CHARACTERIZATION OF PUT1, A POLYAMINE TRANSPORTER FROM PHYTOPHTHORA PARASITICA

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ABSTRACT

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Phytophthora is a genus of oomycetes that causes devastating diseases in both natural and agricultural ecosystems. *Phytophthora parasitica* is a root rot pathogen that can infect over 72 plant genera and thus serves as a model organism for genome study. I chose to investigate polyamine metabolism and transport in this group of plant pathogens, because polyamines are signaling compounds that have critical roles in growth and development in all organisms. BLAST analysis of the *P. parasitica* genome using the polyamine uptake transporter PUT5 of *Arabidopsis thaliana* identified eight homologues. Similar numbers of this family were found in other *Phytophthora* genomes. The phylogenetic analysis indicates that the PUT family in *Phytophthora* is surprisingly more diverse than plant PUTs given that the oomycete morphology is less complex than that of plants. *PPTG_00424* (*PUT1*) was chosen for further analysis since its transcript was highly expressed in swimming zoospores which are the primary dispersal propagules of oomycetes. To determine the subcellular localization of this protein, a plasmid vector containing a *PPTG_00424* fused with green fluorescent protein (GFP) was constructed and transformed into *P. sojae* protoplasts. Confocal microscopy showed that PUT1 is localized to the endoplasmic reticulum (ER). To investigate if *PUT1* could account for the exogenous uptake of polyamines from the environment, the gene was heterologously expressed in yeast, *Saccharomyces cerevisiae*. In yeast complementation assays, PUT1 protected yeast cells from toxic accumulation of spermidine (Spd) indicating that this protein is involved in the cellular efflux of Spd. In yeast, TPO5 is required for export of excess polyamines and expression of

PUT1 in TPO5 mutants partially complemented the WT phenotype. Collectively, our data indicates that *PUT1* codes for an ER-resident PA transporter that is involved in the transport of spermidine. Its high level of expression hints at the importance of polyamine homeostasis in zoospores. Polyamines may be present in the soil environment and zoospore actively uptake polyamines. *PUT1* is thus the first characterized polyamine transporter in *Phytophthora*.

This work is dedicated to my family and my mentors for their encouragement and support

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1. INTRODUCTION TO POLYAMINE BIOLOGY

1.1 Polyamines

Polyamines (PAs) were first discovered by Antonie van Leeuwenhoek in human semen which led to the names spermine and spermidine (Alcázar et al. 2010). Polyamines are N-containing low-molecular-weight aliphatic compounds that play fundamental roles in growth and development of all living organisms (Kusano et al. 2018; Pál et al. 2015; Tiburcio et al. 2014). They constitute the cellular polycation pool mainly the cytoplasm and nuclei, together with Ca^{2+} and Mg^{2+}. Similar to other cations such as Mg^{2+}, PAs can also bind to intracellular polyanions such as ATP, nucleic acids and proteins to regulate various cellular functions (Igarashi and Kashiwagi 2006). Polyamines are involved in fundamental biological functions, which include regulation of gene expression, cell growth and proliferation and regulation of cellular stress (Miller-Fleming et al. 2015).

Most metazoans (E.g. animals) have the diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm). Higher plants contain thermospermine (Tspm) in addition to Put, Spd and Spm (Fig. 1). In a plant cell, PAs are present in the cytoplasm, cell wall as well as in organelles such as vacuole, plastid and mitochondria and nucleus (Mustafavi et al. 2018). They exist either in free, covalently conjugated or non-covalently conjugated forms. The covalently conjugated PAs are divided into two groups; perchloric acid ($HClO_4$)-solubles and insolubles. The majority of plant PAs are perchloric acid-soluble covalently conjugated PAs. These are formed by free PAs covalently combining with small compounds such as phenolic compounds which generate secondary metabolites that mostly regulate local allergic reactions to external stressors and plant morphogenesis (Chen et al. 2019; Subramanyam et al. 2015). Perchloric acid-insoluble covalently conjugated PAs, on the other hand, are formed by free PAs

covalently binding to macromolecules, such as nucleic acids and proteins by ionic and hydrogen bonds.

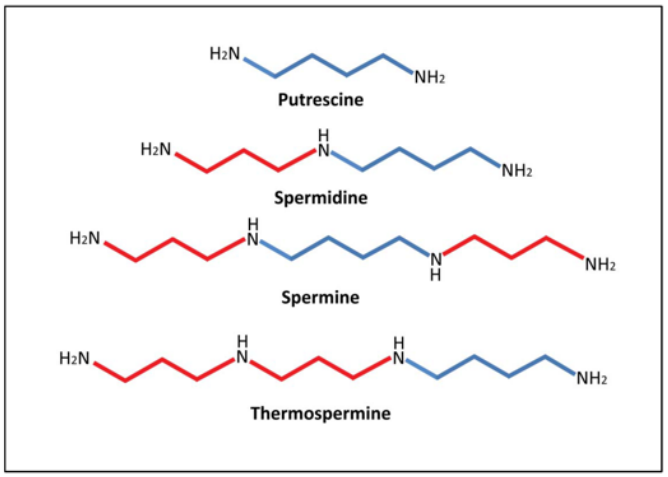

Fig. 1. Chemical structure of four major biologically relevant polyamines (PAs): putrescine, spermidine, spermine and thermospermine (Bitrián et al. 2012). Spermidine, spermine and thermospermine are formed by the addition of aminopropyl moieties (shown in red) to the linear four carbon backbone of putrescine (shown in blue).

Being positively charged and fully protonated at physiological pH, and having a low molecular weight, free PAs can electrostatically bind to negatively charged nucleic acids, proteins and membrane phospholipids producing non-covalently conjugated PAs. These electrostatically bound PAs cause conformational stabilization/destabilization of DNA, RNA and protein (Alcázar et al. 2010; Mustafavi et al. 2018). Thus they are involved in regulation of DNA replication, transcription, RNA processing, protein synthesis and protein processing, and thereby regulate cellular functions such as signal transduction and membrane stability (Igarashi & Kashiwagi, 2000, 2015). Usually, PAs that have more amino groups have stronger physiological activity (Chen et al. 2019). One of the examples of polyamine-involvement in cellular regulation

the post-translational modification called hypusination of the eukaryotic translation elongation factor “eIF5A” (Saini et al. 2009).

In plants, polyamines have been involved in diverse developmental and physiological processes such as organogenesis, flower and fruit development, embryogenesis, leaf senescence and biotic and abiotic stress response (Chen et al. 2019; Kusano et al. 2008 and 2018). Studies that investigated loss and gain of function of polyamine metabolism show that polyamines are essential for above functions in plants (Alcázar et al. 2010). In animals, PAs were found to be regulating angiogenesis, embryonic development, implantation, placentation, apoptosis and oxidative stress while functions of PAs were studied in digestive, reproductive, endocrine and other systems (Hussain et al. 2017; Kalac, 2014; Lenis et al. 2017). The contribution of PA metabolism in the life cycle of fungi has also been explored by using polyamine analogues and enzyme inhibitors (DFMO) and gene replacement (Crespo-Sempere et al. 2015; Wallace & Fraser, 2003; Wallace and Niiranen, 2007). In fungi, PAs are found to be involved in dimorphism, spore germination, appressorium formation and conidiation (Khurana et al. 1996; Ruiz-Herrera, 1994). However, the precise molecular mechanisms underlying these functions of polyamines are not yet known.

1.2 Polyamine metabolism

1.2.1 Polyamine biosynthesis

Intracellular PA level is tightly regulated as at a certain level they enhance growth and proliferation while at excess levels PAs are cytotoxic. Multiple mechanisms maintain PA homeostasis which include biosynthesis, degradation, conjugation and transport (Miller-Fleming et al. 2015). Polyamines are synthesized in the cell cytoplasm via decarboxylation of amino acids. The main carbon skeleton of PAs derives from the amino acids ornithine, arginine and

lysine while the aminopropyl moieties of Spd and Spm are supplied by methionine. In all free-living eukaryotes, PA biosynthesis is initiated with the synthesis of the diamine putrescine (Put), the precursor of all higher PAs (Fig. 1). All eukaryotes synthesize Put directly from ornithine via ornithine decarboxylase activity (ODC pathway) (Alcazar et al. 2010). The animal ODC is a pyridoxal-5'-phosphate (PLP) dependent enzyme which exists as a homodimer of 107 kDa. The active site of the enzyme is constructed by residues of both monomers therefore only the dimer is catalytically active (Kern et al. 1999). The ODC enzyme removes the carboxyl group of ornithine producing Put and CO_2. However, an alternative pathway called arginine decarboxylase (ADC) pathway is used by plants and bacteria to produce Put from arginine via either two step or three step process (Fig. 2). In the two-step process, arginine is converted to agmatine and then into putrescine by ADC and agmatinase enzymes, respectively (Patel et al. 2017). In the three- step process, this conversion occurs through the action of ADC, agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase activity (Fig.2) (Wuddineh et al. 2018). Arabidopsis expresses two ADC genes and they were known to convert arginine into agmatine. It has been recently reported that one of these ADC genes is also involved in synthesis of N-acetylputrescine (Fig.2).

It is hypothesized that utilization of different pathways to synthesize PAs reflect their different evolutionary origins. Arginine decarboxylase, agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase in plants may have derived from the cyanobacterial ancestor of the primary plastid endosymbiosis (Illingworth et al. 2003). Ornithine decarboxylase present in all eukaryotes may have derived from the bacterial genes of the common ancestor of plants and animals that engulfed the cyanobacterial endosymbiont (Illingworth et al. 2003).

Putrescine that is synthesized via ODC and/or ADC pathway, serves as a precursor to form Spd and Spm by addition of aminopropyl groups by aminopropyltransferases called Spd synthase and Spm synthase respectively (Fig. 2). The functions of these two enzymes are very similar but they are highly specific to their corresponding substrates. Spd synthase is highly specific for its substrate Put while Spm is highly specific for Spd (Wu et al. 2007). A third class of aminopropyltransferase was also identified in the extreme thermophilic bacteria where Spd is synthesized from aminopropylagmatine by an ureohydrolase (Ohnuma et al. 2005). The distribution of Spd synthases is wider than that of Spm synthase. Spm synthase is found only in eukaryotes (Tiburcio et al. 1997). The donor of aminopropyl groups for Spd/Spm synthases is decarboxylated S-adenosyl methionine which is generated by decarboxylaion of S-adenosyl methionine (SAM) via SAM decarboxylase (Slocum et al. 1984). SAM decarboxylase is an enzyme which depends on a covalently bound pyruvate as a prosthetic group. Polyamine biosynthesis has been extensively studied and the genes for most of the steps have been identified in *E. coli*. The ADC and agmatinase enzymes are encoded by the genes *speA* and *speB*, respectively while the ODCs are encoded by *speC* and *speF* in *E. coli* (Satishchandran and Boyle, 1986; Schneider and Wendisch, 2011). The *E. coli* SAM decarboxylase is encoded by *speD* while Spd synthase is encoded by *speE*, both of which located in the same operon (Tabor et al. 1986). Cadaverine is a diamine found in some bacteria and some higher plants and is involved in regulating stress response, cell signaling, growth and development and herbivore resistance (Jancewicz et al. 2016; Samartzidou et al. 2003). Cadaverine is synthesized via lysine decarboxylation and there is no evidence of cadaverine biosynthesis from other PAs.

Apart from the common PAs that are found in prokaryotes and eukaryotes (Put, Spd and Spm), some uncommon long-chain and branched PAs are found in organisms such as

thermophilic bacteria, archaea (Oshima, 1979; 2007) and in higher plants. Thermospermine is a structural isomer of spermine abundantly found in the plant kingdom and is synthesized from spermidine by the enzyme Tspm synthase (Knott et al. 2007). Homospermine, norspermine and norspermidine are some other uncommon PAs that have been found in distinct plant species and are synthesized under special conditions (Hamana et al. 2000).

