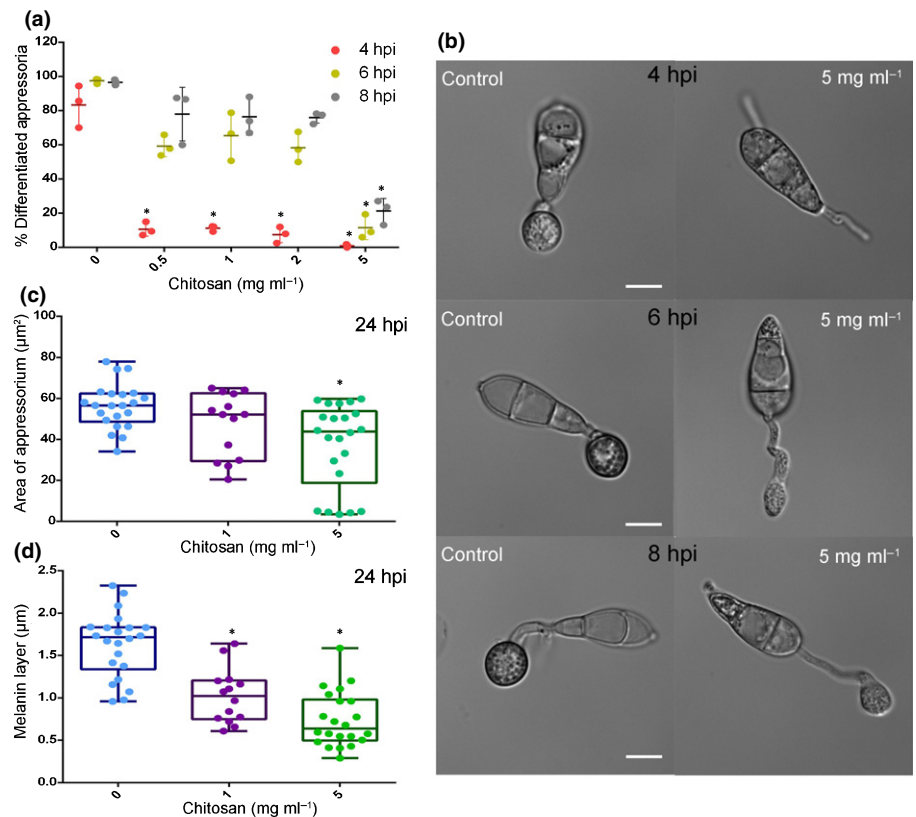


Fig. 2 Chitosan exposure reduces the ability of *Magnaporthe oryzae* to undergo appressorium differentiation and invasive growth. (a) Graph to show frequency of appressorium development at 4, 6 and 8 h following exposure to chitosan (0.5–5 mg ml⁻¹). Plots show mean, SD and data points. (b) Micrographs showing *M. oryzae* development after 4, 6 and 8 h with chitosan (5 mg ml⁻¹) compared with untreated controls. Bars, 10 µm. (c) Box and whisker plot to show effect of chitosan on *M. oryzae* appressorium size (mean, $n > 20$). Error bars are \pm SD. (d) Box and whisker plots of the thickness of the appressorium melanin layer following exposure to 1 and 5 mg ml⁻¹ chitosan. Asterisks (*) indicate significant differences. Multifactorial analysis ANOVA was used to compare treatments (*, $P < 0.05$). Error bars are \pm SD. hpi, h post-inoculation.

no organization into a ring structure (Fig. 3a). Similarly, when the actin-binding protein gelsolin was visualized by expression of gelsolin-GFP, this did not form a ring conformation following exposure to chitosan (Fig. 3b). Consistent with the observed effect of chitosan exposure on septin organization at the appressorium pore, localization of Chm1-GFP, which encodes a kinase which phosphorylates septins, was also affected by exposure to chitosan (Fig. 3c). Polarity determinants, such as Tea1, an F-actin-plasma membrane protein with a C-terminal actin-binding domain and N-terminal ezrin, radixin and moesin domain (Gilden & Krummel, 2010), are also disorganized by exposure to chitosan (Fig. 3d). When appressoria were visualized at 24 h, chitosan exposure was still sufficient to impair septin and F-actin ring organization in *M. oryzae* appressoria (Figs S3–S5). Quantitative analysis confirmed that chitosan treatment reduced the frequency of development of intact septin rings and associated polarity determinants (Fig. 3e–h).

In order to establish the stage at which septin organization was perturbed by chitosan exposure, we decided to apply chitosan to *M. oryzae* germlings in a time-course experiments at 4, 8, 14 and 16 h after conidial germination. Chitosan exposure at early stages of appressorium development, between 4 and 8 h after conidial germination, prevented septin ring organization (Fig S6). There is therefore a window of activity during which chitosan exerts its effect, before 8 h. After this point, the septin ring had already formed and chitosan exposure had no effect on its organization (Fig. S6). We conclude that exposure to chitosan prevents septin-dependent cytoskeletal changes necessary for plant infection by the rice blast fungus.

Chitosan permeabilizes plasma membrane and perturbs the regulated synthesis of ROS during appressorium development

The main cellular change previously reported to occur following chitosan exposure is plasma membrane permeabilization (Palma-Guerrero *et al.*, 2009; Palma-Guerrero *et al.*, 2010). To determine if this occurs upon exposure of *M. oryzae* to chitosan, the lipophilic styryl dye, FM4-64, was applied to germinating conidia in the presence or absence of chitosan (Fig. 4a). Large-scale plasma membrane permeabilization was evident based on widespread FM4-64 fluorescence detected in appressoria in the chitosan-treated samples at 10 min after chitosan treatment (Fig. 4b).

