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Isolation of Natural Fungal Pathogens from *Marchantia polymorpha* Reveals Antagonism between Salicylic Acid and Jasmonate during Liverwort–Fungus Interactions

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The evolution of adaptive interactions with beneficial, neutral and detrimental microbes was one of the key features enabling plant terrestrialization. Extensive studies have revealed conserved and unique molecular mechanisms underlying plant–microbe interactions across different plant species; however, most insights gleaned to date have been limited to seed plants. The liverwort *Marchantia polymorpha*, a descendant of early diverging land plants, is gaining in popularity as an advantageous model system to understand land plant evolution. However, studying evolutionary molecular plant–microbe interactions in this model is hampered by the small number of pathogens known to infect *M. polymorpha*. Here, we describe four pathogenic fungal strains, *Irpex lacteus* Marchantia-infectious (MI)1, *Phaeophlebiopsis peniophoroides* MI2, *Bjerkandera adusta* MI3 and *B. adusta* MI4, isolated from diseased *M. polymorpha*. We demonstrate that salicylic acid (SA) treatment of *M. polymorpha* promotes infection of the *I. lacteus* MI1 that is likely to adopt a necrotrophic lifestyle, while this effect is suppressed by co-treatment with the bioactive jasmonate in *M. polymorpha*, dinor-*cis*-12-oxo-phytodienoic acid (dn-OPDA), suggesting that antagonistic interactions between SA and oxylipin pathways during plant–fungus interactions are ancient and were established already in liverworts.

Introduction

Acquisition and evolution of adaptive mechanisms providing resistance to biotic and abiotic stresses enabled land plants to conquer terrestrial habitats (Delwiche and Timme 2011, Delwiche and Cooper 2015, Rensing 2018). Land plants have coevolved with a large variety of microbes since their colonization of land more than 450 million years ago (Bonfante and Genre 2010, Field et al. 2015, Martin et al. 2017, Field and Pressel 2018, Hoysted et al. 2018, Mathieu et al. 2018, Morris et al. 2018, Spribille 2018, Strullu-Derrien 2018), and it has been hypothesized that mutualistic symbiotic interactions with fungi facilitated plant terrestrialization (Field et al. 2015, Rensing 2018). Given that most microbes are not beneficial to plants and because most plants are resistant to the majority of potentially pathogenic microbes, establishment of a sophisticated immune system probably also played a crucial role in plant terrestrialization (de Vries et al. 2018).

Extant bryophytes, comprising liverworts, hornworts and mosses, are the earliest diverging lineages of land plants, and, as such, represent key plant species for understanding the origin and evolution of molecular systems in plants (Rensing 2017, Puttick et al. 2018). Bryophytes are non-vascular gametophyte dominant plants and develop root-like anchoring structures called rhizoids (Jones and Dolan 2012, Duckett et al. 2014, Harrison 2017). Rhizoids are utilized to take up nutrients and water and also serve as entry sites for symbiotic fungi (Ligrone et al. 2007, Pressel et al. 2008, Humphreys et al. 2010, Pressel et al. 2010, Silvani et al. 2012, Field et al. 2016). Since bryophytes and seed plants have notable differences in their body plans, microbes may have applied different approaches to interact with these plant species. Conversely, bryophytes and seed plants may have followed a unique evolutionary trajectory that has allowed interaction with a large variety of microbes.

Establishment of the moss *Physcomitrella patens* as a model plant paved the way for the study of evolutionary molecular

plant–microbe interactions (EvoMPMI), although there is no evidence of mycorrhiza-like associations in mosses (Rensing et al. 2008, Lehtonen et al. 2009, Pressel et al. 2010, Ponce de León 2011, Castro et al. 2016, Ponce de León and Montesano 2017, Upson et al. 2018, Vigneron et al. 2018). Several studies have shown that pathogenic microbes including bacteria, fungi and oomycetes isolated from other plant species can cause disease in *P. patens* (Andersson et al. 2005, Ponce de León et al. 2007, Oliver et al. 2009, Ponce de León and Montesano 2013, Reboledo et al. 2015, Overdijk et al. 2016). A study on the evolutionarily conserved LysM receptor kinase CERK1 in *P. patens* demonstrated that the LysM-mediated chitin-triggered immune system was established early in evolution and remained well conserved thereafter (Bressendorff et al. 2016). Regarding natural infection in mosses, the fungal pathogens *Irpex* sp. and *Fusarium* sp. were isolated from the moss *Racomitrium japonicum*, and the mosses *Aulacomnium palustre*, *Hylocomium splendens* and *Polytrichum juniperinum* are native hosts of the fungal pathogen *Atradidymella muscivora* (Davey et al. 2009, Lehtonen et al. 2009). Further, *tobacco mosaic virus* or its related virus was detected in the Antarctic mosses *Polytrichum* and *Barbilophozia*, and a necrotrophic fungus *Scleroconidioma sphagnicola* causes disease in the peat moss *Sphagnum fuscum* (Tsuneda et al. 2001, Polischuk et al. 2007).

The thalloid liverwort *Marchantia polymorpha* is emerging as another promising bryophyte model system (Berger et al. 2016, Ishizaki et al. 2016, Shimamura 2016, Delwiche et al. 2017), and molecular genetic tools and techniques for *M. polymorpha* are now well established (Ishizaki et al. 2008, Ishizaki et al. 2013, Kubota et al. 2013, Ishizaki et al. 2015, Flores-Sandoval et al. 2016, Nishihama et al. 2016, Sugano et al. 2018). Importantly, *M. polymorpha* probably did not undergo whole-genome duplication, with the consequence that genetic redundancy is low (Bowman et al. 2017). The overall simplicity of gene families in the species holds advantages for molecular genetic analysis (Eklund et al. 2015, Kato et al. 2015, Lockhart 2015). The majority of immune-related genes characterized in seed plants, including *nucleotide-binding leucine-rich repeat (NLR)* genes, have been identified in the *M. polymorpha* genome (Xue et al. 2012, Bowman et al. 2017). However, *M. polymorpha* has probably lost its symbiotic genes and cannot form beneficial associations with mycorrhizal fungi as in the case of *P. patens*. It is worth noting that most thalloid liverworts including related *Marchantia* species are able to form mycorrhizal associations (Russell and Bulman 2004, Ligrone et al. 2007, Bidartondo and Duckett 2010, Humphreys et al. 2010, Silvani et al. 2012). The study of plant–microbe interactions in *M. polymorpha* requires the establishment of proper and diverse pathosystems. However, information on pathogenic microbes that cause disease in *M. polymorpha* or liverworts remains very limited (Carella and Schornack 2018). Recently, the hemi-biotrophic oomycete pathogen *Phytophthora palmivora*, which is virulent for the root and leaf tissues of a broad range of flowering plants, was shown to infect and cause disease in *M. polymorpha* (Carella et al. 2018). Interestingly, *P. palmivora* could form haustoria-like intracellular infection structures in *M. polymorpha* as in flowering plants, which was not observed in *P. patens* (Overdijk et al. 2016, Carella et al. 2018). Besides, a large

number of fungal endophytes were isolated from wild populations of *M. polymorpha* (Nelson et al. 2018, Nelson and Shaw 2019). Individual inoculation of axenic *M. polymorpha* with the isolated fungi suggested that some of the fungi, namely *Xylaria cubensis*, *Hypoxylon submonticulosum* and *Colletotrichum* sp., were rather pathogenic to *M. polymorpha*.