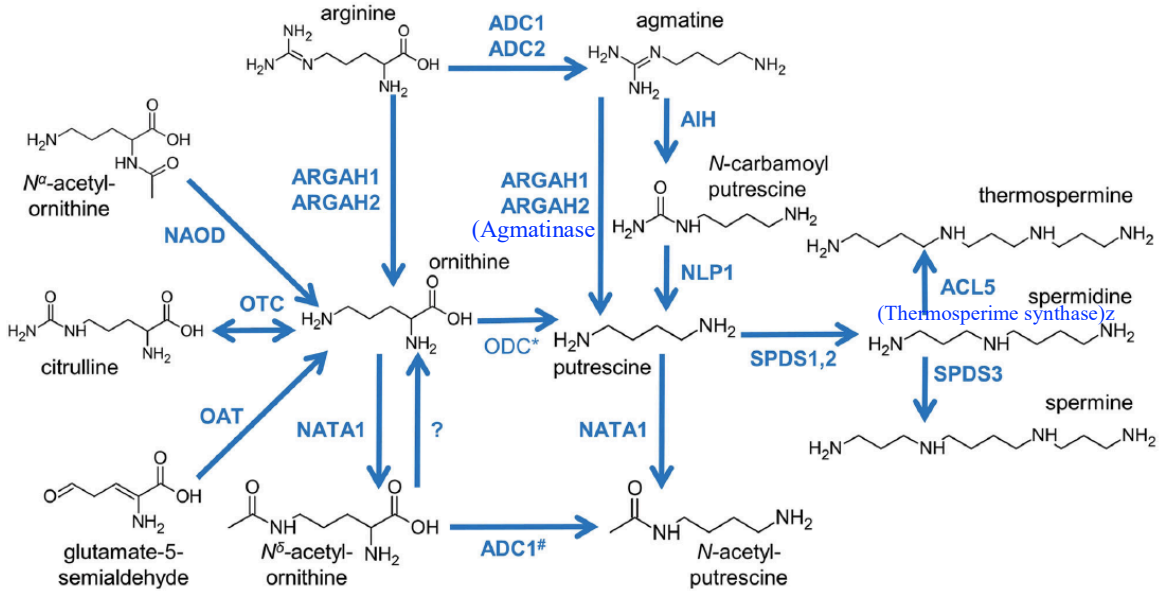

Fig. 2. Biosynthetic pathways of polyamines in plants. Abbreviations refer to following enzymes. ODC=ornithine decarboxylase, ADC= arginine decarboxylase, ARGAH= arginine aminohydrolase (arginase), NATA= N-acetyltransferase activity, OCT= ornithine-carbamoyl transferase, AIH= agmatine iminohydrolase, SPDS= spermine/spermidine synthase, NAOD= N $^{\alpha}$ -acetylornithine deacetylase, ACL= thermospermine synthase (aucaulis), NLP= N-carbamoylputrescine amidohydrolase (nitrilase-like protein). Figure was adapted from Lou et al (2020).

Polyamines could also be converted to another PA depending on the need for each PA. The interconversion is important for non-dividing cells. Spermidine and Spm are first formed via the Spd and Spm synthases as described above. Spermine can be interconverted to Spd and Spd can be interconverted to Put via a process called acetylation catalyzed by Spd/Spm acetyltransferase. The resulting acetyl-Spm/Acetyl-Spd are subjected to oxidative splitting via PA oxidase releasing Spd or Put and 3-acetamidopropanal which will subsequently be transformed to β-alanine. β-alanine would be further catabolized by transamination. Spd/Spm acetyltransferase acts as the rate-limiting enzyme in the interconversion of PAs (Urdiales et al. 2001). Although the PA interconversion has been well described in mammalian cells and plants, there is no clear *in-vivo* evidences of such interconversion in other eukaryotic organisms such as fungi and oomycetes (Moschou et al. 2008; Tavladoraki et al. 2006).

1.2.2 Polyamine conjugation

Polyamines also exist as conjugated forms by conjugating to hydroxycinnamic acid amides or to proteins. Polyamines that are conjugated with hydroxycinnamic acids such as coumaric acid, ferulic acid or caffeoyl acid are widely found in many plant families (Bagni and Tassoni 2001; Martin-Tanguy 1997). The majority of polyamines conjugate with monomers of these hydroxycinnamic acids (perchloric acid-soluble fraction) while they also can conjugate with dimers of hydroxycinnamic acids (perchloric acid-insoluble fraction). Hydroxycinnamoyl transferase and acyltransferase are enzymes that are known to be involved in PA conjugation process (Alcazar et al. 2010). These conjugate reservoirs regulate PA concentration inside the cell. They are also implicated in multiple functions including detoxification of phenolic compounds and plant defense responses against pathogens and insects (Takano et al. 2012;

Tanabe et al 2016; Tiburcio et al 2014). Polyamine conjugates represent a major pool of polyamines in oomycetes (Chibucos and Morris, 2006).

1.2.3 Polyamine transport

Cellular PA level is also controlled by movement of PAs from the external environment into cells and between organelles. Membrane PA transport systems are extensively described in bacteria and unicellular eukaryotes but not very many are characterized in other eukaryotic groups such as fungi or oomycetes. In *E. coli*, PA uptake is mediated mainly by two ATP binding cassette (ABC) type transporters (Fig. 3). One is a Spd-preferential uptake system which consists of an ATPase (PotA), Channel forming proteins (PotB and C) and a substrate binding protein (PotD). The other uptake system, PotFGHI, has a similar conformation and is Put specific (Igarashi & Kashiwagi, 2000). *Escherichia coli* also has a third system of PA transport which includes the two antiporters, PotE and CadB (Fig. 3). PotE is a Put-Orn (putrescine-ornithine) antiporter while CadB is a Cad-Lys (cadaverine-lysine) antiporter. PotE and CadB proteins consist of 12 transmembrane domains with the NH_2- and the COOH-termini located in the cytoplasm (Igarashi & Kashiwagi, 2000, 2010). Additionally, two other PA transport systems have been identified in *E. coli*. One is a protein complex MdtJI, belonging to the multidrug resistance family that excretes Spd (Fig. 3) (Higashi et al 2008). The other transporter is a Put importer PuuP (Fig. 3), which is a part of a Put utilization pathway that involves gamma-glutamyl intermediates (Kurihara et al. 2009).

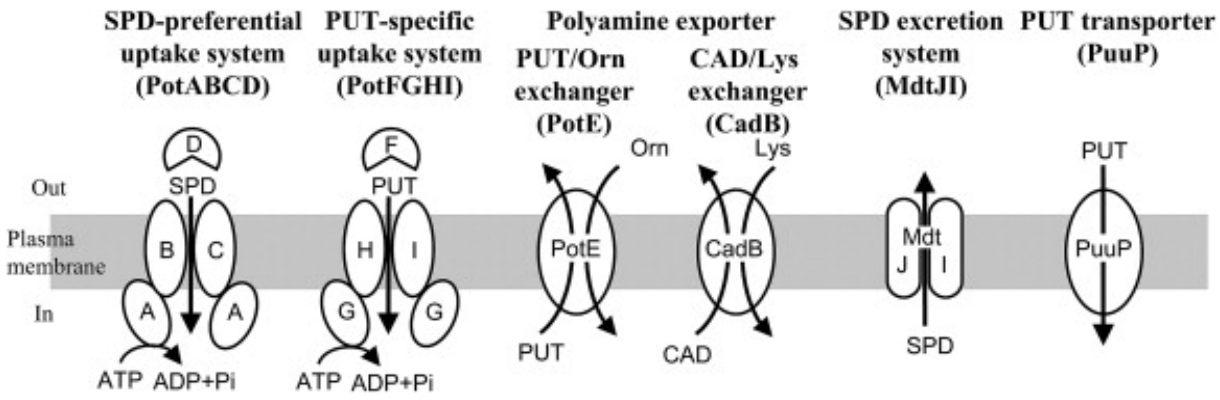

Fig. 3. Polyamine transporters of *E.coli* (Adapted from Igarashi & Kashiwagi, 2010). Spd = spermidine, Put = putrescine, Orn=ornithine, CAD = cadaverine, Lys = lysine.

In yeast, there are at least 10 transmembrane proteins that are known to transport PAs (Miller-Fleming et al. 2015). Three transporters located in the plasma membrane are involved in PA uptake namely DUR3, SAM3 and GAP1, all of which are members of APC superfamily (Fig. 4). Of these, Dur3 and Sam3 function as main importers. DUR3 imports PAs together with urea while SAM3 was found to import PAs along with SAM, glutamic acid, and lysine (Uemura et al. 2007). GAP1 was characterized as a membrane protein that imports PAs and a wide variety of amino acids (Uemura et al. 2005). UGA4 is a membrane protein located on the vacuolar membrane and is responsible for the uptake of Put and GABA into the vacuole in yeast (Uemura et al. 2004). Aouida et al (2005) reported another plasma membrane protein involved in PA import, AGP2, which was later identified as a plasma membrane sensor that regulates transporter genes based on environmental signals (Aouida et al. 2013).

The PA export occurs through four proton gradient-dependent efflux pumps located on the plasma membrane of yeast; they are TPO1- TPO4 which belong to the H^+/drug antiporter subfamily of the major facilitator superfamily. The TPO permeases generally consist of 12 transmembrane domains and they export PAs and a variety of organic compounds such as quinidine and cycloheximide. A fifth TPO permease, TPO5, has also been characterized which is

located in either Golgi or post-Golgi vesicles. TPO5 is found to be involved in the vesicle-mediated efflux of PAs (Tachihara et al. 2005). The well-studied transporters TPO1 and TPO4 export Spd and Spm while TPO2 and TPO3 exports Spm. TPO5 excretes mainly Put and to a lesser extent, Spd (Miller-Fleming et al. 2015).

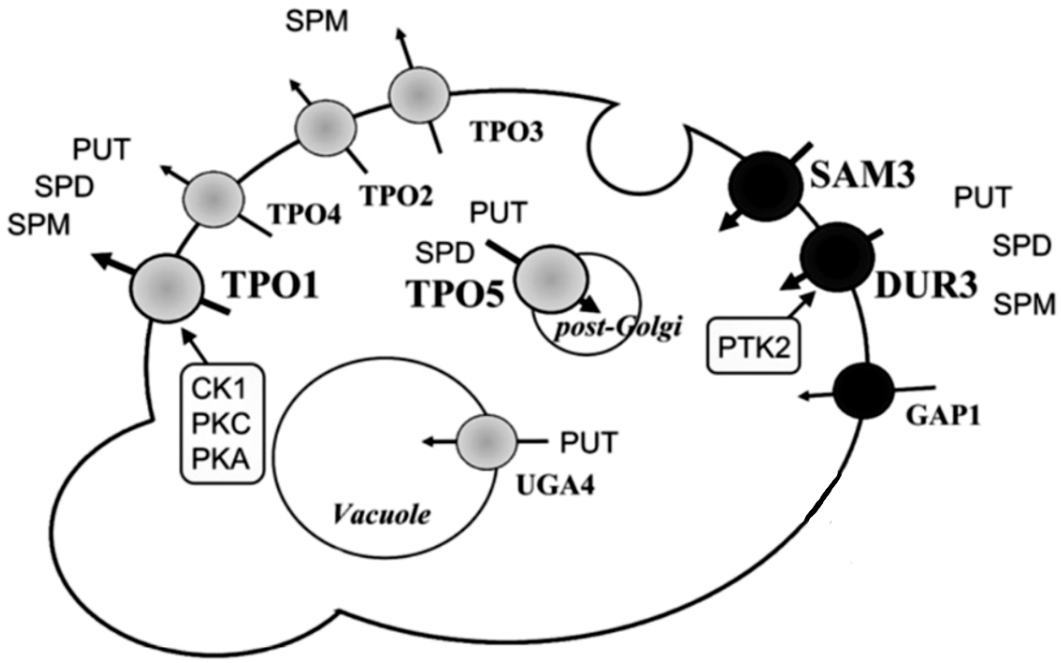

Fig. 4. Yeast polyamine transporters and their localizations (Modified from Igarashi & Kashiwagi, 2010*).*

Polyamine transporters have also been studied in protozoan parasites such as *Trypanosoma cruzi* and *Leishmania major* (Hasne et al. 2005, 2010). Polyamine uptake from external sources is crucial for these organisms since they lack some parts of PA biosynthetic pathways (Miller-Fleming et al. 2015). The knowledge about PA transporters in animal cells is limited. It is proposed that the mechanisms that PA transport in animal cells might be different from those of prokaryotic and other eukaryotic systems. One of the mechanisms through which PA uptake occurs is through endocytosis. PAs are imported by a plasma membrane carrier protein followed by sequestration into PA-sequestering vesicles (Belting et al. 2003). Soulet et al

(2004) proposed that PAs first enter the cytoplasm via a plasma membrane transporter or a channel from the external environment. The cell surface protein named glypican-1 assists the above PA-transporter binding (Belting et al. 2003). Next, PAs are internalized by PA-sequestering vesicles powered by Vacuolar-type H^+ -ATPase and enter the intracellular vesicle trafficking pathway. The sequestered PAs could also be released back to the cytosol (Soulet et al. 2004). A vesicular neurotransmitter transporter was also described by Hiasa et al (2014) in human neuronal cells. This vesicular PA transporter VPAT belongs to the SLC18 transporter family and is involved in the transport and storage of mono-, di-, tri- and tetraamines. In addition to the vesicle-mediated PA export in animal cells, a diamine exporter has been identified by Uemura et al (2008). In this system, Put export is mediated via arginine-Put exchange mechanism. The arginine-diamine exchange occurs via formation of a complex by the two proteins SLC3A2 and SAT1. A few other transporters that have other functions such as SLC7 (Lys/Arg/Orn permeases), CCC9 (an inorganic ion transporter) and OCT6 (cation/anion/zwitterion transporter) are proposed to uptake PAs (Abdulhussein and Wallace, 2014; Poulin et al. 2012) in animal cells.

