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Physcomitrella patens activates reinforcement of the cell wall, programmed cell death and accumulation of evolutionary conserved defence signals, such as salicylic acid and 12-oxo-phytodienoic acid, but not jasmonic acid, upon *Botrytis cinerea* infection

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SUMMARY

The moss *Physcomitrella patens* is an evolutionarily basal model system suitable for the analysis of plant defence responses activated after pathogen assault. Upon infection with the necrotroph *Botrytis cinerea*, several defence mechanisms are induced in *P. patens*, including the fortification of the plant cell wall by the incorporation of phenolic compounds and the induced expression of related genes. *Botrytis cinerea* infection also activates the accumulation of reactive oxygen species and cell death with hallmarks of programmed cell death in moss tissues. Salicylic acid (SA) levels also increase after fungal infection, and treatment with SA enhances transcript accumulation of the defence gene phenylalanine ammonia-lyase (*PAL*) in *P. patens* colonies. The expression levels of the genes involved in 12-oxo-phytodienoic acid (OPDA) synthesis, including lipoxygenase (*LOX*) and allene oxide synthase (*AOS*), increase in *P. patens* gametophytes after pathogen assault, together with a rise in free linolenic acid and OPDA concentrations. However, jasmonic acid (JA) could not be detected in healthy or infected tissues of this plant. Our results suggest that, although conserved defence signals, such as SA and OPDA, are synthesized and are probably involved in the defence response of *P. patens* against *B. cinerea* infection, JA production appears to be missing. Interestingly, *P. patens* responds to OPDA and methyl jasmonate by reducing moss colony growth and rhizoid length, suggesting that jasmonate perception is present in mosses. Thus, *P. patens* can provide clues with regard to the evolution of different defence pathways in plants, including signalling and perception of OPDA and jasmonates in nonflowering and flowering plants.

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INTRODUCTION

Flowering plants have evolved complex signalling and defence mechanisms to protect themselves against pathogen attack. Recognition of the pathogen triggers a large range of inducible defence reactions, including the increase of reactive oxygen species (ROS), synthesis of antimicrobial compounds, reinforcement of the cell wall and induction of defence-related genes. The activation of many of these cellular responses is common to both resistant and susceptible hosts, and the outcome of the interaction will depend on the ability to perceive the pathogen and quickly mount an effective host defence response. One of the most efficient resistance reactions is the hypersensitive response (HR), which is characterized by the rapid death of plant cells in infected tissues. Several studies have shown that plant cell death occurring during the HR is a type of programmed cell death (PCD) (Greenberg and Yao, 2004; Morel and Dangl, 1997). Plant cells undergoing PCD share a number of morphological and biochemical features in common with animal PCD, including chromatin condensation, DNA fragmentation, blebbing of the plasma membrane, accumulation of ROS, release of cytochrome *c* from mitochondria and activation of a number of hydrolytic enzymes, such as serine and cysteine proteases (Greenberg and Yao, 2004; Lam, 2004, 2008). Recently, two major classes of plant PCD have been defined, including vacuolar cell death and necrotic cell death, although the HR falls into a separate modality, as features of both types of cell death are present (van Doorn *et al*., 2011).

The biosynthesis of salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) is induced after pathogen recognition, and these hormones play key roles in plant defence responses. The balance and interplay of these hormones will determine the expression of resistance to particular pathogens (Glazebrook, 2005). In general, SA-mediated responses are associated with resistance to biotrophic pathogens, whereas JA and ET are considered to be important signals in resistance to necrotrophic pathogens. Recent studies have shown that additional hormones are involved in the defence

response to pathogen assault, including abscisic acid (ABA) and auxins (López *et al*., 2008; Robert-Seilaniantz *et al*., 2011). In addition, many pathogens are themselves also capable of producing phytohormones during infection that interfere with the plant defence response (López *et al*., 2008; Robert-Seilaniantz *et al*., 2007).

Bryophytes (mosses, liverworts and hornworts) are nonvascular plants that diverged from flowering plants more than 450 million years ago, representing one of the oldest groups of land plants (Schaefer and Zrÿd, 2001). The first plants that colonized emerged lands needed to adapt to important stresses, including desiccation and UV radiation, as well as infection by microbial pathogens. Although bryophytes interact in nature with a variety of fungal pathogens, some of which infect and cause symptoms (Davey and Currah, 2006), little information exists on the defence mechanisms activated after pathogen assault. We have shown previously that the model moss *Physcomitrella patens* constitutes an excellent model system for the analysis of the infection processes and defence responses activated after microbial infection in an evolutionarily basal land plant (Oliver *et al*., 2009; Ponce de León, 2011; Ponce de León *et al*., 2007). The *P. patens* genome has been sequenced completely (Rensing *et al*., 2008) and functional studies of genes with possible roles in defence responses can be performed by targeted gene disruption, as *P. patens* has a high frequency of homologous recombination, in contrast with flowering plants (Schaefer, 2002).

Broad-host-range necrotrophic pathogens, such as *Botrytis cinerea*, *Pectobacterium carotovorum* ssp. *carotovorum* (*P.c. carotovorum*; formerly named *Erwinia carotovora* ssp. *carotovora*), *Pythium debaryanum* and *Pythium irregulare*, are capable of infecting *P. patens* tissues, causing disease symptoms and activation of defence responses (Oliver *et al*., 2009; Ponce de León *et al*., 2007). Inoculation with *B. cinerea* and both *Pythium* species, as well as treatments with *P.c. carotovorum* cell-free culture filtrates, containing plant cell wall-degrading enzymes which act as elicitors capable of inducing plant defence responses in flowering plants (Vidal *et al*., 1997), induced the expression of defence-related genes, including lipoxygenase (*LOX*), phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*) and pathogenesis-related-1 (*PR-1*). In addition, *P. patens* cells infected with *B. cinerea*, or treated with elicitors of a harpin (HrpN) producing *P.c. carotovorum* strain (SCC1) (Rantakari *et al*., 2001), showed cytoplasmic collapse, the accumulation of autofluorescent compounds and chloroplast breakdown, suggesting that an HR-like response is activated in *P. patens* (Ponce de León *et al*., 2007). Levels of 12-oxo-phytodienoic acid (OPDA), which is the precursor of the defence hormone JA, increased in *P. debaryanum*- and *P. irregulare*-infected moss tissues relative to healthy *P. patens* tissues (Oliver *et al*., 2009). However, the presence of JA or its derivatives, collectively named jasmonates, in *P. patens* is still controversial, as Stumpe *et al*. (2010) could not detect any amount of JA or amino acid conjugates of JA in moss tissues, and only exceedingly low levels of JA were estimated in *Pythium*-inoculated gametophytes (Oliver *et al*., 2009).

In order to expand the knowledge on the different defence mechanisms activated in *P. patens* after pathogen challenge, we continued our studies using *B. cinerea* as a pathogen. *Botrytis cinerea* penetrates plant tissues by releasing hydrolysing enzymes, including cutinases, and pectinolytic enzymes, causing grey mould disease in many crop plants (van Kan, 2006). As part of its invasion strategy, *B. cinerea* promotes PCD in plant cells, leading to pathogen proliferation (Govrin and Levine, 2000). In this study, we show that *B. cinerea* multiplies in *P. patens* tissues and induces defence responses, including the reinforcement of the cell wall, activation of PCD and the accumulation of SA and OPDA. Although we could not detect JA, *P. patens* responds to methyl jasmonate (MeJA) by producing a developmental response in a dose-dependent manner, which is consistent with jasmonate action in other plants (Staswick *et al*., 1992).

RESULTS

Botrytis cinerea multiplies in *P. patens* tissues and activates the reinforcement of the cell wall

We have reported previously that *B. cinerea* infects *P. patens* cells, resulting in necrosis and maceration of host tissues within 2 days of inoculation (Ponce de León *et al*., 2007). In order to quantify mycelium growth during the initial stages of infection, when symptoms are not evident, we isolated total DNA and performed quantitative polymerase chain reaction (PCR) using primers specific for both *B. cinerea* and *P. patens* DNA. As shown in Fig. 1a, *B. cinerea* biomass started to increase after 8 h of inoculation and, after 24 h, *B. cinerea* DNA became predominant in the samples. In addition to quantitative PCR, the colonization of *B. cinerea* was further assessed by staining mycelium in living moss cells with the fluorescent dye solophenyl flavine 7GFE 500 (Hoch *et al*., 2005). Spores germinated and germ tubes elongated at 4 and 8 h post-inoculation (hpi), respectively (Fig. 1b,c). At 24 hpi, hyphae proliferated and different infection stages could be observed in different leaves (Fig. 1d–g). *Botrytis cinerea* hyphal tips attempted to penetrate single moss cells (Fig. 1d) and, once infection occurred, fungal hyphae were observed within the limits of the cell walls (Fig. 1e). Mycelium proliferated and hyphae grew within and on moss leaves (Fig. 1f–g).