Plants are subject to attack by various pathogenic microbes with different lifestyles, namely biotrophs, necrotrophs and hemi-biotrophs (Glazebrook 2005). Biotrophs derive their nutrients from living host tissues, while necrotrophs feed on the contents of dying or dead cells. Hemi-biotrophs switch their lifestyle from biotrophic to necrotrophic during infection. In seed plants, phytohormones play crucial roles in inducing relevant defense responses against these contrasting pathogens (Thomma et al. 1998, Spoel and Dong 2008, Pieterse et al. 2009, Verhage et al. 2010, Berens et al. 2017). The salicylic acid (SA) pathway primarily induces resistance against biotrophic and hemi-biotrophic pathogens, and the jasmonic acid (JA) pathway is chiefly required for resistance against necrotrophic pathogens and herbivorous insects. Crosstalk between the SA and JA pathways, which can spatiotemporally optimize distinct defense responses, is considered to be a fundamental regulatory mechanism for seed plants to deal with simultaneous or consecutive attack by multiple pathogens in natural environments (Koornneef and Pieterse 2008, Betsuyaku et al. 2018, Tsuda 2018). The SA and JA pathways are typically mutually antagonistic, and this SA–JA antagonism has been observed in various seed plants (Thaler et al. 2012). In Arabidopsis, SA is typically prioritized over JA, and the SA receptor NPR1 acts as a negative regulator of SA-induced suppression of the JA pathway (Spoel et al. 2003, Spoel et al. 2007, Leon-Reyes et al. 2010). Although homologs of core signaling components of the SA and JA pathways, including NPR1 and the JA-receptor COI1, were identified in the genomes of non-seed plant species, there is as yet no evidence for SA–JA crosstalk in non-seed plants (Thaler et al. 2012, Berens et al. 2017).

SA and JA are present in a range of bryophytes, although the detected JA levels were imperceptible (Stumpe et al. 2010, Drábková et al. 2015). Importantly, *M. polymorpha* and *P. patens* lack two key enzymes, OPR3 and JAR1, that are needed to produce JA-Ile from 12-oxo-phytodienoic acid (OPDA) in vascular plants (Rensing et al. 2008, Ponce de León et al. 2015, Yamamoto et al. 2015, Bowman et al. 2017). It has been consistently shown that *M. polymorpha* does not accumulate the bioactive JA, JA-Ile, and *M. polymorpha*, *P. patens*, and the hornwort *Anthoceros agrestis* are insensitive to exogenous application of JA or JA-Ile (Yamamoto et al. 2015, Monte et al. 2018). However, bryophytes remain sensitive to exogenous application of OPDA, and the *M. polymorpha* COI1 homolog MpCOI1 was found to be a receptor for dinor-OPDA (dn-OPDA) isomers, which are also catabolic products of OPDA, but MpCOI1 does not perceive JA-Ile (Ponce de León et al. 2012, Ponce de León et al. 2015, Monte et al. 2018). This finding may suggest that dn-OPDA isomers play roles in bryophytes that are similar to those of JA-Ile in seed plants. *P. patens* accumulates OPDA upon inoculation with the necrotrophic pathogens, *Botrytis cinerea*, *Pythium debaryanum* and *Pythium irregulare*, and Mp*coi1* mutants displayed reduced resistance against the generalist

herbivore *Spodoptera littoralis*, suggesting a role of the OPDA-COI1 pathway in defense responses in bryophytes (Oliver et al. 2009, Ponce de León et al. 2012, Monte et al. 2018). However, the contributions of OPDA catabolites and COI1 homologs to resistance against necrotrophic microbes in bryophytes remain uncharacterized. It should be noted that *P. patens* can respond to methyl jasmonate (MeJA) treatment, but it is not clear how *P. patens* perceives MeJA and the biological relevance of this perception remains unknown (Ponce de León et al. 2012, Ponce de León et al. 2015). Regarding the SA pathway in bryophytes, one of the *P. patens* NPR1 homologs was shown to partially complement the Arabidopsis *npr1-1* mutant phenotypes, suggesting that the molecular properties of NPR1 are relatively conserved throughout evolution (Peng et al. 2017). In *P. patens*, SA pretreatment could induce resistance against the bacterial pathogen *Erwinia carotovora* (Andersson et al. 2005). However, SA function in defense responses and the role of NPR1 in the SA pathway in bryophytes remain to be clarified.

Here, we report the isolation of four pathogenic fungal strains from diseased *M. polymorpha*. The four fungal isolates, *Irpex lacteus* Marchantia-infectious (MI)1, *Phaeophlebiopsis peniophoroides* MI2, *Bjerkandera adusta* MI3 and *B. adusta* MI4, displayed different levels of pathogenicity against in vitro grown *M. polymorpha*. Furthermore, by using a newly established *I. lacteus* MI1–*M. polymorpha* pathosystem, we provide first evidence that SA and dn-OPDA play roles in resistance against fungal pathogens, which are presumably necrotrophs, in *M. polymorpha*.

Results

Isolation and morphological characteristics of the MI filamentous microbes

In order to identify the natural pathogens of *M. polymorpha*, plants showing disease-like symptoms were collected, and then filamentous microbes were isolated from the diseased tissues. As a result, we isolated four distinct filamentous microbes and termed them MI1–4 based on the morphology of colonies (Fig. 1A–D). On potato dextrose agar (PDA), MI1, MI3 and MI4 formed white aerial mycelia and filled 9-cm-diameter plates after 6 d of incubation at 26°C in the dark (Fig. 1A, C, D). Scanning electron microscopy (SEM) images of the isolates on PDA are shown in Supplementary Fig. S1. Aerial mycelia of MI3 and MI4 were vigorous with a dense mass of hyphae. However, the aerial mycelium of MI4 was less homogeneous compared to MI3 (Fig. 1C, D). MI2 formed a milky heterogeneous aerial mycelium and its hyphal growth was slower than the other isolates (Fig. 1B). The morphological features of these four isolates were not altered when they were grown under white fluorescent light. After 2 months of culture on PDA, formation of fruiting body-like structures was observed for MI1, MI2 and MI3 (Fig. 1E–G), although none of the four isolates produced spores under our tested growth conditions.

