Using RNA-Seq to identify oospore wall-specific Carbohydrate-Active
Enzyme (CAZyme) coding genes of *Pythium ultimum* var. *ultimum*, an
oomycete plant pathogen

by

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Abstract

Oospores are propagules that result from sexual recombination in oomycetes and act as long-term survival structures. In *Pythium ultimum*, as well as many other oomycete species, the thick, glucan-rich walls of dormant oospores can account for up to 50% of the total volume of the oospore. Despite this considerable thickness, the oospore wall can be digested quickly and used as a food source to propel oospores towards a germinable state. Whether digestion occurs by enzymes resulting from genes transcribed at this stage, or prepackaged transcripts or proenzymes released upon wall rehydration is currently unknown. Since oospore walls are composed predominantly of β -glucans, their enzymatic digestion by glucanases may represent the earliest stage-specific molecular development that occurs at this point in the *P. ultimum* life cycle. In this study we used RNA-Sequencing to investigate the process of oospore conversion and to identify and profile possible genes encoding oospore wall-specific enzyme products. Some enzymes were expressed solely during oospore conversion, and among those, some are only present in *Pythium* species when compared to other oomycetes.

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1. Introduction

The oomycete genus *Pythium* is diverse and cosmopolitan, containing over 150 species, most of which are soil-borne plant pathogens (van der Plaats-Niterink, 1981; Levesque and De Cock, 2004; Uzuhashi *et al.*, 2010). *Pythium ultimum* displays a wide host range and has been recorded on over 30 plant species in Canada and on over 700 plant species worldwide (Farr *et al.*, 2007). The species *Pythium ultimum* var. *ultimum* is one of the most destructive phytopathogens in the *Pythium* genus (Cook *et al.*, 1980; Sumner *et al.*, 1990; Larkin *et al.*, 1995; Schroeder *et al.*, 2006; Snowden, 2008; Schroeder *et al.*, 2013). This necrotrophic species is notorious for its widespread destructive nature, rapid saprophytic growth and pathogenic development in response to germinating seeds and root exudates. It causes seed decay, pre- and post-emergence damping-off or root rot in many plant species and occasionally causes serious damping-off of conifers in Ontario nurseries (Agnihotri and Vaartaja, 1967; Kageyama *et al.*, 2003; Weiland *et al.*, 2013). Seeds, seedlings, and juvenile plant tissues are particularly at risk because their un lignified tissues provide little barrier to protect them from pathogen attack. Damping-off can be observed as non-germinated seeds or as seedlings that fail to emerge from the soil. Newly emerged seedlings may also experience post-emergence damping-off, evident as seedling collapse and death (Weiland *et al.*, 2013). *Pythium*-related disease symptoms are rarely apparent in older seedlings unless the soil is water-logged, in which case root rot can develop (Agnihotri and Vaartaja, 1967; Hering *et al.*, 1987; Fukui *et al.*, 1994; Weiland *et al.*, 2013). On more mature plants, root rot by *Pythium* spp., including *P. ultimum*, can have a significant impact on yield over very large areas (Cook and Haglund, 1982; Cook *et al.*, 1987; Cook and Haglund, 1991; Paulitz *et al.*, 2002).

1.1. Oomycetes

Oomycetes were originally considered members of the fungal kingdom because of their mycelial growth habit and mode of nutrient acquisition via absorption. However, they are now recognized to be more closely related to brown algae and diatoms than fungi and occupy a distinct phylogenetic lineage within the Stramenopiles supergroup (Lee *et al.*, 2012). Many morphological, developmental and biochemical characteristics set this group apart from fungi, such as the occurrence of diploid nuclei in vegetative hyphae, the production of oospores and biflagellate zoospores and the use of several different biochemical pathways, such as in the synthesis of lysine and sterols (Dahlin *et al.*, 2016). Oomycetes also demonstrate unique cell wall composition profiles, consisting primarily of 1,3;1,6- β -glucans and cellulose, whereas in fungal cell walls chitin is the main component (Mélida *et al.*, 2013). In fact, the differences between oomycetes and fungi are so extensive that when they were previously believed to belong to the same kingdom, oomycetes were described as a 'fungal geneticist's nightmare' (Shaw, 1983; Schornack *et al.*, 2009).

The oomycete lineage branches out into two large groups that are not yet formally described as classes but that we will refer to as the “Saprolegniomycetes” and the “Peronosporomycetes” (Thines, 2014), formerly known as the Saprolegnialean and Peronosporalean galaxies (Sparrow, 1960; Sparrow, 1976). A formal proposal is being prepared to elevate Oomycetes to a phylum and elevate to classes the current subclasses Saprolegniomycetidae and Peronosporomycetidae (Lévesque. pers. comm.). There are several orders within these subclasses or “classes” but the main one is the Peronosporales which contains the highest number of plant pathogenic oomycetes, which are highly concentrated within the genera *Phytophthora* and *Pythium* (van West *et al.*, 2003). Among

the new higher level rank proposals, Pythiales is likely to be resurrected as order for the monophyletic *Pythium* species with filamentous sporangia, restricting the Peronosporales to a monophyletic group for species with globose or lemon-shape sporangia, namely, species in the genera *Phytophthora*, *Phytopythium*, *Peronospora*, *Bremia*, and *Hyaloperonospora*, and the ones in clades of *Pythium* species with globose sporangia (Lévesque. pers. comm.).

1.2. Lifestyle and pathogenesis strategy of *Pythium ultimum* var. *ultimum*

Plants rely on constitutive and inducible defenses to protect themselves from pathogens. Examples of constitutive defense include the presence of a waxy cuticle covering the outermost epidermal layer of aerial tissues, and following considerable secondary growth, cell wall lignification and cutin and suberin deposition on outer protective tissues (Freeman and Beattie, 2008). Plants may also deploy inducible defenses in response to detected microbe- or pathogen-associated molecular patterns ranging from localized cell wall fortification, production and release of various toxins and pathogen-degrading enzymes, and deliberate plant cell death at the site of infection to limit pathogen access to nutrients, among others (Freeman and Beattie, 2008). Pathogens with biotrophic lifestyles feed on living plant host tissues, establishing intimate relationships with their hosts to suppress host defenses and promote the slow release of nutrients while necrotrophs tend to inundate plant defenses with toxins and tissue-degrading enzymes in order to promote rapid release of nutrients (Freeman and Beattie, 2008). Plant pathogenic oomycetes employ various attack strategies to circumvent plant defenses and previous studies have suggested that there may be a link between pathogenic lifestyles and expansions of gene families of carbohydrate-active enzymes (CAZy). Unlike phytopathogens of the genus

Phytophthora, *Pythium* phytopathogens lack a biotrophic stage, and tend to exploit necrotrophic lifestyles. They are known to be aggressive saprophytes that tend to be over represented in ecological studies relying on soil or root plating techniques, as illustrated by the numerous growth media providing various amendments to suppress *Pythium* species when trying to isolate *Phytophthora* (e.g. Erwin and Ribeiro, 1996). Because successful attack does not depend on the survival of the plant host, they do not require specialized infection structures or sophisticated effector repertoires typically seen in genomes of *Phytophthora* phytopathogens. In fact, *P. ultimum* var. *ultimum*, which is one of the most successful phytopathogens in the *Pythium* genus, appears to lack an RxLR effector repertoire (Levesque *et al.*, 2010). The genome of *P. ultimum* var. *ultimum* encodes pectinases, candidate α -amylases, glucoamylase and invertase, but appears to lack pectin methylesterases or cutin-encoding genes, precluding it from effectively degrading complex polysaccharides such as xylan and chitin. While it shows limited growth on culture medium wherein citrus pectin is the sole carbon source, it exhibits excellent growth on soluble starch and sucrose. This is consistent with its observed modus operandi as an opportunistic necrotroph targeting young seedlings and plant roots with little or no cuticle or heavily suberized tissue for plant starch and sucrose exploitation (Levesque *et al.*, 2010).

1.3. Lifecycle of *Pythium ultimum* var. *ultimum*

The lifecycle of *P. ultimum* var. *ultimum* consists of both sexual and asexual phases. Unlike other *Pythium* species that produce asexual propagules such as sporangia and zoospores, most *P. ultimum* var. *ultimum* strains have lost the ability to produce zoospores and only produce oospores which are primarily survival propagules for unfavourable environmental conditions that result from sexual recombination. It relies on the outward growth of

mycelium from its primary infection loci to neighbouring plants for its spread during the disease cycle (Trow, 1901; Waterhouse, 1968; Stanghellini and Hancock, 1971; Martin and Loper, 1999; Green and Jensen, 2000)(Figure 1). This makes oospores the single most critical means of long-term survival and disease propagation for this species in climates that experience dry or cold seasonal variation (Levesque *et al.*, 2010).

Pythium ultimum is a homothallic species and exogenous sterols, such as cholesterol and β -sitosterol, serve as hormone precursors for entry into the sexual reproductive phase (Ayers and Lumsden, 1975; Kerwin and Duddles, 1989). Swelling of hyphal tips is the first known response to hormones. These develop into male or female organs, termed antheridia and oogonia, respectively. Once the oogonium has reached its full size, the antheridium emerges immediately below the oogonium on the same thallus and develops rapidly, producing an adhesive required for mating. The oogonium continues to expand, accumulating cytoplasm and nutrient reserves, and presses into the antheridium until it is penetrated and then matures into a pleurotic oospore (Trow, 1901; Marchant, 1968; McKeen, 1975; Hemmes and Stasz, 1984). The diploid nuclei undergo meiosis and when the antheridial nucleus migrates into the oogonium, a diploid zygote is formed and the oogonial cytoplasm becomes enriched with lipid bodies, proteins and glucan polymers. At maturity oospores average 18 μm in diameter (Hemmes and Stasz, 1984). The entire process of oosporogenesis, from the formation of sexual organs to the complete maturation of the oospore requires 24 hours (Trow, 1901; Marchant, 1968; McKeen, 1975). A summary of the stages and timeframes that make up the process of oosporogenesis are presented in Figure 2. These mature oospores possess two distinct layers, a thin (20-40 nm) and dense protective outer wall, and a thick (2-2.5 μm) electron-transparent

polysaccharide inner wall composed of 1,3- β -glucans and 1,3;1,4- β -glucans (Hemmes and Stasz, 1984; Beakes and Bartnicki-Garcia, 1989). Interior to the inner wall, a cortical layer of lipid bodies along with the nucleus and mitochondria, surround the central ooplast which is roughly 10 μ m in diameter (Figure 3). The high lipid content in the outer wall serves as a protective hydrophobic barrier while the thick polysaccharide inner wall provides storage reserves for subsequent oospore germination (Beakes and Bartnicki-Garcia, 1989).

Oospore germination is a critical part of the life cycle that leads to disease outbreaks. Avoiding damping off, primarily caused by *Pythium* spp., is the main reason why potting soil needs to be pasteurized before growing seedlings or why seeds need to be chemically treated in the field (Le *et al.*, 2014). The *Pythium* biomass would exist primarily as oospores in dry soil as the coenocytic mycelium is very sensitive to desiccation (Ayers and Lumsden, 1975; Hancock, 1981). It is a well-known phenomenon that seeds planted in dried unpasteurized soil that has just been wet do get infected within 24 hrs. There must be a fast oospore conversion and germination mechanism that leads to such rapid infection. Root infection of bean and wheat seedlings by *P. ultimum* was observed 24 hours after the seedlings received a sublethal dose of the herbicide glyphosate (Lévesque *et al.*, 1993a; Lévesque *et al.*, 1993b). In this published example, dried unpasteurized soil had been wet a few days before the treatment and the infection could be from mycelium growing saprophytically or from germinating oospores, but the same had been observed with seeds planted in dried soil that had just been wetted (Lévesque, pers. Comm).

Figure 1. Life cycle of *Pythium ultimum* var. *ultimum*.

Figure 2. Approximate time-frames of *Pythium ultimum* var. *ultimum* oosporogenesis stages. Time-frames are based on the microscopic observations described by Trow (1901).

1.4. Oospore dormancy-breaking, conversion and germination

Mature oospores display a period of constitutive dormancy where further development is delayed and will not germinate even when conditions conducive to germination are present (Ayers and Lumsden, 1975; Fukui *et al.*, 1994; Paulitz and Adams, 2003). In *P. ultimum*, as well as many other oomycete species, the thick, glucan-rich walls of dormant oospores can account for as much as 50% of the total volume of the oospore. A taxonomic key based primarily on species variations for this statistic, the wall index, was proposed by Dick (1990), but did not gain much support. Despite this considerable thickness, the oospore wall can be digested quickly and used as a food source to propel oospores towards a germinable, pathogenic state when environmental conditions become favourable and the period of dormancy is done. Thus the enzymatic digestion by glucanases may represent the earliest stage-specific molecular development that occurs at this point in the *P. ultimum* lifecycle.

During conversion to a germinable oospore, the outer oospore wall separates from the thick inner oospore wall and gradual digestion of the thick inner wall due to the production of glucanases and other wall degrading enzymes. At the same time as the inner wall is digested, a new germination wall interior to the thinning inner wall is deposited, representing a very finely controlled process of wall relaxation and cell wall synthesis (Beakes and Bartnicki-Garcia, 1989). Hydrolysis and mobilization of these glucans by glycoside hydrolases 1,3- β -glucanases and 1,3;1,4- β -glucanases during conversion is believed to provide an energy and carbon source for the synthesis of functional cytoplasm. The rate of conversion from dormant, thick-walled oospores to thin-walled, germinable oospores has been observed to occur over a range of as little as two weeks to several

months (Ayers and Lumsden, 1975). In *P. ultimum* var. *ultimum*, full oospore conversion is a pre-requisite for germination (Ayers and Lumsden, 1975; Qian and Johnson, 1987). Conversion rates of culture-produced oospores *in vitro* are rapid compared to field conditions and can be triggered to undergo conversion efficiently and synchronously when suspended in water or non-sterile soil extract under optimal light, temperature, aeration and density conditions (Ayers and Lumsden, 1975; Johnson, 1988). Once converted, oospores cannot revert back to a dormant state, nor will they undergo germination in water-only suspensions. However, germination responses can be induced rapidly and synchronously in converted oospores when glucose or sucrose is added to the suspension (Figure 4)(Hemmes and Stasz, 1984; Giroux, 2014). This enables the controlled separation of conversion and germination responses *in vitro*, facilitating transcriptional profiling studies at this stage of the *P. ultimum* var. *ultimum* lifecycle.

In a previous study by Giroux (2014), dormant oospores that were resuspended in water supplemented with linoleic acid, a sporangial germination trigger (Ruttledge and Nelson, 1997), oospores were not observed to undergo conversion even though other abiotic conditions were favourable (light, aeration, low density of oospores). Instead of halting oospore conversion, oospores lysed when linoleic acid was added to oospores in suspension that had already begun to convert. Additionally, it was extremely difficult to recover oospores from these suspensions, making it impossible to use this as a way to set up negative controls for expression analysis.

Figure 3. Schematic representation of a mature dormant oospore and a converting oospore of *Pythium ultimum*. This schematic is based on electron microscopy images from Hemmes and Stasz (1984). An oospore transitioning from a dormant to a fully converted state undergoes inner oospore wall (IOW) dissolution and concurrent formation of a germination wall. L: lipid bodies, M: mitochondria, N: nucleus, OP: ooplast IOW: inner oospore wall, OOW: outer oospore wall, OW: oogonial wall, GM: germination wall.

Figure 4. Proportion of converted and germinating oospores of *Pythium ultimum* var. *ultimum* in different culture mediums. Data is based on microscopic analysis by Giroux (2014). Conversion and germination in water, glucose, linoleic acid, and glucose:linoleic suspensions over time (mean proportion out of >400 spores; vertical bars = $\pm$ SE).

