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Suppression Subtractive Hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1

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ABSTRACT

Trichoderma species include widespread rhizosphere-colonising fungi that may establish an opportunistic interaction with the plant, resulting in growth promotion and/or increased tolerance to biotic and abiotic stresses. For this reason, *Trichoderma*-based formulations are largely used in agriculture to improve yield while reducing the application of agro-chemicals. By using the Suppression Subtractive Hybridization method, we identified molecular mechanisms activated during the *in vitro* interaction between tomato (*Solanum lycopersicum* L.) and the selected strain MK1 of *Trichoderma longibrachiatum*, and which may participate in the stimulation of plant growth and systemic resistance. Screening and sequence analysis of the subtractive library resulted in forty unique transcripts. Their annotation in functional categories revealed enrichment in cell defence/stress and primary metabolism categories, while secondary metabolism and transport were less represented. Increased transcription of genes involved in defence, cell wall reinforcement and signalling of reactive oxygen species suggests that improved plant pathogen resistance induced by *T. longibrachiatum* MK1 in tomato may occur through stimulation of the above mechanisms. The array of activated defence-related genes indicates that different signalling pathways, beside the jasmonate/ethylene-dependent one, collaborate to fine-tune the plant response. Our results also suggest that the growth stimulation effect of MK1 on tomato may involve a set of genes controlling protein synthesis and turnover as well as energy metabolism and photosynthesis. Transcriptional profiling of several defence-related genes at different time points of the tomato–*Trichoderma* interaction, and after subsequent inoculation with the pathogen *Botrytis cinerea,* provided novel information on genes that may specifically modulate the tomato response to *T. longibrachiatum*, *B. cinerea* or both.

1. Introduction

Soil-borne beneficial microbes, like plant growth promoting rhizobacteria (PGPR) or fungi (PGPF), rhizobia, and mycorrhizal fungi, are well-known plant stimulators and can protect plants from abiotic and biotic stresses (Pozo and Aguilar, 2007; Bonfante and Genre, 2010; Berendsen et al., 2012; Caporale et al., 2014; Ruocco et al., 2015; Vos et al., 2015).

The direct and indirect biocontrol activity of rhizosphere-competent fungi of the genus *Trichoderma* is widely recognized (Harman et al., 2004; Shoresh et al., 2010). Several strains are also

Abbreviations: BCA, biocontrol agent; ET, ethylene; DPI, days post inoculation; ISR, induced systemic resistance; LHC, light-harvesting complex; MAMPS, Microbe-Associated Molecular Patterns; PGP, plant growth promotion; PGPF, plant growth promoting fungi; PGPR, plant growth promoting rhizobacteria; SAR, Systemic Acquired Resistance; SGN, SOL Genomics Network; SSH, Suppression Subtractive Hybridization.

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able to stimulate plant growth (Lorito and Woo, 2015), improve nutrient uptake (Zhao et al., 2014), and contribute to plant hormonal balance and volatile production (Harman et al., 2004; Shoresh et al., 2005; Battaglia et al., 2013; Ruocco et al., 2015). It has also been demonstrated that these PGPF can protect plants from abiotic stresses and affect their direct and indirect resistance to insect pests (Bae et al., 2009; Mastouri et al., 2010, 2012; Battaglia et al., 2013; Brotman et al., 2013; Caporale et al., 2014). Thanks to their proved efficacy, a number of *Trichoderma* strains have been selected for application in agriculture, with a few hundreds of formulations registered worldwide, while several strains have been deeply studied at the laboratory level for their peculiar biological and genetic features (Lorito and Woo, 2015).

The molecular mechanisms that regulate direct *Trichoderma* biocontrol activity have been ascertained (Atanasova et al., 2013), those enabling *Trichoderma* strains to promote indirect defence against pathogens and pests and, even more so, plant growth have not been fully uncovered. Several reports indicate that the ability of *Trichoderma* spp. to activate induced systemic resistance (ISR) against pathogen infections is mediated by jasmonate (JA)- and ethylene (ET)-dependent mechanisms and requires transient expression of defence genes (Shoresh et al., 2005; Korolev et al., 2008). Moreover, long-lasting up-regulation of salicylic acid (SA)-responsive genes was demonstrated in tomato interacting with *Trichoderma harzianum* T22, whose modulation, together with JA-induced gene expression, contributed to increased resistance to the pathogen *Botrytis cinerea* (Tucci et al., 2011). Recently, increasing evidence is accumulating that both JA/ET and SA signalling may be triggered by *Trichoderma* in crop and model plants (Segarra et al., 2007; Tucci et al., 2011; Mathys et al., 2012; Perazzolli et al., 2012; Martinez-Medina et al., 2013). Only a few studies have addressed the molecular mechanisms underlying the promotion of plant growth by *Trichoderma* species. Auxin signalling was demonstrated to be important for biomass production induced by *Trichoderma virens* (Contreras-Cornejo et al., 2009) and increased transcription of IAA-related genes was observed in *Arabidopsis thaliana* roots after *T. harzianum* inoculation (Brotman et al., 2013). Moreover, proteomic approaches indicated increased photosynthesis and carbohydrate metabolism in *Trichoderma*-treated plants (Segarra et al., 2007; Shoresh and Harman, 2008), which were suggested to be related to enhanced growth response.