Regulated synthesis of ROS is essential for appressorium development and necessary for septin organization and penetration peg formation (Ryder *et al.*, 2013). By contrast, plasma membrane permeabilization by chitosan exposure leads to massive ROS generation and cell death (Lopez-Moya *et al.*, 2015). We therefore decided to use a strain of *M. oryzae*, expressing Grx1-roGFP2, which detects changes in glutathione oxidation to measure ROS accumulation in *M. oryzae* appressoria. We observed a significant ($P < 0.05$) reduction in Grx1-roGFP2 fluorescence in appressoria following exposure to 5 mg ml⁻¹ chitosan for 8 h (Fig. 4c,d). We also observed in Guy11 elevated expression of the *NOX2* NADPH oxidase, but a small decrease in *NOX1* expression in response to chitosan treatment (Fig. 4e). To investigate this response further, we then examined the expression of *NOX2* in a $\Delta nox1$ mutant. We observed increased *NOX2* expression in a

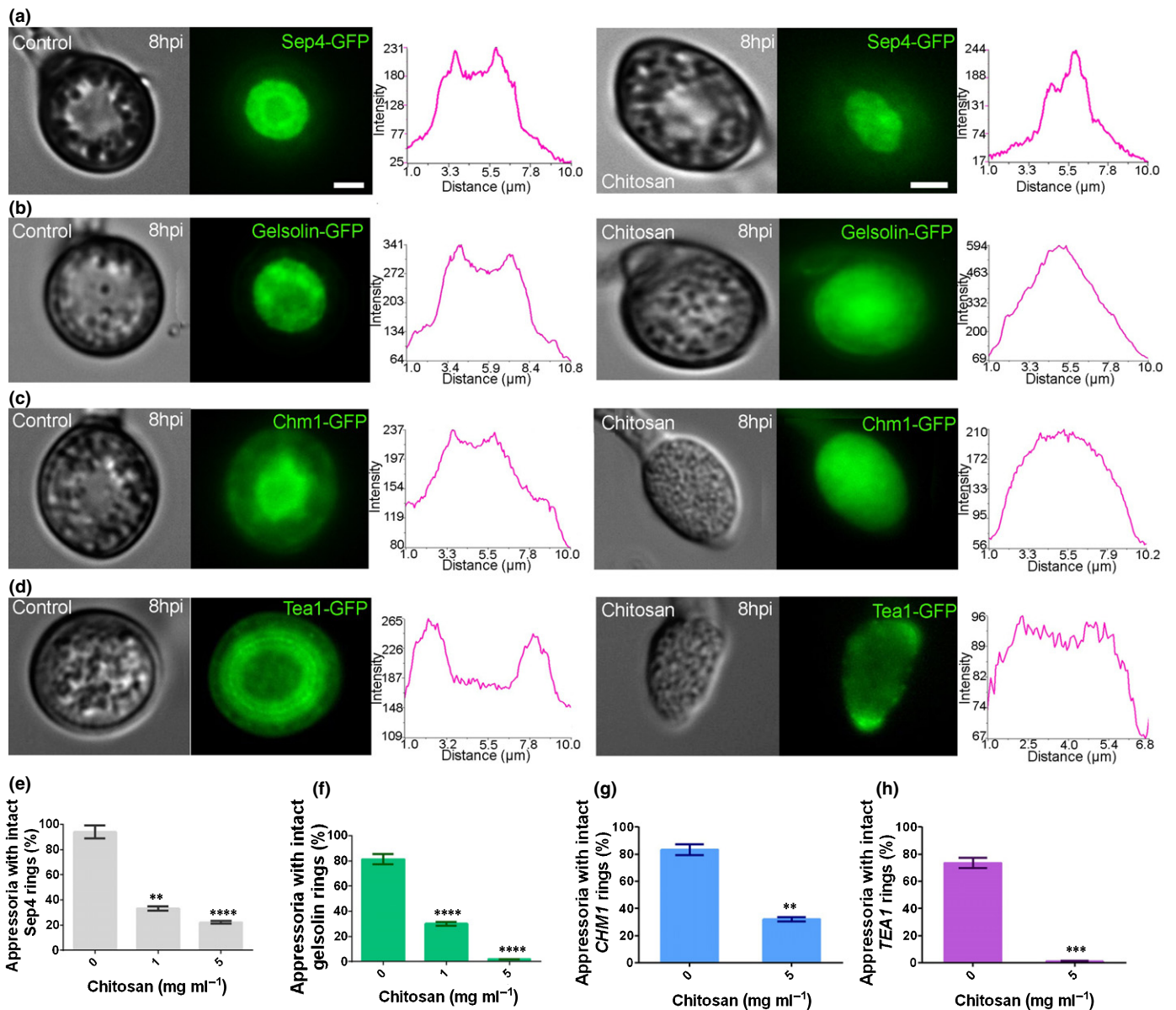


Fig. 3 Chitosan exposure prevents septin recruitment and organization of the appressorium pore. (a) Bright field and epifluorescence micrographs of live cell imaging experiment to show septin-green fluorescent protein (Sep4-GFP) localization during *Magnaporthe oryzae* appressorium development after 8 h in the presence or absence of chitosan (5 mg ml⁻¹). (b) Micrographs showing gelsolin-GFP localization during *M. oryzae* appressorium development after 8 h in the presence or absence of chitosan (5 mg ml⁻¹). (c) Micrographs showing Chm1-GFP localization during *M. oryzae* appressorium development after 8 h in the presence or absence of chitosan (5 mg ml⁻¹). (d) Micrographs showing Tea1-GFP localization during *M. oryzae* appressorium development after 8 h in the presence and absence of chitosan (5 mg ml⁻¹). Transverse line scans for each image on the right to show the distribution of fluorescence in an appressorium. (e–h) Bar charts showing the proportion of appressoria showing ring conformation in the appressorium pore of Sep4-GFP, gelsolin-GFP, Chm1-GFP and Tea1-GFP, in the presence or absence of chitosan. Error bars are \pm SD. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). hpi, h post inoculation.

$\Delta nox1$ mutant in the presence of chitosan (Fig. S7). We conclude that chitosan exposure increases ROS generation and permeabilizes plasma membrane of the rice blast fungus.

Sensitivity to chitosan requires Nox1-dependent NADPH oxidase activity

Given the effect of chitosan exposure to ROS generation and membrane permeability in *M. oryzae*, we decided to investigate

the phenotype of chitosan treatment on $\Delta nox1$, $\Delta nox2$ mutants, lacking the respective catalytic subunits of the NADPH oxidases (Egan *et al.*, 2007). To carry out this experiment, we evaluated the effect of chitosan on appressorium differentiation at 4, 8 and 24 h. At 8 h all untreated strains, including Guy11, $\Delta nox1$, $\Delta nox2$ and $\Delta nox1\Delta nox2$, develop fully differentiated appressoria (Fig. 5a). In the presence of 0.5 and 1 mg ml⁻¹ chitosan, there were mixed phenotypes; however, we noticed that Guy11, $\Delta nox2$ and $\Delta nox1\Delta nox2$ showed significant reduction in the number of