1.5. Cell wall assembly and major types of oomycete cell walls

Cell walls are structures that extend beyond the plasma membrane and are made up of polysaccharide-protein complexes. The composition of these extracellular structures is highly variable across taxonomic groups. Thus, despite major advances in understanding the cell walls of fungi and plants, such as for *Saccharomyces cerevisiae* or *Arabidopsis thaliana*, the information cannot be translated directly to cell walls of oomycetes (Lesage and Bussey, 2006). Nonetheless, however variable the composition and structure of the extracellular matrix may be, all eukaryotes are linked by a highly conserved secretory system that is responsible for synthesizing and sorting relevant cell wall components and other extracellular materials (Novick *et al.*, 1981; Rose and Lee, 2010).

The classical eukaryotic secretion pathway begins with a protein sequence that has an N-terminal signal peptide sequence which directs the ribosomes that are synthesizing the protein to the rough endoplasmic reticulum (ER) whereupon the remainder of the protein is cotranslationally translocated into the ER lumen with concomitant cleavage of the signal peptide (Green *et al.*, 1992). Once inside the ER lumen, a protein destined to be secreted can undergo several modifications while progressively traveling from the rough ER to the smooth ER where they are packaged into Golgi transport vesicles for migration through the Golgi complex to the Golgi cisternae, upon which they are then packaged into secretory or transport vesicles which migrate to the plasma membrane for secretion (Kaiser and Schekman, 1990). Some proteins are secreted constitutively, and once they've migrated through to the Golgi cisternae will be transported in vesicles immediately to the plasma membrane for secretion. Other proteins may undergo regulated secretion where they may be temporarily retained in various compartments along the secretory pathway

(Burgess and Kelly, 1987). Some examples of regulated secretion include proteins packaged in vesicles that require particular stimuli to trigger their fusion and release of contents into the next compartment along the secretory pathway, or inactive proteins that may be retained in various compartments along the secretory pathway until posttranslational proteolytic processing of a proregion of the protein causes it to become enzymatically activated and resume trafficking along the secretory route (Rose and Lee, 2010).

A recent study on the extracellular carbohydrate profiles of oomycetes revealed three general wall types are distributed across species in a manner consistent with the overall phylogenetic history of the oomycete lineage (Mélida *et al.*, 2013). Oomycetes within the “Peronosporomycetes”, of which *P. ultimum* is a member, have been characterized as having Type I wall compositions which lack chitin and contain significant cellulose scaffolding. The chemical composition of oospore walls and the walls in vegetative hyphae have been described as qualitatively similar. However, relative to mycelia, oospore and oogonial walls contain higher amounts of lipid and proteins and have a lower glucan content (Lippmann *et al.*, 1974; Ruben and Stanghellini, 1978). The walls of *Pythium* oogonia and oospores account for up to 47% of the dry weight of the cell and consist predominantly of mycolaminaran, a polysaccharide form of storage in oomycetes consisting predominantly of glucans containing β -1,3 and β -1,6 linkages and cellulose, the amount present being dynamic and specific to oospore growth stages (McLeod *et al.*, 2003; Levesque *et al.*, 2010).

1.6. CAZy families implicated during oosporogenesis and conversion

Glucanases are highly conserved proteins in oomycetes and have also been hypothesized to have originally functioned to promote cell growth and division of unicellular organisms by turning over cell wall β -glucans (McLeod *et al.*, 2003; Levesque *et al.*, 2010). Biochemical studies of glucanases in oomycetes have been conducted in an effort to elucidate their possible role in hyphal tip growth and branching where a delicate balance between cell wall synthesis and hydrolysis is believed to exist, for their role in Ca^{2+} -induced sporulation and for their roles in host-pathogen interactions (McLeod *et al.*, 2003). While many plant, fungal and bacterial glucanases have been characterized, few glucanases have yet been characterized at the molecular level for oomycetes with the exception of *Phytophthora infestans* and *Pythium insidiosum* (McLeod *et al.*, 2003; Keeratijarut *et al.*, 2015; Miller, 2015). However, genes of the endoglucanase family have been described in the *Pythium* genome (Levesque *et al.*, 2010). The CAZy complement in *P. ultimum* var. *ultimum* as well as several other *Pythium* species have been also characterized (Zerillo *et al.*, 2013). However, since this CAZy annotation pipeline was run in 2013, new families have been recognized while others have been updated or removed from the CAZy database (Figure 5).

Figure 5. Total CAZy family HMM models captured across dbCAN release versions. The number of new family models added, removed and updated are included in the table below the graph. Data was obtained from the dbCAN release notes (csbl.bmb.uga.edu/dbCAN/).

1.7. Oospore wall-specific glucanases

There have been few studies detailing the biochemical and structural dissolution of mature oospore wall complexes. This may be due in part to difficulties associated with obtaining large numbers of oospores for study or developing assays that are sensitive enough to be conducted with minimal numbers of oospores. One study conducted by Beakes and Bartnicki-Garcia (1989) investigated the structural changes in the walls of germinating oospores and enzymatically digested oospore wall fragments of *Phytophthora megasperma*, and proposed a mechanism for oospore wall dissolution. Enzymatic hydrolysis of wall

fragments by various exogenous β -glucanases resulted in erosion of the inner oospore wall (IOW), revealing a microfibrillar network, presumably cellulosic, oriented parallel to the plasma membrane. In germinating oospores, similar exposure of the microfibrillar network occurred and digestion of wall components was indicated by the formation of cavities extending parallel to the plasma membrane in a characteristic “scallop” pattern, delimited by radial strands of undigested wall not observed in isolated enzymatically-digested wall fragments. They proposed that the radial strands indicated sites where localized secretion of enzymes or glucanase activators occurred and this formed the basis of two proposed oospore wall digestion mechanisms: 1) Glucanases may be pre-packaged, possibly as translationally repressed transcripts or inactive proenzymes in specific compartments within the oospore wall or within the cytoplasm to be solubilized and released through the concerted influence of environmental and microbial factors in the early growing season; and 2) alternatively, favourable environmental conditions may trigger the breaking of oospore dormancy and de novo synthesis of glucanases. Beakes and Bartnicki-Garcia (1989) made a parallel between wall mobilization in germinating oospores and wall dissolution patterns in seeds and cotyledons of higher plants. They further added that there may be variations in the extent of wall mobilization across species within “Peronosporomycetes” and made reference to the previous findings by Hemmes and Stasz (1984) whereby the entire IOW appeared digested upon germination in *P. ultimum* var. *ultimum*, as opposed to observations with *P. megasperma* oospores where conversion and germination phases overlapped. However, the electron microscopy images presented by Hemmes and Stasz (1984) depicted oospores at advanced stages of conversion where the IOW was almost fully digested and there was an insufficient level of detail in the images

provided to discern any patterning or substructure present in the IOW during its dissolution. The possibility that a wall digestion process exists in *P. ultimum* var. *ultimum* that is similar to that seen in *P. megasperma*, involving the secretion of wall-degrading enzymes into the IOW of converting oospores can't be ruled out. Indeed, the localized and almost overlapping deposition of a new germination wall internal to the IOW indicates the activity of wall-digesting enzymes must be confined and controlled. This supports that a method for their delivery across the IOW is likely. Unlike vegetative growth in mycelium which relies on catabolism of soluble mycolaminaran in the cytoplasm as an energy source, oospores undergoing conversion rely entirely on the catabolism of insoluble glucans from the IOW. This suggests that a unique pathway for glucan metabolism during oospore conversion may exist.

Based on the hypotheses proposed by Beakes and Bartnicki-Garcia (1989), it follows that transcriptional profiling of wall-specific glucanase-encoding genes during oosporogenesis and oospore conversion should follow one of two patterns. If enzymes acting on the IOW are pre-packaged, genes encoding these proteins would be overexpressed during oosporogenesis. It is also possible that translationally-repressed mRNA transcripts encoding these proteins may also be synthesized, in which case the transcriptional profile would still be the same. Translation repression of specific mRNAs has been observed in *Drosophila* embryos (Zhang *et al.*, 1997). In oomycetes, repression in *Phytophthora infestans* may be more general, implicating RNA binding-activity, to protect transcripts in oospores and sporangia until germination. In fungi and plants, spores and seeds contain mRNAs that are preserved by desiccation (Cvitanich and Judelson, 2003). While sporangia of *Phytophthora* may not utilize this mechanism of preservation because

they must stay hydrated, the thick walls of oospores of *P. ultimum* allow these propagules to survive extended periods under drought conditions and it is possible they exploit this mechanism of mRNA preservation as well. If, however, IOW dissolution occurs by glucanases synthesized *de novo*, gene expression profiles of these proteins would be expected to occur during conversion and accompany the observable thinning of the oospore wall. A third hypothesis is that there may exist a combination of mRNA transcription and translational repression of wall-specific enzymes and/or enzyme and proenzyme prepackaging during oosporogenesis, AND *de novo* synthesis of these enzymes during oospore conversion. Under this scenario, over-expression of related genes may be observed during both stages of the lifecycle.

Phylogenetic analysis of CAZy-encoding genes and gene families that are over-expressed during oospore conversion and oosporogenesis in *P. ultimum* var. *ultimum* may offer some insight on the mechanism this unique pathogen uses to gain its success evolutionarily, within the constraints of its lifestyle, and developmentally. Significant expansions of gene families involved in host plant interactions, such as those encoding necrosis-inducing Crinkler-class (CRN) proteins and cytoplasmic effectors (RxLR) which facilitate the infection process, have been observed in phytopathogenic *Phytophthora* species which exploit obligate biotrophic or hemibiotrophic lifestyles (Martens *et al.*, 2008; Haas *et al.*, 2009). If glucanases play a major role in the life cycle of *P. ultimum* by supporting an accelerated rate of conversion of oospores when conditions for causing diseases become favorable, there may also be an expansion of gene families that facilitate this process, or duplication of implicated genes to help in the increased production of these

proteins. Such phylogenetic analyses would require that the most current CAZy family HMM models be used to predict protein family domains (Figure 5).

In this study we aim to identify, using RNA sequencing (RNA-seq) expression analysis, possible candidate genes encoding oospore wall-specific CAZymes which are over-expressed explicitly during the early stages of synchronous oospore conversion, or ahead of time during oosporogenesis, or both. Wall-specific CAZymes are taken to be those CAZy-gene products which are specifically targeted for entry into the secretory pathway and subsequent secretion across the plasma membrane to the extracellular matrix. Such oospore conversion-specific genes are presumed to generate enzymatically active products available for immediate use, while oospore wall-specific genes expressed specifically during oosporogenesis presumably generate products that have intended enzymatic activities that are realized only later when they are needed for oospore conversion. We hypothesize that a dual approach strategy of oosporogenesis pre-packaging and oospore conversion *de novo* RNA synthesis underlies the mechanism of oospore wall-specific CAZyme synthesis. This strategy would enable gene-product RNA precursors to be prepared ahead of time, so as to be readily available to kick-start the conversion process when conditions for disease become favourable, at which time over-expression of the same genes may be initiated to maintain or supplement gene product supplies as the oospore conversion process progresses.

2. Methods

2.1. Growth and culturing of *Pythium*

Stock cultures of *Pythium ultimum* variety *ultimum* (DAOM BR144 = CBS 805.95 = ATCC 200006) maintained in 1.5% agar medium with mineral oil overlay at 16°C were transferred as hyphal masses onto 2% agar in sterile distilled water (SDW) plates and incubated at room temperature under ambient light conditions. Single hyphal tip isolations were transferred to 2.5% V8 vegetable juice agar and incubated at room temperature and ambient light conditions for 2-3 days. Agar plugs obtained from advancing colony edges of mycelia mats grown on these plates represent the starting material for oospore production assays and time-course experiments performed in this study.

2.2. Production of oospore stock

Agar plugs obtained from advancing colony edges of 2-3 day-old culture mats grown on V8-agar were transferred aseptically into Erlenmeyer flasks (250 ml, 500 ml, 1 L, 2 L) containing V8-Cholesterol medium (20% V8-vegetable juice, 30 mg/L 1.5% Cholesterol in 95% EtOH) and incubated without shaking for 4-6 weeks in the dark at 20°C. Culture mats were emptied onto filter paper in Buchner funnels and allowed to air dry under ambient flow conditions in a biological safety cabinet kept on for several days until completely dry. The filter paper containing the dried mats were placed in sterile petri dishes, sealed, and kept for long-term storage at 4°C.

2.3. Oospore dormancy-breaking/conversion time-course experiment

Dried mats with oospores on filter paper were wetted with sterile distilled water to loosen them from the filter paper and transferred to a Waring blender. Sterile distilled water

(SDW) was added and the mats were blended on high speed twice for ~10 sec to break up hyphal tissue and loosen the attached mature dormant oospores. This mixture was subsequently filtered twice through one layer of monofilament open mesh fabric with 50- μ m mesh opening (SefarNitex®, Sefar Filtration, NY, USA, 03-50/31) to remove residual agar and large hyphal fragments. The oospore suspension was collected and concentrated by centrifugation at $5,000 \times g$ for 15 min at 4°C. The final oospore suspension concentration was determined by haemocytometry. Five samples of $\sim 2.0 \times 10^6$ oospores were re-suspended in sterile distilled water and immediately collected and set aside as replicates representing time 0 samples. 250-mL Erlenmeyer flasks containing 30 mL of SDW were inoculated with the concentrated oospore suspension to a final concentration of $\sim 130,000$ oospores/mL. All flasks were inoculated within the same hour as the 0 hour time-point samples were collected. Inoculated flasks were placed randomly on rotating shakers at 190 rpm in a controlled growth chamber incubated at 20°C, under a light exposure of 6 W/m² as described by Johnson (1988). Based on the conversion profile of oospores re-suspended in water from a previous preliminary experiment, oospores were collected after 12, 24, 48, 72, 120, and 240 hours (Figure 6) (Giroux, 2014) (Appendix 1 - Supplemental Methods: Time-point selection for oospore conversion and oosporogenesis experiments). Samples were taken in three or more biological replicates. Tissue samples were immediately immersed in 1.5-mL Eppendorf tubes containing L3-lysis buffer with β -mercaptoethanol and flash-frozen in liquid nitrogen and stored at -80°C until further processing.

2.4. Oosporogenesis time-course experiment

Agar plugs obtained from advancing colony edges of 2-3 day-old cultures on V8-agar plates as described above were transferred aseptically into 250-mL Erlenmeyer flasks containing 20 mL of 20% V8 (0.25% CaCO_3) medium and incubated without shaking at ambient light and temperature conditions for 3 days. For time zero, samples were obtained directly from the 3-day old mats. The remaining mats were then supplemented either with 20 mL 20% V8 medium (untreated control) or 20% V8+cholesterol (30 mg/L) for a final cholesterol concentration of 15 mg/L (treated experimental). Tissue samples were taken from untreated and treated mats at 3, 7, and 24 hours post-treatment (Figure 7) (See Appendix 1 - Supplemental Methods: A1.1. Time-point selection for oospore conversion and oosporogenesis experiments). Samples were taken in three or more biological replicates. Tissue samples were immediately immersed in 1.5-mL Eppendorf tubes containing L3-lysis buffer with β -mercaptoethanol and flash-frozen in liquid nitrogen and stored at -80°C until further processing.

Figure 6. Schematic representation of the oospore conversion time-course set-up and sampling methodology.

Figure 7. Schematic representation of the oosporogenesis time-course set-up and sampling methodology.

