Fungal Genetics and Biology 100 (2017) 33–41

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

A tomatinase-like enzyme acts as a virulence factor in the wheat pathogen *Fusarium graminearum*

Jason Carere *, Aurélie H. Benfield, Mélodie Ollivier, Chunji J. Liu, Kemal Kazan, Donald M. Gardiner

Commonwealth Scientific and Industrial Research Organization (CSIRO) Agriculture and Food, Queensland Bioscience Precinct, Brisbane, Queensland 4067, Australia

ARTICLE INFO

Article history:
Received 6 December 2016
Revised 16 January 2017
Accepted 17 January 2017
Available online 18 January 2017

Keywords:
Tomatine
Saponin
Phytoanticipin
Defense compounds
Secondary metabolites
Fusarium

ABSTRACT

During their interactions with plants, fungal pathogens employ large numbers of pathogenesis-associated molecules including secreted effectors and enzymes that can degrade various defence compounds. However, in many cases, *in planta* targets of pathogen-produced enzymes remain unknown. We identified a gene in the wheat pathogen *Fusarium graminearum*, encoding a putative enzyme that shows 84% sequence identity to FoTom1, a tomatinase produced by the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*. In *F. oxysporum* f. sp. *lycopersici*, FoTom1 is a virulence factor involved in the degradation of tomato defence compound tomatine, a saponin compound. Given that wheat is unknown to produce tomatine, we tested the ability of *F. graminearum* to degrade tomatine and found that *F. graminearum* was unable to degrade tomatine in culture. However, FgTom1 degraded tomatine *in vitro* when heterologously expressed. To determine the possible function of FgTom1 in pathogen virulence, we generated *FgTom1* knockout mutants (ΔTom1). ΔTom1 mutants were not different from wild type when grown in culture but showed significant reduction in pathogen virulence in root rot and head blight assays. In an attempt to identify possible *in planta* targets of FgTom1, the metabolomes of wheat heads infected with wildtype pathogen and ΔTom1 were compared and several peaks differentially abundant between treatments identified. Although the exact identity of these peaks is currently unknown, this result suggested that FgTom1 may have *in planta* targets in wheat, possibly tomatine-like saponin compounds. Overall, our results presented here show that FgTom1 is a new virulence factor in *F. graminearum*.

1. Introduction

Plants have a variety of mechanisms to protect themselves from pathogen attack including the production of physical and chemical barriers as well as a diverse array of secondary metabolites which can act as either antimicrobial compounds or signalling molecules (Pusztahelyi et al., 2015). One of the relatively well known classes of plant defence compounds are the saponins that include avenacin and α-tomatine (tomatine) produced by oats and tomatoes, respectively (Irving et al., 1946; Maizel et al., 1964). Both tomatine and avenacin are known to inhibit multiple pathogens (Irving et al., 1946; Maizel et al., 1964). Saponins are glycoalkaloids consisting of a non-polar triterpenoid or steroidal core termed the aglycone, which is linked to one or more sugar residues (Oleszek, 2002). The antifungal activity of tomatine is thought to be due to its ability to disrupt membranes by binding to 3β-hydroxyl sterols. The removal of the sugar groups is shown to reduce tomatine's ability to bind cholesterol and thus reduce its toxicity (Arneson and Durbin, 1968; Keukens et al., 1995).

To deal with host-derived defence compounds pathogens have typically evolved strategies that may include the modification, degradation, sequestration or efflux of anti-microbial compounds (Maor and Shirasu, 2005). Indeed, various pathogens are able to degrade saponins. For instance, the fungal pathogen *Gaeumannomyces graminis* var. *avenae* produces avenacinase, an enzyme capable of degrading avenacin (Bowyer et al., 1995; Turner, 1961). Strains of *G. graminis* lacking avenacinase are unable to infect oats while still pathogenic on wheat, which does not produce avenacin (Bowyer et al., 1995). Similarly, *Fusarium oxysporum* f. sp. *lycopersici* produces a tomatinase (Tom1) which is able to degrade the tomato saponin tomatine (Roldán-Arjona et al., 1999). A Tom1 homologue called CfTom1 has been shown to be important during the infection of tomato by *Cladosporium fulvum* (Okmen et al., 2013). A potato saponin, α-chaconine, toxic to several fungal species (Fewell and Roddick, 1993) can also be degraded by the potato pathogen, *F. sambucinum* (Becker and Weltring, 1998).

