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Ferricrocin, the intracellular siderophore of *Trichoderma virens*, is involved in growth, conidiation, gliotoxin biosynthesis and induction of systemic resistance in maize

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ABSTRACT

Fungal siderophores are known to be involved in iron acquisition and storage, as well as pathogenicity of mammals and plants. As avirulent plant symbionts, *Trichoderma* spp. colonize roots and induce resistance responses both locally and systemically. To study the role of intracellular siderophore(s) in *Trichoderma*-plant interactions, we have obtained mutants in a non-ribosomal peptide synthetase, TvTex10, that was predicted to be involved in intracellular siderophore(s) biosynthesis. This gene has a detectable basal level of expression and is also upregulated under iron-deplete conditions. This is unlike two other siderophore-encoding genes, which are tightly regulated by iron. Disruption of *tex10* gene using homologous recombination resulted in mutants with enhanced growth rate, reduced conidiation and hyper-sensitivity to oxidative stress as compared to wildtype strain. The mutants also produced reduced levels of gliotoxin and dimethyl gliotoxin but have enhanced ability to colonize maize seedling roots. The mutants were also impaired in induction of induced systemic resistance (ISR) in maize against the foliar pathogen *Cochliobolus heterostrophus*.

1. Introduction

Trichoderma spp. are widely used as biofungicides and agents for enhancing plant health by alleviating abiotic stresses [1,2]. *Trichoderma*-based formulations are reported to occupy about 60% of the total biofungicides market [3]. *Trichoderma virens* is an important member of this group with commercial formulations being available [4,5]. Apart from being a well-known mycoparasite, *T. virens* is also studied extensively for its ability to externally and internally colonize roots and induce systemic resistance (ISR) in plants [6–8]. Several studies show that *T. virens* initiates ISR through the secretion of elicitor-like proteins and secondary metabolites [9]. Vargas et al., [10], demonstrated that symbiotic interactions between *T. virens* and plant roots are driven by plant-derived sucrose. An intracellular invertase and a sucrose transporter are intimate partners in the symbiotic association between *T. virens* and maize [10,11]. Interestingly, the deletion of invertase resulted in enhanced root colonization by *T. virens*. The intracellular siderophore ferricrocin plays an important role in iron homeostasis of filamentous fungi and is known to be involved various Fe-related metabolic processes. In *Aspergillus fumigatus* for example, ferricrocin is involved in storage, intra- and transcellular iron distribution, oxidative stress tolerance, germination and sexual development. Furthermore, this intracellular iron chelator has been shown to be a virulence factor in the pathogenicity of this fungus in mammals [12–15]. Ferricrocin is essential for sexual development in *Cochliobolus heterostrophus* and *Gibberella zeae* [16]. In the insect pathogen *Metarhizium robertsii,* ferricrocin is involved in full virulence against *Spodoptera exigua* [17]. Ferricrocin has been shown to be involved in symbiotic (endophytic) association between *Epichloe*

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festucae and its host ryegrass (*Lolium perenne*) [18]. The beneficial fungus *T. virens* interacts with plants in a symbiosis-like association acquiring nutrients and, in return providing defense against biotic and abiotic stresses [1]. In this study we tested the hypothesis that the intracellular iron storage siderophore is involved in *Trichoderma*-plant interactions leading to its ability to colonize roots and trigger systemic resistance.

2. Methods

Expression of tex10 relative to other siderophore biosynthesis NRPS genes. Iron-free glass ware was prepared according to Oide et al., [16]. Conidial suspensions (10^6/ml) of *T. virens* were inoculated into 100 ml Vogel's minimal medium supplemented with 1.5% sucrose (VMS) without any iron supplement (iron depleted) or supplemented with of 10 μM $FeSO_4$ (iron replete) and incubated shaking at 27 C. After three days, mycelia were harvested, frozen in liquid nitrogen and used for RNA extraction (for expression studies) as well as extraction of intracellular siderophores as described in Oide et al. [16]. RNA was extracted using TriReagent (MRC) and treated with DNA-free reagent (Ambion) for removing traces of DNA. 100 ng of RNA from each sample was subjected to Real-time PCR amplification and the fold increase in mRNA was calculated relative to histone (*h3*) transcript, using the ΔΔCt method [19]. The primers used were FeRTf (CAAGACGCGTTTCACCTTCTTG) and FeRTr (CGCTGTCCATTTGATCTCGC). For determining the expression of other two putative extracellular siderophore encoding NRPSs (tex20/SidD and Tex21/NPS6), SidDRTf (GAACCTTGATAAAACACGAAGAGC), SidDRTr (GGCATCATCTGTCTCACAAACG), NPS6RTf (CAAAGATATCGCCACGGCTG), and NPS6RTr (GGTCGCGATGATGTTTGATTG) were used. The same primers were used for a semi-quantitative RT-PCR where 100 ng cDNA were amplified using Phusion flash polymerase (Thermo) for 22 cycles.

For HPLC analysis, ferrated siderophores were purified by Amberlite XAD-16 column [16]. HPLC was performed in an Agilent Technologies 1200 liquid chromatograph equipped with Phenomenex Prodigy ODS-3 5 μm (4.6 × 250 mm) column and diode array detector. The mobile phase was a linear gradient of mQ-H_2O and acetonitrile, with both containing 0.1% trifluoroacetic acid and the flow rate was 1.00 mL/min. The gradient set points were 6% acetonitrile (0 min), 15% (10 min), 20% (15 min), 30% (20 min), 100% (26 min), 100% (29 min), 6% (30 min), and 6% (34 min). The chromatogram signals were at 254 nm and 435 nm (bandwidth 20 nm), which were referenced to 550 nm (bandwidth 50 nm). The spectra of the peaks were 210–600 nm in 2.00 nm steps. The ferricrocin standard (kind gift from Hubertus Haas) was assayed alongside for identification of the intracellular siderophore.