2.5. RNA extraction

Tissue and oospore samples stored at -80°C from the oosporogenesis and conversion studies were thawed on ice and transferred to Precellys VK01 2-mL lysis tubes with 0.1-mm silica spheres (ESBE Scientific, Markham, ON, Canada, BER-KT0396110052) containing an additional 500 μL of L3-lysis buffer (Ambion™ by LifeTechnologies, ThermoFisher Scientific, 12173022) with β -mercaptoethanol. Tissue was homogenized in a Precellys 24 instrument (Bertin Instruments, ESBE Scientific, Markham, ON, Canada) at a speed of 6,500 rpm for 45 sec, with a 15 sec pause. The samples were placed on dry ice for 2 min and then centrifuged at $12,000 \times g$ for 15 min at 4°C . Following centrifugation, the full volume of the supernatant was split into 100 μL aliquots which were added directly to individual tubes containing 1 mL of TRIzol® reagent. This added step was necessary to reduce the ratio of sample in L3 lysis buffer to TRIzol® reagent below the 1:10 ratio recommended in order to remove the guanidine isothiocyanate salt present in the L3 lysis buffer. Following chloroform-mediated aqueous phase separation and transfer of the aqueous phase to new 1.5-mL Eppendorf tubes, an equal volume of 70% ethanol was added to each tube and then incubated for 10 min at room temperature. Split samples were recombined when the subsamples in ethanol for each sample set were each bound sequentially to a single spin cartridge membrane for total RNA extraction and purification. Total RNA was extracted using the TRIzol®Plus RNA Purification kit (Ambion™ by LifeTechnologies, ThermoFisher Scientific, 12183555) following the steps for lysate preparation for suspension cells followed by binding, washing and elution steps including On-Column PureLink® DNase Treatment using 10- μL RNase-free DNase ($\sim 3 \text{ U}/\mu\text{L}$) (Ambion™ by LifeTechnologies, ThermoFisher Scientific, 12185-010) to remove potential genomic DNA carry-over. The

concentration of RNA was estimated by spectrophotometry using the NanoDrop ND-1000 (ThermoScientific, Wilmington, USA) with optical density ratios ($OD=A_{260}/A_{280}$) and the Qubit® RNA BR and HS Assay kits (Invitrogen™ by LifeTechnologies, ThermoFisher Scientific, Q32853 and Q32854) on a Qubit® 3.0 Fluorometer (Invitrogen™ by LifeTechnologies, ThermoFisher Scientific). Additionally, samples were screened for genomic DNA contamination. For RNA extraction of the oospore conversion time-course samples, modifications to the above described techniques include storing the tubes containing lysate with TRIzol® solution at -80°C before proceeding with on-column RNA purification and an additional post-RNA purification DNase treatment using the DNA-free™ DNA removal kit (Ambion™ by LifeTechnologies, ThermoFisher Scientific, AM1906) according to the manufacturer's protocol for routine DNase treatment.

2.6. cDNA synthesis for qRT-PCR assays

DNase-treated RNA (50 ng) was reverse-transcribed using Superscript™ III Reverse Transcriptase (Invitrogen, 18080-093) in 20-μL reactions using oligo (dT)12-18 primers and treated with 1-μl RNase-Out™ Recombinant Ribonuclease Inhibitor (40U/μl)(Invitrogen™ by LifeTechnologies, ThermoFisher Scientific, 10777019). The reaction was followed by treatment with *E. coli* RNase H (2U/μl) (Ambion™ by Invitrogen, ThermoFisher Scientific, AM2292) and incubated at 37°C for 20 min to remove RNA complementary to the cDNA.

2.7. Identification of gene categories relevant to oospore wall remodeling

In order to focus gene expression analyses in this study to genes encoding enzymes most likely to act upon the IOW, the CAZy-ome of *P. ultimum* var. *ultimum* was reconstructed by

running the CAZy annotation pipeline using the most recently updated version of the dbCAN annotation software and further confining wall-specific candidates to those CAZymes predicted to be secreted using a secretome prediction pipeline. The database for Carbohydrate-Active Enzyme annotation (dbCAN) HMM (hidden Markov Model) version 5.0 (dbCAN-fam-HMMs.txt) was downloaded locally and used to query the genomes for conserved domains using the hmmscan program (hmmscan-parser.sh) in the HMMER package (v3.0, with default parameters) in order to predict and classify the complement of carbohydrate-active enzymes within the genome assemblies of the taxa described in Table 3. The programs SignalP (version 3.0 and 4.1), TargetP (version 1.1), ScanProsite (Prosite release 20.122), and TMHMM (version 2.0) were used with default settings to predict those proteins that were potentially secreted in the genomes of the oomycete taxa described in Table 3 (Sonnhammer *et al.*, 1998; Emanuelsson *et al.*, 2000; Petersen *et al.*, 2011). Proteins were considered secreted if both the neural-network and the hidden-Markov model in both SignalP versions identified signal peptide sequences and were predicted to enter the secretory pathway by TargetP. To prevent false positive assignments, the proteins were scanned for transmembrane domains using TMHMM. If no transmembrane, or only a single transmembrane domain with significant overlap (≥ 10 amino acids; start position of the transmembrane domain with the first 35 amino acids) with the predicted signal peptide, was detected, the protein was retained. If more than a single transmembrane domain was predicted, the protein was discarded. Finally, ScanProsite was used to filter out proteins with predicted ER retention signals (Prosite: PS00014). The final table containing the complete CAZyme complement and secreted proteins predicted for the described genomes is available upon request. A scatter plot was generated for all

secreted CAZY genes using the $\log_{10} + 1$ of the RPKM counts at the time point where it was at the maximum in the cholesterol treatment for oosporogenesis (x-axis) and any of the time points for oospore conversion (y axis)(Figure S3 7). Those genes identified as encoding predicted secreted CAZymes in *P. ultimum* var. *ultimum* were used in subsequent differential expression analysis. Many genes were expressed at a fairly constant rate and the predicted functions of these genes suggested that they were involved in the basic maintenance of cellular function (house-keeping). Given this, the data was analyzed without normalizing for house-keeping genes since it would entail normalizing across a relatively constant number of reads and would not make a difference to the data.

2.8. RNA-seq library preparation and sequencing

For both the oosporogenesis and oospore conversion time-courses RNA-seq libraries were prepared from 50 ng total RNA diluted in RNase-free water. The only exceptions to this total load were two of the four biological replicates from the 12-hour time-point of the oospore conversion time-course where libraries were prepared from 20-32 ng total loads.

For the oosporogenesis time-course, RNA-seq libraries were prepared using the Ovation® Libraries SP+ RNA-Seq DR Multiplex System 1-8 (NuGEN, D-Mark Biosciences, Toronto, ON, Canada, 8161-32) system on the Mondrian SP+ cartridge and Workstation (NuGEN, D-Mark Biosciences, Toronto, ON, Canada).

For the oospore conversion time-course RNA-seq libraries were prepared following protocols for target depletion to remove ribosomal RNA contamination. Depletion primers were designed, as per the NuGen protocol, to target the small, large and 5.8 S ribosomal subunits from the nuclear RNA cistron (scaffold scf1117875581257) and mitochondrial large and small subunit RNA (rns) (from GU138662) of *P. ultimum* var. *ultimum*. These

primers are listed in Table S1. Fragmentation of ds-cDNA was performed using the Covaris M220 system (Covaris, Woburn, MA, USA) with Covaris microTubes (520045) and the M220 holder XTU insert (500489). ds-cDNA samples were diluted up to 130 μ L in 25 mM EDTA and sheared with the following conditions: peak incident power 50 W, duty factor 20%, cycles/burst 200, duration 150 sec, temperature 20°C. Targeted depletion was carried out with custom designed primers on the Sciclone G3 Liquid Handling Workstation (PerkinElmer, Waltham, MA) as controlled by Maestro Workstation Software (PerkinElmer, Caliper LifeSciences, version 6.3). RNA-seq libraries from these depleted targets were prepared using the Ovation Universal RNA-Seq System 1-96 (NuGEN, D-Mark Biosciences, Toronto, ON, Canada, 0402-A01) with the Ovation Universal RNA-Seq on Sciclone NGS protocol for DNase-treated total RNA (NuGEN).

Quantification and quality control of RNA-seq libraries were performed prior to sequencing using Illumina's recommended protocols. Amplicon libraries were quantified using the KAPA SYBR FAST qPCR Library Quantification Kit for Illumina/LightCycler®480 (KAPA Biosystems Inc., Wilmington, MA, USA, KK4854). Library profiles were assessed using the High Sensitivity (HS) DNA LabChip kit (Agilent Technologies, Santa Clara, CA, 5067-4626) on an Agilent 2100 BioAnalyzer with 2100 Expert Software (Agilent Technologies, Santa Clara, CA, G2943CA). The average fragment length of the amplicon libraries was obtained from a smear analysis of the library profiles in order to perform a size-adjustment calculation on the library concentrations obtained from the KAPA library quantification assay prior to equimolar pooling of amplicon libraries. Paired-end sequencing of equimolar pools of individually barcoded libraries was performed using up to 24 samples per sequencing lane on the Illumina Hi-Seq 2000 platform using the PE 100-

bp and v4-PE 125-bp sequencing lanes, for the oosporogenesis and oospore conversion time-courses, respectively (Génome Québec Innovation Centre, Montréal, Canada).

2.9. RNA-seq read processing

Reads were checked for their quality and to provide graphical guidance for filtering, trimming and reformatting using the PRINSEQ-LITE v.0.20.4 and PRINSEQ-graphs v.0.6 software packages (Schmieder and Edwards, 2011). Raw reads were stripped of adapter sequences using SeqPrep v.1.2 with default settings (J. St. John, unpublished, <https://github.com/jstjohn/SeqPrep>) and reads that did not pair were discarded. A stringent approach was taken for read processing. Reads were trimmed using a sliding window approach (window size 3 bases, shift 1 base). From the 3' end, read bases were kept until the average quality score Q of 3 bases was below Q = 32. To trim poly-A and poly-T tails, sequences were scanned and trimmed twice from their ends forward to remove consecutive stretches of 5 or more As or Ts. Reads were additionally trimmed 15 bases and 10 bases from the 5' and 3' ends, respectively. Processed reads were filtered to remove low complexity reads (DUST approach, maximum score 7) or reads that were too short (less than 60 bases). Read pairs reduced to singleton reads during processing were discarded to keep pairs only. Paired-end read sequence order was validated throughout read preprocessing and processing steps using the Fastq Paired-End Validator tool available at http://www.mcdonaldlab.biology.gatech.edu/seqtools_frame.htm.

2.10. Sequence analysis

The *P. ultimum* var. *ultimum* genome contig reference FASTA and annotation (GFF3) files (<http://pythium.plantbiology.msu.edu>) were coordinately modified to simplify an inverted

repeat of the mitochondrial ribosomal cistron and repeats of the nuclear ribosomal cistron resulting in single complete sequences (See Appendix 1 - Supplemental Methods: Reference genome modification to improve alignment mapping during RNA-seq analysis).

2.11. Identification of over-expressed wall-specific CAZy candidates

The log-transformed RPKM values of normalized read counts were used to generate expression plots for each predicted secreted CAZy-coding gene over time for both time-course assays. To make expression patterns over time more easily identifiable across each time-course assay for oospore conversion, discrete time-points were converted to continuous time variables to generate expression trend curves that could be added to the plots. A bell shape curve with the Gaussian equation

$$f(x) = \exp(ae^{-\frac{(x-b)^2}{2c^2}})$$

was fitted to the non-transformed data with non-linear regression (nl) in R (ref), where x is the square root of hours after conversion was initiated, a is the maximum expression counts, b is time where the peak expression is reached, and c is the standard deviation. The untransformed RPKM counts were also analyzed by GLM using the quasipoisson model in R. Additionally, the normalized RPKM counts from a previous transcriptome sequencing study by Lévesque *et al.* (2010) on *P. ultimum* var. *ultimum* with mycelium under various abiotic stressors (i.e., hypoxia, fungicide, cold and heat stress) and in the presence of a host were pooled and the log-transformed RPKM counts were plotted as a single point alongside oosporogenesis and oospore conversion plots to provide additional context for gene expression levels as they relate to general, non-oospore conversion specific processes in mass hyphae. For oosporogenesis studies, gene expression plots were individually

inspected to identify genes that appeared to be overexpressed under oosporogenesis-inducing conditions compared to control conditions. For oospore conversion studies, oospore wall-specific gene candidates were identified by expression profiles that appeared to show a pattern of heightened expression during the early points followed by decreasing expression levels as the time course progressed. For a gene to be considered as being overexpressed at a particular time-point(s) in either study, all replicates at the time-point(s) where elevated expression was suspected had to have non-zero normalized read counts. Expression levels of those genes that appeared to show elevated expression patterns suggestive of oosporogenesis- and/or oospore conversion-specific activity were compared to expression levels of the same genes under various conditions in mass hyphae to further support their elevated expression as stage-specific. Genes identified as coding for proteins having potential oospore wall-specific enzymatic activity were grouped in one of three ways: pre-packaged elevated during oosporogenesis only, de novo elevated expression during oospore conversion only, or a combination of prepackaging and de novo synthesis with elevated oospore conversion and oosporogenesis-inducing activities.

2.12. Phylogenetic analysis of the CAZy families of over-expressed candidates

The CAZy families of *P. ultimum* var. *ultimum* genes that appeared to be most likely over-expressed and oospore wall-specific were identified. The protein sequences of these genes were trimmed to retain the domain portions only based on the positions predicted by the dbCAN annotation pipeline. Similarly, the domain portions of the CAZymes from the selected CAZy families were retrieved for all taxa listed in Table 3. Despite using domain only sequences, the lengths of some CAZyme domains for a few CAZy families were still too variable to generate reliable alignments (GH30, CE13, GH13 and CBM 63), making

phylogenetic analysis of these families impractical. Additionally, initial efforts were directed towards targeting genes that had introns so that carefully-designed qRT-PCR primers and probes could help circumvent challenges related to possible DNA contamination in cDNA samples. Therefore, genes that did not contain exons were deemed less of a priority for phylogenetic analysis. The final CAZy families chosen for phylogenetic analysis were **G**lycoside **H**ydrolase (GH) families 3, 7, 15, 17, 72 and 131, one **C**arbohydrate **B**inding **M**odule (CBM) family 21, and a **S**urface **L**ayer **H**omology (SLH) motif-bearing domain. Multiple alignments of the chosen CAZy families were generated using MAFFT v7.164b using the LINSI algorithm (Katoh and Standley, 2013). Bayesian inferences (BI) were generated using MrBayes v. 3.2.1 x64 (Ronquist *et al.*, 2012) with Metropolis-Couple Markov chain Monte Carlo (MCMCMC) methodology to calculate posterior probabilities of the phylogenetic trees. The program was run for 100 M generations and the first 25% of iterations were discarded as burn-in while every 1000th iteration was sampled from the remainder. The trees were considered to be fully converged when the average standard deviation of split frequencies reached a level less than 0.01. FigTree v.1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to view and edit Bayesian phylogenetic trees. Consensus trees were generated using the 50% majority rule tree criteria.

3. Results

3.1. Identification of secreted CAZyme coding genes

Annotation of putative CAZy-encoding genes in *P. ultimum* var. *ultimum* using the dbCAN annotation pipeline resulted in the identification of 411 putative CAZy-encoding genes spanning a total of 99 unique CAZy families. Some of these genes had matches to more than one non-overlapping CAZy domain. Of these, 126 unique genes belonging to 41 unique CAZy families were predicted to be secreted based on the secretome pipeline used in this study (Table S2).

3.2. Sequencing results

For the oosporogenesis time-course assay, an average of 42.8 (± 6.8) million reads and 8.6 (± 1.4) billion bases per experimental group time-point were collected. Processing of the raw reads *viz.* trimming of adapter sequences, low quality bases and poly-A/T tails, and removal of reads too short or of low complexity yielded an average of 41.0 (± 6.5) million reads and 7.5 (± 1.2) billion bases per experimental group time-point. Averages of 100% of reads were mapped, with a significant percent mapping to ribosomal sequences (92.0 ± 0.5) (Table 1).