Similar to animals, studies on PA transport mechanisms in plants are also limited. Plant PA uptake has also been studied in some plants at cellular and subcellular level. DiTomaso et al (1992) showed that, in maize root cells, Put is transported in and out of the vacuoles across tonoplast and then into the apoplasm across plasma membrane using an energy-dependent and protein mediated system of transport. Plant PA transport systems have been identified by sequence homology to known PA transporters from other organisms. OsPUT1, a Spd-preferential PA uptake protein in rice is the first plant PA transporter that has been functionally characterized (Mulangi et al. 2012b). Subsequent work showed that OsPUT1 is localized to the

ER in Plants (Ahmed et al. 2017). Five Arabidopsis proteins AtPUT1-AtPUT5 have also been identified and the function of AtPUT1-AtPUT3 has been characterized by the same group (Mulangi et al. 2012a and 2012b). OsPUT and AtPUT membrane proteins belong to the L-type amino acid transporter (LAT) family, a member of amino acid-polyamine-organocation (APC) superfamily (Fujita and Shinozaki, 2014). APC membrane proteins possess 12, 13 or 14 transmembrane segments with N and C termini facing the cytoplasm (Hu and King, 1998; Jack et al. 2000). In mammals and bacteria, LATs are Na^+ independent exchangers of common amino acids and facilitate a 1:1 exchange of different amino acids (Errasti-Murugarren et al. 2019). They also function as transporters of PAs and paraquat (a PA analogue) (Fujita et al. 2012). The protein structure of LAT has 12 transmembrane segments in general and sometimes is associated with transmembrane glycoproteins for its activity (Wipf et al. 2012).

1.2.4 Polyamine interconversion and terminal degradation

Catabolism of PAs occur through acetylation and oxidation of PAs, producing either lower polyamines or other PA-derivatives that cannot be recycled back to PAs. Production of lower PAs from higher PAs (Spd and Spm) is the PA interconversion described in 1.2.1 above. The PA interconversion occurs in two steps. First is acetylation of the aminopropyl group of Spd/Spm using acetyl CoA as the acetyl donor producing N^1-acetylspermidine and N^1-acetylspermine (Lou et al. 2016). Acetylation reduces the net positive charge of PAs preventing their interactions with anionic binding sites. These acetyl derivatives are mostly exported from normal cells and only some are used for back conversion of PAs. The second step of PA interconversion is oxidation of Spd or Spm to lower polyamines Put or Spd respectively. The oxidases that catalyze cellular oxidation require different cofactors. Diamine oxidases rely on Cu^{2+} and pyridoxal phosphate as their cofactors. Oxidation of the diamine Put yields H_2O_2, NH_3

and 4-aminobutanal (Zarei et al. 2016). The 4-aminobutanal cyclize to form Δ^1 -Pyrroline which is then converted to GABA by pyrroline dehydrogenase enzyme. Afterwards, GABA is converted into succinate which subsequently enters the tricarboxylic cycle (Chen et al. 2019). Polyamine oxidases (PAOs) are flavin adenine dinucleotide (FAD)-containing enzymes that are involved in final degradation as well as back-conversion of PAs. Unlike diamine oxidases, the substrates of PAOs are higher PAs, such as Spd, Spm and Tspm. Hydrogen peroxide and GABA are two important products of PA oxidation which play significant roles in many biological processes. The H_2O_2 is involved in the signal transduction during plant stress responses (Freitas et al. 2018; Gupta et al. 2013). Final degradation of PAs recycles C and N through tricarboxylic cycle (Wuddineh et al. 2018).

1.3 Regulation of polyamine metabolism

1.3.1 Regulation of biosynthesis

1.3.1.1 Regulation of ornithine decarboxylase

Ornithine decarboxylase is the most studied enzyme that participates in the PA metabolism. In all eukaryotes, Put is biosynthesized via decarboxylation of ornithine by ODC. However, unlike animals, plants exhibit a second mechanism to produce Put via ADC which is an enzyme regulated independently of ODC pathway. Mammalian ODC has a very short half-life and its activity is under a strong feedback control exerted by PAs. This regulation occurs through synthesis of a protein called antizyme which is induced by the presence of excess PAs. The ODC is inactivated by antizyme by a sophisticated mechanism. Mainly, antizyme binds to ODC which exist as a homodimer forming an inactive heterodimer. Upon binding to antizyme, the C terminus of ODC is exposed which is a signal for recognition by 26 proteasomes. Next, the ODC:antizyme heterodimer separates, directing the ODC to degradation (Hayashi et al. 1996;

Kahana 2007). Upon release, the antizyme is recycled to degrade more ODCs. Mammalian antizyme mRNA contains two open reading frames (ORFs) where translation of the antizyme requires a +1 ribosomal frameshifting before reading the stop codon of the first ORF (Bekaert et al. 2010). The structure of antizyme is highly conserved across higher animals thus posing a strong regulatory control on ODC turnover. Furthermore, molecular cloning of mammalian *ODC* gene suggest that the ODC mRNA contains a long 5' upstream untranslated region (5'UTR) is involved in the translational regulation of the enzyme. The GC-rich nature which favors formation of a stem-loop structure and the presence of an upstream open reading frame in the 5' UTR repress the translation initiation of the ODC enzyme (Perez-Leal and Merali 2012).

Studies on plant ODC show that plant ODC is homologous to other eukaryotic ODCs. However, plant ODC is not under the feedback control of PAs (Fuell et al. 2010). Plants do not contain antizyme proteins and the plant ODC does not bind to the human antizyme (Illingworth and Michael 1998). Most plants in general have a single ODC gene in their genomes. Surprisingly, *Arabidopsis thaliana*, most *Brassicaceae* plants and a moss, *Physcomitrella patens* (Fuell et al. 2010) lack ODC from their genomes. In contrast, *Arabidopsis thaliana* and *Brassicaceae* plants have two copies of ADC which arose from a gene duplication event. These evidence suggest that certain plants (E.g. Arabidopsis) are dependent on the ADC pathway for PA biosynthesis and the ODC pathway is not required for their normal growth (Fuell et al. 2010; Carbonell and Blázquez 2009).

1.3.1.2 Regulation of arginine decarboxylase

Arginine decarboxylase (ADC) represents an alternative pathway to biosynthesize Put in higher plants and some bacteria. Arginine decarboxylase was first studied in bacteria and *E. coli.* ADC exists in two forms. First is the biosynthetic form which is constitutively expressed

irrespective of exogenous arginine. Second is biodegradative form which is induced by addition of exogenous arginine (Tiburcio et al. 1997). The biosynthetic ADC of *E. coli* is encoded by the gene *speA*, and synthesized as a 74 kDa precursor which is processed post-translationally to a 70 kDa mature protein (Moore and Boyle 1990). Putrescine and Spd feedback-inhibit ADC by repressing speA gene (Tiburcio et al. 1997) in *E. coli*.

The Plant ADC genes share homology to bacterial ADC genes but the plant protein is longer than the bacterial proteins as the plant ADC proteins contain organellar transit peptides. One of the plant ADCs localizes to chloroplasts while the other is localized to endoplasmic reticulum (Bortolotti et al. 2004; Lou et al. 2020). Plant ADCs contain conserved substrate binding sites present in all eukaryotic ADCs as well as ODCs. The mechanism of eukaryotic ADC activity is also very similar to that of eukaryotic ODCs. The first identified plant ADC gene was from oats (Bell and Malmberg 1990). Unlike E. coli ADC, the oat ADC was synthesized as a 66 kDa precursor which is cleaved to two polypeptides of 24 kDa and 42 kDa in size that are linked by disulfide bonds to form the active ADC enzyme (Malmberg and Cellino 1994). At least one ADC gene is present in all plant genomes but ADC-route to Put biosynthesis has been lost from some green algal lineages (Fuell et al. 2010). It is hypothesized that the eukaryotic ADC may be under the translational and/or post-translational regulation (Tiburcio et al. 1997). For instance, Spm (and to a lesser extent Spd) inhibits post translational processing of ADC precursor in osmotically stressed oat leaves (Borrell et al. 1996). Plant PA biosynthetic enzymes are affected by a wide array of stress reactions and physiological stimuli (Borrell et al. 1996; Borrell and Malmberg 1996). For example, the two *Brassicaceae* ADC genes are regulated differently to stress signals such as methyl jasmonate or wounding (Perez-Amador et al. 2002).

Similarly, in animals, all these PA biosynthetic enzymes are regulated by a wide range of hormonal and cell-growth related cues.

1.3.1.3 Regulation of S-adenosylmethionine decarboxylase

Polyamine metabolism is significantly influenced by S-adenosylmethionine decarboxylase (SAM decarboxylase) enzyme. It performs an important rate-limiting step of synthesizing decarboxylated SAM which serves as the donor of the aminopropyl group for the biosynthesis of the two higher PAs, Spd and Spm, catalyzed by the enzymes Spd synthase and Spm synthase, respectively (Li et al. 2019). Similar to other PA biosynthetic enzymes discussed above, SAM decarboxylase also has a short half-life and its synthesis is controlled by PAs at translational level. Mammalian SAM decarboxylase is well studied and is synthesized as a proenzyme which is then processed into two subunits forming the active enzyme. Similar to the feedback regulation of ODC, SAM decarboxylase is also translationally regulated by polyamines. In both animal and plant systems, Spd and Spm feedback-inhibit SAM decarboxylase while Put stimulates its synthesis (Majumdar et al. 2017; Stanley and Pegg 1991). Both animal and plant SAM decarboxylase mRNAs have two upstream ORFs in their 5’ UTRs. These upstream ORFs are translated in a PA-dependent fashion where the ORFs themselves or their products regulate translation of SAM decarboxylase mRNA (Ivanov et al. 2010). SAM decarboxylase which catalyzes decarboxylation of SAM for synthesizing PAs is also involved in the S recycling pathway called Met cycle or Yang cycle. 5’-methylthioadenosine (MTA) resulting from the transfer of aminopropyl group from dcSAM will be recycled to methionine via Yang cycle (Pommerrenig et al. 2011).

1.3.2 Regulation of polyamine interconversion and catabolism

Regulation of SSAT:

Spermidine/Spermine acetyl transferase is the rate-limiting enzyme in PA interconversion and catabolism. The SSAT acetylates the aminopropyl group of Spd/Spm using acetyl CoA as the acetyl donor. According to the studies that involve animal cell lines, the SSAT levels are regulated tightly at the transcriptional, translational and post-translational level, therefore, the cellular SSAT concentration is generally low (Perez-Leal and Merali 2012). Transcription of the SSAT gene is regulated by binding of two interacting transcription factors: nuclear erythroid factor 2 (NRF2) and polyamine-modulated factor 1 (PMF1) to the polyamine-response element in the promoter region (Wang et al. 2002). Increase in PA level would lead to interaction of this NRF2-PMF1 complex with the polyamine-response element enabling SSAT gene activation. Other non-PA factors and stress stimuli such as hypoxia are also evidenced to activate the SSAT gene (Perez-Leal and Merali 2012). Hyvönen et al (2006) demonstrated that exogenous PAs and PA-analogoues regulate SSAT expression by manipulating alternative splicing to generate a defective protein via exon inclusion which contains a premature stop codon. Increase of PAs and their analogues inhibit the synthesis of defective protein while depletion of PAs promote it. It is also suggested that the free PA level regulates SSAT activity at translational level through a translational repressor (Butcher et al. 2007). Translational control of SSAT expression is poorly understood. Once the cellular mechanisms of SSAT regulation are identified, it will provide new therapeutic potentials for human diseases such as Parkinson disease by modulating SSAT activity (Lewandowski et al. 2010).