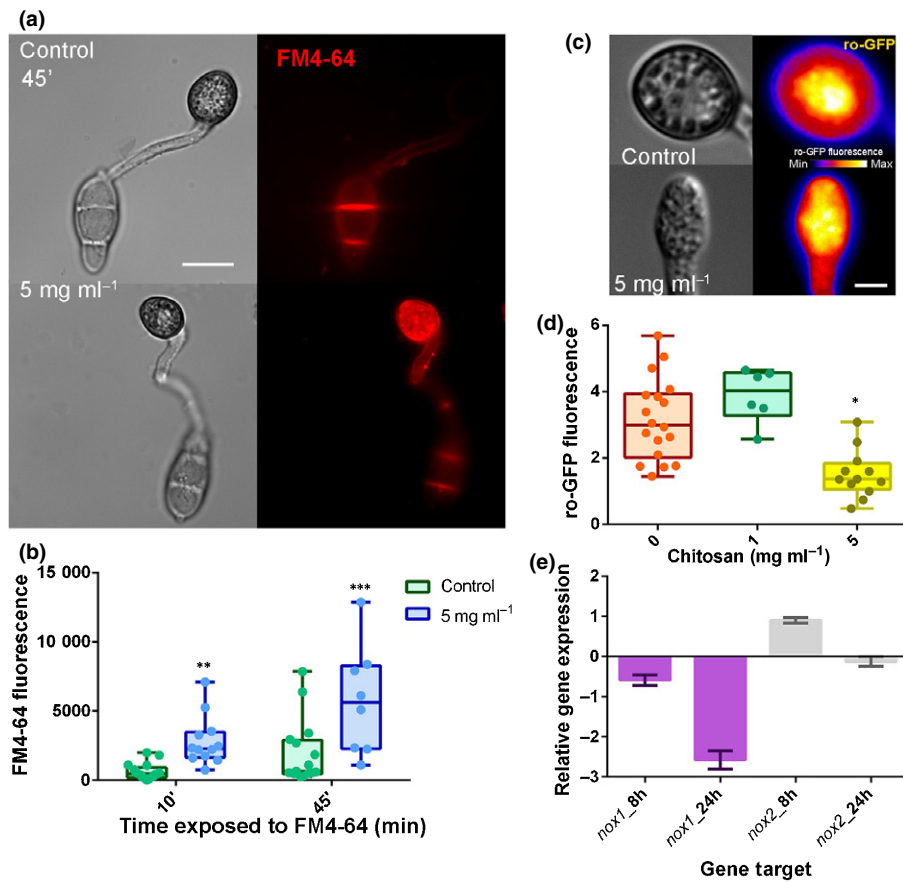


Fig. 4 Chitosan permeabilizes the plasma membrane of *Magnaporthe oryzae* appressoria and induces generation of reactive oxygen species. (a) Bright field and epifluorescence micrographs showing the effect of chitosan on the integrity of plasma membrane in 8 h appressorium of *M. oryzae* after FM4-64 application. Chitosan severely compromises plasma membrane integrity in appressoria exposed to chitosan; FM4-64 fluorescence shows high intensity within the appressorium. Bar, 10 μm . (b) Box and whisker plots of FM4-64 fluorescence within appressoria following chitosan treatment. Plot shows mean \pm SD and data points. (c) Epifluorescence assay showing reduced glutathione by expression of Grx1-roGFP2 in 8-h-old untreated and 5 mg ml⁻¹ chitosan-treated appressoria. Bar, 5 μm . (d) Box and whisker plots showing Grx1-roGFP2 fluorescence quantitation in appressoria in the presence or absence of chitosan. Plot shows mean, SD and data points. (e) Bar charts showing relative gene expression of NADPH oxidase genes *NOX1* and *NOX2* in Guy 11 exposed to chitosan for 8 and 24 h. Error bars are \pm SD. Chitosan treatment represses *NOX1* and initially induces *NOX2* expression. However, after 24 h both *NOX1* and *NOX2* show reduced expression. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

differentiated appressoria formed (Fig. 5b). Appressorium differentiation was not, however, affected in $\Delta nox1$ mutants. In the presence of high chitosan concentrations (5 mg ml⁻¹), few appressoria formed, but $\Delta nox1$ mutants showed increased tolerance to chitosan at all concentrations tested.

We also exposed mycelium of each mutant to 0.5–5 mg ml⁻¹ chitosan and measured vegetative growth in liquid CM cultures, based on DW. We found that chitosan exposure led to accumulation of melanin in culture filtrates of Guy 11 and a $\Delta nox2$ mutant (Fig. 6a). Strikingly, however, $\Delta nox1$ mutants did not secrete excess melanin in the presence of chitosan (Fig. 6a). Consistent with this observation, we found that $\Delta nox1$ mutants were more resistant to chitosan exposure than Guy11 and $\Delta nox2$ mutants (Fig. 6b). We observed that chitosan exposure to a $\Delta nox1 nox2$ double mutant did not cause any reduction in biomass (Fig. 6b). This suggests that absence of the Nox1 catalytic subunit of the NADPH oxidase complex leads to enhanced tolerance to chitosan treatment, which is unaffected by further loss of Nox2. Using growth kinetic analysis, we found that Guy11 had a 3.5-

fold reduction in fungal growth after exposure to a low (0.1 mg ml⁻¹) chitosan dose compared with untreated controls (Fig. S8). The $\Delta nox1$ mutant showed increased resistance to chitosan and was only slightly affected by chitosan at very high doses (> 2 mg ml⁻¹), as shown in Fig. S8.

Sensitivity to chitosan requires the protein kinase C-dependent cell integrity pathway in *M. oryzae*

The $\Delta nox1$ mutant of *M. oryzae* has previously been shown to be more resistant to calcofluor white (Egan *et al.*, 2007), suggesting that Nox1 is critical for cell wall organization. This is consistent with the role of Nox1 in penetration peg elongation and invasive growth (Ryder *et al.*, 2013). We reasoned that resistance to chitosan treatment shown by $\Delta nox1$ mutants may be dependent on correct organization of the fungal cell wall. To test this idea, we evaluated the effect of chitosan on $\Delta mps1$ mutants, impaired the cell integrity pathway (Xu *et al.*, 1998). The Mps1 MAPK is important for hyphal growth, conidiation and appressorium

