A Preliminary Investigation into the Mechanism of Lipoxygenase in Responding to Pathogenic Stress in *Physcomitrella patens* Induced by *Botrytis cinerea*

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Abstract [**Objectives**] To investigate the mechanism of lipoxygenase in responding to pathogenic stress in *Physcomitrella patens* induced by *Botrytis cinerea*. [**Methods**] It used wild-type and NDGA-treated *Physcomitrella patens* as experimental materials to measure the activities of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and cinnamyl alcohol dehydrogenase (CAD), as well as the contents of SA, ABA, and ET after inoculation with *Botrytis cinerea* (0, 6, 12, 24, 48, 72 h). [**Results**] In wild-type plants inoculated with *B. cinerea*, the activities of CHS, PAL, and CAD increased during the early defense stage compared with the control (uninoculated wild-type). In NDGA-treated *P. patens* inoculated with *B. cinerea*, the levels of CHS, PAL, and CAD were lower than those in wild-type plants at the corresponding time points after inoculation, indicating a correlation between LOX activity and the changes in CHS, PAL, and CAD activities during early defense. The contents of SA, ABA, and ET in wild-type plants inoculated with *B. cinerea* were higher than those in the control (uninoculated wild-type). In NDGA-treated *P. patens* inoculated with *B. cinerea*, the levels of SA, ABA, and ET were lower than those in wild-type plants at the corresponding time points after inoculation, suggesting that the synthesis of SA, ABA, and ET is correlated with LOX activity during early defense. [**Conclusions**] This study will lay a foundation for further molecular-level research on the disease resistance mechanisms of LOX in bryophytes.

Key words *Physcomitrella patens*, *Botrytis cinerea*, Lipoxygenase, Resistance mechanism

0 Introduction

Oxylipins are a large class of structurally diverse oxidized fatty acids that participate in various plant processes, including development[1], senescence[2-3], and defense responses against pathogen infection, insect herbivory, and mechanical wounding[4-5]. Lipoxygenase (LOX, EC 1.13.11.12), also known as fat-oxidizing enzyme, fat oxygenase, or lipoxygenase, serves as a key enzyme in the biosynthesis pathways of numerous oxylipins.

Studies have shown that the LOX activity in postharvest tomato fruits infected with *Botrytis cinerea* significantly increased 7 d after inoculation, indicating a clear positive correlation between pathogen infection and fruit LOX enzyme activity[6]. Enzyme activity assays and high-performance liquid chromatography analyses revealed that LOX activity in cucumber leaves increased after pathogen induction, accompanied by a substantial accumulation of jasmonic acid (JA), with LOX and JA showing a significant positive correlation[7]. Inoculation with *Fusarium oxysporum* f. sp. *melonis* resulted in the relative expression levels of LOX gene family members in the oriental melon reaching their peak at 3 - 5 d, alongside a significant decrease in root activity, an increase in electrolyte leakage, and elevated LOX activity in both leaves and roots. These findings suggest that CmLOXs may be involved in defense responses[8].

In this study, the previously screened effective LOX inhibitor NDGA was applied to *Physcomitrella patens* to inhibit its LOX activity. The aim was to investigate changes in pathological indicators of *P. patens* during *B. cinerea* infection at the physiological and biochemical levels, thereby elucidating the potential defense response mechanisms involving LOX and laying a foundation for further research on the disease resistance mechanisms of mosses.

1 Materials and methods

1.1 Experimental materials The *Physcomitrella patens* was kindly provided by Professor He Yikun from Capital Normal University. The *Botrytis cinerea* strain c023 was obtained from Professor Xu Ling of East China Normal University.

1.2 Phenolic substance staining Two days after *B. cinerea* infection, *P. patens* were subjected to safranin staining, and the infection status was observed under an optical microscope.

1.3 Enzyme activity assay of chalcone synthase (CHS) in *P. patens* Plant material treatment: collect plants (*P. patens*) inoculated with *B. cinerea* for 0 - 3 d, and rinse them three times each with sterile water and Tween 80, then blot dry. Weigh 0.2 g of plant material, wash with 5 mL of PBS, blot dry, and transfer to a 5 mL homogenization tube. Add homogenization medium at a 1 ∶ 9 ratio (*w*/*v*) to prepare a 10% tissue homogenate in an ice bath. Transfer the homogenate to a centrifuge tube and centrifuge at 3 000 r/min for 10 min. Collect the supernatant for subsequent analysis. Enzyme activity is determined according to the instructions of the ELISA kit.

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1.4 Enzyme activity assay of phenylalanine ammonia-lyase (PAL) in *P. patens* The plant materials were processed using the same method as described above. The PAL enzyme activity was measured according to the instructions provided in the phenylalanine ammonia-lyase assay kit.

