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Molecular Biology of *Plasmodiophora brassicae*

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Abstract Initially, molecular techniques were used to detect and distinguish *Plasmodiophora* pathotypes in soil. Meanwhile, chromosomes from 2.2 Mb to 680 kb are characterized and the total genome size is estimated to be approximately 20 Mb. Furthermore, the genomic gene structure and the cDNA structure of several genes have been revealed, and the expression of those genes has been linked to development of clubroot to some extent. In addition, the sequence data have reinforced the inclusion of the plasmodiophorids within the Cercozoa. The recent successes in molecular biology have produced new approaches for clubroot research.

Keywords *Plasmodiophora brassicae* · Clubroot · Chromosome · Gene structure · cDNA · Molecular detection · Systematics

The History of Research Obstacles Presented by *Plasmodiophora brassicae* Woronin

Plasmodiophora brassicae is the most studied member of the plasmodiophorids. Clubroot galls provide large amounts of plasmodiophorid material for experiments and *P. brassicae* infects the model plant *Arabidopsis thaliana*. These features make *P. brassicae* a good model organism for plasmodiophorid research. Nevertheless, our understanding of the molecular and cellular biology and biochemistry of *P. brassicae* remains limited despite the economic importance of the pathogen and a long history of its study (Woronin 1878). A review of *P. brassicae* molecular biology studies could easily be dominated by descriptions of the difficulties encountered during experiments with this organism. Nonetheless, for researchers new to *P. brassicae*, a coherent description of the pitfalls to be encountered when studying *P. brassicae* is a valuable starting point for this review.

As an obligate biotroph, *P. brassicae* has remained impossible to grow in axenic culture and the typical experimental systems for working with *P. brassicae* are comparatively unsophisticated (see Kageyama and Asano this issue). Unlike some biotrophic plant pathogens, none of the developmental stages of *P. brassicae* permit the isolation of material that is free from either host plant or microbial contamination. The most commonly isolated stage is mature resting spores isolated from clubroot galls; although these preparations are highly enriched with plasmodiophorid material, unspecific microbial contaminations are present and poor yields of RNA are obtained from the spores. This has so far precluded simple EST (expressed sequence tags) sequencing from this material. In combination with an ability to identify *Brassica* genome sequences, the assembly of a *P. brassicae* genome sequence from preparations of resting spores would probably be achievable; however, to date, the cost of undertaking an analysis of the entire genome of *P. brassicae* has prevented such an approach.

There are other possible points in the life cycle where *P. brassicae* EST sequencing could be applied, but the

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principle developmental stages where gene expression occurs, the plasmodia, continue to be very difficult to purify. Instead of EST sequencing from pure *P. brassicae* plasmodia, most isolations of *P. brassicae* gene-coding sequences have been made from whole clubroot galls, especially those from the host *Arabidopsis thaliana*. Unfortunately, using this as starting material means that the *P. brassicae* sequences are accompanied by large numbers of host plant genes. The availability of the high-quality *Arabidopsis* genome sequence, however, makes removal of these plant genes simple, whereas surface sterilization of healthy gall tissue means that the degree of microbial contamination in these studies is much reduced.

Aside from considerations of developmental stages, two other features of *P. brassicae* deserve mention. First, *P. brassicae* exists as mixtures of pathotypes in the soil and even in single clubbed roots (Jones and others 1982a, b; Fähling and others 2003). To work with genetically homogeneous material requires the generation of single spore lines, but this process is time-consuming and has been completed by only a few laboratories. Most *P. brassicae* samples used for experimentation are likely to be heterogeneous mixtures of genotypes. Second, *P. brassicae* is a notoriously fickle subject; stored spore suspensions may sporadically expire or plant infections fail to develop, stressing the essential needs for standardization of test conditions and infection controls with susceptible lines in every test.

