ARTICLE

NON-HOST RESISTANCE ACTIVITIES OF *Arabidopsis thaliana* INDUCED BY METHANOL EXTRACT OF MYCELIA FROM *Phytophthora infestans*

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ABSTRACT

Defense system of a non-host pathosystem involving the model plant *Arabidopsis thaliana* and methanol extract of mycelia (MEM) elicitor from *Phytophthora infestans* are presented in this article. *A. thaliana* leaves were analyzed for the induction of reactive oxygen species (ROS), hypersensitive reaction (HR) like cell death as well as defense gene expression by MEM elicitor. MEM elicitor induced O_2^- generation in *A. thaliana* leaves with dose dependent nature. HR occurred during the elicitation of *A. thaliana* challenged with MEM elicitor. Responses of intact *A thaliana* seedlings to MEM elicitor caused the retardation of growth of seedlings. Both plant biomass and growth of primary roots was markedly reduced by the treatment of MEM. MEM elicitor induced the expression of defense genes such as pathogenesis related proteins, *PR1*, *PR2*, *PR4 and PR5*, and *rbohF* encoding a ROS generation. These results suggested that MEM elicitor induced non-host defense responses in *A. thaliana*.

Keywords: methanol extract of mycelia, defense activities, *Phytophthora infestans*, *Arabidopsis thaliana*, elicitor

Abbreviations:
DMSO, Dimethyl sulfoxide; HWC, Hyphal wall components; L-012, 8-Amino-5-chloro-7-phenylpyridol[3,4-d] pyridazine-1,4(2H,3H) dione sodium salt; MES, 2-(*N*-morpholino)ethanesulfuric acid; MOPS, 3-(N-morpholino)propanesulphonic acid; MS medium, Murashige-Skoog basal medium; HR, Hypersensitive response; PCD, Programmed cell death; Rboh, Respiratory burst oxidase homolog; ROS, Reactive oxygen species.

INTRODUCTION

Plants are challenged by numerous pathogens throughout their life cycles and yet are able to fend off most infections. The native resistance of most plant species against a wide variety of pathogens is known as non-host resistance (NHR), which confers durable protection to plant species. Recognition of non-self is the key to the activation of innate defense mechanisms in plants in response to microbial attacks. The innate defense system depends on the detection of microbial elicitors that induces a cascade of defense responses that may include MAP kinase activity, the up-regulation of antimicrobial compounds, elicitation of inflammatory responses, the production of cytokines or mucins, apoptosis, nitric oxide-mediated responses, and the transcription of defense related genes (Chisholm *et al*, 2006; Medzhitov, 2007; Schwessinger and Zipfel, 2008).

The most notorious oomycetes are *Phytophthora* species, arguably the most devastating pathogens of dicot plants (Kamoun, 2003). *Phytophthora infestans* causes late blight, a devastating disease that results in multibillion-dollar losses in potato and tomato production (Garelik, 2002; Smart and Fry, 2001). Most plants, such as weeds and various crops, are resistant to *P. infestans*, and grow unimpaired in or next to fields with a high incidence of late blight (Kamoun *et al*., 1999). Understanding the molecular basis of non-host resistance to *P. infestans* will provide insight into a key molecular process and will offer novel perspectives for engineering durable late blight resistance in crop plants.

Species-specific elicitors have been described in *P. infestans* and other *Phytophthora* species and can trigger defense responses in non-

host plants. An extracellular transglutaminase that is conserved in *P. infestans* and other *Phytophthora* species induces defense responses in the non-host parsley (Brunner *et al*., 2002; Nurnberger and Brunner, 2002). *Arabidopsis thaliana* has emerged as a valuable model to study NHR in plants (Holub and Cooper, 2004; Yun *et al*., 2003). A series of penetration mutants were identified in the model plant *A. thaliana*, allowing considerable progress towards understanding NHR. Huitema *et al*. (2003) reported that active defense responses associated with non-host resistance of *A. thaliana* to the oomycete pathogen *P. infestans*.

