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RESEARCH PAPERS

Macromolecular Toxins Secreted by *Botrytis cinerea* Induce Programmed Cell Death in Arabidopsis Leaves[1, 2]

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Abstract—*Botrytis cinerea* causes grey mold disease in crops and horticultural plants. It is suspected to kill plant cells via secreted toxins and to derive nutrients from dead or dying cells. However, whether macromolecular phytotoxins (MPs) secreted by *B. cinerea* induce necrosis or also trigger a programmed cell death (PCD) remains to be determined. We have previously partially characterized MPs secreted by *B. cinerea*. Here we isolated MPs from *B. cinerea* culture and applied them to leaf cells, assessing PCD over the following 120 h. Cell death was assessed by propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) staining. Catalase (CAT), peroxidase (POD) activity and the cytochrome *c*/*a* ratio were assessed by spectrophotometer. POD isomers were measured using the benzidine acetate method. In *Arabidopsis thaliana* (L.) Heynh. exposed to *B. cinerea* MPs, we observed chromatin condensation and marginalization, nuclear substance leakage and accumulation of autofluorescent materials in the cell wall. Furthermore, *B. cinerea* MPs induced release of cytochrome *c* from the mitochondria into the cytosol. Moreover, CAT and POD activity was upregulated and the POD isoenzyme pattern was altered. In conclusion, *A. thaliana* exposed to *B. cinerea* MPs exhibits multiple hallmarks of PCD, suggesting that *B. cinerea* induces PCD in host cells through secreted macromolecules.

Keywords: Arabidopsis thaliana, *Botrytis cinerea*, macromolecule phytotoxins, programmed cell death

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INTRODUCTION

Botrytis cinerea is a necrotrophic fungus capable of infecting over 200 plant species, including dicotyledonous, monocot and fern. This fungal pathogen significantly impacts the profitability of food crops including vegetables, berries and grapes and horticultural plants [1]. *B. cinerea* infects the flowers, fruits, leaves and stems of plants, causing water soaking and soft rot accompanied by collapse of the parenchyma. These symptoms are followed by the rapid appearance of grey masses of conidia, producing the characteristic appearance of gray mold. In response to pathogen invasion, plant host cells can produce reactive oxygen species (ROS) which may induce oxidative burst of infected cells, limiting spread of the infection to healthy cells. This strategy is termed the hypersensitive response (HR), and could be considered a form of programmed cell death (PCD) [2]. Thus, pathogen-triggered HR can be considered both an important component of phytotoxicity and a plant defense mechanism. HR is commonly observed in the plant-biotrophic pathogen interaction, often visible as necrotic lesions [3]. In contrast, necrotrophic pathogens seek to kill host cells and extract nutrients from dead cells, and thus HR may promote susceptibility to these pathogens [2]. *B. cinerea* has been documented to produce ROS. ROS was reported to accumulate in germinating conidia during early stages of infection of French bean leaves [4]. Hydrogen peroxide (H_2O_2) derived from *B. cinerea* has been reported to induce host cell death and favor fungal infection [5]. In addition to synthesizing ROS, *B. cinerea* has also been reported to secrete a variety of nonspecific small molecule phytotoxic metabolites including sesquiterpene-botrydial, polyketidebotcinic acid and oxalic acid [6]. *B. cinerea* also synthesizes phytotoxic proteins including non aspartic proteinase and endopolygalacturonase [7, 8]. These phytotoxins likely induce host cell death and facilitate further invasion of plant hosts.

[1] The article is published in the original.

[2] Supplementary materials are available for this article at 10.1134/S1021443718040131 and are accessible for authorized users.

Abbreviations: PCD—programmed cell death; MPs—macromolecular phytotoxins; PI—propidium iodide; DAPI—4',6-diamidino-2-phenylindole; CAT—catalase; POD—peroxidase; HR—hypersensitive response; PDA—potato dextrose agar; Cyt *c*/*a*—cytochrome *c* and *a*.

However, although *B. cinerea* may produce some phytotoxic compounds, recent research has indicated that these compounds act as cofactors to pathogenesis, rather than as direct phytotoxic agents [9]. Additionally, the typical morphology and biochemistry of *B. cinerea* secretion-induced cell death has not yet been described. We previously identified phytotoxins secreted by *B. cinerea* [10]. The necrotic lesions caused by application of these proteinic phytotoxins to *Arabidopsis thaliana* leaves were similar to those caused by *B. cinerea* infection, suggesting an important role of these secreted proteins in *B. cinerea* disease.

Here, we further characterized the cellular and molecular mechanisms by which macromolecule phytotoxins (MPs) secreted by *B. cinerea* induce *A. thaliana* leaf cell death.

MATERIALS AND METHODS

Plant materials and growth conditions. The *Arabidopsis thaliana* (L.) Heynh. materials used in this experiment (accession Col-0) was previously described by Yang [11]. *Arabidopsis* seeds were obtained from Lehle Seeds (United States) and pre-germinated on agar plates with Murashige and Skoog (MS) medium under conditions of 12 h light/12 h dark, 22~23°C and 70% relative humidity. The seedlings were transplanted into potting soil for further growth under the same conditions as for seed germination.