For the oospore conversion time-course assay, an average of 44.6 (± 22.0) million reads and 11.1 (± 5.5) billion bases per time-point were collected. Sequencing of libraries from the 24 hour time-point resulted in a much lower sequencing yield compared to the other time-points. Processing of the raw reads *viz.* trimming of adapter sequences, low quality bases and poly-A/T tails, and removal of reads too short or of low complexity yielded an average of 40.2 (± 20.0) million reads and 7.6 (± 3.6) billion bases. An average of

59.2 (± 13.6)% of reads were mapped, of which an average of 7.5 (± 5.5)% mapped to ribosomal sequences. The percent of reads mapped was lowest and most varied for earlier time-points and increased in later time-points (Table 2). There was significant contamination of sequencing data which was most pronounced during the earliest time-points of this assay when sample material was most limiting. The top hits of a blast search of the unmapped reads were to *Paenibacillus* species. The extent of read contamination was determined by mapping unmapped reads from all samples to the *Paenibacillus* contigs generated by the assembly of contaminating reads from one of the sequencing library replicates as described in Appendix 1 - Supplemental Methods: Analysis of read contamination. Assembly of contaminating reads generated 250 contigs that matched *Paenibacillus polymixa* at $\geq 90\%$ sequence similarity. A good number of the contigs were larger than 5000 bp and spanned several genes and intergenic regions.

Contrary to library samples for the oospore conversion assay, the library preparation method used for oosporogenesis samples did not include a ribosomal depletion treatment. There was a greater representation of reads mapping to genes in sequencing libraries that were treated for ribosomal depletion, irrespective of sequence contamination. Overall, observed gene expression for most biological replicates clustered according to time-points.

3.3. Differential gene expression analysis

The *P. ultimum* var. *ultimum* reference genome comprises 15,285 nuclear and 24 mitochondrial non ribosomal gene models. After combining the transcriptional profiles of all biological replicates across all time-points for both oosporogenesis and oospore conversion assays, we obtained transcriptional evidence for 14,800 expressed genes. For

oosporogenesis and oospore conversion assays, we detected 13,644 and 14,731 expressed genes, respectively.

The entire transcriptome was analyzed for genes with RPKM-normalized counts that appeared stable across all time-points for both time-course assays and that also showed stable expression levels from a previous transcriptome sequencing study by Lévesque *et al.* (2010) on *P. ultimum* var. *ultimum* with mycelium under various abiotic stressors and in the presence of a host. Because genes with stable expression levels were found throughout the *P. ultimum* var. *ultimum* genome, including candidates that were identified as coding for secreted CAZymes (GT20 PYU1_T000005 and GH81 candidate secreted PYU1_T006546), it wasn't necessary to convert CAZyme-coding gene expression levels to ratios of stably-expressed reference genes (Table S4). Additionally, this analysis provided a way to evaluate the adequacy of previously chosen reference gene candidates used to validate candidate CAZyme-coding gene expression by qRT-PCR (See Appendix 2 – Supplemental Tables: Table S3) and provides a resource for future selection of reference gene candidates.

During the counting of mapped reads, a significant number of reads were considered to have non-unique alignments due to mapping to more than one gene in the reference *P. ultimum* var. *ultimum* assembly, and thus did not contribute to overall gene counts. However, the only genes in the CAZyme-coding set that appeared affected belonged to the GH5 CAZy family, and only one candidate gene in this set was predicted to be secreted (PYU1_T006483, PYU1_T006484, PYU1_T006485 (predicted secreted), PYU1_T011452, PYU1_T011453). Because this CAZy family is very large, it was deemed

unwieldy for the purposes of our study and so was not pursued for further phylogenetic analysis.

Of the 411 predicted to code for CAZymes, we obtained transcriptional evidence of 394 expressed genes for the combined transcriptional profiles of both time-course assays. Individually, we detected 378 and 390 expressed candidate CAZyme-coding genes in oosporogenesis and oospore conversion assays, respectively. While there does not appear to be a difference between the combined overall expression levels of CAZyme-coding genes predicted as secreted and not secreted, there is a difference in the CAZy family domains represented in predicted secreted and not secreted gene sets. Subset analysis of CAZy families identified in the genome of *P. ultimum* var. *ultimum* that have genes encoding putative CAZyme products that are predicted as secreted, not secreted, or both within each of the major CAZyme classes is shown in Figure 6. The following CAZy domains were found only in genes of *P. ultimum* var. *ultimum* that were predicted to be secreted: CE13, CBM21, CBM25, GH2, GH7, GH13, GH28, GH38, GH43, GH53, GH89, GH131, GH43, PL4 and AA9. Note that genes of *P. ultimum* var. *ultimum* in the following CAZy families CE13, CBM21, CBM25, GH7, GH13, GH131, and SLH all passed the secretome pipeline, but that there are candidates not predicted as secreted in the other oomycete reference species included in these dbCAN annotation for these families. For example, for the SLH domain in the dbCAN annotation, there are genes in this family from other *Pythium* species, but both *P. ultimum* var. *ultimum* genes in this family are secreted. Similarly, while most genes with predicted GH131 domains are secreted, there are some genes from the genera *Phytophthora* and *Pythium* that were not predicted to be secreted using our secretome prediction pipeline. In such cases where there are large gene families with the vast majority of genes predicted to

be secreted and only a few not passing the secretion pipeline, it may be that those not passing may still be secreted through non-classical secretion pathways.

Bar plots of predicted secreted CAZyme-coding genes that displayed patterns of elevated expression consistent with assumptions for oospore wall-specific activity are presented in Figure 7 and Figure 8 and their domain structures and descriptions are outlined in Figure 10. With the exception of one candidate with a pattern of elevated expression specific to oosporogenesis (Figure 8), all potentially over-expressed oospore wall-specific candidates displayed patterns of elevated expression during oospore conversion only, or during oospore conversion and under oosporogenesis-inducing treatments. The relationship between the heightened expression patterns of the oospore wall-specific CAZyme-coding gene candidates identified by RNA-seq and the proposed mechanisms of temporal transcriptional activity that underlie the synthesis of CAZymes with oospore wall-specific activity is illustrated in Figure 9.

3.4. Identification and phylogenetic analysis of CAZy family domains in oomycetes and oomycete outgroups

Phylogenetic analysis of CAZy families pertaining to candidate over-expressed oospore wall-specific CAZymes was conducted for those CAZy families for which phylogenetic analysis was manageable.

Genes with CBM21 domains were detected only in genomes of *Pythium* species (all predicted to be secreted) and fungi and together they make up a very small family from the species profiled in this study (Figure 11). *P. ultimum* var. *ultimum* is the only *Pythium* species in this set with an apparent duplication of this gene. Genes with CAZy CBM25 family domains were detected only within oomycete genomes and were uni-modular

without associated catalytic domains, or associated with GH13, GH31, and/or GH3 catalytic domains. CBM63 domains were present as uni-modular genes without associated catalytic domains with the exception of one gene from *P. sojae* where it was associated with a GH45 catalytic domain.

Genes with GH3 domains were detected as uni-modular sequences or associated with GH31 and/or CBM25 domains. No genes with GH3 domains were detected in *S. cerevisiae* genomes. All genes in the genomes of *A. thaliana* and *D. discoideum* with this domain were uni-modular with only one GH3 domain detected per gene. Overall there appeared to be a large number of GH3 gene expansions within *Phytophthora* species, for both predicted secreted and not secreted genes (Figure S3-3). There appeared to be a clustering of *Phytophthora* only and *Pythium* only clades. The candidate over-expressed PYU1_T012530 gene is located in a clade with approximately one gene per species in *Pythium* and *Phytophthora*. There are some *A. thaliana* genes and one *E. huxleyi* gene with this domain. The other over-expressed candidate is located in a much larger clade, dominated by *Phytophthora* expansions.

Genes with GH7 domains were detected only in genomes of oomycetes, *E. huxleyi* and *D. discoideum*. The majority of genes detected were also predicted to be secreted. Genes within this family appear to be expanded in the genomes of the *Phytophthora* species profiled compared to genomes of *Pythium* species and only one gene with this domain was detected in *P. ultimum* var. *ultimum* (Figure 12).

Genes with GH15 family domains were either uni-modular or were associated with an additional CBM21 module as observed for one gene from each of the *P. ultimum* var. *ultimum* and *P. iwayame* genomes. Genes with this domain were detected in the *Pythium*,

but not *Phytophthora* species profiled (Figure 13). Additionally, genes with this domain were absent in the genomes of *A. thaliana* and *P. pseudonana*, but present in the *D. discoideum* and *S. cerevisiae*.

Genes with GH17 domains were either uni-modular or associated with one or more CBM13 modules. In *E. huxleyi* and *S. cerevisiae*, all genes with this domain were uni-modular while in *A. thaliana*, the vast majority of genes with GH17 modules were also associated with CBM43 modules. No genes with GH17 modules were detected in the *D. discoideum* genome. This gene family appears to be expanded in *Phytophthora* compared to *Pythium* species (Figure S3-4).

The GH72 family is divided into two classes, those with an additional CBM43 domain, and those without. All of the oomycetes and diatom genes with GH72 domains did not have a detected additional CBM domain, however, two of the five *S. cerevisiae* genes with detected GH72 domains had additional CBM43 domains. The tree shows two major clades - a small distant clade that has genes from *T. pseudonana* and oomycete genomes with only a single representative gene present for each of the oomycete species profiled, none of which are predicted to be secreted, and a large clade that splits into two branches with representatives from *Phytophthora* and *Pythium* species in each (Figure S3-5). In this larger clade, *Pythium* and *Phytophthora* clusters are evident with the majority of genes predicted to be secreted. Both over-expressed candidates *P. ultimum* var. *ultimum* appear in this larger set.

Genes with GH131 family domains were only detected in oomycetes, the majority of which belonged to genes from *Phytophthora* species. The vast majority of these genes were also predicted to be secreted by our secretome pipeline. No genes with this domain were

detected in the genome of *S. cerevisiae*. The only gene duplication observed in this family occurred in the genome of *P. ultimum* var. *ultimum*, with the duplication appearing absent in its sister variety *P. ultimum* var. *sporangiiferum* (Figure 14).

Genes with SLH motifs were only detected in oomycetes, specifically, *H. arabidopsidis* and pythiums (*P. aphanidermatum*, *P. iwayame*, *P. ultimum* var. *sporangiiferum*, *P. ultimum* var. *ultimum*). Genes with this module were not associated with other modules. There appears to be duplications of this gene in the genomes of each of the species they were detected (Figure 15).

Table 1. Summary of raw and processed sequence data obtained by Illumina Hi-Seq paired-end sequencing of oosporogenesis time-course libraries not treated to remove ribosomal contamination.

Time (hours) per Experimental Group	Oosporogenesis time-course									
	Total Raw Reads	Total Raw Bases	Total Reads (processed)	Total Bases (processed)	%GC	Reads Mapping to <i>P. ultimum</i> Nuclear or Mitochondrial Ribosomal Genes	% Ribosomal Reads Over All Mapped Reads	Reads Mapping to <i>P. ultimum</i> Nuclear Genes	% <i>P. ultimum</i> Coding Genes Reads over All Mapped Reads	% <i>Poenibacillus</i> Contigs Reads Over All Mapped Reads
0 (pre-treatment)	4.45E+07	8.90E+09	4.24E+07	7.65E+09	41.9	3.89E+07	91.8%	4.31E+05	1.0%	0%
Experimental										
3	3.26E+07	6.53E+09	3.12E+07	5.67E+09	41.9	2.87E+07	91.9%	1.66E+05	0.5%	0%
7	3.64E+07	7.27E+09	3.49E+07	6.36E+09	41.2	3.20E+07	91.7%	2.89E+05	0.8%	0%
24	4.27E+07	8.53E+09	4.07E+07	7.41E+09	42.1	3.79E+07	93.1%	2.11E+05	0.5%	0%
Control										
3	4.23E+07	8.46E+09	4.05E+07	7.36E+09	41.9	3.70E+07	91.5%	4.12E+05	1.0%	0%
7	4.87E+07	9.73E+09	4.66E+07	8.49E+09	41.6	4.28E+07	91.9%	4.96E+05	1.1%	0%
24	5.24E+07	1.05E+10	5.05E+07	9.23E+09	41.7	4.65E+07	92.2%	3.99E+05	0.8%	0%

Table 2. Summary of raw and processed sequence data obtained by Illumina Hi-Seq paired-end sequencing of oospore conversion time-course libraries treated to remove ribosomal contamination.

Time (hours)	Oospore Conversion									
	Total Raw Reads	Total Raw Bases	Total Reads (processed)	Total Bases (processed)	%GC	Reads Mapping to <i>P. ultimum</i> Nuclear or Mitochondrial Ribosomal Genes	% Ribosomal Reads Over All Mapped Reads	Reads Mapping to <i>P. ultimum</i> Nuclear Coding Genes	% <i>P. ultimum</i> Coding Genes Reads over All Mapped Reads	% <i>Poenibacillus</i> Contigs Reads Over All Mapped Reads
0	7.73E+07	1.93E+10	6.96E+07	1.30E+10	51.3	1.12E+06	4.3%	6.51E+04	0.3%	95%
12	7.37E+07	1.84E+10	6.57E+07	1.23E+10	51.2	4.10E+05	1.3%	1.52E+05	0.5%	97%
24	2.04E+07	5.09E+09	1.88E+07	3.55E+09	51.9	1.46E+05	1.2%	1.58E+05	1.3%	97%
48	4.06E+07	1.01E+10	3.50E+07	6.56E+09	50.7	2.74E+06	10.3%	3.82E+06	14.3%	63%
72	3.77E+07	9.44E+09	3.48E+07	6.64E+09	51.5	2.01E+06	7.9%	3.10E+06	12.2%	69%
120	3.17E+07	7.92E+09	2.97E+07	5.69E+09	50.9	2.36E+06	12.8%	1.84E+06	10.0%	70%
240	3.10E+07	7.74E+09	2.76E+07	5.17E+09	50.2	2.22E+06	14.9%	1.65E+06	11.1%	66%

Figure 8. Venn diagrams of CAZy family domains in genes of *Pythium ultimum* var. *ultimum* predicted to be secreted, not secreted or having representatives in both. Families in red type have oospore wall-specific over-expressed gene candidates. Note that the CAZy family domain SLH which is present in two genes is not included.

Figure 9. Gene expression of oospore wall-specific CAZy candidates. Overall expression trends are shown for oospore conversion plots. For oosporogenesis plots, black=negative control, blue=cholesterol to induce oosporogenesis. For mycelium, asterisk denotes significant at 0.01 level (i.e. 0.00008 after Bonferroni correction for the 127 secreted genes tested) for overall mean different than zero, otherwise NS for not significant. Cholesterol treatments were not significantly different than controls for those selected genes.

Figure 9. (ctn'd)

Figure 9. (ctn'd)

Figure 9. (ctn'd)

Figure 10. Gene expression of the oospore wall-specific CAZy family GH31 candidate PYU1_T006194 displaying a pattern of elevated expression specific to oosporogenesis. For oosporogenesis plots, black=control, blue=experimental. For oosporogenesis, T₂₄, the p-value of the difference between control and cholesterol was 3.21×10^{-03} .

Figure 11. Correlation plot showing the expected relationship of oospore wall-specific CAZyme-coding genes under different synthesis scenarios. *Various refers to expression under various conditions mass hyphae of *P. ultimum* var. *ultimum* were grown under in the study by Lévesque *et al.* (2010)(i.e., hypoxia, hot and cold stress, fungicide, and growth in the presence of a host).

Figure 12. *Pythium ultimum* var. *ultimum* CAZyme-coding genes with apparent oospore wall-specific over-expression.