Several plant defense responses are induced during the NHR response. Many defense responses are similar to those induced during gene-for-gene or host resistance. A rapid and transient production of reactive oxygen species (ROS), termed 'oxidative burst' is a hallmark of successful recognition of plant pathogens and the oxidative burst could have a direct effect on pathogen or the defenses caused in plant because of its reactivity (Torres, 2010). ROS are produced during both gene-for gene and NHR, even though the timing and amount might slightly vary between the two (Huckelhoven, 2001, Able *et al*., 2003).

ROS also associated with the hypersensitive reaction (HR), a localized response at the site of pathogen attack that displays programmed cell death and that could contribute to limit the spread of the pathogens or be a source of signals for establishment of further defenses (Mur *et al*., 2008). HR is associated with both host and non-host resistance to *P. infestans* (Vleeshouwers, *et al*., 2000). HR occurs at the infection site with apoptosis-like features. Despite controversial debate as to the role of ROS in the plant HR, they are generally regarded as causes of hypersensitive cell death (Delledonne *et al*., 2001, Torres *et al*., 2005).

In this study, we used methanol extract of mycelia (MEM) from *P. infestans* for the study of induced resistant reactions in non-host model plant *A. thaliana*.

MATERIALS AND METHODS

Plant materials

A. thaliana ecotype Columbia-0 (Col-0) plants were grown at 23°C and 70% humidity under a 16 h photoperiod and a 8 h dark period in environmentally controlled growth cabinets and 3-4 weeks old leaves were used for the experiments. For the study of plant growth, seeds were surface sterilized with 1% sodium hypochlorite, rinsed in water, and placed at 4°C for 3-4 days in darkness. Seeds were then plated on Murashige and Skoog (MS) media supplemented with 3% sucrose and solidified with 0.1% Phytagel. MEM elicitor was dissolved in water by adding 3% DMSO and was added separately into the medium and mixed well before gelation. Seeds were plated on medium containing MEM elicitor (or control medium) and effects assayed 6 days later.

Pathogenic isolate *Phytophthora infestans*

The pathogenic isolate [*Phytophthora infestans* (Mont.) De Bary], race 1.2.3.4 was used to culture for mycelia collection and were maintained at Plant Pathology laboratory of the Graduate School of Bioagricultural Sciences, Nagoya University. The fungi used as inocula for liquid culture were grown on rye-seed extract agar medium in a test tube at 18°C in the dark condition for 2 weeks. Parts of the growing mycelia were placed in 100 ml Erlenmeyer flasks containing 20 ml of rye liquid nutrient medium of rye seed-extract (60 g rye seed), 20 g of sucrose and 2 g of yeast extract per 1 liter of distilled water and incubated in dark condition for 2 weeks at 18°C to allow growth of the mycelia. The mycelial mats grown in the liquid medium were washed thoroughly with running tap water on a mesh (3 × 3 mm) to remove the growth medium and zoosporangia and finally washed with distilled water. To remove excess water, water was filtered through filter paper (Toyoroshi No. 2) under reduced pressure and then frozen at -20°C for preparation of MEM elicitor.

Preparation of MEM elicitor from *Phytophthora infestans*

The collected mycelia of *P. infestans* were frozen with liquid nitrogen. The frozen mycelia were ground by a mortar and pestle under a freezing temperature using liquid nitrogen. The ground mycelia were transferred to falcon tube (50 ml) containing methanol at the rate of 10 ml of methanol per 1 g mycelia. The mycelia suspension were finely ground using a polytron type homogenizer (HG30, Hitachi Koki, Japan) for 2 min. After centrifugation at 4°C, 3000 × g for 30 min, the supernatant was collected and dried by using an evaporator, and was used as MEM elicitor.

Measurement of ROS production

The relative intensity of ROS generation was measured by counting photons from L-012 (Waku, Osaka, Japan)-mediated chemiluminescence. In *A. thaliana* leaves, ROS measurement was done as described by Kobayashi *et al*. (2007) with little modification. For the detection of ROS production in leaf tissues, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was used to infiltrate in *Arabidopsis* leaves via a syringe without needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in the dark chamber at 20°C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan) and quantified using the U7501 program (Hamamatsu Photonics).