Generation of tex10 loss-of-function mutants. Loss-of-function mutants were generated by single cross-over homologous recombination as described earlier for *T. virens* [20]. Briefly, part of the *nps2* ORF (+4.5 kb to +6.5 kb) was amplified using the primer pair FeSals (CAG GGA GCA GTC GAC GAA G) and FeKpnas (GCA TGG CAG GTACCTGAA CTG), digested with SalI and KpnI and integrated into pATBS (harboring the hygromycin resistance cassette under the TrpC promoter) predigested with the same set of enzymes. The resulting plasmid (pFeSCO) was used to transform *T. virens* protoplasts with selected colonies screened by PCR with FeOuts (CAA TAC ATG CTG CAG TAA CCA C), a primer located upstream of FeSals, and M13F (a primer located within pBlueScript). This strategy identifies homologous recombination events in the *tex10* locus. The putative recombinants were further purified by repeated single-spore isolation, and the purity of the mutants confirmed by PCR using FeOuts and FeOutas (CTG CGA TCT GAG CCG ATA TCT C, located downstream of FeKpnas). The conditions used for this PCR reaction (Taq polymerase and 3 min extension) does not yield any product for homologous recombinants, but amplifies a 2 kb band in wild type. The mutation was further confirmed by Southern hybridization and HPLC analysis of the intracellular siderophores of WT and the mutants.

Chemical analysis of compounds. For analysis by LC-MS, *T. virens* Gv 29-8 wild type and mutant strains were cultivated in liquid minimal medium. The medium composition was as follows: glucose (0.5%,m/V)), L-asparagine (0.5%, m/V), K_2HPO_4 (0.08%, m/V), KNO_3 (0.07%, m/V), $MgSO_4.7H_2O$ (0.05%, m/V), $CaCl_2.2H_2O$ (0.02%, m/V), $MnSO_4.4H_2O$ (0.001%, m/V), $ZnSO_4.7H_2O$ (0.001%, m/V), $CuSO_4.5H_2O$ (0.0005%, m/V) and chloramphenicol (30 ppb, m/V). For each strain three independent biological cultivations with four technical replicates (each inoculated from the same spore suspension) were performed. For inoculation, conidia were obtained from colonies grown 2–3 days on malt extract agar (2% m/v) and suspended in water containing 0.03% (v/v) Tween 80. The spore suspension was adjusted to a transmission of 0.31 using BIOLOG (Biolog Inc. Hayward, CA, USA) turbidimeter at O.D. 590 nm. Cultivation (25 °C, 12 h light/dark cycle) was done using 24-well plates (Greiner, Germany) containing 2 mL liquid minimal medium and 100 μL conidial suspension. Mycelial growth was measured at O.D. 750 nm after 24, 48, 72 and 96 h. Samples were harvested after 96 h by filtration through disposable syringe filters (0.45 μm cellulose, Asahi Glass co., LTD., Japan) and samples were immediately prepared for LC-HRMS analysis. A detailed protocol of the $^{54}Fe/^{56}Fe$ isotope pattern assisted screening approach for siderophores has been described elsewhere [21]. In brief, 10 μL of aqueous $FeCl_3$ solution [2% (m/V) $FeCl_3$, 10% (v/v) formic acid] were added to 990 μL of culture filtrate prior to full scan LC-HRMS measurements. Ten μl of this sample solution were injected into the HPLC system (Accela, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a reversed-phase Atlantis dC18 analytical column, 150 × 2.1 mm i.d., 3 μm particle size (Waters, Vienna, Austria) and a C18 4 × 3 mm i.d. security cartridge (Phenomenex, Torrance, CA, USA). The column temperature was held at 25 °C. Eluent A was ultrapure water, eluent B was methanol, both containing 0.1% (v/v) formic acid. For chromatographic separation (flow rate 200 μL min^{-1}), 100% A was held constant for 1 min, followed by a linear gradient to 100% B for 35 min. This final condition was held for 4.5 min, followed by 4 min column re-equilibration at 100% A. The HPLC system was coupled to an Accela PDA (scan wavelength 200–600 nm, bandwidth 1 nm, scan rate 20 Hz) and subsequently to an LTQ Orbitrap XL (both Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) interface (operated in positive ionization mode). The full scan data were then automatically screened for the characteristic iron isotopic pattern by the aid of a recently developed in-house software programme. Subsequently, Pearson correlation coefficients were calculated for the extracted ion current (EIC) chromatograms of the putative iron chelators (^{56}Fe and ^{54}Fe containing ion species) and had to exceed a value of 0.75 in order to be further considered as a potential siderophore. For confirmation by siderophore reference standards, the HPLC calibration kits coprogens & fusarinines and ferrichromes were purchased from EMC Microcollections (Tuebingen, Germany).

Colony morphology, conidiation and sensitivity to H_2O_2. Colony morphology, radial growth and conidiation were studied on potato dextrose agar (PDA, Difco, US). The sensitivity of the conidia to oxidative stress was examined by plating conidia on VMS plates amended with 2 mM H_2O_2. After 3 days of incubation, the germination frequency relative to non-amended VMS was calculated as follows: Number of colonies in H_2O_2 amended plates/Number of colonies in non-amended plates.

Biosynthesis of gliotoxin. Ability of wild type and mutants to produce gliotoxin was assessed by growing the strains in Weindling minimal medium [22] for three days and extracting the filtrate with

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