Arabidopsis thaliana, A New Host for Polymyxa Species

Madeleine J. Smith, Michael J. Adams and Elaine Ward

Department of Plant Pathology and Microbiology, Rothamsted Research, Harpenden,
Hertfordshire, AL5 2JQ, UK.

Corresponding Author: Dr. Elaine Ward
Department of Plant Pathology and Microbiology
Rothamsted Research,
Harpden,
Hertfordshire,
AL5 2JQ,
UK.
Tel: + 44 (0) 1582 763133 ext.2495
Fax: +44 (0) 1582 760981
E-mail: elaine.ward@bbsrc.ac.uk

Running Title: Arabidopsis, A New Host for Polymyxa Species

Key Words: Polymyxa graminis, Polymyxa betae, Soil-borne cereal mosaic virus,
Arabidopsis, Plasmodiophorida
Abstract

Polymyxa species 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 two 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 species 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. *P. betae* transmits *Beet necrotic yellow vein virus*, the cause of Rhizomania, to sugar beet.

A number of subgroups (ribotypes) of *Polymyxa* spp. have been identified according to rDNA sequence data (Ward et al., 1994; Ward & Adams, 1998; Legrève et al., 2002; Ward et al., 2005). Some of these 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 *Polymyxa graminis* isolates are found in temperate regions, ribotype I (f. sp. *temperata*) and ribotype II (f. sp. *tepida*). All ITS rDNA sequences for *P. betae* reported to date fall into two types which differ by only one base pair (Ward & Adams, 1998; Legrève et al., 2002).

Due to their obligate nature and relatively long life-cycle, *Polymyxa* species 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-fertilise, 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 mutagenised lines, 8)
ease of transformation and, 9) the large number of ecotypes exhibiting natural variation available (Meyerwitz, 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 e.g. from suppressive subtractive hybridisation experiments to study gene structure and expression (Bulman et al., 2006; Bulman et al., 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 visualise infection biology of *P. brassicae* (Mithen & Magrath, 1992). The availability of synteny maps between *Arabidopsis* and *Brassica* species has allowed identification of resistance loci in *Brassica* species first identified in *Arabidopsis* (Suwabe et al., 2006). Global analysis of host gene expression at different time points post infection has been possible using *Arabidopsis* genome arrays and this has allowed 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 utilising the bank of insertion lines available in *Arabidopsis* (Puzio et al., 2000; Siemens et al., 2006).

Many of the host plants that *Polymyxa* species infect are not well characterised 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 visual
detection of Polymyx in roots difficult. Therefore, if infection of Arabidopsis by
Polymyx species can be demonstrated, this could be a valuable tool in increasing our
understanding of plant-Polymyx interactions. This study aimed to look at the
potential for infection of Arabidopsis by Polymyx spp. under controlled environment
conditions using Polymyx infested soils.

Materials and Methods

Arabidopsis thaliana ecotypes Landsberg erecta (Ler-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 four days in the dark at 4°C. Pots were then removed and
placed in a greenhouse under short day length conditions (8 hr day at 20°C, 16°C
night, light levels 200 - 300 µmol.m⁻².sec⁻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. Two UK soils were used, one from Wiltshire which was
infested with SBCMV (Lyons et al., 2008) and one from Woburn where Polymyx
was present but no associated virus had ever been identified (Ward et al., 2005, R.
Lyons, (Rothamsted Research) pers. com). For each soil, 5 seedlings of each ecotype
were planted. Plants were then allowed to grow for two months. Flowering bolts were
removed upon development to prolong vegetative growth.
Once roots had been removed from pots and undergone vigorous washing in sterile, distilled water, three sets of 3 cm bunches of root, one from the base of the plant, one from the middle of the root mass and one from the root tip, were examined using an Axiophot (Zeiss) light microscope and bright field illumination. Portions of root were mounted in sterile water under a coverslip.

DNA was extracted from root material 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 et al., 1994). 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 & Bruns et al., 1990), were used to amplify rDNA. Each reaction mix (50 µL) contained: 0.2 µM primers, 1U Taq DNA polymerase (MBI), 0.2 mM deoxyribonucleoside triphosphates (Sigma), 1x PCR buffer NH₄ (MBI), 0.02 mg/µL BSA. Cycling conditions were 2 min at 95°C, then 30 cycles of: 94°C for 30s, 50°C for 1 min, 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, US). Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen, Crawley, UK) and sequenced using the ABI PRISM™ Big-Dye version 1.1 kit using primers M13SeqF (5’ GCC AGG GTT TTC CCA GTC AC G A 3’) and M13SeqR (5’ GAG CGG ATA ACA ATT TCA CAC AG 3’) and run at the Geneservice sequencing facility (http://www.geneservice.co.uk).
Results and Discussion

Examination by microscopy showed the presence of Polymyxa-like spores in numerous root hairs (but not the main root) of Arabidopsis ecotype Ler-0 plants grown in the Woburn soil (Figure 1). Two of the Col-0 plants grown in the Woburn soil contained structures that resembled Polymyxa zoosporangia (Figure 2). Three of these structures were seen in total and they were all located in the main root system rather than the root hairs. No spore clusters were observed. In the root sections examined from Arabidopsis plants grown in the Wiltshire soil, no Polymyxa-like spores or zoosporangia were identified.