The Size and Structure of the *P. brassicae* Genome

Plasmodiophora brassicae has small chromosomes that are impossible to distinguish by conventional cytogenetics. Braselton (1982) estimated 20 synaptonemal complexes in *P. brassicae* using serial sections for electron microscopy. Pulse-field gel electrophoresis (PFGE) can determine karyotypes (chromosome size and number) of organisms that cannot be studied using cytologic methods or classical linkage analysis (Howlett 1996). This technique has been used by a number of researchers to determine karyotypes for *P. brassicae* (Ito and others 1994; Bryan and others 1996; Graf and others 2001, 2004; Fähling and others 2004; Siemens and others 2009). Ito and others (1994) used spheroplasts as starting material and distinguished 13 chromosomal bands in the range of 1.9 Mb to 750 kb. Bryan and others (1996) started with isolated plasmodia and could separate six bands in the range of 1.7 Mb to 680 kb. Graf and others (2001) described 16 chromosomal bands of *P. brassicae* in the range of 2.2 Mb to 680 kb that corresponded with the chromosomal sizes estimated by Ito and others (1994), but with three additional chromosomal bands. When three single-spore isolates of *P. brassicae* were analyzed by PFGE, chromosome polymorphisms between isolates were identified, with no isolates having more than 16 chromosomal bands (Graf and others 2004). These chromosome polymorphisms might explain the differences in results between Ito and others (1994) and Graf and others (2001). The number of chromosomes estimated by PFGE, however, was less than the 20 synaptonemal complexes estimated by electron microscopy (Braselton 1982), suggesting that some chromosomal bands might not be completely separated by PFGE. Taking the chromosome number and size from the PFGE studies into account, the total genome size of *P. brassicae* was estimated to be 18–20.3 Mb (Graf and others 2004).

In the first steps toward creating a genetic map, Southern hybridization has been used to map several *P. brassicae*-specific DNA fragments with chromosomes separated by PFGE. Repetitive elements were suitable for visualizing most of the chromosomes and were localized on 14 (H4), 8 (E56), or 5 (RFM8) chromosomes of the single-spore isolate 'e_3' (Graf and others 2001, 2004; Fähling and others 2004). The DNA-encoding ribosomal subunits were localized to a number of chromosomal bands of *P. brassicae* (Graf and others 2004) and may well be involved in chromosome rearrangement because tracts of rRNA genes are frequently associated with chromosome variations (Davière and others 2001). Chromosome polymorphism between single-spore isolates may well hinder gene localization to specific chromosomal bands, but studies of such polymorphisms may also reveal new information behind the high variations in pathogenicity between *P. brassicae* isolates. In some organisms, chromosomal rearrangements have been associated with changes in pathogenicity, such as a loss of toxin production; for example, the loss of pathogenicity in the apple pathogen *Alternaria alternata* (Johnson and others 2001).

Recombination in *P. brassicae*

Synaptonematal complexes (Braselton 1982) and reinfection by dikaryotic plasmodia (Kobelt 2000) have been taken as evidence of sexual recombination in *P. brassicae*. As noted earlier, *P. brassicae* exists as mixtures of pathotypes in the soil and even in single-clubbed roots (Jones and others 1982a, b; Fähling and others 2003). Sexual recombination could explain the high degree of variation in field populations. To investigate the genetics of *P. brassicae*, Fähling and others (2004) attempted to establish a cross between *P. brassicae* lines. Plants were inoculated with two different single-spore isolates, 'e_3' and 'e_6', and then new single-spore isolates were extracted from the resulting new generation of clubs. No sexual recombination was observed by examining repetitive DNA from the

progeny, but a chromosome rearrangement was identified in one of the new single-spore isolates. This appeared to be a recent event occurring during the infection cycle (Fähling and others 2004). This suggests an ability of *P. brassicae* to rearrange chromosomes without sexual recombination and questions the concept of whether genetically stable lines can be established by single-spore production. New sexual recombinants might have been missed in these experiments because of a lower propagation potential in the ECD05 host plant. As with almost all *P. brassicae* lines (see Faggian and Strelkov this issue), however, no information is available for the propagation efficiency of the two parent strains in this plant host. Experiments of this kind with single-spore lines and new molecular markers will be important areas for future research. They will provide evidence about whether sexual recombination exists and is a frequent event in *P. brassicae* clubs.