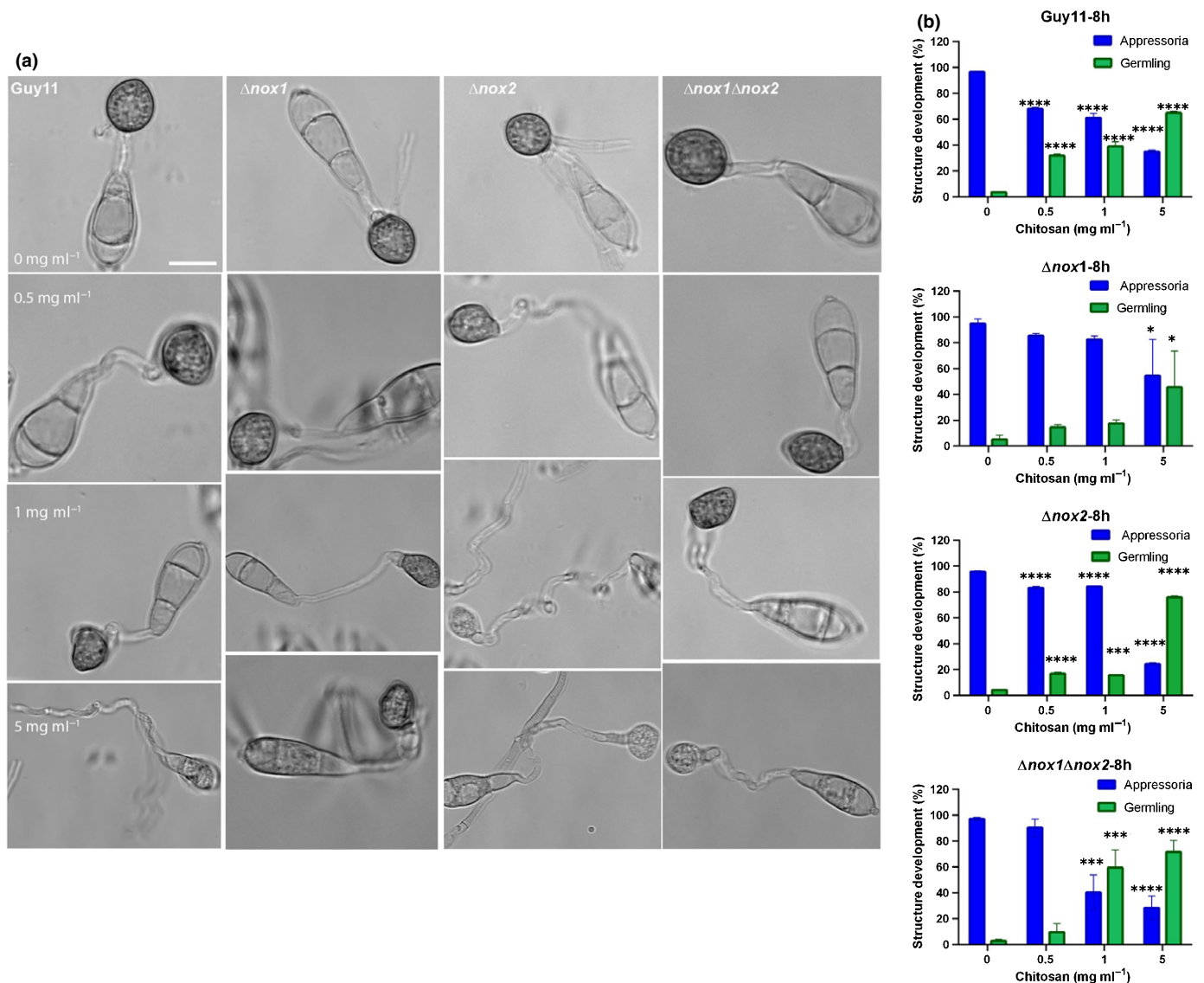


Fig. 5 Sensitivity to chitosan requires *Nox1*-dependent NADPH oxidase activity during appressorium differentiation. (a) Micrographs of *Magnaporthe oryzae* Guy11, $\Delta nox1$, $\Delta nox2$ and $\Delta nox1no x2$ mutants undergoing appressorium development at 4, 8 and 24 h in the presence of chitosan (0.5–5 mg ml⁻¹) compared with untreated controls. Bar, 10 μ m. (b) Bar charts showing the frequency of appressorium differentiation by Guy11, $\Delta nox1$, $\Delta nox2$ and $\Delta nox1no x2$ mutants following 4, 8 and 24 h exposure to chitosan (0.5–5 mg ml⁻¹). Error bars are \pm SD. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

function, and $\Delta mps1$ mutants show defects in the regulation of cell wall biogenesis (Xu *et al.*, 1998). We observed that $\Delta mps1$ mutants showed enhanced resistance to chitosan exposure, growing as well as a $\Delta nox1no x2$ double mutant in the presence of high concentrations of chitosan (Figs 6b, S8).

The cell integrity pathway is regulated by protein kinase C (Levin, 2011) and in *M. oryzae*, *PKC1* is an essential gene required for cell viability (Penn *et al.*, 2015). To test whether chitosan sensitivity requires Pkc1-dependent signalling, we therefore used an analogue-sensitive mutant of Pkc1, which is sensitive to the ATP analogue 4-amino-1-tert-butyl-3-(1'-naphthyl) pyrazolo[3,4-d] pyrimidine (NA-PP1). The *pkc1*^{AS} mutant was generated by mutation of the gatekeeper residue of the ATP-binding pocket of Pkc1 and targeted allelic

replacement to provide a mutant in which Pkc1 activity can be specifically inhibited by application of 1NA-PP1 (described by Penn *et al.*, 2015). This mutant therefore fails to grow in the presence of 1NA-PP1. We incubated the *pkc1*^{AS} mutant in the presence or absence of both chitosan and 1 μ M NA-PP1. The lethal effect of 1NA-PP1 was partially remediated by the presence of chitosan, as shown in Fig. 6(c). This result is consistent with absence of the Pkc1-dependent cell integrity pathway leading to enhanced resistance to chitosan. However, partial remediation of the lethal effect of losing Pkc1 activity by chitosan may also point to a more direct role for chitosan in stabilizing the cell wall when affected in composition by impairment of the cell integrity pathway. We were conscious of only using a single transformant of the *pkc1*^{AS} mutant in