PCR with the Polymyxa-specific primers Pxfwd1/Pxrev7 demonstrated the presence of Polymyxa species in the roots of all four combinations of Arabidopsis ecotypes and soils (Figure 3). A total of 28 clones were sequenced following amplification of rDNA products from Arabidopsis roots using primers Pxfwd1/ ITS4. Eleven of these sequences showed significant identity to P. graminis F1 ITS ribosomal DNA (Table 1) and one to P. betae F67 ITS rDNA. Of the remaining sequences, nine showed 98-100% nucleotide identity to Arabidopsis rDNA, two to uncultured Basidiomycetes, one to an uncultured Helotiales, one to Urostyla grandis, one had very partial identity to Anguina agrapyri, another had partial identity to an Ectomycorrhizal fungus and one had no known homology 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 were aligned with existing *Polymyxa* rDNA sequences and phylogenetic analyses were performed in MEGA4 (Figure 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 (AY12824, 99% support from bootstrapping). There was strong bootstrap support (99%) separating the Col-0 Woburn clone 3 sequence from the other sequences in this clade.

Collectively, our results indicate that *Arabidopsis* is susceptible to infection by *Polymyxa* spp. *Polymyxa*-like spore clusters were identified in root hairs of *Arabidopsis* Ler-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*, were aggregated together in clusters whereas *Plasmodiophora* spores do not form aggregates. In addition no galls were observed in the roots of these plants, as would occur in *Plasmodiophora* infections.

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 Ler-0 plants is interesting as Polymyxa species are not routinely reported infecting root hairs, although this has been observed infrequently (M. Smith, M. J. Adams, unpublished). However, it is not unreasonable to expect changes in morphology and tissue colonisation in this alternative host.

One way to absolutely determine the organism producing the structures observed in the roots of the Arabidopsis plants would be to use a technique such as laser capture micro-dissection although this would be technically challenging (Emmert-Buck et al., 1996; Kerk et al., 2003; Day et al., 2005).

Further experiments would be required to optimise the system, 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.
Acknowledgements

The authors would like to thank A. Cuzick for providing seed and A. Tymon and K. Kanyuka for assisting with soil sampling. MJS was supported by a BBSRC PhD studentship; John Walsh is thanked for his supervision and encouragement. Rothamsted Research receives grant aided support from the Biotechnology and Biological Sciences Research Council.

References


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

<table>
<thead>
<tr>
<th>Arabidopsis ecotype</th>
<th>Soil</th>
<th>Clone No.</th>
<th>BLAST Hit&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genbank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landsberg</td>
<td>Woburn</td>
<td>LeWob34</td>
<td>98% <em>P. graminis</em> F1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FN393973</td>
</tr>
<tr>
<td>Landsberg</td>
<td>Wiltshire</td>
<td>LeWil3</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393974</td>
</tr>
<tr>
<td>Landsberg</td>
<td>Wiltshire</td>
<td>LeWil7</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393967</td>
</tr>
<tr>
<td>Landsberg</td>
<td>Wiltshire</td>
<td>LeWil34</td>
<td>99% <em>P. betae</em>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>FN393976</td>
</tr>
<tr>
<td>Landsberg</td>
<td>Woburn</td>
<td>LeWob8</td>
<td>97% <em>P. graminis</em> F1</td>
<td>FN393968</td>
</tr>
<tr>
<td>Columbia</td>
<td>Woburn</td>
<td>CoWob3</td>
<td>93% <em>P. graminis</em> F1</td>
<td>FN393971</td>
</tr>
<tr>
<td>Columbia</td>
<td>Woburn</td>
<td>CoWob10</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393972</td>
</tr>
<tr>
<td>Columbia</td>
<td>Woburn</td>
<td>CoWob11</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393966</td>
</tr>
<tr>
<td>Columbia</td>
<td>Woburn</td>
<td>CoWob29</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393975</td>
</tr>
<tr>
<td>Columbia</td>
<td>Wiltshire</td>
<td>CoWil1</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393969</td>
</tr>
<tr>
<td>Columbia</td>
<td>Wiltshire</td>
<td>CoWil7</td>
<td>99% <em>P. graminis</em> F1</td>
<td>FN393970</td>
</tr>
</tbody>
</table>

<sup>a</sup>CLOSEST MATCH SHOWING PERCENTAGE NUCLEOTIDE IDENTITY BETWEEN THE QUERY AND DATABASE SEQUENCES. <sup>b</sup>ACCESSION NUMBER: AY12824. <sup>c</sup>ACCESSION NUMBER: Y12827
**Figure legends**

Figure 1. Root hair from *Arabidopsis Ler*-0 plant grown in Woburn soil containing plasmodiophorid-like spore clusters.

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

Figure 3. Amplification of *Polymyxa*-specific rDNA products from *Arabidopsis* roots. Lane 1, 100 bp size marker (Fermentas); lane 2, healthy uninfected *Arabidopsis* Col-0; lane 3, healthy uninfected *Arabidopsis* Ler-0; lane 4, *Arabidopsis* Col-0 from Woburn soil; lane 5, *Arabidopsis* Ler-0 from Woburn soil; lane 6, *Arabidopsis* Col-0 from Wiltshire soil; lane 7, *Arabidopsis* Ler-0 from Wiltshire soil; lane 8, no DNA control; lane 9, *Polymyxa* DNA positive control.

Figure 4. Phylogenetic relationships between *Polymyxa* ITS rDNA sequences from *Arabidopsis* and other isolates. Sequences PgF1 (*P. graminis* ribotype I, Accession No. Y12824), Pg51 (*P. graminis* ribotype II, Y12826) and PbF67 (*P. betae*, Y12827) were reported in Ward and Adams, 1998), other sequences were obtained in the current study. The Neighbor-Joining method (Maximum composite likelihood distances) was used in MEGA4 (Tamura *et al.*, 2007) with 10000 bootstrap replications.