RESEARCH LETTER

Evidence that *Polymyxa* species may infect *Arabidopsis thaliana*

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Abstract

Polymyxa spp. are obligate biotrophs belonging to the plasmodiophorid group, responsible for transmitting a large number of plant viruses to many crop species. Their obligate nature makes them difficult to study. Controlled environment experiments were used to investigate the potential of infection of *Arabidopsis thaliana* by *Polymyxa* spp. to provide a more tractable system. Two ecotypes of *Arabidopsis*, Columbia and Landsberg *erecta*, were grown in soils known to be infested with *Polymyxa*. At the end of a 2-month growth period, both ecotypes were found to harbour *Polymyxa*-like structures or spores. These findings were confirmed by *Polymyxa*-specific PCR tests and rDNA sequencing, which positively identified the presence of *Polymyxa* in the roots of both ecotypes of *Arabidopsis*. Both *Polymyxa graminis* and *Polymyxa betae* were identified. This is the first report of infection of *Arabidopsis* by *Polymyxa* spp. and shows the possibility of using this system for studies of infection biology and host–parasite interactions.

Introduction

Polymyxa spp. are a group of obligate root-infecting organisms belonging to the plasmodiophorid group that are important plant–virus vectors (Kanyuka *et al.*, 2003). *Polymyxa graminis* transmits viruses such as soil-borne cereal mosaic virus (SBCMV), soil-borne wheat mosaic virus and wheat spindle streak mosaic virus to cereals. *Polymyxa betae* transmits beet necrotic yellow vein virus, the cause of rhizomania, to sugar beet. *Polymyxa graminis* has a wide host range including wheat, barley, rye, rice, sorghum, groundnut and various grasses, whereas *P. betae* is mostly restricted to beet and other plants in the family *Chenopodiaceae*.

A number of subgroups (ribotypes) of *Polymyxa* spp. have been identified according to rDNA sequence data (Ward *et al.*, 1994, 2005; Ward & Adams, 1998; Legrève *et al.*, 2002). Some of the *P. graminis* ribotypes appear to differ in host range and temperature requirements, leading to the suggestion that they should be classified as *formae speciales* (Legrève *et al.*, 1998, 2002). Two groups of *P. graminis* isolates are found in temperate regions: ribotype I (f. sp. *temperata*) and ribotype II (f. sp. *tepida*). All internal transcribed spacer (ITS) rDNA sequences for *P. betae* reported to date fall into two types that differ by only one base pair (Ward & Adams, 1998; Legrève *et al.*, 2002).

Because of their obligate nature and relatively long life cycle, *Polymyxa* spp. have been difficult to study. The development of a model system for studying *Polymyxa*–plant interactions would be extremely useful. *Arabidopsis thaliana* is an invaluable model system for several reasons: (1) short generation time, (2) the ability to grow large numbers in a relatively small space, (3) its ability to self-fertilize, (4) the large number of progeny that can be produced from a single plant, (5) its small haploid genome containing a relatively small number of repetitive genetic elements, (6) the availability of a fully sequenced genome, (7) the availability of mutagenized lines, (8) ease of transformation and (9) the large number of ecotypes exhibiting natural variation available (Meyerowitz, 1989). These features are in contrast to many crop species such as cereals, where genetic resources are less well advanced.

Arabidopsis has already been used very successfully to study the interactions of another plasmodiophorid: *Plasmodiophora brassicae* (Koch *et al.*, 1991). The ability to separate host sequences from those of *Plasmodiophora* by bioinformatics analysis has simplified the interpretation of data, for example from suppressive subtractive hybridization experiments to study gene structure and expression (Bulman *et al.*, 2006, 2007). Sources of resistance and factors important for the infection of *Plasmodiophora* have been studied by exploring the responses to both natural and induced (mutagenic) variation in host genes affecting infection (Siemens *et al.*, 2002; Alix *et al.*, 2007). *Arabidopsis* has been used to visualize infection biology of *P. brassicae* (Mithen & Magrath, 1992). The availability of synteny maps between *Arabidopsis* and *Brassica* spp. has allowed the identification of resistance loci in *Brassica* spp. first identified in *Arabidopsis* (Suwabe *et al.*, 2006). Global analysis of host gene expression at different time points postinfection has been possible using *Arabidopsis* genome arrays, and this has allowed the identification of host genes that may be important for infection by *Plasmodiophora* (Siemens *et al.*, 2006). Genes of interest can then be studied further by transforming into *Arabidopsis* or by utilizing the bank of insertion lines available in *Arabidopsis* (Puzio *et al.*, 2000; Siemens *et al.*, 2006).

