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Transcriptomic response of *Arabidopsis thaliana* after 24 h incubation with the biocontrol fungus *Trichoderma harzianum*

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ABSTRACT

Trichoderma harzianum is a fungus used as biocontrol agent using its antagonistic abilities against phytopathogenic fungi, although it has also direct effects on plants, increasing or accelerating their growth and resistance to diseases and the tolerance to abiotic stresses. We analyzed *Arabidopsis thaliana* gene expression changes after 24 h of incubation in the presence of *T. harzianum* T34 using the Affymetrix GeneChip *Arabidopsis* ATH1. Because this microarray contains more than 22,500 probe sets representing approximately 24,000 genes, we were able to construct a global picture of the molecular physiology of the plant at 24 h of *T. harzianum–Arabidopsis* interaction. We identified several differentially expressed genes that are involved in plant responses to stress, regulation of transcription, signal transduction or plant metabolism. Our data support the hypothesis that salicylic acid- and jasmonic acid-related genes were down-regulated in *A. thaliana* after 24 h of incubation in the presence of *T. harzianum* T34, while several genes related to abiotic stress responses were up-regulated. These systemic changes elicited by *T. harzianum* in *Arabidopsis* are discussed.

Introduction

Alternative and ecological strategies are necessary and in demand for disease management in order to reduce the use of pesticides in agriculture. Thus, the use of biological control agents and enhancers of plant defenses such as plant growth-promoting rhizobacteria (PGPR) or several strains of the beneficial fungus *Trichoderma* spp. is a good approach to reach this healthy and environmentally adequate objective.

Some *Trichoderma* strains are commonly used as biocontrol agents in agriculture for their abilities to produce antibiotics (Vinale et al., 2008; Rubio et al., 2009) or/and hydrolytic enzymes (Benitez et al., 2004) that directly antagonize plant-pathogenic fungi. Some *Trichoderma* strains also promote plant growth and development, and thus increase yields (Harman et al., 2004; Lorito et al., 2010), with *Trichoderma harzianum* being the most frequently cited species as an active matter in a variety of commercial biopesticides and biofertilizers (Monte, 2001; Harman et al., 2004; Harman, 2011; Vinale et al., 2008).

In addition to their role as biocontrol agents, *Trichoderma* spp. also have other beneficial effects on plants, including stimulation of defenses, root development, growth promotion and activation of seed germination or amelioration of abiotic stresses (Shoresh et al., 2010). It has been reported that the presence of *Trichoderma* primes the systemic resistance system (Tucci et al., 2011), a complex signaling mechanism involving jasmonic acid (JA)/ethylene (ET)-induced systemic resistance (ISR) and/or salicylic acid (SA)-dependent systemic acquired resistance (SAR) pathways, although priming may not occur universally in plant-*Trichoderma* interactions (reviewed by Shoresh et al., 2010).

The proteome and transcriptome of plants change as a consequence of the interaction with *Trichoderma* metabolites (Marra et al., 2006) or root colonization (Alfano et al., 2007; Segarra et al., 2007; Shoresh and Harman, 2008; Bae et al., 2011). Thus, the fungi reprograms plant gene expression, resulting in changes of plant responses to their environment (Harman, 2011). The availability of microarrays and an annotated genome provide the possibility of obtaining genome-wide insight into the trascriptomic response of a given plant under interaction with other organisms. Because *Trichoderma* spp. trigger rapid transcriptomic responses in the plant

Abbreviations: ISR, induced systemic resistance; PGPRs, plant growth-promoting rhizobacteria; SAR, systemic acquired resistance.

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tissues, more information about how the gene expression in plant is modulated in relation to the presence of these biocontrol agents is needed to contribute to a global picture of the molecular physiology of the plant during the interaction with *Trichoderma*. In this work, we used a transcriptomic approach to analyze systemic responses induced in *Arabidopsis thaliana* after 24 h of incubation in the presence of *T. harzianum* T34, and we discuss the systemic changes in *Arabidopsis* genes related to biotic and abiotic stress conditions.

Materials and methods

Fungal strain

Trichoderma harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) (referred to as T34 along the paper) was the strain used in this work. *T. harzianum* T34 was grown on Potato Dextrose Agar (PDA, Difco Becton Dickinson, Sparks, MD, USA) and spores were maintained at −80 °C in a 30% glycerol solution.

Plant material

Arabidopsis thaliana Col-0 ecotype plants were used throughout this work. Seeds were surface sterilized in 70% (v/v) ethanol and 1% (v/v) Triton X-100, and finally, washed four times in sterile distilled water. Stratification of the seeds was conducted during 3 days at 4 °C. Afterwards, they were grown in a growth chamber under 40% humidity, a temperature of 22 °C, and with a 16 h light/8 h dark photoperiod from 80 up to 100 $\mu E\, m^{-2}\, s^{-1}$ in pots containing a 1:3 vermiculite/soil mixture. Twenty five-days-old Arabidopsis plants were cultured in a 10-mL Erlenmeyer flask containing 8 mL of liquid Murashige and Skoog (MS) medium containing 10^5 conidial germlings mL^{-1} of *T. harzianum* T34, which were incubated in an orbital shaker at 80 rpm at 25 °C for 24 h. In parallel, *Arabidopsis* plants were cultured in MS medium without fungus (control). Five Erlenmeyer flasks (3 *Arabidopsis* plants per flask) were used for each condition. Conidial germlings were obtained from 15 h-old cultures of strain T34 in 200 mL of minimal medium (MM) (Penttilä et al., 1987) shaken at 200 rpm and incubated at 25 °C. After 24 h of *T. harzianum*-plant interaction or control culture, the aerial parts were collected from *Arabidopsis* plants, frozen, and kept at −80 °C until total RNA extraction. In addition, after 6 h of interaction *Arabidopsis* roots were collected to analyze the expression of PR-1 gene in this part of the plant.

