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Potential role for saccharopine reductase in swainsonine metabolism in endophytic fungus, *Undifilum oxytropis*

Suman MUKHERJEE[a,*,1], *Angus L. DAWE*[a,c], *Rebecca CREAMER*[a,b]

[a]*Molecular Biology Program, New Mexico State University, Las Cruces, NM 88003, USA*
[b]*Department of Entomology, Plant Pathology, and Weed Science, New Mexico State University, Las Cruces, NM 88003, USA*
[c]*Biology Department, New Mexico State University, Las Cruces, NM 88003, USA*

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ABSTRACT

Locoweed plants in the southwestern United States often harbour a slow-growing endophytic fungus, *Undifilum oxytropis* (Phylum: *Ascomycota*; Order: *Pleosporales*), which produces a toxic alkaloid, swainsonine. Consumption of *U. oxytropis* by grazing animals induces a neurological disorder called locoism for which the toxic alkaloid swainsonine has been reported to be the causal agent. Little is known about the biosynthetic pathway of swainsonine in endophytic fungi, but previous studies on non-endophytic ascomycetous fungi indicate that pipecolic acid and saccharopine are key intermediates. We have used degenerate primers, Rapid amplification of cDNA ends (RACE)-PCR and inverse PCR to identify the gene sequence of *U. oxytropis* saccharopine reductase. To investigate the role of this gene product in swainsonine metabolism, we have developed a gene deletion system for this slow-growing endophyte based on our recently established transformation protocol. A strain of *U. oxytropis* lacking saccharopine reductase had decreased levels of saccharopine and lysine along with increased accumulation of pipecolic acid and swainsonine. Thus, saccharopine reductase influences the accumulation of swainsonine and its precursor, pipecolic acid, in *U. oxytropis*.

Introduction

Locoweeds (*Astragalus sp.* and *Oxytropis sericea*) are perennial flowering plants found frequently in the rangelands of the western United States, Asia, and South America (Kingsbury 1964; Molyneux & James 1982; James & Nielson 1988; Cook *et al.* 2009). Consumption of locoweeds by cattle, sheep, and horses induces a neurological condition termed locoism (James & Panter 1989). The etiological agent of locoism, swainsonine (1, 2, 8-trihydroxyindolizidine), is produced by fungal endophytes that reside within the locoweeds (Braun *et al.* 2003). The common endophyte of the *O. sericea* Nutt. Locoweed was recently classified as *Undifilum oxytropis*, belonging to the phylum *Ascomycota* and order *Pleosporales* (Cook *et al.* 2009; Graham *et al.* 2009; Pryor *et al.* 2009).

Undifilum oxytropis can be isolated from stems, seeds, and leaves of locoweed plants (Ralphs *et al.* 2002; Braun *et al.* 2003). The fungus is transmitted from one generation to the next through the seed coat (James & Panter 1989) (Kingsbury 1964). When *U. oxytropis* grown in pure culture was fed to rats symptoms of locoism were induced (McLain-Romero *et al.* 2004).

* *Corresponding author*. Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), 8, Center Drive, Bethesda, MD 20892-0830, USA. Tel.: +1 301 451 3771; fax: 1 301 402 0240.
E-mail addresses: suman.mukherjee@nih.gov, dawe@nmsu.edu, creamer@nmsu.edu
[1] Present address: Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0830, USA.
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Swainsonine, an alkaloid, is known to be produced by three groups of fungi, *U. oxytropis* (Braun *et al.* 2003; Cook *et al.* 2009; Pryor *et al.* 2009; Mukherjee *et al.* 2010), *Rhizoctonia leguminicola* (Smalley *et al.* 1962), and *Metarhizium anisoplae* (Sim & Perry 1997). A water-soluble trihydroxyindolizidine compound, swainsonine was first isolated from the Australian legume *Swainsona canescens* (Colgate *et al.* 1979). It inhibits lysosomal α-mannosidase, which is involved in the catabolism of glycoproteins (Harris *et al.* 1988) and golgi α-mannosidase II (Broquist 1985) and this activity has been shown to be the cause of locoism in cattle (Sim & Perry 1997). Swainsonine also has potential therapeutic benefits. It can inhibit growth and movement of tumour cells (Hino *et al.* 1985) and can prevent the processing and expression of cell surface complex oligosaccharides in tumour cells (Tulsiani *et al.* 1990).