Figure 10. (ctn'd)

Table 3. Species used for CAZy repertoire comparisons.

Species	Taxonomy ID	Strain/Race	Coding Genes: Gene Transcripts	Genome Assembly	Spp.Abr.†	Supergroup	Database	
<i>Pythium ultimum</i> var. <i>ultimum</i>	431595	DAOM BR144	15,290 : 15,462	GCA_000143045.1	pyuu	Stramenopiles	Oomycetes	1*, 2a
<i>P. ultimum</i> var. <i>sporangiferum</i>	1223559	DAOM BR650	14,098	GCA_000387525.2	pyus		Oomycetes	1*
<i>P. aphanidermatum</i>	1223555	DAOM BR444	12,305 : 12,370	GCA_000387445.2	pyap		Oomycetes	1*, 2a
<i>P. arrhenomanes</i>	1223556	ATCC 12531	13,805 : 13,857	GCA_000387505.2	pyar		Oomycetes	1*, 2a
<i>P. irregulare</i>	1223557	DAOM BR486	13,804 : 13,870	GCA_000387425.2	pyir		Oomycetes	1*, 2a
<i>P. iwayamai</i>	1223558	DAOM BR242034	15,254 : 15,352	GCA_000387465.2	pyiw		Oomycetes	1*, 2a
<i>P. vexans</i>	1223560	DAOM BR484	11,957 : 11,992	GCA_000387545.2	pyve		Oomycetes	1*, 2a
<i>Phytophthora infestans</i>	403677	T30-4	17,785 : 25,638	ASM14294v1	Phin		Oomycetes	2a
<i>P. nicotianae</i>	4790	race 0	17,348 : 17,348	ASM148301v1	Phni		Oomycetes	2a
<i>P. parasitica</i>	1317065	P1569	23,159 : 28,372	GCA_000365505.1	Phpa		Oomycetes	2a
<i>P. sojae</i>	67593	P6497	26,489 : 28,142	GCA_000149755.2	Phso	Oomycetes	2a	
<i>P. ramorum</i>	164328	Pr102	15,605 : 15,955	ASM14973v1	Phra	Oomycetes	2a	
<i>H. arabidopsidis</i>	559515	Emoy2	14,321 : 14,609	GCA_000173235.2	Ha	Oomycetes	2a	
<i>Thalassiosira pseudonana</i>	296543	CCMP1335	11,672 : 11,788	GCA_000149405.2	Thaps	Diatoms	2a	
<i>Phaeodactylum tricornutum</i>	556484	CCAP 1055/1	12,178 : 12,392	GCA_000150955.2	Phatr	Brown algae	2a	
<i>Emiliania huxleyi</i>	280463	CCMP1516	38,544 : 38,800	GCA_000372725.1	Ehux	Haptophytes	2a	
<i>Arabidopsis thaliana</i>	3702	Columbia	27,655 : 54,013	GCA_000001735.1	Ath	Archaeplastida – land plants	2b	
<i>Dictyostelium discoideum</i>	352472	AX4	13,243 : 13,753	GCA_000004695.1	Ddi	Amoebozoa – dictyostelids	2a	
<i>Saccharomyces cerevisiae</i>	559292	S288C	6,002 : 6,445	GCA_000146045.2	Sce	Opisthokonts – fungi	3	

1=Pythium Genome Database, www.pythium.plantbiology.msu.edu;2=Ensembl, a:www.protists.ensembl.org, b:www.plants.ensembl.org;3=Saccharomyces Genome Database, www.yeastgenome.org, genome release R64-2-2;

†=Species Name Abbreviations; *= Web server database no longer publically available.

Figure 13. CBM21 CAZy family domain gene tree.

Figure 14. GH7 CAZy family domain gene tree.

Figure 15. GH15 CAZy family domain gene tree.

Figure 16. GH131 CAZy family domain gene tree.

Figure 17. SLH CAZy family domain gene tree.

4. Discussion

4.1. Gene expression and experimental design.

In a previous study by Lévesque *et al.* (2010) transcriptional analysis of mats of *P. ultimum* var. *ultimum* subjected to a wide range of conditions not related to the sexual stage of the life cycle revealed evidence of gene expression for 10,164 genes out of the total 15,325 coding genes predicted. While there may have been a very small proportion of oospores produced by hyphae under these various conditions, as well as a small proportion of oospores undergoing conversion, oospore conversion-specific expression responses would not be detectable above background expression due to overwhelming signals from transcription in the mass hyphae. In our study, gene expression evidence was detected for 13,644 and 14,731 genes for oosporogenesis and oospore conversion experiments, respectively, and the conditions profiled represent a much more narrow set of conditions which may not trigger gene expression related to the extreme condition ranges profiled in the study by Lévesque *et al.* (2010). There is little concern that ribosomal DNA contamination or other possible contaminating reads overwhelmed reads and limited capture of the *P. ultimum* transcriptome as the tremendous output of reads generated from sequencing libraries on the Illumina Hi-Seq platform more than compensated for the low proportion of reads mapping to coding genes obtained for some of the samples.

Furthermore, genes with stable levels of expression were identified that were common to all time-points for both time-course experiments as well as all treatments profiled in the previous study by Lévesque *et al.* (Levesque *et al.*, 2010). This set of genes consisted of genes with low but stable levels of expression, as well as genes with stable moderate to high levels of expression, and the consistency of the expression profiles support that the

sequencing depth and reads obtained in this study adequately provide a full snap-shot of the transcriptome under the conditions tested. Moreover, while expression of genes were compared between oosporogenesis and oospore conversion time-courses, as well as to expression in mass hyphae under various conditions, at no point did we conduct formal statistical comparisons of expression levels between the time-course experiments, even though they were normalized together.

Hyphae of *P. ultimum* var. *ultimum* can be induced to produce oospores rapidly and abundantly when cholesterol, a hormone precursor for oomycetes, is added to the growth medium. *P. ultimum* is a prolific oospore producer. In this study, hyphae were grown in V8-vegetable juice, and this tomato-based medium is not devoid of plant sterols (Ayers and Lumsden, 1975; Beecher, 1998). As nutrients become depleted in the growth media, hyphae will begin to produce oospores even when the media is not supplemented with cholesterol. For this reason, the oosporogenesis time-course experiment was started when mats were only three days old and growing in an abundant volume of nutrient-rich media. The time-course began when additional nutrient media was added to both control and experimental sets so that nutrient depletion would not be a factor leading to oosporogenesis in control sets. Sterols in the soil may also be a trigger to initiate pathogenesis responses as it may signal the presence of a plant host (Haskins *et al.*, 1964; Nelson, 1991; Uren, 2007). Other responses such as germ tube elongation in the direction of the source sterols, or hyphal growth in this direction may occur in addition to oosporogenesis induction.

We have identified candidates that are either expressed only in the early stage of conversion in converting oospores, or both in converting oospores and mats under

oosporogenesis-inducing conditions. While there may be important CAZymes overexpressed during oosporogenesis without being over-expressed in converting oospores, it's not possible to resolve these from CAZymes involved in other processes during oosporogenesis, such as oogonial wall and septum formation, or the release of CAZymes as pathogenicity factors due to association of exogenous sterols (used to stimulate oosporogenesis) with susceptible host proximity. Nonetheless significant insight was gained in CAZyme gene expression during oosporogenesis and oospore conversion.

4.2. Cell wall-degrading enzymes (CWDE) are a broad class of enzymes that play a critical role in the breakdown of complex polysaccharides. Enzymes of this class are broken down into categories based largely on the glycosidic bonds they act upon (Cantarel *et al.*, 2009). Glycoside hydrolases (GH), carbohydrate esterases (CE) and polysaccharide lyases (PL) are categories of CWDE that include major plant polysaccharide degradation (PDD) families, however, it seems more likely that enzymes within these classes function to control oomycete wall remodelling and that the functional overlap as PDD reflects similar wall polysaccharide components between oomycete and plant cell wall polysaccharide composition (Zerillo *et al.*, 2013). For example, cellulose, which consists of 1,4- β -glucans is a primary component of plant cell walls and one of the most abundant components of oomycete cell walls. We identified three oospore wall-specific candidates with CAZy domains known to be involved in cellulose metabolism, two of them with GH3 domains and the other with a GH7 domain (Figure 10). While expression of cellulase-related genes is supported by RNA-seq analysis in this study, as well as previous studies by Lévesque *et al.* (2010) and Adhikari *et al.* (2013), studies of *P. ultimum* var. *ultimum* incubated on minimal media plates containing cellulose as the sole carbon growth show only limited growth,

indicating that while there may be sufficient production of cellulases for self-wall remodelling, there is insufficient production to support the complete digestion of plant cellulose for use as a carbon source (Zerillo *et al.*, 2013). The CAZy database provides a regularly updated portal into the evolving world of CAZymes and currently annotated protein CAZy family members from various species. However, the collection of information on CAZymes from organisms of interest as well as annotation and computational analysis are complicated by the inability to use the database to download sequences of interest or for users to query their own protein sequences for CAZy domains or for similar protein sequences. An excellent study on the CAZy complement of key oomycete plant pathogens and deployment of these enzymes based on pathogen lifestyle, including *P. ultimum* var. *ultimum* was conducted by Zerillo *et al.* (2013). However, since the pipeline was run by Zerillo *et al.* in 2013, there have been several reclassifications made to these enzyme classes. For example, a new class of enzymes called “Auxiliary Activities” (AA), has been described, which cover redox enzymes that act in conjugation with CAZymes. This reflects a reclassification of enzymes in the carbohydrate-binding module (CBM) 33 and GH61 families, which have been found to be actually lytic polysaccharide mono-oxygenases (LPMO), together with the families of lignin degradation enzymes as they often act in concert together. One of the prediction tools used by Zerillo *et al.* (2013) for CAZymes annotation was the dbCAN program (release 2.0). Since the paper was published, three new releases of this program have been made, reflecting two removed and 42 new CAZyme HMM models (Figure 5). However, due to limitations of the CAZy database, we couldn’t use the database to efficiently retrieve all the newest annotated CAZymes for the species we wanted to profile for phylogenetic analysis, nor could we use it to annotate our own protein

sequences. In order to obtain the CAZy annotations based on the most up-to-date classifications, we had to re-run the pipeline using the open-source program offered by dbCAN. We took this opportunity to apply our CAZyme prediction pipeline to a broader range of outgroup taxa for phylogenetic analyses of CAZy families and to impose additional stringencies on protein secretion predictions. While our secretory pipeline was similar to that used by Zerillo *et al.* (2013) and aimed to identify candidate secreted proteins that migrated through the classical secretory pathway, it included additional steps to eliminate proteins that were predicted to target other organelles, such as mitochondria, or contained ER retention signals. Performing our CAZy and secretome pipeline on our protein sequences resulted in sets of proteins that were similar, but not identical to the sets predicted for *P. ultimum* var. *ultimum* by Zerillo *et al.* (2013). Some CAZymes predicted to be secreted by Zerillo *et al.* were eliminated in our secretome pipeline, and several new CAZy families were detected. It's important to note that not all secreted proteins migrate through the classical secretory pathway and oospore wall-specific CAZyme candidates may have skipped detection from our secretory pipeline. Additionally, wall-related proteins may have other functions and be localized to different areas, despite our current predictions. Unlike true Fungi that have cell walls built upon chitin scaffolds, cell walls of oomycetes are built upon microfibrillar scaffolds of type IV cellulose, which is a disorganized form of cellulose (Bulone *et al.*, 1992; Helbert *et al.*, 1997; Fugelstad, 2011). The composition of oomycete cell walls varies across taxonomic groups, with β -glucans and cellulose representing the most abundant components. Previous studies by Mérida *et al.* (2013) revealed the existence of three major wall types clustered across oomycete species in a manner consistent with the overall phylogenetic lineage. Assuming

this overlap of general wall types with overall phylogenetic lineage, oomycetes within the Peronosporalean galaxy, including the genus *Pythium*, would be expected to share Type 1 wall compositions characterized by the absence of chitin and composed primarily of β -glucans (>85%), of which approximately one third was presumed to be cellulose. Lesser amounts of mannan, uronics and other carbohydrates complete the Type I wall composition profile. Studies of the alkali soluble and insoluble fractions of Type 1 wall polysaccharides in Peronosporalean species revealed higher position 6 branching on 1,3-glucans, with a lower degree of polymerization, compared to the other wall types (Mélida *et al.*, 2013). Of the 17 genes identified in this study as encoding possible oospore wall-specific degrading enzymes, the majority belonged to CAZymes involved in cellulose (GH7, 3), starch (GH13, 15, 31, CBM20, 21, 25), 1,3- and 1,6- β -glucan (GH16, 17, 30, 72, 131), pectin (CE13), xyloglucan (GH31) and xylan (GH3) metabolism (Zerillo *et al.*, 2013). No overexpressed oospore wall-specific candidates encoding CAZymes involved in cutin, chitan and chitosan, and galacto/gluco-mannan metabolism were detected. These results are consistent with the overall abundance profile of oomycete Type 1 walls and the view that deployment of secreted CAZymes in *P. ultimum* var. *ultimum*, which exploits an opportunistic necrotrophic lifestyle, target self-walls rather than plant host cell walls. With the exception of one gene candidate that displayed oosporogenesis-specific expression patterns, all candidates displayed patterns that included expression during early stages of oospore conversion which coincides with thinning of the inner oospore wall. The types of polysaccharides typically metabolized by the CAZyme families of over-expressed candidates are consistent with the hypothesis that the mobilization of glucans within the inner oospore wall by glycoside hydrolases 1,3- β -glucanases and 1,3;1,4- β -

glucanases provides the energy and carbon source required to propel oospores out of dormancy towards a germinable, pathogenic state.

4.3. Phylogenetic analysis was conducted for some of the CAZyme-encoding gene families wherein potential oospore wall-specific genes were detected. A domain-only approach was taken as the high variability in the lengths of genes within many of the CAZy families prevented proper sequence alignment. Despite this approach, domain sequences of some families still could not be adequately aligned or were too large for phylogenetic analysis to be practical. Of those CAZy families profiled, a few displayed interesting patterns such as clustering by species, representation in particular groups of species and prevalence of sequences predicted as secreted. CAZy families that contained genes from *Phytophthora* and *Pythium* species generally showed expansions of genes in *Phytophthora* not apparent for *Pythium* species (i.e., GH7, 131). However, there were some CAZy families that were detected in *Pythium* oomycetes only (i.e., CBM21, GH15). Below we describe the structure of phylogenetic trees and CAZyme characteristics for those families that had the most interesting and distinct patterns, namely, CAZy families CBM21, GH7, 15 and 131, and SLH.

4.3.1. Carbohydrate-binding module family 21 are collectively known for their role in conferring glucoamylase and α -amylase with the ability to bind raw-starch. Atomic force microscopy studies have demonstrated that binding of CBM21 alters the ultrastructure of amylose (Jiang *et al.*, 2012). Fungal glucoamylases with CBM21 domains are often associated with GH families 15 and 13, with the CBM domain preceding the catalytic GH family domain (Machovič *et al.*, 2005; Chen *et al.*, 2012). In our study we identified one

CBM21-GH15 secreted CAZyme-coding gene that demonstrated apparent over-expression specifically during oospore conversion stages. Interestingly, genes detected with the CBM21 family domain were observed only in some *Pythium* species, namely *P. ultimum* var. *ultimum*, *P. ultimum* var. *sporangiiiferum*, *P. irregulare*, and *P. iwayame* and in more distant genes in *S. cerevisiae*. There appears to be duplicates of genes in *P. ultimum* var. *ultimum* and *P. irregulare*. Genes of *P. ultimum* var. *ultimum* and *P. iwayame* that have CBM21 domains also have GH15 family domains proceeding the CBM domain. Distantly-related genes with CBM21 domains in *S. cerevisiae* were uni-modular and predicted to have putative protein phosphatase regulator activities, and only Sce_YOR178C had a eukaryotic signal peptide sequence that was detected by InterProScan.