***Botrytis cinerea* culture and isolation of MPs.** *Botrytis cinerea* (isolated from tomato) was kindly provided by Dr. L.J. Qu (University of Beijing, China). Spores were grown in potato dextrose agar (PDA) medium for 8 days. 100 mL potato dextrose liquid medium was inoculated with 0.5 cm^2 of agar-grown *B. cinerea* and incubated in a 500 mL flask at 25°C with 200 rpm shaking for 20 days, after which MPs were extracted via Sarpeleh's method [12]. Mycelial mats were removed from the culture medium by filtering through four layers of cotton gauze. The filtrate was centrifuged at 4000 rpm for 15 min, and the supernatants were further filtered through rapid filter paper No. 1 and medium speed filter paper No. 2 to remove spores. The resultant filtrates were loaded into dialysis bags (exclusion 14 kD) and dialyzed against double distilled water at 4°C, then passed through a 0.45-μ millipore filter. The protein content of this filtrate was determined spectrophotometrically by the Bradford protein assay.

Leaf infiltration and visualization of cell death. *B. cinerea* MPs were infiltrated into the leaf mesophyll using a Hagborg device through the stomata on the epidermal layer of intact Arabidopsis leaves with slight modifications to minimize mechanical harm to the leaves [12–14]. The Hagborg device was constructed in house as described by Deng [14]. Intact leaves were selected from 5-week-old plants, and 10 μL of phytotoxin was gently infiltrated into each leaf. Control plants were administered the same volume of sterile distilled water. Treated plants were kept in the tissue culture room at 22~23°C with 90% relative humidity in the dark for 24 h, followed by a 12 h light/12 h dark photoperiod. Leaves were collected 48, 60, 72, 84, 96, 108 and 120 h post infiltration. Collected leaves were stained with 5 μg/mL cell permeable stain 4',6-diamidino-2-phenylindole (DAPI) and 5 μg/mL the membrane-impermeable stain propidium iodide (PI) to assess cell death. Boiled leaves were used as a positive control for cell death. The fluorescence of DAPI and PI was measured at 365 and 530 nm respectively by an epifluorescence microscopy (LEICA DMI3000B, Germany).

Detection of endogenous fluorescence in leaf cells. Autofluorescence of Arabidopsis leaves was assessed by immersing leaves into a fixative solution (of formaldehyde, acetic acid, absolute ethyl alcohol and water at a ratio of 5 : 1 : 9 : 8, v/v/v/v) for 15 min, and dehydrating leaves in 50% ethanol for 15 min, then in 95% ethanol overnight. The fixed leaves were mounted on microscope slides in 70% glycerin in water and autofluorescence observed using ultraviolet epifluorescence (LEICA DMI3000B, excitation wavelength 470 nm). Leaves infiltrated without *Botrytis* MPs or boiled for 30 min were served as controls.

Assays of catalase and guaiacol peroxidase activities. Leaves (0.3 g) were homogenized at 0–4°C in 1 mL of 50 mM phosphate buffer, pH 7.8. The homogenate was centrifuged at 15000 rpm for 15 min and the supernatant enzyme extract was collected. Catalase (CAT) activity was assessed by monitoring the consumption of H_2O_2 at 240 nm in a spectrophotometer. The 3 mL reaction mixture contained 1 mL of 0.3% H_2O_2, 1.95 mL of H_2O and 0.05 mL of enzyme extract. At 1-min interval the absorbance of the solution was recorded against a blank containing every ingredient except the enzyme. The initial straight-line part of the curve was used to determine the enzyme activity which was expressed as decrease in absorbance at 240 nm per min per gram fresh leaf weight and calculated as following:

$$\text{Activity [U/g fr wt]} = \Delta OD_{470} \times D/0.01 \times \text{fr wt} \times t,$$

where U—0.01 decrease in OD per minute, ΔOD_{240}—absorbance in reaction time, g fr wt—leaf fresh weight, *t*—reaction time, D—dilution time of enzyme extract.

Peroxidase (POD) activity was measured colorimetrically with guaiacol as a substrate. The 4 mL reaction mixture contained 1 mL of 50 mM phosphate buffer (pH 7.0), 2 mL of 0.3% H_2O_2 and 0.95 mL of 0.2% guaiacol. Then 0.05 mL enzyme extract was added to start the reaction. The reaction mixture was incubated at 25°C. At 1-min interval the absorbance of the solution was read at 470 nm against a blank (same as CAT activity measurement). The POD activity was expressed as the increase in absorbance at 470 nm/(g fr wt min) and calculated as CAT activity.

Isomers assays of POD. POD isozymes of Arabidopsis leaves pre-treated with or without MPs were

isolated by vertical polyacrylamide gel electrophoresis (PAGE), and revealed by benzidineacetate staining as previously described [15] with slight modifications. The Arabidopsis leaf POD enzyme preparation followed the procedure described above. A 15-μL sample of enzyme extract was loaded on the gel (Invitrogen, United States) and run for 3–4 h at 90 V in 10% separating gel and 200 V in 3.75% concentrating gel on Power/pAc (Bio-Rad). After PAGE isolation, the gel was immersed in benzidineacetate to staining bands. The benzidineacetate staining was prepared following the formula: 5 mL of 2% benzidin solution (1 g benzidin in 9 mL acetate and 36 mL distilled water) and 0.2 mL 30% H_2O_2 added to 94.8 mL distilled water. The gels were stained in the dye liquid for 5–10 min with gentle shaking, then washed in distilled water 3–5 times to stop staining. Images were captured using a camera (LEICA D-LUX4, Germany).

