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**A family of small tyrosine rich proteins is essential for oogonial and
oospore cell wall development of the mycoparasitic oomycete *Pythium
oligandrum***

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Running Title: Cell wall proteins from *Pythium oligandrum* oogonia

Keywords: gene silencing; mycoparasite; oospore formation; oomycete sexual reproduction; TEM; ultrastructure.

Abstract

The mycoparasitic oomycete *Pythium oligandrum* is homothallic, producing an abundance of thick walled spiny oospores in culture. After mining a cDNA sequence dataset, we identified a family of genes that code for small tyrosine rich (PoStr) proteins. Sequence analysis identified similarity between the PoStr proteins and putative glycine-rich cell wall proteins from the related plant pathogenic oomycete *Pythium ultimum*, and mating induced genes from the oomycete *Phytophthora infestans*. Expression analysis showed that *PoStr* transcripts accumulate during oospore production in culture and immunolocalization indicates the presence of these proteins in oogonial and oospore cell walls. PoStr protein abundance correlated positively with production of oogonia as determined by antibiotic-mediated oogonia suppression. To further characterise the role of PoStr proteins in *P. oligandrum* oospore production, we silenced this gene family using homology-dependent gene silencing. This represents the first characterisation of genes using gene silencing in *Pythium* species. Oospores from silenced strains displayed major ultrastructural changes and were sensitive to degradative enzyme treatment. Oogonia of silenced strains either appeared to be arrested at the mature oosphere stage of development or in around 40% of the structures, showed a

complete suppression of oospore formation. Suppressed oogonia were highly vacuolated and the oogonium wall was thickened by a new inner wall layer. Our data suggest PoStr proteins are probably integral structural components of the normal oospore cell wall and play a key role in oospore formation.

1. INTRODUCTION

The oomycete phylum is a large group of ubiquitous eukaryotic microorganisms. It includes both saprophytes and pathogens of plants, algae, insects, fish, nematodes and vertebrates (Grenville-Briggs and van West, 2005; Grenville-Briggs *et al.* 2011; Hardham 2005; Phillips *et al.* 2008), as well as several mycoparasitic species including *Pythium oligandrum*. One of the defining features of the oomycetes, is the thick walled oospore, which is the result of sexual reproduction. These spores are important to the life cycle of most oomycetes both to maintain fitness (providing a means of genetic variation), and as resistant structures that can withstand freezing, desiccation, drought, and microbial degradation, ~~as well as persisting~~ and persist in a resting state in the soil for many years (Judelson 2009). They are the primary inoculum source for *Aphanomyces euteiches* infection of legumes (Dyer and Windels 2003) and *Plasmopara viticola* infection of grape vine (Galbiati and Longhin 1984; Rossi *et al.* 2008). *Peronosclerospora sorghi* also relies primarily on oospores to cause downy mildew on sorghum (Jeger *et al.* 1998). Oospores are also an important inoculum source in *Phytophthora sojae* root rot of soybean, where most of the primary

1 lesions arise from these overwintered resting spores (Schmitthenner 1999). The
2 arrival in Europe, 30 years ago, of the A2 mating type of *Phytophthora infestans*
3 (the causal agent of potato late blight) has meant that oospore derived late blight
4 infections have become increasingly important (Cooke *et al* 2007). This is
5 particularly the case in Nordic countries where climatic conditions favour oospore
6 survival (Brurberg *et al.* 2011; Lehtinen and Hannukkala 2004; Widmark *et al*
7 2007).

8
9 *P. oligandrum* is both a mycoparasite and an endophyte (Le Floch *et al.* 2005) ,
10 colonising the root rhizosphere of many crop plant species (Martin and Hancock
11 1987). Its presence reduces soil-borne diseases of fungal, oomycete and
12 bacterial origins, both directly through antagonism, of eukaryotic pathogens and
13 indirectly through the activation of plant defences. *P. oligandrum* has been
14 commercialised as a biocontrol agent for the protection of a wide variety of plant
15 species (Brozova 2002). It is a fast growing (~35 mm/day) homothallic species,
16 amenable to laboratory culture, producing abundant spiny oogonia *in vitro*.

17 Oospores are an important means of reproduction for *P. oligandrum*, since
18 many strains appear to have completely lost the ability to form asexual
19 zoospores. Oogonia of *Pythium* species are plerotic, developing a single
20 oospore within each oogonium. Many strains appear to have completely lost the
21 ability to form asexual zoospores. *In vivo*, oogonium production is triggered upon
22 mycoparasitic contact with host fungal or oomycete hyphae, and oogonia are
23 even formed within both the macroconidia of *Fusarium culmarum* (Davenlou *et al.*

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1999) and sclerotia of *Botrytis cinerea* (Rey *et al.* 2005). Following inoculation with *P. oligandrum*, oospores are also formed in abundance over the surface of tomato roots and within both epidermal cells (Rey *et al.* 1998) and vascular tissue (Le Floch *et al.* 2005).

The details of sexual reproduction and oosporogenesis in oomycetes vary greatly between species. Oogonia of *Pythium* species are plerotic, developing a single oospore within each oogonium. However, the oospore is usually clearly separated from the outer oogonium wall, as a result of lysis of the intervening periplasmic layer (Beakes 1981). Early studies of *Pythium acanthicum*, a closely related mycoparasitic species, report that most oogonia develop parthenogenetically, although occasionally antheridia are seen attached to oogonia, (Haskins *et al.* 1976). The ultrastructural cytoplasmic changes that accompany oogonium differentiation have been described for several *Pythium* species, including the closely related mycoparasite *P. acanthicum* and *Phytophthora* species. These studies show that both the oogonium and oospore walls are complex, multilayered structures (Beakes 1981; Beakes and Bartnicki-Garcia 1989; Haskins *et al.* 1976; Hemmes and Bartnicki-Garcia 1975; McKeen 1975). In *Pythium* the relatively thin electron dense outer oospore wall complex (OOW) separates the oospore from the surrounding periplasmic space and oogonium wall. This dense OOW layer is unique to all oospores and its formation, following fertilization or nuclear fusion, marks the transition from oosphere to resting oospore (Beakes 1980; Beakes 1981). Once this layer is laid

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1 down the immature oospores become much more difficult to fix and embed,
2 (Beakes *et al.* 1986) which suggests this layer prevents access of fixative agents
3 to the oospore interior and is thus probably hydrophobic in nature. The much
4 thicker and generally electron-lucent inner oospore wall (IOW) (Beakes 1981;
5 Haskins *et al.* 1976; McKeen 1975; Ruben and Stanghellini 1978) that is laid
6 down inside of the OOW is the repository of the glucans that are mobilised at the
7 onset of germination (Beakes and Bartnicki-Garcia 1989; Hommes and Stasz
8 1984; Ruben and Stanghellini 1978).