1.3.3. Regulation of polyamine transport

Cells are equipped with mechanisms to uptake and export PAs from and to their external environment. Polyamine transport has been thoroughly studied in bacteria. *Escherichia coli* exhibits five types of transport mechanisms. Two of them are ABC transporters which are responsible for uptake of either Put or Spd (PotABCD and PotFGHI, respectively). The two ABC transporters consist of subunits that have a ATP-binding site as well as a PA binding site. The PA uptake decreases upon accumulation of PAs in the *E. coli* cells. The inhibitory effect of Spd on the ATPase activity of PotA was considered as one of the reasons for PA-mediated inhibition of PA uptake. A second scenario was described by Antognoni et al (1999) where the Spd-binding protein, PotD, functions as a transcriptional regulator by binding close to the transcriptional initiation site of the operon which encodes the four subunits of Spd uptake system. It has been also suggested that more than the PotD protein itself, PotD precursor is responsible for the transcriptional inhibitory effect.

The third type of transporters are PotE and CadB exchangers (antiporters) that mediate transport of either Put or Cad, respectively (Igarashi and Kashiwagi, 2010). These exchangers can mediate both uptake and excretion of Put/Cad (Fig. 5). *CadB* gene, which encodes CadB exchanger and *cadA* gene which encodes an inducible lysine decarboxylases are located in one operon, *cadBA* (Meng and Bennett, 1992). The PA uptake occurs at neutral pH while excretion occurs at acidic pH by antiporter activity (Igarashi and Kashiwagi, 2010). The regulation of CadB seemed to occur at transcriptional level through the product of the *cadC* gene located adjacent to the *cadBA* operon. CadC protein senses the pH and the presence of lysine and as a result, CadC activates transcription of the *cadBA* operon. It has also been proposed that LysP, a lysine transporter negatively regulates *cadBA* operon in the absence of lysine (Neely and Olson,

1996). At acidic pH CadB along with inducible lysine decarboxylase neutralize the medium and produce Cad and CO_2. The proton gradient will be generated by consumption of protons by lysine decarboxylase resulting in an increase of ATP in cells.

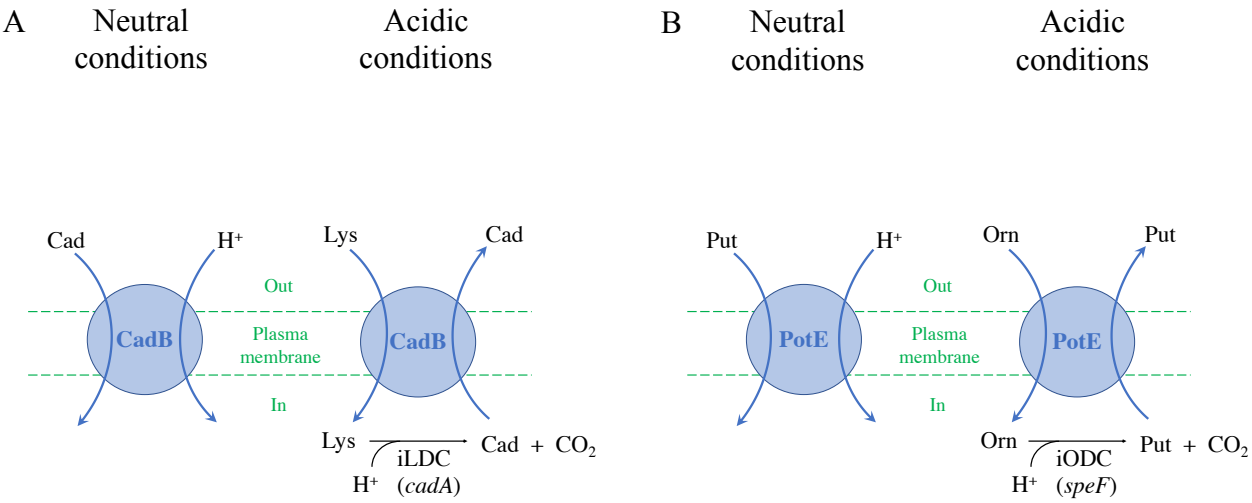

Fig. 5. A) Function of A) CadB, cadaverine transporter and B) PotE, Putrescine transporter, under neutral and acidic conditions. *cadA* = inducible lysine decarboxylase gene, iLDC = inducible lysine decarboxylase, *speF* = inducible ornithine decarboxylase gene, iODC = inducible ornithine decarboxylase.

Similar to the CadB system, the *speF* gene, which encodes inducible ornithine decarboxylase, and *potE* gene, which codes for PotE transporter, form a single operon. Acidic pHs induces the expression of *speF-potE* operon at transcriptional level. Regulation of *speF-potE* operon is found out to be mediated by RNase III, an endonuclease that is necessary for processing precursors of a variety of noncoding RNAs such as ribosomal RNA and small interfering RNA. It also cleaves incorrectly processed mRNAs serving certain regulatory functions (Nicholson 2014). *E. coli* RNase III cleaves the 5' UTR of the *speF-potE* mRNA and thus enhances the expression of inducible ornithine decarboxylase (Kashiwagi et al. 1994). However, it is not clear how RNase III is activated at acidic pHs. The presence of ornithine is

also required for the induction of *speF-potE* operon but the mechanism behind it remains to be sought.

In eukaryotes, it is unclear whether the PA transport and regulation is carried out by a single protein or several proteins. Several studies have explored the regulation of PA uptake in yeast and it has been suggested that one of the mechanisms for regulation of PA uptake is phosphorylation and dephosphorylation by kinases. Genetic screens in the yeast systems have identified three serine/threonine kinases, PTK, PTK2 and NPR1(Kakinuma et al. 1995; Kaouass et al. 1997, 1998) and one serine-arginine protein kinase, SKY1 (Erez and Kahana, 2001). For instance, PTK2 has been shown to phosphorylate tyrosine and serine residues of DUR3 to regulate the PA uptake in yeast (Igarashi & Kashiwagi, 2010). Phosphorylation is a mechanism that is extensively adopted by cells to regulate cellular functions such as transport. However, the kinases could serve as either substrates or inhibitors of transporters (I Stolarczyk et al. 2011).

The function of TPO1 to TPO4 is similar to that of *E. coli* CadB and PotE. The PA uptake occurs alkaline pHs (~8) while excretion occurs at acidic pHs (~5). Since yeast cells generally grow at acidic pHs, the TPO1 to TPO4 serve as exporters. However, the regulation of these TPOs may be different from that of PotE and CadB as TPO1 protein contains a longer hydrophilic NH2-terminus which includes serine and threonine residues, indicating that these TPOs is regulated by kinases. The phosphorylation of serine and threonine residues has enhanced the TPO1 transport activity as well as sorting of TPO1 from endoplasmic reticulum, suggesting that kinases positively regulated the TPOs (Uemura et al. 2004).

The plasma membrane protein AGP2 in yeast is shown to be involved in regulatory function in addition to its transport function. It is proposed that AGP2 could sense molecules such as PAs and modulate transcription of multiple genes including genes that encode main two

high affinity PA transporters SAM3 and DUR3 and regulatory kinase SKY1 (Aouida et al. 2013). Transcription factors that are responsible for activating above downstream genes in response to ligand binding of AGP2 have not been identified yet.

Mammalian PA transport is reported to be regulated by several factors such as internal PA levels, insulin and other growth factors (Kano and Oka 1976). PA uptake is enhanced when intracellular PA level is low. Not many studies have been done to seek regulatory mechanisms behind mammalian PA uptake. Antizyme, the regulatory protein that accelerates degradation of ODC is also involved in inhibition of PA uptake in mammalian cells (Mitchell et al. 1994). Synthesis of antizyme is induced by the presence of excess PAs. Antizyme negatively regulates PA uptake by inhibiting the PA transport system at the plasma membrane (Ramos-Molina et al. 2018). However, the mechanism by which antizyme inhibits the transporters is still an enigma. In addition to antizyme-mediated regulation, it has been reported that a protein termed TATA-binding-protein-associated factor 7 encoded by *TAF7* gene serves as a regulator for mammalian PA transport (Fukuchi et al. 2004).

Similar to all other eukaryotes, polyamines have been instrumental in growth, development and stress responses in plants. Unlike animals, plants are unable to escape harsh environments and therefore they are evolutionarily adapted to carry a wide array of protective metabolites such as PAs (Liu et al. 2015). The key plant-PA biosynthetic gene ADC and several other PA-biosynthetic genes are upregulated resulting accumulation of PAs under stress conditions (Liu et al. 2015). Five LAT family transporters have been identified in Arabidopsis, AtPUT1-AtPUT5 (PUT1-PUT5), while three of them (PUT1-PUT3) are known to transport PAs (Fujita and Shinozaki 2014; Mulangi et al. 2012a). In eukaryotes, cellular membrane proteins regulate their functions in response to environmental cues, and some membrane proteins form

complexes with each other upon ligand binding in order to regulate their transport activity. Calcineurin B-like protein-interacting protein kinases (CIPK) are identified as essential kinases involved in plant stress-responsive pathways such as salt overly sensitive (SOS) pathway. SOS2 is a CIPK kinase identified in Arabidopsis, which phosphorylates the Na^+/H^+ antiporter SOS1 to enhance salt tolerance (Quintero et al., 2002). Chai et al 2020 demonstrated that Arabidopsis PUT3 is also phosphorylated by SOS2 kinase as a response to heat stress. They propose that SOS2 is activated by heat stress by an unknown mechanism and as a result, SOS2 forms a complex with the two membrane proteins SOS1 and PUT3. As a result of these protein-protein interactions and phosphorylation of PUT3 by SOS2 kinase, the PA transport activity of PUT3 is enhanced. Increased concentration of PAs in the cytosol might be involved in stabilizing molecules such as mRNAs. Abiotic stress factors cause accumulation of ROS (reactive oxygen species) in cells causing cellular damage. It is suggested that increased PA level through the SOS2-SOS1-PUT3 pathway might act as anti-ROS molecules to mitigate oxidative stress. Sequence analyses have shown that Arabidopsis SOS2 (CIPK24) and some other CIPKs share sequence similarity to the kinase PTK2 in yeast (Chai et al. 2017). Therefore, SOS2-PUT3 activation pathway could be a conserved mechanism across eukaryotes for regulating PA uptake (Chai et al. 2017).

Polyamine transport has been extensively studied in the fungi *Neurospora crassa* and *S. cerevisiae* (Hoyt and Davis, 2004). However, the role of PA transport and its regulation in plant-pathogen interaction is one of the avenues that has been least studied. Several PA biosynthesis inhibitors have been used as a strategy to control phytopathogenic fungi (Barker et al. 1993; Gamarnik et al. 1994; Walters et al. 1995). Although this was perceived as a good method of disease control, some phytopathogenic fungi were less sensitive to these inhibitory effects

(Galston and Weinstein, 1988; Pieckenstain et al., 2001; West and Walters, 1989). A possible reason for the failure of these treatments could be due to increased uptake of PAs. For the first time, West and Walters (1991) reported that the soilborne fungal pathogen *Fusarium culmorum* could uptake ^{14}C-labelled polyamines in a pH dependent and biphasic fashion. Subsequently, very few reports on PA uptake by other phytopathogenic fungi and oomycete pathogens have been published (Chibucos and Morris, 2006). Exploration of PA transport mechanisms in phytopathogens leads to development of new disease control strategies possibly in combination with PA biosynthesis inhibitors. In light of this, identification and characterization of PA transport proteins especially in inferiorly-studied phytopathogens such as oomycetes is necessary. The major goal of this study is to explore PA biology in oomycetes and to characterize PA transporters in *Phytophthora* pathogens. The focus of this research is to identify a key PA transporter in *Phytophthora* species and to characterize its function using a yeast system.

1.4 Objectives

- To identify putative genes involved in polyamine metabolism in *Phytophthora*.
- To identify a putative polyamine transporter that is expressed in the zoospores of *P. parasitica.*
- To determine subcellular localization of the PA transporter PPTG_00424 using *P. sojae* as a model system.
- To demonstrate that PPTG_00424 is a PA transporter using a yeast system.

