

Involvement of a Class III Peroxidase and the Mitochondrial Protein TSPO in Oxidative Burst Upon Treatment of Moss Plants with a Fungal Elicitor

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Production of apoplastic reactive oxygen species (ROS), or oxidative burst, is among the first responses of plants upon recognition of microorganisms. It requires peroxidase or NADPH oxidase (NOX) activity and factors maintaining cellular redox homeostasis. Here, PpTSPO1 involved in mitochondrial tetrapyrrole transport and abiotic (salt) stress tolerance was tested for its role in biotic stress in *Physcomitrella patens*, a nonvascular plant (moss). The fungal elicitor chitin caused an immediate oxidative burst in wild-type *P. patens* but not in the previously described $\Delta Prx34$ mutants lacking the chitin-responsive secreted class III peroxidase (Prx34). Oxidative burst in *P. patens* was associated with induction of the oxidative stress-related genes *AOX*, *LOX7*, and *NOX*, and also *PpTSPO1*. The available $\Delta PpTSPO1$ knockout mutants overexpressed *AOX* and *LOX7* constitutively, produced 2.6-fold more ROS than wild-type *P. patens*, and exhibited increased sensitivity to a fungal necrotrophic pathogen and a saprophyte. These results indicate that Prx34, which is pivotal for antifungal resistance, catalyzes ROS production in *P. patens*, while PpTSPO1 controls redox homeostasis. The capacity of TSPO to bind harmful free heme and porphyrins and scavenge them through autophagy, as shown in *Arabidopsis* under abiotic stress, seems important to maintenance of the homeostasis required for efficient pathogen defense.

Plants sense their environment and react to abiotic and biotic stimuli with a wide range of physiological and biochemical responses. Apoplastic reactive oxygen species (ROS) are among the first molecules produced upon recognition of microorganisms by the plant (Nanda et al. 2010; Torres 2010). Rapid ROS generation, known as the oxidative burst, is observed commonly upon pathogen attack (Apel and Hirt 2004; Wojtaszek 1997) and results in fast systemic activation of pathogen defense in the plant (Miller et al. 2009). In addition to their signaling effect, ROS can be toxic to the invading microbes (Angelova et al. 2005), mediate production of phytoalexins and other compounds that limit the growth of pathogens, and be positive or negative regulators of the hypersensitive response (HR) associ-

ated with cell death (Torres 2010). Although HR may restrict the pathogen to the initial infection site, necrotrophic fungal pathogens can take advantage of the HR-associated cell death and use it as a means to colonize the host plant (Govrin and Levine 2000).

The origins of extracellular ROS seem to vary depending on the plant–pathogen combination and have been attributed to, for example, an apoplastic peroxidase (Bindschedler et al. 2006) and a NADPH oxidase localized in the plasma membrane (Torres et al. 2002). Peroxidases utilize hydrogen peroxide (H_2O_2) for oxidative power in the peroxidative cycle but they are also capable of producing highly reactive free radicals such as superoxide in the hydroxylic cycle for defense purposes (Kawano 2003).

The signaling pathways involving the peroxidase or NADPH oxidase-catalyzed superoxide production in vascular plants include common factors (Fujita et al. 2006; Walley et al. 2007). One of them is the heme co-factor that is required by peroxidases and NADPH oxidases for electron transfer in ROS production (Keller et al. 1998; Schuller et al. 1996). Heme is produced via the tetrapyrrole biosynthesis pathway in plastids (Mochizuki et al. 2010). Peripheral-type benzodiazepine receptor and the bacterial tryptophane-rich sensory protein are involved in the transport of intermediates of the tetrapyrrole biosynthesis pathway, and a gene encoding a homologous protein (PpTSPO1) has been described in a moss (*Physcomitrella patens* (Hedw.) B. S. G., family Funariaceae), a nonvascular plant (Frank et al. 2007). The PpTSPO1 protein is localized to mitochondria, induced by drought and abscisic acid, and essential for adaptation of the moss to salt stress. The *PpTSPO1* knockout mutants are impaired in their mitochondrial protoporphyrin IX uptake and produce elevated levels of intracellular ROS, implying a role of PpTSPO1 in redox homeostasis (Frank et al. 2007). Recently, the homolog of *PpTSPO1* has been characterized in *Arabidopsis thaliana*, in which it contributes to salt stress tolerance (Balsemão-Pires et al. 2011) as in *P. patens*, and is involved in scavenging unbound heme and porphyrins (Vanhee et al. 2011).

Mosses produce ROS, including H_2O_2 , under abiotic stress (Frank et al. 2007; Mayaba et al. 2002; Proctor et al. 2007) but little is known about production of ROS during biotic stresses in mosses. *P. patens* encodes a class III peroxidase (Prx34) that is released immediately to the extracellular space and culture medium upon treatment of the moss with a chitosan preparation (CHN) (Lehtonen et al. 2009). The CHN preparation contains chitin, a fungal cell wall component that elicits pathogen

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defense in plants, and which is deacetylated to 90% in order to make it soluble in water (Knaul et al. 1999; Shibuya and Minami 2001). In the first and, thus far, only study that has utilized knockout mutants of *P. patens* to address questions related to pathogen defense and biotic stresses (Lehtonen et al. 2009), the two copies of the *Prx34* gene were deleted utilizing the highly efficient homologous recombination in *P. patens* and the dominant haploid lifestyle to reveal the phenotypes (Cove 2005; Strepp et al. 1998). Results showed that the 11 independent $\Delta Prx34$ mutants tested had lost resistance to pathogenic and saprotrophic fungi isolated from mosses. However, i) whether or not apoplastic oxidative burst occurs in mosses as a defense response to infection (biotic stress) and ii) whether *Prx34* is involved is not known. Similarly, iii) whether the functions of the mitochondrial protein TSPO are important in biotic stress has not been reported. The aim of this study was to answer the three aforementioned question using *P. patens* as the model system.