4.3.2. Glycoside hydrolase family 7 are a group of enzymes with endo- β -1,4-glucanase and cellobiohydrolase (exo-cellulase) activity and are known for their role in metabolizing cellulose (Henrissat *et al.*, 1989; Gilkes *et al.*, 1991). Genes of oomycete species dominated this gene tree, with most genes predicted to be secreted. Genes with GH7 family domains were detected in all of the oomycete species profiled, and were clustered into *Phytophthora* and *Pythium* clades, with expansions apparent in the former. This pattern is consistent with the description of this CAZy family across plant pathogenic oomycetes provided by Zerillo *et al.* (2013) where it was also suggested, based on the predicted cellular location of CAZymes within this family, that they are associated with oomycete cell wall metabolism as opposed to targeting plant cell walls. Among the species profiled for this CAZy family domain, distantly-related genes were observed only in *Emiliania huxleyi* (Haptophyte) and *Dictyostelium discoideum* (Ameobozoa) outgroups. It is interesting that no genes with GH7 domains were detected in the *Arabidopsis thaliana* genome profiled. Perhaps the type of

cellulose metabolized by these CAZymes exhibits cellulose substrate specificities that relate back to cellulose type, which has been described as different in the walls of oomycetes compared to plants (Helbert *et al.*, 1997). This would be consistent with the hypothesis that overexpression of this CAZyme in *P. ultimum* var. *ultimum* supports its necrotrophic lifestyle and should not be mis-characterized as being used for potential PDD activity. Only one gene with this domain was detected for *P. ultimum* var. *ultimum*, and this gene displayed expression patterns highly suggestive of oospore conversion-specific activity.

4.3.3. Glycoside hydrolase family 15 are a group of exo-acting enzymes that hydrolyze the non-reducing ends of α -glucosides and are known for their role in metabolizing starch (Sierks *et al.*, 1990; Zerillo *et al.*, 2013). The *P. ultimum* var. *ultimum* oospore wall-specific gene candidate detected in this family had a domain typical of GH15 enzymes with glucoamylase activities and also had an N-terminal non-catalytic CBM21 domain. Starch-binding activity and glucan hydrolysis of an *Aspergillus niger* CBM21-GH15 dual module CAZyme with the ability to hydrolyze not only 1,4- α -glycosidic bonds, but also to a lesser extent 1,6-, 1,3-, and 1,2- α -glycosidic bonds has been characterized (Frandsen *et al.*, 1994). With the exception of the *P. ultimum* var. *ultimum* over-expressed candidate and one gene in *P. iwayame* that had N-terminal CBM21 domains associated with the GH15 domain, all other GH15 genes detected occurred as single module GH15 CAZymes. Interestingly, no genes within this family were detected in the genomes of the *Phytophthora* species profiled nor were any detected within the other genomes profiled except for *Saccharomyces cerevisiae* and *Dictyostelium discoideum* and the representation of genes across species in the gene tree did not resemble what we know of the species tree. There appears to be a

few duplications of GH15 genes within the *P. ultimum* var. *ultimum* genome as well as a relatively recent expansion of this family within *Pythium*.

4.3.4. Glycoside hydrolase family 131 encode broad specificity bifunctional exo- β -1,3/1,6-glucanases with endo- β -1,4-glucanase activity acting on cellulose and hemicellulose substrates (Lafond *et al.*, 2012). Often genes encoding CAZymes with GH131 domains are associated with additional CBM domains, however, in this study, genes encoding CAZymes with GH131 domains were detected only in the genomes of the oomycete species sampled and all of them were uni-modular with the vast majority predicted to be secreted (Lafond *et al.*, 2012; Blackman *et al.*, 2015). It's possible that some of the genes not predicted as secreted using our secretome pipeline may have features for non-classical secretion. The bifunctional exo- and endo-glucanase activity appears to be unique to this CAZyme class. Two candidate over-expressed oospore wall-specific CAZymes were identified with GH131 domains, and both displayed clear patterns of oospore conversion-specific expression. Given the high cellulose composition within oomycete cell walls, employment of a bifunctional enzyme that can hydrolyze the nonreducing end of β -1,3;1,6-branched glucans as well as having endo- β -1,4-glucanase activity would offer an efficient and rapid breakdown of a major component of the oospore cell wall.

4.3.5. Surface layer homology (SLH) domains are mostly known for their group I S-layer proteins which function to anchor the bacterial cellulosome to cell surfaces (Engelhardt and Peters, 1998). However, group II of these glycoside hydrolase-associated motifs include extracellular enzymes and proteins mostly involved in polysaccharide degradation

and their presence is not restricted to bacteria (Engelhardt and Peters, 1998; Zona and Janecek, 2005; Carlton *et al.*, 2007; Peer *et al.*, 2009; Ferro *et al.*, 2010). In this study, CAZyme-coding genes with SLH domains were detected only in genomes of *Pythium* species and the *Phytophthora* species *H. arabidopsidis*. Most genes with this domain were predicted to be secreted. One potentially over-expressed oospore wall-specific candidate was identified for *P. ultimum* var. *ultimum* with apparent oospore-conversion specific activity and protein structure predictions using InterProScan identified an associated subtilisin-related domain with proteolysis and serine-type endopeptidase activity on a region overlapping the detected SLH domain. Bioinformatic studies by Peer *et al.* (2009) revealed strong support for horizontal transfer of cellulosomal-like components, such as SLH-motif domains, cohesin and dockerin modules from bacteria to archaea and eukaryotic genomes and suggested they may serve as modular-like appendages that may play roles in processes unrelated to classical cellulosomal function. SLH motifs may enhance activities of cell-wall directed proteins owing to the strong and specific protein-protein interaction made possible by the lock-and key multiprotein complexes formed by the interaction of proteins with SLH motifs, cohesin and dockerin modules.

4.4. Challenges. Several challenges typical of working with non-model organisms had to be overcome for stage-specific expression analysis by RNA-Seq to be successful in this study. Oosporogenesis time-course libraries were the first samples sequenced and the library preparation methods used for this step were produced on the Mondrian platform, which did not allow for the incorporation of a ribosomal depletion treatment. The Illumina Mi-Seq platform available in-house was used for initial sequencing efforts, however, the vast majority of transcriptome sequences mapped to ribosomal reads, leaving on a small

proportion of mRNA reads that mapped to genes. In order to mitigate challenges related to sequencing samples with very low proportions of mRNA to total RNA, we reverted to outsourcing sequencing of RNA-Seq libraries on the Illumina Hi-Seq platform to obtain sufficient amounts of reads mapping to coding genes. High levels of ribosomal reads and low levels of mRNA transcripts that account for less than 5% of total transcripts sequenced are not uncommon in transcriptomics studies (Islam *et al.*, 2012; Conesa *et al.*, 2016). Initially, read counts were complicated by the presence of an inverted repeat in the mitochondrial ribosomal cistron and ribosomal sequences occurring on the tail ends of some scaffolds in the annotation file that had not been updated since it was generated by Lévesque *et al.* (2010), resulting in reads that mapped to more than one location being categorized among reads with non-unique alignments. Only once the annotation GFF file was modified to remove the mitochondrial inverted repeat and the nuclear ribosomal reads were removed from the ends of scaffolds and consolidated into a single separate scaffold was the full extent of the overwhelming proportion of ribosomal reads realized. Once familiar with the ribosomal RNA to messenger RNA proportions typical of our RNA extractions, we used an alternative library preparation method that included a ribosomal RNA depletion step for oospore conversion time-course samples. Optimization of library preparation methods was critical for this time-course experiment as tissue samples for this study were extremely limiting and would require methods sensitive to ultra-low input DNA. Using the modified annotation file we were able to design ribosomal depletion primers which were included in the library preparation steps for oospore conversion time-course samples. The difference in the proportion of reads mapping to coding genes in ribosomal depleted and non-depleted samples can be appreciated from the summary

statistics described in Table 1 and Table 2 and in the abundance profiles generated in Figure S3-2.

Sequence library contamination by introduced microbial DNA presented a significant challenge in this study, particularly for the oospore conversion time-course samples. Contamination was most intense in sequencing libraries from the earliest time-points (0, 12, 24 hours), and alleviated in libraries from 48 hours onwards. Sequencing library contamination is known to be especially problematic when dealing with low-input DNA samples where contaminating DNA becomes dominant in sequence libraries, effectively swamping the low amounts of actual starting material and occupying the larger fraction of reads sequenced (Salter *et al.*, 2014). Such problems are further amplified when sequencing projects require PCR amplification of libraries. Common sources for introduction of contaminating DNA during sample preparation include molecular grade water and laboratory reagents such as PCR reagents and DNA extraction kits (Maiwald *et al.*, 1994; Kulakov *et al.*, 2002; Mohammadi *et al.*, 2005; Weiss *et al.*, 2014). Although a range of contaminants may be present in commonly used DNA extraction reagents, the taxa most commonly identified are those that inhabit water or soil environments, frequently having nitrogen fixation abilities (Salter *et al.*, 2014). During the earliest stages of oospore conversion, the challenge was to extract sufficient amounts of RNA, of sufficient quality, from oospores that still had very thick walls and low transcriptional metabolic activity. Harsh lysing and homogenization techniques were required to break open thick-walled oospores so that RNA could be made accessible and extracted. As oospore conversion progressed, RNA yield and quality increased following extraction increased, and this is apparent in the proportion of reads mapping. Nonetheless, sample material was still very

limiting, especially considering that only a fraction of the initial concentration of 130,000 oospores/mL inoculated per flask could be recovered for subsequent RNA extraction. The decision to include a ribosomal RNA-depletion step during library preparation, and to use the Illumina Hi-Seq for maximal sequencing throughput rescued this experiment. Additionally, having a genome to align the sequencing reads to, along with paired-end sequencing and very conserved mapping criteria, allowed contaminating reads to be effectively washed out from further sequencing analyses. An analysis of the source of the contaminating reads revealed that the majority mapped to the genome of *Paenibacillus polymixa*, which are among the species listed in the taxa most commonly identified from DNA extraction kits by Salter *et al.* (2014). A good number of the contigs were larger than 5000 bp and spanned several genes and intergenic regions, supporting that contamination was most likely reagent-derived. However, to properly investigate the possibility of reagent contamination, samples would need to be taken from the various reagents used through this study and independently tested for presence of genomic material.

4.5. Future studies. While oospore-wall-specific gene candidates have been identified in this study, additional studies are required to determine if the gene products of these candidates have the enzymatic activity and oospore wall specificity predicted.

Some possibilities for future studies include using an *in vitro* approach whereby candidate genes are cloned and heterologously expressed in an expression vector, such as *Pichia pastoris*, for mass production followed by the direct application of purified enzymes to oospores and oospore wall fragments to see if enzymatic activity is detected and if microscopic analysis reveals alterations of the fine structure of oospore walls.

Alternatively, a possible approach to study the activity of these gene products in vivo may be to use the CRISPR interference (CRISPRi) system to repress expression of target genes and gene families using guide RNA complementary to the nontemplate DNA of unique or conserved regions of particular CAZy family sequences, respectively (Qi *et al.*, 2013). Engineered CRISPR-cas9 systems represent a rapidly evolving toolset for biotechnological applications. Under this approach, samples of *P. ultimum* var. *ultimum* could be transformed with a modified Cas9 (dCas9) lacking endonuclease activity with a doxycycline (DOX)-inducible promoter co-expressed with custom guide RNA to generate a DNA recognition complex that specifically interferes with gene transcription but does not alter the targeted gene sequence (Qi *et al.*, 2013; Dow *et al.*, 2015). Gene transcription may be controlled by applying a DOX treatment to suppress transcription and relieve transcriptional suppression by washing off the applied DOX treatment (Dow *et al.*, 2015). If transcription can be controlled by applying DOX treatments to induce CRISPRi gene suppression at particular time-points during oosporogenesis or oospore conversion, it may be possible to monitor the effects of improperly developed or converting oospores. Additionally, this method of controlled and inducible transcriptional repression allows the study of genes that may be essential and whose complete suppression may lead to loss of viability and growth.

Transformation of some *Pythium* and *Phytophthora* species has been well-studied and can be accomplished in different ways (i.e., with different selective markers and by different plasmid incorporation techniques – electroporation, PEG-mediated, microprojectile bombardment and *Agrobacterium tumefaciens*-mediated transformation). A recent study by Vijn and Govers (2003) used *Agrobacterium tumefaciens*-mediated

transformation (ATMT) to transform *P. ultimum* var. *sporangiferum* sporangia to geneticin resistance. Optimizing such a method for *P. ultimum* var. *ultimum* oospore transformation, possibly for use on oospores that have converted and no longer possess thick inner walls, may simplify transformation and circumvent the need to produce protoplasts or deal with challenges in transforming ceonocytic hyphae where random transgenic nuclei are generated.

4.6. Conclusion. Identifying genes that are only expressed during certain parts of the *P. ultimum* var. *ultimum* life cycle may be valuable for developing diagnostic markers to measure virulence of test soil samples. Environmental metagenomics is becoming an important research area and environmental transcriptomics is being used to understand functions at play in complex environments such as soil. Johnson and Arroyo (1983) demonstrated in cotton that certain pathogenic *Pythium* species, including *P. ultimum*, can be present without causing diseases. One would expect that the candidate CAZyme-coding genes that appear to be over-expressed only during oospore conversion would present an early warning of disease. The candidate oospore wall-specific genes identified in this study appear to show a strong correlation with the oospore conversion stage of the *P. ultimum* var. *ultimum* lifecycle and offer a set of genes that could be used to profile soils to detect for oospores undergoing conversion.

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Appendix 1 - Supplemental Methods

A1.1. Time-point selection for oospore conversion and oosporogenesis experiments

A1.1.1. Oospore conversion

The time-points selected for RNA extraction during oospore conversion were based on previous conversion studies where oospores were recovered and observed microscopically for wall thinning and germination after resuspension in different media treatments over time under optimal conversion conditions similar to those described by Johnson (1988). From these studies, oospores were observed to convert rapidly when re-suspended in water while also showing very little germination (Figure 4)(Giroux, 2014). In order to characterize gene expression during conversion and not during germination, a time-course experiment was performed in water resuspension (Giroux 2014). Selected time-points ranged from 0 hours (dried oospore stock re-suspended in concentrated oospore-water suspension), to 240 hours (10 days), with most time-points concentrated from 0-72 hours as it was during this period where the conversion profile had the steepest slope in Giroux (2014). Samples were taken from oospore suspensions at each time-point and inspected by microscopy to confirm oospore conversion was progressing as expected.