CHAPTER 2. BIOINFORMATIC ANALYSIS OF POLYAMINE BIOSYNTHESIS, TRANSPORT AND ITS REGULATION IN OOMYCETES

2.1 Introduction: Polyamine metabolism in oomycetes

2.1.1 What are oomycetes?

Oomycetes are a distinct group of fungus-like filamentous microorganisms that belong to the kingdom Straminopila (also known as heterokonts), in the supergroup Chromalveolata (Cavalier-Smith and Chao 2006; Thines 2014). Chromalveolata is a recent refinement of the former kingdom Chromista. Chromalveolates are proposed to be originated through multiple endosymbiotic events (Fig. 6). The primary endosymbiosis event explains the origin of green algae, glaucophytes and land plants, where a mitochondrial eukaryote acquired a cyanobacterial endosymbiont. Chromalveolates which include stramenophiles are believed to have originated from a series of endosymbiotic events in which a non-photosynthetic eukaryotic cell first engulfed a green algal cell, followed by another endosymbiotic capture of a red algae (Bhattacharya et al. 2007; Prihoda et al. 2012). These events involve depletion of the consumed endosymbiont and transfer of many endosymbiont genes to the host nucleus as well as loss of genes from the endosymbiont (Cavalier-Smith and Chao 2006; Rogers, 2011) (Fig. 6). Heterokonts are a major group of chromalveolates, and are characterized by two flagella of unequal length. Heterokonts consist of multiple groups of photosynthetic algae such as diatoms, yellow-green algae, brown algae and golden-brown algae and heterotrophic oomycetes such as Saprolegniales and Peronosporales. Molecular timescale analyses suggest that oomycetes have diverged as a separate group from the common ancestor of diatoms approximately 400 million years ago (Matari and Blair, 2014).

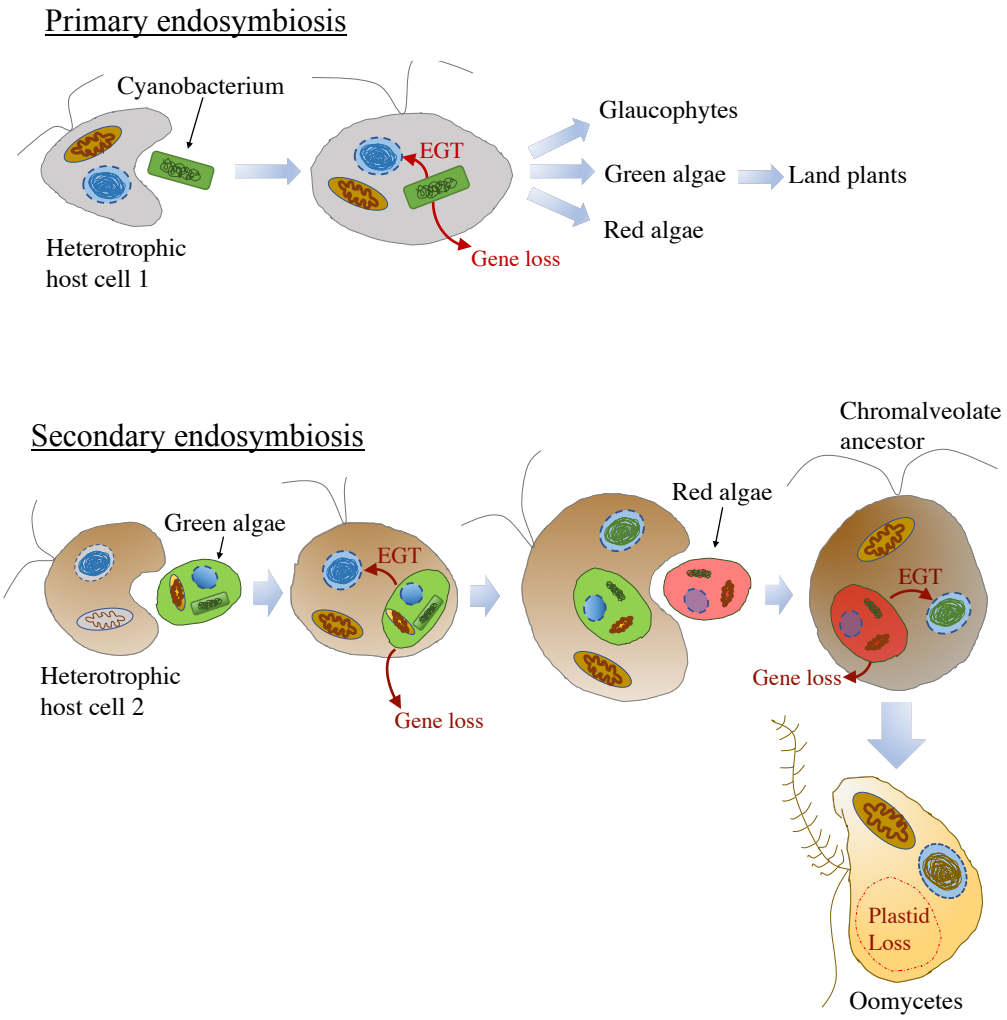

Fig. 6. A visual representation of the endosymbiotic events of evolution. The upper panel shows the primary endosymbiosis giving rise to green algae and land plants where the lower panel shows the secondary endosymbiosis leading to evolution of oomycetes. EGT = endosymbiotic gene transfer (modified from Prihoda et al. 2012).

The organelles transferred via the endosymbiotic events have been lost from the oomycetes, however, genomic footprints of the endosymbiont genomes were found in oomycete genomes. A study by Tyler et al (2006) reports that 855 putative oomycete genes originate from its photosynthetic ancestor, either the red algal or the cyanobacterial endosymbiont. A surprising feature of oomycete genomes is the large number of fusions and rearrangements of the genes involved in the primary metabolic pathways such as amino acid biosynthesis. The metabolic gene repertoire is diverse and varies across oomycete lineage (Rodenburg et al. 2020). It has been suggested that most of this diversification probably occurred after the separation of oomycetes from diatoms. These gene fusion events have led to the presence of multifunctional proteins that increase the efficiency of metabolic pathways. Several studies report that horizontal transfer of genes from prokaryotes as well as eukaryotes had significantly contributed to the oomycete genome complexity (Richards and Talbot, 2007). Tyler et al. (2006) show that horizontal gene transfers may have occurred from the primary cyanobacterial endosymbiont genome as well as from other independently acquired bacterial genomes. Richards and Talbot (2007) show that the genomes of the oomycetes *P. sojae* and *P. ramorum* contain genes that descend from at least three eukaryotic lineages; the initial heterokont ancestor, red algal endosymbiont and true fungi. It has been proposed that this horizontal acquisition of fungal genes may have led to the osmotrophic and filamentous behavior of oomycetes (Richards and Talbot, 2007).

2.1.2 Polyamine metabolism in oomycetes

Polyamines (PAs) are equally important for growth and proliferation of oomycete pathogens as disruption of polyamine metabolism had implicated in their growth retardation. In the last few decades, compounds that inhibit PA biosynthesis have been tested as a means of

controlling fungal and oomycete diseases in plants. Most widely used inhibitors were L-alpha-difluoromethylornithine (DFMO) and 1-4 diamino butanone (DAB), both of which inhibit ODC enzyme. When these ODC inhibitors are added, only the pathogens' PA biosynthesis is compromised while the plant host can synthesize the needed PAs via the alternate ADC pathway. Barker et al (1993) and Walters et al (1995) reported that inhibition of polyamine biosynthesis using DFMO resulted in significant hyphal growth reduction of *Phytophthora infestans.* Furthermore, this growth reduction could also be reversed by the addition of polyamines externally.

To date, how PAs are being metabolized in oomycetes and their role in regulating cellular processes is largely unknown. Multiple evidence suggests that oomycetes are able to synthesize at least four PAs including the three common PAs; Put, Spd, Spm and Tspm; a major plant PA (Valdés-Santiago et al. 2012). These authors reported bioinformatic evidence for the presence of ODC, Spd synthase, Spm synthase and PAO in few oomycetes. *Phytophthora* is a genus of oomycetes that causes devastating diseases in both natural and agricultural systems. Some *Phytophthora* members such as *P. sojae* and *P. infestans* have a very narrow host range while others such as *P. ramorum* and *P. parasitica* have a very broad host range such as. There have been very few studies focusing on PA metabolism in *Phytophthora* species and the role of PAs in *Phytophthora*-host interaction.

Since *Phytophthora* is a group of pathogens that is facultatively parasitic on plants, uptake of essential polyamines from either their host or environment must be crucial to them. Similar to all oomycetes, *Phytophthora* produce motile spores called zoospores which is the primary mode of infecting a host. Chibucos and Morris (2006) showed that zoospores of *P. sojae* can take up polyamines from the external environment. The same group suggest that *P. sojae*

expresses at least two high affinity polyamine transporters at the zoospore stage and the zoospore survival and fitness in the soil depend on the internal polyamine stores as well as the uptake (Chibucos and Morris, 2006). Therefore, PA uptake system could be a strategy to manipulate oomycete PA metabolism, thus providing a way to control oomycete diseases. Previous phylogenomic studies suggest that oomycete genomes contain putative PA transporters with significant homology to plant PA transporters (Mulangi et al. 2012b).

2.2 Hypothesis and research goals

Numerous studies have investigated the PA metabolism of plants in the context of plant-pathogen relationships, but only a few studies have investigated the PA biology from the side of the pathogen. Even fewer reports have analyzed the PA metabolism in oomycete pathogens. This study was carried out with the hypothesis that *Phytophthora* genomes contain genes that are involved in PA metabolism. The major goal of this part of the project is to identify a potential PA transporter expressed in *P. parasitica* zoospores. The long term goal of this project is to provide insights into PA metabolism in *Phytophthora* with special emphasis on PA transport. This may lead to identification of potential targets for controlling oomycete diseases.

2.3 Materials and methods

2.3.1 Identification of putative genes related to polyamine metabolism in *Phytophthora* spp.

To determine if oomycete genomes contain genes involved in PA metabolism, a BLASTP analysis was performed using known Arabidopsis PA genes that encode ornithine decarboxylase (ODC), arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), N-carbamoylputrescine amidohydrolase (NCPAH), Spd, Spm and Tspm synthases, polyamine oxidase (PAO) and S-adenosylmethionine decarboxylase (SAMDC) against genomes of five *Phytophthora* species: *P. infestans*, *P. sojae*, *P. ramorum*, *P. capcisi* and *P. parasitica*.

Saccharomyces cerevisiae ODC was used for searching ODC homologs in *Phytophthora* as *Arabidopsis* genome lacks ODC genes. Spd and Spm synthases were considered as a single class of enzymes (aminopropyltransferases) due to their high degree of sequence similarity.

2.3.2 Identification of a putative polyamine transporter that is expressed in zoospores of *P. parasitica*

A comparative genomics approach was adopted to identify candidate PA transporters in *Phytophthora* spp. The protein sequence of Arabidopsis PUT5 encoded by the gene *AT3G19553*, was used as a query sequence for a homology search. First, a BLASTP search was performed with Arabidopsis PUT5 against *P. parasitica* proteome on FungiDB (the Fungal and Oomycete Genomics Resource). Percent identity = 30% (E value = 10^{-20}) was used as the cut off for selecting the highly homologous genes among BLASTP hits. Next, the selected genes were identified in *P. parasitica* transcriptome data obtained from the Broad Institute (MA) in order to prove existence of protein coding genes as well as to observe their expression pattern. Out of the identified protein coding genes, *PPTG_00424* was chosen as a gene candidate for further analysis considering several factors including presence of cross-species homologs and considerably high expression at zoospore stage. A multiple sequence alignment of the *PPTG_00424* homologs in *P. parasitica* was performed using TM-coffee server (Chang et al. 2012.

To identify cross-species members of this protein family, sequences homologous to PPTG_00424 in other *Phytophthora* species were selected via BLASTP search (E-value < 10^{-20}) on FungiDB. The sequences that shared significant homology *PPTG_00424* (based on E value) and have characteristics of a standard coding sequence were chosen for phylogenetic analysis.