Botrytis cinerea-infected leaves were stained with safranin-O and toluidine blue to measure the incorporation of phenolic compounds. Water-treated leaves (Fig. 2a) and uninfected tissues were not stained, whereas *B. cinerea*-infected tissues were positively stained, reflecting a cell wall fortification mechanism (Fig. 2b–f). Plant cell walls in contact with *B. cinerea* hyphae incorporated phenolic compounds, visualized by staining with both dyes. Fungal hyphae on the surface of the leaves were also stained (Fig. 2c,f).

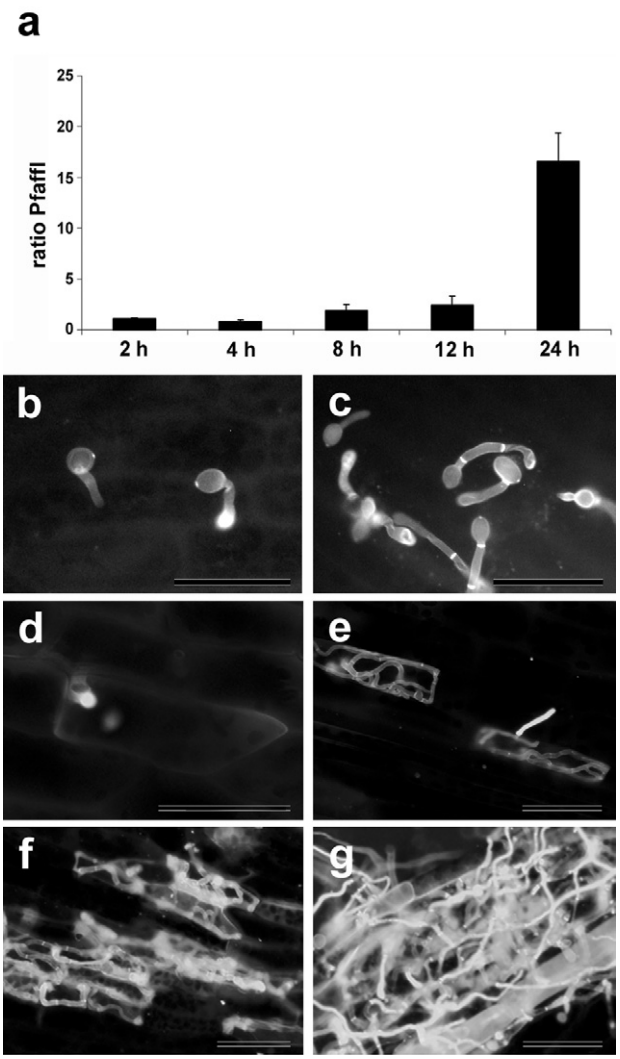

Fig. 1 Progress of *Botrytis cinerea* colonization in *Physcomitrella patens* tissues. (a) *Botrytis cinerea* DNA levels were estimated by quantitative polymerase chain reaction (qPCR) adapted from Pfaffl (2001), normalizing against plant DNA content as an endogenous reference and relative to time zero of the infection time course. The results and standard deviation of three independent triplicate experiments are shown. (b–g) Staining of fungal hyphae with the fluorescent dye solophenyl flavine 7GFE 500, showing different infection stages of moss colonization by *B. cinerea*. (b) Spore germination at 4 h post-inoculation (hpi). (c) Germ tube elongation at 8 hpi. (d) Hyphal tip approaching a moss cell at 24 hpi. (e) Hyphae growing within cell wall limits at 24 hpi. (f, g) Proliferation of mycelium within and on moss tissues at 24 hpi. The scale bar represents 20 μm.

Previously, we have identified a gene encoding a dirigent-like (DIR) protein in a cDNA library obtained from *P. patens* tissues treated with elicitors of *P.c. carotovorum* (A. Alvarez and I. Ponce de León, personal observation). DIR proteins are considered to mediate the free radical coupling of monolignol plant phenols to yield the cell wall polymers lignans and lignins (Davin and Lewis, 2000). Expression pattern analysis showed that *P. patens DIR*-like gene is induced within 4 h of *B. cinerea* inoculation, and high levels of transcript accumulation persist until 48 h (Fig. 2g). The results suggest that induced expression of a *DIR*-like gene could lead to the modification of the cell wall polymer content and contribute to the reinforcement of the cell wall after *B. cinerea* infection.

Botrytis cinerea infection causes ROS accumulation and PCD in *P. patens*

We evaluated whether ROS accumulates after the inoculation of *P. patens* colonies with *B. cinerea*. ROS production was observed in a single cell showing an approaching hypha (Fig. 3b) and in fungal invaded cells (Fig. 3c–e), while no fluorescence could be detected in water-treated leaves (Fig. 3a). As the infection progressed, intracellular ROS were distributed homogeneously in the cytoplasm (Fig. 3e). Since ROS are involved in the generation of plant cell death, we quantified cell death occurring in *B. cinerea*-inoculated tissues with Evans blue staining. As shown in Fig. 3f, the number of dead cells started to increase after 1 dpi, reaching a three-fold increase after 2 dpi in comparison with water-treated tissues.

We have shown previously that *P. patens* cells infected with *B. cinerea* exhibit hallmarks of PCD (Ponce de León *et al*., 2007). Here, using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay, we confirmed that *B. cinerea* induced PCD in moss tissues, particularly in cells colonized by fungal hyphae (Fig. 3g–j), indicating the occurrence of DNA fragmentation. No fluorescence was detected in water-treated tissues or in tissues corresponding to the negative control in which the terminal transferase was omitted (data not shown). DNA laddering characteristics of nucleosomal breakdown were not detected in *B. cinerea*-infected *P. patens* tissues (data not shown), or in moss tissues infected with other pathogens (Lawton and Saidasan, 2009). Taken together, these results show that *B. cinerea* infection caused ROS production in infected *P. patens* tissues and activation of an HR-like response.

Physcomitrella patens induces the accumulation of SA and OPDA, but not JA, after *B. cinerea* infection

To examine the involvement of different hormones in the response of *P. patens* to *B. cinerea* inoculation, SA, JA, OPDA, ABA and indole-3-acetic acid (IAA) were analysed in *B. cinerea*-inoculated and control plants. The results showed a seven-fold induction in SA concentration 4 h after inoculation with *B. cinerea* (Fig 4a). However, *B. cinerea* induced only a two-fold increase in ABA levels at 1 dpi, and the IAA content decreased at 4 and 8 hpi (Fig. 4a). To analyse the possible role of SA in the activation of moss defence responses, plants were pretreated with different concentrations of SA and water, and symptom development was compared