Detection of cell death

To detect cell death on the plant, elicitors were infiltrated by using a syringe without needle from opposite surface of the leaves. Cell death was measured by an electrolyte leakage method, which was adapted from the method described by Yeom *et al*. (2011). Leaves were infiltrated by elicitors under the leaf surface, and leaf discs (1 cm in diameter) were collected from the leaf 12, 24 and 48 h after treatment. Seven leaf discs were floated on 7 ml of distilled water for 2 h at room temperature and electrical conductivity was measured using a conductivity meter (Horiba, Kyoto, Japan).

Microscopic observation of cell death

To visualize plant cell death and colonization of *P. infestans*, leaves of *A. thaliana* were stained with lactophenol trypan blue as described by Takemoto *et al*. (2003) with a minor modification. Briefly, infected leaves were cleared in methanol overnight, and then the cleared tissue was boiled for 2 min in lactophenol trypan blue stain (10 ml H_2O, 10 ml lactic acid, 10 ml glycerol, 10 g phenol, and 10 mg trypan blue). After the leaves had been allowed to cool at room temperature for 1 h, the stain was replaced with 1 g/m1 chloral hydrate. Stained leaves were monitored using an Olympus microscope BX51 (Olympus).

RNA preparation and RT-PCR

Total RNAs were isolated from MEM treated frozen *Arabidopsis* leaves using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed as previously described (Kato *et al*., 2008). Expression of *Arabidopsis* defense related-genes after the treatment of elicitors was analyzed by RT-PCR. RT-PCR was conducted using a commercial kit (ReverTra-Plus-; Toyobo, Osaka, Japan). The cDNA was synthesized from total RNA (1 μg) with an oligo (dT) primer. After the cDNA synthesis reaction, the PCR was performed with denaturing, annealing, and extension temperatures of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, respectively. Gene-specific primers for each sequence were as follows:

AtUBQ5 forward primer, 5′-GTGGTGCTAAGAAGAGGAAGA-3′, *AtUBQ5* reverse primer, 5′-TCAAGCTTCAACTCCTTCTTT-3′, *AtPR1* forward primer, 5′-GTAGGTGCTCTTGTTCTTCCC-3′, *AtPR1* reverse primer, 5′-CACATAATTCCCACGAGGATC-3′, *AtPR2* forward primer, 5′-CTACAGAGATGGTGTCA-3′, *AtPR2* reverse primer, 5′-AGCTGAAGTAAGGGTAG-3′, *AtPR3* forward primer, 5′-AACAACCCTGACCTTGTTGC-3′, *AtPR3* reverse primer, 5′-

ACGTCCACACTCCAATCCAC-3′, *AtPR4* forward primer, 5′-CTTGTCCCGGTAACATCTGC-3′, *AtPR4* reverse primer, 5′-TGGAGCAATAAGCACTCACG-3′, *AtPR5* forward primer, 5′-CACATTCTCTTCCTCGTGTTC-3′, *AtPR5* reverse primer, 5′-TAGTTAGCTCCGGTACAAGTG-3′, *AtrbohD* forward primer, 5′-CATGAACTTGGGATTCTACGAGG-3′, *AtrbohD* reverse primer, 5′-AGACCTTTGAGTGCGTGGATGG-3′, and *AtrbohF* forward primer, 5′-GACGATGACACAATCGTTCTTCGT-3′, *AtrbohF* reverse primer, 5′-TTGAGCGAAATCGGAGCGATAGAT-3′.

RESULTS AND DISCUSSION

O_2^- producing activity

MEM elicitor contains relatively low molecular weight compounds because of limited solubility of high molecular weight compounds or protein in methanol. The oxidative burst (rapid and transient production of O_2^-) in *A. thaliana* plant was detected after MEM elicitor treatment. MEM was infiltrated into *A. thaliana* leaves at the concentration of 0, 0.25, 0.5, 0.75 or 1 mg/ml and incubated for 24 h. O_2^- producing activity was measured by using O_2^- unique luminous reagent L-012 (Fig. 1). MEM is a high O_2^- producing elicitor with dose dependent nature (Fig. 1A and B). Three % DMSO was used as a negative control because MEM elicitor was dissolved in 3% DMSO. Thus, MEM elicitor induced O_2^- generation in non-host *A. thaliana* as an early response and recognition of elicitor by plant.