Preparation of mitochondrial and cytoplasmic extracts. Mitochondria were isolated from Arabidopsis leaves as described by Kelly [16] with modifications. Leaves (0.2 g fr wt) were homogenized on ice for 3–5 min in 1.5 mL of mitochondria extraction buffer (0.5 mM sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.2% BSA, 5 mM cysteine, 0.3% β-mercaptoethanol, and 0.3% polyvinylpyrrolidone). All subsequent steps were carried out on ice. The homogenate was centrifuged at 1000 rpm for 5 min, and the pellet was resuspended in mitochondria extraction buffer. The supernatant was centrifuged again at 1000 rpm for 5 min. The supernatant was collected and further centrifuged at 4000 rpm for 10 min. The supernatant was centrifuged at 12000 rpm for 10 min. The resultant pellet was resuspended in 1.5 mL mitochondria extraction buffer and spun at 10000 rpm for 10 min. This step was repeated once more. The resultant pellet was resuspended in 1.5 mL precooled acetone and centrifuged again at 13000 rpm for 5 min. This step was repeated once more. The final pellet, containing mitochondria protein, was air dried on ice. Supernatants from 12000, 10000 and 13000 rpm centrifugations were collected and stored at −80°C as cytoplasmic extract for further assay.

Isolation of mitochondria- and cytoplasm-special proteins. To assess the integrity of the isolated mitochondria, mitochondrial and cytoplasmic proteins were isolated by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The mitochondrial and cytoplasmic extracts from Arabidopsis leaves described above were taken up in a suitable volume of 1 M NaOH. These extracts were mixed with sample buffer, boiled for 5 min, and loaded to the gel for electrophoresis. SDS-PAGE was carried out with a Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, United States).

Analysis of mitochondrial ratio of cytochrome *c* and *a* (Cyt *c*/*a*). The mitochondrial pellets described above were resuspended in 0.2% (w/v) BSA buffer and adjusted to 0.5 mg/mL. The absorbance at 550 nm (the absorption peak of cytochrome *c*) and 630 nm (the absorption peak of cytochrome *a*) was measured by a spectrophotometer at MPs treatment time of 24, 36, 48, 60, 72, 84, 96 and 108 h. The ratio of the absorption values of the two wavelengths is Cyt *c*/*a*.

All the experiments were repeated in triplicate.

RESULTS

A. thaliana Cellular Morphology after Infiltration of B. cinerea Culture Filtrate

The MPs secreted by *B. cinerea* were isolated from cultures by filtration and dialysis. *A. thaliana* leaves were infiltrated with MPs as previously described by Hagborg et al. [13, 14]. Control leaves were infiltrated with sterile distilled water or boiled for 30 min. The first visible symptoms, translucence of the epidermis, appeared from 48 h post-infiltration onwards. Within 72 h, epidermal cells in the infiltrated zones were completely white. The complete infiltrated region including the abaxial mesophyll tissue turned to brown within a week. A microscopic examination was carried out after 48–120 h and leaves were stained with DAPI or PI alone, or a mixture of DAPI and PI to distinguish between living and dead cells. MPs induced cell death 48 h after infiltration. Microscopic observations of the transition zone between healthy and infiltrated regions showed an accumulation of PI in nuclei within the infiltrated region from 72 h post-infiltration onwards. Cell nuclei in non-infiltrated leaved were stained with DAPI, but remained PI-negative, indicating uncompromised cell membranes (Figs. 1a and 1b). DAPI-stained euchromatin was clearly present around the chromomeres (Fig. 1c). In contrast, apoptotic nuclei appeared orange/red when stained with a mixture of PI and DAPI (Fig. 1a yellow arrow), and apoptotic nuclei exhibited compartmentalization and chromatin condensation when stained with DAPI alone (Figs. 1d and 1e). Apoptotic nuclei further disintegrated over time (Figs. 1f and 1g). The nuclei of boiled leaves were misshapen and debris was observed by fluorescence microscope (Figs. 1h and 1i).

Endogenous Autofluorescence of Apoptotic Cells

Many plant tissues have been observed to autofluoresce. The characteristics of autofluorescence vary between plant species, tissues and physiological status. We observed autofluorescence in leaf cells exposed to *B. cinerea* MPs by fluorescent microscopy (Fig. 2). In leaf cells exposed to water, background autofluorescence was observed in trichomes, stomata guard cells and leaf veins, as previous reported by Rossi et al. [9], and these cells retained structural integrity (Figs. 2a–2c). Similar fluorescent signals were detected in leaves