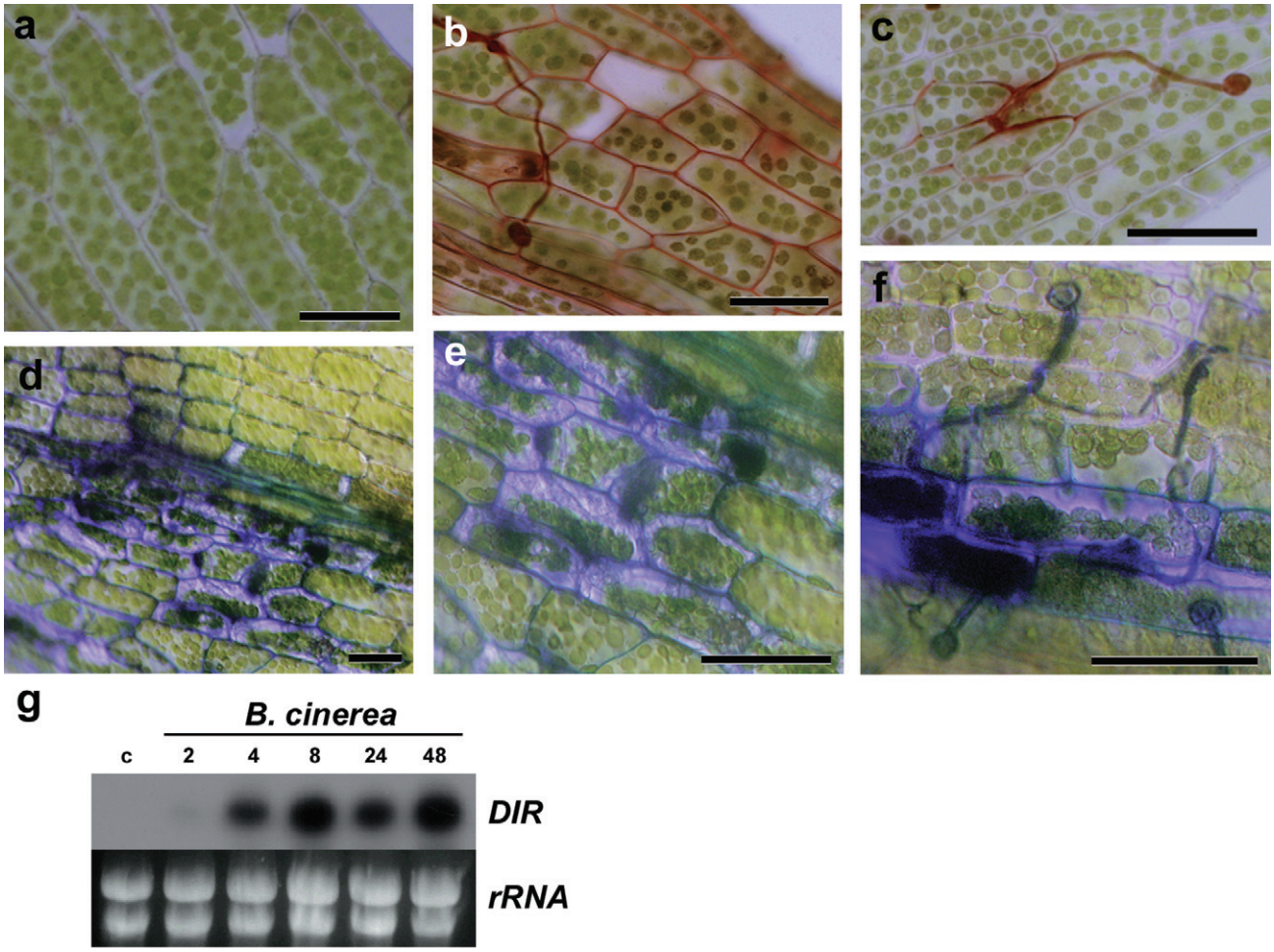

Fig. 2 Cell wall-associated defence responses in *Botrytis cinerea*-infected tissues. (a) Water-treated leaf stained with safranin-O. (b, c) *Botrytis cinerea*-infected leaf stained with safranin-O. (d–f) *Botrytis cinerea*-infected leaves stained with toluidine blue. Photographs of representative samples were taken 1 day after inoculation. The scale bar represents 20 μm. (g) Expression of dirigent-like (*DIR*) gene after *B. cinerea* inoculation. Plants treated with water were used as controls. Moss samples were harvested at the indicated times (hours) after treatment. Ten micrograms of total RNA were separated on formaldehyde–agarose gels, transferred to a nylon membrane and hybridized to a ^{32}P-labelled partial cDNA *DIR* probe corresponding to *Physcomitrella patens DIR* gene Phypa_170601. Ethidium bromide staining of rRNA was used to ensure equal loading of RNA samples. Experiments were repeated thrice with similar results.

after *B. cinerea* inoculation. We could not observe clear differences in disease symptoms caused by *B. cinerea* in SA- or water-treated plants (data not shown). However, the expression of *PAL* (Phypa_180561) was induced at 4 h after SA application, showing the potential of SA to modulate defence gene expression.

The results also showed that the free OPDA content increased 66-fold 24 h after *B. cinerea* inoculation, which correlated with an increase in the level of free linolenic acid (LNA) (Fig. 5a, Table S1, see Supporting Information). However, no C12 jasmonate analogues could be detected in *B. cinerea*-infected tissues. We also analysed free LNA, OPDA and JA content in *P. patens* plants infected with *P. irregulare* or *P. debaryanum* or treated with elicitors of *P.c. carotovorum*. The results showed that, although LNA and OPDA increased in response to both *Pythium* species, and LNA increased after treatment with elicitors of *P.c. carotovorum*, no jasmonates could be detected in any of the samples analysed (Table S1).

We have shown previously that LOX1 (Anterola *et al*., 2009) transcript levels increase after *B. cinerea* inoculation in *P. patens* tissues (Ponce de León *et al*., 2007). In order to analyse in more detail the expression pattern of genes potentially involved in OPDA synthesis, we analysed the expression pattern of an additional *LOX* (*LOX6*; Anterola *et al*., 2009) and an allene oxide synthase (*AOS*) in response to *B. cinerea* infection. A gene with high identity to OPDA reductase-encoding genes (*OPR*) of flowering plants was also included in the expression analysis. As shown in Fig 5b, the expression of *LOX*, *AOS* and *OPR* increased after fungal inoculation. *LOX* transcript accumulation started at 2 h and

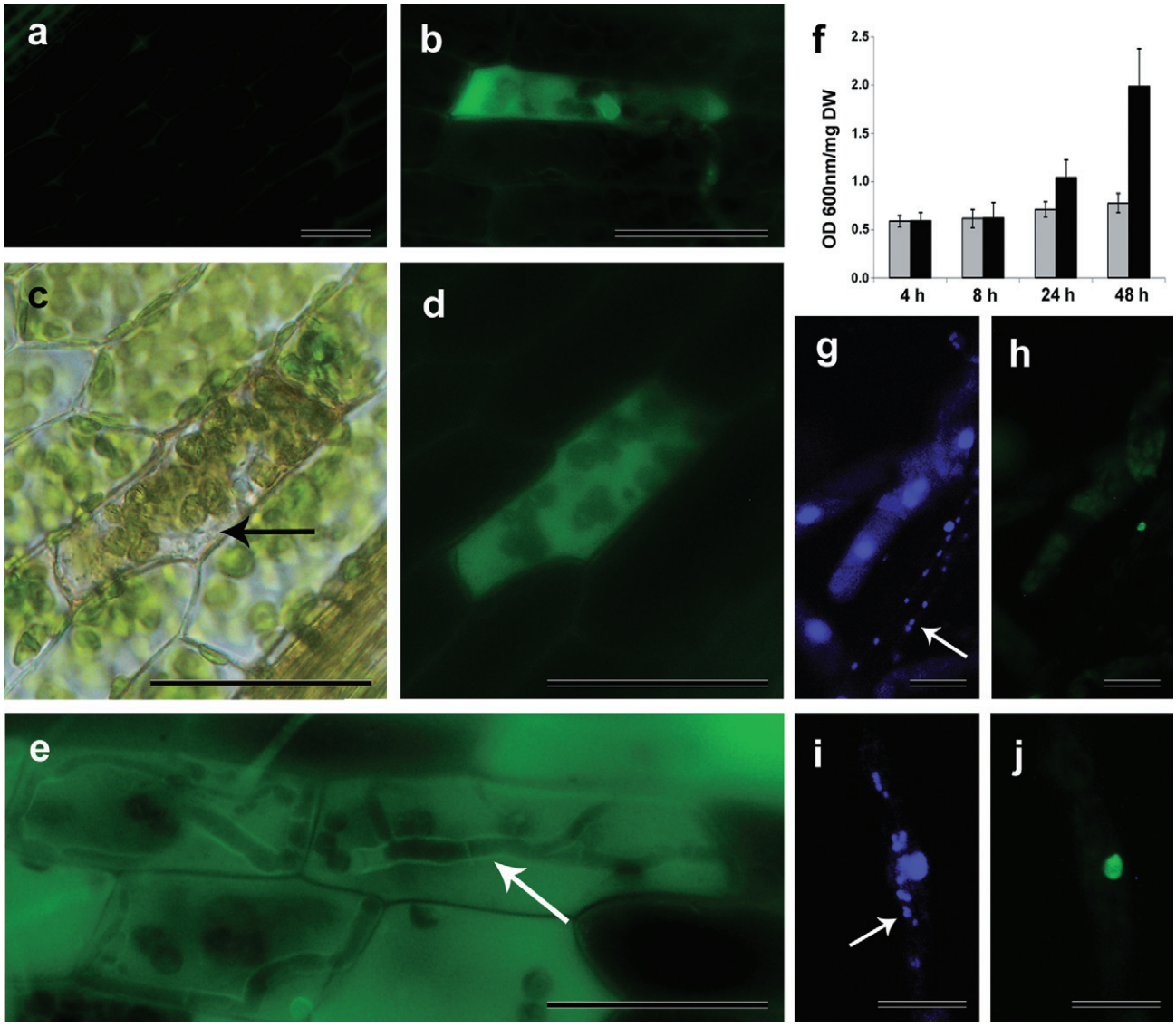

Fig. 3 Reactive oxygen species (ROS) production and programmed cell death in *Botrytis cinerea*-infected *Physcomitrella patens* tissues. (a) ROS production in water-treated leaf (control). (b) ROS production in *B. cinerea*-inoculated leaf. (c) Bright field of a *B. cinerea*-infected leaf showing a *P. patens* cell containing fungal hyphae. (d) Same picture as (c) showing ROS accumulation. (e) ROS distributed homogeneously in the cytoplasm of a *B. cinerea*-infected leaf. The arrows in (c) and (e) point to fungal hyphae. (f) Measurement of cell death by Evans blue staining 4, 8, 24 and 48 h after inoculation in water-treated colonies (grey bars) and *B. cinerea*-inoculated colonies (black bars). Data were expressed as the optical density (OD) at 600 nm per milligram of dry weight (DW). Values in (f) are means with standard deviations of six independent replicate moss samples. (g, i) Nuclei of protonemal filaments inoculated with *B. cinerea* and stained with 4′,6-diamidino-2-phenylindole (DAPI). Fungal nuclei are indicated with an arrow. (h, j) Nuclei positively stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). Experiments were repeated thrice with similar results. Photographs of representative samples were taken 1 day after inoculation. The scale bar represents 20 μm.

was maintained during 4 and 8 h after spore treatments. The level of *AOS* and *OPR* expression increased at 2 h in fungal inoculated tissues, reaching a maximum level at 24 h. Taken together, the results show that the synthesis of SA and OPDA is induced in *P. patens* tissues after *B. cinerea* infection, whereas JA could not be detected in any of the infected or elicitor-treated tissues analysed.