A1.1.2. Oosporogenesis

In order to study oospore production in mats growing in V8 media supplemented with cholesterol compared to V8 media alone, agar plugs were taken from advancing colony edges of 2-3 day old culture mats growing on V8-agar plates and transferred to V8-only medium, to grow mycelium without oospores, or V8-cholesterol medium, to grow mycelium with oospores. Oospores were purified from hyphae 3, 5, 7 and 14 days post

inoculation for quantification by haemocytometry. Also, tissue samples were taken from mats with oospores at each time point and were visually inspected by microscopy for the presence of oogonia and oospores. Only mats growing in V8-cholesterol media showed significant oosporogenesis responses within the first 3 days post inoculation. Oospores were observed in mats growing under both treatments by 7 days post inoculation, however, there was significantly greater production of oospores in mats growing in cholesterol-supplemented media (results not shown). Despite mats demonstrating rapid oosporogenesis in response to cholesterol treatment, oospores could not be purified from mats until at least 3 days post treatment as the oospores were too fragile to be quantified without lysing during isolation and purification. To aid in the selection of specific time-points to sample from for expression studies by RNA-seq that would overlap the 24 hours following the earliest observance of oosporogenesis response, guided by the developmental time-frame of oosporogenesis to the formation of mature oospores in *P. ultimum* described by Trow (1901), 3-day old mats growing in V8-media were subjected to a V8-only or V8-cholesterol supplementation treatment. Tissue was sampled from these mats immediately prior to supplementation (0 hours) and at 1, 3, 5, 7, 24 and 48 hours post treatment application for microscopic observation of oospore production. Mats treated with cholesterol-supplemented media displayed rapid and ample oosporogenesis responses as noted by the presence of oogonia and oospore production, while mats treated with V8-only media did not (results not shown). In order to get a general idea of the growth patterns of mats under these treatment conditions, tissue samples were taken from mats at these time points and the expression of a candidate glucanase gene (GH5 PYU1_T002515) relative to two candidate reference genes (NADP PYU1_T009706 and 40S

PYU1_T009816) was measured by real-time quantitative PCR (qRT-PCR)(Table S3). Over-expression of PYU1_T002515 was observed in time-points immediately following treatment application, with little difference of expression between experimental and control treatments, indicating that expression of this gene is implicated in general growth (Figure S3 1). The profile suggests a wave phenomenon may exist for stimulus and induction of a logarithmic-type response over time. The time-points 3, 7, and 24 hours are similar to the multiples of 3 (3, 9, 27 ...). There appeared to be an inverse response to treatment type, with PYU1_T002515 expression increasing under control and experimental conditions from the time of application to 1 hour post treatment, followed by a dramatic drop in expression under control conditions at 3 hours, and a convergence of expression in control and experimental conditions after 5 hours post treatment. From this point onwards, expression under control conditions appears to be consistently higher than under experimental conditions, with expression generally decreasing over time under both treatments. This is consistent with the idea that there is rapid growth in response to nutrient addition, which wears off as nutrients are depleted. Based on these trends, the time-points chosen for expression profiling by RNA-seq analysis were 0 (control, pre-treatment), 3, 7 and 24 hours post treatment application.

Figure S3-1. Expression of a GH5 gene relative to two reference genes obtained by qRT-PCR during a preliminary study of oosporogenesis in *Pythium ultimum* var. *ultimum*. *P. ultimum* var. *ultimum* hyphal mats were cultured in V8-only (control) and V8+Cholesterol (experimental) media over time and the following genes were profiled: target gene PYU1_T002515 (GH5) and reference genes PYU1_T009816 (40S) and PYU1_T009706 (NADP).

A1.2. Reference genome modification to improve alignment mapping during RNA-seq analysis

Preliminary mapping of reads indicated a high percentage of unaligned and not unique reads. This was largely due to an inverted repeat in the mitochondrial cistron causing reads to map twice. Similarly, nuclear ribosomal DNA repeats for the small subunit (18S) and large subunit were also present at the ends of several scaffolds. To improve alignment mapping a modified reference genome was made by removing the inverted repeat of the mitochondrial cistron and the partial nuclear ribosomal sequences present on other scaffolds. We then generated a modified GFF3 file to make this consistent.

A1.3. Analysis of read contamination

One of the most contaminated of the RNA-Seq samples (oospore conversion, 48-hour time-point replicate) was assembled de novo using Megahit (Li *et al.*, 2015). 250 contigs were generated from short reads that matched *Paenibacillus polymixa* at $\geq 90\%$ sequence similarity. A good number of the contigs were larger than 5000 bp and spanned several genes, supporting that contamination was most likely reagent-derived as template reads spanned intergenic regions.

Megahit command:

```
$ megahit --k-min 21 --k-max 255 --k-step 28 - 1 T48-1_BC21_a1.5processed.R1.fastq T48-1_BC21.5processedMerged.fastq - 2 T48-1_BC21_a1.5processed.R2.fastq -o Megahit_assembly/T48-1_BC21__a1_HiSeq_B
```


Appendix 2 – Supplemental Tables

Table S1. Custom-designed ribosomal depletion primers used during RNA-seq library preparation.

Gene Target	Name	Primer Sequence (5' -> 3')
<i>Pythium ultimum</i> var. <i>ultimum</i> small subunit (nuclear)	scf1117875581257_SSU_20_11	ATCCTGCCAGTAGTCATACGC
	scf1117875581257_SSU_95_80	GTGAAACTGCGAATGGCTCAT
	scf1117875581257_SSU_170_163	CTAATACATGCACAAATACCCAACTG
	scf1117875581257_SSU_230_222	CCAATGCAGTCTTCGGGCT
	scf1117875581257_SSU_305_293	CGATTGAGTTTCTGCCCTATCAG
	scf1117875581257_SSU_365_353	CGGGTAACGGGGAATTAGGG
	scf1117875581257_SSU_425_411	ATCCAAGGAAGGCAGCAGG
	scf1117875581257_SSU_485_473	AATAACAATGCTCTGGCTCTTCG
	scf1117875581257_SSU_545_535	CGAGGATCAATTGGAGGGCA
	scf1117875581257_SSU_620_613	CAGTTAAAAAGCTCGTAGTTGGATTT
	scf1117875581257_SSU_680_664	CTCTGGGAGTGTGTACTTGTGA
	scf1117875581257_SSU_740_726	AGTTGGTGGTTGAATAGACTTGC
	scf1117875581257_SSU_800_784	AGGCGTTTGCTCATTGAATACAT
	scf1117875581257_SSU_860_856	CACACCAGGGTAATGATTAATAGGG
	scf1117875581257_SSU_935_918	AATTCTTGGATCGCTGAAAGATGA
	scf1117875581257_SSU_995_976	TTAATCAAGAACGAAAGTTAGGGGAT
	scf1117875581257_SSU_1040_1027	TCTTAACCATAAACTATGCCGACTC
	scf1117875581257_SSU_1115_1104	AAAGTCTTTGGGTTCCGGGG
	scf1117875581257_SSU_1190_1186	CCTGCGGCTTAATTTGACTCAA
	scf1117875581257_SSU_1280_1263	TTCTTGATTCTATGGGTGGTGGT
	scf1117875581257_SSU_1340_1332	TTAACGAACGAGACCTCCGC
	scf1117875581257_SSU_1415_1395	TCTTAGAGGGACTTTTGGGTAATCA
	scf1117875581257_SSU_1475_1460	CTTAGATGTTCTGGGCCGCA
	scf1117875581257_SSU_1535_1522	CGATAGGTCTGGGTAATCTTTTGAA
	scf1117875581257_SSU_1610_1600	TCCTAGTAAATGCAAGTCATCAGC
	scf1117875581257_SSU_1670_1665	CGCACCTACCGATTGAATGAC
	scf1117875581257_SSU_1730_1715	TTGCTTTATTGCGAGTGGGTTTA
	scf1117875581257_SSU_1790_1779	CGTAACAAGGTTTCCGTAGGTG
5.8S (nuclear)	scf1117875581257_5_8S_20_10	ATGTCTAGGCTCGCACATCG
	scf1117875581257_5_8S_80_68	GCAGAATTCAGTGAGTCATCGAA
	scf1117875581257_5_8S_140_124	CTGGAAGTATGTCTGTATCAGTGTC
Large subunit (nuclear)	scf1117875581257_LSU_20_16	GACAAGACTACCCGCTGAACT
	scf1117875581257_LSU_95_83	TAGTAACGGCGAGTGAAGCG
	scf1117875581257_LSU_155_138	TGCATGGCGAATTGTAGTCTATAG
	scf1117875581257_LSU_230_226	TCCCGTTTGTGCCTGAGTAG
	scf1117875581257_LSU_305_295	CGCAAAGTAGGTGGTAAATTCCA
	scf1117875581257_LSU_380_361	CGTGAGGGAAAGATGAAAAGAACT
	scf1117875581257_LSU_455_434	CGTATCCAGTGTCTATAATCTGTAGC
	scf1117875581257_LSU_500_496	GAAGTGGTTTTCTGCTCTTTGTG

Table S1. Custom-designed ribosomal depletion primers used during RNA-seq library preparation.

Gene Target	Name	Primer Sequence (5' -> 3')
Large subunit (nuclear)	scf1117875581257_LSU_590_573	TTCGTATGCTGTGAGAAATGGC
	scf1117875581257_LSU_665_658	GGACTGAGGTGCCTACAATGT
	scf1117875581257_LSU_740_725	TTGCTATGCTGACGGAGTGAT
	scf1117875581257_LSU_800_783	GACTTTGACGAAATGGAGCGAT
	scf1117875581257_LSU_860_848	CAAGTGTTAGGGTGTGGAAACC
	scf1117875581257_LSU_935_922	TTTCTTCTGAGAGGTGGCACTAT
	scf1117875581257_LSU_1010_1001	CCCGAAAGATGGTGAACCTATGC
	scf1117875581257_LSU_1070_1061	TCGTAGCGATCTGACGTGC
	scf1117875581257_LSU_1145_1132	GTAGCTGGTTCCCTCCGAAAT
	scf1117875581257_LSU_1220_1204	AATGATTAGAGGTCTGGGGGAAG
	scf1117875581257_LSU_1280_1273	GGTTTCTTAATTGAACTTGTGGATGT
	scf1117875581257_LSU_1355_1351	GATGATCCGAACGCAAAGTTAAAG
	scf1117875581257_LSU_1415_1411	TGTTGGTTCATTTAGACAGCAGG
	scf1117875581257_LSU_1475_1470	TGTAACAACCTCACCTGCCGAA
	scf1117875581257_LSU_1535_1522	GCGAGTTACTTATACTTTGCCGT
	scf1117875581257_LSU_1610_1596	GTTGTGAAGAAGCTCGTGCC
	scf1117875581257_LSU_1670_1650	TTGGTGGTAGTAGCAAATATTCAAATG
	scf1117875581257_LSU_1745_1730	CGGGTGAGTCGATCCTAAGAG
	scf1117875581257_LSU_1805_1789	TGGGTTTCGGCTCATATTTTGTAT
	scf1117875581257_LSU_1865_1850	CCTGAACCGAGACGTGGATAA
	scf1117875581257_LSU_1910_1906	GTGTGCCCAGGGAAGAGTT
	scf1117875581257_LSU_1985_1974	CGATAGGGAGGAAGCCTTGG
	scf1117875581257_LSU_2045_2036	GCCCTTGAAAATCCTGGTTAGAG
	scf1117875581257_LSU_2120_2104	AAGGTTAGCAGCCTCTAGTTGATA
	scf1117875581257_LSU_2180_2170	TTCGGGAAAAGGATTGGCTCT
	scf1117875581257_LSU_2255_2242	GCTGTGTTTGGACCTTTCGG
	scf1117875581257_LSU_2330_2326	TTGCACTGGTGGCTTGTTTC
	scf1117875581257_LSU_2420_2414	AAGCATTGTGATGGCCGGA
	scf1117875581257_LSU_2480_2462	CAGTGCTCTGAATGTCAAAGTGA
	scf1117875581257_LSU_2540_2533	TAAGGTAGCCAAATGCCTCGT
	scf1117875581257_LSU_2615_2604	TATCTACTGTCTAGCGAACCCAC
	scf1117875581257_LSU_2675_2663	AAGAAGACCCTGTTGAGCTTGA
	scf1117875581257_LSU_2735_2720	TAGGATAAGTGGGAGGCTTTGC
	scf1117875581257_LSU_2795_2791	GTAGCATCTAGCCGTCCTCG
	scf1117875581257_LSU_2855_2851	GGTGAGCTACGTGGAAGACAT
	scf1117875581257_LSU_2930_2912	ATGATAACACAGGTGTCCTAAGGT
	scf1117875581257_LSU_2990_2981	GCTCACTTGATTTTGTATTTTCAAGTATG
	scf1117875581257_LSU_3065_3059	TAAGCTAGAGGTGTCAGAAAAGTTAC
	scf1117875581257_LSU_3140_3135	TTTGATTCTTCGATGTCGGCTC
	scf1117875581257_LSU_3215_3210	TAGGGAACGTGAGCTGGGT

Table S1. Custom-designed ribosomal depletion primers used during RNA-seq library preparation.

Gene Target	Name	Primer Sequence (5' -> 3')
	scf1117875581257_LSU_3305_3289	ATCCAACCCAGTACGAGAGGA
	scf1117875581257_LSU_3380_3373	CTGTAGGATTATGGCTGAACGC
	scf1117875581257_LSU_3455_3449	GCGAATAGCGATAGATGTCTTTTG
	scf1117875581257_LSU_3530_3520	TGTGGTAGTGGAGAGTATGCTG
	scf1117875581257_LSU_3590_3579	GTAGACGACTAAATACAGAACGGG
	scf1117875581257_LSU_3635_3624	TCTTGTACTACGATCTGCTGAGAT
mitochondrial	GU136662_SSU_20_15	GATCCTGGCTCAGAATAAATGCTAT
	GU136662_SSU_95_85	AGCGAACGGGTGAGTAACG
	GU136662_SSU_185_181	CGTTAAAAGATGAGTTTGCCTAAGAT
	GU136662_SSU_260_244	ATCTTTAGCTGGTTTGAAAGAATGATC
	GU136662_SSU_320_313	CAGCAGTGGGGAATTTTGGAC
	GU136662_SSU_395_382	ATTAGGTTGTAAAGCTCTTTCATTAAGG
	GU136662_SSU_455_445	TAATACCCGTGCCAGCAGC
	GU136662_SSU_530_510	AAAGGGTTTGTAGGTGGTTTTTTAAG
	GU136662_SSU_620_602	AGAGGATAATGGAATTTCTATTGGAGG
	GU136662_SSU_680_663	AGCGAAGGCAATTATCTGGGTA
	GU136662_SSU_740_735	CCCGGTAGTCCATGCTGTAAA
	GU136662_SSU_815_810	CCGCCTGAAAAGTATAATCGCA
	GU136662_SSU_890_877	AGCATGTGGTTTAATTCGATAATACG
	GU136662_SSU_965_946	ATTTAATCTAAACAGGCGTTGCATG
	GU136662_SSU_1025_1021	GCAACCCCTATTATTAGTTGCTAATTTA
	GU136662_SSU_1100_1082	CAATGATAGGTTGAAGGAAGGTAGG
	GU136662_SSU_1175_1154	CTACAATGATAATTACAATGAGAGGCAA
	GU136662_SSU_1235_1227	GGATTATGGGCTGAAACTCGC
	GU136662_SSU_1295_1286	ATGCTGCGGTGAATATGTTACTAG
	GU136662_SSU_1340_1325	CCCGTCACATCAAGGAAGTTAAA
	GU136662_SSU_1460_1450	TTAGTAACCTTTGATGAAGTCGTAACAAG
	GU136662_LSU_50_36	GCTGAATACCTTACGATACTTACACT
	GU136662_LSU_125_118	TGGTTTCTTACTTAGATGCTTTCAGT
	GU136662_LSU_215_209	CCCTTCTCGGTCCTCTCGT
	GU136662_LSU_290_274	GAACCAAACGTCTCACGACG
	GU136662_LSU_350_335	CCGAACCCTTGGAACCTTCT
	GU136662_LSU_395_382	TCGAGGTGCCAAACGACTC
	GU136662_LSU_470_454	ATCCGTTGAGCGATAATCTTTCC
	GU136662_LSU_530_525	TGTCTATCTTACAGTCAAGCAAGC
	GU136662_LSU_620_616	CTCTTTAGGAGGTGACCGCC
	GU136662_LSU_710_697	AGAGTGGTATTTCAATGTTATCTAAACA
	GU136662_LSU_800_790	TAAAGATGTAGTAAAGGTTACGGG
	GU136662_LSU_875_870	GAGTTTATATTGGAGACAGCGGG
	GU136662_LSU_950_942	TCGCTACCTTAGGACCGTCA

Table S1. Custom-designed ribosomal depletion primers used during RNA-seq library preparation.

