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Development of a transformation system in the swainsonine producing, slow growing endophytic fungus, *Undifilum oxytropis*

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ABSTRACT

Undifilum oxytropis (Phylum: *Ascomycota*; Family: *Pleosporaceae*) is a slow growing endophytic fungus that produces a toxic alkaloid, swainsonine. This endophyte resides in locoweeds, which are perennial flowering legumes. Consumption of this fungus by grazing animals induces a neurological disorder called locoism. The alkaloid swainsonine, an α-mannosidase inhibitor, is responsible for the field toxicity related to locoism. Little is known about the biosynthetic pathway of swainsonine in endophytic fungi. Genetic manipulation of endophytic fungi is important to better understand biochemical pathways involved in alkaloid synthesis, but no transformation system has been available for studying such enzymes in *Undifilum*. In this study we report the development of protoplast and transformation system for *U. oxytropis*. Fungal mycelia required for generating protoplasts were grown in liquid culture, then harvested and processed with various enzymes. Protoplasts were transformed with a fungal specific vector driving the expression of Enhanced Green Florescent Protein (EGFP). The quality of transformed protoplasts and transformation efficiency were monitored during the process. In all cases, resistance to antibiotic hygromycin B was maintained. Such manipulation will open avenues for future research to decipher fungal metabolic pathways.

1. Introduction

Locoweeds commonly refer to a group of perennial flowering legumes that are found in the dry and arid regions of United States. Common genera of locoweed found in the western USA are *Oxytropis sericia, Oxytropis lambertii and Astragalus mollissimus* (Allred, 1991). Aerial parts of locoweeds can be poisonous when consumed by grazing animals (James and Panter, 1989). Consumption of locoweed by cattle causes a neurological disorder called locoism, which is characterized by loss of muscular coordination and staggered gait (Broquist, 1985). In contrast, other locoweed species, such as *Astragalus crassiparus* or some populations of *O. lambertii* are not toxic to grazing animals (Ralphs et al., 2002).

Toxic locoweeds are found to be associated with a slow growing endophytic fungus, *Undifilum oxytropis* (Pryor et al., 2009). *U. oxytropis* is an *Embellisia*-like fungus that does not sporulate or produce external mycelia on the host plant. The fungus is found to be in a symbiotic relationship with the locoweeds and found in associated with seed coat, stem and leaves (Ralphs et al., 2002). When cultured *in vitro*, the fungus grows 0.03–0.34 mm/day with thin septate hyphae (Braun et al., 2003) on potato dextrose agar (PDA, Difco, Detroit, MI) plates at room temperature. Incubation of cultures for more than 30 days is often required to attain a colony diameter of at least 5 mm. After colonies reached 5–6 mm in diameter, continued radial growth is extremely slow or arrested. These endophytic fungi also produce a polyhydroxy alkaloid, swainsonine, (1, 2, 8-trihydroxyindolizidine), naturally and in culture (Braun et al., 2003; Harris et al., 1988).

Levels of swainsonine in locoweeds correlate well with presence or absence of endophytic fungi in plants (Braun et al., 2003). Production of swainsonine is dependent on various factors such as environmental conditions and the specific fungal isolate (Braun, 1999; Oldrup, 2005). Swainsonine is a very stable compound that can induce toxic reactions even when the locoweed plants have been dried for many years. Swainsonine inhibits lysosomal α-mannosidase (involved in the biosynthesis and catabolism of glycoproteins) and golgi α-mannosidase II (Elbein, 1991; Stegelmeier et al., 1999).

The metabolic pathway of swainsonine has been partially determined in *Rhizoctonia leguminicola* and *Metarhizium anisopliae* (Sim and Perry, 1997; Wickwire et al., 1990). Biochemical details of the swainsonine pathway in alkaloid producing fungi such as *U. oxytropis* have not been deciphered, principally due to the absence of methods for genetic manipulation.

The primary step to such manipulation would be to establish methods for generation of protoplasts and develop a transformation

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system. In related Ascomycetes like *Alternaria alternata* and *Penicillium chrysogenum*, preparation and regeneration of protoplasts and transformation system have been established. (Akamatsu et al., 1997; Fierro et al., 1993). There are reports of protoplast isolation from slow growing grass endophytes (Panaccione et al., 2001, Wang et al., 2004). However, there is no established protocol for generating protoplasts from swainsonine producing slow growing endophytic fungi like *U. oxytropis*. The aim of this study was to establish a protocol for generation of protoplasts and develop a transformation system for *U. oxytropis*.

In the current study, protoplasts of *U. oxytropis* were prepared and transformed with a fungus specific vector, pPd-EGFP driving the expression of Enhanced Green Florescent Protein (EGFP) (Suzuki et al., 2000). The quality of transformed protoplasts and transformation efficiency were monitored during the process by visualizing GFP in the transformed protoplasts. Screening of the regenerated fungal mycelia for stable expression of GFP was performed.

2. Materials and methods

2.1. Fungal strains and culture conditions

U. oxytropis isolate 25-1 was isolated from leaves of *Oxytropis sericea* (white locoweed), which were collected from sites around Green River, WY, USA. Samples were pressed to dry the intact plant for subsequent isolation and culturing of the endophyte. The greenest tissues were selected and 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). Fungal hyphae were transferred to potato dextrose agar (PDA) plates and grown at room temperature (Ralphs et al., 2008) for at least 14 days. Hyphal mass of the recovered endophytes were transferred onto PDA plates and maintained at 18 °C. The 25-1 isolate described above has been preserved as desiccated mycelia and stored at both 4 °C and −80 °C at the New Mexico State University Center for Natural History Collections (NMSU-CNHC).

