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Methyl jasmonate and ethylene induce partial resistance in *Medicago truncatula* against the charcoal rot pathogen *Macrophomina phaseolina*

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ABSTRACT

The soil-borne necrotrophic fungal pathogen *Macrophomina phaseolina* causes charcoal rot disease in many plant species. There are no effective control methods for this disease and no resistant host cultivar for *M. phaseolina* has been identified. Moreover, the host–pathogen interaction has not been investigated previously at the molecular level. In this study, we established a pathosystem for charcoal rot disease using the model legume *Medicago truncatula*. Using quantitative real-time PCR, we analyzed the expression of selected *M. truncatula* genes in response to *M. phaseolina* infection. Genes involved in flavonoid and isoflavonoid biosynthesis were strongly up-regulated in the shoot; however, activation of these genes in the root was not as dramatic. In addition, some genes in jasmonates (JAs) or ethylene (ET) pathways were not strongly induced in infected root tissue. Treating plants with methyl jasmonate (MJ) or ET induced partial resistance in *M. truncatula* plants. These results indicate that modifying JA/ET signalling pathways may improve plant resistance to *M. phaseolina* infection. This study provides a basis for further investigation of the molecular interactions between *M. Phaseolina* and its plant hosts.
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1. Introduction

Charcoal rot is a plant disease caused by the soil fungus *Macrophomina phaseolina*. This fungus has a wide range of plant hosts, readily invading over 500 different monocotyledonous and dicotyledonous species including various important crops such as soybean, sorghum, maize, alfalfa, etc [1]. *M. phaseolina* exists in soil as compact mass of hardened mycelial structures called sclerotia, which can remain dormant in soil for many years. Under appropriate conditions, hyphae germinated from the sclerotia infect the roots of plant hosts by penetrating the plant cell wall [2]. Interestingly, *M. phaseolina* is favored by hot and dry conditions, and the fungus can rapidly grow into the stalk and multiplies in the pith of the host when plants become stressed in hot and dry summers [3]. The disease symptoms range from leaf yellowing and wilting to plant death. After decay of infected plants, sclerotia are released into the soil and the infection cycle can start again in following years. This disease can also be transmitted through infected seeds [4]. Charcoal rot disease can result in severe loss in hot and dry years and drastic weather changes due to global warming pose a serious threat to crops that are susceptible to *M. phaseolina*. Charcoal rot disease used to be seen mostly in southern states in the US, but now it has been found in northern states as well, such as Iowa, Ohio, Illinois [5]. The common strategies that are currently applied to control the disease are crop rotation and irrigation; however, these approaches are not always effective. The search for naturally existing resistance cultivars resulted in little success [6], and lack of knowledge of the molecular processes occurred during *M. phaseolina*–host interactions prevents us to engineer resistant plants.

In order to better understand the host responses to *M. phaseolina* infection at the molecular level, we established a model pathosystem for charcoal rot using *Medicago truncatula*. *M. truncatula* is the primary model legume species for genomic and functional genomic research because of its relatively small genome size and available genomic resources, such as high-density genetic and physical maps [7,8], almost completely sequenced genome [9], and different types of mutant populations, including EMS, fast-neutron deletion and insertion mutants that are available for genetic screen [10,11]. The Affymetrix Medicago Gene Chip and Medicago Gene Atlas are available for high-throughput transcriptome studies [12]. In addition, *M. truncatula* is relatively easy to transform, which makes it suitable for reverse genetics experiments.

Abbreviations: dpi, day-post-inoculation; JAs, jasmonates; MJ, methyl jasmonate; ET, ethylene; SA, salicylic acid; SAR, systemic acquired resistance; RT-qPCR, quantitative real-time PCR.

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To study the host–pathogen interaction at the molecular level, we examined the expression of 14 *M. truncatula* genes in response to *M. phaseolina* infection using real-time quantitative PCR (RT-qPCR). These genes represent various disease response pathways, including flavonoid/isoflavonoid biosynthesis, reactive oxygen species (ROS) homeostasis, salicylic acid (SA) induced systemic acquired resistance (SAR) pathway, jasmonates (JAs) and ethylene (ET) response pathways. We also examined the effects of methyl jasmonate (MJ) and ethephon on *M. truncatula*'s response to *M. phaseolina*. The data suggest that MJ and ethephon could induce partial resistance in *M. truncatula* against the charcoal rot pathogen. To our knowledge, this is the first study to examine the molecular interactions between *M. phaseolina* and its plant host. We hope the gained knowledge and future research in this area will bring hope to engineering resistant plants to *M. phaseolina*.

2. Materials and methods

2.1. Plant materials

M. truncatula cv. *Jemalong A17* seeds were treated with concentrated sulfuric acid for 8 min, rinsed five times with distilled water, followed by surface sterilization with 20% bleach. After sterilization, the seeds were rinsed three times with sterilized distilled water and germinated on half-strength Murashige and Skoog (MS) medium (1% agar) (Sigma–Aldrich, St. Louis, MO, USA) at room temperature in the dark for 3 days. The seedlings were then transplanted to plastic pots filled with wet sterilized soil (Sun Gro Horticulture, Bellevue, WA, USA) and grown in the growth chamber (27 °C, 12-h photoperiod, 44% of relative humidity [RH], photon flux density 150–200 $\mu mol\ m^{-2}\ s^{-1}$). When the plants were 4 weeks old, they were inoculated with the fungal pathogen as described below. To minimize variations caused by soil-grown conditions, plant materials used in RT-qPCR were grown in Magenta boxes containing half-strength MS medium (1% agar) (pH 5.7) supplied with Gamborg vitamins (Phytotechnologies Laboratories, Shawnee Mission, KS, USA) and 1% sucrose.