1.5 Enzyme activity assay of cinnamyl alcohol dehydrogenase (CAD) in *P. patens* The plant materials were processed using the same method as described above. The CAD enzyme activity was measured according to the instructions provided in the cinnamyl alcohol dehydrogenase assay kit.

1.6 Measurement of Ethylene (ET) Content in *P. patens* The plant materials were processed using the same method as described above, and their contents were determined using an ELISA kit (ET) method.

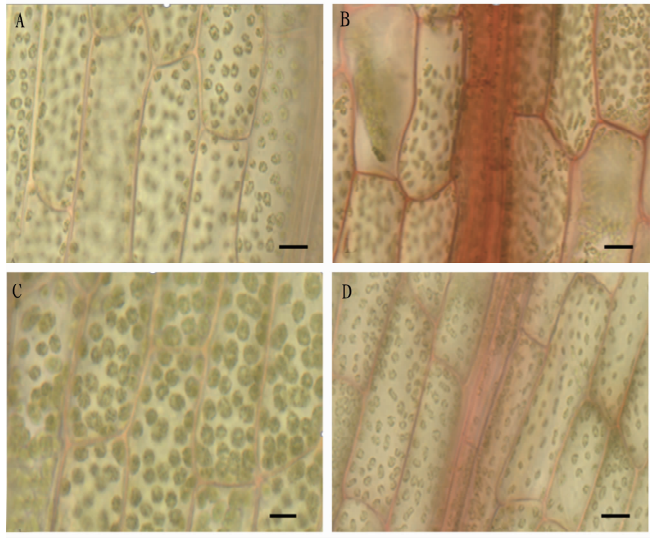
1.7 Determination of Salicylic Acid (SA) Content in *P. patens* Sample collection and cleaning: The plants (*P. patens*) inoculated with *B. cinerea* for 0 – 3 d were collected and washed with sterile water to remove surface-attached hyphae and vermiculite. After absorbing excess moisture, the samples were air-dried for subsequent analysis. Approximately 0.1 g of sample was thoroughly ground into a fine powder, and 1 mL of extraction solution (90% methanol) was added. The mixture was incubated overnight at 4 ℃, followed by centrifugation at 8 000 g for 10 min. The supernatant was transferred to a new EP tube. The residue was re-extracted with 0.5 mL of extraction solution for 2 h, centrifuged again, and the two supernatants were combined for analysis. The salicylic acid (SA) content was determined according to the method described by Bowling *et al*. [9] The liquid chromatography conditions were as follows: mobile phase A: methanol; mobile phase B: 0.1% acetic acid in water; ratio A ∶ B = 35 ∶ 65; injection volume: 10 μL; flow rate: 0.8 mL/min; column temperature: 35 ℃; run time: 40 min. Peak heights and peak areas were recorded.

1.8 Determination of abscisic acid (ABA) content in *P. patens* Sample collection and cleaning were performed as described above. The cleaned samples were ground into a paste at low temperature and transferred to centrifuge tubes, followed by the addition of 1 mL of pre-cooled 70% – 80% methanol solution (pH = 3.5). The mixture was extracted overnight at 4 ℃, then transferred to a centrifuge tube and centrifuged at 12 000 g for 10 min at 4℃ to collect the filtrate. The extraction was repeated three times, and the filtrates were combined. The combined filtrate was concentrated under reduced pressure at 40 ℃ to one-third of its original volume, after which an equal volume of petroleum ether was added. The mixture was allowed to stand for phase separation, and the de-coloration step was repeated 2 – 5 times. The pH was adjusted to 8.0 with triethylamine, and polyvinylpolypyrrolidone (PVPP) was added. The mixture was shaken and incubated for 20 min. After centrifugation under the same conditions, the supernatant was transferred to a new EP tube. The pH was adjusted to 3.0 with hydrochloric acid, and extraction was carried out three times with ethyl acetate. The ester phases were combined and evaporated to dryness under reduced pressure at 40 ℃. The residue was dissolved in the mobile phase by vortex mixing, filtered, and then subjected to content analysis. The instrument conditions were as follows: mobile phase A: 100% methanol; mobile phase B: 0.1% acetic acid aqueous solution; A ∶ B = 55 ∶ 45; injection volume: 20 μL; flow rate: 0.8 mL/min; column temperature: 30 ℃. Quantification was performed using the external standard peak area method.

1.9 Effects of ABA and salicylic acid (SA) treatments on plant recovery *B. cinerea* inoculation was performed on plants treated with WT and NDGA after spraying with ABA and SA. Phenotypic observations were conducted to examine whether the two hormones could restore the plant phenotype.