Many of the host plants that *Polymyxa* spp. infect are not well characterized genetically, have fewer genetic tools available and they have long generation times. Also, the roots of cereals can be difficult to visualize by microscopy as they are thicker in diameter than those of *Arabidopsis*. This can sometimes make the visual detection of *Polymyxa* in roots difficult. Therefore, if infection of *Arabidopsis* by *Polymyxa* spp. can be demonstrated, this could be a valuable tool in increasing our understanding of plant–*Polymyxa* interactions. This study aimed to look at the potential for infection of *Arabidopsis* by *Polymyxa* spp. under controlled environment conditions using *Polymyxa*-infested soils.

Materials and methods

Arabidopsis thaliana ecotypes Landsberg *erecta* (L*er*-0) and Columbia (Col-0) were used for this study (supplied by A. Cuzick, Rothamsted Research, UK). These ecotypes were chosen because they are genetically distinct and mapping populations are available. Seeds were sown into sterile Levingtons No. 2 compost containing sand and stratified for 4 days in the dark at 4 °C. Pots were then removed and placed in a greenhouse under short-day length conditions (8 h day at 20 °C, 16 °C night, light levels 200–300 $\mu mol\, m^{-2}\, s^{-1}$). Once the seedlings had produced their first true leaves, they were transferred to 10 cm pots containing infectious soils diluted 1 : 2, soil to sterile sand and grown as before. Two UK soils were used: one from Wiltshire, which was infested with SBCMV (Lyons *et al.*, 2008), and one from Woburn, where *Polymyxa* was present, but no associated virus had ever been identified (Ward *et al.*, 2005; R. Lyons, pers. commun.). For each soil, five seedlings of each ecotype were planted. Plants were then allowed to grow for 2 months. Flowering bolts were removed upon development to prolong vegetative growth.

Roots were removed from pots and vigorously washed in sterile, distilled water. Portions of root were then mounted in sterile water under a coverslip and examined using an Axiophot (Zeiss) light microscope with bright field illumination. Roots were separated into three 3-cm sections: one from the base of the plant, one from the middle of the root and one from the root tip. Around 20 pieces of each section of root were examined for each of the five plants from each ecotype–soil combination (i.e. approximately 60 root pieces per plant).

DNA was extracted from approximately 0.5 g freeze-dried and ground root material (one root system for each ecotype–soil combination) as described by Ward *et al.* (2005). *Polymyxa*-specific rDNA primers Pxfwd1 (5′-CTG CGG AAG GAT CAT TAG CGT T-3′) and Pxrev7 (5′-GAG GCA TGC TTC CGA GGG CTC T-3′) were used in PCR (Ward & Adams, 1998). *Plasmodiophora*-specific PCR was performed as in Cao *et al.* (2007) using primers TC1F/TC1R. For sequencing studies, the *Polymyxa*-specific forward primer Pxfwd1 and the generic fungal ITS4 reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.*, 1990) were used to amplify rDNA. Each reaction mix (50 μL) contained 0.2 μM primers, 1 U *Taq* DNA polymerase (MBI), 0.2 mM dNTPs (Sigma), 1 × PCR buffer NH_4 (MBI) and 0.02 mg μL^{-1} bovine serum albumin. Cycling conditions were 2 min at 95 °C, and then 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, followed by 72 °C for 10 min. Products were analysed in 1% agarose gels.

PCR products were cloned into the pGEM®-T Easy vector (Promega Corporation, Madison, WI). Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Crawley, UK) and sequenced using the ABI PRISM™ BigDye version 1.1 kit using M13 sequencing primers and run at the Geneservice sequencing facility (http://www.geneservice.co.uk). ITS rDNA sequences were aligned by CLUSTALX and manually adjusted. Phylogenetic analysis was performed using the neighbour-joining method (maximum composite likelihood distances) in MEGA4 (Tamura *et al.*, 2007) with 10 000 bootstrap replications.

Results and discussion

Examination by microscopy showed the presence of *Polymyxa*-like spores in numerous root hairs (but not the main root) of all five *Arabidopsis* ecotype L*er*-0 plants grown in the Woburn soil (Fig. 1). Two of the Col-0 plants grown in the Woburn soil contained structures that resembled *Polymyxa* zoosporangia (Fig. 2). Three of these structures were seen in total and they were all located in the main root system rather

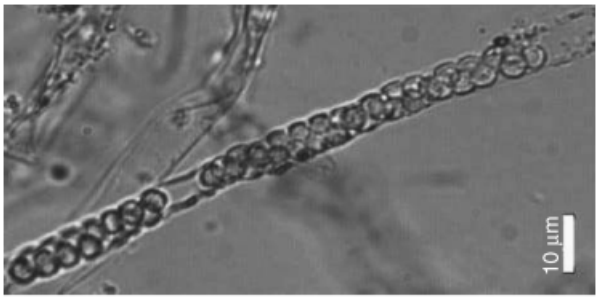

Fig. 1. Root hair from *Arabidopsis* L*er*-0 plant grown in Woburn soil containing plasmodiophorid-like spore clusters.