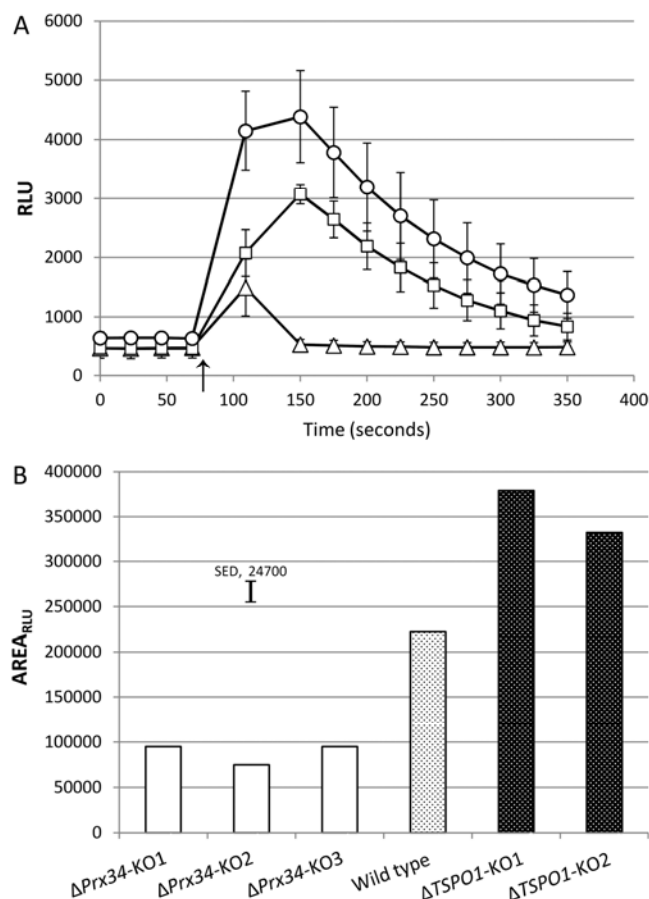


Fig. 1. Production of superoxide ($O_2^{\bullet-}$) in *Physcomitrella patens* upon elicitation with a chitosan preparation (CHN). **A**, $O_2^{\bullet-}$ measured as relative light units (RLU) based on chemiluminescence of the luciferin analog 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2- α)pyrazin-3-one at different time points following addition of CHN to the liquid culture medium in four independent experiments. Arrow indicates the time point of CHN addition. Symbols: □ = wild-type (WT) *P. patens*; ○ = $\Delta PpTSPO1$, a knockout mutant that is lacking the *TSPO1* gene and exhibits sensitivity to salt stress (Frank et al. 2007); △ = $\Delta Prx34$, a knockout mutant lacking the CHN-responsive class III peroxidase gene and rendered susceptible to fungal infection (Lehtonen et al. 2009). Error bars indicate standard deviation ($n = 4$). **B**, Mean cumulative $O_2^{\bullet-}$ production in three independent experiments at 3 min after addition of CHN to the culture medium. Three independent $\Delta Prx34$ knockout mutants, WT *P. patens*, and two independent $\Delta PpTSPO1$ knockout lines were tested. Differences between the $\Delta Prx34$ and $\Delta PpTSPO1$ knockout lines and WT *P. patens* are statistically significant ($P < 0.01$). The error bar indicates standard error of difference (SED; $df = 220$).

RESULTS

Superoxide production induced by CHN in *P. patens*.

There is little information available from nonvascular plants as to whether or not they respond to pathogens or pathogen elicitors with oxidative burst. It was tested in this study by application of CHN, a water-soluble hydrolysate of the fungal cell wall component chitin, to the liquid culture of *P. patens*. An immediate burst of ROS in the medium was observed (Fig. 1A). Superoxide production also was detected in the axenic cultures of *Sphagnum capillifolium* (Ehr.) Hedw. (Supplementary Fig. S1), an unrelated moss species (family Sphagnaceae), upon treatment with CHN. These results indicate that oxidative burst is a general response of bryophytes to pathogens or pathogen elicitors.

Production of superoxide increased in a dose-dependent manner and reached the maximum level with application of CHN in the culture medium at the final concentration of 1 mg ml^{-1} (Supplementary Fig. S2A). However, the oxidative burst was not observed in the protoplasts of *P. patens* treated with CHN, suggesting that the secreted peroxidase is deposited to cell walls that are removed in the process of protoplast preparation.

Dependence of the ROS generation on *Prx34* was tested by including the previously described $\Delta Prx34$ knockout mutants, in which the gene for a CHN-induced, excreted peroxidase *Prx34* has been knocked out (Lehtonen et al. 2009). There was no oxidative burst observed with $\Delta Prx34$ knockout mutants in four independent experiments (Fig. 1A), indicating that *Prx34* is an essential catalyst of ROS production.

The total amount of superoxide that accumulated during the first 3 min postapplication of CHN in three independent experiments indicated that superoxide production in the two independent, previously described *PpTSPO1* knockout mutant lines ($\Delta PpTSPO1$) (Frank et al. 2007) was 1.6-fold higher than that in the wild-type (WT) *P. patens* (Fig. 1B). On the other hand, superoxide production in the three independent $\Delta Prx34$ mutants tested was only 40% of that in WT *P. patens* (Fig. 1B) and was not affected by CHN treatment (Fig. 1A). The consistent response among the independent knockout mutant lines in repeated experiments and the statistically significant differences compared with WT *P. patens* (Fig. 1) indicated that the increased or decreased superoxide production resulted from the deletion of *PpTSPO1* and *Prx34* genes, respectively.

Superoxide production correlates with peroxidase activity.

Because the results on the $\Delta Prx34$ knockout mutants indicated that the oxidative burst upon treatment with CHN requires *Prx34* (Fig. 1), we tested whether the higher ROS production in $\Delta PpTSPO1$ also was associated with higher peroxidase activity. Indeed, peroxidase activity in the culture medium of the $\Delta PpTSPO1$ mutants was approximately 2.6-fold higher than that of WT *P. patens* following treatment with CHN. The difference was statistically significant in the three independent experiments carried out (Fig. 2). These results suggested that the $\Delta PpTSPO1$ mutants are not able to control ROS production, compared with WT *P. patens*.

Elevated transcript levels of stress-associated genes in $\Delta PpTSPO1$ mutants.

Mosses (bryophytes) consist of approximately 10,000 species (Cuming 2009) but, still, only a very few studies have examined the molecular defense responses in *P. patens* following pathogen infection (Lawton and Saidasan 2009; Oliver et al. 2009; Ponce de León et al. 2007) or treatment with defense elicitors, such as salicylic acid (Andersson et al. 2005) or CHN (Lehtonen et al. 2009).