2.3.3 Construction of phylogenetic trees

Two phylogenetic trees were generated. The first phylogenetic tree included only the identified family members of PPTG_00424 in *P. parasitica.* The relative expression of PPTG_00424 family members in zoosporic, mycelial and infective stages were represented in a heatmap along with this phylogenetic tree. The heatmap displays increase and decrease in the expression of each gene based on the normalized read counts (log10) obtained from the *P. parasitica* INRA-310 RNA sequencing project carried out by the Broad Institute, MA (http://www.broadinstitute.org/). This phylogenetic and heatmap analysis included eight amino acid sequences. The heatmap was constructed using Microsoft Excel. The second phylogenetic tree involved homologous protein sequences of PPTG_00424 in close relatives of *P. parasitica* which include *P. capsici, P. cinnamomi, P. infestans, P. ramorum* and *P. sojae.* The three published rice PA transporters OsPUT1-OsPUT3, five Arabidopsis PA transporters AtPUT1-AtPUT5 (Mulangi et al. 2012a) and two protozoan PA transporters LmPOT1 and TcPAT12 (Hasne et al. 2005, 2010) were used as outgroups for the second phylogenetic tree. This phylogenetic tree involved 56 amino acid sequences including the outgroups. To generate both phylogenetic trees, multiple sequence alignments were conducted using ClustalW (Thompson et al. 1994). The phylogenetic trees were constructed by MEGA7 (Kumar et al. 2016) usiang Maximum-likelihood method with 100 bootstrap replicates. Branch lengths represent the evolutionary distances. The evolutionary distances were computed using the Jones et al. w/freq. model and are in the units of the number of amino acid differences per site.

2.3.4 *In silico* analysis of PPTG_00424

To determine the topology of PPTG_00424 protein, *in silico* analyses were carried out using the amino acid sequence. Three different prediction programs were used to predict the

secondary structure of the PPTG_00424 protein: TMHMM (Sonnhammer et al. 1998), Phobius (Käll et al. 2007) and Protter (Omasits et al. 2014).

To determine structural and sequence conservation of this protein family in other *Phytophthora* members, a multiple sequence alignment was performed using amino acid sequences on TM-coffee server. The cross-species homologs that are closely related to PPTG_00424 were selected from the phylogenetic tree generated in 3.4.3.2. These proteins formed a single cluster in the phylogenetic tree and they belong to the species *P. ramorum*, *P. capsici*, *P. cinnamom*i, *P. sojae* and *P. palmivora*.

2.4 Results and discussion

2.4.1 Identification of genes associated with polyamine metabolism in *Phytophthora*

To determine if oomycete genomes contain key enzymes involved in PA metabolism, a homology search was performed using BLASTP (E value$<10^{-20}$). Results showed that all five *Phytophthora* species examined contained putative ODC, Spd/Spm synthases amd SAMDC genes (Table 1). Proteins homologous to Tspm synthase and PAO could not be identified from *P. ramorum* and *P. capsici* genomes, respectively. Although no statistically significant matches were found for these enzymes in some *Phytophthora* species, chances are that there might be distant homologs that share structural similarity to the query protein (Pearson, 2013). No homologs were found for ADC and AIH in any *Phytophthora* species examined.

Table 1. Putative enzymes that are involved in PA biosynthesis and oxidation in five *Phytophthora* genomes.

Enzyme	*P. infestans*	*P. parasitica*	*P. ramorum*	*P. sojae*	*P. capsici*
Ornithine decarboxylase	PITG_02303	PPTG_05180	PSURA_71540 PSURA_49709	PHYSODRAFT_474019	PHYCA_12789
Spd/Spm synthases	PITG_10866	PPTG_17569	PSURA_72107	PHYSODRAFT_477587	PHYCA_505369
Tspm synthase	PITG_17608	PPTG_18910	NA	PHYSODRAFT_518702	PHYCA_543889
N-carbamoylputrescine amidohydrolase	PITG_12837	PPTG_07189	PSURA_93988 PSURA_93988	PHYSODRAFT_561825	PHYCA_502634
Polyamine oxidase	PITG_04877	PPTG_04694 PPTG_01485	PSURA_79768	PHYSODRAFT_559981	NA
S-adenosylmethionine decarboxylase	PITG_01062	PPTG_10301	PSURA_82460	PHYSODRAFT_308542	PHYCA_77644

Note: Characterized protein sequences of *Arabidopsis thaliana* were used as queries for homology search via BLASTP against reference proteomes available in FungiDB (Fungal and Oomycete Genomics Resource (https://fungidb.org). Proteins that showed significant homology (E value < 10^{-20}) were selected. NA represents no homologous proteins found using the BLASTP search. The Genbank accession number is provided for each putative gene.

Ornithine decarboxylase (ODC) is the universal pathway for Put biosynthesis in all eukaryotes where Spd is synthesized through addition of aminopropyl groups to Put by Spd synthase using aminopropyl groups donated by SAMDC in all eukaryotes. Plants possess an additional route for Put biosynthesis through ADC, AIH and NCPAH, and this pathway was believed to be derived from the cyanobacterium that became the chloroplast in the primary endosymbiotic event (Illingworth et al. 2003). It is evident that oomycetes rely on the core PA biosynthetic pathway (ODC pathway) that has been derived from the last eukaryotic common ancestor (Michael, 2016). Table 1 shows that oomycetes contain ODC for synthesis of Put and Spd synthase and SAMDC for the synthesis of Spd from Put. Oomycetes also contain Spm synthase gene which was not derived from the last common ancestor of all eukaryotes, but evolved independently in a more recent ancestor of metazoans via duplicated and diverged copy of Spd synthase (Pegg and Michael 2010). The presence of NCPAH in oomycete genomes suggests that oomycetes may have acquired the ADC pathway from the ancestral algal endosymbionts. However, pathogenic oomycetes may have lost most biosynthetic genes that constitute the ADC pathway due to their parasitic behavior. Partial or complete loss of genes involved in PA biosynthetic pathways is a notable phenomenon of eukaryotic parasites (Suzuki and Kusano, 2015). A similar example is the loss of ODC in some *Trypanosoma* species, which exhibit an intracellular parasitic lifestyle. However, some African *Trypanosoma* species have later reaquired a new ODC gene copy from a vertebrate source via horizontal gene transfer (Steglich and Schaeffer, 2006).

The ADC and AIH genes apparently have been lost from the oomycete genomes and thus the ODC pathway may be the only route for PA biosynthesis in oomycetes. Oomycetes have

probably lost ADC pathway after the split between diatom and oomycete lineages because AIH homologs have been found in the diatom genome (Lin and Lin, 2019).

Proteins homologous to Tspm synthase were also found in the examined *Phytophthora* genomes. However, it is not known if any of these Tspm synthases are functional in *Phytophthora*. This observation is supported by Pegg and Michael (2010) who reported that oomycetes and some other chromalveolates retained Tspm synthase-like genes that have been initially acquired from the cyanobacterial endosymbiont. Polyamine oxidases (PAO) are associated with two types of oxidative reactions. The first is back-conversion of Spm and Spd to lower PAs Spd and Put, while the second is terminal degradation of amines and their acetyl derivatives to end products such as H_2O_2. Direct back-conversion of PAs occurs in mammalian cells and plants (Moschou et al. 2008). However, it is not yet known if oomycetes are able to back convert PAs directly.

2.4.2 Identification of a putative polyamine transporter that is expressed in zoospores of *P. parasitica*

A BLASTP analysis was performed using AtPUT5 against *P. parasitica* proteome via FungiDB database. Eleven genes were identified which could be potential PA uptake transporters with significant similarities to AtPUT5. The selected genes were validated with *P. parasitica* transcriptome data obtained from the Broad Institute (MA) in order to recognize if they are protein coding genes as well as to observe their expression pattern at different life stages. Multiple strategies have been developed to delineate protein coding genes from non-coding regions in eukaryotic genomes. Complex computational methods are being used by current databases to create assemblies of protein coding genes eliminating spurious ORFs (Clamp et al. 2007). However, transcriptome data is one of the most reliable ways to determine

transcribed genes (Harrow et al. 2009). Out of the 11 *P. parasitica* genes identified by BLASTP, transcriptome data indicated that three of them are pseudogenes. Out of the eight protein-coding genes, six genes were upregulated at the zoospore stage (Table 2). Out of these six *P. parasitica* genes, *PPTG_00424* was chosen as a gene candidate for further functional analyses since it contained characteristics of a well-defined gene model which include obvious start and stop codons, presence of ORF exceeding ~300 bp and most importantly, presence of cross-species homologs. *PPTG_00424* was a 1732 bp long gene which corresponds to a single transcript of 1662 bp consisting of two exons (Fig. 7). The predicted protein of the transcript was 501 amino acids long.

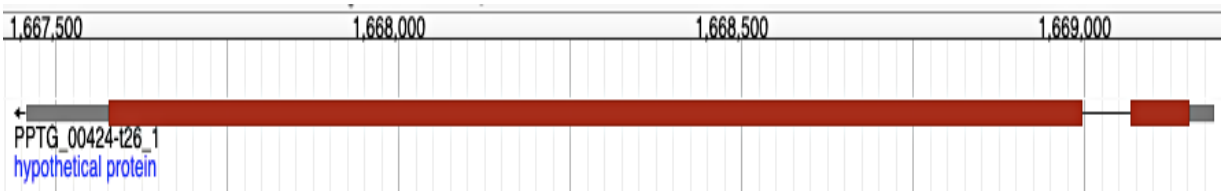

Fig. 7. Gene structure of *PPTG_00424* as shown on JBrowse Genome Browser. Exons are in red boxes whereas introns are the thin lines between exons. Untranslated regions are in gray.

PPTG_00424 protein had at least six homologs in most other *Phytophthora* species namely *P. capsici, P. cinnamomi, P. palmivora* and *P. sojae.* The conservation of PPTG_00424 across multiple species suggested that the gene plays important biological role/s in *Phytophthora* pathogens. *PPTG_00424* is highly expressed in the zoospore stage according to the RNA-seq data of *P. parasitica*, with the second highest log2-transformed RPKM value among other transporters in this gene family (Table 2).

Table 2. RNA-seq data of the putative polyamine transporters identified in *P. parasitica* assembly published by the Broad Institute Genome Sequencing Platform (http://www.broadinstitute.org/).

	Gene ID	Annotation	Relative expression of the genes (Log2-transformed RPKM values)		
			Mycelia	Infection	Zoospores
1	PPTG_00433	Amino_Acid-Polyamine-Organocation_(APC)_family	-0.16	0	4.2
2	PPTG_00424	Hypothetical_protein	0.00	-2.71	1.53
3	PPTG_03297	Amino_Acid-Polyamine-Organocation_(APC)_family	0.00	-0.06	1.49
4	PPTG_01481	Hypothetical_protein	0.00	-0.55	1.31
5	PPTG_12482	amino_Acid-Polyamine-Organocation_(APC)_family	0.00	-0.08	0.72
6	PPTG_03166	Amino_Acid-Polyamine-Organocation_(APC)_family	-0.40	0	0.53
7	PPTG_13502	Hypothetical_protein	2.01	0	-1.71
8	PPTG_09866	Amino_Acid-Polyamine-Organocation_(APC)_family	0.00	0.37	-2.87

Note: The genes are sorted from highest to lowest expression at the zoospore stage. RPKM = reads per kilobase, per million mapped reads.