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9 The chemical composition of oomycete oogonial and oospore cell walls is
10 qualitatively similar to that of vegetative mycelium and sporangial cell walls.
11 Oogonial and oospore walls are predominantly composed (80%) of non-cellulosic
12 β 1-3 linked glucans, and contain less than 10% cellulose (Lippman *et al.* 1974).
13 In addition to glucose, minor amounts of mannose and glucosamine are present
14 and in comparable quantities to mycelial cell walls (Lippman *et al.* 1974). R
15 However, relative to mycelium, oospore and oogonial walls contain increased
16 higher amounts of lipid and protein levels and have a lower glucan content
17 (Lippman *et al.* 1974; Ruben and Stanghellini 1978). It is likely that this higher
18 lipid content confers hydrophobicity to these spores to allow dormancy and
19 subsequent germination. T Additionally, the oogonial and oospore cell wall
20 accounts for up to 47% of the dry weight of the cell, one of the highest
21 percentages of fungal or oomycete cell walls, indicating that a highly thickened
22 cell wall is the major component of oomycete oospores (Lippman *et al.* 1974).
23 This thick multi-layered oospore cell wall, together with the lipid-rich cytoplasmic

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1 | matrix, reduced ~~organelle~~-cytoplasmic organelle content (Beakes *et al.* 1986) and
2 | low metabolic rate all contribute to making the mature oospore an effective
3 | resting structure.

4 | Molecular analysis of oomycete sexual reproduction and oosporogenesis is
5 | largely derived from work on the heterothallic species, *Phytophthora infestans*,
6 | where a microarray study identified over a hundred genes induced during mating
7 | (Prakob and Judelson 2007). Most of these genes were also highly transcribed
8 | during oosporogenesis in the homothallic species *Phytophthora phaseoli*. Several
9 | of these genes encoded regulatory proteins such as protein kinases, protein
10 | phosphatases, cell cycle regulators and transcription factors (Prakob and
11 | Judelson 2007). Structural components of the oomycete cell wall, have so far, not
12 | been analysed at the molecular level. We previously identified a gene family
13 | (*PoStr*) from a *P. oligandrum* mycoparasitic cDNA library that showed
14 | characteristics of extracellular cell wall proteins (Horner *et al.* 2012). In the
15 | current paper we describe expression analysis, immunolocalization, and gene
16 | silencing of the *PoStr* gene family and show that the *PoStr* proteins are structural
17 | components of *P. oligandrum* oogonia and are required for spore integrity. This
18 | study represents the first report of functional characterisation of genes in a
19 | *Pythium* species by gene silencing, and the first functional study of oomycete
20 | oospore-related proteins.

22 | 2. MATERIALS AND METHODS

2.1 *In vitro* growth of *Pythium oligandrum*

P. oligandrum strain CBS 530.74 was obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) and maintained on V8 medium at 24°C as described in (Rahimian and Banihashemi 1976). All strains were grown under the Scottish Executive Environment and Rural Affairs licence number PH/37/2010.

2.2 Effects of G418 on oospore production by *P. oligandrum*

P. oligandrum strain CBS 530.74 was grown in liquid V8 medium supplemented with either 0.1 mg/l, 0.5 mg/l or 2.0 mg/l G418 antibiotic for 5 days and the production of oospores observed. After 5 days of growth, proteins were extracted for Western blot analysis.

2.3 Sequence Analysis

Predicted protein sequences were obtained by using the online programme, OrfPredictor (Min *et al.* 2005). Similarity searches were performed using either the blastall or netblast programmes (Altschul *et al.* 1997). The Predict Protein webserver (Rost *et al.* 2004) was used to predict the protein characteristics of PoStr5. ProtScale (Gasteiger *et al.* 2005) was used to produce hydrophobicity plots and the potential disorder of PoStr5 was analysed by the prediction

1 | ~~program GlobPlot (Linding *et al.* 2003) using the Russell/Linding algorithm.~~

2 | ~~Protein repeats were predicted using RADAR (Heger and Holm 2000).~~

4 | **2.34 RT-PCR**

5 | Total RNA was extracted from approximately 100 mg of mycelia using the
6 | RNeasy Plant Mini Kit (Qiagen). Total RNA was checked on an agarose gel for
7 | integrity. cDNA was created from total RNA using the First-Strand cDNA
8 | Synthesis Kit (Amersham) according to the manufacturers instructions.
9 | Approximately 1 µg of RNA was used per reaction. Reverse transcriptase was
10 | primed by the addition of 1:25 dilution of 5 µg/µl *NotI*-d(T)₁₈ primer. cDNA was
11 | used in subsequent PCR reactions at 0.5 µl per 50 µl reaction with 300 nM of
12 | forward and reverse primers.

13 | ~~SCyber~~-green quantitative PCR was carried out as described (Grenville-Briggs *et*
14 | *al.* 2008). The tubulin alpha gene from *P. oligandrum* was used as a constitutively
15 | expressed control and the expression of *PoStr1*-5 was determined relative to
16 | tubulin alpha as described by (Grenville-Briggs *et al.* 2008). Primers used for
17 | both semi-quantitative and cyber green real-time RT-PCR are listed in Table 1.