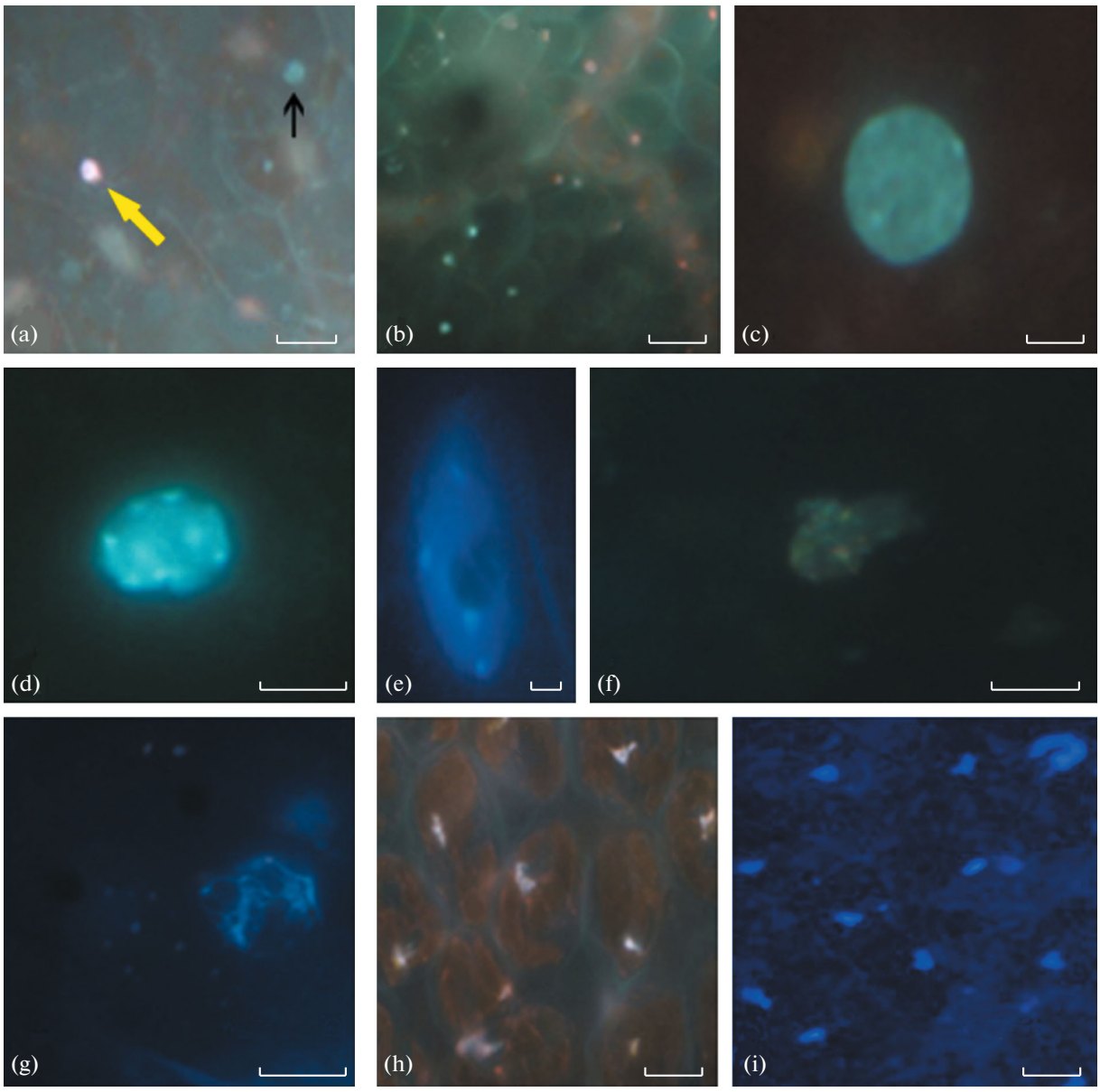

Fig. 1. *B. cinerea* macromolecular phytotoxins (MPs > 14 kD) induced programmed cell death (PCD) in *A. thaliana* leaves. (a) *A. thaliana* leaves administered *B. cinerea* MPs for 72 h were stained with nuclei with PI and DAPI. Apoptotic nuclei were identified by PI staining (red fluorescence, yellow arrow). Regular nuclei are identified by DAPI staining (blue fluorescence, black arrow). Scale bar 150 μm. (b) The border of the leaf area administered *B. cinerea* MPs for 72 h (the right half of figure). Scale bar 200 μm. (c) *A. thaliana* leaves administered only water for 72 h exhibit only DAPI staining. Scale bar 50 μm. (d) 60 h after *B. cinerea* MP administration DAPI-stained DNA condensation was observed. Scale bar 50 μm. (e) After 72 h alveolar cavities were observed. Scale bar 15 μm. (f) After 84 h chromatin marginalization was observed. Scale bar 20 μm. (g) After 108 h nucleus dissolution was observed. Scale bar 20 μm. (h, i) In leaves boiled for 30 min nuclei were stained with PI. Scale bar 100 μm.

exposed to *B. cinerea* MPs after 24 h (Fig. 2g), however leaf samples boiled for 30 min exhibited dulled autofluorescence, detected only in trichomes (Figs. 2d–2f). Cell wall thickening was observed in leaf tissues exposed to *B. cinerea* MPs after 48 h under both fluorescent and bright field (Figs. 2h and 2i). Strong, isolated spots of autofluorescence were detected in leaf cell walls 60 and 84 h after exposure to *B. cinerea* MPs (Figs. 2j and 2k). These fluorescent signals faded and disappeared after 108 h (Fig. 2l) and 120 h (Fig. 2m) after exposure to *B. cinerea* MPs, by which time structural damage to the cell wall was observed (Fig. 2m), while autofluorescence was maintained in the trichomes, stomata guard cells and leaf veins exposed only to water (Figs. 2n and 2o).