2 Results and analysis

2.1 Staining of phenolic compounds in *P. patens* In *P. patens* plants not treated with NDGA, safranin staining was notably deeper following *B. cinerea* inoculation (Fig. 1B). In contrast, NDGA-treated plants showed lighter safranin staining in the intercellular spaces and vein regions compared with the control plants (wild-type WT/untreated with NDGA) after inoculation with *B. cinerea* (Fig. 1D).



NOTE A: Wild type stained with safranin; B: Safranin staining of wild type after inoculation with *B. cinerea*; C: plants treated by NDGA stained with safranin; D: Safranin staining of plants treated by NDGA after inoculation with *B. cinerea*. (A, B, C and D Bar = 0.05 mm.)

Fig. 1 Safranin staining of wild type and plants treated by NDGA after inoculation with *Botrytis cinerea*

2.2 Enzyme activity analysis of chalcone synthase (CHS) in *P. patens* Experimental results indicate that the CHS enzyme activity in wildtype plants inoculated with *B. cinerea* showed an increase compared to the control (noninoculated wildtype plants). The smallest increase was observed at 12 h after inoculation, while the largest increase occurred at 6 h, reaching approximately 21%. When *P. patens* was pretreated with NDGA and then inoculated with *B. cinerea*, the CHS enzyme activity gradually decreased over

0 – 3 d, reaching its lowest level at 72 h after inoculation. Compared with the control group (noninoculated wildtype/without NDGA treatment), the activity decreased by about 30% (Fig. 2).

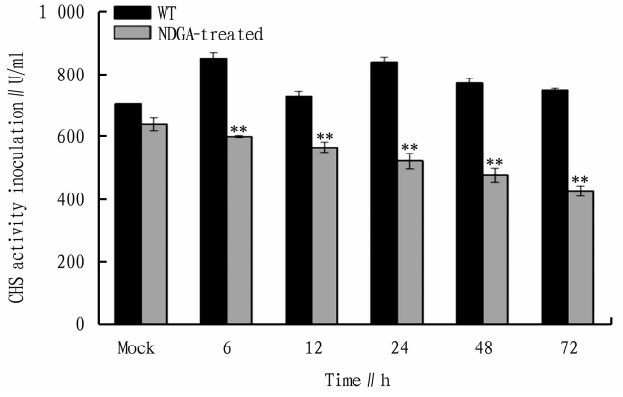


Fig. 2 Changes in CHS activity of *Physcomitrella patens* inoculated with *Botrytis cinerea*

2.3 Enzyme activity analysis of phenylalanine ammonia-lyase (PAL) in *P. patens* Under pathogen stress, the phenylpropanoid metabolic pathway in plants is activated[10]. PAL acts as the rate-limiting enzyme in this pathway, catalyzing the deamination of phenylalanine to produce cinnamic acid. Through a series of enzymatic reactions, cinnamic acid is further metabolized to synthesize various polyphenolic compounds, such as lignans, flavonoids, and stilbenes[11-13], which enhance plant defense against *B. cinerea*.

As shown in Fig. 3, after inoculation with *B. cinerea*, the PAL enzyme activity in wildtype plants gradually increased within 0 – 12 h, reaching its peak at 12 h, which was 1.6 fold higher than that of the control group (noninoculated wildtype). From 12 to 72 h after infection, the PAL activity gradually declined, dropping to 15 (OD_{290}/min/gFW) at 72 h, which was only 24% of the control group's enzyme activity. After NDGA treatment, the PAL activity showed a significant decreasing trend compared with the control group (noninoculated wildtype/without NDGA treatment) during 0 – 24 h, reaching its lowest point at 12 h with a reduction of 37.5% relative to the control. Between 48 and 72 h, no significant difference in PAL activity was observed between the NDGA-treated group and the control group.

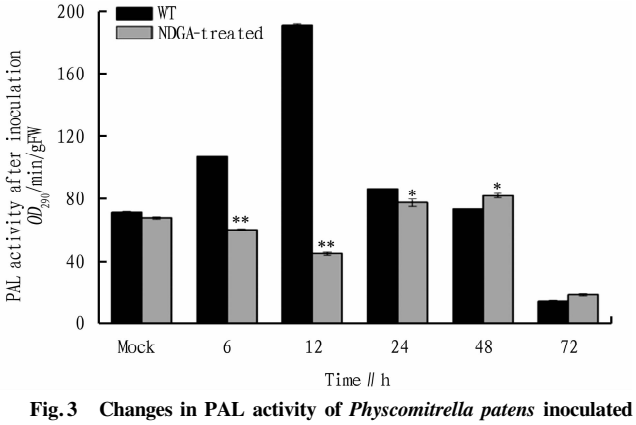


Fig. 3 Changes in PAL activity of *Physcomitrella patens* inoculated with *Botrytis cinerea*