19 | **2.45 Cloning of *PoStr1* and production of constructs for silencing**

20 | The full-length cDNA sequence of *PoStr1* was cloned into the *EcoRV* site of the
21 | oomycete expression vector pTOR (Blanco and Judelson 2005; Whisson *et al.*

2007) in a forward orientation to produce pTorSTR1F and in reverse orientation to produce pTorSTR1-R (Suppl. Fig. S13). The following primers were used for cloning: PoFSTR1F; AATCCCGGCATTTCCTTC, PoFSTR1R; GTCTTTGTGTGATGTGAAAAACGAC. Since there is significant similarity between *PoStrs*1-5 it was anticipated that pTorSTR1-R would achieve silencing of this family of 5 genes. pTorSTR1-R was therefore transformed into *P. oligandrum* as described below.

2.56 Transformation of *P. oligandrum*

Stable transformation of *P. oligandrum* was carried out according to (Horner *et al.* 2012), based on the protocols of (Judelson *et al.* 1991; van West *et al.* 1998; van West *et al.* 1999a; van West *et al.* 1999b) with the following minor modifications. Forty-eight hour old mycelium from 8-10 plates of V8-liquid grown cultures was washed three times with KC-osmoticum (0.6M KCl, 0.2M CaCl₂) and incubated with 125 mg cellulase (Sigma C8546) and 75 mg lysing enzymes from *Trichoderma harzianum* (Sigma L1412) at room temperature with 30 rpm shaking for 90 min. Protoplasts were filtered through a 70 µm mesh, pelleted at 700 g, washed once in KC osmoticum, once in KC/MT (0.6 M KCl, 0.2 M CaCl₂/0.8 M Mannitol, 10 mM Tris-HCl) and re-suspended in MT buffer (0.8 M Mannitol, 10 mM Tris-HCl pH 7.5) containing 25 mM CaCl₂ at a concentration of 0.1-1 x 10⁷ protoplasts per ml. A mixture containing 30 µg vector DNA (circular) plus 60 µg Lipofectin reagent was pre-incubated at room temperature and added to 1 ml of

protoplast solution. A 1 ml solution of PEG-Ca (50 % w/v PEG 3350, 25 mM CaCl₂, 10 mM Tris-HCl pH 7.5) was slowly added to the protoplast mixture and incubated at room temperature for 4 min. A solution of 2 ml of clarified V8 broth containing 0.8 M mannitol (V8M) was added to the DNA/protoplast mixture, inverted and incubated for 1 min. A further 1 ml of V8M was added to the mixture, which was then transferred into a solution of 25 ml V8M with the addition of antibiotics (100 µg/ml ampicillin, 50 µg/ml vancomycin). *In vitro* growth of *P. oligandrum* strain CBS 530.74 on V8 agar amended with geneticin (G418) determined the minimum inhibitory concentration (MIC) at 30 µg/ml. Protoplasts were allowed to regenerate at 24°C for between 15-18 hrs, after which time they were pelleted at 700 g, resuspended in 3 ml V8M and spread onto V8 agar supplemented with 30 µg/ml G418 antibiotic. Colonies appeared within 2-3 days and were subsequently propagated on V8 agar with the addition of G418.

2.7 Southern blot analysis

6 µg of *P. oligandrum* genomic DNA was digested with 10 U enzyme, electrophoresed and blotted onto a nitrocellulose membrane. The probe derived from a *Nco*I/*Spe*I digest of pTorSTR1F. Labelling and detection was carried out using an ECL direct nucleic acid labelling and detection kit (GE Healthcare) according to the manufacturers instructions.

2.68 Isolation of *P. oligandrum* proteins

Protein extraction was performed as described previously (Grenville-Briggs *et al.* 2005). Briefly, proteins were solubilised in 7 M Urea, 2M thio-urea, 4 % CHAPS 1% DTT with the addition of a protease inhibitor cocktail (Roche). Solubilised proteins were subjected to SDS-PAGE using the NuPAGE™ (Invitrogen) system, according to the manufacturers specifications. Pure PoSTR1 peptide was synthesised by Sigma.

2.79 Western blotting

Western blotting was carried out as described previously (van West *et al.* 2010). Briefly, protein samples were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4 °C in phosphate-buffered saline + 0.2 % Tween-20 (PBS-T) and 5 % skimmed milk powder. After washing the membrane several times in PBS-T, it was incubated for 1 h with pre-immune serum or final bleed antibody, diluted 1 : 400 in PBS. Membranes were washed several times in PBS-T and incubated for 1 h in secondary horseradish peroxidase-conjugated anti-rabbit antibody (Sigma-Aldrich, No. A0545), diluted 1 : 16000 in PBS-T. After several washes, membranes were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific), according to the manufacturer's protocol. Membranes were exposed to a Kodak BioMax XAR film (Amersham Biosciences). For the time course expression analysis, cellular proteins from hyphae and spores were isolated using the urea lysis buffer described above. Secreted proteins were

1 acetone precipitated from the culture media. Proteins were isolated every day,
2 over a five-day period.

3 4 **2.849 Immunolocalization and transmission electron microscopy**

5 A polyclonal PoStr-antiserum was raised in rabbits against a peptide consisting of
6 15 aa present in the N-terminus of PoStr1-5 (CGYDDYDYGKNDY) (peptide 1)
7 (Sigma-Genosys) and specificity was tested on the purified PoStr peptide. Five-
8 day old mycelium and oospores from *P. oligandrum* were fixed in 4 %
9 paraformaldehyde/PBS for 24 hr. Samples for electron microscopy were
10 processed conventionally (Wastling *et al.* 1992) and sectioned as described
11 previously (Grenville-Briggs *et al.* 2008). Samples were viewed at an
12 accelerationed voltage of 80 KV using a Philips CM10 TEM fitted with a Gatan
13 bioscan CCD camera.

