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Reactive oxygen species promote chloroplast dysfunction and salicylic acid accumulation in fumonisin B1-induced cell death

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ABSTRACT

We report a novel regulatory mechanism by which reactive oxygen species (ROS) regulate fumonisin B1 (FB1)-induced cell death. We found that FB1 induction of light-dependent ROS production promoted the degradation of GFP-labeled chloroplast proteins and increased phenylalanine ammonia lyase (PAL) activity, *PAL1* gene expression and SA content, while pretreatment with ROS manipulators reversed these trends. Moreover, treatment with H_2O_2 or 3-amino-1,2,4-triazole increased PAL activity, *PAL1* gene expression and SA content. PAL inhibitor significantly blocked FB1-induced lesion formation and SA increase. Our results demonstrate that light-dependent ROS accumulation stimulates the degradation of chloroplastic proteins and up-regulates PAL-mediated SA synthesis, thus promoting FB1-induced light-dependent cell death.

1. Introduction

Plants have developed a complex immune system to resist pathogen attack. Hypersensitive response (HR) is a form of programmed cell death (PCD) as it leads to rapid, localized cell death at infection sites triggered by pathogens. Elicitor-induced hypersensitive cell death is a pathogen strategy for infection, whereas localized cell death at the site of infection is a plant defense strategy against pathogen attack [1,2]. Moreover, HR produced during some pathogen infection in plants has been recognized requiring the light [3,4]. However, further studies are needed to illustrate the underlying molecular mechanism of light-dependent HR cell death.

It is known that reactive oxygen species (ROS), in particular hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$), are versatile molecules mediating a variety of cellular responses in both the animal and plant kingdoms. In animals, mitochondria have been recognized playing vital roles in ROS-dependent apoptotic cell death [5]. For instance, ROS act upstream of mitochondrial membrane depolarization, Bax relocalization, cytochrome *c* release, executing caspase activation and nuclear fragmentation [6,7]. In plants, besides mitochondria, chloroplasts are also important ROS suppliers, and they may generate intermediate signals involved in PCD. For example, the role of chloroplastic polyunsaturated fatty acids and phytochrome signaling during HR process suggest the participation of chloroplastic factor in the pathway leading to the HR cell death [4,8]. Our early studies showed that protoplasts exhibited an increase of ROS levels in a light-dependent manner after UV-C irradiation [9]. Recently, some studies demonstrated that various stimuli, such as aluminum toxicity, methyl jasmonate treatment and pathogen attack, can cause chloroplast dysfunction and photosynthetic damage as well as chloroplastic ROS production [10–12]. Studies showing the function of ROS in signal transduction networks during plant PCD process have started to emerge [13–15]. Although ROS generated in various cellular compartments of plants exposed to pathogens or elicitors are essential for the progress of HR. There are many functional roles of ROS, especially the signaling pathway leading to plant HR, remain largely unclear. For instance, it is not clear whether they interact with hormone signaling in triggering HR. Also, the direct role of ROS produced in different plant compartments needs to be illustrated.

Previous studies have utilized fumonisin B1 (FB1), a programmed cell death–eliciting mycotoxin produced by the *Fusarium moniliforme*, as a model system to investigate the role of cell death in plant–microbe interactions [16–18]. However, the exact

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mechanism by which FB1 regulates light-dependent plant PCD is still largely unknown. Salicylic acid (SA) is a hormone signaling molecule functioning in plant-microbe interactions in the induction of HR. In addition, SA synthesis is influenced by the light condition and closely associated with the chloroplasts [19], indicating that chloroplasts play an important role in SA-mediated, light-dependent HR. Biochemical studies using isotope feeding demonstrate that a number of plants could synthesize SA from cinnamate, synthesized by phenylalanine ammonia lyase (PAL) from phenylalanine. However, whether SA is influenced by the early accumulation of ROS during the formation of HR and how ROS exert its regulating function are still unclear.

This study focuses on the investigation of the roles of ROS signals derived from chloroplasts on the oxidative damage to chloroplast morphology and function, as well as SA hormone signaling defenses in Arabidopsis (*Arabidopsis thaliana*) in order to reveal a novel molecular mechanism of fumonisin B1 (FB1)-triggered light-dependent cell death.

2. Materials and methods

2.1. Plant material and treatments

Plants of wild-type, transgenic Arabidopsis expressing stroma-targeted fluorescent protein (GFP) were all in the ecotype Columbia (Col-0) background. Plants were grown in soil culture with 16/8 h light/dark cycle (120 μmol quanta m^{-2} s^{-1}) and 82% relative humidity at 22 ± 1 °C. 4 weeks old plants were used for experiment. Stock solution of FB1 (7 mM) was prepared in methanol, and a final concentration of 10 μM (diluted in water) was used for treatments, with an equivalent volume of 0.14% methanol as controls.

For FB1 treatment, WT leaves were infiltrated with 10 μM FB1 or 0.14% methanol (control) using a 1 ml syringe without a needle and maintained in the presence or absence of light for indicated times. To manipulate ROS levels, we first treated plant leaves with ROS manipulators for 2 h by spraying 100 U ml^{-1} catalase (CAT), 1 mM ascorbic acid (AsA) or 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), then infiltrated with 10 μM FB1 for 4 d under the same growth conditions as described above.