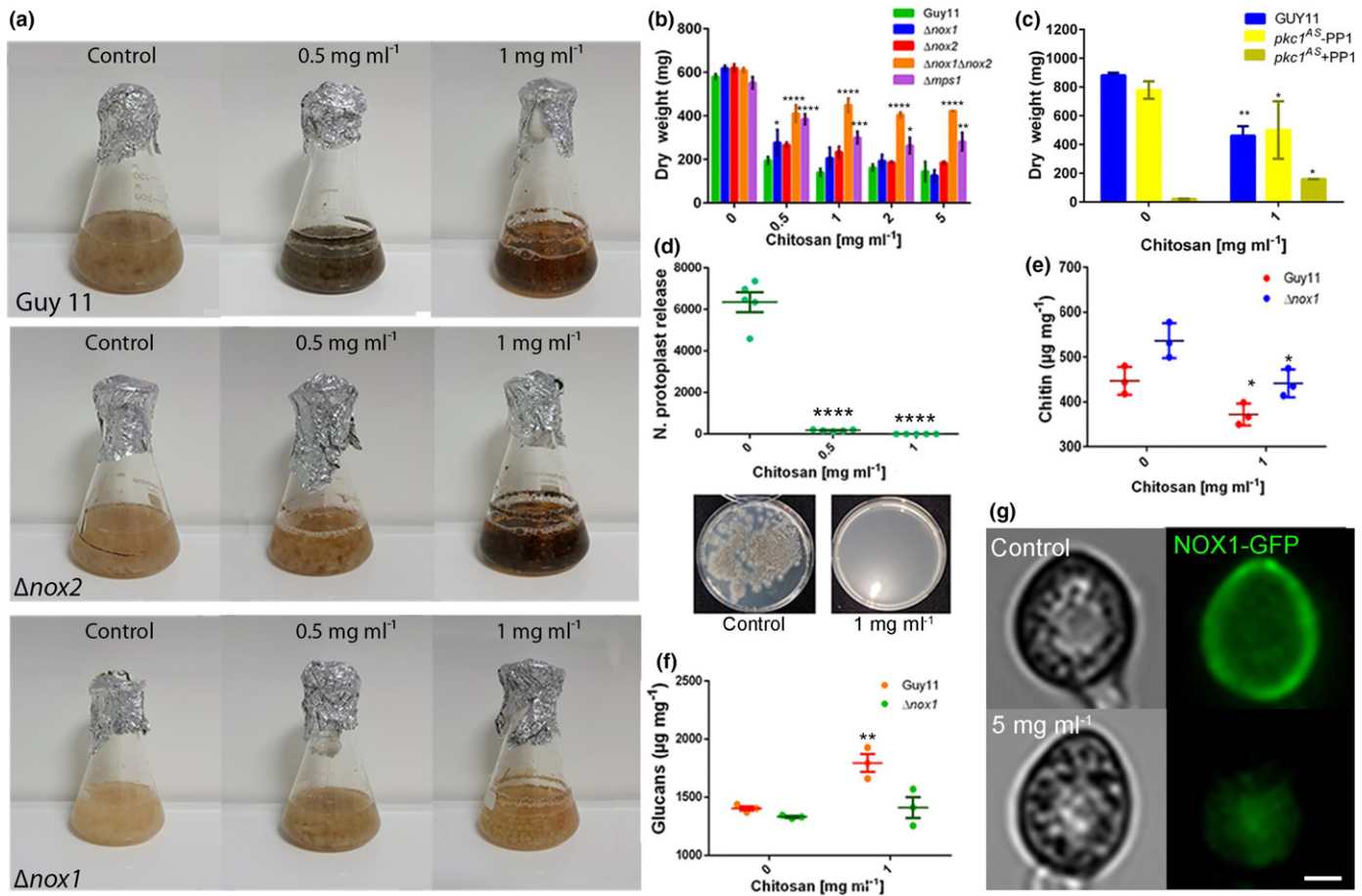


Fig. 6 The sensitivity of *Magnaporthe oryzae* to chitosan requires *NOX1* and the cell integrity pathway. (a) Liquid cultures of *M. oryzae* Guy11, $\Delta nox1$, $\Delta nox2$ and $\Delta nox1 nox2$ following exposure to chitosan (0.5 and 1 mg ml⁻¹). Cultures were grown at 24°C for 48 h. (b) Bar charts showing DW of mycelium recovered from liquid cultures grown in the presence or absence of chitosan (between 0.5 and 5 mg ml⁻¹ for Guy11, $\Delta nox1$, $\Delta nox2$, $\Delta nox1 nox2$ and $\Delta mps1$ mutants). (c) Bar charts showing DW of Guy 11 and the *Pkc1*^{AS} analogue-sensitive mutant in the presence or absence of NA-PP1. (d) Scatterplots showing frequency of protoplast generation following incubation of Guy11 mycelium with Glucanex, following exposure to chitosan. Error bars are \pm SD. (e) Scatterplots showing chitin content of cell wall extracts from mycelium of Guy11 and $\Delta nox1$ mutants after exposure to chitosan. The $\Delta nox1$ mutant has excess chitin compared with Guy11 in untreated mycelium. Chitosan exposure leads to a reduction of chitin in cell walls of both strains. Error bars are \pm SD. (f) Scatterplots showing glucan content of cell wall extracts of Guy11 and $\Delta nox1$. Exposure to chitosan increases the glucan content of Guy 11 and, to a lesser extent, of $\Delta nox1$ mutants. Error bars are \pm SD. (g) Micrographs show Nox1-green fluorescent protein (Nox1-GFP) localization in *M. oryzae* appressorium after 8 h in the presence or absence of chitosan. Bar, 2.5 μ m. Multifactorial ANOVA was used to compare treatments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

these experiments, but this was selected as being consistent with a set of transformants of this mutant, as previously reported (Penn *et al.*, 2015). As a critical control experiment, we tested whether chitosan was able to sequester 1NA-PP1 directly. For this, we used an analogue-sensitive mutant of the Pmk1 MAPK, *pmk1*^{AS} (Sakulkoo *et al.*, 2018). The *pmk1*^{AS} mutant also contains a point mutation that modifies its ATP binding pocket conferring susceptibility to NA-PP1 (Bishop *et al.*, 2000; Sakulkoo *et al.*, 2018). When 1NA-PP1 is applied for between 0 and 1 h, the *pmk1*^{AS} mutant is unable to develop appressoria (Xu & Hamer, 1996; Sakulkoo *et al.*, 2018). To test whether chitosan sequesters 1NA-PP1, we inoculated spores of the *pmk1*^{AS} mutant and treated them 1 or 5 mg ml⁻¹ chitosan and 5 μ M 1NA-PP1 (Fig. S9). We observed that these mutants were not able to develop appressoria in the presence of Na-PP1, either in the presence or

absence of chitosan. The 1NA-PP1 is therefore active, providing strong evidence that chitosan does not sequester 1NA-PP1. We conclude that chitosan exerts an effect on the cell wall that prevents the normal lethality associated with loss of Pkc1.