Gene Discovery in *P. brassicae*

Pathogen DNA Detection and Distinguishing Races

As with several plant pathogens, an ongoing incentive for studying *P. brassicae* DNA sequences has been a desire for rapid molecular detection assays from soil or water. Non-molecular tests for the presence of *P. brassicae* in soil are time consuming and require significant space for plant growth (see Faggian and Strelkov this issue).

As with many other pathogen assays, several DNA detection assays for *P. brassicae* have used PCR primers designed in the sequence of ribosomal internal transcribed spacer (ITS) sequences (Faggian and others 1999; Wallenhammar and Arwidsson 2001; Kageyama and others 2003). These standard PCR-based assays have been highly sensitive for spore detection but are not quantitative. Recently, a real-time PCR test based on the ribosomal ITS sequences has been designed. In combination with measurements of the fatty acid biomarker arachidonic acid, quantification of *P. brassicae* infection is now possible (Sundelin 2008).

Many DNA detection studies have been combined with surveys of molecular diversity among *P. brassicae* field or single-spore isolates. In particular, there is the hope that molecular markers will eventually allow the rapid identification of virulence patterns among isolates. In some of the earliest experiments of this type, distantly related isolates of *Plasmodiophora* with divergent virulence were characterized by PCR amplification patterns using arbitrary primers (Buhariwalla and others 1995; Möller and Harling 1996; Yano and others 1997) and sequence-generated primers (Buhariwalla and others 1995; Ito and others 1999a; Klewer and others 2001). The number of distinguishing patterns was low, however, and virulence patterns and PCR patterns could not be correlated unequivocally. Early successes in the cloning of repetitive DNA elements of the pathogen were achieved when small *P. brassicae* DNA libraries were established (Buhariwalla and Mithen 1995; Buhariwalla and others 1995; Ito and others 1998; Klewer and others 2001). Difficulties in obtaining high-quality genomic *P. brassicae* DNA limited the library size in these studies to between 27 and 150 clones.

In the most comprehensive study of its kind to date, Manzanares-Dauleux and others (2001) applied 19 random primers and 9 sequenced-derived primers to obtain 103 polymorphic bands among 46 related isolates. This demonstrated the very high level of genetic diversity among isolates from one field (Manzanares-Dauleux and others 2001). Two RAPD markers (Manzanares-Dauleux and others 2001) and a sequence-characterized amplified region (SCAR) marker (Manzanares-Dauleux and others 2000) were correlated to isolates of pathotype group 1. In a similar set of experiments, Klewer and others (2001) examined the molecular diversity of single-spore isolates originating from a German field isolate ‘e’. Reliable differentiation of isolates was produced with sequence-derived primers and RFLP analysis using repetitive elements as hybridization probes. Using three highly repetitive *P. brassicae* probes and combinations of restriction enzymes, the number of polymorphic bands in these fingerprint patterns was increased to more than 100 and the patterns of different single-spore isolates showed fewer common bands (Fähling and others 2003). In recent experiments, isolate-specific banding patterns have been obtained using a combination of sequence-derived primers from the borders of highly repetitive elements of *P. brassicae* (sequence-anchored primer) and primers for genomic walking (random primer) (Rehn and Siemens unpublished).

Overall, the first simple molecular techniques to characterize isolates are now established but there is as yet no standard set of markers to describe an isolate. A widespread screening of pathotypes by standardized molecular techniques would make it easier to describe pathotypes and map their distribution. Molecular techniques that rapidly describe the virulence characteristics of *P. brassicae* field populations would be highly valuable for studies of population structure and dynamics in *Brassica* crops and thereby benefit growers and farmers.