It is generally assumed that establishment of the plant–*Trichoderma* interaction triggers an extensive transcriptional reprogramming of genes involved in defence, growth and secondary metabolism, though the alteration of gene expression levels is often quantitatively small (Alfano et al., 2007; Shoresh and Harman, 2008; Moran-Diez et al., 2012). In search for genetic components of this beneficial response that undergo small transcriptional changes, we used PCR-Select cDNA Suppression Subtractive Hybridization (SSH), which has been proven useful for the identification of rare differentially expressed transcripts, to study transcriptome remodelling of tomato plantlets interacting with the MK1 strain of *Trichoderma longibrachiatum*. This *Trichoderma* strain was selected since it has been well characterised *in vitro* and has a demonstrated ability to increase plant growth and enhance pathogen resistance (Battaglia et al., 2013; Ruocco et al., 2015). Moreover, it is a rich source of bioactive molecules (Ruocco et al., 2015), some of which are the subject of a few patent applications.

Our results identified several plant genes activated by *T. longibrachiatum* strain MK1, which may mediate, at least in part, the ability of this PGPF to stimulate plant growth as well as ISR against pathogen infections. These genes were fully annotated and available transcriptomic data of tomato and *Arabidopsis* were exploited to obtain indications on their possible role and regulation by different signals. Further transcriptional characterisation showed that some defence-related transcripts are induced by the PGPF but not by inoculation with the pathogen *B. cinerea* and suggested that alleviation of disease symptoms in *Trichoderma*-treated plants may be achieved by boosting the expression of some components of the plant defence machinery while maintaining others at a low transcription level.

The repository of differentially expressed genes produced in this study can therefore be useful to acquire further knowledge on the mechanisms activated by *Trichoderma* strains to stimulate ISR, while also contributing to unravel the less known plant growth promotion (PGP) activity, thus supporting the development of more effective biostimulator formulations. Moreover, this gene catalogue represents a useful tool for designing new breeding strategies for the selection of crop varieties with improved ability to benefit from the interaction with *Trichoderma*.

2. Materials and methods

2.1. Fungal strain and plant material

T. longibrachiatum PGPF strain MK1 (referred to as MK1 along the paper), isolated from tomato roots, was obtained from the collection of the Department of Agricultural Sciences of the University of Naples Federico II, Italy and grown on potato dextrose agar. Colonies were allowed to sporulate at 25 °C in the dark for 7 days, then collected by washing the plates with sterile distilled water and brought to a concentration of 10^9 mL^{-1}.

Solanum lycopersicum cv. 'Crovarese' seeds were kindly provided by La Semiorto Sementi, Sarno (SA, Italy). The seeds, sterilized in 2% sodium hypochlorite for 20 min and washed in sterile distilled water, were germinated *in vitro* on Murashige and Skoog (MS) solid medium (Duchefa Biochemie, Haarlem, Netherlands) with 3% (w/v) sucrose at 24 °C and 16 h light/8 h dark photoperiod with an irradiance of 80 μmol m^{-2} s^{-1} until second true leaf appearance.

In vitro-grown plantlets were then transferred to petri dishes (12 × 12 cm) on half strength MS medium with Mk1 pre-germinated spores and grown as above indicated. Inoculum was diluted at a concentration of 1×10^5 spores mL^{-1} and applied at a rate of approximately 0.02 mL per plant. Mock inoculated plantlets were treated using the same conditions, but with sterile water. Control and treated shoots were collected at 1, 2 and 3 days post inoculation (DPI) and immediately frozen and kept at −80 °C until total RNA extraction. To reduce the impact of biological variation, at least six plantlets at 2 DPI were pooled for the construction of the subtractive library. Moreover, at least three biological replicates of 3–4 plantlets each were collected at each time point for the qRT-PCR analyses.

For *B. cinerea* infection, sterilized tomato seeds were incubated in a 10^6 mL^{-1} fresh MK1 spore suspension (treated) or in water (control) according to Tucci et al. (2011). Germinated seeds were transferred to pots in soil and grown in the greenhouse of the CNR-IBBR in Portici for two months.

2.2. Botrytis cinerea *infection*

B. *cinerea* strain 309, isolated from tobacco, was obtained from the culture collection available at the Department of Agricultural Sciences, University of Naples Federico II, Italy. Untreated control and MK1-treated 2-month-old tomato plants were infected with *B. cinerea* by inoculating the third true leaf with 10 μL of a 10^6 mL^{-1} spore suspension of the pathogen as reported in Tucci et al. (2011). Three plants per treatment were used for the inoculation. Immediately before infection, the fourth leaf of control and MK1-treated plants of each replicate were collected as the uninfected control. At 48 h after inoculation, the fifth or sixth leaf from each replicate