Interestingly, degradation products of tomatine, β-tomatine, (Bouarab et al., 2002) tomatidine and lycotetraose (Ito et al.,

* Corresponding author at: Agriculture and Agri-Food, Canada.
E-mail address: Jason.Carere@Canada.ca (J. Carere).

http://dx.doi.org/10.1016/j.fgb.2017.01.007
1087-1845/

2004) were proposed to suppress plant defence. Although a recent study in which tomatidine and lycotetraose were incubated with tomato cells failed to confirm the findings of Ito et al. (2004), it was shown that external application of tomatidine in tomato leads to cell death and necrosis (Okmen et al., 2013). Therefore, tomatine degradation seems to serve multiple beneficial purposes for the pathogen.

In this study, we found that the *F. graminearum FG05_04856* gene encodes FgTom1, a homologue of the Tom1 tomatinase enzyme from *F. oxysporum* f. sp. *lycopersici*. This gene has previously been shown to be expressed during infection of wheat but expression is quite low during growth in axenic culture which lead to the belief this gene plays a role in infection (Stephens et al., 2008). The heterologously expressed FgTom1 was able to degrade tomatine. In addition, *F. graminearum* FgTom1 mutants showed reduced virulence on wheat. Together, these results suggest that FgTom1 acts as a virulence factor during wheat-*F. graminearum* interaction possibly by modifying a tomatine-like defence molecule in wheat.

2. Results

2.1. Comparative genomics identifies a gene encoding a tomatinase-like enzyme in the genome of a cereal infecting pathogen F. graminearum

During the comparative analysis of the genomes of the *Fusarium* pathogens, we identified a gene (*FG05_04856*) in the genome of the *F. graminearum*, which encodes a protein sharing 89% sequence similarity to the FoTom1 tomatinase from the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (Fig. 1). We named *FG05_04856*, *FgTom1*, as this *F. graminearum* open reading frame encodes a homologue of FoTom1. FoTom1 was shown to degrade tomato defence compound tomatine during the wilt disease caused by *F. oxysporum* f. sp. *lycopersici* on tomato (Roldán-Arjona et al., 1999). However, wheat is not known to produce tomatine although the saponin, avenacin, is known to be present in a related cereal species (oats) (Osbourn, 2003). As previously mentioned this gene is more highly expressed during infection of wheat compared to growth in axenic culture (Fig. S1) (Stephens et al., 2008). We therefore examined the potential roles of FgTom1 in *F. graminearum*.

2.2. Heterologously expressed FgTom1 shows tomatinase activity

To determine if *F. graminearum* is able to degrade tomatine, *F. graminearum* and *F. oxysporum* f. sp. *lycopersici* were grown in three different media (PDB and defined media containing either $(NH_4)_2HPO_4$ or $NaNO_3$ as a nitrogen source) supplemented with tomatine, which is known to induce tomatinase activity in *F. oxysporum* f. sp. *lycopersici* (Lairini et al., 1996). The liquid chromatography mass spectrometry analysis of the extracellular fraction of the cultures showed that *F. oxysporum* f. sp. *lycopersici* converted all tomatine in the media to tomatidine under all conditions tested (Fig. 2). In contrast, no tomatidine was detected in any type of media when inoculated with *F. graminearum*. This result was somewhat unexpected, given that FgTom1 shares high sequence identity with FoTom1.