2.2. M. phaseolina isolate and preparation of inoculum

The *M. phaseolina* isolate used in this study was kindly provided by Dr. Nancy Brooker (Pittsburg State University, Pittsburg, KS, USA). The isolate was cultured on potato dextrose agar (PDA) at 27 °C for 3 days to obtain actively growing mycelium. Then, 3 agar pieces of roughly 1 cm^2 in size were cut and placed in a beaker containing 250 ml of potato dextrose broth (PDB). The liquid culture was incubated at 27 °C for 14 days. The fungal mat grown on top of the PDB was collected by using a sterile spatula and air dried for 24 h at room temperature. The dried materials containing mostly sclerotia were grinded to fine powder and stored at 4 °C. For preparing wheat seeds used in inoculating plants grown in Magenta box, agar pieces containing *M. phaseolina* were used to inoculate sterilized wheat seeds prepared in a 250 ml flask and the seeds were incubated at 27 °C (44% relative humidity) for 4 days until they were evenly colonized.

2.3. Inoculation procedure

For soil assay, 4-week-old *M. truncatula* plants were un-potted, and rinsed with distilled water to remove dirt on the root. The plant roots were submerged in *M. phaseolina* sclerotia suspension (1 g of dried sclerotia in 10 ml 0.015% agarose) for 30 s, then re-potted. The low percentage of agarose helps to evenly suspend the sclerotia and also keeps the sclerotia sticking onto plant roots. Control plants were dipped in 0.015% agarose only. To better control the amount of inoculum among different samples used in RT-qPCR, 2-week-old plants grown in Magenta boxes were inoculated with a single wheat seed colonized by *M. phaseolina* near the root. Control plants were mock inoculated with one sterile wheat seed.

2.4. Microscopic analyses

Plant roots were placed on a glass slide and cut into 2–3 mm in length. The roots were first soaked in 0.5 N NaOH for 3 min to soften the tissue. Then a drop of lactophenol cotton blue was added onto the root tissue to stain the fungal hypha. A cover slip was placed on the top and the samples were gently pressed to crush the roots. The prepared slides were observed under the microscope and images were taken with the attached digital camera.

2.5. Chemical preparation and application

Four-week-old plants were sprayed with either 0.1% (v/v) MJ (Sigma–Aldrich, St. Louis, MO, USA), or 3.0 mM ethephon (2-chloroethyl phosphonic acid) (Sigma–Aldrich, St. Louis, MO, USA), or the combination of both for three days before inoculation. MJ solution was prepared freshly by diluting the stock solution in water containing 0.01% of Tween-20. Ethephon acts as a direct source of ET once applied to plants. It was freshly prepared by dissolving in water containing 0.01% Tween-20 to a final concentration of 3.0 mM. Mock treatment was water with 0.01% Tween-20. For SA treatment, 4-week-old plants were sprayed with 100 mM methyl salicylate containing 0.01% Tween-20 for three consecutive days before inoculation. Plants sprayed with the chemicals were covered with a plastic dome and kept in a separate area from untreated plants.

2.6. Large scale hormone treatment

One hundred twenty 4-week-old plants were divided into four groups, each group was treated with either mock control, MJ, ET or MJ plus ET for three days prior to inoculation. Disease symptoms were monitored from 1 dpi to 7 dpi using digital imaging and a scoring matrix in the scale of 0–6 (0: no detectable symptom; 1: 1–10% chlorotic or 1–5% necrotic; 2: 10–20% chlorotic or 5–10% necrotic; 3: 20–40% chlorotic or 10–20% necrotic; 4: 40–60% chlorotic or 20–40% necrotic; 5: 60–80% chlorotic or 40–60% necrotic; 6: >80% necrotic or dead plant). Data were analyzed using Two-way ANOVA in GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

2.7. Real-time quantitative PCR

Total RNA was isolated from each sample using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction, and the samples were treated with TURBO DNase (Applied Biosystems, Foster City, CA, USA) to remove DNA contamination. To make cDNA, 1 μg of total RNA from each sample was mixed with 1 μl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 1 μl RNasin (Promega, Madison, WI, USA), 2 μl DTT (100 mM), 1 μl Oligo dT primer (20 μM), 4 μl 5× reaction buffer in a 20 μl reaction. Primers used in RT-qPCR were kindly provided by Dr. Srinivasa Rao Uppalapati (The Samuel Roberts Noble Foundation, Ardmore, OK, USA) [13], and the primer concentration was optimized using control samples. RT-qPCR reactions were set up as 10 μl reaction containing 5 μl of Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA), 1 μl of each primers (100 nM), and 1 μl diluted cDNA sample (1:20). All real-time PCR reactions were performed in a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA, USA). *β-Tubulin* (*TUB*) was used