Physcomitrella patens responds to OPDA and MeJA

As jasmonates are not detected in *P. patens* tissues (healthy and infected tissues), suggesting that they are not synthesized in this moss, we addressed the question of whether moss cells could perceive and respond to OPDA or MeJA. Moss colonies were grown in the presence of different concentrations of OPDA and MeJA and,

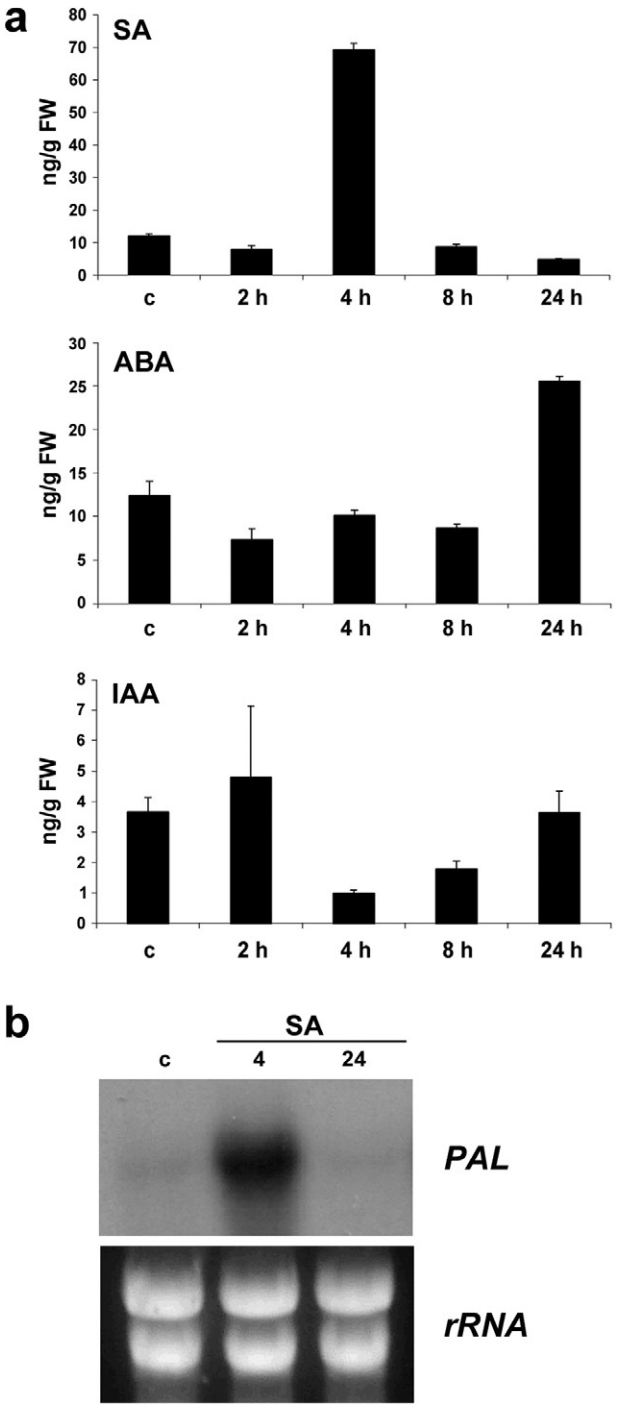

Fig. 4 Salicylic acid (SA), abscisic acid (ABA) and auxin accumulation and expression analysis of a phenylalanine ammonia-lyase (*PAL*) gene in response to SA. (a) Endogenous SA, ABA and indole-3-acetic acid (IAA) levels [ng/g fresh weight (FW)] in water-treated and *B. cinerea*-inoculated tissues were analysed at the indicated time points. Values are means and standard errors of three independent experiments. (b) Expression of *PAL* after 0.5 mM SA treatment at 4 and 24 h. Ten micrograms of total RNA were separated on formaldehyde–agarose gels, transferred to a nylon membrane and hybridized to a ^{32}P-labelled partial cDNA *PAL* probe corresponding to *Physcomitrella patens PAL* gene Phypa_180561. Ethidium bromide staining of rRNA was used to ensure equal loading of RNA samples. Expression analysis was repeated thrice with similar results.

after 21 days, the diameters of the colonies were measured. The results showed a reduction in the colony diameter of between 34% and 35% after the growth of moss tissues with 5, 10, 25 and 50 μM OPDA compared with control plants (Fig. 6a,b). When moss tissues were grown in the presence of MeJA, reductions of 25% and 36% in colony diameter were observed with 25 and 50 μM MeJA, respectively, compared with control plants, whereas 5 and 10 μM MeJA had no significant effect on moss development (Fig. 7a,b). In addition, a significant reduction in rhizoid length was observed when plants were grown in the presence of 5, 10, 25 and 50 μM OPDA (40%–53%) and 25 and 50 μM MeJA (46% and 53%), when compared with control plants (Figs 6 and 7). Rhizoids were more abundant in gametophores grown in the presence of OPDA compared with rhizoids in the control condition (Fig. 6e). These results show that OPDA and MeJA affect moss development and, although jasmonates are not detected in this moss, *P. patens* is able to perceive and respond to OPDA and MeJA.

DISCUSSION

To gain further insight into the defence mechanisms acquired against airborne pathogens during the transition of plants from an aquatic to a terrestrial environment, we continued our studies on the interaction of the bryophyte *P. patens* with the wide-host-range fungus *B. cinerea*. *Physcomitrella patens* is susceptible to *B. cinerea* infection, and we show that *B. cinerea* multiplies after 8 and 12 hpi, and that disease symptoms are visible after 24 hpi when *B. cinerea* DNA becomes predominant in the samples.

Modification of the plant cell wall by the incorporation of phenolic compounds is an important defence mechanism operating in the defence response of flowering plants against *B. cinerea* (Asselbergh *et al*., 2007; Curvers *et al*., 2010). *Botrytis cinerea*-infected *P. patens* tissues incorporate phenolic compounds in the cell wall, detected by safranin-O and toluidine blue staining. This type of fortification mechanism occurs during lignification and suberization in vascular plants (Lucena *et al*., 2003; Mellersh *et al*., 2002). The cell wall composition of bryophytes is different from that of vascular plants, especially with regard to the process of lignification (Edelmann *et al*., 1998; Lee *et al*., 2005). From an evolutionary perspective, mosses represent the first lineage across the plant kingdom with the complete lignin biosynthesis pathway, with the exception of ferulate 5-hydroxylase (F5H), which converts G (guaiacyl) monolignol to S (syringyl) monolignol (Xu *et al*., 2009). In green algae, only certain lignin biosynthesis genes have been found and no lignin and monolignol-like molecules have been detected (Xu *et al*., 2009). The occurrence of lignins in bryophytes is still controversial. Instead of lignin, mosses may contain other phenolic compounds, such as lignin-like polymers (Popper, 2008).