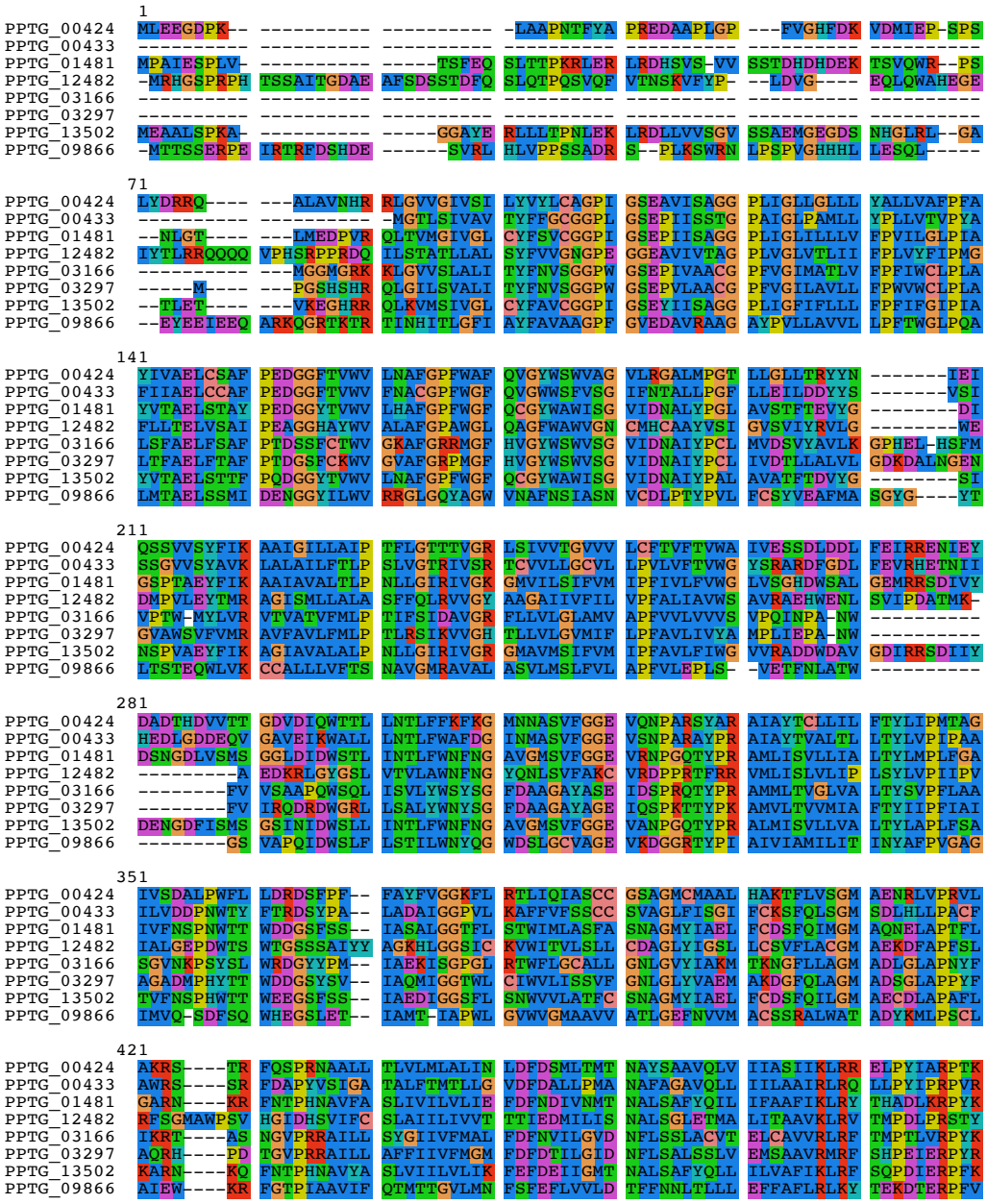

Fig. 8. Multiple sequence alignment of PPTG_00424 and its homologs from *P. parasitica*. Protein sequences were obtained from FungiDB and were aligned using SeaView (Gouy et al. 2010). Amino acids are presented in a range of colors from red to blue, where blue is the most hydrophilic and red is the most hydrophobic.

Protein sequence alignment of the *P. parasitica* homologs show that the N termini and C termini are highly variable (Fig. 8). Furthermore, the residues between 270-290 which correspond to the hydrophilic region between the 3rd and 4th transmembrane domains are also comparatively variable. Presence of largely variable regions suggest that these *P. parasitica* proteins are likely to have a broader substrate specificity or have a differential regulation pattern. Immediately after the highly variable N terminus, few hydrophobic residues show a stronger sequence conservation through all *P. parasiti*ca proteins between the residues 100-140. Proline and glycine residues are more conserved compared to other amino acids which was a characteristic of integral membrane proteins (Jacob et al. 1999). Presence of glycine and proline induce the formation of kinks in the transmembrane helices facilitating helical packing (Dong et al. 2012).

2.4.3 Phylogenetic analysis

2.4.3.1 Phylogenetic and heatmap analysis of *PPTG_00424* in *P. parasitica* genome

The first phylogenetic tree was made using PPTG_00424 and its other paralogs in *P. parasitica* genome that have been identified in the RNA-seq data (Fig. 9). This phylogenetic analysis shows that PPTG_00424 represents a group of eight members in *P. parasitica* (Fig. 9). The membrane proteins of this group range from 451 to 515 residues in size. In order to determine the structures of these *P. parasitica* proteins, the topology was predicted by the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). The hydropathy plots (data not shown) reveal that this group of transporters contains 9-12 transmembrane domains. According to the phylogenetic tree (Fig. 9), PPTG 00424 and PPTG_00433 proteins are more related to each other with a high bootstrap support and they form a cluster together with PPTG_01481 and PPTG_13502. These four proteins (PPTG_00424, 00433, 01481 and 13502) cluster separately from the rest and all of them are comprised with 12 transmembrane segments. PPTG_03166 and

PPTG_03297 group together and away from the other proteins and are composed of 10 transmembrane domains. PPTG_12482 and PPTG_09866 exist singly and are composed of nine transmembrane segments (Fig. 9). It can be hypothesized that all these proteins belong to a diverse family and be potentially involved in PA transport since they all show significant homology to Arabidopsis PUTs. However, since these membrane proteins have significant sequence variability among each other, they may have different specificities to different PAs and they may transport other molecules such as amino acids and organic cations.

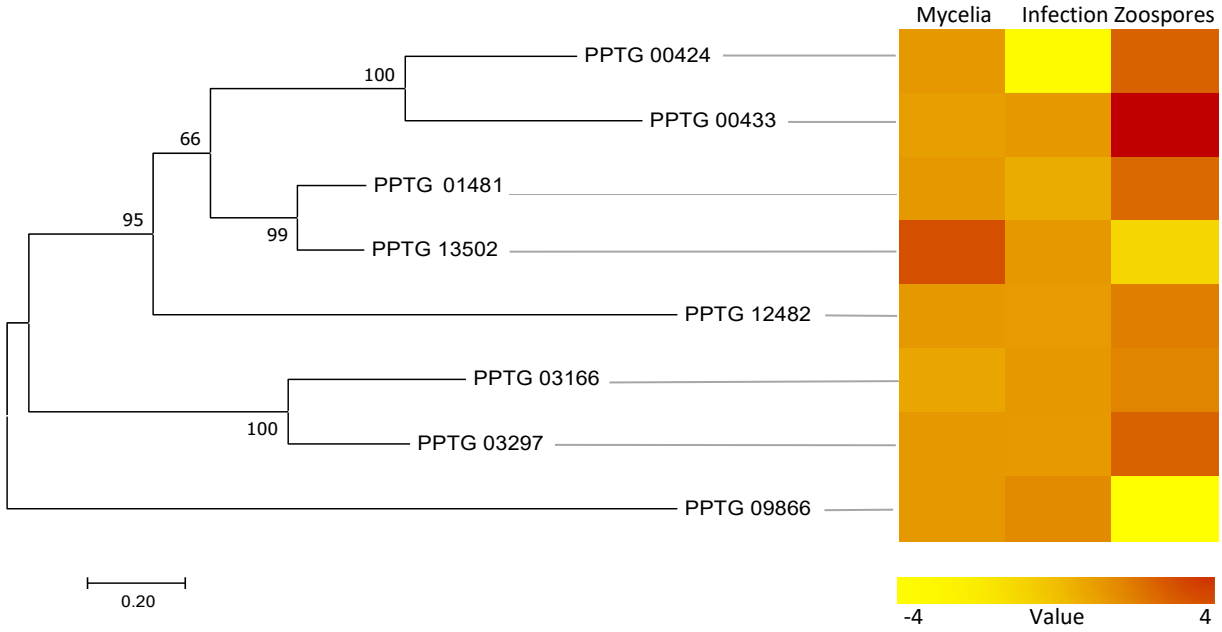

Fig. 9. Phylogenetic tree and heatmap of the paralogs of PPTG_00424 in *P. parasitica*. The phylogenetic tree was constructed by MEGA7 using Maximum-likelihood method. Bootstrap values are shown next to the branches. The evolutionary distances were computed using the Jones et al. w/freq. model and the branch lengths represent the number of amino acid differences per site. 100 bootstrap replicates were used to estimate the confidence. The bootstrap values are shown in the tree. The heatmap was constructed using Microsoft Excel, based on the relative expression (in log2-transformed RPKM values) at mycelial, zoospore and infective stages of *P. parasitica* published by The Broad Institute Genome Sequencing Platform (http://www.broadinstitute.org/).

The expression profile shows that the identified putative membrane transporters of *P. parasitica* have differential expression at different life stages. PPTG_01481, 00424, 00433 and 03297 are highly expressed at the zoospore stage compared to other two life stages. Only PPTG_13503 and 09866 were downregulated at the zoospore stage whereas they were highly expressed in mycelial and infective stages respectively. PPTG_00424 along with 03297, 01481 and 12482 show up-regulation at zoospore stage while being down-regulated during infection and not expressed in mycelial stage. PPTG_00424 was shown to be highly expressed in zoospores second only to PPTG_00433 (Fig. 10 and Table 2). The structural differences of the proteins and the expression pattern of this family of transporters suggest that they are most likely involved in sequestration of one or more PAs and may have tissue-specific regulation in *P. parasitica*.

2.4.3.2 Phylogenetic analysis of *PPTG_00424* in *Phytophthora* genus

To identify cross-species members of this protein family, a BLASTP search was performed using PPTG_00424 against *Phytophthora* genus on the FungiDB database. A total of 46 genes were obtained from this BLASTP search. A multiple sequence alignment was performed followed by construction of a phylogenetic tree using these protein sequences. The phylogenetic analysis shows that PPTG_00424 belongs to a diverse family of membrane transporters across *Phytophthora* members (Fig. 10). According to the phylogenetic tree, these membrane transporters can be further divided into seven clusters. The rice and Arabidopsis PUTs formed a monophyletic group along with *Leishmania major* and *Trypanasoma cruzi*. All *Phytophthora* sequences formed six distinct clusters indicating that the ancestral *Phytophthora* genome might have had six members of this family. Two of these *Phytophthora* clusters grouped together and are more related to rice and Arabidopsis PUTs while the other four remained as

separate groups (Fig. 10). The phylogenetic tree indicated that the multiple sequences from the same *Phytophthora* species did not always group together in the same cluster except few *P. palmivora* sequences. They are PHYPALM 28099, PHYPALM 5901 and PHYPALM 310. However, this was not the case for plant PUTs where all the Arabidopsis PUTs were highly homologous (Fig. 10). The putative *Phytophthora* transporters included in this phylogenetic tree show a greater variation within each *Phytophthora* genome compared to that of Arabidopsis PUTs (Fig. 10). It can be hypothesized that the reasons for this variability might include differential membrane targeting (plasma membrane/ ER/Golgi), variations in substrate specificity and specialization in terms of function (import and export mechanism).