The fungal isolates belong to the order Polyporales

For molecular identification of the isolates, genomic DNA was extracted from liquid cultures and the rDNA-ITS region was PCR-amplified and sequenced. Phylogenetic analysis of the rDNA ITS1-5.8S-ITS2 regions revealed that all four fungal isolates belong to *Irpicaceae* or *Phanerochaetaceae* families in the Polyporales order (Fig. 1H). The sequenced rDNA-ITS region of MI1 was 100% identical to *I. lacteus*; MI2 was 99% identical to *Phaeophlebiopsis peniphoroides*; and MI3 and MI4 were 99% identical to *B. adusta*. Therefore, we named the isolates *I. lacteus* MI1, *P. peniphoroide* MI2, *B. adusta* MI3 and *B. adusta* MI4 (Fig. 1H). Many species within the Polyporales are saprotrophic, and *Irpicaceae* and *Phanerochaetaceae* fungi are known to produce a white rot by degrading lignin, suggesting that these fungal isolates are necrotrophic pathogens of *M. polymorpha*.

Pathogenicity of the isolated fungi for *M. polymorpha*

In order to establish new pathosystems to study the immune system of *M. polymorpha*, we tested whether the isolated fungi can infect and cause disease on *M. polymorpha* growing axenically in vitro on Gamborg's B5 agar plates. We grew the fungi in liquid potato dextrose broth medium, fragmented them using a homogenizer and adjusted the OD_{600} for inoculation because we were not able to obtain spores from these fungi. Fourteen-day-old thalli of *M. polymorpha* Tak-1 (male) and *M. polymorpha* Tak-2 (female) were challenged with two different concentrations of fungi (Fig. 2A). The Marchantia thalli inoculated with *I. lacteus* MI1, *B. adusta* MI3 and *B. adusta* MI4 exhibited macroscopic disease symptoms, which developed in a concentration-dependent manner (Fig. 2C and Supplementary Figs. S2–S4). Among these three fungi, *I. lacteus* MI1 exhibited the strongest pathogenicity for *M. polymorpha* and had completely killed the Marchantia thalli by 5 to 6 d postinoculation (dpi) (Fig. 2C). Although *B. adusta* MI3 and *B. adusta* MI4 are closely related (Fig. 1H), the pathogenicity of *B. adusta* MI4 was slightly higher compared to *B. adusta* MI3 (Fig. 2C). In contrast, we did not observe any obvious *P. peniophoroides* MI2 infection on Marchantia thalli under these experimental conditions up to 11 dpi (Fig. 2C). It should be noted that observed differences between Tak-1 and Tak-2 strains for their susceptibility to the fungi could be due to their differential growth or development of thalli. Based on these observations, we classified the pathogenicity of these fungi on *M. polymorpha* as shown in Table 1. We decided to use *I. lacteus* MI1 for further analyses because of its robust pathogenicity for the Marchantia thalli in our experimental system.

SA and/or OPDA treatments alter the susceptibility of *M. polymorpha* to *I. lacteus* MI1 infection

Irpex lacteus is one of the most common wood-decaying necrotrophs, and, therefore, the *I. lacteus* MI1–*M. polymorpha* pathosystem could be used as a model for studying necrotrophic fungus–liverwort interactions. In seed plants, the JA pathway is primarily required for resistance against necrotrophic pathogens and herbivorous insects, and its activity is often suppressed by the SA pathway as in Arabidopsis. In *M. polymorpha*, dn-OPDA rather than JA-Ile functions as a phytohormone, which binds to the MpCOI1 receptor. Mp*coi1* mutants exhibit enhanced susceptibility to the generalist herbivore *S. littoralis* (Monte et al. 2018), suggesting a possible role of dn-OPDA in
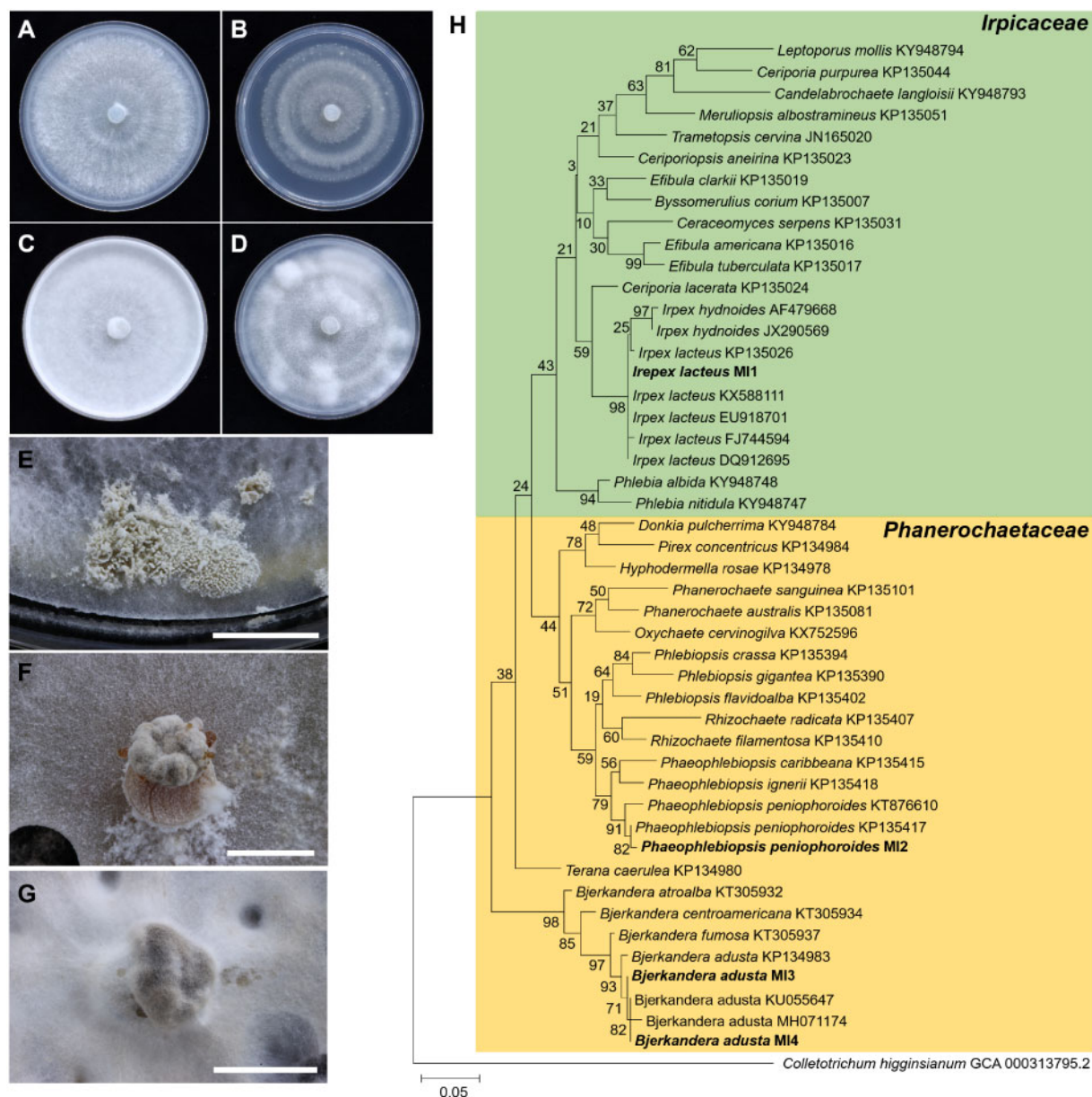
Fig. 1 Fungal strains isolated from diseased *Marchantia polymorpha* Tak-1. (A–D) Colonies of *Irpex lacteus* MI1 (A), *Phaeophlebiopsis peniophoroides* MI2 (B), *Bjerkandera adusta* MI3 (C) and *Bjerkandera adusta* MI4 (D) grown on PDA media in a 9-cm Petri dish for 6 days at 26°C in the dark. (E–G) Fruiting bodies of *I. lacteus* MI1 (E), *P. peniophoroides* MI2 (F) and *B. adusta* MI3 (G) grown for 2 months at 26°C in the dark. Scale bars = 1 cm. (H) Maximum likelihood tree of fungal strains from *Phanerochaetaceae* and *Irpicaceae* families based on the ITS1-5.8S-ITS2 region. MI1, MI2, MI3 and MI4 are isolates from diseased *M. polymorpha* Tak-1. The sequence of *Colletotrichum higginsianum* was used as an outgroup. Numbers on the branches represent bootstrap values obtained from 1,000 replications.

