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Relevance of trichothecenes in fungal physiology: Disruption of *tri5* in *Trichoderma arundinaceum*

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ABSTRACT

Trichothecenes are sesquiterpenoid mycotoxins produced mainly by *Fusarium* species. Harzianum A (HA), a non-phytotoxic trichothecene produced by *Trichoderma arundinaceum*, has recently been found to have antagonistic activity against fungal plant pathogens and to induce plant genes involved in defense responses. In the present work, we have shown that disruption of the *T. arundinaceum tri5* gene, which encodes a terpene synthase, stops the production of HA, alters the expression of other *tri* genes involved in HA biosynthesis, and alters the expression of *hmgR*, *dpp1*, *erg9*, *erg1*, and *erg7*, all genes involved in terpene biosynthetic pathways. An increase in the level of ergosterol biosynthesis was also observed in the *tri5* disrupted transformant in comparison with the wild type strain. The loss of HA also resulted in a drastic reduction of the biocontrol activity of the transformants against the phytopathogenic fungi *Botrytis cinerea* and *Rhizoctonia solani*. Finally, the effect of *tri5* gene disruption on the regulation and balance of intermediates in terpene biosynthetic pathways, as well as the hypothetical physiological role of trichothecenes, both inter- and intracellularly, on regulation and biocontrol, are discussed.

1. Introduction

Trichoderma is well known for the ability of some of its strains to act as important biocontrol agents against phytopathogenic fungi (Harman et al., 2004; Lorito et al., 2010). In interactions with plants, it has been shown that some *Trichoderma* strains can act as biofertilizers, as inducers of plant defense responses, and also can increase tolerance to abiotic stresses (Shoresh et al., 2010; Hermosa et al., 2012). Many *Trichoderma* strains produce primary or secondary metabolites and enzymes with diverse industrial interest. Among the metabolites produced by *Trichoderma,* trichothecenes have attracted attention in recent years because they are described as important mycotoxins, with phytotoxicity as well as animal and human toxicity (Desjardins, 2006; McCormick et al., 2011). *Trichoderma* species of the Brevicompactum clade, *Trichoderma brevicompactum* and *Trichoderma arundinaceum*, produce two different trichothecenes, trichodermin and Harzianum A (HA), respectively. While trichodermin is known to be phytotoxic, Harzianum A is not. Thus, HA serves as a model of a non-phytotoxic trichothecene produced by a fungal strain that shows a significant biocontrol activity (Malmierca et al., 2012), and can be used to study the impact of trichothecenes in fungal physiology.

The genes involved in the biosynthesis of trichothecenes (*tri* genes) in *T. brevicompactum and T. arundinaceum* have been recently cloned and characterized (Cardoza et al., 2011; Tijerino et al., 2011; Malmierca et al., 2012). It was shown that the *tri* genes are clustered, similar to that which occurs in *Fusarium*, but these two genera have important differences in the genetic organization of these genes. The *Fusarium tri* genes are located at three different *loci*, with the main *locus* including 12 genes with *tri5* located at a central position. The *tri5* gene in both *Fusarium* and *Trichoderma* encodes trichodiene synthase, a terpene cyclase that catalyzes the first step of the trichothecene biosynthetic pathway, converting farnesyl diphosphate (FPP) to trichodiene (Hohn and Van Middlesworth, 1986; Hohn and Desjardins, 1992; Cardoza et al., 2011). In contrast, the "main cluster" of *tri* genes in *Trichoderma* does not contain the *tri5* gene, and includes only seven genes with a marked difference in their relative organization in comparison with that of the *tri* genes of *Fusarium* (Cardoza et al., 2011). This is the only trichothecene producer described so far in which *tri5* is not located in the main *tri* cluster. In *Trichoderma*, trichodiene is oxygenated at the C2, C11, and C12 positions by the Tri4 protein (trichodiene monooxygenase) giving rise to isotrichodiol, which is non-enzymatically converted to 12,13-epoxytrichothecene (EPT),

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and afterwards oxygenated at C4 by the Tri11 protein (trichothecene monooxygenase) to produce trichodermol. The latter is finally acylated by the Tri3 protein (trichodermol acyltransferase) that adds a 2,4,6 octatriendioyl side chain to the C-4 hydroxyl group to produce HA (Fig. 3B) in *T. arundinaceum* or an acetyl to the C-4 hydroxyl group in *T. brevicompactum* to produce trichodermin (Cardoza et al., 2011; Tijerino et al., 2011). Thus, *tri5* and the gene/s hypothetically involved in the biosynthesis of the octatrienedioyl side chain, the latter of which has not yet been cloned, would be in other genomic locations.

Previously we described the characterization of a *tri4* gene disrupted transformant (Malmierca et al., 2012). Surprisingly, this transformant showed traces of HA production as well as production of 12,13-epoxytrichoene-2-ol derivatives, the production of which may have been due to one or more non-specific P450 oxygenases. In that *tri4* disrupted transformant, an increase in total chitinase activity and a reduction in biocontrol activity were detected. However, it was not determined if these observed results were due to the disruption of the *tri4* gene alone or from the production of the additional 12,13-epoxytrichoene-2-ol derivatives.