Discovery of the extracellular ROS production in response to CHN treatment in this study was followed by studying the possible induction of ROS-responsive and defense-signaling-related genes (Torres 2010), namely those encoding alternative oxidase (*PpAOX*), NADPH-oxidase (*PpNOX*), and lipoxygenase (*PpLOX7*). *PpNOX* and *PpAOX* are probable orthologs. These genes were identified in the genome sequence of *P. patens* (Rensing et al. 2008) and specific primers designed for quantitative reverse-transcription real-time polymerase chain reaction (PCR) analysis of gene expression. In the three experiments, the transcript levels of the three oxidative stress-associated genes were enhanced in WT *P. patens* treated with CHN (Fig. 3).

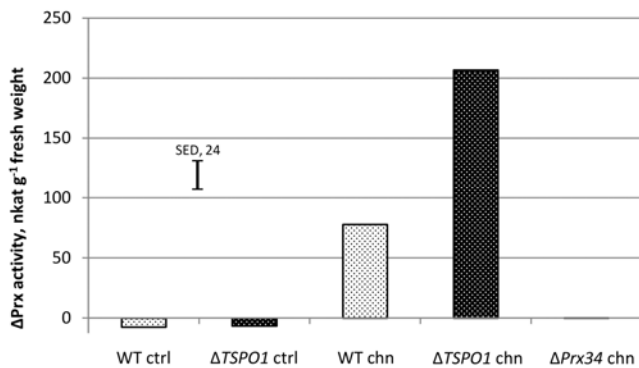


Fig. 2. Chitosan preparation (CHN)-induced peroxidase activity in moss lines. Bars indicate the change in peroxidase activity (activity $T_{15}-T_0$) measured in the liquid culture medium 15 min following addition of CHN (chn) or water (ctrl) to liquid cultures of wild-type (WT) *Physcomitrella patens*, $\Delta PpTSPO1$ mutant, and $\Delta Prx34$ mutant in three independent experiments. Differences between the three types of moss lines are statistically significant ($P < 0.01$). The error bar indicates standard error of difference (SED; $df = 27$).

PpAOX, *PpNOX*, and *PpLOX7* transcript levels were slightly increased by 15 min and significantly increased by 30 and 90 min posttreatment with CHN (Fig. 3B to D). However, transcript levels of *PpTSPO1* were elevated significantly as soon as 15 min posttreatment with CHN but returned to the basal level by 90 min after the CHN treatment (Fig. 3A).

Because the $\Delta PpTSPO1$ mutants showed elevated ROS levels upon CHN treatment, we also analyzed transcript levels of the oxidative stress-related genes in these mutant lines. Data indicated that the transcript levels of *PpAOX* and *PpLOX7* were constitutively overexpressed in the non-CHN-treated $\Delta PpTSPO1$ mutant compared with WT *P. patens* (Fig. 4). In contrast, expression levels of *PpNOX* and *Prx34* in the $\Delta PpTSPO1$ mutants were slightly elevated but not significantly different from WT *P. patens* (Fig. 4).

Resistance to fungi is reduced in $\Delta PpTSPO1$.

The higher constitutive expression of defense-related genes in the salt-sensitive $\Delta PpTSPO1$ mutant was anticipated to also reflect higher levels of resistance to pathogens, which was tested by inoculation of the plants of $\Delta PpTSPO1$ and WT *P. patens* with two fungi representing a saprophytic minor pathogen (*Irpex* sp.) and a severe pathogen (*Fusarium avenaceum*), both isolated from moss (Akita et al. 2011; Lehtonen et al. 2009).

Fungal inoculum was placed next to the moss colony and hyphae were allowed to grow in contact with the moss protonema. Differences in the response between $\Delta PpTSPO1$ and WT *P. patens* could be observed with a microscope as soon as the initial contact between fungal hyphae and the moss protonema (filamentous) tissue occurred (Fig. 5A and C). In $\Delta PpTSPO1$, discoloration of the transverse cell walls was pronounced at the contact points with *F. avenaceum* (Fig. 5D), whereas no discoloration was observed in WT *P. patens* (Fig. 5B). Later, at 12 days postinoculation, discoloration and death

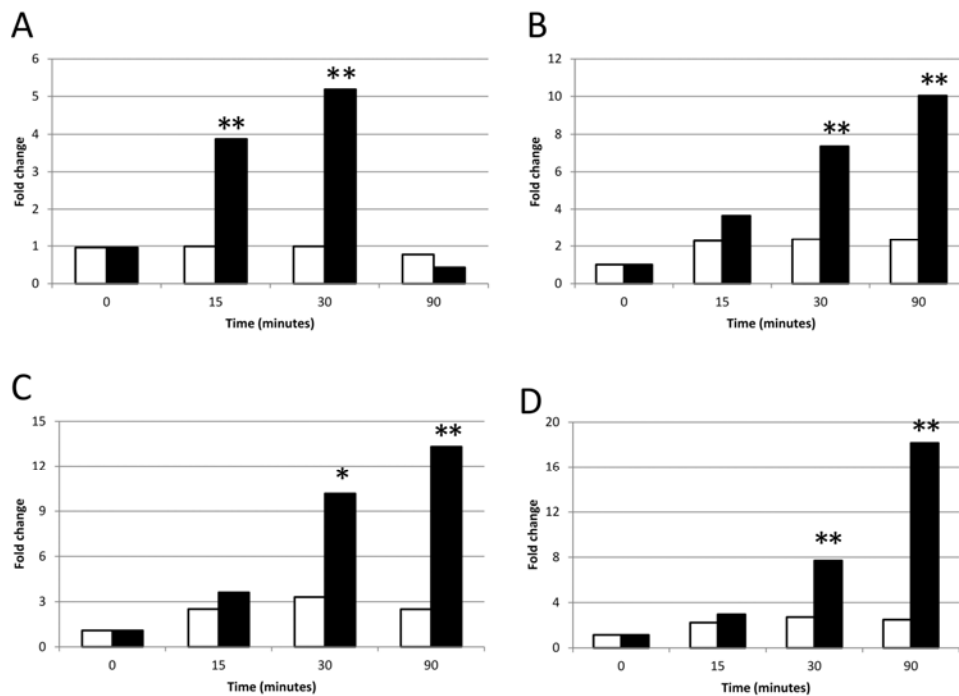


Fig. 3. Induction of *TSPO1* and three reactive oxygen species (ROS)-responsive and defense signaling genes in *Physcomitrella patens* following treatment with a chitosan preparation (CHN). Transcript levels of the genes A, *PpTSPO1*; B, *PpAOX*; C, *PpNOX*; and D, *PpLOX7* were determined by quantitative reverse-transcription real-time PCR (qRT-PCR) in WT *P. patens* treated with CHN (black bars) or water (control, white bars) at various times posttreatment. Bars indicate the mean fold changes in gene expression, compared with the expression level just before treatment (time = 0 min), in three independent experiments. Asterisks indicate that the mean is significantly different from the non-treated control ($P < 0.05^*$; $P < 0.01^{**}$). (ANOVA, $F_{6,14} = 21.1$ in A, 54.0 in B, 41.4 in C, and 109.1 in D; $\alpha = 0.001$). Note that the fold change scale differs between charts.