2.4 Enzymatic activity analysis of cinnamyl alcohol dehydrogenase (CAD) in *P. patens* This study determined the changes in CAD enzyme activity in plant materials following *B. cinerea* infection (Fig. 4). In wild-type plants, CAD enzyme activity gradually increased from 0 to 48 h after inoculation with *B. cinerea*, reaching a maximum value of 3.4 (nmol/min/gFW) at 48 h, and then showed a declining trend from 48 to 72 h. The CAD enzyme activity in NDGA-treated plants infected with *B. cinerea* exhibited a consistent trend with the wild-type control, increasing gradually from 0 to 48 h before declining, with a peak value of 3.2 (nmol/min/gFW) at 48 h. Moreover, the CAD enzyme activity in NDGA-treated plants was consistently lower than that in the corresponding wild-type plants, with the largest difference observed at 24 h between NDGA-treated plants and the control.

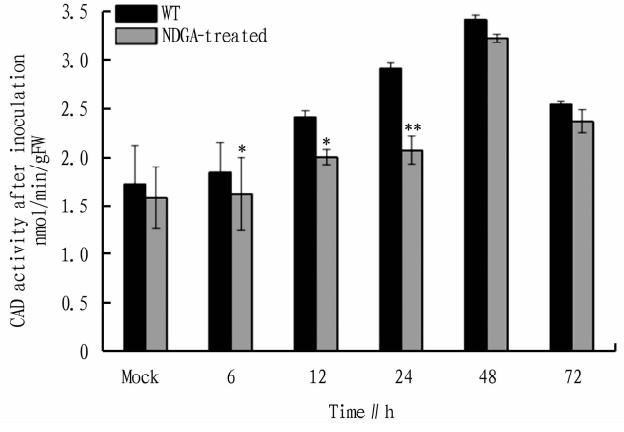
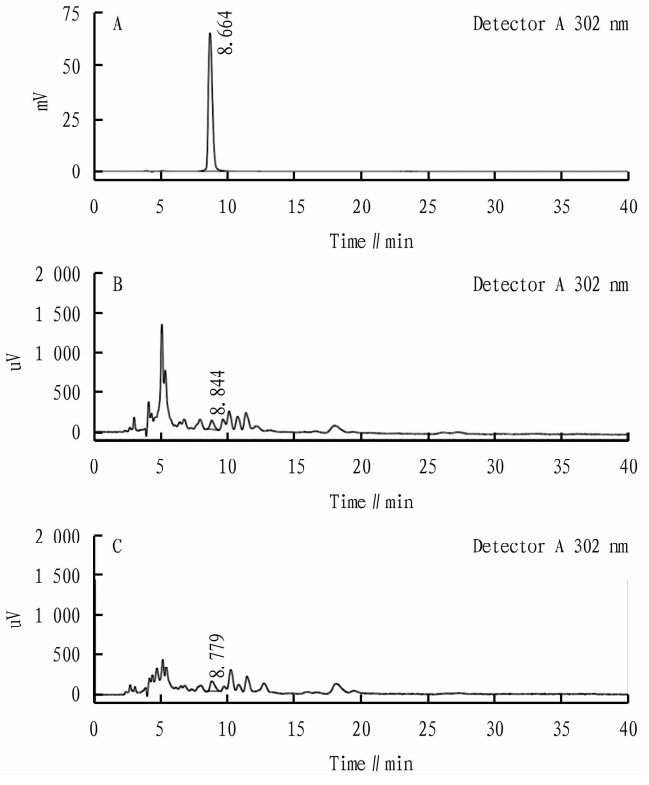


Fig. 4 Changes in CAD activity of *Physcomitrella patens* inoculated with *Botrytis cinerea*

2.5 Analysis of salicylic acid (SA) content in *P. patens* In this study, SA standard solutions were prepared at different concentrations, and the peak areas were measured to establish a standard curve: $y = 25\ 709x - 722.5$, with $R^2 = 0.999\ 7$. The changes in SA content in two types of *P. patens* materials (WT and NDGA-treated) from 0 to 72 h were determined using high-performance liquid chromatography (Fig. 5 and Fig. 6). In wild-type plants infected with *B. cinerea*, the endogenous SA content increased from 0 to 72 h, reaching a maximum of 0.805 μg/g FW at 6 h, then gradually decreased from 12 to 72 h. At 72 h, the SA level was at its lowest value but remained higher than that of the control. In NDGA-treated plants, the SA content showed a significant decrease compared to the control group from 0 to 72 h, with a minimum value of 0.17 μg/g FW observed at 24 h.