Examining the Evolutionary Position of *P. brassicae* and Other Plasmodiophorids

One of the most significant advances coming from the study of the molecular biology of *P. brassicae* has been a clarification of plasmodiophorid evolution. As with many

other eukaryotic microbes, the first DNA target amplified and sequenced from *P. brassicae* was the ribosomal small subunit gene (SSU) (Castlebury and Domier 1998). Unlike all other plasmodiophorids examined to date, the *P. brassicae* SSU was found to contain self-splicing type I introns. The SSU sequence was used by Cavalier-Smith and Chao (1996/97) to provide the first robust phylogenetic evidence of linkage between the plasmodiophorids and cercozoan protists. To understand cercozoan evolution further, Archibald and Keeling (2004) used degenerate PCR to amplify sequences of plasmodiophorid actin and polyubiquitin genes, including from *P. brassicae*. Phylogenies constructed with the actin amino acid sequences again showed a relationship between the plasmodiophorids and cercozoans. Even more convincingly, *P. brassicae* was shown to share with other cercozoans a characteristic amino acid insertion between ubiquitin monomers (Archibald and others 2003).

Subsequent analyses of these sequences using larger data sets have reinforced the inclusion of the plasmodiophorids within Cercozoa (for example, Bass and others 2005). For a long time the systematic placement of *P. brassicae* has been unclear, and the organism is still often referred to historically as a fungus. The more accurate evolutionary positioning of *P. brassicae* provides an essential basis for understanding many aspects of *P. brassicae* biology. For example, it will be more rational to compare the *P. brassicae* genome with those of other cercozoans—as they become available—than with the currently available completely sequenced fungal genomes.

cDNA Cloning Approaches

In early studies, only a small number of partial genes were isolated from *P. brassicae*. Ito and others (1997) isolated a single-copy DNA sequence unique to *P. brassicae*. From subtractive cDNA libraries, Graf and others (2004) isolated a few partial cDNAs that had no significant homology to GenBank sequences, whereas Ito and others (1998) found a single-copy fragment with high similarity to the yeast RNA polymerase II gene. Brodmann and others (2002) isolated a short partial sequence for a trehalose-phosphate synthase (*Pb*TPS). Ando and others (2006) designated a full-length *P. brassicae* cDNA clone of a single-copy gene *Pb*STKL1 due to a serine/threonine kinase-like domain at the C-terminal region. An acceleration of the hunt for *Plasmodiophora* genes was achieved when more than 70 cDNA clones of the pathogen were isolated by suppression subtractive hybridization (SSH) or by direct EST sequencing (Bulman and others 2006). More than 40 of these cDNA sequences were full-length. This study showed that working with a pool containing both plant and *P. brassicae* cDNA was an acceptable starting point for plasmodiophorid gene discovery. Again using SSH, but starting with *Brassica rapa* rather than *Arabidopsis* clubroot galls, Sundelin (2008) isolated about 140 genes. Half of these gene sequences originated from the pathogen; of these, ten clones were newly characterized *P. brassicae* genes. The remainder of the *P. brassicae* genes had been previously identified, particularly from the work of Bulman and others (2006), showing the consistency of results between these two SSH studies. Moreover, multiple copies of some *P. brassicae* genes (for example, *Pb*GST1, *Pb*SUR2, *Pb*SHSP1) were sequenced in both studies, indicating a high transcription of these genes or perhaps preferential amplification of the transcripts.