Induction of cell death

HR-like cell death was examined in *A. thaliana* leaves. Ion leakage has been observed as an indicator of plant cell death (Rizhsky *et al*., 2002). In this experiment, average-sized *A. thaliana* leaves (21 days after germination) were selected and infiltrated by MEM elicitors by using syringe without needle from lower surface of the leaves. Leaf discs collected from treated leaves were floated on distilled water and shaken for 2 h at room temperature. Higher degree of ion leakage was detected in MEM elicitor-treated leaves after 12, 24 and 48 h compared to DMSO-treated leaves (Fig. 2A). The interaction between MEM elicitor and *A. thaliana* was examined using light microscopy after staining with lactophenol trypan blue of inoculated leaves because staining of plant cells with trypan blue is indicative of cell death. The densely stained epidermal and mesophyll cells were observed in the MEM treated leaves from 3 days after inoculation (Fig. 2B). On the other hand, no stained areas were developed in DMSO treated *A. thaliana* leaves. These results confirm that MEM causes cell death in non-host *A. thaliana* plant.

Root growth is arrested in response to MEM elicitor

To study the effects of MEM elicitor on root growth, we germinated seeds in the presence of the MEM elicitor and examined the effects on the roots after 6 days. Inhibition of growth of the *A. thaliana* seedlings treated with MEM elicitor was observed (Fig. 3A). Retardation of plant growth was apparent irrespective to seedling biomass and root growth (Fig. 3B and C). Thus, it was indicated that the treatment with MEM elicitor sensitized the plant which causes internal changes, and consequently root growth was markedly retarded.

Expression of defense-related genes

To investigate whether the defense-related genes are induced by MEM elicitor, total RNAs from MEM-treated *A. thaliana* leaves were extracted and were analyzed by RT-PCR (Fig. 4). We checked salicylic acid associated defense gene *PR* (pathogenesis-related protein) namely *AtPR1*, *AtPR2*, *AtPR3*, *AtPR4 and AtPR5*. High expression of *AtPR1*, *AtPR2*, *AtPR4* and *AtPR5* genes was found by MEM elicitor treatment. MEM elicitor also induced ROS production regulatory genes, *Rbohs* (respiratory burst

oxidase homologue). Expression of *StrbohF* gene was observed in *A. thaliana*. Therefore, MEM-induced gene expression of *AtrbohF* and PR gene series, *AtPR1*, *AtPR2*, *AtPR4* and *AtPR5*, are involved in non-host defense response in *A. thaliana*.

One of the most rapid defense reactions to pathogen attack is the so-called oxidative burst, which constitutes the production of O_2^- at the site of attempted invasion (Apostol *et al*., 1989). In the present study, it was shown that MEM elicitor from *P. infestans* induced O_2^- production in non-host *A. thaliana* (Fig. 1). The involvement of ROS in non-host response has been found in various plant-pathogen systems (Wen *et al*., 2011, Hückelhoven and Kogel, 2003, Nimchuk *et al*., 2003). ROS are produced during both gene-for gene and nonhost resistance, even though the timing and amount might slightly vary between the two (Huckelhoven, 2001, Able *et al*., 2003). These results, in conjunction with the present study, provide evidences that MEM elicitor from *P. infestans* induced ROS in non-host *A. thaliana*-elicitor or -pathogen system.

An elicitor can be recognized by the plant surveillance system and a defense reaction leading to HR will be activated (Mysore and Ryu, 2004). MEM elicitor induced HR in non-host *A. thaliana* (Fig. 2). Chloroplast-generated ROS play a major role in localized cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria* (Zurbriggen *et al*., 2009). Several lines of evidence from various plant species suggest that the sources of ROS are different during non-host response and during the HR, but these sources may interact with each other (Bindschedler *et al*., 2006, Soylu *et al*., 2005, Torres *et al*., 2005). These results indicate that both host and non-host specific interactions can provide similar kind of HR response even though the reduced degree of response appears in non-host interaction.