Influence of B. cinerea Culture Filtrate on A. thaliana POD and CAT Activity and POD Isoenzyme Pattern

In leaf homogenates collected between 12 and 120 h after administration of *B. cinerea* MPs, POD activity was measured spectrophotometrically and isoenzyme

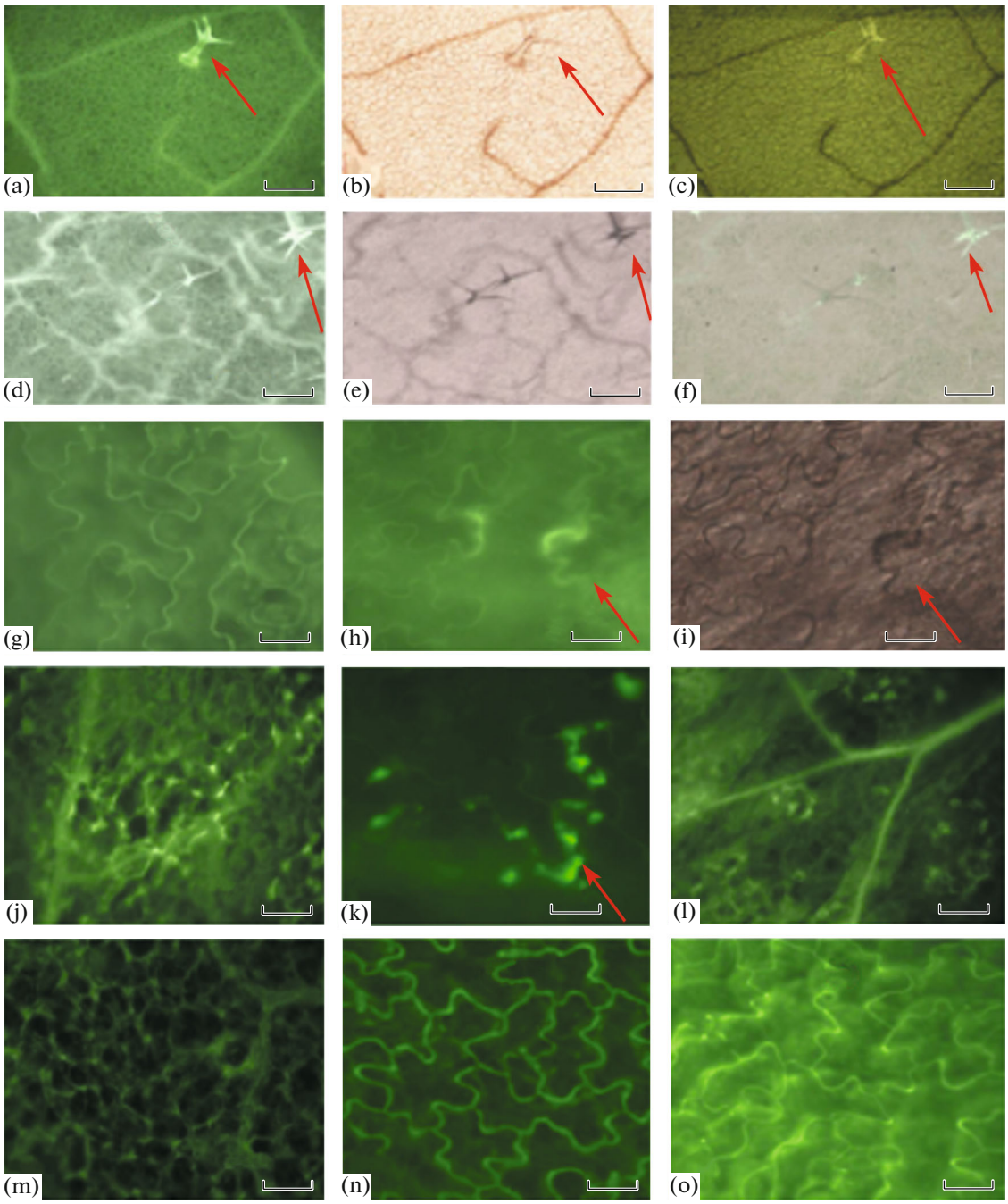

Fig. 2. *A. thaliana* leaf autofluorescence after the administration of *B. cinerea* MPs. In leaves infiltrated with sterile water, only the trichome exhibited spontaneous fluorescence; (a) fluorescent microscopy; (b) bright field microscopy; (c) merged images of a and b leaves boiled for 30 min; (d) fluorescent microscopy; (e) bright field microscopy; (f) merged images of d and e. Leaves infiltrated with *B. cinerea* MPs exhibited cell wall structural integrity at 24 h. After 48 h, cell wall thickening was observed (arrow) by fluorescent microscopy (g) and bright field (i). After 60 and 84 h spontaneous fluorescence was observed to accumulate in the cell wall (j and k). After 108 h, cell wall deformation was observed (l). After 120 h no autofluorescence was observed on the leaf surface (m). In leaves treated only with water, the cell structural integrity was preserved after 48 and 84 h (n and o, respectively). Scale bars in a, b, c, d, e and f: 400 μm; g, h, i, n and o: 200 μm; j, k, l and m: 100 μm.

pattern was assessed by electrophoresis. POD activity was elevated in *A. thaliana* leaves 48–108 h after administration of MPs, peaking at 72 h post-MP administration (Fig. 3a). The POD isoenzyme pattern was also observed to alter after MPs infiltration (Figs. 3b and 3c). Four POD isoenzymes were detected in *A. thaliana* control leaves, named POD1, 2, 3 and 4 (Fig. 3b). In leaves treated with MPs 12 or 24 h, no change was observed in isoenzyme distribution, however, between 36 and 60 h post-MP treatment, the POD1 and

