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Trehalose 6-phosphate phosphatase is required for development, virulence and mycotoxin biosynthesis apart from trehalose biosynthesis in *Fusarium graminearum*

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ABSTRACT

Trehalose 6-phosphate synthase (TPS1) and trehalose 6-phosphate phosphatase (TPS2) are required for trehalose biosynthesis in yeast and filamentous fungi, including *Fusarium graminearum*. Three null mutants Δtps1, Δtps2 and Δtps1–Δtps2, each carrying either a single deletion of *TPS1* or *TPS2* or a double deletion of *TPS1–TPS2*, were generated from a toxigenic *F. graminearum* strain and were not able to synthesize trehalose. In contrast to its reported function in yeasts and filamentous fungi, TPS1 appeared dispensable for development and virulence. However, deletion of *TPS2* abolished sporulation and sexual reproduction; it also altered cell polarity and ultrastructure of the cell wall in association with reduced chitin biosynthesis. The cell polarity alteration was exhibited as reduced apical growth and increased lateral growth and branching with increased hyphal and cell wall widths. Moreover, the TPS2-deficient strain displayed abnormal septum development and nucleus distribution in its conidia and vegetative hyphae. The Δtps2 mutant also had 62% lower mycelial growth on potato dextrose agar and 99% lower virulence on wheat compared with the wild-type. The Δtps1, Δtps2 and Δtps1–Δtps2 mutants synthesized over 3.08-, 7.09- and 2.47-fold less mycotoxins, respectively, on rice culture compared with the wild-type. Comparative transcriptome analysis revealed that the Δtps1, Δtps2 and Δtps1–Δtps2 mutants had 486, 1885 and 146 genotype-specific genes, respectively, with significantly changed expression profiles compared with the wild-type. Further dissection of this pathway will provide new insights into regulation of fungal development, virulence and trichothecene biosynthesis.

1. Introduction

Fusarium graminearum is an ascomycete that causes Fusarium head blight (FHB) in wheat, maize and other small grain cereal crops worldwide. FHB epidemics in wheat occur frequently in central China, especially along the middle and lower reaches of the Yangtze River (Chen et al., 2000; Qu et al., 2008; Zhang et al., 2013). Recently, global climate change has aggravated the spread and severity of FHB to even wider regions, encompassing more than 10 provinces that are primary agricultural areas in China (Yuan et al., 2007). Since the mid-1990s, FHB has re-emerged as a serious problem for agriculture in North America and Europe (Parry et al., 1995; Windels, 2000). FHB pathogens produce various trichothecene mycotoxins that are harmful to humans, domestic animals and plants (D'Mello et al., 1999). Trichothecene type B mycotoxins such as deoxynivalenol (DON) are considered the main toxic compounds in contaminated wheat grains entering food/feed chains (Chen et al., 2000; Zhang et al., 2013). Mycotoxicosis caused by consumption of flour made from FHB-affected wheat has been reported in China (Chen et al., 2003) and continues to pose a serious threat to human health.

Trehalose is a nonreducing disaccharide formed by two glucose molecules linked by a 1α–1α bond that is widely present in bacteria, fungi, plants and invertebrates (Avonce et al., 2006). Many functions have been described for trehalose. For instance, trehalose is frequently used as a compatible solute to contend with osmotic stress in prokaryotes (Purvis et al., 2005; Strom and Kaasen, 1993). In yeast and filamentous fungi, trehalose can be used as a reserve source of carbon (Gancedo and Flores, 2004; Thevelein, 1984)

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and for an adaptive response to various stress conditions including dehydration, oxidative stress, heat and cold treatment, and freezing stress (Cao et al., 2008; Elbein et al., 2003; Kandror et al., 2002; Sasano et al., 2012; Zakharova et al., 2012). Furthermore, trehalose is capable of stabilizing and protecting membranes and proteins, allowing anhydrobiotic organisms to survive cycles of dehydration and rehydration (Singer and Lindquist, 1998). Thus, trehalose has received considerable attention regarding the molecular regulation of its biosynthetic and metabolic pathways.

Molecular characterization of trehalose biosynthetic pathways and the corresponding genes in different organisms has revealed that there are at least five biosynthetic pathways for trehalose (Avonce et al., 2006; Elbein et al., 2003). The most widely distributed pathway, studied in greatest detail in *Saccharomyces cerevisiae*, involves two enzymatic steps catalyzed by trehalose 6-phosphate synthase (TPS1) and trehalose 6-phosphate phosphatase (TPS2). TPS1 catalyzes the transfer of glucose from uridinediphospho (UDP)-glucose to glucose 6-phosphate to generate trehalose 6-phosphate (T6P), while TPS2 catalyzes the dephosphorylation of T6P to form trehalose (Bell et al., 1992; Vuorio et al., 1993).

More efforts have been devoted to TPS1 than TPS2 for functional analyses revealing various roles in development, pathogenicity and stress responses in yeast and higher fungi in addition to a role in trehalose synthesis (Al-Bader et al., 2010; Arisan-Atac et al., 1996; Gancedo and Flores, 2004; Flores et al., 2011; Zaragoza et al., 1998; Bell et al., 1992; Foster et al., 2003; Wolschek and Kubicek, 1997). For example, TPS1 mutants of *Magnaporthe oryzae* and *Fusarium verticillioides* had reduced pathogenicity and secondary metabolism (Fernandez and Wilson, 2011; Fernandez et al., 2012; Wilson et al., 2007, 2010; Boudreau et al., 2013). TPS2 mutants in *Aspergillus* and *Candida* had reduced cell wall integrity, chitin synthesis and infectivity (Thevelein and Hohmann, 1995; Borgia et al., 1996; Elliott et al., 1996; Maidan et al., 2008; Puttikamonkul et al., 2010). Some of these effects have been attributed to impaired glycolysis via the inhibition of hexokinase activity. Since the fungal core trehalose biosynthetic genes appear to be absent in mammals and share minimal similarity to plant homologs, disruption of trehalose synthesis may serve as a promising target for the development of new strategies to control fungal diseases (Li et al., 2010a; Puttikamonkul et al., 2010; Tinoco et al., 2010).