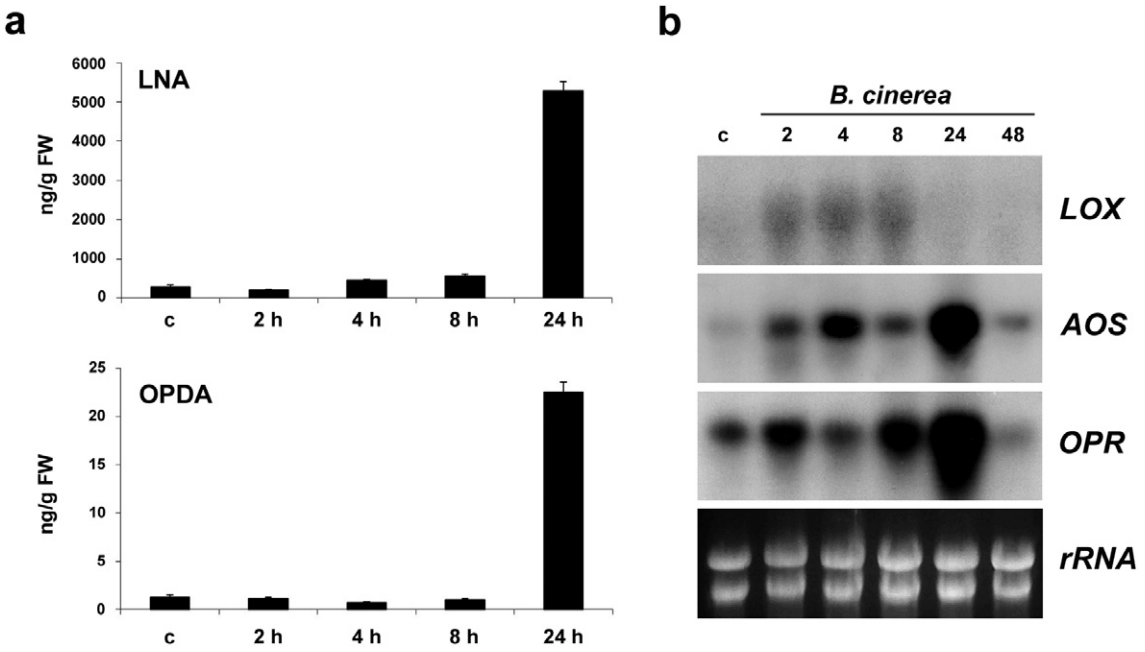

Fig. 5 Linolenic acid (LNA) and 12-oxo-phytodienoic acid (OPDA) accumulation and expression analysis of lipoxygenase (*LOX*), allene oxide synthase (*AOS*) and OPDA reductase-encoding (*OPR*) genes on *Botrytis cinerea* inoculation. (a) Endogenous free LNA and OPDA levels [ng/g fresh weight (FW)] in water-treated and *B. cinerea*-inoculated tissues were analysed at the indicated time points. Values are means and standard errors of three independent experiments. (b) Expression of *LOX*, *AOS* and *OPR* after *B. cinerea* inoculation at the indicated times (hours). Ten micrograms of total RNA were separated on formaldehyde–agarose gels, transferred to nylon membranes and hybridized to ^{32}P-labelled partial cDNA probes corresponding to *Physcomitrella patens* genes *LOX* (Phypa_117739), *AOS* (Phypa_214061) and *OPR* (Phypa_158472). Ethidium bromide staining of rRNA was used to ensure equal loading of RNA samples. Expression analysis was repeated thrice with similar results.

Espiñeira *et al*. (2011) have shown that *P. patens* cell walls contain lignin-like polyphenols, although lignin could not be found by solubilizing the β-*O*-4 lignin core, which is exclusive to lignins. In addition, methoxyl groups and the methoxylated ring carbon atoms in the monolignol units of lignified materials are absent in the moss *Rhacocarpus purpurascens* (Edelmann *et al*., 1998). Nevertheless, peroxidase enzymes, which are typically involved in lignin biosynthesis, and are capable of oxidizing monolignols, have been detected in *P. patens* (Espiñeira *et al*., 2011). DIR proteins are thought to mediate the coupling of monolignol plant phenols to yield lignans and lignins (Davin and Lewis, 2000). In this article, we have shown the induced expression of a gene encoding a DIR-like protein in *B. cinerea*-infected moss tissues. The *P. patens* genome contains eight additional putative *DIR*-like genes with identities of 39.7%–96.7% to the *DIR*-like gene used in this study. As the *DIR* cDNA fragment used as a probe (Phypa_110421) and two other *DIR*-like genes (Phypa_170601 and Phypa_48771) exhibit high identity (81.1%–96.7%), and encode transcripts of similar sizes, Northern blot results could represent the expression pattern of these three *DIR* genes. Transcriptional profiling has revealed the induced expression of some *DIR* genes in flowering plants during the disease resistance response (Chakravarthy *et al*., 2010; Coram *et al*., 2008), suggesting that they could play important roles in plant–pathogen interactions. The expression pattern of different pathogen-inducible *DIR*-like genes may vary depending on the pathosystem. In wheat, a similar expression pattern of two *DIR*-like genes was reported during the course of infection with *Puccinia striiformis* (Coram *et al*., 2008). However, the expression pattern of two *DIR*-like genes from grapevine was different after *Erysiphe necator* assault (Fung *et al*., 2008). Consistent with a possible role of DIR proteins in defence, DIR-overexpressing transgenic plants show enhanced resistance after fungal assault (Wang and Fristensky, 2001; Wang *et al*., 1999). Our results suggest that changes in cell wall composition occur after infection as part of a defence mechanism, leading to the reinforcement of the well wall. Although cell wall fortification mechanisms are activated in *P. patens* after *B. cinerea* infection, they are not sufficient to stop fungal colonization, leading, finally, to plant decay. Bhuiyan *et al*. (2009) have demonstrated that S lignin plays a pivotal role in plant defence against fungal penetration. The lack of S monolignols in *P. patens* could contribute to the high susceptibility observed in moss tissues after *B. cinerea* infection. Hence, *P. patens* represents an interesting model system to analyse lignin-like material composition after pathogen assault, and to evaluate the role of DIR-like proteins in the coupling of monolignols during plant defence responses.

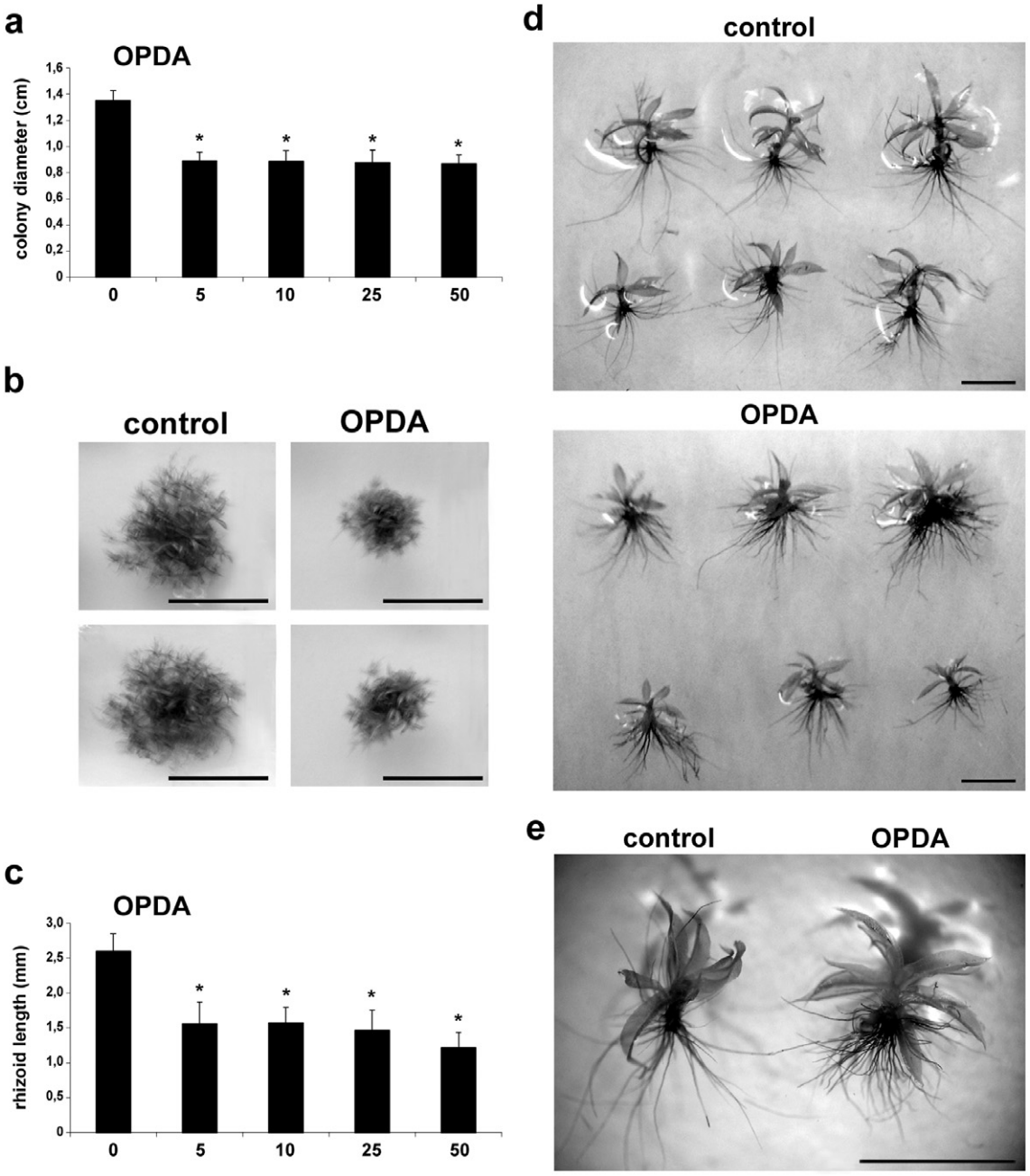

Fig. 6 12-Oxo-phytodienoic acid (OPDA) induces reduction of colony growth and rhizoid length. (a) Moss colonies grown on different concentrations of OPDA, measured as size (diameter in centimetres) of single moss colonies relative to control moss colonies grown on 0.5% ethanol. Results and standard deviation corresponding to 16 colonies per sample are shown. (b) Individual colonies showing the typical phenotype 21 days after growing plants on 0.5% ethanol (control) or 50 μM OPDA-containing medium. (c) *Physcomitrella patens* gametophores grown on 0.5% ethanol or 5, 10, 25 and 50 μM OPDA were taken from the edge of the moss colonies, stained with toluidine blue and the rhizoid lengths were measured (length in millimetres). Results and standard deviation corresponding to 24 gametophores are shown. (d, e) Individual gametophores grown for 21 days on 0.5% ethanol (control) or 50 μM OPDA-containing medium. Rhizoids were stained with toluidine blue and their lengths were measured (mm). Experiments were repeated at least three times with similar results and representative photographs are shown. Scale bars represent 1 cm in (b) and 0.2 cm in (d, e). Asterisks for plants grown on 5, 10, 25 and 50 μM OPDA indicate that the values are significantly different from control plants according to Kruskal–Wallis test: $P < 0.0001$.