of protonema and also shoots were observed in the two $\Delta PpTSPO1$ lines tested (Fig. 6E, F, H, and I). Symptoms were more severe than in WT *P. patens* (Fig. 6B and C). These differences between the $\Delta PpTSPO1$ lines and WT *P. patens* were consistent, no matter whether the *Irpex* sp. (Fig. 6B, E, and H) or *F. avenaceum* (Fig. 6C, F, and I) was used. Furthermore, the

growth of mycelia of both fungal species was more abundant on the $\Delta PpTSPO1$ lines than on WT *P. patens* (Fig. 6). These differences were found consistently in the four independent experiments carried out. Taken together, unexpectedly, $\Delta PpTSPO1$ was less resistant to both fungi compared with WT *P. patens*.

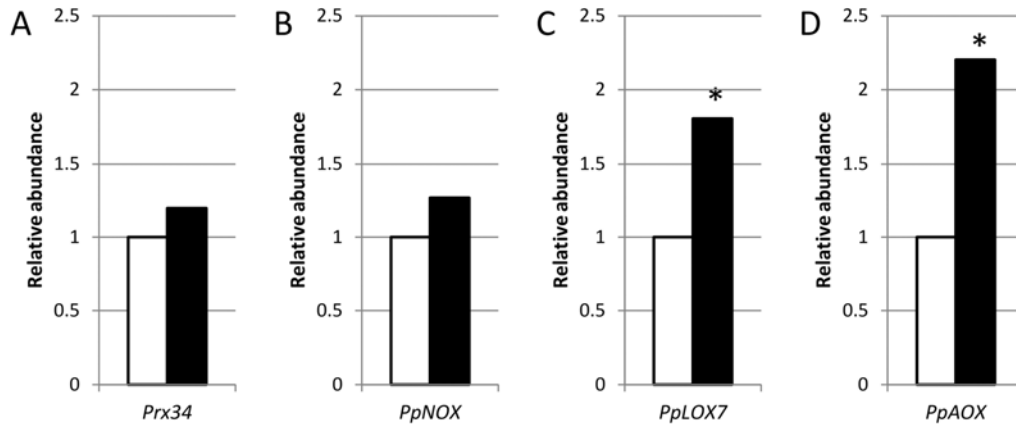


Fig. 4. Expression of stress-associated genes in wild-type (WT) *Physcomitrella patens* (white bar) as compared with expression in the $\Delta PpTSPO1$ mutant lines (black bars) under normal growth conditions. Transcript levels were determined using quantitative reverse-transcription polymerase chain reaction and data normalized by comparison to *EF1 α* and *L21* transcripts. Bars indicate the mean of three independent experiments. Asterisks (*) indicate that the mean of $\Delta PpTSPO1$ is significantly different from WT *P. patens* ($P < 0.01$). Analysis of variance: $F_{1,4}$ equals **A**, 2.11, **B**, 3.29, **C**, 25.1, and **D**, 83.3.