resistance against necrotrophic pathogens as well. A number of bryophytes including liverworts have been shown to accumulate SA (Drábková et al. 2015), and we detected SA in *M. polymorpha* Tak-1 (**Supplementary Table S1**). However, the contribution of SA to *M. polymorpha* immunity and possible crosstalk between SA and dn-OPDA pathways has not yet been clarified. To address these questions, we tested whether exogenous applications of SA and/or OPDA, the dn-OPDA precursor, to *M. polymorpha* could influence *I. lacteus* MI1 infection.

Thalli of 14-day-old *M. polymorpha* Tak-1 were soaked in water (mock), 500 μM SA, 1,000 μM SA or 50 μM OPDA for 3 h and then inoculated with *I. lacteus* MI1 (**Fig. 3A**). Macroscopic disease symptoms appeared at 5 dpi, and the SA treatments resulted in a significant promotion of lesion development at 6 and 7 dpi compared to the mock treatment (**Fig. 3B, C** and **Supplementary Fig. S5**). In contrast, the OPDA treatment showed no effect on the lesion development, which is reminiscent of the inability of exogenous JA treatments to protect wild-type Arabidopsis plants against certain necrotrophic pathogens (Thomma et al. 1998). The SA or OPDA treatments did not affect plant health (**Supplementary Fig. S5**), and we confirmed that SA does not promote but rather suppresses *I. lacteus* MI1
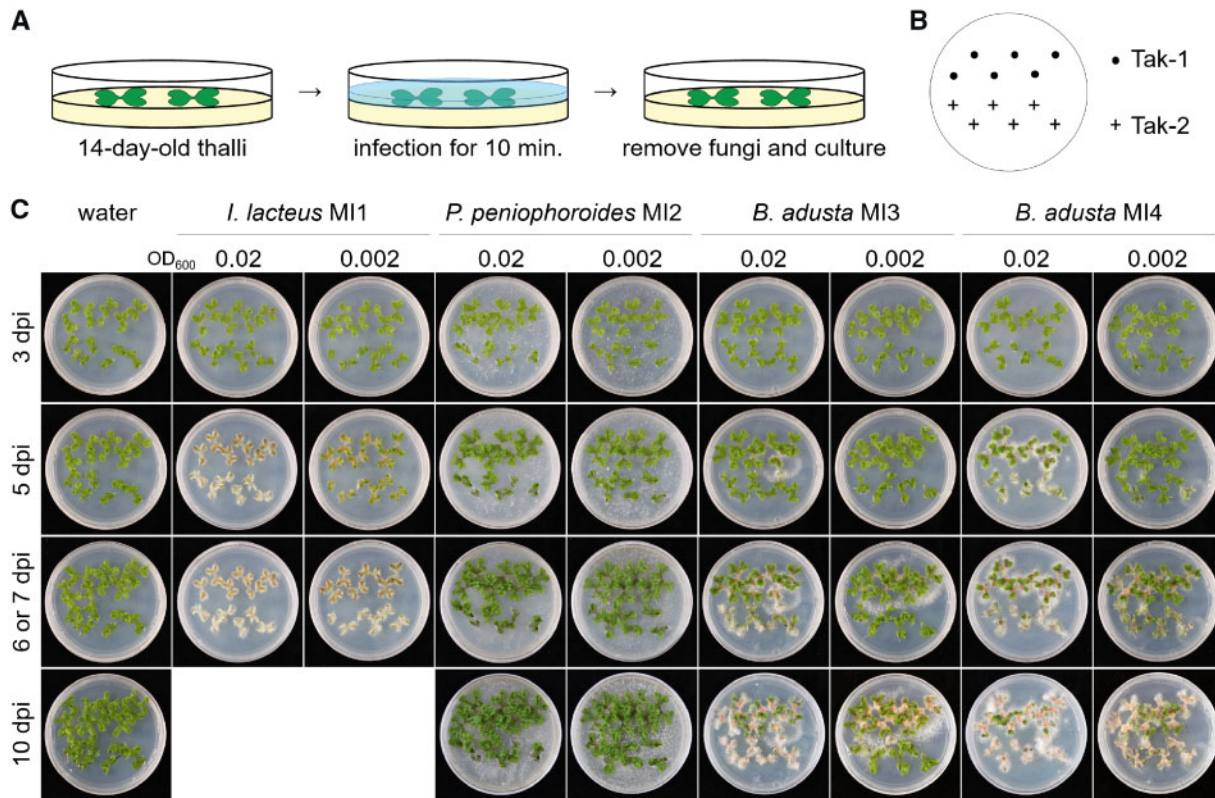
Fig. 2 Pathogenicity of the fungal isolates for *Marchantia polymorpha*. (A) Schematic presentation of fungal inoculations of *M. polymorpha*. (B) Arrangement of *M. polymorpha* Tak-1 and Tak-2 on plates. (C) Inoculation of *M. polymorpha* with the fungal isolates. For *I. lacteus* MI1 infection, the image was taken at 6 days postinoculation (dpi). For the rest, images are from 7 dpi.