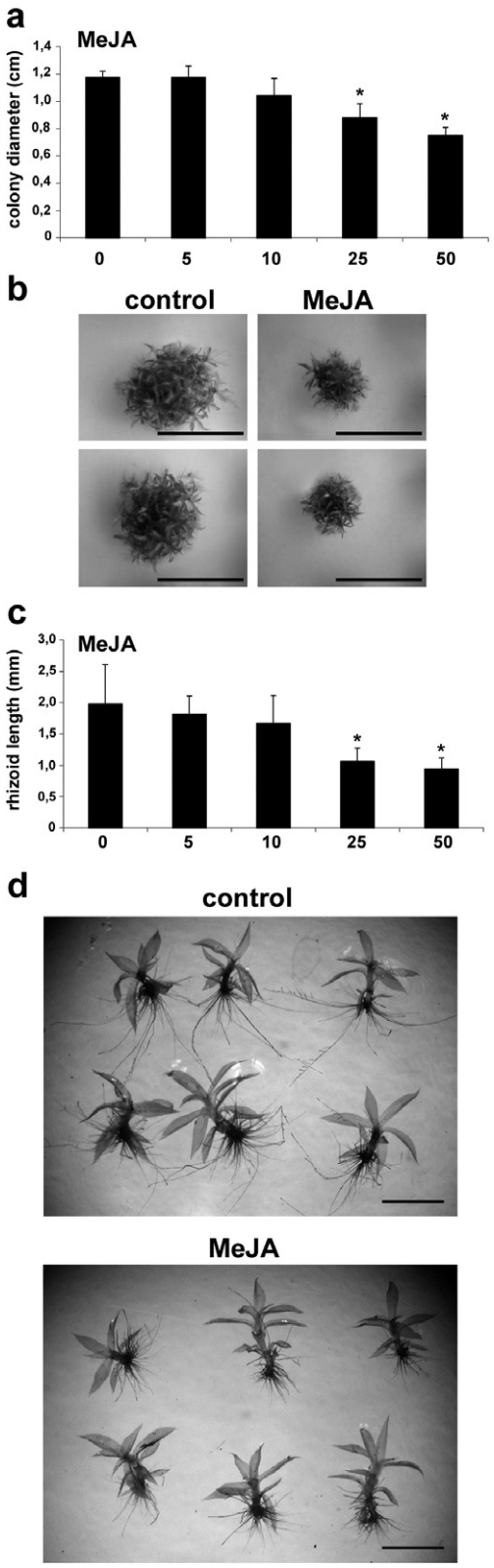

Fig. 7 Methyl jasmonate (MeJA) induces reduction of colony growth and rhizoid length. (a) Moss colonies grown on different concentrations of MeJA, measured as size (diameter in centimetres) of single moss colonies relative to control moss colonies grown on 0.5% ethanol. Results and standard deviation corresponding to 16 colonies per sample are shown. (b) Individual colonies showing the typical phenotype after 21 days of growth on 50 μM MeJA-containing medium in comparison with control plants grown on 0.5% ethanol. (c) *Physcomitrella patens* gametophores grown on 0.5% ethanol or 5, 10, 25 and 50 μM MeJA were taken from the edge of the moss colonies, stained with toluidine blue and the rhizoid lengths were measured (mm). Results and standard deviation corresponding to 24 gametophores are shown. (d) Individual gametophores grown for 21 days on 50 μM MeJA-containing medium relative to control plants grown on 0.5% ethanol. Rhizoids were stained with toluidine blue and their lengths were measured (mm). Experiments were repeated at least three times with similar results and representative photographs are shown. Scale bars represent 1 cm in (b) and 0.2 cm in (d). Asterisks for plants grown on 25 and 50 μM MeJA indicate that the values are significantly different from control plants according to Kruskal–Wallis test: $P < 0.0001$.

ROS production is an important component of *B. cinerea* virulence, and increased levels of ROS in plant cells seem to cause more aggressive infection of *B. cinerea*, resulting in an accelerated colonization of host tissue (Govrin and Levine, 2000). Consistently, we detected ROS generation in *B. cinerea*-infected *P. patens* tissues which showed increased cell death after fungal colonization. Single moss cells respond rapidly to hyphal contact by generating ROS, suggesting that, like vascular plants (Asai and Yoshioka, 2009; Govrin and Levine, 2000), the oxidative burst is probably induced before and during *B. cinerea* invasion. Intracellular ROS were detected homogeneously in the cytoplasm and may contribute to the observed cell wall strengthening by oxidative cross-linking of wall components. Previously, we have shown the occurrence of protoplast shrinkage, the accumulation of autofluorescent compounds and chloroplast breakdown in *B. cinerea*-infected cells, which are indicative of an HR-like response (Ponce de León *et al*., 2007). ROS accumulation and protoplast shrinkage are typical features of necrotic plant PCD (van Doorn *et al*., 2011). These results, together with TUNEL-positive staining of *P. patens* nuclei invaded by *B. cinerea* hyphae, confirm the activation of an HR-like response after *B. cinerea* infection. Studies in tobacco and *Arabidopsis* suggest that *B. cinerea* needs HR cell death to achieve full pathogenicity (Dickman *et al*., 2001; Govrin and Levine, 2000). *Arabidopsis* mutants with an accelerated cell death response are more susceptible to *B. cinerea*, whereas mutants with reduced or delayed cell death are generally more resistant (van Baarlen *et al*., 2007). *Physcomitrella patens* therefore represents an interesting model plant to perform functional studies of the key components involved in PCD and can provide clues to the evolution of PCD in plants.

Signal molecules, including SA, JA, ABA and auxins, are involved in the defence response of flowering plants against pathogens, and the balance between these hormones will influence the

outcome of the interaction (López *et al*., 2008; Robert-Seilaniantz *et al*., 2011). In the present study, we observed an increase in SA content in *B. cinerea*-inoculated moss tissues relative to control plants. To our knowledge, this is the first time that an SA increase has been reported in a moss after pathogen assault. The rapid and transient increase in SA levels was observed 4 h after the inoculation of moss tissues with *B. cinerea* spores. In *B. cinerea*-infected flowering plants, SA levels have been measured at later time points. In infected tomato and *Arabidopsis* plants, increased SA levels were observed at 12–24 hpi and 48 hpi, respectively (El Oirdi *et al*., 2011; Veronese *et al*., 2006). The *P. patens* genome contains 14 putative genes encoding PALs (Wolf *et al*., 2010) and several putative homologues of isochorismate synthases, supporting the synthesis of SA in this moss. In addition, the treatment of *P. patens* colonies with SA induces the expression of *PAL* (Phypa_180561), suggesting that the endogenous rise of SA leads to enhanced expression of this defence gene. Consistent with our findings, Andersson *et al*. (2005) demonstrated that SA application to *P. patens* colonies induced defence mechanisms and increased resistance to *P.c. carotovorum*. The ability to perceive and transduce the SA signal was also observed in the moss *Funaria hygrometrica*, in which SA treatments caused an effect on plant growth and development (Christianson and Duffy, 2002). Taken together, these results suggest that the ability to synthesize, perceive and transduce the SA signal is an ancient feature in plant evolution.

The role played by SA signalling in the resistance of flowering plants to *B. cinerea* infection is complex. Although SA-deficient NahG *Arabidopsis* and tomato plants are more susceptible than wild-type plants to *B. cinerea* (Audenaert *et al*., 2002; El Oirdi *et al*., 2011; Ferrari *et al*., 2003), NahG and wild-type *Nicotiana benthamiana* plants respond similarly to *B. cinerea* infection (Asai *et al*., 2010). Recently, El Oirdi *et al*. (2011) have demonstrated that *B. cinerea* produces an exopolysaccharide, which acts as an elicitor of the SA pathway, antagonizing the JA signalling pathway in tomato to enhance disease symptoms. Contradictory results have been obtained in tomato, as the pretreatment of plants with 0.5 mM SA reduced slightly (Vicedo *et al*., 2009) or enhanced (El Oirdi *et al*., 2011) disease development caused by *B. cinerea* relative to control plants. This difference could be a result of the activation or not of the SA/JA antagonism by *B. cinerea*, which could be strain and host dependent (El Oirdi *et al*., 2011). In *Arabidopsis*, *B. cinerea*-induced necrotic lesions were similar in SA-treated and untreated plants (Govrin and Levine, 2002), and several authors have suggested that, in *Arabidopsis*, SA contributes to resistance against *B. cinerea*, although it does not play a major role (Ferrari *et al*., 2003; Glazebrook, 2005). Similar to *Arabidopsis*, we could not observe clear differences in disease symptoms caused by *B. cinerea* after the pretreatment of moss plants with different concentrations of SA or water (data not shown). Taken together, the results suggest that SA may contribute to the activation of defence responses against *B. cinerea*. The generation of *P. patens* plants impaired in SA accumulation or perception will certainly provide valuable insights into the role of SA in moss defence against *B. cinerea* and other pathogens.