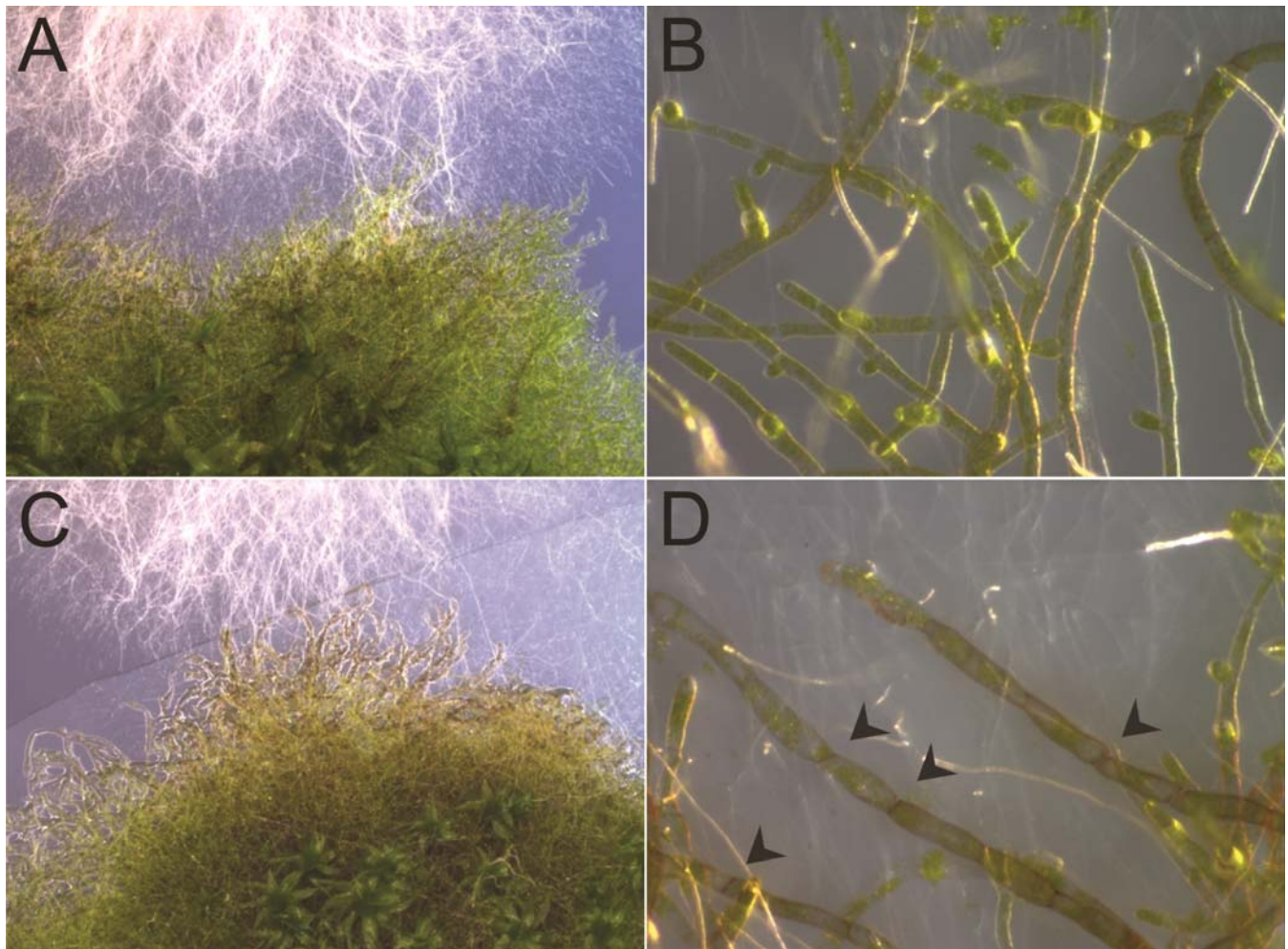


Fig. 5. Phenotypic responses of **A** and **B**, wild-type *Physcomitrella patens* and **C** and **D**, the $\Delta PpTSPO1$ mutant to a pathogenic strain of *Fusarium avenaceum* at the time of initial contact with the hyphae. Inoculum was placed next to the moss colony and the fungus allowed to grow in contact with the moss protonema tissue. Arrowheads in **D** point out transversal cell walls that are discolored in the $\Delta PpTSPO1$ mutant, in contrast to the wild-type moss in **B**.

DISCUSSION

Results of this study showed that treatment with CHN caused an oxidative burst in WT *P. patens*, whereas no such response was observed in the $\Delta Prx34$ knockout mutants. Release of the peroxidase Prx34 (Lehtonen et al. 2009) and the oxidative burst occurred within seconds after treatment of *P. patens* with CHN. These results indicate that production of superoxide, which was analyzed specifically using a quantitative chemiluminescence-based method (Tarpey et al. 2004; Uehara et al. 1993), involves Prx34 upon recognition of chitin. Prx34 is a class III peroxidase, for which two identical genes exist in the genome of *P. patens*. Both copies of the gene have been deleted in the $\Delta Prx34$ knockout mutants, which makes them susceptible to fungal pathogens and saprophytes (Lehtonen et al. 2009). Previous studies have shown that Prx34 is pivotal

to antifungal defense in *P. patens* (Lehtonen et al. 2009), and the present study revealed a plausible mechanism by which Prx34 confers resistance to fungal invaders in *P. patens*. Furthermore, the results revealed that PpTSPO1 is required for maintenance of redox homeostasis during the oxidative burst elicited by CHN because the $\Delta PpTSPO1$ knockout mutant plants released more peroxidase activity to culture medium and created a stronger oxidative burst upon CHN treatment than was observed with WT *P. patens*. Previous studies (Frank et al. 2007; Vanhee et al. 2011) have not addressed the role of TSPO in the context of biotic stress. Thus, our results point out a novel host factor that is important to the maintenance of homeostasis in plants which are attacked by pathogens.

P. patens contains 45 putative genes for class III peroxidases but, remarkably, only *Prx34*, encoding a 35-kDa (330 amino acids) peroxidase protein, is responsive to CHN treat-

