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Agrobacterium-mediated transformation of *Sclerotinia sclerotiorum*

Richard J. Weld [a,*], Colin C. Eady [a,b], Hayley J. Ridgway [a]

[a] *National Centre for Advanced Bio-Protection Technologies, PO Box 84, Lincoln University, Canterbury, New Zealand*
[b] *New Zealand Institute for Crop and Food Research, Lincoln, New Zealand*

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Abstract

Ascospores from the phytopathogenic fungus *Sclerotinia sclerotiorum* were transformed to hygromycin B resistance by co-cultivation with *Agrobacterium tumefaciens*. Transformed spores germinated and grew on PDA supplemented with 100 ug/ml hygromycin B. The presence of mitotically stable *hph* gene integration at random sites in the genome was confirmed by PCR and Southern blot analysis. A transformation frequency of 8×10^{-5} was achieved in five separate experiments. This study is the first report of success co-cultivating *A. tumefaciens* with *S. sclerotiorum*. This report of a reproducible *Agrobacterium*-mediated transformation method should allow the development of T-DNA tagging as a system for insertional mutagenesis in *S. sclerotiorum* and provide a simple and reliable method for genetic manipulation.

Keywords: *Agrobacterium*-mediated transformation; *Sclerotinia sclerotiorum*; Hygromycin B resistance

1. Introduction

Sclerotinia sclerotiorum (Libert) de Bary is an ubiquitous, necrotrophic, ascomycetous fungus that infects over 400 plant species including many economically important crops (Purdy, 1979; Boland and Hall, 1994). During its lifecycle this highly successful pathogen can form hardy resting structures called sclerotia that allow it to over-winter in the soil. Sclerotia are formed when environmental conditions become unfavourable for fungal growth and in favourable conditions (usually the following Spring), they germinate to produce mycelia or carpogenically to disperse abundant infective ascospores that initiate new infections.

During the infection of plants, *S. sclerotiorum* secretes oxalic acid and a wide range of lytic enzymes, killing and macerating the plant tissue. Valuable knowledge about the molecular basis of *S. sclerotiorum*'s life cycle and pathogenicity could be gained from genetic studies such as gene knock-out and insertional mutagenesis. However, efforts to understand the

* Corresponding author. Tel.: +64 03 325 2811; fax: +64 03 325 3864.
E-mail address: weldr@lincoln.ac.nz (R.J. Weld).

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genetic basis of *S. sclerotiorum* pathogenicity have been limited by the lack of a suitable, easily reproducible transformation method.

A standard polyethylene glycol-mediated transformation system has been used previously in a gene knock-out and a gene expression study in *S. sclerotiorum* (Rollins, 2003; Chen et al., 2004). However, the protoplast-based transformation of *S. sclerotiorum* has not been routinely reproduced by other groups. Difficulties achieving transformation of *S. sclerotiorum* have recently led to studies on regulation of the *S. sclerotiorum acp1* gene being performed in the related, and more easily transformable fungus, *Botrytis cinerea* (Rolland et al., 2003).

Agrobacterium tumefaciens naturally causes crown gall in plants by transferring part of a tumour-inducing plasmid (the T-DNA) which integrates into the recipient genome. This DNA transfer system has been adapted to transfer DNA in vitro to a wide range of organisms including filamentous fungi (de Groot et al., 1998; Covert et al., 2001; Malonek and Meinhardt, 2001; Mikosch et al., 2001; Mullins et al., 2001). In plants, T-DNA generally integrates via illegitimate recombination, and therefore at random sites, into the recipient genome (Mayerhofer et al., 1991). In fungi, when the T-DNA has sequence similarity to the recipient genome, integration into the genome can be by homologous or illegitimate recombination (Bundock et al., 1995; de Groot et al., 1998). If the transferred DNA lacks homology to the recipient genome, *Agrobacterium*-mediated transformation usually results in, low-copy number integrations at random sites in the fungal genome and thus is an ideal system for insertional mutagenesis.

Agrobacterium-mediated transformation of *S. sclerotiorum* has been tried unsuccessfully by several groups and the recalcitrance of *S. sclerotiorum* hyphae to T-DNA transformation has been recently described (Rolland et al., 2003). Asexual conidia, widely used in the transformation of other filamentous fungi, are not produced by *S. sclerotiorum*. Production of sclerotia, apothecia and ascospores in the laboratory has been described for some *S. sclerotiorum* strains (Henson and Valleau, 1940; Mylchreest and Wheeler, 1987; Phillips, 1987; Mitchell and Wheeler, 1990; Clarkson et al., 2003). No previous published work has tested the ability of *Agrobacterium* to transform these tissues.

Agrobacterium-mediated transformation has proven itself to be a simple and reproducible fungal transformation method in many instances. Unlike protoplast transformation, *Agrobacterium*-mediated transformation does not rely on cell wall degrading enzymes and avoids the widely reported inconsistencies that arise through differences in activity of enzyme batches. DNA transfer from *A. tumefaciens* has been used for both gene knock-out and gene transformation studies in filamentous fungi (Bundock et al., 1995; de Groot et al., 1998; Zwiers and De Waard, 2001; Zeilinger, 2004) and is being developed as a system for insertional mutagenesis in other filamentous fungi (Combier et al., 2003; Mullins et al., 2001). The successful development of a reproducible *Agrobacterium*-mediated transformation system for *S. sclerotiorum*, reported in this manuscript, will facilitate genetic studies such as gene knock-out in this organism and may allow the development of an insertional mutagenesis system through T-DNA tagging.

2. Materials and methods

2.1. Strains and culture conditions

S. sclerotiorum, strain GL (Rabeendran, 2000), sclerotia were grown on sterilized wheat grain inoculated with mycelial plugs as previously described (Alexander and Stewart, 1994). Mature sclerotia were stored at 4 °C or used immediately without storage. For ascospore production, sclerotia were incubated on water-saturated, sterile sand for several weeks at 17 °C with 12 h light/dark until mature apothecia were formed. Ascospores were harvested by submerging apothecia from about 500 sclerotia in 50 ml water in a plastic tube and applying −100 kPa of vacuum for 10–15 min.

A. tumefaciens strain EHA105 containing binary vector pYT6 was routinely grown on Luria Bertani Agar (LBA) supplemented with rifampicin (25 μg/ml) and kanamycin (50 μg/ml). pYT6 (pCAMBIA1380 with a *Hin*dIII/*Xho*I fragment, containing the hygromycin B-resistance gene (*hph*) under the control of the *Aspergillus glaA* promoter and the *trp*C terminator, ligated into the T-DNA) was kindly provided by Prof. Barry Scott, Massey University, New Zealand.