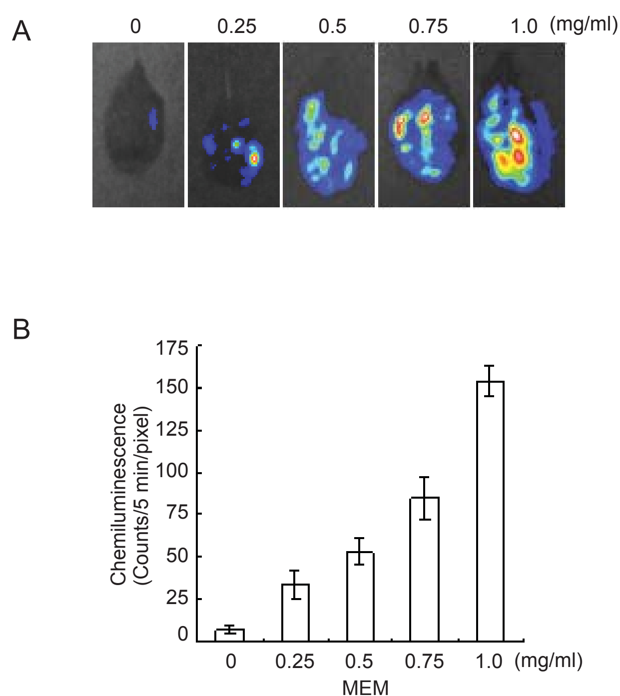

Fig. 1. O2- inducing activity of MEM in *A. thaliana* leaves. A, Leaves of *A. thaliana* ecotypes (Col-0) were treated with 0, 0.25, 0.5, 0.75 or 1.0 mg/ml MEM elicitor in 3% DMSO. Production of O2- was detected 24 h after treatment as L-012 mediated chemiluminescence. Chemiluminescence images shown were obtained using CCD camera. B, Intensities of chemiluminescence were quantified with photon image processor. Data are means standard deviation from three independent experiments.

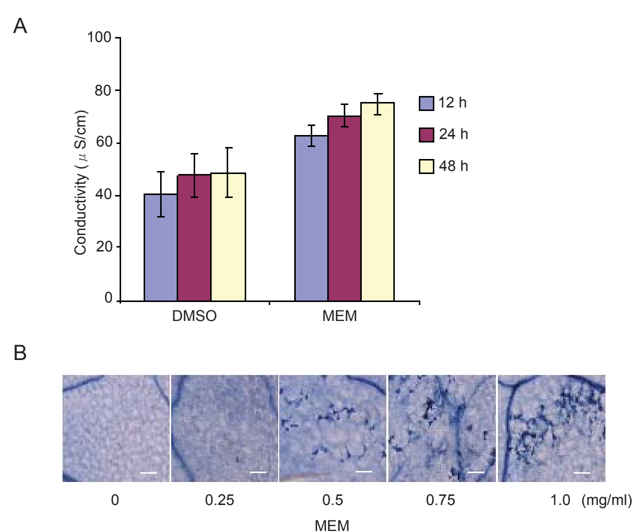

Fig. 2. Induction of cell death by MEM in *A. thaliana* leaves. A, Electrolyte leakage of dead cells in *A. thaliana* leaves. Leaves were infiltrated with 1.0 mg/ml of MEM and incubated for 12, 24 and 48 h. After treatment, leaf discs were floated on distilled water and shaken for 2 h at room temperature. Electrical conductivity was measured using a conductivity meter. Data are means standard deviation from five replications. B, Microscopic observation of hypersensitive cell death induced by MEM elicitor in *A. thaliana* ecotype (Col-0). Leaves of *A. thaliana* were treated with 0 l, 0.25, 0.5, 0.75 or 1.0 mg/ml MEM. Hypersensitive cell death was detected by trypan blue staining at 3 days after treatment. Bars = 200 mm

Root growth is highly responsive to environmental signals. To access the response of intact and growing plants to MEM elicitor, we examined the morphogenic changes in *A. thaliana* seedlings treated with the elicitor. Our results demonstrated that MEM induced the growth retardation (Fig. 3) concomitantly with the defense responses in *A. thaliana* seedlings. Kawaguchi *et al*. (2011) reported that fungal elicitor-induced retardation and its restoration of root growth in tobacco seedlings.