14 15 **3. RESULTS AND DISCUSSION**

16 **~~3.1 Identification of a gene family from a *P. oligandrum* cDNA library and~~** 17 **~~bioinformatic analysis~~**

18 ~~Mining of two *P. oligandrum* cDNA libraries revealed a family of five small~~
19 ~~transcripts that we have called *PoStr* (*Pythium oligandrum* small tyrosine rich).~~
20 ~~cDNA sequences of these genes have been deposited in Genbank (EV245172,~~
21 ~~EV244447, EV245146, EV245340, EV244818). The protein-coding regions of the~~

Comment [LG2] : I have deleted all the in silico stuff, based on reviewer 2's point 2 and although rev 1 did not ask for it to be deleted they also commented on it, and perhaps on reflection it was something better suited just to the thesis and I don't think it adds that much to the paper, given everything else we have done!

transcripts are highly similar, with all of the predicted proteins showing highly similar N-terminal regions (Fig.1) whereas C-terminal regions are more divergent. *PoStr2* was found to have an identical protein-coding sequence to *PoStr1*. The predicted protein sequences are rich in tyrosine and glycine residues (Table 2). BLAST searches, revealed high similarity to putative glycine-rich cell wall proteins (PYU1_T000208; 1×10^{-12}), from the related pathogenic oomycete *Pythium ultimum* (Levesque et al. 2010). Further BLAST searches performed without a low complexity filter, also revealed similarity to M96 mating-specific proteins (*P. infestans* top hit E value = 2×10^{-14}), P48 eggshell protein (*Schistosoma mansoni* E value = 8×10^{-15}), salivary glue protein (*Microsporium canis* E value = 1×10^{-16}), and thread matrix protein (*Mytilus galloprovincialis* E value = 1×10^{-12}). All these proteins are expected to be structural components of either the cell wall or the extracellular matrix.

A Southern blot performed with *P. oligandrum* genomic DNA, using *PoStr1* as a probe, indicated that the *PoStr* genes comprise a large family (Fig. 2).

PoStr5, having the longest predicted polypeptide sequence, was chosen for secondary structure analysis and analysed with the Predict Protein webserver, which incorporates several prediction programs (Rest et al. 2004). The protein was predicted to form a non-globular structure by the GLOBE program with 107 of 110 residues predicted as solvent exposed. No stable secondary structure was predicted using NORSq or PROF programs. A hydrophobicity plot produced by ProtScale (Gasteiger et al. 2005) showed that the whole of the mature *PoStr5* protein is predicted to be hydrophilic (Suppl. Fig. S1). The potential disorder of

PoStr5, defined as the propensity of a protein to have no stable structure, was analysed by the prediction program GlobPlot using the Russell/Linding algorithm (Linding *et al.* 2003). PoStr5 was predicted to be composed of almost entirely disordered regions (Suppl. Fig. S2). PoStr5 was also predicted by the *de-novo* repeat detection software, RADAR (Heger and Holm 2000), to be composed of three repeating subunits (Suppl. Fig. S3). These results indicate that PoStr proteins may have a highly flexible structure, are composed of repeating subunits, and have similarity to known extracellular structural proteins.

3.12 PoStr gene expression correlates with oospore presence

We have previously identified a family of putative cell wall proteins in *P. oligandrum* that we have named *PoStr* (*Pythium oligandrum* small tyrosine rich) (Horner *et al* 2012). Five of these proteins (PoStr1-5) are highly similar to one another (85-99 % sequence similarity), small, and rich in both tyrosine and glycine residues (Genbank: EV245172, EV244447, EV245146, EV245340, EV244818). A sixth family member, PoStr6, was not included in the present study since it is more divergent from the other family members (36-39 % sequence similarity) and not recognisable by our peptide antibody or qPCR primers. We therefore focused our studies on the five highly similar members of the family, PoStr1-5. Due to the similarity of these PoStr proteins to cell wall, mating-induced and eggshell proteins (Horner *et al* 2012), we hypothesised that these proteins may be a component of the *P. oligandrum* spinose oogonia. Using primers that

Comment [LG3]: Not sure why we only mentioned 5 initially, I know the 6th is longer, but I think we should talk about the family as having 6 members

Comment [LG4]: Pieter, please check this to see if it is an OK justification for disregarding PoStr6 and change if necessary! I can't find any evidence Neil tested the silenced lines for Postr6 expression and he hasn't much work on it overall, so my feeling is just drop it and try to justify why we just worked on PoStr1-5, but I am happy for you to rewrite/change this!

specifically amplified each gene, or primers that were predicted to amplify all five the five -PoStr ORFs (Table 1), semi quantitative RT-PCR showed that PoStr transcripts were abundant in three-day-old cultures (Fig. 13A), coinciding with the production of oogonia. Using a polyclonal, PoStr peptide-derived antiserum, PoStr proteins isolated from mycelial/oogonial cellular fractions became detectable by immunoblotting in four-day-old cultures, whereas no signal was detectable in the culture medium (Fig. 13B). Interestingly, in 21-day-old-cultures, PoStr proteins were no longer detectable (data not shown) Sub-lethal concentrations of anti-oomycete compounds have been used to suppress sporulation in *P. infestans* in order to correlate gene expression with oogonia production (Cvitanich *et al.* 2006). Similarly, we found that *P. oligandrum* cultures grown in V8 media containing antibiotic G418 (Geneticin) suppressed oogonium production in a dose-dependent manner (Fig. 24). Liquid grown cultures treated with 0.1 mg/l G418 produced oospores profusely, in those supplemented with 2.0 mg/l G418 virtually no oogonia were visible, and cultures treated with 0.5 mg/l G418 showed intermediate amounts of oogonia (Fig. 24A - C). Western blots performed using protein extracts from these G418 grown cultures showed a correlation between the production of oogonia and PoStr protein abundance, (Fig. 24D). Taken together these data suggest that the PoStr proteins may be involved in oospore growth or development, are not secreted into the culture medium, and are associated with oospores.

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3.23 PoStrs are localised in the oospores

Antiserum Po1 was used to localise the PoStr proteins in 5 day-old *P. oligandrum* fixed sections (Fig. 35). The gold particles were almost exclusively detected in vesicle-like structures, approximately 100-200 nm in diameter that were located at periphery of immature oospores (Fig. 35A - C) which appear similar to the small grey vesicles observed in comparable stained sections (Fig. 69D). Many of these vesicles were observed in close association with the inner side of the oospore wall. Gold particles were not observed in significant amounts in any other part of the oogonia or in the hyphae. As immunoblotting had shown that PoStr proteins were not detectable in 21-day-old cultures, we decided to see if the proteins were also absent in sections. Surprisingly, the oospore (IOW) and oogonial (OG) wall including the spines, were strongly labelled with Po1 antiserum, but vesicle-like structures containing the gold particles that were previously visible were now absent (Fig 35 D - E). These data suggest that the PoStr proteins are transported in vesicles to the oospore cell wall, and are incorporated into the oospore cell wall during the early stages of oospore formation and maturation.