To investigate the effect of chitosan on cell wall composition in *M. oryzae*, we carried out a protoplast release assay to evaluate the sensitivity of the cell wall to enzymatic degradation. We treated fungal mycelium, grown in the presence or absence of chitosan (0.5 and 1 mg ml⁻¹), with Glucanex (Sigma Aldrich), a commercial preparation of glucanases and chitinases, to release protoplasts. Mycelium grown in the presence of chitosan was more resistant to cell wall-degrading enzymes, showing significantly ($P < 0.0001$) less protoplast release (Figs 6d, S10). We also investigated the effect of chitosan in combination with caspofungin, a lipopeptide inhibitor of β -1,3 glucan synthase. We exposed homogenized Guy11 mycelium to 0.1 μ g ml⁻¹

caspofungin in combination with increasing chitosan concentrations (0.1–2 mg ml⁻¹). Low chitosan exposure (0.1 mg ml⁻¹) in combination with caspofungin completely arrested fungal growth compared with when the same dose of chitosan was applied in the absence of caspofungin (Fig. S11). To investigate these effects on cell wall integrity, we next determined the chitin and glucan content of cell walls of mycelium of *M. oryzae*, grown in the presence or absence of chitosan (Fig. 6e,f). Chitosan exposure led to a significant decrease in chitin content and an increase in glucan in fungal cell walls. Interestingly, when we repeated this assay in a $\Delta nox1$ mutant, we observed an elevated chitin content and reduced glucan content in the mutant, when grown in the absence of chitosan. Chitosan treatment led to a decrease in chitin content of a $\Delta nox1$ mutant, but only to the same as in those in untreated Guy11 (Fig. 6). Furthermore, there was little effect on glucan content in the $\Delta nox1$ mutant when exposed to chitosan (Fig. 6e,f). These observations suggest that $\Delta nox1$ mutants may be resistant to chitosan as a result of their distinct glucan/chitin cell wall ratio. Consistent with the importance of Nox1 function in the response to chitosan, live cell imaging experiments of a *M. oryzae* strain expressing Nox1-GFP revealed mislocalization of the NADPH oxidase upon exposure to chitosan. Nox1-GFP is normally located at the appressorium cortex in 24 h appressoria. However, in appressoria exposed to a high chitosan dose (5 mg ml⁻¹), Nox1-GFP was located in the central appressorium vacuole (Fig. 6g). When considered together, our observations suggest that the cell integrity pathway is critical to the fungicidal activity of chitosan and this may be linked to the glucan : chitin ratio of fungal cell walls.

Exposure to chitosan causes repression of cell wall integrity pathway gene expression

To characterize the link between the cell wall integrity pathway and the response of *M. oryzae* to chitosan, we studied the expression of genes encoding components of the cell wall integrity pathway in mycelium grown in the presence or absence of chitosan. We observed that chitosan exposure led to repression in the expression of *MPS1*, *PKC1*, *RHO1* and *SWI6* in Guy 11 (Fig. 7). In all cases the effect was most pronounced after 8 h exposure, but some repression was maintained even after 24 h exposure to chitosan. When the same genes were analysed in a $\Delta nox1$ mutant in the presence or absence of chitosan, we observed that chitosan exposure led to elevated expression of *PKC1*, *MPS1*, *RHO1*, *SWI6* and *CHM1* after 24 h exposure (Figs 7, S12). *MPS1* expression was initially reduced in the $\Delta nox1$ mutant when exposed to chitosan for 8 h, but then showed elevated expression by 24 h, while *CHM1* expression was elevated even in the absence of chitosan. The overall pattern of gene expression suggests that chitosan treatment normally leads to repression of the cell wall integrity pathway. By contrast, the absence of the Nox1 NADPH oxidase prevents this repression mediated by chitosan exposure and instead leads to elevated expression. Taken together, these results are consistent with the cell wall integrity pathway function being essential for the fungicidal activity of chitosan.

Discussion

Chitosan has considerable potential as a naturally occurring anti-fungal agent. It can be readily produced by partial deacetylation of chitin, one of the most common biopolymers, a constituent of the cell walls of fungi, and the exoskeletons of arthropods and crustaceans (Allan & Hadwiger, 1979). Commercially, chitosan can be produced in large quantities, for example, from crab shell waste (Kumar, 2000). In addition to being a highly toxic cationic inhibitor of the growth of many fungal species, including important plant pathogens, chitosan is not toxic to mammals, including humans (Lopez-Moya *et al.*, 2015), or to plants leaves, where it can act instead as a plant defence-inducing compound (Trotel-Aziz *et al.*, 2006). Chitosan therefore has considerable potential to treat plant diseases, but its mode of action is still not completely clear.

In this study we set out to determine the effects of chitosan treatment on the rice blast fungus *M. oryzae*, one of the world's most devastating crop pathogens, which causes very substantial losses to the annual rice harvest (Wilson & Talbot, 2009; Martin-Urdiroz *et al.*, 2016). The infection mechanism of *M. oryzae* has also been intensively studied, making it an excellent model for understanding how chitosan might act in the context of perturbing plant infection by a phytopathogenic fungus.

First of all, we demonstrated that chitosan is able to inhibit the growth of *M. oryzae* and its ability to cause rice blast disease. We also showed that not only does chitosan exhibit anti-penetrant activity, but it also affects fungal invasive growth. In leaf sheath bioassays, chitosan inhibits penetration peg formation and invasive hyphae development. It is known that chitosan forms part of the differentiated cell wall of *M. oryzae* during appressorium-mediated plant infection (Geoghegan & Gurr, 2016), but the fungicidal action of exogenously applied chitosan to *M. oryzae* has not previously been shown. We observed that chitosan blocks appressorium-mediated plant infection by the fungus and is able to prevent organization of the septin ring, which is necessary for F-actin reorganization at the appressorium pore. Four core septins, Sep3, Sep4, Sep5 and Sep6, are essential for generating a heterooligomeric ring structure that bounds the appressorium pore and is essential for plant infection (Dagdas *et al.*, 2012). Formation of this ring structure is pivotal to penetration peg development and therefore rupture of the rice cuticle. The septin ring acts as a scaffold to organize F-actin at the point of infection and as a lateral diffusion barrier to hold polarity determinants, such as Tea1, Cdc42 and Las17, at the centre of the pore, from where formation of the penetration peg occurs (Dagdas *et al.*, 2012; Ryder *et al.*, 2013). Chitosan prevents these developmental changes from occurring and polarity of the appressorium is thus impeded, preventing plant infection. Septins are well-known regulators of polarity and fungal morphogenesis in filamentous fungi, such as *Aspergillus nidulans* and *Neurospora crassa* (Berepiki & Read, 2013), and yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe* (Gladfelter *et al.*, 2005; Hernandez-Rodriguez & Momany, 2012). Consistent with this effect on septin recruitment and organization, previous transcriptional profiling experiments in *N. crassa* show that chitosan exposure represses