The trehalose biosynthesis pathway has also been shown to have critical impacts on fungal virulence in human pathogenic yeasts *Cryptococcus neoformans* (Petzold et al., 2006), *Cryptococcus gattii* (Ngamskulrungroj et al., 2009) and *Candida albicans* (Zaragoza et al., 1998, 2002; Van Dijck et al., 2002); human invasive mold *Aspergillus fumigatus* (Al-Bader et al., 2010; Puttikamonkul et al., 2010); and filamentous fungal plant pathogens *Magnaporthe grisea* (Foster et al., 2003; Wilson et al., 2007) and *Stagonospora nodorum* (Lowe et al., 2009). However, there has been no investigation of the trehalose biosynthesis pathway in devastating fungal phytopathogens of the *F. graminearum* clade, nor of a role for a trehalose biosynthetic gene in trichothecene mycotoxin biosynthesis in toxigenic fungi. In this study, we determined the role of trehalose biosynthesis in the development, virulence and mycotoxin biosynthesis of *F. graminearum* by comparatively characterizing the three null mutant strains Δtps1, Δtps2 and Δtps1–Δtps2, together with two complementation strains, TPS1C and TPS2C, and the wild-type progenitor strain. We found that in contrast to reports on yeasts and other filamentous fungi, neither the loss of *TPS1* nor *TPS2* had an effect on hexokinase activity, and *TPS1* appeared dispensable for development and virulence. Instead, single deletion of *TPS2* abolished sporulation and sexual development, and caused altered cell polarity and virtual loss of virulence on wheat. Moreover, our data indicated that the Δtps2 mutant had a more significant reduction in mycotoxin production than the Δtps1 and Δtps1–Δtps2 mutants, and it contained 1885 genotype-specific genes with changed expression patterns, including several key regulators from the G-protein pathway. This is the first demonstration that single deletion of *TPS2* causes such a wide range of alterations in a fungal species.

2. Materials and methods

2.1. Strains and culture conditions

F. graminearum wild-type (WT) strain 5035 and its derivatives were cultured at 28 °C on potato dextrose agar (PDA) and in potato dextrose broth (PDB) for mycelium growth, and in CMC broth (7.5 g l^{-1} of carboxymethyl cellulose, 0.5 g l^{-1} of NH_4NO_3, 0.5 g l^{-1} of $KHPO_4$, 0.25 g l^{-1} of $MgSO_4{\cdot}7H_2O$, and 0.5 g l^{-1} of yeast extract) for conidiation (Duvick et al., 1992). The Δtps1 and Δtps2 mutants were selected on PDA plates containing hygromycin B (50 μg ml^{-1}) while the Δtps1–Δtps2 double mutant and TPS1C and TPS2C complementation strains were selected on PDA containing both hygromycin B (50 μg ml^{-1}) and G418 (30 μg ml^{-1}). All strains are available upon request from the Molecular Biotechnology Laboratory of Triticeae Crops, Huazhong Agricultural University, Wuhan, China.

2.2. Nucleic acid manipulations

DNA from mycelia grown on PDA for 5 days was extracted by the CTAB method (Nicholson et al., 1997). Total RNA was isolated with TRIZOL Reagent (Invitrogen, USA) followed by reverse-transcription with Superscript II (Invitrogen, USA) and an oligo-dT_{20} primer. Polymerase chain reaction (PCR) was carried out in a PTC-100™ Thermal Cycler (MJResearch, USA) with gene-specific PCR primers (S. Table 1) designed according to the *Fusarium* Comparative Database at Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html). PCRs were carried out as previously described (Xu et al., 2010).

2.3. Generation of null mutant and complementation strains

The trehalose 6-phosphate synthase (TPS1) and trehalose 6-phosphate phosphatase (TPS2) genes in *F. graminearum* were obtained by Blast algorithms with *S. cerevisiae* Tps1p and Tps2p protein sequences, respectively. The deduced TPS1 and TPS2 proteins encoded by FG06051.1 and FG07926.1 showed 97% and 95% identity with Tps1p and Tps2p from *S. cerevisiae*, respectively. Three null mutant strains, Δtps1, Δtps2 and Δtps1–Δtps2, were generated by disruption of the *TPS1* gene (FG06051.1) (Δtps1), the *TPS2* gene (FG07926.1) (Δtps2), and both *TPS1* and *TPS2* genes (Δtps1–Δtps2), while two complementation strains TPS1C and TPS2C were generated by reintroducing a *TPS1* gene into the Δtps1 mutant (TPS1C) and a *TPS2* gene into the Δtps2 mutant (TPS2C), respectively. *Agrobacterium tumefaciens*-mediated transformation (Xu et al., 2010) was used for homologous recombination to generate four strains (Δtps1, Δtps2, Δtps1–Δtps2 and TPS1C), while protoplast transformation (Maier et al., 2005) was used to generate the TPS2C strain. A *PLS1* gene (FG08695) encoding a tetraspanin that is dispensable in *F. graminearum*, served as the target site for integration of the *TPS1* or *TPS2* genes in their respective deletion mutant strains. Details of the construction of all strains are illustrated in Fig. S1. All the generated mutant and complementation strains were molecularly verified by PCR and Southern blot analyses, as described in Figs. S1 and S2.