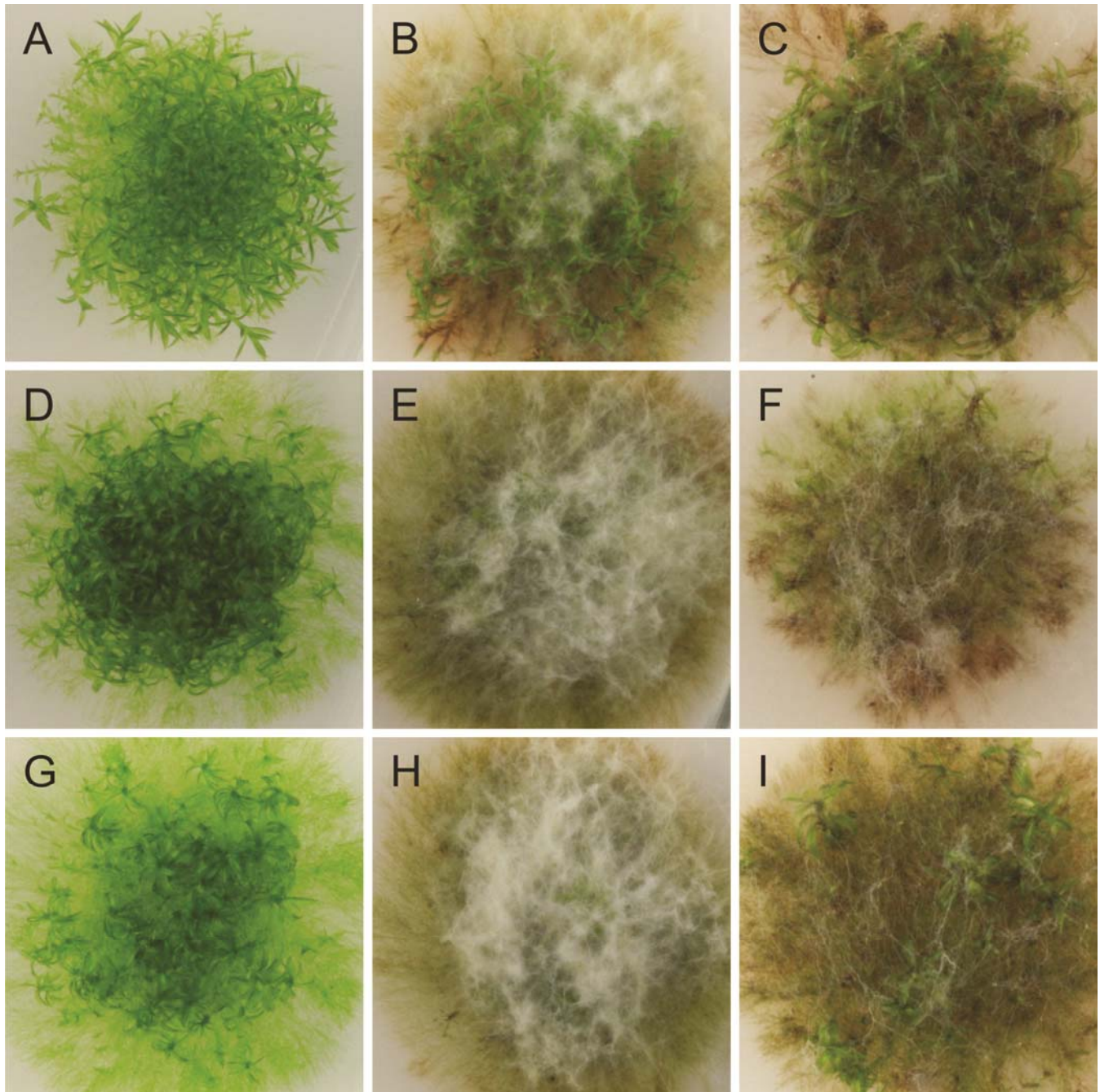


Fig. 6. Phenotypic response of **A** to **C**, wild-type *Physcomitrella patens* and **D** to **F** and **G** to **I**, two $\Delta PpTSPO1$ mutant lines to **B**, **E**, and **H**, a saprotrophic fungus (*Irpex* sp.) and **C**, **F**, and **I**, a pathogenic fungal isolate (*Fusarium avenaceum*) at 12 days postinoculation. **A**, **D**, and **G**, Untreated controls.

ment (Lehtonen et al. 2009). This gene belongs to a *P. patens*-specific *Prx* subfamily, as indicated by phylogenetic comparison of the putative class III peroxidase genes predictable in the genome sequences of *P. patens*, *A. thaliana*, and *Oryza sativa* (rice) (Lehtonen et al. 2009). In vascular plants, class III peroxidases are part of the strategically positioned reservoirs of antimicrobial compounds which prevent pathogens from colonizing the tissue. They are stored in vacuoles or cell walls to be ready for excretion or release upon pathogen recognition (Almagro et al. 2009). Our results suggest that Prx34 is stored in cell walls because no oxidative burst was detected in protoplasts, in contrast to the gametophyte tissue used as a source of them. In another type of nonvascular plant, the liverwort *Dumortiera hirsuta* (Marchantiophyta), a cell wall peroxidase is attributed to the production of apoplastic superoxide under drought stress (Beckett et al. 2004; Li et al. 2010). Furthermore, as we observed that treatment with CHN also elicits ROS production in *S. capillifolium*, a moss unrelated to *P. patens*, the data collectively suggest that a quick apoplastic oxidative burst is a common stress response in nonvascular plants.

The oxidative stress-related genes, such as *AOX1*, respond to increased endogenous or exogenous concentrations of ROS, which can be used as an indicator for a variety of stresses (Maxwell et al. 1999; Van Aken et al. 2009). We tested by quantitative (q)PCR whether *PpAOX1* is induced in WT *P. patens* upon treatment with CHN and results were positive, which seems to be a novel observation in nonvascular plants. *LOX* gene activation is observed under abiotic and biotic stresses such as salt stress (Molina et al. 2002; Rodriguez-Rosales et al. 1999) and fungal infection (Hano et al. 2008). Previous studies have reported induction of *PpLOX1*, a gene for an arachidonic acid lipoyxygenase, in *P. patens* infected with fungi (Oliver et al. 2009; Ponce de Leon et al. 2007) and exhibiting intracellular accumulation of ROS revealed by nonquantitative histological staining (Olivier et al. 2009). In this study, CHN treatment was observed to induce *PpLOX7* that encodes a type-2 lipoyxygenase located in plastids and preferring α -linolenic acid as a substrate to produce 13(S)-hydroperoxides (Anterola et al. 2009). Furthermore, we observed induction of the gene coding for PpNOX that is homologous to the mammalian gp91^{phox} respiratory burst oxidase (Torres 2010). NOX localizes in the plasma membrane and is needed for ROS accumulation and signaling in biotic and abiotic stresses (Miller et al. 2009; Torres et al. 2002). These data widened the knowledge of molecular responses to pathogen elicitors in nonvascular plants.

The $\Delta PpTSPO1$ knockout mutants were slightly but detectably and consistently more susceptible to fungal infection than WT *P. patens* in four independent experiments. In fact, the opposite was expected because the $\Delta PpTSPO1$ plants produced significantly higher amounts of peroxidase activity and extracellular superoxide than WT *P. patens*. Explanations were sought from the physiological alterations described for the $\Delta PpTSPO1$ mutants (Frank et al. 2007). These mutants are sensitive to abiotic stress and, under high salt concentration, suffer from enhanced lipid peroxidation and cell death. Our results indicated that the expression levels of the oxidative stress-related gene homologs, such as *PpAOX1* and *PpLOX7*, were constitutively higher in $\Delta PpTSPO1$ mutants than WT *P. patens* under standard growth conditions. Furthermore, the TSPO knockout mutants of *Arabidopsis* are also unusually sensitive to salt stress, and the higher induction of salt-responsive genes than in WT *Arabidopsis* indicates disturbed homeostasis (Balsemão-Pires et al. 2011). Hence, the evidence indicates that the TSPO-deficient plants are under permanent oxidative stress and suffer from disturbed redox homeostasis.

Recent data of Vanhee and associates (2011) shed light on the mechanism by which the TSPO of *Arabidopsis* regulates

redox homeostasis. Regulation is based on scavenging unbound heme and porphyrins that produce radicals under exposure to light and, consequently, damage the cellular organelles and structures (Vanhee et al. 2011). Consistent with the findings of Vanhee and associates (2011), our study shows that expression of *PpTSPO1* is induced in WT *P. patens* upon CHN treatment (i.e., as a response to ROS accumulation), and the levels of oxidative stress-related genes are constitutively higher in the *PpTSPO1* knockout mutants (i.e., in absence of the TSPO-mediated heme and porphyrin scavenging mechanism). The scavenging mechanism in *Arabidopsis* requires that TSPO binds heme and, also, the autophagy-related protein Atg8 via a specific Atg8 family interacting motif. The complex is subsequently degraded by autophagy (Vanhee et al. 2011). A similar role for PpTSPO1 in *P. patens* is conceivable because PpTSPO1 is implicated in translocation of protoporphyrin precursors to mitochondria for heme formation (Frank et al. 2007) and, according to our observation, the PpTSPO1 amino acid sequence (National Center for Biotechnology Information database accession number ABG37902) contains five putative Atg8 binding sites (W/Y-x-x-L/V/I) (Noda et al. 2010). Further studies are needed to show which site is active in Atg8 binding but, collectively, the findings suggest a plausible mechanism by which PpTSPO1 may suppress oxidative stress and regulate homeostasis in *P. patens*.

