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Detection of putative peptide synthetase genes in *Trichoderma* species: Application of this method to the cloning of a gene from *T. harzianum* CECT 2413

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Abstract

Some of the secondary metabolites produced by *Trichoderma*, such as the peptaibols and other antibiotics, have a peptide structure and in their biosynthesis are involved proteins belonging to the Non-Ribosomal Peptide Synthetase family. In the present work, a PCR-mediated strategy was used to clone a region corresponding to an adenylation domain of a peptide synthetase (PS) gene from 10 different strains of *Trichoderma*. In addition, and using the fragment isolated by PCR from *T. harzianum* CECT 2413 as a probe, a fragment of 19.0 kb corresponding to a PS-encoding gene named *salps1*, including a 1.5 kb fragment of the promoter, was cloned and sequenced. The cloned region of *salps1* contains four complete, and a fifth incomplete, modules, in which are found the adenylation, thiolation and condensation domains, but also an additional epimerization domain at the C-terminal end of the first module. The analysis of the Salps1 protein sequence, taking into consideration published data, suggests that it is neither a peptaibol synthetase nor a protein involved in siderophore biosynthesis. The presence of two breaks in the open reading frame and the expression of this gene under nitrogen starvation conditions suggest that *salps1* could be a pseudogene.

Keywords: *Trichoderma*; Non-ribosomal peptide synthetase; Degenerated-PCR; Antibiosis; Pseudogene

1. Introduction

Trichoderma species are used as biological control agents of important plant-pathogenic fungi. Some species of this genus are active as mycoparasites and have been tested in field experiments and successfully shown to be effective against a range of economically important aerial and soil-borne plant pathogens [1].

Mycoparasitic strains of *Trichoderma* produce cell wall-degrading enzymes (CWDEs) and antibiotics. One of the main modes of action of *Trichoderma* is the production of a large variety of secondary metabolites: volatile (e.g. ethylene, hydrogen cyanide, alcohols, aldehydes and ketones up to C_4 chain-length), and non-volatile compounds, including peptide antibiotics (e.g. peptaibols) [2]. Peptaibols, a class of linear peptides with 5–20 residues, have three structural characteristics:

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(a) a high proportion of α,α' dialkylated amino acids with an abundance of α-isobutyric acid (Aib), (b) a N-acyl terminus, usually acetyl, and (c) a C-terminal amino alcohol, such as phenyl-alaninol or leucinol [3,4]. Peptaibols generally exhibit antimicrobial activity against Gram-positive bacteria and fungi [5]. Peptaibols have been shown to act synergistically with CWDEs to inhibit the growth of fungal pathogens [6,7].

Another well-known mechanism of action of *Trichoderma* species is the competition for nutrients. Under iron starvation, as a potential means of competition in the soil, most fungi excrete at least one type of siderophore in order to solubilize the environmental iron. Fungal siderophores are typically short peptides containing also non-proteinogenic amino acids [8]. Iron-chelating siderophores have been described as biocontrol tools in some strains of *Trichoderma* [9], but they have not been characterized. In contrast, three types of hydroxamate siderophores have been described in *T. virens*: a monohydroxamate (*cis*- and *trans*-fusarinines), a dipeptide of *trans*-fusarinine (dimerum acid), and a trimer disdepsipeptide (copragen) [10].

Peptide antibiotics with an unusual amino acid content (like peptaibols) and siderophores, in bacteria and fungi, are often produced non-ribosomally by large multifunctional peptide synthetases (NRPSs). These large multifunctional enzymes assemble compounds from a wide range of precursors (including non-proteinogenic amino acids and hydroxy or carboxyl acids) [11].

NRPSs are organized into repetitive synthase units or modules, each of which has the functions required to complete a different single amino acid elongation step in the synthesis of the peptide product. Each module can be further partitioned into distinct adenylation (A), thiolation (T) and condensation (C) domains, which together represent a minimal repeating unit of NRPSs [12]. The functions of each unit include ATP-dependent activation to form a peptide bond (A), transfer of the acyl adenylates to specific thiols located in the enzyme-bound cofactors (4′-phosphopantetheine) (T), and condensation to form a peptide bond (C). The modification of the incorporated monomers (e.g. by epimerization or N-methylation) or the peptide backbone (e.g. by acylation, glycosilation or heterocyclization) can further functionalize the peptide product. These tailoring reactions are catalyzed by specialized domains or by fusion to polyketide synthase (PKS) modules [11,13–15]. In most NRPSs, the organization and order of the modules maps in a 1:1 manner to the amino acid sequence of the peptide products (co-linearity rule) [16].

In *Trichoderma* species, only one entire peptide synthetase gene (*tex1*) has been cloned and characterized (the largest NRPS so far), an 18-module peptaibol synthetase from *T. virens* [17]. A partial sequence (almost identical to *tex1*) from the final module of a putative siderophore synthetase has also been obtained from another *T. virens* strain [18].

The conservation of consensus sequence motifs in the domains provides a tool for detecting and cloning peptide synthetase genes in *Trichoderma* and other species using a degenerate PCR-based approach [17,18].

Analysis of the phenylalanine adenylation domain of the gramicidin synthetase, *Grs*A, has been used to determine the key residues responsible for A-domain specificity in substrate recognition. These have been called signature sequences [16,19]. It is hoped that a sufficiently large collection of verified signature sequences will provide a NRPS "codon" table allowing prediction of amino acids substrates based on the signature sequences in uncharacterized NRPSs.

In this article, we describe a method for the detection of peptide synthetase genes in different *Trichoderma* species through a PCR-based approach, and its application to the cloning of a partial peptide synthetase gene from the *T. harzianum* CECT 2413 (T34).

2. Materials and methods

2.1. Fungal isolates

Ten strains were used in this study: *Trichoderma asperellum* T3 (International Mycological Institute, Egham, UK, IMI 20179), *T. atroviride* T11 (IMI 352941), *T. harzianum* T14 (IMI 306222), *T. harzianum* T24 (IMI 352940), *T. asperellum* T25 (IMI 296237), *T. harzianum* T34 (Spanish Type Culture Collection, Valencia, Spain, CECT 2413), *T. atroviride* T35 (IMI 281112), *Trichoderma* sp. T37 (IMI 296235), *T. longibrachiatum* T44 (IMI 304058) and *T. longibrachiatum* T52 (NewBioTechnic, Sevilla, Spain, NBT52). Cultures were maintained on Potato Dextrose Agar (PDA, Difco) at 25 °C.

2.2. DNA and RNA manipulations

Mycelia for DNA extraction were grown in liquid cultures (200 rpm) at 28 °C in potato dextrose broth (PDB, Difco). Hyphae were collected by filtration, washed with distilled water, frozen and lyophilized. Fungal genomic DNA was isolated according to previously described protocols [20].

For Northern analysis, mycelia were grown in minimal medium (MM) [21] containing 2% glucose as carbon source (200 rpm) at 28 °C for 36–48 h. Then, the mycelia were harvested by filtration, washed with sterile water and placed into fresh MM containing different carbon sources: 2% glucose, 0% glucose for absence of carbon source, 1.5% chitin (*N*-acetylglucosamine polymer, Sigma), or 0.5% fungal cell walls from the strawberry pathogen *Colletotrichum acutatum*. Nitrogen starvation conditions corresponded to a 100-fold decrease in the