2.2. HygromycinB selection

In this study, transformation of *U. oxytropis* was performed using pPd-EGFP vector. pPd-EGFP, is a fungal specific expression vector that confers resistance to antibiotic hygromycin B (Hyg B) because the vector expresses the HygB phosphotransferase gene driven by the *Aspergillus nidulans* trpC-promoter (Suzuki et al., 2000). Once transformed, the vector expresses EGFP driven by the *Cryphonectria parasitica* glyceraldehydes-3-phosphate dehydrogenase (gpd) promoter. To evaluate the sensitivity of *U. oxytropis* to HygB, the fungi was grown in presence of varying concentrations of HygB (MP Biomedicals, OH), 0 μg/ml to 40 μg/ml. The cultures were grown for 14 days at room temperature. Measurement of hyphal growth was performed by counting the length of radial growth in mm beyond a point of reference point (start of culture on day zero). Hygromycin selection was performed on three independent fungal cultures grown on PDA plates for each concentration.

2.3. Preparation of protoplasts

Fungal protoplasts were prepared based on methods described by Churchill et al. (1990) with modifications as described below. Liquid suspension cultures of *U. oxytropis* were started in 100 ml of Potato Dextrose Broth (PDB) and allowed to shake for two weeks on a platform shaker at 200 rpm at room temperature. Hyphal suspensions were harvested by filtration using miracloth (EMD Biosciences, San Diego, CA) in a Buchner funnel. Mycelial mass was resuspended in 100 ml of digestion buffer and incubated at room temperature on an orbital shaker for 3 h. Digestion buffer consisted of 1 ml of β-glucuronidase (Sigma Aldrich, St. Louis, MO), 75 mg lysing enzyme (Sigma Aldrich, St. Louis, MO), 800 mg β-D-Glucanase G (Sigma Aldrich, St. Louis, MO), 600 mg Bovine Serum Albumin (Sigma Aldrich, St. Louis, MO) dissolved in 100 ml osmotic medium (1.2 M $MgSO_4$ with NaH_2PO_4, pH 5.8). After incubation, 8 ml of the digested mycelial mass was collected in 30 ml corex tubes and combined with 10 ml of Trapping Buffer (0.4 M Sorbitol in 100 mM Tris–HCl, pH 7.0). Thereafter, the solution was centrifuged at 6000 rpm at 4 °C for 15 min. Protoplasts were collected at the interface of the two layers and 2 volumes of 1 M sorbitol were added. The trapped protoplasts were centrifuged at 6000 rpm at 4 °C for 5 min. Pellets were collected after decanting the supernatant and collected protoplasts were suspended in STC buffer (1 M sorbitol in 100 mM Tris–HCl, pH 8.0, 100 mM $CaCl_2$). Protoplasts suspended in STC buffer were kept on ice for transformation experiments. Protoplasts generated were counted using a hemocytometer.

2.4. Transformation and regeneration of protoplasts

Freshly prepared protoplasts in a volume of 100 μl were transferred to pre-cooled 50 ml tubes on ice. Starting protoplasts concentration was 10^9/ml. Five μg of pPd-EGFP vector diluted in 10 μl of Tris–EDTA (TE) buffer was added to the protoplast solution and incubated on ice for 30 min. For the control reaction, 10 μl of the pPdEGFP vector was replaced by TE buffer. Thereafter, 1 ml of PTC buffer (PTC buffer: 40% Poly Ethylene Glycol, 4000 Molecular Weight, 100 mM Tris–HCl, pH 8.0 and 100 mM $CaCl_2$) was added to each tube, and incubated for an additional 25 min at room temperature. The transformation mixture was evenly distributed as 2, 20, and 200 μl droplets onto petri dishes. 12.5 ml of regeneration medium composed of 1 M of sucrose, 0.001 w/v of yeast extract, 0.001 w/v of casein hydrolysate and 0.016% of Bacto Agar was added to each plate. Further 12.5 ml of regeneration media containing 60 μg/ml HygB was overlaid on each plate. The concentration of HygB was more than double the initial lethal concentration (20 μg/ml) for *U. oxytropis*. High concentration of HygB was added to each plate in order to avoid dilution of the antibiotic. Further experiments were supplemented with 20 μg/ml HygB. Concentration of protoplasts was maintained constant throughout the process. Plates were incubated for four days at room temperature to observe hyphal growth. Fungal cultures produced after regeneration were transferred to fresh PDA–HygB containing plates. Transfer of fungal cultures was performed after three weeks of growth, which correlates to one fungal passage. Control transformation was also transferred to PDA plates and overlaid with media without HygB to compare phenotype of putative transformants. *U. oxytropis* protoplasts were also regenerated on hygB- negative PDA plates as an additional control. Transformation experiments were repeated four times.

2.5. Sporulation and hyphal tipping

To generate mononucleate cultures, mycelia were transferred to water agar plates for sporulation. Single spores and single hyphal tips were collected from water agar plates to obtain a pure mononucleate culture. Single spore and single hypha were grown separately in different PDA plates again for three weeks to visualize EGFP expression microscopically.

2.6. Microscopy

Zeiss (Thornwood, NY) inverted florescence microscope, Axiovert 200 M was used to capture florescence images at a combined objective and eyepiece magnification of 40× and 10×, respectively. Nuclear DNA was stained using 4′, 6-diamidino-2-phenylindole