Higher susceptibility of the $\Delta PpTSPO1$ knockout mutants to the saprotrophic and necrotrophic fungi tested in this study was observed as enhanced discoloration and necrotization of the protonema and gametophore tissue following contact with the fungal hyphae. The response may be associated with autophagy, whose “pro-death” functions provide an essential mechanism for removal of damaged membranes, organelles, oxidized proteins, and other cellular “garbage” and are vital for maintenance of homeostasis (Hofius et al. 2011). Autophagy can also serve “anti-death” functions, which are important in innate immunity because they limit the spread of cell death during infection with necrotrophic fungal pathogens (Lenz et al. 2011). However, when the innate immunity and autophagy are insufficient to control infection and remove garbage, respectively, accumulation of host-cell-derived damage-associated molecular patterns (DAMPs) may trigger accumulation of salicylic acid and pathogenesis-related proteins (Krol et al. 2010). The DAMPs per se can be harmful, and the responses induced by them may enhance stress and cause damage to membranes (Hofius et al. 2011). These consequences from DAMP accumulation should be challenging to control in the $\Delta PpTSPO1$ mutants that are intrinsically stressed and limited in their abilities to control ROS and homeostasis. Indeed, treatment of the $\Delta PpTSPO1$ plants with the fungal elicitor CHN enhanced ROS production. The salicylic-acid-mediated signaling for resistance to necrotrophic pathogens (Andersson et al. 2005) and induction of pathogenesis-related proteins by fungal elicitors (Lehtonen et al. 2009; Oliver et al. 2009; Ponce de León 2007; this study) have been reported in *P. patens*. It seems that, in the $\Delta PpTSPO1$ mutants, additional ROS produced upon pathogen recognition may cause damage to the membranes and organelles and result in severe damage and cell death, which is advantageous to the necrotrophic fungi (Govrin and Levine 2000).

Taken together, our results point out TSPO as a novel host factor that is needed to maintain homeostasis in plants attacked by pathogens. TSPO seems to ensure that cells will retain their ability to operate the broad spectrum of defense responses against the invader despite the potentially very harmful burst of radical oxygen species as the first defensive response. Furthermore, enhanced susceptibility of plants to pathogen infections under drought and salt stress has been documented (Audenaert et al. 2002; Brosché et al. 2010; DiLeo et al. 2010). Future

studies on TSPO, which appears as a new link in abiotic and biotic stress signaling, may help to resolve this phenomenon.

MATERIALS AND METHODS

Plant material.

Protonemal tissue of *P. patens*, ecotype Gransden (Ashton and Cove 1977), was grown in petri dishes (9 cm in diameter) on a cellophane membrane placed on BCD medium (1 mM MgSO₄, 1.85 mM KH₂PO₄ [pH 6.5, adjusted with KOH], 10 mM KNO₃, 45 μM FeSO₄, 0.22 μM CuSO₄, 0.19 μM ZnSO₄, 10 μM H₃BO₄, 0.10 μM Na₂MoO₄, 2 μM MnCl₂, 0.23 μM CoCl₂, and 0.17 μM KI) (Ashton and Cove 1977) supplemented with 1 mM CaCl₂, 45 μM ethylenediaminetetraacetic acid disodium salt (Na₂-EDTA), and 5 mM ammonium tartrate ([NH₄]₂C₄H₄O₆), and solidified with 0.8% agar. The cultures were grown in a growth chamber (Model 3755; Forma Scientific, Marietta, OH, U.S.A.) at 23°C (photoperiod, 12 h; light intensity, 60 μmol m⁻² s⁻¹) and subcultured weekly.

Generation of the $\Delta PpTSPO1$ and $\Delta Prx34$ gene knockout mutants by a gene-replacement technique based on homologous recombination has been described previously (Frank et al. 2007; Lehtonen et al. 2009). Three independent $\Delta Prx34$ mutants and both $\Delta PpTSPO1$ mutants, all shown to have only a single integration of the knockout construct at the homologous locus and no additional insertions (Frank et al. 2007; Lehtonen et al. 2009), were included in this study.

CHN treatment.

According to the manufacturer, the product called chitosan used in this study contains chitin deacetylated to 90% (M_w < 5000) (Kraeber GmbH & Co, Ellerbek, Germany). It was dissolved in Milli-Q water at 10 mg ml⁻¹, prefiltered twice through a 1.6-μm glass fiber filter (VWR International, West Chester, PA, U.S.A.), filter-sterilized using a 0.2-μm cellulose acetate filter (Whatman GmbH, Dassel, Germany), and stored at -20°C until use. Stock solution of CHN (10 mg ml⁻¹) was added to 1-week-old moss liquid cultures (2 ml) to the final concentration needed in the experiment and the moss grown as above. Sterile Milli-Q water was used as a control treatment.

Superoxide production assay.

Superoxide production in liquid moss culture medium was measured by chemiluminescence of a luciferin analog 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one (MCLA) (Sigma-Aldrich, St. Louis). Pieces of 2-week-old protonemal mats (approximately 5 mm in diameter) covering the LIA-plate (Lumitrac 200; Greiner Bio-One, Frickenhausen, Germany) were placed in the wells containing 5 μM MCLA (final concentration) in liquid culture medium (see above). The

plate was incubated in the dark at 22°C for 30 min before starting the measurements. Chemiluminescence was measured before addition of CHN and at intervals of 25 to 1,200 s after addition of CHN. The measurement lasted for 1 s at each time point and was carried out with a VICTOR³ multilabel reader (Perkin-Elmer, Waltham, MA, U.S.A.). Differences in superoxide production in WT and knockout moss lines were calculated based on the area under the curve of measurement values in function of time (Fig. 1) and were tested by the analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$) (SPSS, v. 15.0; SPSS Inc., Chicago).