Table 1 Fungal strains isolated in this study

Species	Isolate	Isolation locality	Pathogenicity to *M. polymorpha*[a]
Irpex lacteus	MI1	Kitashirakawa, Kyoto	++
Phanerochaete peniophoroides	MI2	Kitashirakawa, Kyoto	±
Bjerkandera adusta	MI3	Kitashirakawa, Kyoto	+
Bjerkandera adusta	MI4	Kitashirakawa, Kyoto	+

[a]Pathogenicity under the experimental conditions used in this study.

growth on Gamborg's B5 agar (**Supplementary Fig. S6**). These results indicate that, as in many seed plants, activation of the SA pathway compromises resistance against necrotrophic pathogens in *M. polymorpha*. This suggests the existence of antagonistic crosstalk between the SA and dn-OPDA pathways in *M. polymorpha*.

As the possible effects of a single OPDA treatment can be very transient, we next added 500 μM SA and/or 1 μM dn-*cis*-OPDA to the Gamborg's B5 agar plates, thus continuously exposing the Marchantia thalli to these phytohormones during infection (**Fig. 4A**). The stimulatory effect of SA on *I. lacteus* MI1 infection was confirmed under these conditions, but sustained application of dn-*cis*-OPDA did not affect the lesion development as we previously observed (**Fig. 4B, C** and **Supplementary Figs. S7, S8**). Intriguingly, however, the ability of SA to promote *I. lacteus* MI1 infection was compromised by co-treatment with

dn-*cis*-OPDA (**Fig. 4B, C** and **Supplementary Figs. S7, S8**). These results suggest that dn-*cis*-OPDA suppresses the SA pathway and thereby contributes to resistance against necrotrophic pathogens in *M. polymorpha*.

To further verify the involvement of the OPDA pathway in resistance against necrotrophic pathogens, we challenged the Mp*coi1-2* mutant allele (male) (Monte et al. 2018) with *I. lacteus* MI1. The Mp*coi1-2* mutant displayed enhanced susceptibility against *I. lacteus* MI1 compared to *M. polymorpha* Tak-1 (**Fig. 5B, C** and **Supplementary Figs. S9, S10**), which suggests that the OPDA pathway positively regulates resistance against necrotrophic pathogens in *M. polymorpha*.

