Available online at www.sciencedirect.com
ScienceDirect
Fungal Genetics and Biology 44 (2007) 258–268
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata*

Heriberto Vélëz [a], Norman J. Glassbrook [a], Margaret E. Daub [b,*]

[a] *Department of Plant Pathology, NC State University, Raleigh, NC 27695, USA*
[b] *Department of Plant Biology, NC State University, Raleigh, NC 27695, USA*

Received 14 April 2006; accepted 27 September 2006
Available online 7 November 2006

Abstract

Mannitol metabolism in fungi is thought to occur through a mannitol cycle first described in 1978. In this cycle, mannitol 1-phosphate 5-dehydrogenase (EC 1.1.1.17) was proposed to reduce fructose 6-phosphate into mannitol 1-phosphate, followed by dephosphorylation by a mannitol 1-phosphatase (EC 3.1.3.22) resulting in inorganic phosphate and mannitol. Mannitol would be converted back to fructose by the enzyme mannitol dehydrogenase (EC 1.1.1.138). Although mannitol 1-phosphate 5-dehydrogenase was proposed as the major biosynthetic enzyme and mannitol dehydrogenase as a degradative enzyme, both enzymes catalyze their respective reverse reactions. To date the cycle has not been confirmed through genetic analysis. We conducted enzyme assays that confirmed the presence of these enzymes in a tobacco isolate of *Alternaria alternata*. Using a degenerate primer strategy, we isolated the genes encoding the enzymes and used targeted gene disruption to create mutants deficient in mannitol 1-phosphate 5-dehydrogenase, mannitol dehydrogenase, or both. PCR analysis confirmed gene disruption in the mutants, and enzyme assays demonstrated a lack of enzymatic activity for each enzyme. GC–MS experiments showed that a mutant deficient in both enzymes did not produce mannitol. Mutants deficient in mannitol 1-phosphate 5-dehydrogenase or mannitol dehydrogenase alone produced 11.5 and 65.7 %, respectively, of wild type levels. All mutants grew on mannitol as a sole carbon source, however, the double mutant and mutant deficient in mannitol 1-phosphate 5-dehydrogenase grew poorly. Our data demonstrate that mannitol 1-phosphate 5-dehydrogenase and mannitol dehydrogenase are essential enzymes in mannitol metabolism in *A. alternata*, but do not support mannitol metabolism operating as a cycle.

Keywords: Mannitol cycle; Mannitol dehydrogenase; Mannitol 1-phosphate 5-dehydrogenase; Polyols; Fungi

1. Introduction

Polyhydroxy alcohols or sugar alcohols are produced by many organisms including bacteria, plants, and fungi (Lewis and Smith, 1967; Jennings, 1984). In fungi, mannitol is the most common polyol, found in large quantities in spores, fruiting bodies, sclerotia, and mycelia (Lewis and Smith, 1967). In *Agaricus bisporus*, mannitol can contribute up to 20% of the mycelium dry weight and increases dramatically to 30–50% in differentiating sporophores, while in *Aspergillus niger* conidiophores, mannitol makes up to 10–15% of the dry weight (Stoop and Mooibroek, 1998; Ruijter et al., 2003).

Mannitol is purported to have different roles in fungi, including osmoregulation, serving as a storage or translocated carbohydrate, serving as a source of reducing power, regulating coenzymes, and regulating cytoplasmic pH by acting as a sink or source for protons (Lewis and Smith, 1967; Jennings, 1984). More recently, mannitol has been shown to quench reactive oxygen species (ROS) both *in vitro* and *in vivo* (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Voegele et al., 2005). *Cryptococcus neoformans* has been reported to produce and secrete mannitol to protect itself against oxidative killing mechanisms of phagocytic cells (Chaturvedi et al., 1996). A role for mannitol in

* Corresponding author. Fax: +1 919 515 3436.
E-mail address: margaret_daub@ncsu.edu (M.E. Daub).

1087-1845/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2006.09.008

antioxidant defense has also been supported by experiments with *Saccharomyces cerevisiae* (Chaturvedi et al., 1997), *Alternaria alternata* (Jennings et al., 1998; Jennings et al., 2002), and the rust fungus *Uromyces fabae* (Voegele et al., 2005).

The mannitol cycle was proposed by Hult and Gatenbeck (1978) from studies of cell-free extracts of the fungus *A. alternata* (Fig. 1). Cell-free extracts from both an alternariol-producing and a non-producing strain were prepared from mycelia grown in Czapek-Dox medium. Specific activities of mannitol dehydrogenase, mannitol 1-phosphate 5-dehydrogenase, mannitol 1-phosphatase, hexokinase, glucose 6-phosphate dehydrogenase, glucose phosphate isomerase, phosphofructokinase and aldolase were measured to obtain information on the regulation of the cycle. In the proposed mannitol cycle, the enzyme mannitol 1-phosphate 5-dehydrogenase (MPDH; EC 1.1.1.17) would catalyze the reduction of fructose 6-phosphate to mannitol 1-phosphate using the cofactor NADH. mannitol 1-phosphatase (M1Pse; EC 3.1.3.22), presumed to be specific for mannitol 1-phosphate (Yamada et al., 1961; Ramstedt et al., 1986), would hydrolyze mannitol 1-phosphate to mannitol and inorganic phosphate. Mannitol would then be oxidized to fructose by mannitol dehydrogenase (MtDH; EC 1.1.1.138) using the cofactor $NADP^+$. Finally, fructose would be phosphorylated to fructose 6-phosphate by a hexokinase (EC 2.7.1.1). The cycle as proposed goes in one direction with the production of mannitol by MPDH and its utilization by MtDH. Thus, NADPH is produced at the expense of one molecule of ATP and NADH. Hult and Gatenbeck (1978) found no differences on the specific enzyme activities between the two strains of *A. alternata*, that could explain why the non-producing strain would synthesize more fat and oxidize more mannitol than the alternariol-producing strain. They further postulated that the cycle was regulated by the availability of the coenzymes NADH and $NADP^+$.