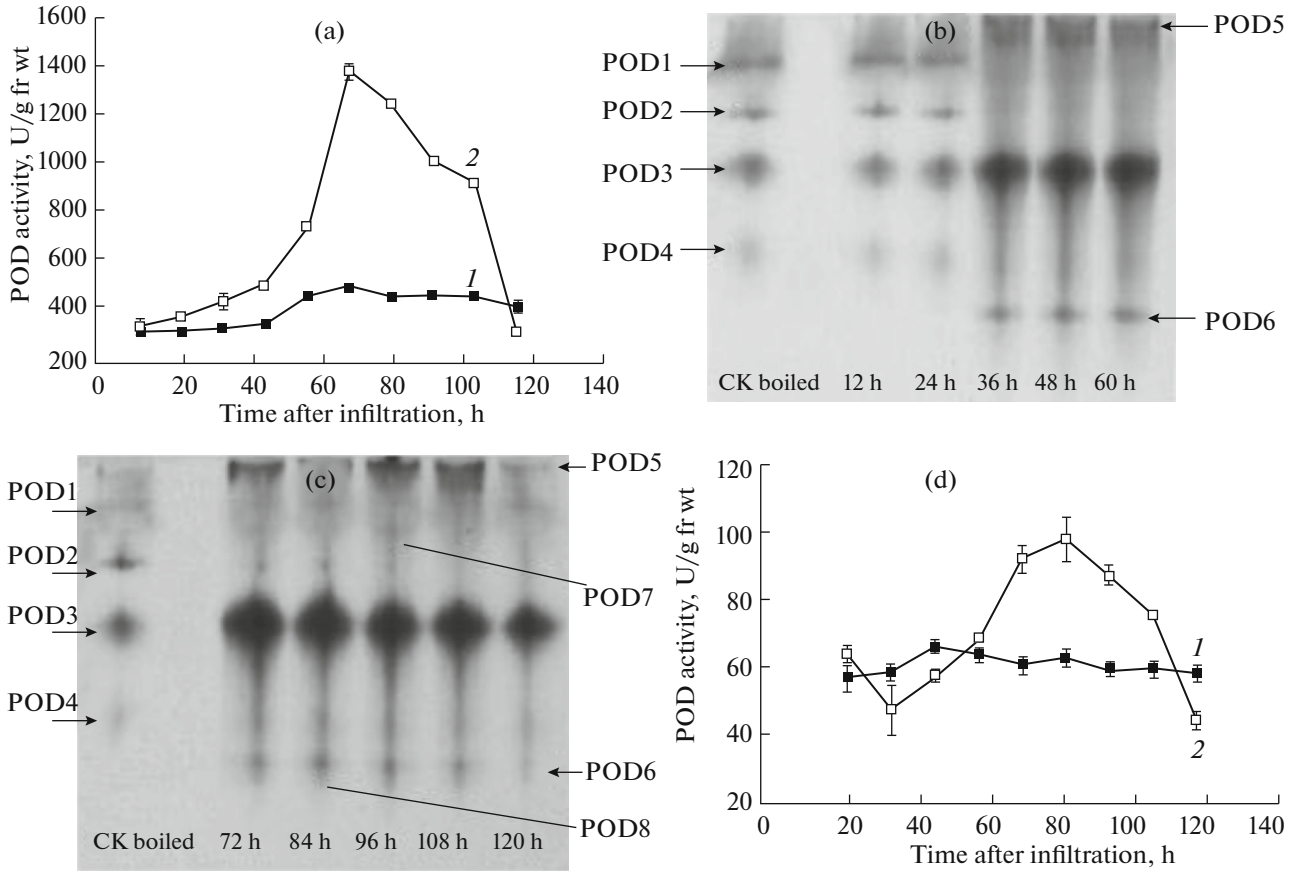

Fig. 3. Guaiacol peroxidase (POD) activity and isoenzymes and catalase (CAT) activity in *A. thaliana* leaves infiltrated with *B. cinerea* MPs. (a) The activity of guaiacol POD in *A. thaliana* leaves administered *B. cinerea*. MPs were assessed by electrophoresis; (b), (c) POD isoenzyme pattern in leaves infiltrated with *B. cinerea* MPs; (d) CAT activity in leaves infiltrated with *B. cinerea* MPs was assessed by monitoring H_2O_2 consumption at 240 nm with a spectrophotometer. *1*—untreated control; *2*—infiltration with *B. cinerea* MP.

two bands disappeared, and two new bands – POD5 and POD6 – appeared. Between 72 and 96 h post-MP administration POD6 and seven bands were visible in the gel, but the POD6 band faded. After 108 and 120 h, POD7 and POD6, respectively, could not be detected. The level of POD5 was also decreased by 120 h.

CAT activity was assessed by monitoring H_2O_2 consumption (Fig. 3d). CAT activity was depressed 36 h after MP administration, but begun to increase after 60 h, and peaked at 84 h, and the CAT activity of treated and control leafs did not differ at 120 h. These results suggested that qualitative and quantitative changes in the activity of scavenger enzymes including peroxidase and catalase isolated from plants exposed to *B. cinerea* MPs. The time courses of appearance were almost similar in both peroxidase and catalase after 12–120 h of administration of *B. cinerea* MPs. And the activity of POD was increased slightly fast than CAT.