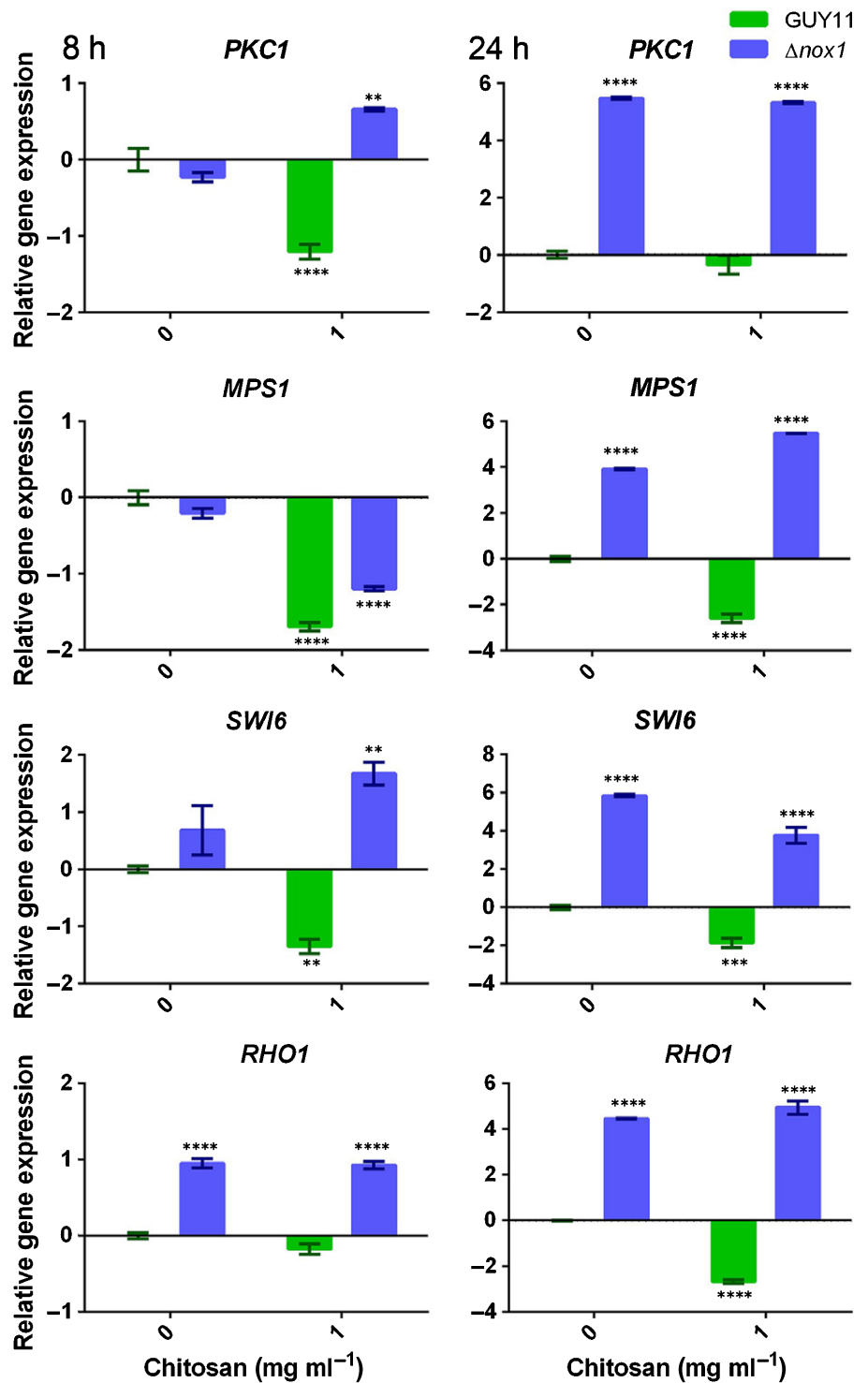


Fig. 7 Chitosan represses the cell integrity pathway in a *NOX1*-dependent manner. Bar charts to show the effect of chitosan exposure (8 and 24 h) on gene expression of cell integrity pathway genes *PKC1*, *MPS1*, *SWI6* and *RHO1* in Guy11 and an isogenic $\Delta nox1$ mutant. Error bars are \pm SD. A multifactorial ANOVA was used to compare treatments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

expression of the core septin-encoding genes *CDC10*, *CDC11* and *CDC12* (Lopez-Moya *et al.*, 2016). The inhibition of septin recruitment was also shown to impair F-actin organization during appressorium differentiation and the localization of Tea1 and Chm1. This is consistent with chitosan preventing appressorium function because of an inability to carry out the rapid septin-dependent, actin polymerization at the base of the infection cell, necessary for repolarization. In *N. crassa*, proteins associated with

actin polymerization proteins are also repressed by chitosan (Lopez-Moya *et al.*, 2016). These results support the hypothesis that one of the consequences of chitosan exposure is to disrupt cytoskeletal organization.

To investigate the primary mode of action of chitosan we decided to evaluate its effect on the plasma membrane and cell wall. Previous studies have shown that chitosan exposure causes plasma membrane permeabilization (Palma-Guerrero, *et al.*,

2009; Jaime *et al.*, 2012; Lopez-Moya, *et al.*, 2015). It is known that chitosan permeabilizes plasma membrane in an ATP-dependent manner in *N. crassa* (Palma-Guerrero, *et al.*, 2009) and that its toxicity is dependent on membrane fluidity. A fatty acid desaturase mutant, Δods , showing reduced plasma membrane fluidity exhibited increased resistance to chitosan (Palma-Guerrero *et al.*, 2010). Chitosan-resistant fungi, such as the nematophagous fungus *Pochonia chlamydosporia*, also showed reduced polyunsaturated fatty acid content in the plasma membranes (Palma-Guerrero *et al.*, 2010). We observed that chitosan exposure led to plasma membrane permeabilization in *M. oryzae*, consistent with these previous studies. Permeabilization and disruption of membrane integrity may lead to the changes in septin organization observed, given the importance of the plasma membrane cytoskeleton scaffolding (Porter & Day, 2015).