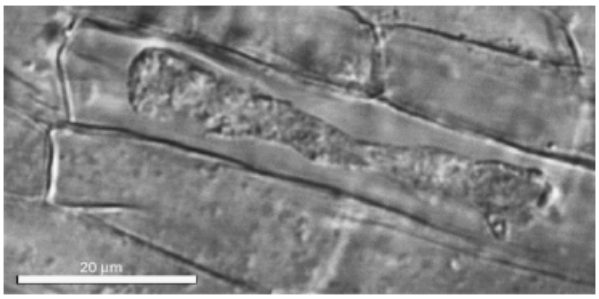

Fig. 2. *Polymyxa*-like zoosporangial structure in a main root cell of an *Arabidopsis* Col-0 plant grown in Woburn soil.

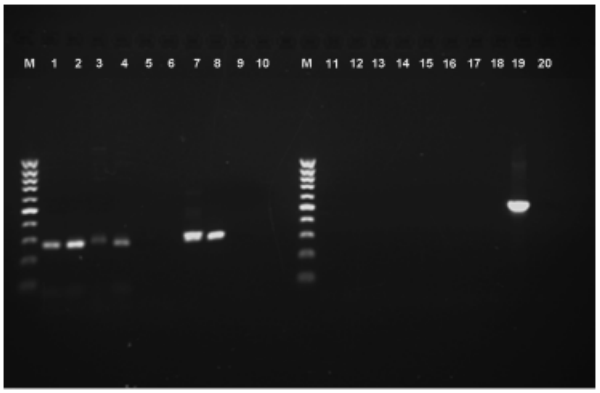

Fig. 3. Testing of DNA from *Arabidopsis* plants and soil with specific PCRs for *Polymyxa* (lanes 1–10) and *Plasmodiophora* (lanes 11–20). Lanes 1 and 11, *Arabidopsis* L*er*-0 from Wiltshire soil; lanes 2 and 12, *Arabidopsis* Col-0 from Wiltshire soil; lanes 3 and 13, *Arabidopsis* L*er*-0 from Woburn soil; lanes 4 and 14, *Arabidopsis* Col-0 from Woburn soil; lanes 5 and 15, healthy uninfected *Arabidopsis* L*er*-0; lanes 6 and 16, healthy uninfected *Arabidopsis* Col-0; lanes 7 and 17, soil from Woburn; lanes 8 and 18, *Polymyxa* DNA control (F1); lanes 9 and 19, *Plasmodiophora* DNA control (isolate N); lanes 10 and 20, no DNA control; M, size marker (100 bp ladder, Fermentas).

than the root hairs. No spore clusters were observed. In the root sections examined from *Arabidopsis* plants grown in the Wiltshire soil, no clusters of *Polymyxa*-like resting spores or zoosporangia were identified.

PCR with the *Polymyxa*-specific primers Pxfwd1/Pxrev7 demonstrated the presence of *Polymyxa* spp. in the roots of all four combinations of *Arabidopsis* ecotypes and soils (Fig. 3). Using a *Plasmodiophora*-specific PCR assay, we also demonstrated that *Plasmodiophora* was not present in these samples (Fig. 3). A total of 28 clones were sequenced following the amplification of rDNA products from *Arabidopsis* roots using primers Pxfwd1/ITS4. These included representatives of all of the restriction fragment length polymorphism types obtained by prescreening the clones using DdeI. Eleven of these sequences showed significant identity to *P. graminis* F1 ITS ribosomal DNA (Table 1), one to *P. betae* F67 ITS rDNA and nine to *Arabidopsis* rDNA. For the remaining seven sequences, the closest matches were to uncultured *Basidiomycetes* (two clones) and an uncultured *Helotiales*, and there were partial matches (short regions of high identity in a limited part) to *Urostyla grandis*, *Anguina agropyri* and an ectomycorrhizal fungus. The nucleotide sequence of one clone showed no significant identity to any sequence in GenBank. The identification of *Arabidopsis* and other non-*Polymyxa* sequences in the roots is not unexpected, as only one of the primers used (Pxfwd1) is *Polymyxa* specific, whereas the ITS4 primer is a generic, 'fungal' rDNA primer.