P. brassicae Gene Structure

The presence of introns in plasmodiophorid genes was apparent during cloning of short *P. brassicae* gene fragments (for example, Brodmann and others 2002). The first full structure of a *P. brassicae* protein-coding gene was provided when Ando and others (2006) sequenced both the cDNA and the genomic DNA of the *Pb*STKL1 gene. By comparing these sequences, the *Pb*STKL1 gene was observed to contain 14 intron sequences. Subsequently, Bulman and others (2007) obtained a genomic DNA sequence corresponding with 24 *P. brassicae* cDNAs, whereas Sundelin (2008) confirmed the genomic DNA sequence corresponding with five newly characterized genes. These included the cDNA and genomic sequence of the first ATPase gene (*Pb*ATP1) cloned from a plant parasitic protozoan species. The *Pb*ATP1 protein contained at least eight transmembrane helixes and a number of protein domains characteristic for P-type ATPases. Although the total number of genomic DNA sequences is small, the structure of *P. brassicae* genes appears to be consistent between these studies. On the whole, *P. brassicae* genes are intron-rich. The great majority of the introns were bordered by the consensus border sequences 5′ GT and AG 3′ and contained consensus branch point sequences, indicating that the splicing event in *P. brassicae* proceeds in a similar way to that in the majority of eukaryotes to date. Examination of the promoter and transcription starter sites of genes indicated that *P. brassicae* transcription is likely to begin from initiator elements, as no TATA box-containing promoters were found. Where neighboring genes were confirmed, intergenic distances were found to be short, ranging from 44 to 470 bp, but a number of larger DNA fragments of up to 2.2 kb were also found and these contained no obvious genes (Bulman and others 2007).

Calculations made using a mean gene size of 1500 bp and a mean intersection size of 500 bp mean that a *Plasmodiophora* genome of approximately 20 Mbp has the potential to contain 10,000 genes. Consequently, 99% of

the coding sequences on the genetic map of *P. brassicae* remain unknown.

Examining the Plant-*P. brassicae* Interaction

Because *P. brassicae* continues to resist routine manipulation in the laboratory, finding functions for isolated genes remains very challenging. Genomic studies of *P. brassicae* are hampered by both the small number of *P. brassicae* genes isolated and the limited homology of many *Plasmodiophora* sequences with known genes. To date, approximately 100 partial cDNA fragments or full-length clones of *P. brassicae* have been isolated. For about half of these *P. brassicae* genes, a biochemical function is strongly indicated by DNA or amino acid similarities with characterized genes of other organisms, but until now no function for a *P. brassicae* gene has been proven experimentally. The remaining DNA fragments from *P. brassicae* reveal little or no similarity to sequence data in the databases, reflecting the paucity of genomic data available for protists from the Cercozoa (Keeling and others 2005). Nucleotide or amino-acid-sequence similarity with known genes from other organisms can give a hint as to gene function, but the presence of a protein domain in a sequence might also be misleading.

The small roots of *A. thaliana* facilitate the observation of clubroot symptoms in this host plant, revealing a reasonably reproducible pattern of disease development under controlled environmental conditions (Kobelt 2000; Kobelt and others 2000; Siemens and others 2002, 2006a). Correlating transcription with distinct stages of symptom development caused by the parasite will be an opportunity to define landmarks and milestones of disease development and a possibility for gaining insights into the function of *P. brassicae* genes. A prominent actin cytoskeleton has been detected by immunohistochemistry over that part of the life cycle after secondary infection (Kobelt 2000). The *Pb*Actin and *Pb*GST products appeared strongly expressed and well detectable over the whole infection cycle within *A. thaliana* roots and could therefore be used as indicators of an actively growing pathogen (Siemens and others 2009).

Two unidentified single-copy genes, *Pb*Brip9 and *Pb*CC249, were strongly expressed at disease stages corresponding with the occurrence of sporulating plasmodia (Siemens and others 2009), whereas *Pb*STKL1 transcription coincided with resting spore formation (Ando and others 2006). The expression of gene fragment Y10 was exclusively correlated with the vegetative plasmodial stage (Ito and others 1999b), and the transcription of *Pb*TPS correlated with an accumulation of trehalose (Brodman and others 2002). Finally, preliminary evidence indicates that the genes *Pb*PP2A (protein phosphatase 2A) and *Pb*HMG (DNA binding protein with high-mobility group box) are preferentially expressed in the early phases of infection in *B. rapa* roots (Sundelin 2008). Altogether, the very first molecular landmarks of clubroot disease development in *Arabidopsis* are established. For the first time, whole-genome transcription data from the host plant *A. thaliana* has revealed new insights into clubroot development (Siemens and others 2006a, b; Jubault and others 2008; Ludwig-Müller and others this issue). Combining *Arabidopsis* microarray data with expression analyses of *P. brassicae* genes will provide a strong new approach to clubroot research in the future.