Detection of Trichoderma in plant tissue

To verify that *T. harzianum* T34 is able to colonize the *Arabidopsis* roots but not leaves, we performed an experiment following the procedure described by Segarra et al. (2009). Briefly, root and leaf segments, from 25-day-old *Arabidopsis* plants incubated for 24 h in the presence or absence of *T. harzianum* as above indicated, were surface sterilized by immersing in 95% ethanol (2 min), followed by sodium hypochlorite (4% available chlorine; 5 min) and then washed three times with sterile water. Samples were then allowed to dry on sterile filter paper for 10 min in a sterile laminar flow chamber. Segments were placed horizontally on selective medium and plates were incubated for 7 days in order to check the presence or absence of strain T34 from *Arabidopsis* tissues.

RNA isolation, cDNA synthesis and microarray hybridization

Total RNA was obtained from aerial parts of *Arabidopsis* plants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. RNA from five biological replicates was pooled. The integrity and pureness of the RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). cDNAs were synthesized from 1 μg of total RNA using the Superscript Choice System for synthesis kit (Invitrogen, Gaithersburg, MD, USA), and used for hybridization to Affymetrix GeneChip ATH1 genome array using the GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) and according to protocols described in the Gene Expression Analysis Technical Manual (http://www.affymetrix.com). Three technical replicates were performed.

Microarray hybridization data analysis: normalization and differential gene expression

Digitalization of the fluorescent signals emitted after the hybridization was performed with the Gene Array Reader (Affymetrix) and the Genespring Software GX11 (Agilent Technologies). RMA algorithm was used in normalization of samples. We compare two groups (3n) using control samples as reference. BoxWhisker graph and PCA analysis were used in order to validate all samples in the study. A T Test paired with a 0.05 corrected *p*-value cut-off using an asymptotic *p*-value computation and no Multiple Testing Correction was used to discriminate genes between both groups. GO analyses were performed using the same software.

Real-time PCR analysis

Quantitative real-time PCR was performed using an AB PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Roche) as previously described in Montero-Barrientos et al. (2010). The following specific primers were used and checked for dimer formation: *PR-1*, forward: 5′-GGCTAACTACAACTACGATG-3′ and reverse: 5′-GGCTTCTCG TTCACATAATTC-3′; *SEN1*, forward: 5′-CCACTGCTTTTAACACAAC-ATCAC-3′ and reverse: 5′-TGTCGTTGCTTTCCTCCATCGG-3′; ABC transporter, forward: 5′-CATATCGGTGAGATGACTGTTCG-3′ and reverse: 5′-CATCACATTTGTTTTTTCACCTGC-3′; IAA amidase, forward: 5′-GTCGCACTGACCGTGAGACTTTC-3′ and reverse: 5′-TCTC-CTGTCTAGCTCCTCTTCG-3′; cytochrome P450 (*CYP79B2*), forward: 5′-TGTGGCTATAACCTTAGTGAT-3′ and reverse: 5′-GCCGTTAGAGA-GGATCTTCTG-3′; *RAB18*, forward: 5′-GGAGAAGTTGCCAGGTCATC-3′ and reverse: 5′-ACCGGGAAGCTTTTCCTTGAT-3′; *RD29*, forward: 5′-AGAAGGAATGGTGGGGAAAG-3′ and reverse: 5′-CAACTCACTT-CCACCGGAAT-3′; and actin, forward: 5′-CTCCCGCTATGTATGTC-GCC-3′, reverse: 5′-TTGGCACAGTGTGAGACACAC-3′.

Results and discussion

Trichoderma is widely used as biocontrol agent because of the multiple beneficial effects on plant growth and disease resistance (Harman et al., 2004), producing molecular cross-talk between the plant and the fungus (Tucci et al., 2011). This molecular dialogue is beginning to be understood and transcriptomic and proteomic approaches (Alfano et al., 2007; Bailey et al., 2006; Shoresh and Harman, 2008; Segarra et al., 2009) provide information about changes induced in plant by these beneficial fungi.

Transcriptomic analysis through microarrays is a powerful tool to study gene expression profiling. Thus, we performed microarray analysis using *Arabidopsis* as a plant model system and the Affymetrix GeneChip *Arabidopsis* ATH1 Genome Array, which contains more than 22,500 probe sets representing approximately 24,000 genes, in order to ascertain into physiological and biochemical changes in the host plant elicited by the beneficial fungus *T. harzianum* T34. Fig. 1 shows an optical microscope image of *Arabidopsis* roots colonized by strain T34 obtained from a 24 h plant-fungus culture.