2.6 Determination of ABA levels in *P. patens* In this study, a concentration gradient of ABA standard was diluted, and the absorption peak was detected at a wavelength of 254 nm (as shown in Fig. 7). The standard curve equation obtained was $y = 50\ 665x - 238.6$, with $R^2 = 1$. The ABA content in two materials of *P. patens* (WT and NDGA treated) was determined by HPLC. The results are shown in Fig. 8: both WT and NDGA-treated plants showed absorption peaks at 254 nm after inoculation with *B. cinerea* from 0 to 72 h. Compared with the control, the ABA content

in WT plants increased from 6 to 72 h, with minor changes at 6 and 24 h. The ABA content reached its highest level at 48 h, reaching 0.47 μg/g · FW, which was approximately 6-fold higher than that of the control. In NDGA-treated plants, the ABA content decreased significantly compared with the control from 12 to 72 h, with a value of 0.07 μg/g · FW at 48 h, taking up only 17% of the ABA content in the control group at 48 h.



NOTE A: Chromatogram of SA standard; B: HPLC chromatogram of wild-type *P. patens* material at 0 h after inoculation; C: HPLC chromatogram of NDGA-treated *P. patens* material at 0 h post-inoculation.

Fig. 5 HPLC chromatogram of SA in different types of *Physcomitrella patens* after its inoculation with *Botrytis cinerea*

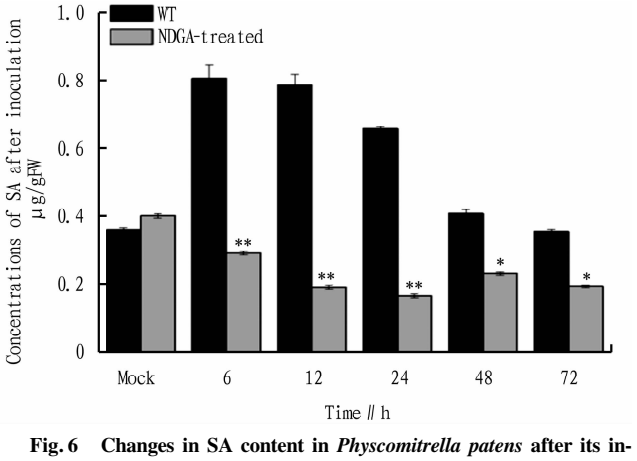
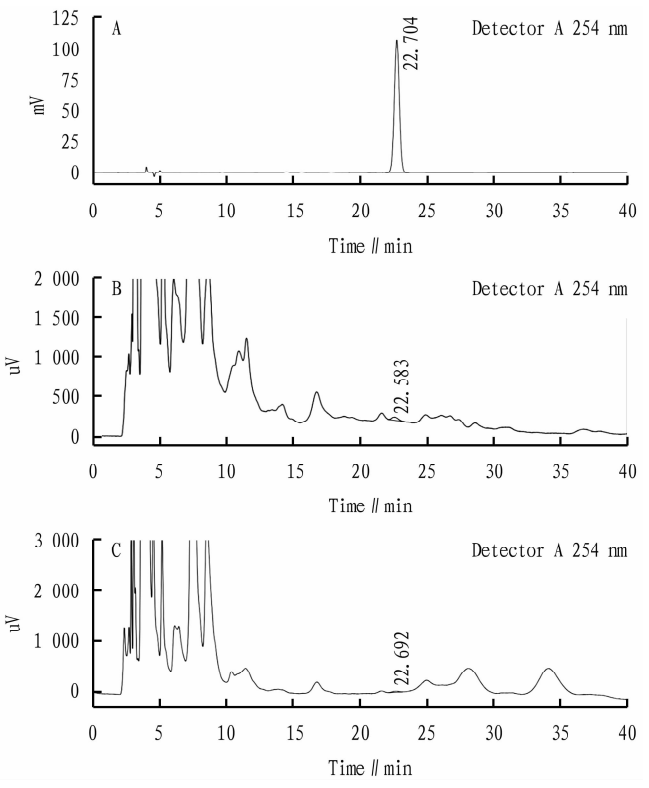


Fig. 6 Changes in SA content in *Physcomitrella patens* after its inoculation with *Botrytis cinerea*



NOTE A: Chromatogram of ABA standard; B: HPLC chromatogram of wild-type *P. patens* at 0 h after inoculation; C: HPLC chromatogram of NDGA-treated *P. patens* at 0 h after inoculation.

