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A putative α-glucoside transporter gene *BbAGT1* contributes to carbohydrate utilization, growth, conidiation and virulence of filamentous entomopathogenic fungus *Beauveria bassiana*

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Abstract

Carbohydrate transporters are critical players mediating nutrient uptake during saprophytic and pathogenic growth for most filamentous fungi. For entomopathogenic fungi, such as *Beauveria bassiana*, assimilation of α-glucosides, in particular, trehalose, the major carbohydrate constituent of the insect haemolymph, has been hypothesized to represent an important ability for infectious growth within the insect hemocoel. In this study, a *B. bassiana* α-glucoside transporter homolog was identified and genetically characterized via generation of a targeted gene disruption mutant. Trehalose utilization was compromised in the mutant strain. In addition, inactivation of the α-glucoside transporter resulted in decreased conidial germination, growth, and yield on various carbohydrates (α-glucosides, monosaccharides and polyols) as compared to the wild-type strain. Insect bioassays revealed decreased mean lethal mortality time using both topical and intrahemocoel injection assays, although final mortality levels were comparable in both the mutant and wild type. Gene expression profiles showed altered expression of other putative transporters in the knockout mutant as compared to the wild type. These results highlighted complex sugar utilization and responsiveness in *B. bassiana* and the potential role for trehalose assimilation during fungal pathogenesis of insects.

Keywords: *Beauveria bassiana*; α-Glucoside transporter; Virulence; Entomopathogen

1. Introduction

Broad host range entomopathogenic fungi, such as *Beauveria bassiana*, have long been considered as alternatives to chemical pesticides for the control of insect pests (Fan et al., 2012; Feng et al., 1994). Unlike insect pathogenic bacteria and viruses that require ingestion and/or specialized modes of entry, fungal infection by *B. bassiana* begins with conidial adhesion to the host cuticle, germination and growth on the insect surface, and penetration of cuticle to the integument (Fang et al., 2009; Holder and Keyhani, 2005; Zhang et al., 2012). As a consequence of cuticle penetration, the fungal hyphae reach the insect hamocoel where specialized cells termed *in vivo* blastospores or hyphal bodies are produced (Lewis et al., 2009; Wanchoo et al., 2009). Hyphal bodies are able to evade the insect immune system and proliferate within the hemocoel, utilizing the nutrient constituents of the hemolymph (Pendland et al., 1993). Trehalose [α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside], the non-reducing disaccharide of glucose, is the principal circulating hemolymph sugar of most insects (Thompson, 2003), and hence trehalose utilization is a potentially important attribute during fungal infection of insects (Xia et al., 2002a). In addition to its

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pathogenic life cycle, *B. bassiana* can grow as a saprophyte and can form endophytic relationships with certain plants (Vega, 2008). In the latter case, plants are considered to provide carbohydrate substrates for fungal growth and in turn, the fungus appears to be able to provide nitrogen to the host plant (Behie et al., 2012).

Fungi have evolved two mechanisms for trehalose utilization. The first strategy is to secrete trehalase enzymes that hydrolyze extracellular trehalose into glucose, followed by uptake and assimilation of the resultant glucose. An alternative route is the direct uptake of trehalose via active transporter and the subsequent intracellular catabolism of the carbohydrate. Secreted trehalases are often referred to as acid trehalases, reflecting their lower pH optima as compared to intracellular or "neutral" trehalases. Targeted gene knockout mutants of the acid trehalase from *Aspergillus nidulans* grow poorly on trehalose but still retain an ability to assimilate this carbohydrate presumably via transport and intracellular catabolism (d'Enfert and Fontaine, 1997). A wide diversity of carbohydrate transporters has been described in fungi. The *Saccharomyces cerevisiae AGT1* gene is one of the best-studied examples of an α-glucoside transporter capable of transporting trehalose, maltose and other polysaccharides (Han et al., 1995; Malluta et al., 2000). Multiple maltose/malto-oligosaccharide transporter genes including *MAL1-6*, *MPH2*, and *MPH3* have also been characterized in yeast (Han et al., 1995; Jespersen et al., 1999; Klein et al., 1996). These transport systems, including Agt1p, mediate the active uptake of α-glucosides via H^+-symport, which in turn couples the electrochemical proton gradient across the plasma membrane to sugar transport (Hollatz and Stambuk, 2001; Dietvorst et al., 2010). Growth on trehalose and trehalose uptake is compromised in yeast *AGT1* mutants (Plourde-Owobi et al., 1999). Intriguingly, natural *AGT1* mutants have been reported to potentially distinguish ale versus lager yeast brewing strains. Ale yeast strains appear to encode functional Agt1p transporters, but certain lager strains contain premature stop codons in the *AGT1* open reading frame that result in non-functional transporters (Vidgren et al., 2009). Lager yeast *AGT1* mutants can be complemented using the *AGT1* gene from ale yeast, and the resultant strains displayed improved fermentation performance on the α-glucoside, maltose (Vidgren et al., 2009). Although significant research has been done regarding *AGT1* function in yeast, to date, *AGT1* homologs in filamentous fungi have yet to be characterized.