Our results also show that the ABA content increases in *P. patens* tissues infected with *B. cinerea* relative to water-treated plants. This increase was observed 24 h after the inoculation of *P. patens* colonies, when mycelium had colonized heavily the plant tissues and thus ABA could have been produced by *B. cinerea* itself. This is consistent with the fact that *B. cinerea* produces ABA that may contribute to the infection process (Siewers *et al*., 2004). In flowering plants, ABA-deficient mutants are more resistant to *B. cinerea* (AbuQamar *et al*., 2006; Asselbergh *et al*., 2007; Curvers *et al*., 2010). In contrast, increased ABA levels contribute to the development of grey mould in tomato (Asselbergh *et al*., 2007; Ton *et al*., 2009). The ABA increase observed in *B. cinerea*-infected *P. patens* plants could promote susceptibility to this pathogen by interfering negatively with SA-dependent defence, as has been reported previously for flowering plants (Audenaert *et al*., 2002; Yasuda *et al*., 2008). Recently, *P. patens* microarray expression profiles in response to *B. cinerea* inoculation revealed differential expression of ABA-inducible transcription factors and several genes with predicted ABA-responsive promoters (C. Neu and S. Rensing, Albert-Ludwigs-Universität, Freiburg, Germany, personal communication). Thus, *P. patens* can provide new insights into the evolution of ABA as a defence signalling molecule in land plants.

Fungal pathogen infection perturbs auxin homeostasis, either by the pathogen production of auxin and/or by the alteration of host auxin biosynthesis (Maor *et al*., 2004; Tanaka *et al*., 2011). In this study, we observed a decrease in auxin content in *B. cinerea*-inoculated moss tissues relative to control plants. In flowering plants, *B. cinerea* infection causes the repression of auxin signalling (El Oirdi and Bouarab, 2007; Llorente *et al*., 2008). The greatest decrease in IAA in moss tissues was detected 4 h after fungal inoculation, which correlates with an increase in SA levels. Wang *et al*. (2007) have demonstrated that SA inhibits the auxin signalling pathway as part of the plant defence mechanism. Further studies need to be performed to understand the interaction between these two hormonal pathways, as well as the role of auxins, in *P. patens*–microbe interactions.

Jasmonate biosynthesis is typically initiated with the release of LNA from membrane glycerolipids and conversion to OPDA by the sequential action of the plastid enzymes LOX, AOS and allene oxide cyclase (AOC). The second part of the pathway takes place in peroxisomes, where OPDA is reduced by OPDA reductase (OPR3) to give 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), followed by three rounds of β-oxidation involving three enzymes to yield (+)-7-iso-JA. This molecule epimerizes to the more stable (–)-JA, generally known as JA (Browse, 2009). It has been demonstrated recently that the active form of the hormone is (+)-7-iso-JA-L-isoleucine (Fonseca *et al*., 2009). Our

results show that, like flowering plants, *P. patens* responds to *B. cinerea* by increasing the endogenous levels of free LNA and OPDA (Vicedo *et al*., 2009; Yang *et al*., 2007). Accordingly, the expression of genes encoding enzymes involved in the synthesis of OPDA, including LOX and AOS, is enhanced in *B. cinerea*-infected tissues. In addition, free LNA and OPDA levels increase in moss tissues infected with *P. irregulare* and *P. debaryanum*, and increased LNA levels are detected after treatment with *P.c. carotovorum* elicitors. However, we could not detect JA in healthy, pathogen-infected or elicitor-treated *P. patens* tissues, suggesting that oxylipins are not further metabolized to JA in *P. patens*.

We have reported previously that OPDA levels and very small amounts of JA (1–7 ng/g fresh weight) increase in *P. patens* tissues infected with *P. irregulare* and *P. debaryanum* (Oliver *et al*., 2009). However, other groups have shown that OPDA, but not JA or JA-isoleucine, accumulates in healthy or wounded *P. patens* gametophytes (Browse, 2009; Stumpe *et al*., 2010). In the current study, our re-examination found no evidence for JA or related metabolites, suggesting that the trace gas chromatography/mass spectrometry (GC/MS) peaks, bordering the limits of detection, which were previously attributed to JA, actually correspond to other unrelated metabolites.

We have also observed the induced expression of an OPR-encoding gene in response to *B. cinerea*. The *P. patens* genome contains several *OPR*-like genes (Breithaupt *et al*., 2009; Li *et al*., 2009) and, although one of the gene products has been identified as a candidate for OPR3-like activity, which is the only enzyme that converts *cis*-(+)-OPDA to JA (Breithaupt *et al*., 2009), enzymatic activity is missing. Studies with the *Arabidopsis opr3* mutant have indicated that OPDA is active as a defence signal against pathogens and regulates defence gene expression (Browse, 2009; Stintzi *et al*., 2001; Taki *et al*., 2005). Consistently, moss colonies treated with OPDA show an enhanced expression of a defence gene *PAL* (Oliver *et al*., 2009). This PAL-encoding gene (Phypa_156018) is different from the SA-inducible *PAL* gene (Phypa_180561) and differential expression could lead to the production of different metabolites with distinct roles in defence. JA increases in *Arabidopsis* upon *B. cinerea* infection (Yang *et al*., 2007), and it has been shown recently that JA plays an important role in defence against this pathogen (Chehab *et al*., 2011). In mosses, other oxylipins, including OPDA, may play primary roles in defence against *B. cinerea*, either by activating gene expression and/or by its antimicrobial activity against this fungus (Prost *et al*., 2005).

The *P. patens* genome contains six putative genes encoding the JA-isoleucine receptor COI (coronatine insensitive) and six encoding the repressor JAZ (Chico *et al*., 2008). It remains an open question as to which molecule(s) is/are the ligand(s) of *P. patens* COI-like proteins and how this signalling pathway is perceived. Several explanations are possible, including the perception of OPDA or OPDA-isoleucine or other jasmonates by *P. patens* COI-like proteins, although there is currently no experimental evidence of the interaction of OPDA with the COI1–JAZ complex in flowering plants (Chini *et al*., 2009).

OPDA affects moss development, evidenced by a reduced growth of *P. patens* colonies and decreased rhizoid length. Although jasmonates were not detected in *P. patens* tissues, this moss also responds to MeJA by reducing the growth of the colonies and the length of the rhizoid in a dose-dependent manner. This is consistent with previous results obtained in *Arabidopsis*, where the growth of seedlings and roots was reduced on OPDA- and MeJA-containing medium (Mueller *et al*., 2008; Staswick *et al*., 1992; Vellosillo *et al*., 2007; Yan *et al*., 2007). It has been suggested that growth inhibition might occur at the level of the cell cycle, as JA and OPDA block cell cycle progression (Mueller *et al*., 2008; Swiatek *et al*., 2002). Although higher levels of MeJA relative to OPDA were needed to alter moss development, both oxylipins were perceived, leading, finally, to the activation of similar responses. Accordingly, at the level of gene expression, OPDA, JA and MeJA induce the accumulation of the defence gene *PAL* (Phypa_156018) (Oliver *et al*., 2009). A central question is how these jasmonates are perceived by *P. patens* cells. It is tempting to speculate that numerous components utilized by the jasmonate perception system could exist as ancient features already present in mosses. In addition, the different *P. patens* COI-like proteins could have different specificity and recognize different oxylipins, allowing the binding of a wider range of ligands. In conclusion, further studies are needed to obtain a more complete scenario with regard to the evolution of different defence pathways in plants, including the signalling and perception of OPDA and jasmonates in nonflowering and flowering plants, and *P. patens* represents an excellent basal plant to perform these studies.

EXPERIMENTAL PROCEDURES

Physcomitrella patens and pathogen growth conditions

Physcomitrella patens Gransden WT isolate (Schaefer *et al*., 1991) was provided by the IMSC International Moss Stock Center (Freiburg, Germany), and grown on cellophane overlaid agar BCDAT medium. Protonemal cultures and moss colonies were generated as described previously (Oliver *et al*., 2009). Plants were grown at 22 °C under a photoperiod of 16 h light, and 3-week-old colonies were used for all the experiments. *Botrytis cinerea*, *P. irregulare* and *P. debaryanum* were cultivated on 24 g/L potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 22 °C; *P.c. carotovorum* was propagated on Luria–Bertani (LB) medium at 28 °C and culture filtrates containing the elicitors were prepared according to Ponce de León *et al*. (2007).

Pathogen inoculation and *B. cinerea* staining

Botrytis cinerea inoculations were performed as described by Ponce de León *et al*. (2007) using a suspension (2×10^5 spores/mL) in water. Both

Pythium species were inoculated according to Oliver *et al*. (2009). The culture filtrate of *P.c. carotovorum* was applied by spraying the moss colonies. *Botrytis cinerea* tissues were stained with 0.1% solophenyl flavine 7GFE 500 in water for 10 min, rinsed in water and visualized with epifluorescence (Hoch *et al*., 2005). All photographs were taken at 1 day after inoculation, unless otherwise indicated, and representative photographs are shown.