Fig. 7 HPLC chromatogram of ABA in different types of *Physcomitrella patens* after its inoculation with *Botrytis cinerea*

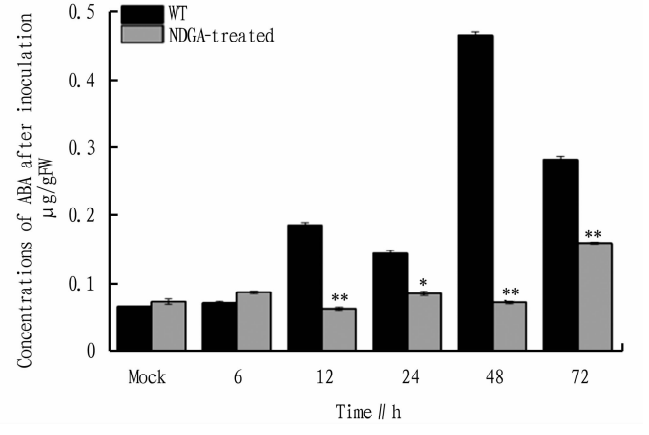
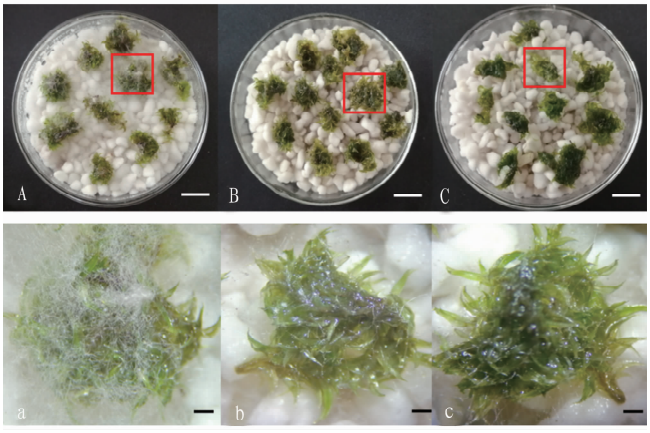


Fig. 8 Changes in ABA content in *Physcomitrella patens* after its inoculation with *Botrytis cinerea*

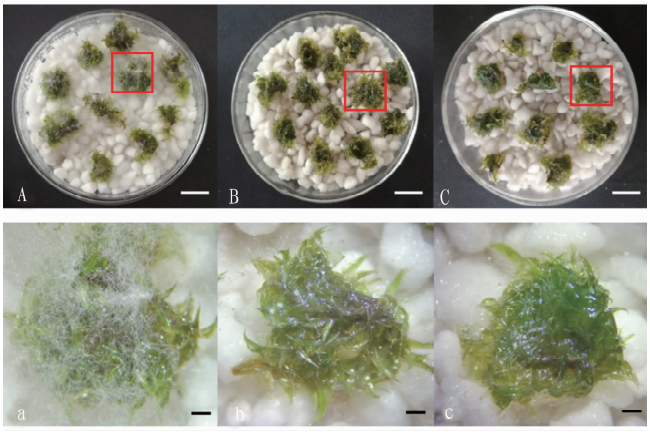
2.7 Recovery of plant phenotypes by ABA and SA Different concentrations of ABA and SA were prepared and sprayed onto the surfaces of plants pretreated with NDGA, followed by inoculation with *B. cinerea*. The results are shown in Fig. 9 and Fig. 10. Three days after inoculation with *B. cinerea*, it was observed that the phenotype of NDGA-treated plants sprayed with ABA or SA recovered to the level comparable to that of wild-type plants. The amount of surface hyphae was lower than that on NDGA-treated plants without ABA or SA application (Fig. 9C and Fig. 10C).

External application of ABA or SA restored the plant's resistance to *B. cinerea*.



NOTE A and a: NDGA-treated plants inoculated with *B. cinerea*; B and b: Wild-type (WT) plants inoculated with *B. cinerea*; C and c: Plants pretreated with NDGA followed by exogenous ABA application and *B. cinerea* inoculation. (A, B, C Bar = 1cm; a, b, c Bar = 1 mm).

Fig. 9 Effect of exogenous ABA spray on plant phenotype following *Botrytis cinerea* infection



NOTE A and a: NDGA-treated plants inoculated with *B. cinerea*; B and b: Wild-type (WT) plants inoculated with *B. cinerea*; C and c: Plants pretreated with NDGA followed by exogenous SA application and *B. cinerea* inoculation. (A, B, C Bar = 1 cm; a, b,c Bar = 1 mm).