In the present work, we describe the isolation of a *T. arundinaceum* mutant carrying a disrupted *tri5* gene. This mutant allowed us to characterize the effect of a total lack of trichothecene production on several phenotypic characteristics of *Trichoderma*, as well as genotypic influences on the level of expression of other genes. Five genes in the terpene biosynthetic pathway were chosen for the study. *dpp1* is involved in the dephosphorylation of several isoprenoid phosphates, including FPP conversion to farnesol (Faulkner et al., 1999), a compound that acts as an important regulator of the cellular levels of FPP. *hmgR*, encodes hydroxymethylglutaryl CoA (HMG-CoA) reductase that catalyzes the production of mevalonate from HMG-CoA. This gene/enzyme is regulated at several levels, including transcription, translation, and enzyme degradation (Goldstein and Brown, 1990; Meigs and Simoni, 1997; Leichner et al., 2011). *erg9* encodes squalene synthase, the enzyme that is responsible for the first specific step of ergosterol biosynthesis, converting FPP into squalene. *erg9* has been described as a key target in the regulation of ergosterol biosynthesis (Asadollahi et al., 2010). *erg1* encodes squalene epoxidase, an enzyme involved in the oxygenation of squalene giving rise to oxidosqualene in a reaction that implies the entry of the squalene in an energy costly branch that produces ergosterol (Leichner et al., 2011). Lastly, *erg7* encodes oxidosqualene cyclase, which is involved in the cyclization of oxidosqualene to produce lanosterol (Corey et al., 1994).

Finally, we describe the effect of *T. arundinaceum tri5* disruption on antifungal activity.

2. Materials and methods

2.1. Strains, culture media, and culture conditions used in the present work

T. arundinaceum IBT 40837 (=Ta37) (IBT Culture Collection of Fungi at the Department of Biotechnology, Technical University of Denmark) was kindly provided by Ulf Thrane. For trichothecene analysis and RNA isolation, *T. arundinaceum* strains were grown using a two-step procedure in CM (0.5% malt extract, 0.5% yeast extract, 0.5% glucose) followed by growth in PDB modified medium as described previously (Cardoza et al., 2011). For ergosterol and squalene quantification, Ta37 and TaΔTri5 spores were inoculated in CM medium and incubated at 28 °C, 250 rpm, for 24 h. Then, 4 g (wet weight) of filtered mycelia were added to 100 ml of PDB modified medium and incubated at 28 °C, 250 rpm, for 24 and 96 h.

Phytopathogens *Botrytis cinerea* 98, isolated from diseased strawberry plants, *Rhizoctonia solani* CECT 2815, *Myrothecium roridum* ATCC 52485, and *Fusarium sporotrichioides* CECT 20166 were used as targets in the dual confrontation assays. *B. cinerea* and *R. solani* were also included in growth assays on membranes.

All fungal strains were routinely maintained on PDA (2.4% PDB, 2% agar), except *B. cinerea* and Ta37, which were maintained on MEA (2% glucose, 2% malt extract, 1% peptone, 2% agar, pH 5.6) and PPG (2% mashed potatoes, 2% glucose, 2% agar), respectively.

Three *Solanum lycopersicum* varieties: var. Marmande (Semillas Battle S.A., Barcelona, Spain), var. Tres Cantos and var. Muchamiel (Rocalba S.A., Girona, Spain) were used for fungal-plant interaction studies.

2.2. Construction of pΔtri5 and pUStri5 plasmids

A 784 bp fragment (GenBank accession number FR715494) of the *tri5* gene (from 54 to 837 bp) of Ta37 was amplified using iProof High-Fidelity DNA Polymerase (BioRad, Hercules, CA) and primer pair 2100/2101 (Table S1a, Supplementary data) (the latter primer with an *Asc*I restriction site at the end) using genomic DNA as template. The amplicon was band-purified with the Ultraclean DNA purification kit (MoBio, Carlsbad, CA) and cloned into pCR™4 Blunt-TOPO® (Invitrogen, Carlsbad, CA). The resulting plasmid was cut with *Asc*I and ligated with a chimeric hygromycin B gene (2.5 kb) (Turgeon et al., 1987), which contained *Asc*I restriction sites at both ends of the chimera, leading to the final 7.3 kb pΔtri5 vector (Fig. S1A, Supplementary data).

For construction of the pUStri5 plasmid, intact *tri5* was amplified by PCR using *Pfu* polymerase and the primer pair 2114/2115 (Table S1a, Supplementary data). The 1.2 Kb DNA fragment was gel purified and cloned into the *Bam*HI restriction site of pAN52.1 (Punt et al., 1987). The resulting plasmid was digested with *Stu*I and *Hind*III and the 3.8 Kb band that contained the *tri5* expression cassette was gel purified and cloned into the *Eco*RI restriction site of pUSR0 (Cardoza et al., 2006a), thus obtaining the 17.1 Kb plasmid pUStri5 with the phleomycin resistance gene as selectable marker (Fig. S2B, Supplementary data).

2.3. Transformation of T. arundinaceum

The host fungus was transformed with plasmid pΔtri5 using a protoplast transformation protocol as described previously (Malmierca et al., 2012). Transformants were selected by hygromycin B (100 μg/ml) resistance. The selected transformants were analyzed by PCR using the primer pairs 2114/2115 and 2114/T7 (Table S1a, Supplementary data) using the Terra™ PCR Direct Polymerase Mix (Clontech, Mountain View, CA) and by Southern hybridization to detect those with the pΔtri5 vector inserted into the *tri5* gene.

Strain TaΔTri5 was transformed with pUStri5 plasmid by using *Agrobacterium*-mediated transformation as described previously (Cardoza et al., 2006a). The transformants were selected by phleomycin (30 μg/ml) resistance, and after two rounds of growth in selective medium, transformants were analyzed by PCR using the primer pair 2114/pAN52-trpC (Table S1a, Supplementary data) and the same procedures as above.

2.4. Extraction and chemical analysis of HA

Cultures of wild-type and transformants were analyzed by high-performance liquid chromatography (HPLC) for HA as previously described (Cardoza et al., 2011).

2.5. Antifungal assays

2.5.1. Direct confrontation assay

In vitro confrontation assays between *Trichoderma* strains (Ta37 and the disruptant strain TaΔTri5) and the pathogens *R. solani*,