3.34 Silencing of *PoStr*-gene expression results in aberrantly-formed oospores

We recently developed a stable transformation protocol for *P. oligandrum* (Horner *et al.* 2012), which we employed to silence expression of the [five most similar PoStr transcriptss](#) in order to gain an insight into their function. *P. oligandrum*

1 | was transformed with an antisense *PoStr1* cDNA construct (Suppl. Fig. S14).
2 | ~~Silencing of up to~~ Reductions in mRNA levels of up to 91 % ~~were~~ achieved in
3 | some lines compared to non-silenced controls transformed with empty vector
4 | constructs (Fig. 46). Western blots using whole protein extracts from five-day old
5 | cultures confirmed attenuation of expression (data not shown).

6 |
7 | Silenced strains and control strains produced equal numbers of oospores in five-
8 | day old cultures. Strain PoStrS29 was chosen for further characterisation, since it
9 | exhibited the lowest levels of *PoStr* expression (Fig. 46). Preparations of hyphal
10 | material containing oospores were treated with cellulase and lysing enzymes, in
11 | order to digest hyphae and hence remove them from samples. Thick-walled
12 | oospores from wild-type strains were resistant to this procedure. However,
13 | purification of oospores from PoStrS29, using this method, resulted in a dramatic
14 | reduction in oospore recovery compared to control strains. Oospores from
15 | PoStrS29 were seen to lyse during this treatment (Fig. 57C, D), and after 2 hours
16 | of treatment had been almost completely digested (Fig 57E), whereas oospores
17 | from control strains remained intact (Fig 57A, B) with internal lipid and ooplast
18 | vacuoles still visible. Furthermore, oospores prepared in this manner from
19 | PoStrS29, were seen to leak material from the oospore (Fig. 57C), suggesting a
20 | loss of structural stability of the cell wall. This leaking phenomenon was not
21 | observed in the wild type or the control strain PoStrT4. Since resistance to
22 | enzymes is likely a property of the normal oospore wall, we conclude that
23 | silencing PoStr1-5 suppressed normal oospore cell wall formation. ▲

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3.45 Ultrastructural analysis of *PoStr*-silenced strains reveals changes in the oospore cell wall

To gain further insights into the effects of silencing the *PoStr* genes, we used transmission electron microscopy (TEM) to examine oogonia fixed after 5 days of growth. ~~The cytological changes which normally accompany oospore formation in this species are summarised in the micrograph tracings in Fig 8A-F (adapted from Beakes (1981)). The young oogonium is decorated by distinctive spine-like papillae, although these contain few of the major organelles, such as mitochondria, nuclei, fingerprint/dense body vesicles or lipid, that occupy the main body of the developing oogonium (Fig 8A-B). Following synchronous meiosis a narrow cleavage envelope (furrow) forms to delimit the uninucleate oosphere cytoplasm from a layer of peripheral periplasm, containing most of the supernumerary nuclei (Fig 8C-D). The thinner outer oospore wall layers are made up of an outer amorphous, grey OOWa layer (Fig 9B) and an inner electron dense layer, labelled OOWb in Fig 9B. The formation of the electron dense outer oospore wall (OOWb) coincides with the lysis of the periplasm (Fig 8E-F). At the same time the spore contents becomes more dense in appearance (Fig 9A). It is at this stage that the empty space between the developing oospore and the oogonium wall (Figs, 8D-E, 9A) becomes apparent as a result of the complete breakdown of the periplasmic cytoplasm. In immature oospores a single centrally~~

located nucleus is surrounded by abundant small cytoplasmic lipid droplets and interspersed fingerprint/dense body vesicles are randomly intermixed (Fig 8E, 9C). Mature oospores (Figs 8F, 9A,B) are characterised by the thick inner oospore wall layer (IOW) which, in this species, appears to be of moderate electron density but has a uniform amorphous granular texture (Fig 9B). The cytoplasm of the mature oospore packed with lipid bodies and has a prominent electron dense vacuole, (Figs 8F, 9A) which has variously been referred to as the ooplast (Beakes *et al.* 1986) or central globule (Ruben and Stanghellini 1976). At this stage very few organelles can be recognized and even the peripheral nucleus, is only distinguishable as a region free of lipid globules (Fig 9A).

The ultrastructural effects that were visible in PoStr silenced oospores were variable. The PoStrS29 silenced oogonium shown in Fig 69C, D appears to be a fairly typical late oosphere stage/immature- ~~oospore. oospore (intermediate between Fig 8B and 8C).~~ The oosphere is well delimited (Fig 69C) and the periplasm is in an advanced state of disintegration (Fig 69D). However, the spore appears to be surrounded by just an amorphous layer of intermediate electron density, which is similar to the OOWa layer seen in the control unsilenced spores (Fig. 69B). There is no evidence of the more electron dense OOWb layer, which is normally laid down at this stage (Beakes *et al.* 1981; ~~unpublished observations~~Beakes and Bartnicki-Garcia 1989). The spore cytoplasm is densely packed with lipid bodies with the relatively small electron-dense finger print/dense body vesicles squeezed in between-~~(as in Fig 8B and 8C).~~ Dense body vesicles and other membrane-bound cytoplasmic organelles

are still clearly visible in adjacent cytoplasm (Fig. 69D), all of which are features typical of mature oospheres. These observations reinforce the conclusion that it is the electron dense OOWb layer that is the main protective layer and is the structure that differentiates an oospore from an oosphere as proposed by Beakes (1980).