Sequences from these experiments (approximately 430–500 bp) were aligned with existing *Polymyxa* rDNA sequences and phylogenetic analyses were performed in MEGA4 (Fig. 4). With the exception of LeWil clone 34, which grouped with *P. betae*, all of the other *Polymyxa* sequences obtained from *Arabidopsis* root samples formed a clade with the *P. graminis* F1 (ribotype I) isolate (Y12824, 96% support from bootstrapping). There was strong bootstrap support (98%) separating the Col-0 Woburn clone 3 sequence from the other sequences in this clade. The sequence identity between *P. graminis* type I sequences and those of *P. betae* was around 80%.

The range of *Polymyxa* sequences obtained from the *Arabidopsis* roots was diverse, but not unexpected as previous work has demonstrated that plants can contain more than one ribotype of *Polymyxa* in their roots (Ward *et al.*, 2005; Vaïanopoulos *et al.*, 2007; Smith, 2008). The diversity seen could also be due to the heterogeneity between rDNA repeat units in the same *Polymyxa* spore or cell.

Collectively, our results indicate that *Arabidopsis* is susceptible to infection by *Polymyxa* spp. *Polymyxa*-like spore clusters were identified in root hairs of *Arabidopsis* L*er*-0 plants and structures resembling young *Polymyxa*-like zoosporangia in the roots of Col-0 plants. The putative zoosporangium is not like that of any of the other plasmodiophorid genera. Although these structures were not observed in all plants, it is possible that they were present in parts of the root system other than those examined by microscopy. The spores, although similar in appearance to *Plasmodiophora*,

Table 1. Results of BLAST hits for sequenced clones with significant nucleotide identity to *Polymyxa* rDNA

Arabidopsis ecotype	Soil	Clone no.	BLAST hit* (DISTMAT value†)	GenBank accession no.
Landsberg	Woburn	LeWob34	PgF1‡ 98% (99.4%)	FN393973
Landsberg	Wiltshire	LeWil3	PgF1 99% (99.4%)	FN393974
Landsberg	Wiltshire	LeWil7	PgF1 99% (99.2%)	FN393967
Landsberg	Wiltshire	LeWil34	PbF67§ 99% (99.4%)	FN393976
Landsberg	Woburn	LeWob8	PgF1 97% (98.2%)	FN393968
Columbia	Woburn	CoWob3	PgF1 93% (94.7%)	FN393971
Columbia	Woburn	CoWob10	PgF1 99% (99.0%)	FN393972
Columbia	Woburn	CoWob11	PgF1 99% (99.4%)	FN393966
Columbia	Woburn	CoWob29	PgF1 99% (99.4%)	FN393975
Columbia	Wiltshire	CoWil1	PgF1 99% (99.2%)	FN393969
Columbia	Wiltshire	CoWil7	PgF1 99% (99.6%)	FN393970

*Closest match showing the percentage nucleotide identity between the query and the database sequences.
†Percentage nucleotide identity calculated from the CLUSTALX alignment using the EMBOSS program DISTMAT (Rice *et al.*, 2000).
‡Accession number: Y12824.
§Accession number: Y12827.

