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Gene expression profiling of *Macrophomina phaseolina* infected *Medicago truncatula* roots reveals a role for auxin in plant tolerance against the charcoal rot pathogen

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ABSTRACT

The soil-borne necrotrophic fungal pathogen *Macrophomina phaseolina* causes charcoal rot disease in a wide range of plant species. There are no effective control methods for this disease and no genetic resistance for *M. phaseolina* has been identified in most field crops. Currently, little is known about the molecular mechanisms involved in host susceptibility and resistance to *M. phaseolina*. Using *Medicago truncatula* as a model, we analyzed the global gene expression profile of *M. truncatula* roots infected by *M. phaseolina*. MAPMAN analyses identified genes involved in jasmonic acid and ethylene pathways, which are important for plant resistance against necrotrophs. In addition, genes involved in auxin homeostasis, polar auxin transport and auxin signalling were also regulated by the infection process. The differential expression patterns of these auxin-related genes suggested that the host susceptibility maybe partly due to the suppression of auxin response in the host by *M. phaseolina*. In additional, *M. truncatula* plants treated with exogenous auxin gained partial resistance against *M. phaseolina*, further suggesting the potential role for auxin in plant defense against this necrotrophic pathogen.

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1. Introduction

The soil-borne fungus *Macrophomina phaseolina* (*M. phaseolina*) is a necrotrophic fungal pathogen that causes charcoal rot disease in over 500 different plant species, including various important crops such as soybean, sorghum, maize, alfalfa. Charcoal rot has a wide geographical distribution, and the disease has been reported in southern and north-central regions of the United States [1,2], and in tropical and subtropical regions of the world [3–5]. *M. phaseolina* overwinters as sclerotia in the soil and infected plant debris and can remain viable for several years. Under favorable conditions (e.g. higher soil temperatures and low water potential), the sclerotia germinate and colonize the plants [6]. *M. phaseolina* can grow rapidly in infected plants and produce large amount of sclerotia that clog the vascular tissue, resulting in disease symptoms ranging from leaf yellowing, wilting to plant death [7]. Charcoal rot can result in severe crop losses due to reduced yield and low seed quality. For instance, the soybean yield reduction due to charcoal rot in the United States was valued at $173.8 million in year 2002 alone [8]. Currently, there is no effective management approach available for charcoal rot. Moreover, development of resistant plants via genetic engineering is not feasible due to the lack of knowledge on the molecular processes occurred during *M. phaseolina*–host interactions.

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* [9]. *M. truncatula* is closely related to the important forage crop alfalfa (*Medicago sativa*), which is often infected by *M. phaseolina* in North America [10]. *M. truncatula* plants infected with *M. phaseolina* show disease symptoms such as wilting and leaf yellowing at 1 day-post-inoculation (dpi), and most plants die by 4 dpi. Microscopic examination of infected roots showed that initial entry occurred within 24 h, and colonization happened rapidly around 36–48 h-post-inoculation (hpi) [9].

In current study, we analyzed the global gene expression profiles of *M. truncatula* roots at initial entry and colonization stages of *M. phaseolina* infection process using Affymetrix Medicago Genome Array. The study has identified differentially expressed genes belonging to several metabolic pathways. Like many other necrotrophs, *M. phaseolina* induced the expression of genes in jasmonic acid (JA) and ethylene (ET) pathways, which is consistent

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with the findings in our previous study [9]. Interestingly, the gene expression profiling also revealed that *M. phaseolina* modulates the expression of several auxin-related genes at the initial entry stage, indicating a possible role for auxin in establishing the compatible interaction. The expression analyses of auxin signalling-related genes indicated that the host susceptibility to *M. phaseolina* is perhaps partially due to suppression of the auxin response by the pathogen. In addition, plants treated with the active auxin, indole-3-acetic acid (IAA), were more tolerant to *M. phaseolina*. Furthermore, the effect of IAA was blocked by auxin polar transport inhibitor 2, 3, 5-triiodobenzoic acid (TIBA). These results suggest that auxin maybe another hormone that is involved in the host defense response against *M. phaseolina*.

2. Materials and methods

2.1. Plant materials

M. truncatula cv. Jemalong A17 was used in the study. Seed treatment and plant growth conditions were described previously [9].