Approximately 40% of the PoStrS29 silenced spores however, appeared very different, exhibiting a large central vacuole (Fig. 69E,F, Suppl. Fig. S25), which was not present in the non-silenced transformant T4 spores (Fig. 69A,B, Suppl. Fig. S25). These abnormal PoStrS29 oogonia have a huge central vacuole containing electron-dense globules (which are derived from the reabsorbing ooplast as described in germinating oospores: (Beakes 1980) and a narrow peripheral layer of lipid rich cytoplasm. The lipid droplets are much less uniform in size than in a normal oospore (compare Fig 6 (E, F with A, B). There is no sign of an oospore wall having been formed, instead there is an additional layer of wall material deposited inside the more electron dense oogonium wall (Fig. 69F).

This new layer has papilla like wall ingrowths (that were previously termed lomasomes; (Beakes 1980)), which often form in situations where localised wall deposition exceeds surface expansion (Fig. 69F). The normal OOWb and IOW oospore wall layers formed in control strains, were completely absent from these vacuolate PoStrS29 oogonia, suggesting a complete suppression of oospore differentiation. Instead the phenotype is much more reminiscent of the cytoplasm observed in germinating oospores in oomycetes such as Pythium

1 aphanidermatum (Ruben and Stanghellini 1976), *Pythium ultimum* (Hemmes and
2 Stasz 1984) and *Saprolegnia ferax* (Beakes 1980). In all of these species the
3 thick inner oospore wall dissolves and a new germination wall layer forms around
4 the protoplast that is continuous with the germ tube wall. Aberrant oogonia from
5 *PoStr*-silenced strains, analysed in our study, show a similar inner wall layer
6 deposited directly against the more electron dense oogonium wall (Fig. 6F). This
7 inner wall layer also exhibits localised ingrowths as described in the germination
8 wall layers of *S. ferax* (Beakes 1980). The presence of small electron-dense
9 globules, within the large vacuole of these aberrant oogonia, is also similar to
10 vacuoles described in germinating oospores of *S. ferax*, where these globules are
11 believed to be derived from the breakdown and reabsorption of the electron-
12 dense, ooplast vacuole contents. In the case of these spores, this material is
13 presumably derived from the fingerprint/dense body vesicles that would, in
14 normal oospore differentiation, have given rise to the central oopore globule
15 (ooplast).

17 **4. CONCLUSIONS**

18 **4. DISCUSSION**

19 ~~We have identified and characterised a family of proteins from *P. oligandrum*~~
20 ~~oospore cell walls; showing that their presence is necessary for full structural~~
21 ~~integrity of the oospore. The *PoStr* genes likely represent a large gene family in~~
22 ~~*P. oligandrum* with at least 5 members of the family (*PoStr*1-5) showing high~~

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1 levels of sequence similarity to one another. As previously suggested for the M96
2 protein family (Cvitanich *et al.* 2006), with which *PoStrs* show similarity, the high
3 tyrosine content could provide structural integrity to complexes formed by these
4 proteins. Glycine residues are also abundant within the *PoStr* proteins, which
5 may also contain pseudo-repeats. Taken together, these data suggest that the
6 formation of flexible, higher order structures of *PoStr* proteins is likely, as was
7 also suggested for the M96 proteins from *P. infestans* (Cvitanich *et al.* 2006).

8 Expression analysis by RT-PCR and Western blot analysis shows that *PoStr1-5*
9 transcripts accumulate from three days post-inoculation onwards.

10 Correspondingly, *PoStr1-5* proteins are detectable in cultures from four days
11 post-inoculation. Under conditions which prevented oospore formation, we were
12 unable to detect *PoStr* proteins on Western blots, however in cultures containing
13 high numbers of oospores, *PoStr* proteins were present. Additionally,

14 Immunolocalization of *PoStr1-5* with polyclonal antiserum Po1 showed an
15 oogonial specific localization. Peripheral vesicles were seen to accumulate *PoStr*
16 in younger cultures. In older cultures, containing mature oospores, the *PoStr1-5*
17 proteins localised most strongly to the spikes (Fig. 5H) and outermost layer of the
18 oospore cell wall (Fig. 5G). Interestingly, at this stage *PoStr* proteins could not be
19 detected on Western blots. It is possible that the *PoStr* proteins have become
20 strongly integrated into the cell wall of the oospores, for example by oxidative
21 cross linking, which would prevent their release or solubility in the protein lysis
22 buffer. Therefore we conclude that the *PoStr* proteins are constituents of the *P.*
23 *oligandrum* oogonial and oospore cell walls.

To further characterize the precise role of the PoStr proteins in *P. oligandrum* oogonia and oospore cell walls, we produced *P. oligandrum* strains silenced for *PoStr1-5*. Having recently developed a stable transformation protocol for *P. oligandrum* (Horner *et al.* 2012), we utilized this method to produce strains of *P. oligandrum* that were stably silenced for *PoStr1-5*. Using this method we were able to achieve up to 91% silencing in individual lines. Although the resulting silenced strains still produced oogonia in culture in numbers similar to the wild-type, the cell walls of these spores were more susceptible to degradative enzymes than wild type spores. This is probably because resistance to enzymes is a property of the normal oospore wall, the formation of which appears completely suppressed in *PoStr1-5* silenced strains. This conclusion is further supported by observations that oogonia from silenced strains appeared to undergo plasmolysis and ultimately cytorrhysis, after cellulose and lysis enzyme treatments that had no impact on control strains. Oogonia from silenced line *PoStrS29* were also seen to leak cellular contents into the surrounding media, through breaches in the cell wall (Fig. 7C). In mature *PoStrS29* silenced oospheres/immature oospores (Fig. 9C, D) the only 'oospore wall' layer formed appears to be the OOWa layer that is probably derived from the periplasm. These observations reinforce the conclusion that it is the electron dense OOWb layer that is the main protective layer and is the structure that differentiates an oospore from an oosphere as proposed by Beakes (1980). Electron microscopy revealed that approximately 40 % of *PoStrS29* oogonia contained a large central vacuole and that normal oospore formation had been