Quantitative PCR

Total DNA was isolated from moss colonies inoculated with *B. cinerea* using the DNeasy kit (Qiagen, Hilden, Germany). Samples were frozen in liquid nitrogen immediately after inoculation (time zero) and after 2, 4, 8, 12 and 24 h. DNA was isolated from triplicate independent experiments. Each sample corresponds to 16, 3-week-old *P. patens* colonies sprayed with a spore suspension containing 2×10^5 spores/mL. The oligonucleotides used to amplify *P. patens* DNA corresponded to the single copy 3′-end of an elongation factor gene (Fwd: 5′-TTTGGGATTGAAATGTCGTG-3′; Rev: 5′-TGAGCATGAGAAATTGGGTCT-3′); the PCR product size was 180 bp, the PCR efficiency was 0.95 with an R^2 value of 0.9983. The oligonucleotides used to amplify *B. cinerea* DNA corresponded to the multiple copy ITS region (Fwd: 5′-CTGTTCGAGCGTCATTTCAA-3′; Rev: 5′-CCTACCTGATCCGAGGTCAA-3′); the PCR product size was 184 bp, the PCR efficiency was 1.01 with an R^2 value of 0.9975. Quantitative PCR was performed using the QuantiMix Easy SYG kit (Biotools, Madrid, Spain) based on Sybr Green technology and the Rotor-Gene 6000 cycler (Corbett Life Science, Sydney, Australia). The annealing temperature was 55 °C. The relative amount of *B. cinerea* DNA was normalized to plant DNA and expressed relative to the calibrator samples taken at time zero. After 2 dpi, plant tissues were macerated, decreasing the plant DNA yield and preventing the use of this quantitative PCR approach. The relative quantification was performed according to Pfaffl (2001), a mathematical model that includes the real-time PCR efficiencies of both the target and internal reference genes, and does not require any calibration curves with pure genomic DNA (Gachon and Saindrenan, 2004).

Visualization of plant cell wall-associated defence responses

To detect cell wall modifications, safranin-O staining was performed according to Lucena *et al*. (2003). Tissues were incubated with 0.01% safranin-O in 50% ethanol for 5 min. The accumulation of phenolics was also detected by staining tissues with 0.05% toluidine blue in citrate–citric acid buffer (50 mM, pH 3.5; Mellersh *et al*., 2002).

Bright field microscopy and fluorescence microscopy were performed with an Olympus BX61 microscope (Shinjuku-ku, Tokyo, Japan), and all images shown in this study were captured with the MICROSUITE software package (Olympus).

ROS production and cell death detection

The intracellular production of ROS was analysed by incubating moss tissues with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 15 min in 0.1 M phosphate buffer (pH 7.5) in the dark. Leaves were visualized with epifluorescence. For the detection of cell death, moss colonies were incubated for 2 h with 0.05% Evans blue and washed four times with deionized water to remove excess and unbound dye. Dye bound to dead cells was solubilized in 50% methanol with 1% sodium dodecylsulphate (SDS) for 45 min at 50 °C, and the absorbance was measured at 600 nm (Levine *et al*., 1994). Each sample consisted of four colonies incubated in 6 mL of a methanol–SDS mixture. Six samples were analysed per experiment, which was repeated at least three times and expressed as the optical density (OD) per milligram of dry weight. Dry weight was measured after drying plant colonies for 18 h at 65 °C.

TUNEL assay

Water-treated and *B. cinerea*-inoculated protonemal tissues were fixed in 10% formalin and 50 mM 1,4 Piperazine bis (2-ethanosulfonic acid) (PIPES) buffer (pH 6.8) at 4 °C overnight, dehydrated through a graded ethanol series (50%, 70%, 80%, 95%), washed twice with phosphate-buffered saline and permeabilized with Triton X100 solution [10% Triton X100 and 4′,6-diamidino-2-phenylindole (DAPI) staining solution containing 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.8) and 150 mM NaCl] overnight at 4 °C. Nuclear DNA fragmentation was identified by TUNEL staining, which detects free 3′-hydroxyl groups of degraded nuclear DNA. The TUNEL assay (TUNEL *in situ* cell death detection kit-fluorescein, Roche Applied Science, Mannheim, Germany) was carried out according to the manufacturer's instructions. To reduce the nonspecific signal, the reaction buffer was diluted three times in dilution buffer (Roche Applied Science). Terminal transferase was omitted for the negative control. After washing with DAPI staining buffer, samples were incubated in DAPI staining buffer containing 0.1 μg/mL at room temperature for 15 min. Excitation and emission filters were as follows: for DAPI, excitation was between 353 and 377 nm and emission was above 397 nm; for fluorescein, excitation was between 450 and 490 nm and emission was between 515 and 565 nm.

RNA gel blot analysis

Total RNA was isolated from water-treated and *B. cinerea*-inoculated tissue using standard procedures based on phenol–chloroform extraction followed by LiCl precipitation. Each sample consisted of 48 colonies. Ten micrograms of total RNA were separated, transferred to nylon membranes, hybridized and washed as described previously (Ponce de León *et al*., 2007). A partial cDNA clone of 506 bp, which showed a high level of similarity to DIR-encoding genes from flowering plants (Phypa_110421), was cloned into pGem®T-Easy (Promega, Madison, WI, USA) and used as a probe to amplify by PCR the corresponding cDNA using universal primers. A second partial cDNA clone of 374 bp was obtained with a high level of similarity to PAL-encoding genes from flowering plants; in order to obtain a larger fragment for use as a probe, primers were used (PAL-Fwd: 5′-CACGACCCCATGCTTGTGGACTCC-3′; PAL-Rev: 5′-AATCCTGTCAGCATG AGAAGGGTC-3′) to amplify a cDNA of 2144 bp (Phypa_180561), which was cloned into pGEM (Promega). Partial cDNAs were obtained from a suppression subtractive hybridization cDNA library constructed from RNA from *P. patens* tissues treated with *P.c. carotovorum* strain SCC1 (Rantakari *et al*., 2001) elicitors as the tester and RNA from water-treated moss tissues as the driver (A. Alvarez and I. Ponce de León, personal observa-

tion). In addition, the *P. patens* cDNA clones from RIKEN used as probes were obtained by digesting the plasmid harbouring the corresponding cDNA, including *LOX* (clone pphn31p10; Phypa_117739), *AOS* (clone pphb27o22; Phypa_214061) and *OPR* (clone pphb29k02; Phypa_158472) (Nishiyama *et al*., 2003). PCR and digested fragments were purified using Qiaquick columns (Qiagen), and labelled with [α^{32}P]-dCTP using the Rediprime II Random Prime labelling system (GE Healthcare, Buckinghamshire, UK). The amount of RNA loaded was verified by the addition of ethidium bromide to the samples and photography under UV light after electrophoresis. The blots shown are representative examples of the results obtained in three independent experiments.

Hormone analysis and treatments

Three-week-old moss colonies were inoculated with a spore suspension of *B. cinerea*. Tissue was collected 2, 4, 8 and 24 h after inoculation, frozen, ground and weighed until hormone quantification. Eight moss colonies were pooled and 200 mg of ground tissue were homogenized, derivatized, vapour phase extracted and analysed by gas chromatography/isobutane chemical ionization mass spectrometry (GC/CI-MS), as described previously (Schmelz *et al*., 2004). This procedure was repeated three times.

Moss colonies were treated by spraying 0.5 mM SA on the tissues, and samples were taken at the indicated time points. Control plants were sprayed with water.

Growth of colonies with OPDA and MeJA

Small pieces of protonema were harvested from protonemal cultures and placed on fresh plates with increasing concentrations of OPDA and MeJA (5, 10, 25 and 50 μM). Control plants were grown on plates containing 0.5% ethanol. For each concentration and each oxylipin, two plates were set up containing 16 colonies each. Plants were observed after 21 days and the diameter of each colony was recorded between the borders of the moss colonies. For the measurement of the rhizoid length, 24 gametophores for each concentration and each oxylipin were taken from the edge of the colonies, stained for 1 min in 0.05% toluidine blue in citrate–citric acid buffer to allow rhizoid staining, and rinsed in water to remove excess dye. The colony diameter and rhizoid length were measured using GIMP 2.6 software. All experiments were repeated at least three times. To compare the significance of the differences between the diameter of the colonies and the length of the rhizoids of plants grown on different concentrations of OPDA and MeJA relative to control plants, a nonparametric Kruskal–Wallis multiple comparison test was performed using STATISTICA 7 software.

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SUPPORTING INFORMATION

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

Table S1 Endogenous free linolenic acid (LNA), 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA) levels [ng/g fresh weight (FW)] were analysed at the indicated time points in *Physcomitrella patens* tissues after inoculation with *Botrytis cinerea*, *Pythium irregulare* and *Pythium debaryanum*, or after treatment with elicitors of *Pectobacterium carotovorum* ssp. *carotovorum*. Potato dextrose agar (PDA)- and water-treated tissues were used as controls. Values are means and standard errors of three independent experiments.

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