Gene Target	Name	Primer Sequence (5' -> 3')
mitochondrial	GU136662_LSU_1010_1006	CATCTCCTATTAATCTTCCAGCACC
	GU136662_LSU_1100_1093	CAGTCGCAACCCCTATTTTG
	GU136662_LSU_1175_1162	GTCATTTTGCCGAGTTCCTTCA
	GU136662_LSU_1235_1220	ATCCACCTGTGTCGGTTTAGG
	GU136662_LSU_1325_1320	ATTATCCCATCAAAATTTGCTTTCGT
	GU136662_LSU_1400_1390	ACATAGAAACCCTTGGATTTACGG
	GU136662_LSU_1475_1452	CCAGCATTTTCTTTTCTGATATCTTCAA
	GU136662_LSU_1550_1533	TTATAAATAAAACTTGCCGCTTCGG
	GU136662_LSU_1610_1596	AACTAGACCAATGAGCTATTACGC
	GU136662_LSU_1670_1654	ACTGGTTGTAATTATTTTTTCACTTCCT
	GU136662_LSU_1730_1725	GGCTGTTTCCCTCTTGACAATAG
	GU136662_LSU_1805_1793	TTCGGAGTTTCTAAGAGTTCAGTAAG
	GU136662_LSU_1880_1867	TAAATGCTCTACTTCAATAGATTTTCGC
	GU136662_LSU_1940_1927	TTCACTCCTAACCACAAATCATCTC
	GU136662_LSU_2015_2011	GTTTCATGGTTAGATCACTCGGTTT
	GU136662_LSU_2120_2104	GCTTGCTATAAAAAATAAACTTGCTGG
	GU136662_LSU_2210_2204	TTTCAAACCTCAAAAGTTCTTTTTTCACC
	GU136662_LSU_2300_2284	GGGTGGTTCCCAATATTCAAAA
	GU136662_LSU_2450_2439	ACTCGTCATTACTCACGGAATCT
	GU136662_LSU_2525_2515	TTTCACAAAGAAAAAGTTTTCTCTAGGA
	GU136662_LSU_2585_2578	GCTTTTCGCATTTTTTACGTCCTA
	GU136662_LSU_2630_2617	CCAAGGCATCCACTTAATGCTT

Table S2. *Pythium ultimum* var. *ultimum* genes predicted to encode secreted CAZymes.

Sequence ID	CAZy Family	Sequence ID	CAZy Family	Sequence ID	CAZy Family
1. PYU1_T000438	GH28	43. PYU1_T004202	GH43	85. PYU1_T007532	PL3
2. PYU1_T000471	CBM63	44. PYU1_T004204	GH43	86. PYU1_T007535	PL3
3. PYU1_T000490	CBM63	45. PYU1_T004531	GH53	87. PYU1_T008222	GT4
4. PYU1_T000955	GH3	46. PYU1_T004532	GH53	88. PYU1_T008740	GH72
5. PYU1_T000957	GH3	47. PYU1_T004661	GH17	89. PYU1_T008966	GH3
6. PYU1_T001030	GH28	48. PYU1_T004665	GH17	90. PYU1_T009144	CE13
7. PYU1_T001031	GH28	49. PYU1_T004680	PL4	91. PYU1_T009211	GH72
8. PYU1_T001053	PL3	50. PYU1_T004681	PL4	92. PYU1_T009220	GH72
9. PYU1_T001170	GH5	51. PYU1_T004970	GT4	93. PYU1_T009223	GH72
10. PYU1_T001346	CBM25/GH13	52. PYU1_T005009	CBM63	94. PYU1_T009228	GH72
11. PYU1_T001495	GH5	53. PYU1_T005010	CBM63	95. PYU1_T009237	GH72
12. PYU1_T001515	AA9	54. PYU1_T005011	CBM63	96. PYU1_T009505	GH28
13. PYU1_T001658	GH5	55. PYU1_T005056	GH6	97. PYU1_T009536	PL3
14. PYU1_T001777	GH16	56. PYU1_T005072	GH6	98. PYU1_T009543	GH18
15. PYU1_T001944	GH131	57. PYU1_T005073	GH6	99. PYU1_T010437	GT2
16. PYU1_T001945	GH131	58. PYU1_T005135	GH17	100. PYU1_T010707	GH16
17. PYU1_T001949	GH131	59. PYU1_T005252	GH13	101. PYU1_T010744	GT96
18. PYU1_T002046	GH81	60. PYU1_T005352	GH31	102. PYU1_T010806	CBM63
19. PYU1_T002299	GH81	61. PYU1_T005443	CBM21/GH15	103. PYU1_T010809	CBM63
20. PYU1_T002412	PL1	62. PYU1_T005445	CBM21/GH15	104. PYU1_T010810	CBM63
21. PYU1_T002413	PL1	63. PYU1_T005497	CE1	105. PYU1_T010978	GH38
22. PYU1_T002414	PL1	64. PYU1_T005498	CE1	106. PYU1_T010982	GH89
23. PYU1_T002415	PL1	65. PYU1_T005968	GH72	107. PYU1_T010997	GH89
24. PYU1_T002416	PL1	66. PYU1_T005971	GH30	108. PYU1_T011896	GH7
25. PYU1_T002417	PL1	67. PYU1_T005972	GH30	109. PYU1_T012179	PL1
26. PYU1_T002418	PL1	68. PYU1_T005973	GH30	110. PYU1_T012180	PL1
27. PYU1_T002420	PL1	69. PYU1_T006015	GH18	111. PYU1_T012442	PL3
28. PYU1_T002421	PL1	70. PYU1_T006030	GH30	112. PYU1_T012446	PL3
29. PYU1_T002541	PL3	71. PYU1_T006194	CBM25/GH31	113. PYU1_T012455	PL3
30. PYU1_T002542	PL3	72. PYU1_T006325	GH17	114. PYU1_T012511	GH5
31. PYU1_T002783	AA8	73. PYU1_T006327	GH17	115. PYU1_T012512	GH5
32. PYU1_T003056	CE5	74. PYU1_T006485	GH5	116. PYU1_T012529	GT41
33. PYU1_T003169	CBM1	75. PYU1_T006546	GH81	117. PYU1_T012530	GH3
34. PYU1_T003170	CBM1	76. PYU1_T006878	GT31	118. PYU1_T012880	GH3
35. PYU1_T003171	CBM1	77. PYU1_T006946	CE13	119. PYU1_T013005	GT10/GT31
36. PYU1_T003173	GH31	78. PYU1_T006947	CE13	120. PYU1_T013244	AA2
37. PYU1_T003498	GH81	79. PYU1_T006952	CE13	121. PYU1_T013496	GH2
38. PYU1_T003515	CE10	80. PYU1_T007490	PL3	122. PYU1_T013821	GH17
39. PYU1_T003536	SLH	81. PYU1_T007523	PL3	123. PYU1_T014584	GH17
40. PYU1_T003553	SLH	82. PYU1_T007525	PL3	124. PYU1_T014660	GH17
41. PYU1_T003928	GH17	83. PYU1_T007528	PL3	125. PYU1_T015021	PL1
42. PYU1_T004063	CE1	84. PYU1_T007530	PL3	126. PYU1_T015040	GH5

Table S3. Primers and probes optimized for use as reference genes for expression profiling by qRT-PCR.

Gene	Primer name	Sequence (5'→ 3')	Direction	[Conc](nM)
GH5 - PYU1_T002515	PYU1_T002515_528_F	GCCCAACTACCTGAGCAAGA	Forward	500
	PYU1_T002515_642_R	GATGCGCATGCCGTTTC	Reverse	1000
	PYU1_T002515_597_P	CATCAAGGGCGTCAACTGGTTC	Probe	150
		<u>GCCCAACTACCTGAGCAAGA</u> ACGGCCAAATCATCGCGCAGTCCAAGAACGGCACCGAGGTCCCGGTTCGC CATCAA		115 bp
		GGGCGTCAACTGGTTC GGCATG GAAAC*CGGCATGCCGCATC		
GT48 - PYU1_T002377	PYU1_T002377_440_F_B	AATTATGGAAATGCTGGGAAACTCAT	Forward	1000
	PYU1_T002377_644_R	TTCCTGCTGTGGATCACGTC	Reverse	1000
	PYU1_T002377_605_P	AGACGCCAATACTGACCACC	Probe	250
		<u>AATTATGGAAATGCTGAAA*CTCAT</u> CGTCGCTTGGTGACATTTTTGATTTCATTGAAACGAACCTTTTGGTTTCCAA		205 bp
		GAAGCCAACGTTGAGAACCAACGCGAGCATGTGCTGCTACTTCTCACAAACTACAAGGAACAGTCTGAAGAAGATA CGGCTCCAGGTCA AGACGCCAATACTGACCACC GACGTGATCCACAGCAGGAA		
40S - PYU1_T009816	PYU1_T009816_29_F	GCAAGAAGAAAGCCGTGCGAC	Forward	500
	PYU1_T009816_154_R	AAGCGATCTTGGTACCGGC	Reverse	1000
	PYU1_T009816_58_P	CGCAAGGACTGGTACGACAT	Probe	250
		<u>GCAAGGAAAGC*CGTCGAC</u> CCCTTTCACC CGCAAGGACTGGTACGACAT TAAGGCGCCAGCCATCTTCTCGGAGCGC		126 bp
		AACGTCGCAAGACCTTGGTGAACCGTACC GCCGGTACCAAGATCGCTT		
NADP - PYU1_T009706	PYU1_T009706_1125_F	GCTCACGAAGGACCTGGC	Forward	1000
	PYU1_T009706_1215_B_R	GGCGATCTTGTCATAAAAGGCAA	Reverse	500
	PYU1_T009706_1151_P	TCTACGAAGACAAGATGGCGC	Probe	250
		<u>GCTCACGAAGGACCTGGC</u> GCTGCTCA TCTACGAAGACAAGATGGCGC GCGAGCACTACTACGAGACT TTGCC*TT		91 bp
		TATTGACAAGATCGCC		
Ubq. PYU1_T008814	PYU1_T008814_328_F	CATCAGGAGTTGCGAGTG	Forward	1000
	PYU1_T008814_422_R	TGCTCTGCGTGGCGATAC	Reverse	1000
	PYU1_T008814_350_P	CGCTCAACATCAATGCCAGT	Probe	250
		<u>CATCAGGAGTTGCG*AGTG</u> TTGT CGCTCAACATCAATGCCAGT GCCGCGTCTCAGTCCCTTCTGGATTGCCTGGAG		96 bp
		ACGTATCGCCACGCAGAGCAC		

Table S4. Summary of RPKM-normalized read counts across all samples.

Sequence ID	mean	min	max	SD
PYU1_T000005	1.755	1.124	2.212	0.248
PYU1_T000007	2.279	1.848	2.627	0.215
PYU1_T000377	2.298	1.833	2.863	0.240
PYU1_T000417	1.941	1.487	2.562	0.242
PYU1_T001122	2.365	1.843	2.607	0.171
PYU1_T002014	1.295	0.806	1.906	0.248
PYU1_T002105	2.188	1.740	2.737	0.237
PYU1_T002975	1.987	1.480	2.399	0.230
PYU1_T003676	1.930	1.510	2.384	0.205
PYU1_T003814	2.123	1.552	2.568	0.221
PYU1_T004067	1.918	1.396	2.443	0.241
PYU1_T004192	1.962	1.321	2.509	0.250
PYU1_T004237	1.601	1.055	2.120	0.249
PYU1_T004468	2.359	1.874	2.713	0.217
PYU1_T005629	2.295	1.510	2.715	0.222
PYU1_T005764	2.148	1.509	2.461	0.226
PYU1_T005904	2.010	1.597	2.415	0.227
PYU1_T006207	2.198	1.600	2.538	0.241
PYU1_T006347	1.895	1.461	2.656	0.249
PYU1_T006546	1.998	1.586	2.324	0.209
PYU1_T006924	2.317	1.691	2.687	0.209
PYU1_T007334	2.327	1.725	2.850	0.247
PYU1_T007542	1.746	1.303	2.352	0.250
PYU1_T007972	3.228	2.461	3.701	0.246
PYU1_T008314	2.083	1.640	2.855	0.234
PYU1_T008632	1.921	1.442	2.605	0.235
PYU1_T010177	1.887	1.420	2.384	0.227
PYU1_T010374	1.843	1.379	2.263	0.225
PYU1_T010478	2.095	1.625	2.493	0.207
PYU1_T010859	2.386	1.690	2.757	0.227
PYU1_T011571	2.512	1.892	2.837	0.235
PYU1_T011708	2.439	1.830	2.816	0.212
PYU1_T011781	3.055	2.094	3.286	0.243
PYU1_T012390	2.492	1.751	2.882	0.235
PYU1_T012538	2.221	1.535	2.518	0.237
PYU1_T012618	2.017	1.523	2.478	0.244
PYU1_T012675	1.784	1.294	2.271	0.239
PYU1_T013739	2.119	1.519	2.577	0.212
PYU1_T013740	1.871	1.495	2.608	0.231
PYU1_T013845	2.097	1.599	2.461	0.195
PYU1_T013945	1.598	1.189	1.933	0.164

Table S4. Summary of RPKM-normalized read counts across all samples.

Sequence ID	mean	min	max	SD
PYU1_T014541	1.772	1.344	2.361	0.234
PYU1_T014614	1.850	1.406	2.460	0.216
PYU1_T014631	2.506	1.814	3.057	0.243
PYU1_T014812	2.240	1.750	2.607	0.217
PYU1_T014853	1.898	1.263	2.289	0.238
PYU1_T015330	2.181	1.413	2.642	0.242

*The summary statistics reflect the RPKM-normalized read counts of each *P. ultimum* var. *ultimum* gene across all time-points for both oosporogenesis and oospore conversion time-course assays as well as their counts from a previous transcriptome sequencing study by Lévesque *et al.* (2010) on *P. ultimum* var. *ultimum* with mycelium under various abiotic stressors (i.e., hypoxia, fungicide, cold and heat stress), and in the presence of a host.

Appendix 3 – Supplementary Figures

Figure S3-2. Total number of reads (RPKM-normalized) in oosporogenesis 7-hour time-point control samples comparing reads mapped in samples treated and not treated with a ribosomal depletion step. T7-c3_BC29 represents samples that were treated with a ribosomal depletion step that used custom designed depletion primers, while T7-c3_BC30 and T7-c3_BC31 represent samples depleted with *Arabidopsis* ribosomal sequence depletion primers or no depletion primers, respectively. The proportion of reads mapping to contaminating *Paenibacillus* genomic material are also included.

Figure S3-3. GH3 CAZy family domain gene tree. One of the oospore wall-specific gene candidates is situated in the block highlighted in green, while the other is in the blue block which is dominated by expansions in *Phytophthora*.

Figure S3-4. GH17 CAZy family domain gene tree. The tree on the right is an expansion of the portion of the tree highlighted in blue on the left.

Figure S3-5. GH72 CAZy family domain gene tree. *T. pseudonana* does not pass the secretome pipeline, but *P. tricornutum* does. Blue bars represent clusters of *Phytophthora* while red bars represent *Pythium* clusters.

Figure S3-6. Genes expressed at relatively constant rates across oospore conversion time-points.

Figure S3-6. (ctn'd)

Figure S3-6. (ctn'd)

Figure S3-6. (ctn'd)

Figure S3-6. (ctn'd)

Figure S3-6. (ctn'd)

Figure S3-7. Scatter plot of secreted CAZYme-coding genes showing the relationship between expression during oospore conversion and under oosporogenesis-inducing conditions.