2.2. Inoculation procedure and disease assessment

The preparation of the fungal inoculum and inoculation procedure were previously described [9]. Disease symptoms were monitored using three different methods. Leaf symptoms were analyzed using a scoring matrix in the scale of 0–6 as previously described [9]. To monitor symptoms in the root, plants were removed from the pots, rinsed clean with tap water and padded dry with paper towel. The lower stem sections and roots were excised and the fresh weights were measured. The weight of inoculated plant was divided by the weight of un-inoculated plant to calculate the relative root weight. The excised root tissues were then used to prepare materials for determining the colony forming unit (CFU) based on the method described by Mengistu et al. [11] with modifications. Basically, roots of three plants with the same treatment condition were pooled together and surface sterilized in 0.6% NaOCl for 3 min and rinsed four times with sterile distilled water. The air-dried roots were ground using a mortar and pestle and thee ground tissue was mixed with 12 ml warm PDA (50 °C) supplemented with carbenicillin (50 μg/ml), and the liquid was then quickly poured on to three different carbenicillin (50 μg/ml) PDA plates. After incubating at 30 °C for three days, CFUs were counted and converted to CFU per gram of fresh tissue. The Student *t*-test was performed using Graphpad Prism (GraphPad Software, La Jolla, CA, USA) with $P < 0.05$.

2.3. RNA preparation

Plants were grown in Magenta boxes containing half-strength Murashige and Skoogsalt (pH 5.7, 1% agar) (Sigma–Aldrich, St. Louis, MO, USA) supplied with 1% sucrose. Two-week-old plants were inoculated with sterile wheat seeds (control) or *M. phaseolina*-colonized wheat seeds. Roots were harvested from control plants or *M. phaseolina*-inoculated plants at 24, 36 and 48 h to prepare RNAs for microarray and the follow-up RT-qPCR experiments. For IAA-treated samples, roots were harvested 5 h after the plants were transplanted to medium supplied with 10 μM IAA in agar-based assay, or 5 h after the plants have been dipped in 100 μM IAA insoil-based assay. Roots from control plants that were not treated with IAA were harvested at the same time. Total RNA for each sample was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. RNA samples were then treated with TURBO DNase (Applied Biosystems, Foster City, CA, USA) to eliminate DNA.

2.4. Microarray experiment

Total RNAs isolated from three individual plants for each treatment were pooled together to represent one biological replicate. Samples were processed from three independent experiments to produce three biological replicates. For microarray experiment, total RNA was further purified using the Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). RNA samples were sent to the microarray facility at the Samuel Roberts Noble Foundation (Ardmore, OK, USA) for the array experiment. RNA was quantified and evaluated for purity using a Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Willington, DE, USA) and Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). For each sample, 10 μg of total RNA was used for the expression analysis of each sample using the Affymetrix GeneChip® Medicago Genome Array (Affymetrix, Santa Clara, CA, USA). Probe labeling, chip hybridization and scanning were performed according to the manufacturer's instructions for one-cycle labeling (Affymetrix). Data normalization between chips was conducted using RMA (Robust Multichip Average) [12]. Presence/absence calls for each probe set were obtained using dCHIP [13]. Gene selections for pairwise comparison were made based on Associative Analysis [14] in Matlab (MathWorks, Natick, MA, USA). In this method, the background noise presented between replicates and technical noise during microarray experiments was measured by the residual presented among a group of genes whose residuals are homoscedastic. Genes whose residuals between the compared sample pairs that are significantly higher than the measured background noise level were considered to be differentially expressed. A selection threshold of 2 for transcript ratios and a Bonferroni-corrected *P* value threshold of 8.15954E-07 were used. The Bonferroni-corrected *P* value threshold was derived from 0.05/*N* in these analyses, where *N* is the number of probes sets (61,278) on the chip. False discovery rate of all significant genes was monitored with Q-Values obtained by EDGE software [15,16]. Genes that showed at least two-fold changes were selected for pathway construction using MAPMAN. MAPMAN ontology was adapted to *M. truncatula* genes represented on the Affymetrix array. The mapping file was generated to group genes on the array into different functional groups with specific BIN numbers. The mapping file then was used to map the affected genes onto different pathways using ImageAnnotator [17]. The data and complete MIAME information are deposited to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) with accession number E-MEXP-3580.

2.5. Real-time quantitative PCR

To make cDNA, 1 μg of total RNA for each sample was mixed with 1 μl Supercript 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 5X reaction buffer in a 20 μl reaction. RT-qPCR reactions were set up using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) with gene-specific primers and diluted cDNA (1:10) samples. Primers used in RT-qPCR were listed in Supplemental file Table 1. The primer concentrations were optimized using control samples. All real-time PCR reactions were performed in a StepOne Plus real-time PCR machine (Applied Biosystems, Foster City, CA, USA). *β-Tubulin* (*TUB*) was used as the reference gene and the control treatment was used as the calibrator. The relative abundance of each gene was analyzed using the comparative C_T method with the formula $RQ = 2^{-\Delta\Delta C_T}$ ($\Delta\Delta C_T = \Delta C_T$ (treatment) $-\Delta C_T$ (calibrator), in which $\Delta C_T = C_T$(test gene) $- C_T$(TUB)). The RQ of the calibrator was set to 1. Primers used