It is known that membrane-localized NADPH oxidases are key to regulating the recruitment and organization of septins during plant infection by *M. oryzae* (Egan *et al.*, 2007; Ryder *et al.*, 2013). We observed that chitosan exposure causes massive ROS synthesis, consistent with its effects on septin recruitment. In this sense, we also observed elevated expression of *NOX2* by chitosan treatment. We also found that a $\Delta nox1$ mutant, which lacks one of the catalytic subunits of NADPH oxidase, is more resistant to chitosan treatment. Previously it has been reported that Nox1 is required for cell wall organization in *M. oryzae*, as $\Delta nox1$ mutants show greater resistance to calcofluor white (Egan *et al.*, 2007). It is also known that Nox1 is necessary for penetration peg elongation and is therefore likely to be a key regulator of cell wall biosynthesis. We observed that $\Delta nox1$ mutants showed a higher chitin content than an isogenic wild-type strain of *M. oryzae* and this may have contributed to their resistance to chitosan, as exposure to chitosan appears to deplete the fungus of chitin in its cell walls. The imbalance in chitin and glucan content caused by chitosan exposure may be an important element of its toxicity and may be associated with the ability of chitosan to traverse the wall effectively to bind to its primary target the plasma membrane (Palma-Guerrero *et al.*, 2009; Palma-Guerrero *et al.*, 2010; Lopez-Moya *et al.*, 2015; Lopez-Moya *et al.*, 2016; Aranda-Martinez *et al.*, 2016). We also observed that chitosan induces melanin secretion from Guy11 mycelium but not in the $\Delta nox1$ mutant. However, during appressorium differentiation chitosan arrests melanin accumulation in appressorial cell walls of both strains. This may be related to the ability of melanin to buffer the stress generated by chitosan. Chitosan induces physiological changes that lead to accumulation of phenols in fungi and plants (Camacho *et al.*, 2017; Lopez-Moya *et al.*, 2017), and the stress generated by chitosan may therefore affect melanin biosynthetic pathways in appressoria.

The importance of the fungal cell wall in conditioning the response of *M. oryzae* to chitosan exposure led us to examine mutants in key components of the cell wall integrity pathway. This pathway is well known to mediate the response to cell wall stress and is therefore associated with osmotic stress adaptation and xenobiotic stresses, including drug treatments (Bahn

et al., 2007; LaFayette *et al.*, 2010; Penn *et al.*, 2015). We found that chitosan exerts its toxicity towards *M. oryzae* through a process dependent on a functional cell wall integrity pathway. A $\Delta mps1$ mutant lacking the cell wall integrity MAPK, was more tolerant to chitosan than the wild-type strain. *MPS1*, *PKC1*, *SWI6* and *RHO1* genes were all repressed in response to chitosan treatment. *RHO1* controls cell wall synthesis and aggregation of actin cables in *M. oryzae* (Fu *et al.*, 2018) and its downregulation in response to chitosan is consistent with the defects in F-actin organization observed after drug treatment. Protein kinase C, which acts as a major control point for operation of the cell wall integrity pathway, is essential in *M. oryzae*, but we found that a conditionally lethal, analogue-sensitive mutant of *PKC1* (Penn *et al.*, 2015) could be partially remediated for growth in the presence of chitosan. This result strongly suggests that chitosan can partially serve to stabilize cell wall integrity in the absence of the PKC signalling pathway in a way that allows some fungal growth to occur. It is clear, however, that when the cell integrity pathway is fully operational, chitosan can permeabilize the cell membrane, affect NADPH oxidase function, disorganize septins and disrupt the actin cytoskeleton. The role of the Nox1 NADPH oxidase in regulating cell wall biogenesis is also clear, because its absence renders the fungus much less sensitive to chitosan, upregulates genes encoding components of the PKC cell wall integrity pathway in the presence of chitosan, and leads to an elevated chitin cell wall content. This change in cell wall composition explains the previously reported resistance of $\Delta nox1$ mutants to calcofluor white (Egan *et al.*, 2007) and reveals the importance of cell wall function to the ability of chitosan to inhibit fungal growth.

Chitosan treatment may therefore be a potential means by which rice blast disease could be controlled in future, given its ability to prevent leaf infection at a very early stage, before cuticle penetration. Furthermore, because chitosan is likely to act at multiple sites in conditioning membrane permeabilization, the chances of selecting specific resistant mutants is likely to be low. Our results show how increased tolerance to chitosan, which might emerge as a result of perturbation of cell wall function, is unlikely to result in strains that could survive under field conditions, because of the essential nature of cell wall composition in growth and development. In conclusion, chitosan serves as a valuable research tool to study fungal morphogenesis and the biology of plant infection by pathogenic fungi, but may also constitute a natural antifungal agent that could be used to control blast disease.







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Author contributions

FL-M: design and performance of research, data collection and data analysis, writing of the manuscript. MM-U: performance of research, technical support and data interpretation. MO-R: performance of research, technical support and data interpretation. VMW: performance of research, technical support and data interpretation. MF: ro-GFP results analysis and other data interpretation. GL: performance of research, technical support with confocal imaging and data interpretation. LVL-L: design and performance of the research, data interpretation, writing the manuscript. NJT: design of the research, data interpretation, writing of the manuscript.

ORCID

Mark D. Fricker  <https://orcid.org/0000-0002-8942-6897>
 George Littlejohn  <https://orcid.org/0000-0002-8768-2598>
 Federico Lopez-Moya  <https://orcid.org/0000-0003-4414-0702>
 Luis V. Lopez-Llorca  <https://orcid.org/0000-0003-4390-3608>
 Magdalena Martin-Urdiroz  <https://orcid.org/0000-0003-4209-5389>
 Miriam Osés-Ruiz  <https://orcid.org/0000-0002-3989-9071>
 Nicholas J. Talbot  <https://orcid.org/0000-0001-6434-7757>
 Vincent M. Were  <https://orcid.org/0000-0002-9885-4877>

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Conidial germination of *M. oryzae* in the presence and absence of chitosan. Bar charts to show frequency of conidial germination.

Fig. S2 Morphometric analysis of the effect of chitosan on appressorium development.

Fig. S3 Chitosan inhibits septin (Sep4) organization in the appressorium pore of *M. oryzae*.

Fig. S4 Chitosan inhibits gelsolin localization in the appressorium pore of *M. oryzae*.

Fig. S5 Chitosan exposure leads to mislocalization of Chm1-GFP and Tea1-GFP in the appressorium pore of *M. oryzae*.

Fig. S6 Chitosan applied at early stages of appressorium development impairs septin organization in the appressorium pore of *M. oryzae*.

Fig. S7 Chitosan exposure increases *NOX2* expression in a *M. oryzae* $\Delta nox1$ mutant.

Fig. S8 $\Delta nox1$ and $\Delta mps1$ show increased tolerance to chitosan exposure in a growth kinetic assay.

Fig. S9 Chitosan does not affect Na-PP1 activity during appressorium development by a *pmk1AS* mutant.

Fig. S10 *M. oryzae* mycelium grown in the presence of chitosan is resistant to cell wall-degrading enzymes.

Fig. S11 Chitosan shows synergistic antifungal activity in the presence of caspofungin.

Fig. S12 Chitosan exposure modulates *CHM1* gene expression in wild-type and $\Delta nox1$ mutants of *M. oryzae*.

Table S1 Physicochemical properties of chitosan (T8).

Table S2 Sequences of primers used in this study.

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