Fig. 10 Effect of exogenous SA spray on plant phenotype following *Botrytis cinerea* infection

2.8 Determination of ethylene (ET) levels in *P. patens* The changes in ethylene content of *P. patens* plants inoculated with *B. cinerea* from 0 to 72 h are shown in Fig. 11. Compared to the control, ethylene release in WT plants increased during the 0 – 72 h after inoculation, with similar levels observed between 6 and 24 h. In NDGA-treated plants, ethylene release showed a significant decrease compared to the control from 6 to 48 h, reaching the lowest level at 48 h.

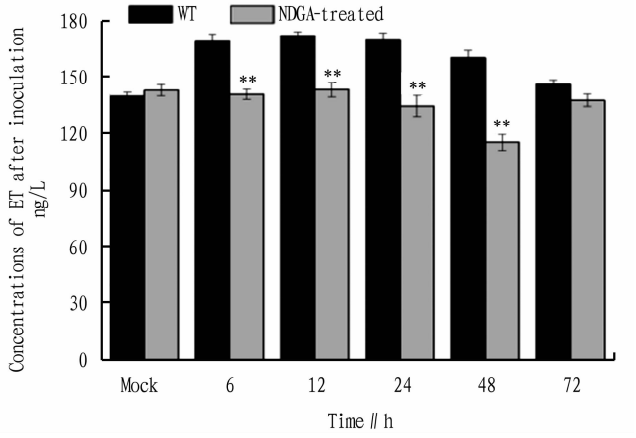


Fig. 11 Changes in ET content in *Physcomitrella patens* after its inoculation with *Botrytis cinerea*

3 Discussion

NDGA is an effective inhibitor of LOX in *P. patens*. After treatment, the catalytic activity of LOX *in vivo* is partially inhibited. This study shows that after NDGA treatment, the plants exhibited increased surface hyphae and greater susceptibility to *B. cinerea* following inoculation, which is consistent with the phenomenon observed in NDGA-treated cucumber leaves[7]. Lignin is a complex phenolic polymer in the cell walls of higher plants and plays an important role in defense responses against pathogen infection. Lignin is also the end product of the phenylpropanoid pathway, and CAD is a key enzyme catalyzing the final step in lignin biosynthesis. Safranin can stain lignified tissues in plants. Our results show that CAD enzyme activity gradually increased from 0 to 48 h after infection with *B. cinerea* in *P. patens*, suggesting that wild-type plants accumulate large amounts of phenolic substances upon infection. Safranin staining confirmed that inoculated plants exhibited deeper red coloration compared to uninoculated plants. In NDGA-treated plants infected with *B. cinerea*, the trend of CAD enzyme activity changes was consistent with that of the wild-type control; however, the CAD enzyme activity in NDGA-treated plants was lower than that in the corresponding wild-type plants at all time points. After infection with *B. cinerea*, safranin staining revealed that the intercellular spaces and leaf vein areas in NDGA-treated *P. patens* plants were lighter in color compared to wild-type plants (as shown in Fig. 1B, 1D), indicating less accumulation of phenolic substances in the former. Thus, partial inhibition of LOX activity resulted in reduced accumulation of phenolic compounds, leading to increased susceptibility to *B. cinerea* infection. It is hypothesized that changes in LOX activity in *P. patens* are positively correlated with the accumulation of phenolic substances.

ABA is a hormone associated with plant maturation, senescence, and defense. Studies have shown that xanthoxin is a relatively direct precursor of ABA biosynthesis[14-15]. The conversion from neoxanthin (the direct precursor of xanthoxin) to xanthoxin

is the rate-limiting step in ABA synthesis. LOX acts on the double bonds of neoxanthin, catalyzing the oxidative cleavage of these bonds to promote xanthoxin formation[16]. Gong Changrong *et al.* [17] demonstrated that under water stress, the accumulation of ABA is positively correlated with LOX activity. Treatment of isolated tobacco leaves with the inhibitor NDGA not only suppressed LOX activity but also blocked the substantial generation of ABA, indicating that LOX likely plays a key role in both the biosynthesis and stress-induced accumulation of ABA. The results of this study show that in wild-type *P. patens* plants infected with *B. cinerea*, ABA levels were higher than in the control from 6 to 48 h after inoculation and decreased from 48 to 72 h, consistent with findings in banana and rice[18-19]. After 72 h, ABA content was again higher than in the control, and the late-stage yellowing, senescence, and decay of leaves in infected plants may be related to the substantial accumulation of ABA in plant tissues. In NDGA-treated plants inoculated with *B. cinerea*, ABA levels were significantly lower than in the control from 12 to 72 h, indicating that NDGA partially inhibited LOX activity, thereby affecting ABA synthesis and accumulation and reducing plant resistance to pathogens. In the resistance recovery experiment, we further confirmed that supplementing NDGA-treated *P. patens* plants with exogenous ABA significantly enhanced disease resistance, suggesting that LOX may influence plant disease resistance by regulating ABA synthesis.