Examination of Mitochondrial Intactness

As a semi-autonomous organelle, mitochondrion has its own DNA and ribosome, and can synthesize some proteins. If mitochondria obtained from Arabidopsis leaves were intact, mitochondrial marker proteins should be retained in the mitochondrial extracts and not released into the cytoplasmic extracts. Here, SDS-PAGE electrophoresis was employed to visualize distribution of mitochondrial-specific and cytoplasm-specific proteins in these extracts. We observed a 54.4 kD mitochondrial marker protein and a 27.5 kD cytoplasmic marker protein in mitochondrial and cytoplasmical fractions, respectively (Fig. S1), indicating that the mitochondrial membranes are intact.

Effect of B. cinerea MPs on Cyt c/a of Mitochondria from A. thaliana Leaves

Cyt *a* and *c* are components of the electron transport chain of the mitochondrial inner membrane. Cellular stress may induce biomembrane lipid peroxidation, which induces the plant mitochondrial permeability transition pore (MPTP) to open, causing depolarization of the mitochondria and damages the mitochondrial membrane. Under these conditions, Cyt *c* translocates from the mitochondrial inner mem-

brane to the intermembrane space. If large amount of swelling occurs, causing disruption of the outer membrane, Cyt *c* will be released from the intermembrane space into the cytosol [17]. Hence, the ratio of Cyt *c/a* indicated whether Cyt *c* has been released from the mitochondria. We measured the Cyt *c/a* ratio in *A. thaliana* leaf tissue after infiltration of *B. cinerea* MPs. The Cyt *c/a* ratio started to decrease 60 h after MPs-treatment and continued to decrease throughout the experiment (Fig. 4), indicating that Cyt *c* was released from the mitochondrial inner membrane into the cytoplasm of cells exposed to MPs.

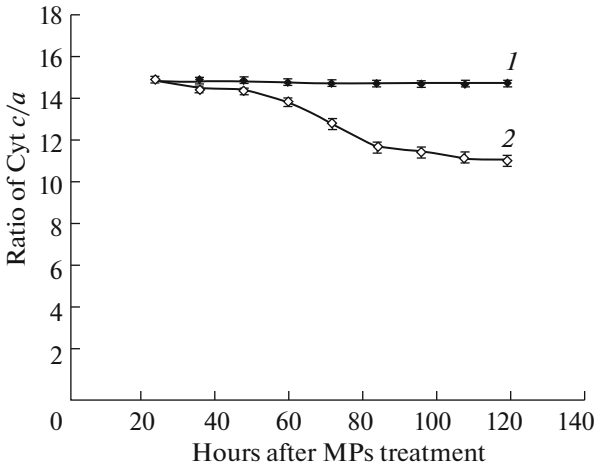

Fig. 4. The effect of *B. cinerea* MPs on *A. thaliana* Cyt *c/a*. *1*—untreated control; *2*—infiltration with *B. cinerea* MPs. The absorptions at 550 nm (for Cyt *c*) and 630 nm (for Cyt *a*) of mitochondria were measured respectively by spectrophotometer after MPs treatment 24 36, 48, 60, 72, 84, 96 and 108 h. The ratio of the absorption values of the two wavelengths is Cyt *c/a*.

DISCUSSION

In response to pathogen invasion plant cells may trigger PCD, limiting spread of infection to healthy cells [2, 18]. The necrotrophic pathogen *B. cinerea* has been previously reported to produce phytotoxins, and thus may potentially benefit from plant hypersensitive reaction. Here we have isolated phytotoxic MPs secreted by *B. cinerea,* and characterized the mechanism by which these macromolecules trigger cell death in *A. thaliana*.

PCD hallmarks are evident in the Arabidopsis leaf cells after infiltration with *B. cinerea* MPs. Staining of the MP-treated leaves with DAPI revealed fluorescent nuclei in the treated area displaying chromatin condensation, which is often associated with PCD (Fig. 2a) [19]. *B. cinerea* MPs also induced increased antioxidant enzyme activity, synthesis of new POD, and Cyt *c* release from mitochondria into the cytosol of host cells. These hallmarks of PCD have not only been observed in plants during the defense response to biotrophic pathogens, but also to necrotrophic pathogens, such as *B. elliptica* [5].

Fungal toxins have been previously reported to induce PCD in bacteria and plants. The host-specific AAL toxins secreted by *Alternaria alternate* f. sp. *lycopersici* induce PCD in tomato plants [20]. Victorin, a host-specific toxin produced by *Cochliobolus victoriae*, also induces PCD in susceptible oat plants [21]. Botrydial, a small molecular toxin secreted by *B. cinerea* is reported to induce PCD in *A. thaliana* [9]. Toxins produced by some bacteria such as *Pseudomonas syringae* pv. *Atropurpurea* and *P. syringae* pv. *coromafaciens*, can also elicit hallmarks of PCD in oat plants [22]. Therefore, PCD is thought to be a common plant response to necrotrophic pathogen toxins. However, here for the first time we have characterized PCD in response to one or more macromolecules, secreted by *B. cinerea*.