Discussion

To date, there are hardly any reports that describe pathogenic microbes that naturally infect and cause disease in *M. polymorpha*, which hinders the use of *M. polymorpha* in EvoMPMI studies (Carella and Schornack 2018). The recent finding that the hemi-biotrophic oomycete pathogen *P. palmivora* can colonize *M. polymorpha* enabled the study of liverwort–pathogen interactions at the molecular level (Carella et al. 2018). Nevertheless, to enable the study of a range of different interactions, other types of *M. polymorpha*-infecting pathogens are needed. In this work, we have isolated four fungal strains, which are most likely necrotrophic pathogens, from diseased *M. polymorpha*, and demonstrated that the *I. lacteus* MI1 isolate can be
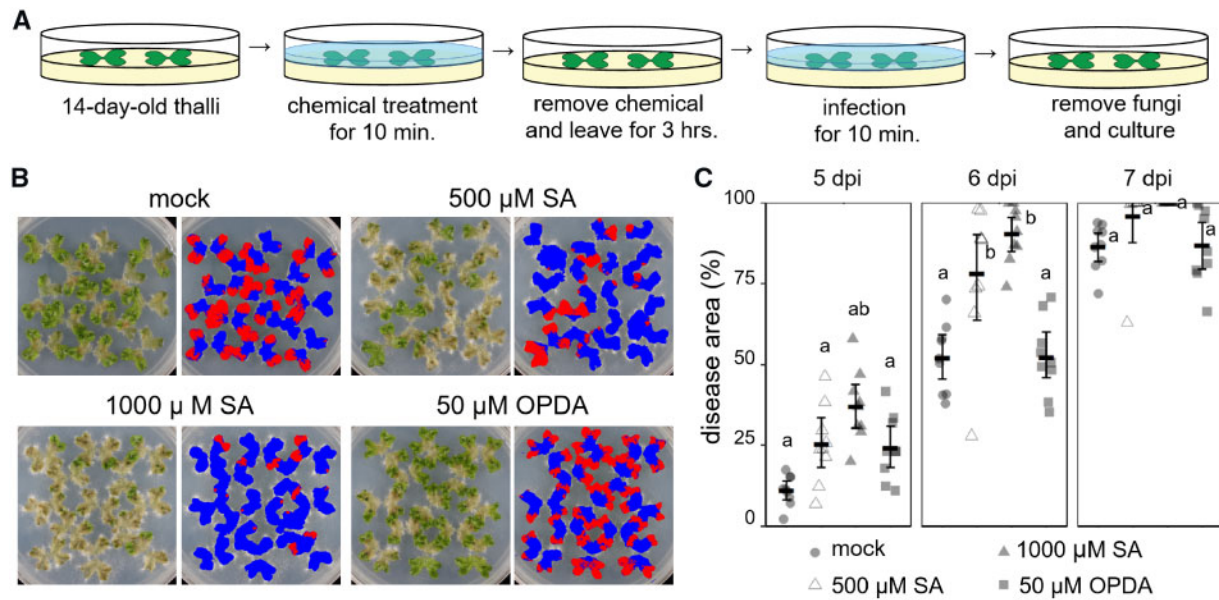
Fig. 3 SA treatment promotes *Irpex lacteus* MI1 infection of *Marchantia polymorpha* Tak-1. (A) Schematic representation of chemical treatment and subsequent fungal inoculation. (B) Images of *M. polymorpha* Tak-1 thalli infected with *I. lacteus* MI1. Treatment conditions are indicated on the top of the panels. Images at 6 dpi (left) and their healthy and lesion-bearing areas detected by the ImageJ-based PIDIQ method (right) are shown. Healthy and lesion-bearing areas are colored in red and blue, respectively. (C) Effects of SA or OPDA on *M. polymorpha* Tak-1 infection with *I. lacteus* MI1. The proportions of the lesion-bearing areas at 5–7 dpi are shown. Gray circles, white triangles, gray triangles and gray squares represent individual data points for each condition indicated. Within each group, the middle horizontal bar is the mean, and the error bars reflect 95% confidence intervals. Different letters indicate significant differences between treatments at each dpi ($n = 9$, $P < 0.01$, Tukey's HSD test).
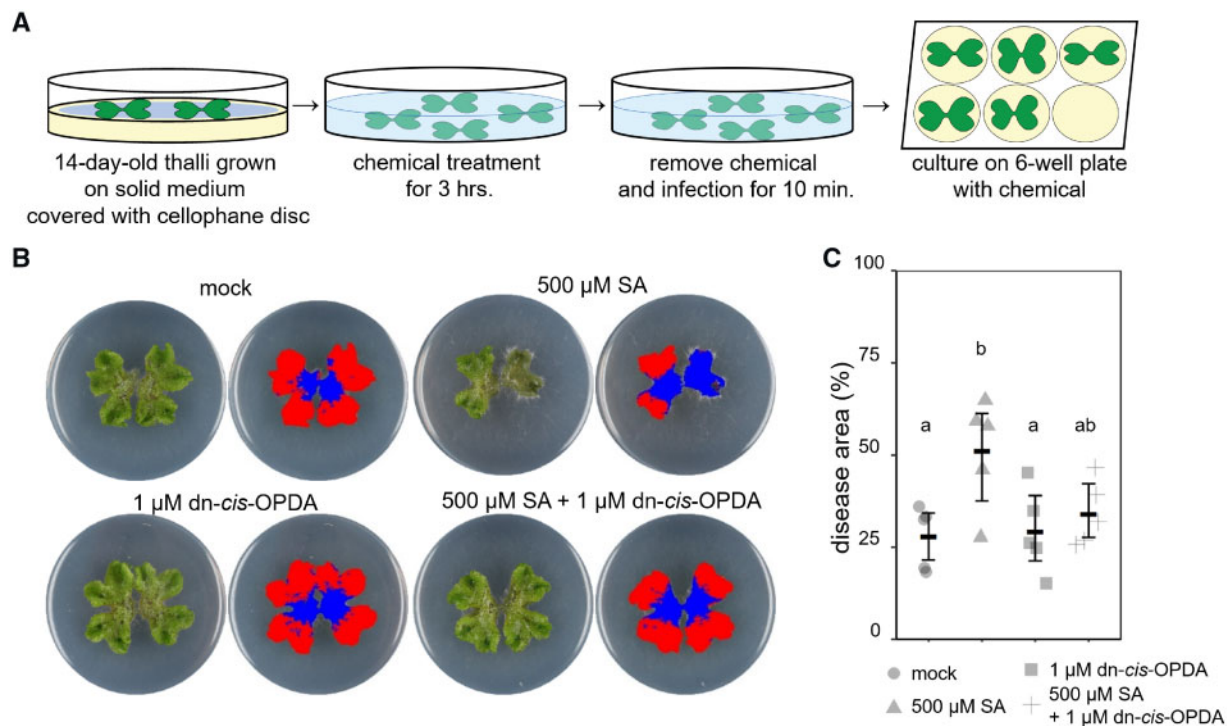

Fig. 4 SA effect is suppressed by dn-*cis*-OPDA co-treatment. (A) Schematic representation of chemical treatment and fungal inoculation. (B) Images of *M. polymorpha* Tak-1 thalli infected with *I. lacteus* MI1. Treatment conditions are indicated on the top of the panels. Representative images at 4 dpi (left) and their healthy and lesion-bearing areas detected by the ImageJ-based PIDIQ method (right) are shown. Healthy and lesion-bearing areas are colored in red and blue, respectively. (C) Effects of SA or dn-*cis*-OPDA on *M. polymorpha* Tak-1 infection with *I. lacteus* MI1. The proportions of the lesion-bearing areas at 4 dpi are shown. Circles, triangles, squares and plus signs represent individual data points for each condition indicated. Within each group, the middle horizontal bar is the mean, and the error bars reflect 95% confidence intervals. Different letters indicate significant differences between treatments at each dpi ($n = 5$, $P < 0.05$, Tukey's HSD test).
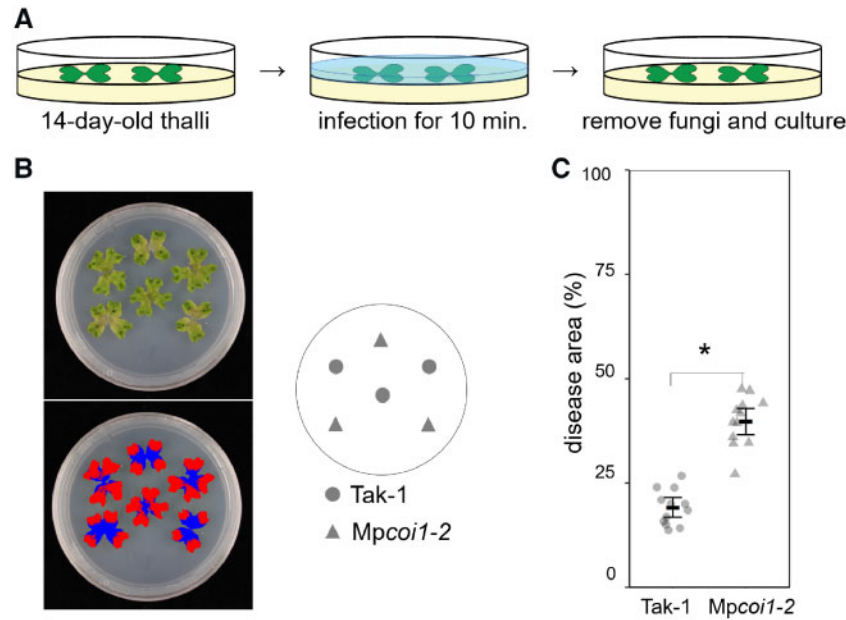
Fig. 5 Mp*coi1* mutant is susceptible to *Irpex lacteus* MI1. (A) Schematic presentation of fungal inoculations of *M. polymorpha*. (B) Images of *M. polymorpha* Tak-1 and Mp*coi1-2* thalli infected with *I. lacteus* MI1. Representative images at 6 dpi (upper left) with healthy and lesion-bearing areas detected by the ImageJ-based PIDIQ method (lower left) are shown. Healthy and lesion-bearing areas are colored in red and blue, respectively. Arrangement of *M. polymorpha* Tak-1 and Mp*coi1-2* on the plate (right). (C) Infection of *M. polymorpha* Tak-1 and Mp*coi1* with *I. lacteus* MI1. The proportions of the lesion-bearing areas at 6 dpi are shown. Circles and triangles represent individual data points for each genotype indicated. Within each group, the middle horizontal bar is the mean, and the error bars reflect 95% confidence intervals. Asterisks indicate significant differences between *M. polymorpha* Tak-1 and Mp*coi1-2* ($n = 12$, $P < 0.01$, Student's *t*-test).