The flavonoid biosynthesis is a plant-specific metabolic pathway and a crucial branch of the phenylpropanoid pathway. CHS is a key enzyme in plant flavonoid biosynthesis, playing roles in influencing flower color formation, defending against pathogenic bacteria, and preventing UV damage. This study shows that the CHS enzyme activity in wild-type plants inoculated with *B. cinerea* increased compared to the control (non-inoculated wild-type plants). After NDGA soaking treatment of *P. patens* followed by inoculation with *B. cinerea* for 0-3 d, the CHS enzyme activity gradually decreased, reaching its lowest level at 72 h after inoculation. Compared to the control group (non-inoculated wild-type/NDGA-untreated plants), the activity decreased by approximately 30% (as shown in Fig. 2). Thus, both CHS and LOX activities showed a declining trend. The synthesis of flavonoids in plants is regulated by hormones[20]. Abscisic acid (ABA) treatment can significantly enhance anthocyanin synthesis in cherry fruits[21]. LOX activity is positively correlated with the synthesis and accumulation of ABA, suggesting that in *P. patens*, LOX may regulate flavonoid biosynthesis through ABA.

ET in plants is typically induced and synthesized in response to environmental stress, senescence, and fruit ripening. The induction of the gene encoding the rate-limiting enzyme for ethylene synthesis, ACC synthase, leads to an increase in ethylene release[22]. Research has shown that in postharvest tomato fruits infected with *B. cinerea*, there is a significant positive correlation between ethylene release and the activities of ACC synthase and LOX enzymes[6]. Studies by Lynch *et al.* [23] indicated that LOX is directly or indirectly involved in ethylene biosynthesis, primarily because hydroperoxides produced via the LOX pathway promote the conversion of ACC to ethylene. If LOX activity is inhibited, ethylene synthesis decreases. This study found that in wild-type *P. patens* infected with *B. cinerea*, ET content remained relatively stable within 6-24 h, showing an increase compared to the control, and decreased after 24 h. After infection by pathogens, ethylene in *P. patens* increased briefly in the short term. As the infection progressed, the plant leaves turned yellow and decayed, leading to a reduction in ethylene release. Treatment with NDGA resulted in lower ethylene release compared to the control, indicating that partial inhibition of LOX activity affects ACC synthase activity, reduces ethylene synthesis in the plant, and consequently impairs the early disease resistance of *P. patens* against *B. cinerea*.

SA, a small phenolic compound ubiquitously found in plants, can induce plant resistance against pathogens[24]. In tobacco infected with Tobacco mosaic virus, SA content increases around 24 h after infection[25]. SA biosynthesis in plants involves PAL pathway and the isochorismate synthase pathway. PAL, the first key enzyme in the SA biosynthetic pathway, also serves as the rate-limiting enzyme in the phenylpropanoid metabolic pathway. This study found that in wild-type plants infected with *B. cinerea*, SA content increased from 6 to 48 h compared to the control group, peaking between 6 and 12 h before declining, which is consistent with findings in tobacco and cotton[26-27]. Therefore, during the early stage of infection, *P. patens* may participate in defense responses by producing SA. When LOX activity was inhibited by NDGA, SA content was significantly reduced compared to the control group, suggesting that LOX may promote SA synthesis and thereby enhance plant resistance. After inoculation with *B. cinerea*, PAL activity in plants increased at 6-12 h and decreased at 24-72 h. Thus, in the early defense stage following *B. cinerea* infection, PAL activity in *P. patens* was enhanced. However, when *P. patens* was treated with the LOX inhibitor NDGA, PAL activity showed a significant decreasing trend from 0 to 24 h compared to the control group (uninoculated wild-type/untreated with NDGA), reaching its lowest point at 12 h with a 37.5% reduction relative to the control. This indicates that changes in LOX activity may influence PAL activity. The variation in PAL activity corresponded with changes in SA content, suggesting that during *B. cinerea* infection, PAL catalyzes SA synthesis to participate in the defense response. The PAL activity in NDGA-treated plants was significantly lower than in the control group, indicating that LOX may affect SA synthesis by modulating PAL activity. Treatment of infected rice with SA or JA enhances lignin synthesis[28-29]. Inhibition of LOX activity reduces the synthesis of SA and JA, thereby affecting disease resistance in *P. patens*.

4 Conclusions

In summary, LOX may participate in the defense response of

P. patens against *B. cinerea* by influencing the synthesis of phenolic compounds, the phenylpropanoid pathway, flavonoid production, and the synthesis of signaling molecules.

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