As with other biotrophic pathogens, it is likely that *P. brassicae* secretes proteins into the host cell to effect modifications of cell structure. Predicted proteins with signal peptides from *P. brassicae* are thus of much interest. Surprisingly, a recent attempt to specifically clone such genes was not successful (Sundelin 2008). Of the *P. brassicae* sequences with signal peptides known to date, two matched with functionally characterized genes with roles in pathogenicity, namely, *Pb*PPI1, which contains an FKBP family peptidyl prolyl *cis-trans* isomerase domain, and *Pb*PDA1, which contains a "NodB-type" chitooligosaccharide deacetylase domain (Bulman and others 2006). Surface-exposed or secreted PPIase domain-containing proteins have been found in several intracellular pathogens (Engleberg and others 1989; Moro and others 1995). The chitooligosaccharide deacetylase family includes members known to deacetylate substrates, including xylan and chitin, which might play roles in cell wall penetration during myxamoeboid movement of secondary plasmodia.

Examples of RNA interference (RNAi) and related RNA-silencing phenomena are widespread in nematodes, plants, and fungi. Overexpression of parasite genes in the host plant has been used successfully to induce resistance against root-knot nematodes (Huang and others 2006). The intracellular parasite *P. brassicae* lives in close contact with the host; therefore, using a plant-derived RNAi strategy might work when an important parasitism gene of *P. brassicae* is identified. A first RNAi approach of this type has been undertaken, but no evidence for induction of RNA silencing in *P. brassicae* has emerged so far (Bulman 2006).

Outlook

These recent general successes in molecular biology have produced new approaches for clubroot research. The approximately 100 known genes of *P. brassicae* provide new opportunities for examining plasmodiophorid biology. We see a number of new techniques that hold promise for

future *P. brassicae* research. Transient gene expression has been used to analyze genes of some obligate leaf pathogens (Cai and others 2007). It seems likely that transient gene expression can be achieved in *P. brassicae*-infected plant cells and probably also in *P. brassicae* secondary plasmodia by particle bombardment. Thus, in the future, promoter studies or gene function analysis in *P. brassicae* might become possible. Some cellular features of clubroot infection conditions can be simply observed in slides of a gall. Time limitations to the viability of gall sections might be overcome by the dual culture systems of secondary plasmodia in suspension cells (Asano and Kageyama 2006), where movement and division effects can be observed more simply compared with the normal development of disease in roots. Hormone-induced callus cultures have long been established for *P. brassicae* (for example, Buczacki 1983); perhaps in the near future these will fulfill some of their promise as reproducible experimental tools for plasmodiophorid experimentation. Up to now the lack of good observable phenotypes in clubroot has limited these studies.

Tissue microdissection has been used successfully as a starting point for several genomic and metabolite studies of specific cell types (Nelson and others 2006). Preliminary laser microdissection of different plasmodial stages of *P. brassicae* has already been established (A. Schuller, Dresden personal communication). In a parallel approach (Siemens unpublished), *P. brassicae* plasmodia have been gathered by combining root protoplast isolation and tissue-specific GFP expression lines of *A. thaliana.* Birnbaum and others (2003) originally established this technique for the isolation of specific root cells by fluorescence-assisted cell sorting (FACS) and to establish a root expression map (www.arexdb.org). Further progress with these dissection techniques will improve analyses of *P. brassicae* development by linking gene expression to a specific host or pathogen cell type.

The genome of *P. brassicae* is small. With continuing reductions in sequencing costs and the huge capacity of sequencing facilities worldwide, a complete genome sequence of the parasite is surely not far away. With the arrival of new pyrosequencing approaches, studies of the *P. brassicae* transcriptome will probably speed up in the near future.

Over the past century the plasmodiophorids have been synonymous with problems for biologists. Nonetheless, some dramatic progress has been made recently and several important problems have been solved. There are still clouds on the horizon in understanding the molecular biology of this agriculturally important pathogen, but the forecast is becoming brighter.

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