used as a model to understand interactions between necrotrophic fungi and liverworts. We anticipate that further bacterial pathogens or biotrophic pathogens of *M. polymorpha* will be identified in the future.

The white rot fungus *I. lacteus* is a cosmopolitan species, which produces hydrolases including lignin-degrading enzymes that decompose plant cell walls facilitating its colonization (Novotný et al. 2009). Because of its biotechnological potential, *I. lacteus* has been studied for many years, and the genomes of the *I. lacteus* strains F17 and CD2 have been sequenced (Yao et al. 2017, Qin et al. 2018). Interestingly, *I. lacteus* was previously isolated from the moss *R. japonicum* growing in a field in Japan and was shown to cause disease in *P. patens* (Lehtonen et al. 2009). Therefore, *I. lacteus* might be pathogenic for a wide range of bryophytes, and could therefore be generally used as a model necrotroph to study interactions between bryophytes and fungi. In *P. patens*, the class III peroxidase Prx34 is secreted upon chitosan treatment, and *prx34* knock-out mutants displayed susceptibility to the *I. lacteus* isolate (Lehtonen et al. 2009). Determining the roles of the class III peroxidases during *I. lacteus* infection in *M. polymorpha* would contribute to our understanding of the evolution of immune mechanisms within bryophytes.

When we inoculated the *I. lacteus* MI1, *B. adusta* MI3 or *B. adusta* MI4 isolates to *M. polymorpha* Tak-1 thalli using our method, we could hardly observe fungi growing on the surface of Marchantia thalli. Interestingly, we found that hyphae of the fungi are coming out from air chambers (**Supplementary Figs. S2E, F, S3B, D**), which suggests that the fungal isolates

enter air chambers for their successful growth and infection. These observations are very interesting because the hemibiotrophic oomycete pathogen *P. palmivora* was shown to take similar approach to infect *M. polymorpha* (Carella et al. 2018). These findings suggest that air chambers are crucial battlefields between the host and invading microbes.

JA plays a pivotal role in defense responses against necrotrophic pathogens in seed plants. In *M. polymorpha*, OPDA is expected to play a similar role as JA in seed plants (Monte et al. 2018). However, OPDA treatment of *M. polymorpha* failed to boost resistance against the *I. lacteus* MI1 isolate under our experimental conditions. This observation is consistent with the fact that JA treatment can boost resistance against the necrotrophic fungus *Alternaria brassicicola* only in *pad3* mutants but not in wild-type Col-0 plants in Arabidopsis (Thomma et al. 1998, Spoel et al. 2007). In Arabidopsis, the JA-insensitive *coi1* mutants also display susceptibility to *A. brassicicola*, and, therefore, either JA-dependent defense activation or PAD3-dependent camalexin synthesis seems to be sufficient for the resistance against necrotrophic pathogens. Accordingly, the increased susceptibility of the OPDA-insensitive Mp*coi* mutant to *I. lacteus* MI1 (Monte et al. 2018) indicates that OPDA-dependent defense activation plays a role in resistance against necrotrophic fungi in *M. polymorpha*.

SA levels in 3-week-old thalli of *M. polymorpha* Tak-1, which were grown on Gamborg's B5 agar plates, were relatively low compared to non-stressed Arabidopsis but were comparable to other analyzed liverworts (Drábková et al. 2015). Interestingly, 6-day-old gemmae of *M. polymorpha* Tak-1 that were grown in

Gamborg's B5 liquid media with shaking accumulated more SA compared to the thalli on the plates (**Supplementary Table S1**). SA levels may differ in different developmental stages. It is also possible that growth in liquid media, a rather stressful condition for gemmae, may have induced SA accumulation. It would be interesting to determine whether biotic stresses affect SA levels in *M. polymorpha* as in seed plants. We could detect only very low levels of SA glucoside (SAG) in the samples we analyzed (**Supplementary Table S1**), and we expect that further investigations into SA biosynthesis and metabolism in *M. polymorpha* will improve our understanding of the physiological roles of SA in liverworts.

The striking observation that SA treatment promoted *I. lacteus* MI1 infection implies the existence of SA-OPDA crosstalk in *M. polymorpha*, which is similar to the antagonistic SA–JA crosstalk in seed plants. In line with this observation, OPDA co-treatment resulted in suppression of the SA-dependent stimulatory effect. This suggests that OPDA is prioritized over SA in *M. polymorpha*, which is opposite to the case in Arabidopsis (Spoel *et al.* 2003, Spoel *et al.* 2007, Leon-Reyes *et al.* 2010). Further molecular studies are needed to draw solid conclusions regarding the SA–OPDA crosstalk in *M. polymorpha*. For instance, generation and use of a transgenic *M. polymorpha* that expresses NahG (Gaffney *et al.* 1993), which degrades endogenous SA to catechol may give us more insight into this crosstalk. Considering the importance of SA–JA antagonism in seed plants in dealing with simultaneous or consecutive attack by multiple contrasting pathogens, we speculate that SA treatment might boost the resistance of *M. polymorpha* against biotrophic and hemi-biotrophic pathogens. Intriguingly, it was shown that SA treatment could induce resistance against the necrotrophic bacterial pathogen *E. carotovora* in *P. patens* (Andersson *et al.* 2005). Therefore, the roles of SA in defense responses may differ between mosses and liverworts.