To further examine if FgTom1 has tomatinase or tomatinase-like activity, an attempt was made to heterologously express FgTom1 in *E. coli* and analyse its activity against tomatine. Heterologous expression and subsequent purification of FoTom1 from *E. coli* was successful with SDS-PAGE analysis showing a highly enriched band at the expected molecular weight of 37.1 kDa (Fig. S2). In contrast, all constructs, in two separate strains of *E. coli* failed to express FgTom1 (data not shown); the expression of codon optimized FgTom1 produced only a faint band of the expected size (Fig. S2). However, the 150 mM imidazole elution fraction from the column corresponding to this band was able to convert tomatine to tomatidine (Fig. 3), suggesting that FgTom1 is able to use tomatine as a substrate. As expected, both crude lysate from *E. coli* containing empty vector pET28a and the boiled FgTom1 lysate showed no activity toward tomatine (Fig. 3), suggesting that the observed effect was specific to FgTom1.

2.3. FgTom1 contributes to F. graminearum virulence

In order to access the role FgTom1 in *F. graminearum*, we replaced *FgTom1* with a nourseothricin-resistance gene (*nat*) in this species. A PCR assay confirmed the successful deletion of *FgTom1* (Fig. S3). The individual transformants referred to as ΔTom1.1, ΔTom1.2 and ΔTom1.3 did not show any obvious growth defects compared to the parental strains when grown on ½ PDA (Fig. S4), suggesting that FgTom1 is not necessary for normal axenic growth. There was no difference in the inhibitory effects of tomatine on ΔTom1 and the wild type strain in liquid culture (Fig. S4).

To assess the potential contribution of FgTom1 to the virulence of *F. graminearum*, root rot and head blight assays were performed in wheat. In root rot assays, the shoots of plants inoculated with *F. graminearum* ΔTom1 mutants were significantly longer than those inoculated with wild type *F. graminearum* (Fig. 4), suggesting that fungal virulence was attenuated. There was also less stem browning in the ΔTom1 inoculated plants than those inoculated with the parental strain (Fig. S5). This experiment was independently replicated and similar results observed (Fig. S5).

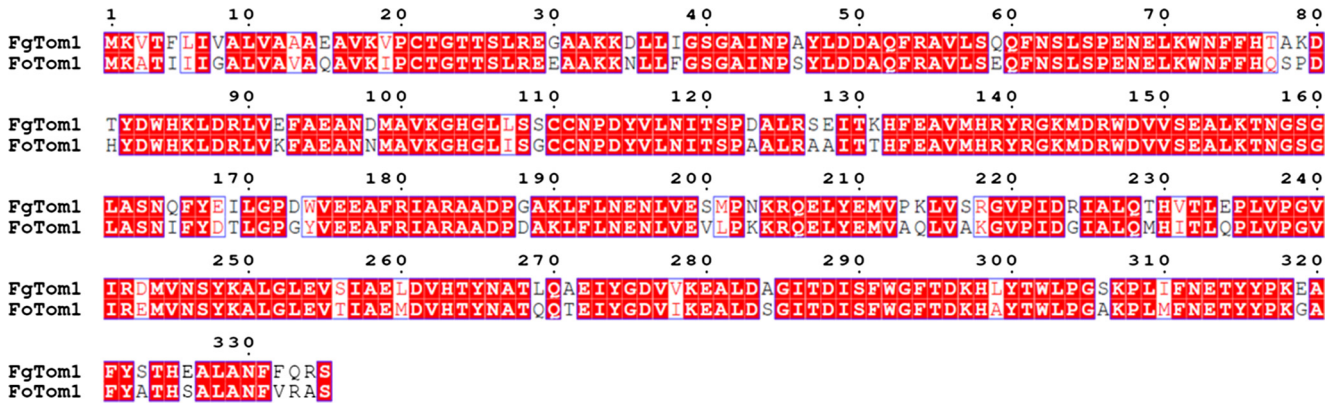

Fig. 1. Sequence alignment of FoTom1 and FgTom1 (*FG05_04856*). FoTom1 shares 89% sequence similarity to FgTom1. Sequences shown here lack the signal sequence. The sequences were aligned with ClustalX (Larkin et al., 2007) and visualized using ESPript (Robert and Gouet, 2014).