Entomopathogenic (filamentous) fungi including *Metarhizium anisopliae* and *B. bassiana* encode for both neutral and acid trehalases and contain homologs of the *AGT1* α-glucoside transporter. These data suggest that entompathogenic fungi have a diversified strategy for the assimilation of α-glucosides and trehalose in particular. The conditions under which each pathway, i.e. production of extracellular (acid) trehalase and uptake of the resultant hydrolysis products versus uptake of the disaccharide and internal hydrolysis of the carbohydrate (via neutral trehalase activity), predominates especially within the context of the insect infection process remains unknown, and the contribution of α-glucoside transport processes to fungal development and virulence has not been explored. It has been reported, however, that *M. anisopliae* up-regulates neutral trehalase expression and activity during the early stage of infection (Xia et al., 2002b). This result suggests that Agt1p mediated transport activities could play an important role in early infection processes and potentially during growth on the trehalose-rich hemolymph once the fungus has penetrated through to the hemocoel.

An expressed sequence tag (EST) analysis of *B. bassiana* grown under various conditions had previously revealed a partial nucleotide sequence encoding a general α-glucoside transporter protein (Cho et al., 2006). In this report, the role of an *AGT1* α-glucoside transporter homolog in the filamentous fungus, *B. bassiana* was characterized. Targeted gene disruption of *B. bassiana AGT1* resulted in a strain impaired in growth and development on a variety of α-glucosides including sucrose, trehalose, and maltose. *BbAGT1* also contributes to germination, vegetative growth and conidial yield on various carbohydrate carbon sources. The *ΔBbAGT1* strain displayed reduced virulence in both topical and intrahemocoel injection insect bioassays, which suggesting that it is involved in cuticle penetration events and assimilation of nutrients from the host. Changes in the expression pattern of 36 putative carbohydrate transporters identified in the *B. bassiana* genomes as a function of growth carbohydrate and compared between the *ΔBbAGT1* and wild type strains was also noted.

2. Materials and methods

2.1. Fungal strains and growth conditions

The fungal strain *B. bassiana* 2860 (ARSEF 2860, RW Holley Center for Agriculture and Health, Ithaca, NY, USA) was conserved as dry conidia mixed with sterile sands at −76 °C. *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used for propagation of plasmids and was cultured in Luria-Bertani medium supplemented with 50 μg ml^{-1} kanamycin or 100 μg ml^{-1} ampicillin as needed. Sabouraud dextrose broth and agar (SDB/SDA, 4% glucose, 1% peptone, 1% yeast extract, with 1.5% agar) was used for routine culturing of fungal strains. Czapek-Dox agar (CZA) was used for fungal transformation and screening. Minimal media was based upon Czapek-Dox lacking sucrose (the carbon source normally found in CZA), supplemented with individual carbohydrates including; glucose, sucrose, trehalose, maltose, fructose, mannose, mannitol and glycerol at a final concentration of 3.0%. These media were used to evaluate the effect of various carbohydrate carbon sources on fungal conidiation, conidial germination and vegetative growth.

2.2. Nucleic acid manipulations for gene disruption and complementation

Primers used in this study are given in Supplemental Tables S1 and S2. The *BbAGT1* targeted gene disruption vector was constructed using a one step method (García-Pedrajas et al., 2008) with some modifications (detailed in Supplemental