The biochemical pathway for swainsonine production has been partially characterized in *R. leguminicola* and *M. anisopliae* (Wickwire *et al.* 1990; Sim & Perry 1997; Naranjo *et al.* 2004). In another ascomycete, *Penicillium chrysogenum*, which does not produce swainsonine, saccharopine oxidase acts on saccharopine to produce 1-piperideine6-carboxylic acid (P6C) that in turn, leads to the formation of L-pipecolic acid in presence of pipecolate oxidase (Naranjo *et al.* 2004). Saccharopine reductase (EC number 1.5.1.10) converts P6C to form saccharopine, thereby impacting the accumulation of saccharopine and L-lysine in the cell (Naranjo *et al.* 2004). In *R. leguminicola*, pipecolic acid is formed by the catabolism of L-lysine that can lead to the synthesis of the alkaloids slaframine and swainsonine (Wickwire *et al.* 1990).

Naranjo *et al.* reported that the disruption of the *lys7* gene, which encodes for saccharopine reductase in *P. chrysogenum*, results in the accumulation of large amounts of pipecolic acid (Naranjo *et al.* 2004). Pipecolic acid is known to ultimately impact swainsonine production. Thus, saccharopine reductase, although involved in formation of saccharopine, may play a role in the metabolism of swainsonine and lysine, two major end products of the lysine metabolic pathway in the alkaloid producing fungus *U. oxytropis*. However, little is known about the biosynthetic pathway of swainsonine produced by *U. oxytropis* despite its potential importance.

The aim of this study was two-fold; first to identify the gene sequence of *U. oxytropis* saccharopine reductase and second to disrupt the function of saccharopine reductase using our established transformation system (Mukherjee *et al.* 2010) and evaluate the resulting levels of biochemical products of the pathways related to swainsonine metabolism. We report the identification of the saccharopine reductase gene sequence from *U. oxytropis* and that disruption of the gene led to high accumulation of P6C, swainsonine and pipecolic acid, along with a decrease in the levels of saccharopine and lysine suggesting the involvement of saccharopine reductase in the swainsonine and lysine metabolic pathways.

Materials and methods

Strains, media, and culture condition

Undifilum oxytropis was cultured from leaves of *Oxytropis sericea* (white locoweed), which was collected from Green River, WY, USA (hereafter referred to as isolate 25-1 of *U. oxytropis*). Intact plant samples were pressed and dried for subsequent isolation and culturing of the endophyte. The tissues were surface sterilized for 30 s in 70 % ethanol, followed by 3 min in 20 % bleach, and then 30 s in sterile water. Tissues were dried on sterile paper towels and plated on water agar media. Plates were stored at room temperature (25 °C) for future use. Fungal hyphae were transferred to potato dextrose agar (PDA) plates and grown at room temperature for at least 14 d. Hyphae from the recovered endophytes were transferred onto PDA plates and maintained at 18 °C (Ralphs *et al.* 2008; Mukherjee *et al.* 2010). The 25-1 isolate described above has been preserved as desiccated mycelia and stored at both 4 °C and −80 °C. The pressed *Oxytropis sericea* 25-1 specimen was stored at room temperature at the New Mexico State University-Center for Natural History Collections (NMSU-CNHC).

Nucleic acid isolation from Undifilum oxytropis

Fungal genomic DNA was extracted using the DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). *Undifilum oxytropis* DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and analyzed on a 1 % agarose gel. The purified DNA was used for PCR, cloning, and sequencing as described below.

Degenerate PCR

Saccharopine reductase coding sequences from *Magnaporthe grisea* (nts 1350-2303, accession number: AF144424) and *Penicillium chrysogenum* (nts 395-1348, accession number: XM_002564566) were aligned and degenerate primers sacred_seq-F and sacred_seq-R (Table 1) were designed from the alignment. *Undifilum oxytropis* DNA was amplified using PCR and the degenerate primers. PCR was performed using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The resulting PCR product was cloned in pGEMTeasy cloning vector (Promega, Madison, WI, USA) and sequenced using Li-Cor 4200 Global IR 2 system at the New Mexico State University Molecular Biology Sequencing Facility.

Rapid amplification of cDNA ends (RACE)-PCR

RACE-PCR was used to obtain additional 3′ sequence of saccharopine reductase. One μg of total RNA was extracted using the Plant RNeasy kit (Qiagen, Valencia, CA, USA). The isolated RNA was treated according to manufacturer's instructions of the First Choice RLM-RACE-PCR kit (Ambion, Austin, TX, USA) and processed to amplify the cDNA ends. The amplified product was cloned into a pGEMTeasy cloning vector (Promega, Madison, WI, USA) and the product was sequenced using universal M13 forward and reverse primers using the Li-Cor system as described above.

Inverse PCR

Inverse PCR was used to obtain sequence of the 5′ end of saccharopine reductase. Five μg of *Undifilum oxytropis* DNA was digested with 10 units of *Sal*I (Promega, Madison, WI, USA).