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Genomics of Biotrophic, Plant-infecting Plasmodiophorids Using In Vitro Dual Cultures

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The plasmodiophorids are a phylogenetically distinct group of parasitic protists that infect plants and stramenopiles, causing several important agricultural diseases. Because of the obligate intracellular part of their lifecycle, none of the plasmodiophorids has been axenically cultured. Further, the molecular biology of the plasmodiophorids is poorly understood because pure cultures are not available from any species. We report on an in-vitro dual culture system of the plasmodiophorids *Plasmodiophora brassicae* and *Spongospora subterranea* with their respective plant hosts, *Brassica rapa* and *Solanum tuberosum*. We show that these plasmodiophorids are capable of initiating and maintaining stable, long-term plant cell callus cultures in the absence of exogenous plant growth regulators. We show that callus cultures harbouring *S. subterranea* provide an excellent starting material for gene discovery from this organism by constructing a pilot-scale DNA library. Bioinformatic analysis of the sequences established that almost all of the DNA clones from this library were from *S. subterranea* rather than the plant host. The *Spongospora* genome was found to be rich in retrotransposable elements, and *Spongospora* protein-coding genes were shown to contain introns. The sequence of a near full-length non-LTR retrotransposon was obtained, the first transposable element reported from a cercozoan protist.

Key words: callus; genomic sequence; *Plasmodiophora*; powdery scab; retrotransposons; *Spongospora*.

Introduction

Plasmodiophorida is an enigmatic order of protists containing several important plant-pathogens (Braselton 1995, 2001). These include *Spongospora subterranea* which causes powdery scab disease of potato (Merz and Falloon 2009), *Plasmodiophora brassicae* which causes clubroot of Brassicaceae plants (Dixon 2009), and *Spongospora nasturtii* which causes crooked root of water-cress. *Spongospora* and the *Polymyxa* species *P. graminis* and *P. betae*, also serve as vectors for many plant viruses such as Barley yellow mosaic virus, Potato mop top virus and Beet soilborne mosaic virus (e.g. Kanyuka et al. 2003). Both the plasmodiophorid and virus diseases are serious problems for crop production worldwide. The plasmodiophorids are considered to be members of Rhizaria (Archibald and Keeling 2004), a super-assemblage of mostly amoeboid unicellular eukaryotes (Bass et al. 2005; Nikolaev et al. 2004). The position of Rhizaria relative to other groups of eukaryotes remains an active area of research, with a number of recent phylogenomic studies suggesting a larger grouping of at

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least the Stramenopila, Alveolata and Rhizaria (Burki et al. 2008). Unlike other major groups of eukaryotes, little is known about the genomes of organisms in Rhizaria (Keeling and Slamovits 2005), with the first genome sequence from a rhizarian, *Bigelowiella natans*, being in assembly (http://www.jgi.doe.gov/sequencing/why/50026.html; John Archibald pers comm).

The history of plasmodiophorid research has been strongly shaped by their obligate biotrophic nature. Plasmodiophorids persist in the environment as resistant resting spores. Stimulated by the presence of a host, resting spores germinate to release flagellated zoospores. Upon encountering the host root, these attach, and then infect cells via a complex extrusome. The plasmodiophorid then completes a biphasic period of development within the host cell, featuring cruciform nuclear divisions and multi-nucleate plasmodia. In the secondary phase of infection by some plasmodiophorids, formation of hyperplastic galls is driven by substantial fluxes in phytohormone levels (Devos et al. 2005).

Most experimental plasmodiophorid infections continue to be made in pot and glasshouse conditions. For example, experiments with *P. brassicae* are typically undertaken with slurries of resting spores prepared from rotted club root galls; plant infection is achieved by flooding potting mix with these spore suspensions (e.g. Bulman et al. 2006).

While their axenic growth is not yet possible, two techniques for gnotobiotic propogation of plasmodiophorids (principally *P. brassicae*) have been developed (Kageyama and Asano 2009). First, techniques for in vitro infection of plant hairy root cultures have been developed for *P. brassicae* (Mugnier 1987), and these have recently been adopted for *S. subterranea* (Qu and Christ 2006). Much of the natural *P. brassicae* infection cycle, such as galling and plant re-infection, can be reproduced during infection of hairy roots (Asano et al. 1999; Asano and Kageyama 2006), but these techniques have not been widely adopted. The second approach for gnotobiotic *P. brassicae* culturing began with a series of experiments in the 1960s, when it was discovered that treatment of clubroot gall sections with plant growth regulators led to the establishment of *P. brassicae*-infected callus (Ingram 1969a,b; Strandberg et al., 1966; Tommerup and Ingram 1971; reviewed by Buczacki 1980). These cultures offer large amounts of comparatively homogenous infected material, but, despite an initial flurry of enthusiasm, experimentation since the 1970s with *P. brassicae* callus cultures has been sporadic. Little use has been made of callus cultures, because they do not replicate the typical plasmodiophorid growth forms, and the use of exogenous growth supplements makes the interaction with the plant cell appear artificial.

In the present study, we show that *P. brassicae*-containing *Brassica rapa* cultures can be established and maintained in the absence of exogenous plant growth regulators. Importantly, we extend these studies to demonstrate that potato callus cultures harbouring *S. subterranea* are easily established. The usefulness of these cultures for plasmodiophorid genomic studies was demonstrated by the collection of DNA sequences from *S. subterranea*.

Results

Generation of Callus Cultures Harbouring Plasmodiophorids

Our first establishment of plasmodiophorid-plant callus cultures was a serendipitous discovery. As part of another experiment, surface-sterilised sections of *B. rapa* clubroot galls were incubated on MS culture medium to monitor for the presence of contaminating micro-organisms. After extended periods it became apparent that not only were contaminants absent from some gall sections, but that the plant cells were multiplying. White to white/green callus grew from the edges of the tissues (Fig. 1A). When these growing callus sections were transferred to a fresh medium, they multiplied, yielding calli up to 2–3 cm across. As the callus aged, the older sections changed from white to brown in colour (Fig. 1B). *Plasmodiophora brassicae*–induced *B. rapa* calli ranged from fast growing and friable to slow growing and compact. The friable callus quickly turned brown and died within two weeks if not sub-cultured, whereas the compact cultures were more easily maintained through sub-culturing. Transfers of fresh white/green callus to new plates were made monthly. *Plasmodiophora brassicae*–induced *B. rapa* callus was maintained through repeated subculture for four years, although eventually the samples were lost to contamination and/or reduced vigour. The *Plasmodiophora*–induced *Arabidopsis* callus was more difficult to establish than from the *B. rapa* host. This may have been because the sterilisation protocol damaged the smaller tissue sections, but there have also been prior reports that (uninfected) *Arabidopsis* cell cultures are more difficult to work with than those from other brassicas (e.g. Mathur et al. 1995). The *Arabidopsis* cultures