completely suppressed. The residual cytoplasmic contents, mainly large lipid globules were instead located at the periphery of the oogonium, which appear to have aborted without forming oospores. The normal OOWb and IOW oospore wall layers formed in control strains, were completely absent from these vacuolate *PoStrS29* oogonia, suggesting a complete suppression of oospore differentiation. Instead the phenotype is much more reminiscent of the cytoplasm observed in germinating oospores in oomycetes such as *Pythium aphanidermatum* (Ruben and Stanghellini 1976), *Pythium ultimum* (Hemmes and Stasz 1984) and *Saprolegnia ferax* (Beakes 1980). In all of these species the thick inner oospore wall dissolves and a new germination wall layer forms around the protoplast that is continuous with the germ tube wall. Aberrant oogonia from *PoStr*-silenced strains, analysed in our study, show a similar inner wall layer deposited directly against the more electron dense oogonium wall (Fig. 9F). This inner wall layer also exhibits localised ingrowths as described in the germination wall layers of *S. ferax* (Beakes 1980). The presence of small electron dense globules, within the large vacuole of these aberrant oogonia, is also similar to vacuoles described in germinating oospores of *S. ferax*, where these globules are believed to be derived from the breakdown and reabsorption of the electron-dense, ooplast vacuole contents. In the case of these spores, this material is presumably derived from the fingerprint/dense body vesicles that would, in normal oospore differentiation, have given rise to the central oospore globule (ooplast). Therefore the ultrastructural evidence suggests that normal oospore differentiation is completely suppressed in *PoStr*-silenced strains and points to

1 this gene family being key regulators of resting spore formation. This hypothesis
2 requires further testing, but may have exciting possibilities in terms of
3 suppressing the formation of resting spores, which are often key to the success
4 of these important plant pathogens. Our results show that PoStr proteins are an
5 important structural component of the oomycete oospore cell wall, without which
6 oospores are unable to differentiate. PoStr proteins were not seen to localise to cell
7 walls from other structures, such as sporangia or hyphae (data not shown),
8 indicating that oospore cell walls have unique characteristics compared to cell
9 walls produced at other stages of the life cycle. Therefore, whilst the chemical
10 composition of oospores and mycelium is largely similar (Lippman *et al.* 1974),
11 lipid and protein levels are significantly higher (Lippman *et al.* 1974; Ruben and
12 Stanghellini 1978) allowing these spores to withstand freezing, desiccation,
13 drought, microbial degradation, and persist in a resting state in soil for many
14 years (Judelson 2009). Since up to 47 % of the dry weight of oogonial and
15 oospores is accounted for by the cell wall, the PoStr proteins, described here, are
16 likely to play an integral role in this process. Future detailed structural analysis of
17 the oospore cell wall in wild type and silenced lines may further illuminate the role
18 of the PoStr proteins in these processes.

19 Our results have also shown, for the first time, that *P. oligandrum* is amenable to
20 gene silencing in a similar manner to other oomycetes. The gene silencing
21 methodology described here is thus, an important molecular tool, opening the
22 way to future gene characterisation studies in this important mycoparasite. We
23 have characterised a family of five PoStr proteins, correlating gene expression

1 [with the formation of oospores in culture, localising these proteins to the oogonial](#)
2 [cell wall and showing that oospore differentiation is dependent on the presence](#)
3 [of these proteins. We therefore conclude that PoStr proteins are an important](#)
4 [structural component of the oomycete oospore cell wall, without which oospores](#)
5 [are unable differentiate. Highly similar putative cell wall and mating-induced](#)
6 [proteins are present in related phytopathogenic species such as *Pythium ultimum*](#)
7 [and *Phytophthora infestans* \(Levesque et al 2010; Cvitanich et al 2006\). Our](#)
8 [ultrastructural evidence suggests that normal oospore differentiation is](#)
9 [completely suppressed in *PoStr*-silenced *P. oligandrum* strains and points to this](#)
10 [gene family being key regulators of resting spore formation. This hypothesis](#)
11 [requires further testing, but may have exciting possibilities in terms of](#)
12 [suppressing the formation of resting spores, which are often key to the success](#)
13 [of these important plant pathogens.](#)

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7. FIGURE LEGENDS

Figure 1. Protein alignment of four of the most similar PoStr predicted proteins.

Predicted protein sequences were aligned by MAFFT. Bottom black line indicates the predicted signal peptide. The top black line indicates the sequence used to synthesize the synthetic peptide for antibody production.

Figure 2. *PoStr* Southern blot analysis of *P. oligandrum*

Genomic DNA of *P. oligandrum* was digested with various restriction enzymes, run on an agarose gel, blotted and probed with a DNA fragment of *PoStr1*. Multiple hybridising bands were found indicating that a large gene family of *PoStr* exists in *P. oligandrum*.

Figure 13. Expression analysis of the *PoStr* family.

(A) Time course RT-PCR using primers specific for *PoStr1*, *PoStr1-5*, or *PoStr6* with tubulin as an internal control.

(B) Western blot using a *PoStr*-specific antibody on proteins extracted from *P. oligandrum* mycelia/spores cellular fractions or extracted from culture media.

Figure 24. Reduced oospore formation and *PoStr* protein presence in *P. oligandrum* cultures treated with geneticin.

(A, B and C) *P. oligandrum* cultures were treated with various concentrations of antibiotic G418. The oogonia production was proportional to the G418 amount.

(D) After five days of growth, proteins were isolated and a Western blot was labelled with the anti-*PoStr* antiserum.

Figure 35. PoStr proteins localize to peripheral vesicles, oospore and oogonial walls and the oospore spikes.

(A-C) five-day-old cultures were sectioned and immunogold labeling was performed with the antiserum Po1. Labeling is visible in vesicles at the periphery of the oospores. (D-E) 21-day-old spores samples were sectioned. Labeling is visible in the oospore walls and spikes. (F) control experiment in which primary antiserum was omitted. OG, Oogonium wall; OOWa, periplasm derived outer layer of outer oospore wall; OOWb, thin electron dense inner layer of the outer oospore wall; IOW, thick amorphous inner oospore wall oospore wall; ve, vesicle; m, mitochondrion; Og sp, oogonial spike; L, lipid body; DBV, dense body vesicles. Dashed arrows highlight labeled vesicles. Scale bar in A is 5 μ m; in B and F are 0.5 μ m; in C is 0.2 μ m in D is 2 μ m; in E is 1 μ m.