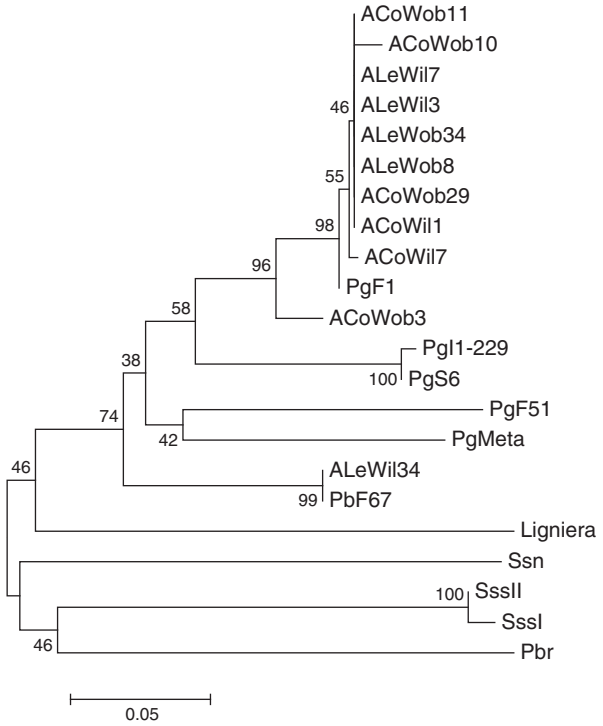

Fig. 4. Phylogenetic relationships between *Polymyxa* ITS rDNA sequences from *Arabidopsis* and other isolates. Sequences previously deposited in public databases are PgF1 (*Polymyxa graminis* ribotype I, accession no. Y12824), PgF51 (ribotype II, Y12826), PgI1-229 (ribotype III, Y12825), PgS6 (ribotype III, AJ311580), PgMeta (ribotype V, AJ010424), PbF67 (*Polymyxa betae*, Y12827), Pbr (*Plasmodiophora brassicae*, Y12831), Ligniera (Y12828), Ssn (*Spongospora subterranea* f. sp. *nasturtii*, AF310907) SssI (*S. subterranea* f. sp. *subterranea* type I, AY604171) and Sss II (*S. subterranea* f. sp. *subterranea* type II, AY604172). Other sequences were obtained in the current study. The neighbour-joining method (maximum composite likelihood distances) was used in MEGA4 (Tamura *et al.*, 2007) with 10000 bootstrap replications.

were aggregated together in clusters, whereas *Plasmodiophora* spores do not form aggregates. Additionally, no galls were observed in the roots of these plants, as would occur in *Plasmodiophora* infections, and a *Plasmodiophora*-specific PCR assay showed that *Plasmodiophora* was not present in the *Arabidopsis* or soil samples.

Using *Polymyxa*-specific PCR assays, *Polymyxa* was detected in all four combinations of *Arabidopsis* ecotypes and soils, and this was confirmed by rDNA sequencing; sequences either had high nucleotide identity to the rDNA sequence from ribotype I *P. graminis* or to *P. betae*. None showed close identity to *P. graminis* type II despite this ribotype being present in both soils (Ward *et al.*, 2005; Lyons *et al.*, 2008). Although temperate ribotypes of *P. graminis* have been shown mainly to infect monocotyledonous plants, *P. betae* and tropical isolates of *P. graminis* have been shown to infect dicotyledonous plants (Barr, 1979; Ratna *et al.*, 1991; Barr & Asher, 1992; Legrève *et al.*, 2000).

The observation of spores in the root hairs of the *Arabidopsis* ecotype L*er*-0 plants is interesting as *Polymyxa* spp. are not routinely reported infecting root hairs, although this has been observed infrequently (M. Smith & M.J. Adams, unpublished data). Because this is a new and distinctive host, it is not unreasonable to expect that that the localization of *Polymyxa* within the plant or aspects of its morphology might differ. This could result for example from spatial constraints within the cells. There is support for this from anatomical studies of *P. graminis* infection in sorghum and wheat (Littlefield *et al.*, 1997).

Unfortunately, we cannot confirm absolutely that the structures observed in the roots of the *Arabidopsis* plants correspond to the *Polymyxa* detected using molecular methods. In hindsight, we should have selected infected root tissue before DNA extraction to provide additional support for this, but conclusive proof would require a technique

such as laser capture microdissection (Day *et al*., 2005). These techniques are technically challenging and have rarely been successfully used in these types of study.

There are problems associated with the use of soil to infect the plants rather than resting spores or zoospores from previously characterized *Polymyxa* isolates. There is a possibility of detection of *Polymyxa* from soil adhering to the root, which could confuse the issue of whether detection in the plant has occurred. However, the roots were washed thoroughly before use and this was facilitated by growth in a mixture of soil and sand (1 : 2), rather than soil alone. Also, from our previous experience of this system, we feel that it is unlikely that loosely attached *Polymyxa* spores would be responsible for the detection. Infection using *Polymyxa*-infected material would also have been superior in that it would have allowed a demonstration of Koch's postulates. However, it is generally more difficult to infect plants using zoospores or resting spores, than using soil and we felt that, to establish the system, it would be better to bait plants with the mixture of ribotypes that are present in the soil, rather than test individually zoospores/resting spores from a wide range of different isolates, some of which may not be well adapted to the new host.

Further experiments would be required to optimize the system, to demonstrate completion of the life cycle and onward transmission to further plants, to establish the range of *Polymyxa* isolates capable of infecting *Arabidopsis* and to determine whether there were any links between the type of infection seen (location and developmental stage) and the *Arabidopsis* ecotype used.

This is the first report to demonstrate that infection of *Arabidopsis* by *Polymyxa* spp. is possible. Both *P. graminis* and *P. betae* sequences were found in infected *Arabidopsis* roots and extends the range of known hosts for both species. This important finding opens up the exciting possibility of using a model system for studying *Polymyxa* infections with a wide range of available tools, and that is much more amenable to study than using sugar beet or cereal hosts.

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