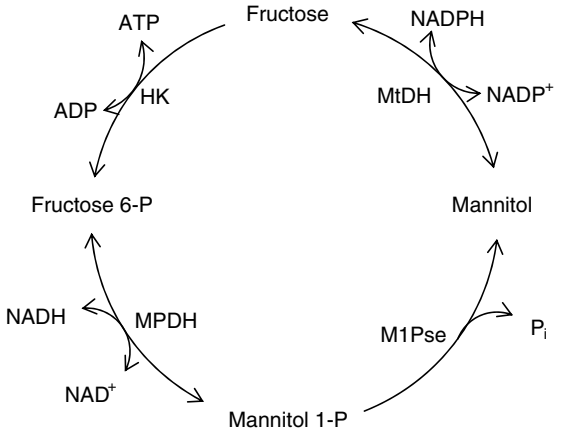

Fig. 1. Proposed mannitol cycle in fungi (Hult and Gatenbeck, 1978). HK, hexokinase; MPDH, mannitol 1-phosphate 5-dehydrogenase (EC 1.1.1.17); M1Pse, mannitol 1-phosphatase (EC 3.1.3.22); P_i, inorganic phosphate; MtDH, mannitol dehydrogenase (EC 1.1.1.138); mannitol 1-P, mannitol 1-phosphate, fructose 6-P, fructose 6-phosphate.

Evidence for MtDH and MPDH enzyme activities have been reported in many fungi, but only very few genomic sequences have been reported (Supplementary Table 1). MtDH genes have been characterized from a number of Ascomycetes as well as *A. bisporus* (Stoop and Mooibroek, 1998) and *U. fabae* (Voegele et al., 2005). The only characterized genes for MPDH are those from *Stagonospora nodorum* (Solomon et al., 2005) and *A. niger* (Ruijter et al., 2003). MPDH was thought to be limited to Zygomycetes and Ascomycetes, hence the cycle was thought to be absent in Basidiomycetes. Recently, however, MPDH activity was found in *Pleurotus ostreatus* and *Cryptococcus neoformans* (Suvarna et al., 2000; Chakraborty et al., 2004).

In spite of the extensive reports on enzyme activities in fungi, questions concerning the importance of the mannitol cycle and whether or not it acts as a cycle have never been definitively answered (McCullough et al., 1986; Solomon et al., 2005). Since the initial report in 1978, the genes encoding the *A. alternata* MtDH and MPDH enzymes have not been reported. Further, only *MPDH* has been disrupted in fungi (Ruijter et al., 2003; Solomon et al., 2005). The goals of this research were to confirm the proposed *Alternaria* mannitol cycle by genetic analysis. Here, we confirm the enzymatic activity of both MtDH and MPDH in a tobacco isolate of *A. alternata*, and report the isolation of the genes, their disruption, and characterization of the mannitol cycle in disruption mutants.

2. Materials and methods

2.1. Strain and media

All studies utilized *A. alternata* strain A5, isolated from brown spot-infected tobacco in Oxford, NC (provided by H. Spurr Jr, Oxford, NC) (Spurr, 1973). For enzyme assays, the fungus was grown in malt extract medium (15 g malt extract, 3 g peptone, and 30 g glucose per L). For protoplast isolation, the fungus was grown on GYB medium (l0 g glucose, 5 g yeast extract per L). Liquid regeneration medium (RM; 0.5 M sucrose, 0.1% yeast extract, 0.1% casein amino acids, and 0.1% mannitol) was used for protoplast regeneration. Solid RM (1 M sucrose, 0.1% yeast extract, 0.1% casein amino acids, 0.1% mannitol and 1.5% agar) with either 150 μg/mL hygromycin B (Roche, Indianapolis, IN) or 10 μg/mL phleomycin (Research Products International Corp., Mt. Prospect, IL) was used for selection of transformants. V-8 medium [300 mL V-8 juice (Campbell Soup Co., Camden, NJ), 4.5 g $CaCO_3$, and 15 g agar per L] was used to grow transformants. Modified Richard's minimal medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 20 g sucrose, 1 g yeast extract, and 15 g agar per L) was used for sporulation. Minimal medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, and 20 g glucose, per L) was used for *in vitro* growth characteristics of wild type and mutants. Glassbrook minimal medium was used to grow the *A. alternata* wild type and mutants to determine the levels of mannitol. This medium