Figure 46. PoStr-expression levels in silenced lines.

Expression of *PoStr*'s relative to the constitutively expressed control tubulin alpha in transformed *P. oligandrum* lines. Primers were used that amplified *PoStr1-5* (Table 1). Black bars: control strains, transformed with an empty vector; white bars: silenced lines, transformed with pTorStr1-R. Error bars represent standard errors of the mean from three replicate experiments.

Figure 57. Morphology of oospores from *PoStr*-silenced *P. oligandrum* lines.

Oogonia were isolated from five-day old cultures after treatment with cellulase and lysing enzymes to remove hyphae. (A-B): Oospores from the wild type (A) and the control strain T4 (B) transformed with empty vector are intact after treatment. (C-E) oospores from silenced line S29 showing abnormal morphology. Oogonia from S29 were lysing and leaking their contents (C); undergoing plasmolysis and cytorrhysis (D) or containing little visible oospore content and appearing devoid of the periplasmic space (E). Scale bars represent 2µm.

Figure 8. A series of electron-micrograph tracings through differentiating oogonia, of a different isolate of *P. oligandrum* summarising the main stages of differentiation.

Nuclei (N) are cross-hatched, lipid globules stippled and fingerprint/dense body vesicles (DBV) indicated in black. **A.** Differentiation begins with the development of papillae. **B.** Pre-meiotic young oogonium, showing spiny outgrowths that are devoid of most of main organelles. The lipid, nuclei (N) and small spherical DBV are more or less randomly interspersed at this stage. **C.** Oosphere cleavage occurs. **D.** Oosphere differentiation phase, showing fully delimited oosphere surrounded by a thin layer of peripheral periplasmic cytoplasm. The oosphere contains two centrally located gametic nuclei (only one shown, N) at this stage, and supernumerary nuclei are also distributed in the periplasm. The dense body

vesicles are interspersed with the lipid globules that typically coalesced and are larger at this stage. ~~E. Immature oospore stage, showing empty periplasmic space between the oogonium wall and the developing oospore. The latter has a single centrally located fusion nucleus (N). The lipid filled cytoplasm is interspersed with coalescing dense body vesicles (DBV). F. Oogonium with a fully differentiated, mature oospore. Note the thick oospore wall, and coalesced centrally located ooplast vacuole (OP) derived from coalesced DBV. There is single peripheral fusion nucleus, and the lipid globules have dispersed into smaller units again. Based on material from an unpublished study, adapted from (Beakes 1981).~~

Figure 69. Ultrastructure of representative oogonia from control and *PoStr*-silenced *P. oligandrum* lines.

~~(A,B)~~ Near median cross sections of T4 oogonium (A,B) (empty vector control strain). ~~(C-F)~~ and of oogonia showing two representative morphotypes (C-F) seen in S29 (the *PoStr*-silenced strain). The thick amorphous inner oospore wall (IOW) was often missing or disintegrated (white arrow heads in C), or aberrantly formed in the silenced line (D). Additionally, the thin electron dense inner layer of the outer oospore wall (OOWb) was also often completely missing in the silenced line (D). Furthermore, debris (indicated by black plusses in C and D) was observed in the area between the oospore and oogonial walls, but not in the non-silenced line (A, B). Approximately 40% of the *PoStr*-silenced oogonia contained

a large central vacuole and the absence a defined oospore (wall) (E,F). The high magnification image (F) reveals a new inner wall layer, with associated papillate ingrowth (asterisk).

OG Oogonium wall; OOWa periplasm derived outer layer of outer oospore wall, OOWb thin electron dense inner layer of the outer oospore wall, IOW, thick amorphous inner oospore wall oospore wall; IW, inner wall layer formed in aberrant vacuolated oogonia. L, Lipid body; V, vacuole; DBV, dense body vesicles; lo, lomasome; black plusses indicate debris in the space between oospore and oogonial walls. Scale bars in C and E are 2µm; in A is 1µm; in F is 0.5µm; in B and D are 0.2µm.

8. TABLES

Table 1. Oligonucleotide Primer Pairs used in semi-quantitative and quantitative real-time RT-PCR

Gene	Primer Name	Primer Sequence
<i>PoStr1-5</i>	PoStrF	GTGCCTATGGCTACGACGAC
	PoStrR	GTGGTGCTTGTGGTGCTTC
<i>PoStr1</i>	PoStr1F	GTTTTTCACATCACACAAAGAC
	PoStr1R	GTCTTTGTGTGATGTGAAAAACG

<i>PoStr2</i>	PoStr2F	GTACGTCTAGCGTTGAAAGTCT
	PoStr2R	TGATGTGAAAAACGACAATACTTTG
<i>PoStr3</i>	PoStr3F	TGATGTCGCTCAAGTGGTGT
	PoStr3R	TAAAAGGCGGCGTTTGACAG
<i>PoStr4</i>	PoStr4F	TGCGTAAAATTATACTGTTTGTTCTTC
	PoStr4R	AAAGCATGGCTCCGTTGTAT
<i>PoStr5</i>	PoStr5F	TGCGTGTCATTGTTAGTTTTGA
	PoStr5R	TGTCACCGTTCAACGTTCTT
<i>PoTUBA</i>	TUBAF	GATGTCGTGCCAAAGGATGTC
	TUBAR	CGAAGGTGGCTGGTAGTTGATAC

1

2 **Table 2. Predicted properties of PoStr proteins and putative homologs after**
3 **signal peptide cleavage.**

Protein	length (aa)	TYR %	GLY %	pI	MW (KDa)
PoStr1	86	32.6	23.3	7.94	10379
PoStr2	86	32.6	23.3	7.94	10379

PoStr3	105	32.4	22.9	6.54	12668
PoStr4	110	30.9	23.6	6.70	1390
PoStr5	110	31.0	24.5	6.63	13138
PoStr6	205	20.5	17.6	4.40	22728
PYU1_T000208	207	21.3	28.0	4.98	22031
M96	260	13.1	13.5	7.80	29238
P48	414	26.1	15.0	6.51	50249