DAPI, as a membrane-permeable stain, can bind nuclei, emitting a bright blue light. In contrast, PI is a membrane-impermeable stain and can only enter dead cells. We observed that MP-treated cells were PI-stained 60 h after MP treatment. At this time, the nuclei of infiltrated cells stained with DAPI also displayed chromatin condensation. These observations suggest that MP-induced cell death is a type of PCD in which nuclear compaction. Our data are consistent with previous reports that *B. elliptica* culture filtrate (CF) induced cell PCD in lily leaves three days post infiltration of CF [5]. *B. cinerea* MPs-induced signs of cell death in Arabidopsis leaves about 72 h after infiltration, almost as observed with *B. elliptica* CF.

Autofluorescence was detected in the infected cells 24 h post-infiltration with *B. cinerea* MPs (Fig. 2). Biosynthesis and deposition of secondary metabolites, including lignin and other phenolic compounds, produced autofluorescence which is coupled tightly to host resistance. We found that the cell walls of *A. thaliana* leaf began to thicken 48 h after exposure to *B. cinerea* MPs (Figs. 2h–2k). Similar phenomena were observed by Soylu in Arabidopsis, reporting that injection of intercellular washing fluid (IWF) caused HR-like cell collapse, which was associated with accumulation of phenolics and lignin-like material in walls of cells undergoing cell death [23]. In broad bean leaves infected with *B. cinerea* exhibited degradation of plant cell walls appeared to precede cell death in advance of colonization by pathogen. These observations suggest that the small pores formed by *B. cinerea* on penetration points of the leaf cell wall were created by enzymatic activity. The close association between changes in wall structure and plant cell death at all stages of infection implies that most cells died as a result of attack by cell wall degrading enzymes [24]. The components of MPs secreted by *B. cinerea* are not yet fully characterized. However, we previously reported that MPs contain laccase [25], and this enzyme could damage plant cell walls. Our results, like those of others, support enzyme

secretion as the main mechanism of induction PCD by *B. cinerea*. Enrichment of the cell walls and wall apposition (papilla) with phenolics or lignins is probably a key mechanism of resistance to the degrading enzymes secreted by *B. cinerea*. When the wall thinking failed to prevent a breech, cell death occurred.

ROS accumulation is a typical signifier of PCD and induced by various cellular stressors [26]. ROS causes oxidative damage and induces cell death. It was reported that *B. cinerea* itself generates ROS, and this capability could contribute to the virulence of the pathogen. In bean leaf tissues H_2O_2 accumulated, which was generated mainly by pathogen during the interaction of *B. cinerea* and its host plant *Phaseolus vulgaris* [4]. We found that the activity of enzymes that remove H_2O_2, catalase and peroxidase, were significantly increased in *B. cinerea* MPs exposed *A. thaliana*. Moreover, the *A. thaliana* isoenzyme distribution of POD was significantly altered by exposure to *B. cinerea* MPs. These results suggest that MPs cause accumulation of ROS, and that *A. thaliana* upregulates catalase and peroxidase to prevent high accumulation of H_2O_2 and other ROS in plant cells.

To assess whether *B. cinerea* MPs stress may induce new POD isoforms, or only induce quantitative changes in the existing isoforms, the isoenzyme spectra of POD was assessed by gel electrophoresis. Four isozyme bands (POD1–POD4) were observed in control Arabidopsis plants, indicating that plants themselves have a certain ability to adapt to changes in environments. The activities of different isozymes began to alter 36 h after MPs treatment. Changes in peroxidase isoforms have been suggested to be involved mainly in growth and development processes via lignin polymerization [27]. Economou group had studied the rooting ability of *Ebenus cretica* cuttings treated with or without hormone indolic-3-butyric acid (IBA) by assaying electrophoretic patterns of peroxidases [28]. Four bands of peroxidase isoforms (namely A_1–A_4) were obtained in both control and IBA-treated plants. The number of isoforms was unchanged, but an increase in peroxidase activity followed by decrease has been found in IBA-treated cuttings. And the study suggested that fast-migrating peroxidase isoform (A3) was likely correlated to lignin synthesis during rooting process [28]. The fast-migrating peroxidase isoforms of tobacco and populous [27] were reported to be involved in the polymerisation of lignin monomer. We also observed that a fast-migrating peroxidase isoform (POD6) appeared before cell wall thickening (36 h post administrated with *B. cinerea* MPs). Our data support the hypothesis that fast-migrating peroxidase isoforms are involved in the polymerisation of lignin monomers during environmental stresses.

Studies point to mitochondria as an important player in the processing and amplification of cell-death signals. One of the early events in PCD, mitochondrial membrane permeability transition (MPT), is induced by multiple independent pathways [29, 30]. Increased MPT may lead to the release from the mitochondrion of a number of cell-death activators, inhibitors and inhibitor depressors, including Cyt *c*, apoptosis-inducing factor and Smac/DIABLO. Among these proteins, Cyt *c* has been shown to regulate the activity of the initiator caspase [29]. We found that the mitochondrial inner membrane Cyt *c* content in the *A. thaliana* leaf was reduced after exposure to *B. cinerea* MP. Osmotic swelling of the mitochondria then causes rupture of the outer mitochondrial membrane, and subsequent release of Cyt *c*. The mitochondrion is involved in signaling and initiating the death execution pathway, regulating PCD in Arabidopsis exposed to *B. cinerea* MPs.

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