An incompatible interaction of a pathogen and a nonhost plant often induces several different defense signaling cascades, including generation of ROS, programmed cell death or HR in infected cells, and induction of PR genes. We examined the expression profiles of typical genes involved in various defense and metabolic pathways. Elicitor induced defense genes such as pathogenesis related proteins (*PR-1*, *PR-2*, *PR-5*) involved in systemic acquired resistance (Rogers and Ausubel, *1997*), and two homologues of the human respiratory burst oxidase (*gp91phox*)

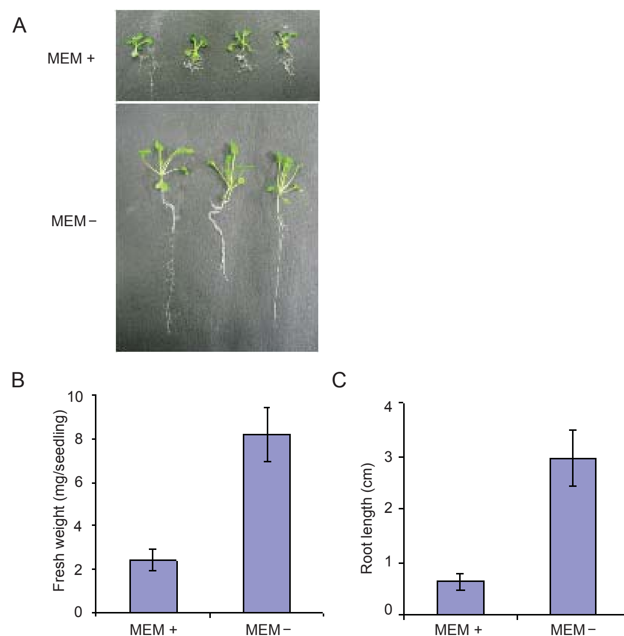

Fig. 3. Effect of MEM elicitor on growth of *A. thaliana* ecotype Col-0. A, *A. thaliana* were grown on MS media supplemented with or without 1.0 mg/ml MEM elicitor for 2 weeks. B, Fresh weight of *Arabidopsis* seedlings in response to 1.0 mg/ml MEM. Data are means SE from at least 10 seedlings. C, Root growth in response to 1.0 mg/ml MEM. Data are means SE from at least 10 seedlings.

such as *rbohD* and *rbohF* (Torres *et al*., *1998*). In our present study, well studied defense genes *AtPR1*, *AtPR2*, *AtPR4 and AtPR5*, and *AtrbohF* encoding ROS generation were induced by MEM elicitor regardless of the ROS levels in *A. thaliana* leaves. Thus, these results suggested that MEM elicitor induced non-host defense responses are activated in *A. thaliana*.

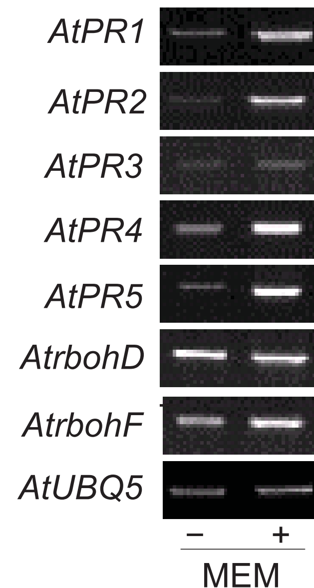

Fig. 4. Gene expression in MEM treated *A. thaliana* leaves. Total RNAs were isolated from *A. thaliana* leaves treated with MEM at concentration of 100 g/m for 24 h. Three % DMSO was used as a control. RNAs were analyzed by RT-PCR using specific primers for *AtPR1*, *AtPR2*, *AtPR3*, *AtPR4*, *AtPR5*, *AtrbohD*, and *AtrbohF*. Equal loads of cDNA were monitored by amplification of constitutively expressed *AtUBQ5*.

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