Materials and Methods

Plant material and growth condition

Marchantia polymorpha, accession Takaragaike-1 (Tak-1; male) and Takaragaike-2 (Tak-2; female) were used for experiments. *M. polymorpha* was cultured at 22°C on half-strength Gamborg's B5 medium (Gamborg *et al.* 1968) containing 1% agar under 50–60 µmol photons m^{-2}s^{-1} continuous white LED (light-emitting diode) light.

Fungal isolation

In vitro grown thalli of *M. polymorpha* Tak-1 were exposed to outside air for a while at Kitashirakawa, Kyoto, and then further cultivated in a growth chamber at 22°C under continuous white light. For isolation of natural pathogens, 3 mm^2 pieces were cut from *M. polymorpha* Tak-1 with disease-like symptoms collected at Kyoto, Japan. Small pieces were soaked in 70% (w/v) ethanol for 1 min, and dipped into 1% (w/v) sodium hypochlorite solution for 3 min. Pieces were then immediately rinsed two times with sterilized water, placed on 1% water agar plates containing 0.01% (w/v) streptomycin sulfate and incubated at 26°C for a few days. After confirming the growth of fungal colonies, a hyphal tip from each developing colony was transferred to a PDA (BD, Franklin Lakes, NJ, USA) plate.

DNA amplification and extraction

Isolates were cultured on PDA plates at 26°C for 7 d. Ten pieces of mycelial plug (1 mm diameter) of MI1, MI3 and MI4 were transferred to 300 ml flasks containing 100 ml of CM liquid medium consisting of 0.3% (w/v) casamino acids, 0.3% (w/v) yeast extract and 0.5% (w/v) sucrose. Ten pieces of mycelial plug of the MI2 isolate were transferred to 300 ml flasks containing 100 ml of liquid medium consisting of 0.3% (w/v) yeast extract and 0.5% (w/v) sucrose. Flasks were shaken at 90 rpm at 26°C for 2–4 d. Mycelia produced in liquid media were filtered, freeze-dried, frozen with liquid nitrogen and ground to powder using a mortar and pestle. Genomic DNA was extracted using DNeasy Plant Kits (QIAGEN, Hilden, Germany), according to the manufacturer's protocol.

PCR amplification

PCR was performed using 5 ng of fungal genomic DNA. ITS1F (5′-GTAACAAGGTYTCCGT-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′) (White *et al.* 1990, Gardes and Bruns 1993) were used for the amplification of the nucleotide sequences of rDNA ITS1-5.8S-ITS2 regions. The PCR-amplified products were purified using Wizard® SV Gels and a PCR Clean-Up System (Promega, Madison, WI, USA) and then sequenced.

Sequence alignment and phylogenetic analysis

Multiple alignment of nucleotide sequences was performed using MUSCLE. Phylogenetic analysis was performed using PhyML (**http://www.atgc-montpellier.fr/phyml/**) with 1,000 bootstrap replications. For multiple genetic genealogy analysis, nucleotide sequences of rDNA ITS1-5.8S-ITS2 regions from Polyporales were downloaded from GenBank (**https://www.ncbi.nlm.nih.gov**).

Inoculation test

Marchantia gemmae were placed on half-strength Gamborg's B5 agar plates and grown for 14 d under continuous white LED light or a 14 h light/10 h dark cycle. For preparation of inoculum, mycelium was cultured in CM liquid medium at 21°C for 7 d on shakers running at 120 rpm. About 1 cm of mycelium ball was rinsed with sterilized water, homogenized with ULTRA-TURRAX T8 (IKA Labortechnik, Staufen, Germany) and suspended into 5 ml sterilized water. Mycelium suspension at an OD$_{600}$ of 0.02 or 0.002 was decanted into the Marchantia culture plate and incubated for 10 min. The mycelium suspension was removed, and then Marchantia thalli were further cultured for the indicated number of days.

For SA or OPDA treatments in **Fig. 3**, sodium salicylate (Sigma-Aldrich, Munich, Germany) and OPDA (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in 1.47% ethanol, and 1.47% ethanol was used for the mock treatment. The chemical solutions were added to the Marchantia culture plates and the 14-day-old thalli were immersed in the chemicals for 10 min. After removal of the chemical solutions, treated thalli were incubated for 3 h, and then the mycelium suspension at an OD$_{600}$ of 0.002 was inoculated as described above.

For SA and/or dn-*cis*-OPDA application to the growth media in **Fig. 4**, Marchantia gemmae were grown on half-strength Gamborg's B5 agar covered with a cellophane disc (Diagonal, Münster, Germany) to avoid rhizoids growing into the media for easy transfer of thalli. SA and dn-OPDA (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in 0.026% ethanol, and 0.026% ethanol was used as the mock control. Fourteen-day-old thalli were dipped in the chemical solutions for 3 h followed by incubation with the mycelium suspension at an OD$_{600}$ of 0.002 for 10 min. Then, treated thalli were cultured on half-strength Gamborg's B5 agar plates supplemented with the chemicals.

Measurement of disease area

To measure healthy and lesion-bearing areas of *M. polymorpha* infected with *I. lacteus* MI1, ImageJ-based macro plant immunity and disease image-based quantification (PIDIQ) was used (Laflamme *et al.* 2016). The 'Threshold Color' value was adjusted as described in the supplemental DATA (**Supplementary ImageJ macro 1–3**). Statistical analysis was performed using Tukey's HSD test with R software version 3.2.3 (**https://www.r-project.org/**).

Quantification of SA

The quantification of SA and SAG was performed as described previously (Seo *et al.* 1995, Betsuyaku *et al.* 2018). Briefly, three-week-old thalli of *M. polymorpha* Tak-1 that were grown on Gamborg's B5 agar plates or 6-day-old gemmae of *M. polymorpha* Tak-1 that were grown in Gamborg's B5 liquid media with shaking

were frozen and ground using liquid nitrogen. SA and SAG were extracted with 90% methanol, and SAG was converted to SA by β-glucosidase treatment. After separation by HPLC (Shimadzu) with an ODS column (μ-Bondasphere C18, 150 mm × ID3.9 mm, 5 μm, 100 A; Waters), SA levels were determined using a fluorescence detector (RF-20A; Shimadzu) with an excitation wavelength of 313 nm and an emission wavelength of 405 nm.

Cryo-SEM

Samples were mounted on copper-made sample holders, shock frozen in liquid nitrogen and sublimated, sputtered with Gold/Palladium mixture (80% Gold/ 20% Palladium) using an Emitech K1250X cryo system, and then images were taken using a Zeiss Supra 40VP scanning electron microscope (Carl Zeiss NTS).

Supplementary Data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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