For H_2O_2 treatment, the plants were excised the base of the stem and maintained in distilled water for 1 h to eliminate wound stress, then the cut ends of the stems were kept in a small growth chamber wrapped with aluminium foil containing 5 mM H_2O_2 for 1–4 days at 22 ± 1 °C. For 3-amino-1,2,4-triazole (3-AT) treatment, plant leaves were sprayed with 15 mM 3-AT for 1–4 days, then the leave samples were collected and immediately frozen with liquid nitrogen for further analysis.

2.2. Histochemical staining

In situ detection of $O_2^{\cdot-}$ and H_2O_2 accumulation was performed using nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) histochemical staining [11], respectively. Leaves after different treatment were detached from plants and submerged in NBT solution (1 mg ml^{-1} NBT plus 10 mM NaN_3 solution in 10 mM potassium phosphate buffer pH 7.8) or DAB solution (1 mg ml^{-1}, pH 5.5). After leaves stained for 40 min (NBT) or 2 h (DAB), then leaves were boiled in 95% ethanol for 15 min to remove the chlorophyll completely and stored in 60% glycerol for observation and photos taking.

To observe the dying cells in FB1-induced Arabidopsis leaves, Evans blue staining was performed as described [20]. Detached leaves were submerged in Evans blue solution (0.25%, w/v) for 5 h. Then the leaves were boiled in 95% ethanol for 15 min to remove the chlorophyll completely for observation and photos taking. The blue precipitates were solubilized with 1% (w/v) SDS in 50% (v/v) methanol at 50 °C for 20 min and quantified by measuring the absorbance at 600 nm.

2.3. GFP fluorescence analysis and chlorophyll quantification

Freshly prepared abaxial leaf strips expressing stroma-targeted GFP were incubated with treatments (10 μM FB1, 10 μM FB1 + 100 U ml^{-1} CAT, 10 μM FB1 + 1 mM AsA) or 0.14% methanol (Control) in 10 mM MES–NaOH (pH 5.5) at 23 °C for 5–48 h. The leaf strips were then placed in a small Petri dish containing 10 mM MES–NaOH (pH 5.5). Then microscopic observations were performed using a Zeiss LSM 510 META laser confocal scanning microscope (Zeiss, Jena, Germany). The GFP signal was visualized with excitation at 488 nm and emission at 500–550 nm using a band pass filter, and chlorophyll fluorescence (488 nm excitation) was visualized at 650 nm with a long pass filter. Various treated leaf strips were placed flat onto a plastic holder and fixed at both ends with silicon grease and the GFP fluorescence signal was measured in a luminescence spectrometer (Perkin–Elmer, LS55, UK), with an excitation wavelength of 488 nm and emission wavelengths between 500 and 600 nm (a slit width of 2.5 nm). The fluorescence intensity at 525 nm was used to quantify the relative GFP fluorescence.

The chlorophyll contents were measured according to Arnon [21]. Samples were ground in 95% ethanol together with 150 mg of washed quartz sand under dim light and homogenized for 5 min at 4 °C. The concentration was determined by spectrophotometric analysis of extracts of leaves in 95% ethanol at 649 and 665 nm.

2.4. Total RNA isolation and quantitative reverse transcript-PCR (qRT-PCR)

Total RNA was extracted according to the manufacturer's specifications using the TRIZOL reagent (Invitrogen). First-strand cDNA was synthesized with the SuperScript II First-Strand Synthesis System (Invitrogen) and cDNA were used as a template in PCR reactions with gene-specific primers (designed and synthesized by Sangon Biotech, Shanghai). Quantitative real time PCR (qRT-PCR) was performed using the Roche lightCycler™ 2.0 Real-time PCR Detection System. The relative abundance of *Actin2* was determined and used as the internal standard. PCR was performed using the following primers.

Actin2: (5′-AACGATTCCTGGACCTGCCTCATCATACTC-3′ and 5′-AGAGATTCAGATGCCCAGAAGTCTTGTTCC-3′).

PAL1 (5′-AACGGAGGAGGAGTGGACG-3′ and 5′-CTTTCATTTGCTC CGCTGC-3′).

2.5. PAL activity assay

PAL activity was determined according to a previous reported method with minor modifications [22]. Crude protein was extracted from samples stored at −78 °C. A 0.1 g sample was ground into a fine powder with liquid nitrogen and homogenized for 5 min at 4 °C in 1 ml of 0.1 M borate buffer (pH 8.8) containing 5% polyvinylpyrrolidone, 20 mM β-mercaptoethanol, and 1 mM EDTA. The mixture was centrifuged at 13400 rpm at 4 °C for 10 min. The supernatant was defined as a crude protein extract. Reaction mixtures consisting of 3.9 mL 0.1 M borate buffer (pH 8.8) and 100 μl enzyme extracts were preincubated at 37 °C (5 min) and the reaction was started by adding 1 mL 50 mM L-phenylalanine (Sigma–Aldrich, China). The mixture was incubated at 37 °C for 2 h and stopped by adding 0.2 mL 6 M HCl. PAL activity was assayed by measuring absorbance at 290 nm because of the release of