Peroxidase activity assay.

The plants for peroxidase activity assay were grown for 1 week on cellophane on the culture medium, transferred onto medium without ammonium tartrate for 1 week, and finally grown in liquid medium containing ammonium tartrate and 1% glucose for 1 week. Peroxidase activity was tested in aliquots (50 μl) of the liquid culture medium spun at 16,000 × g for 1 min. The supernatant was transferred to a clean tube and kept on ice until the measurement. The reaction mixture (1 ml) contained 0.5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Fluka, Buchs, Switzerland) and 200 μM H₂O₂ in phosphate-citrate buffer (29 mM phosphate and 23 mM citrate, pH 4.0) preheated to 35°C in a heated cuvette chamber before adding the sample (40 μl). The absorbance at 405 nm (A₄₀₅) was recorded using a spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan) 15 to 120 s after starting the reaction. Peroxidase activity was calculated based on oxidation of ABTS as moles per liter per second per gram of fresh moss tissue. The A₄₀₅ extinction coefficient of ABTS is 36,800 M⁻¹ cm⁻¹. Three replicates (cultures) of the moss treated or untreated with CHN were included in each of the three independent experiments.

Fungal isolates and inoculation.

A saprophytic fungal isolate of an *Irpex* sp. (Basidiomycota; Polyporales; family Steccherinaceae) and a pathogenic isolate of *F. avenaceum* (Hyphomycetes) were both isolated from the Sunagoke moss (*Racomitrium japonicum* Dozy and Molk.; family Grimmiaceae) growing in the field in Japan and have been described previously (Akita et al. 2011; Lehtonen et al. 2009). Pure cultures of these fungi were grown on potato dextrose agar (Biokar Diagnostics, Pantin Cedex, France) and used for inoculation.

Colonies of the WT and $\Delta PpTSPO1$ mutants of *P. patens* were grown for inoculation on cellophane on agar media as described for plant materials above. When 1 month old, the colonies were inoculated with fungi using a small plug of culture medium excised from the edge of an actively growing fungal colony. Inoculum was placed next to the moss colony, allowing

Table 1. Primers designed for quantitative polymerase chain reaction analysis of gene expression utilizing the genome sequence of *Physcomitrella patens* available at the COSMOSS website

Gene	Joint Genome Institute gene model	Sequence 5' to 3'
<i>EF1α</i>	Phypa_158916	fwd AGGCAGACGTGGGTTACTTGA rev CATTAAATCATCAGGCAGGCATA
<i>L21</i>	Phypa_106900	fwd TGGTGTCTGATGAACAAGC rev CTCAATTCTGAACGTGCAGAC
<i>PpTSPO1</i>	Phypa_148670	fwd CGTGGTTAGTGTGGGCTGA rev AGTAAGCACCGAGAGCGAAA
<i>PpAox</i>	Phypa_89421	fwd TCGTGACGTTAACCATTTCG rev GATTCCCGCAGCTCCTTAC
<i>PpNox</i>	Phypa_204103	fwd CACGATGTTGCAGTCGTTG rev TACGTGCCCTAGTGCCTGA
<i>PpLOX7</i>	Phypa_184078	fwd GTGGCGGTTTGATCAGGA rev CGTTCAGCCATCCCTCTTC
<i>Prx34</i>	Phypa_144797/144819	fwd CAATACGCTACTCGCGACTCTGT rev CGTCTCTTCGACCGCCATA

the hyphae to grow in contact with moss tissue. Inoculated moss colonies were cultured under the conditions described above.

RNA extraction, cDNA synthesis, and qPCR.

Two cultures each of the CHN-treated and untreated $\Delta PpTSP01$ mutant and WT *P. patens* were grown in each experiment and used for extraction of total RNA. Moss tissue was ground to powder under liquid nitrogen. Total RNA was extracted using a Trizol-like reagent (Caldo et al. 2004). Total RNA (1 μ g) was treated with RQ1 RNase-free DNase (Promega Corp., Fitchburg, WI, U.S.A.) according to the manufacturer's instructions.

Expression levels of the genes of interest (Table 1) were estimated using qPCR. For cDNA synthesis, 1 μ g of the DNase-treated RNA was incubated with 1 μ l (200 ng) of random hexamers (Sigma Genosys, Woodlands, TX, U.S.A.) at 70°C for 10 min and added to the cDNA reaction mixture consisting of 4 μ l of 5 \times *Moloney murine leukaemia virus* (M-MLV) reverse-transcription (RT) buffer, 2 μ l of 0.1 M dithiothreitol, 1 μ l of 10 mM dNTPs, 0.5 μ l (20 U) of RNasin (Promega Corp.), and 2 μ l (400 U) of M-MLV RT (Promega Corp.). The reaction mixture was incubated at 37°C for 1 h and, subsequently, at 70°C for 10 min, after which 35 μ l of nuclease-free water was added.

Primers (Table 1) for the target genes were designed utilizing the genome sequence of *P. patens* (Rensing et al. 2008) available online at the COSMOSS website.

cDNA of the two replicate samples from each moss line and treatment were pooled for qPCR analysis. Three technical replicates of each sample (2.5 μ l of cDNA in a total volume of 10 μ l of reaction mix) were included. qPCR was carried out using LightCycler 480 SYBR Green I Master mix (Roche, Basel, Switzerland) and the LightCycler 480 SYBR Green system following the manufacturer's instructions. *EF1 α* and *L21* (Table 1) were used as reference genes to normalize possible variation in the amounts of the cDNA template between samples. Target specificity of the primers was verified by sequencing the amplification products. Gene expression levels were calculated employing relative quantification with efficiency correction (Pfaffl 2001).

Statistical analysis.

ANOVA followed by Tukey's test for significant differences was used to analyze the data on ROS and peroxidase production and the fold values from qPCR analyses. The fold values were log₂-transformed and tested by the between the CHN treated and control samples ($P < 0.05$) (SPSS, v. 15.0, SPSS Inc.). The differences in gene transcript levels between WT *P. patens* and $\Delta PpTSP01$ mutant were tested by a paired-sample *t* test using the same program ($P < 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The COSMOSS moss computational biology toolbox: www.cosmoss.org