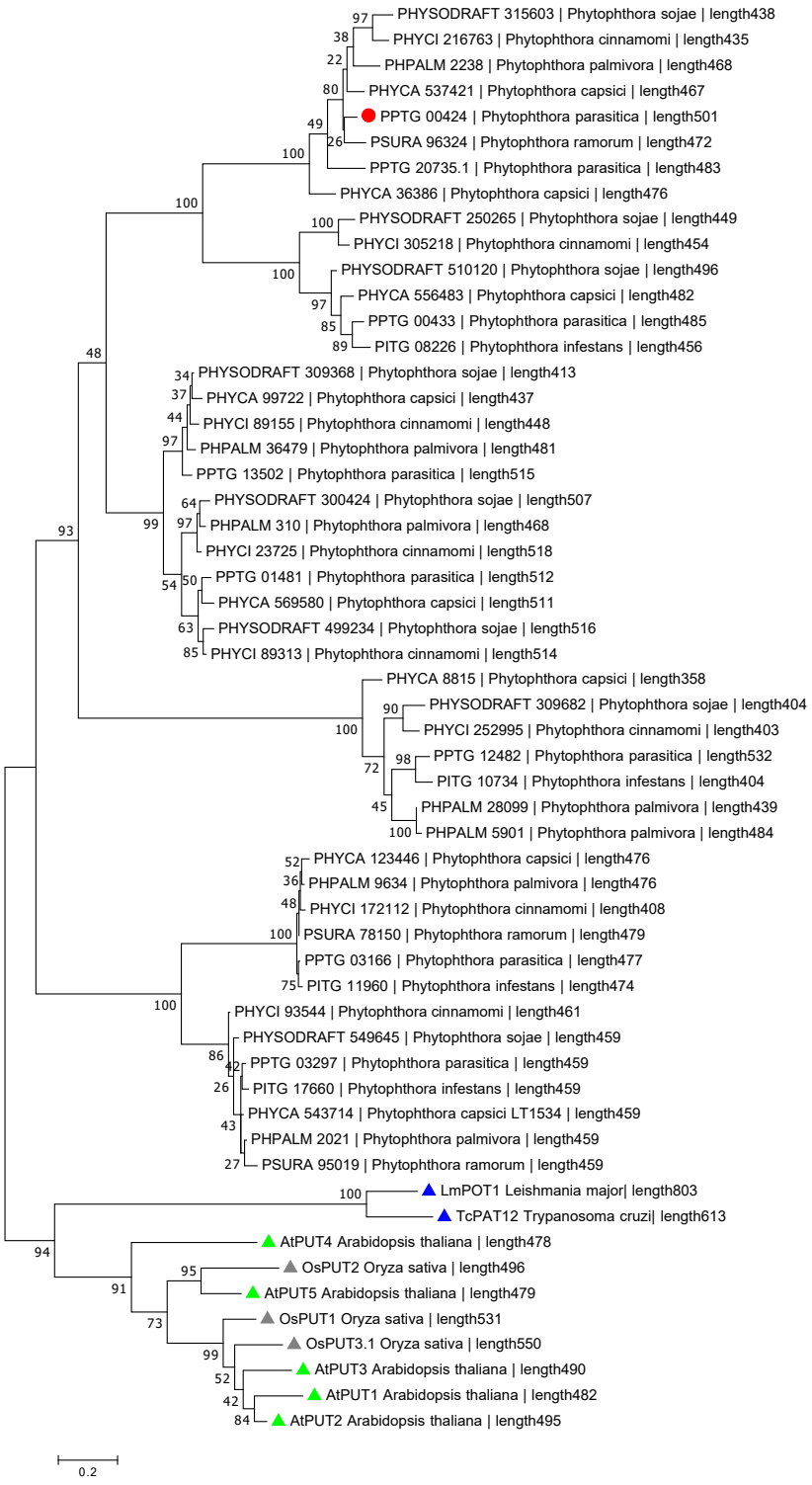

Fig. 10. Evolutionary relationships of PPTG_00424 to its close relatives in other *Phytophthora* members (*P. capsici, P. cinnamomi, P. infestans, P. ramorum, P. sojae*). PPTG_00424 is

indicated by a red circle. Polyamine transporters from Arabidopsis (green triangles), Rice (gray triangles), the two protozoans *Leishmania major* and *Trypanasoma cruzi* (blue triangles) were used as outgroups. The phylogenetic tree was constructed by MEGA7 using Maximum-likelihood method. Bootstrap values (100 replicates) are shown next to the branches. The evolutionary distances were computed using the Jones et al. w/freq. model and the branch lengths represent the number of amino acid differences per site. The analysis involved 56 amino acid sequences in total.

2.4.4 *In silico* analysis of PPTG_00424

To determine the topology of PPTG_00424 protein, *in silico* analyses were carried out using the amino acid sequence. Three different prediction programs were used: TMHMM (Sonnhammer et al. 1998), Phobius (Käll et al. 2007) and Protter (Omasits et al. 2014). These databases predict secondary structure of a given protein by calculating structural propensity and hydrophobicity of amino acids. TMHMM uses a hidden Markov model to predict transmembrane topology. Phobius uses location probabilities to predict both topology as well as signal peptides while Protter also uses predictions made by Phobius. All these programs predicted 12 transmembrane regions in PPTG_00424 and the topology predicted by TMHMM and Protter are shown in Figs 11 and 12A, respectively. According to the predictions made based on the amino acid sequences, PPTG_00424 contains 12 distinct transmembrane helices that are more or less perpendicular to the membrane and each consisting of approximately 20 amino acid residues (Fig. 12A). The protein has a relatively long central intracellular loop between transmembrane domains 6 and 7 (Fig. 12A). Both N and C termini of PPTG_00424 protein were intracellular which was characteristic of LAT family transporters. The closest paralog of PPTG_00424 protein in *P. parasitica* was PPTG_00433 which shares 48% identity. When the

topology of PPTG_00424 was compared with that of PPTG_00433, significant differences could be observed especially in the length and distribution of loops between 3rd and 7th transmembrane regions. These different topologies of PPTG_00424 and its closest paralog PPTG_00433 suggest that they may differ in their respective functions.

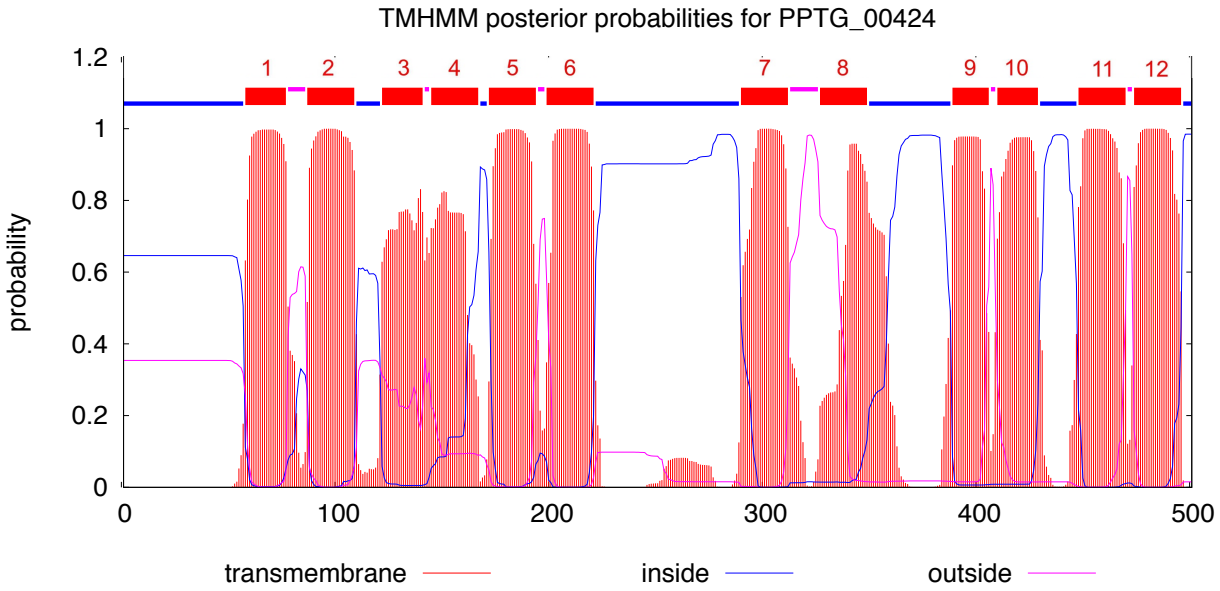

Fig. 11. Predicted transmembrane protein topology of PPTG_00424 using TMHMM algorithm. Number of predicted transmembrane helices was 12 and the expected number of amino acids in the transmembrane helices was 249. The total probability that N-terminus is on the cytoplasmic side of the membrane was estimated to be 0.646. Transmembrane helices are numbered from 1-12.

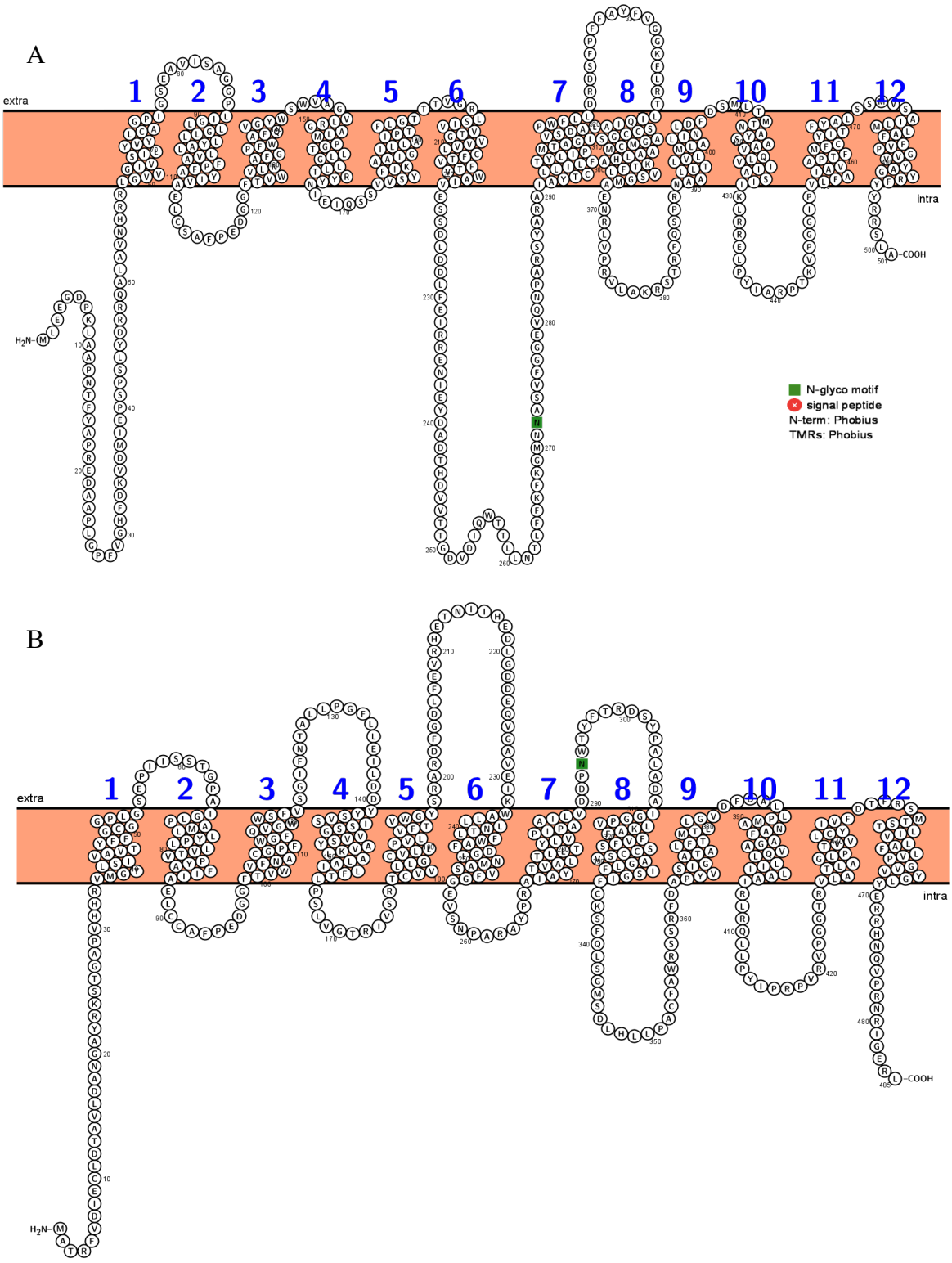

Fig. 12. The amino acid sequence and transmembrane topology of A, PPTG_00424 and B, PPTG_00433 as predicted by the Protter server. Residue numbers delineating each domain are

shown. Transmembrane domains are numbered from 1-12. The word “extra” represents outside of the plasma membrane whereas “intra” represents inside of the membrane.

To resolve the tertiary structure, the amino acid sequence was fed to SWISS-MODEL (Schwede et al. 2003). However, the best-matching template protein that the software adopted was a bacterial amino acid and PA transporter (PDB ID: 6F2G) that shares only 17% homology to PPTG_00424 making the 3D structure prediction less reliable. Since no other well-resolved 3D structures were available for PA transporters, only the secondary structure of the protein was analyzed.

To determine structural and sequence conservation of this protein family in other closely related *Phytophthora* members, a multiple sequence alignment of the amino acid sequences was performed using TM-coffee server (Fig. 13). The cross-species homologs that are closely related to PPTG_00424 were selected from the phylogenetic tree shown in Fig. 10. The six selected protein homologs formed a single cluster with PPTG_00424 in the phylogenetic tree (Fig. 10). Most multiple sequence alignment programs use a heuristic technique termed “progressive alignment” to combine multiple pairwise alignments progressively from the closest to most distant ones. The most popular program for multiple sequence alignment is ClustalW whereas T-coffee is another slower but more accurate especially for distantly related sequences (Notredame et al. 2000; Sun et al. 2014). TM-coffee is an extension of T-coffee which uses a similar progressive alignment algorithm and includes a hidden Markov model for topology prediction for display purposes. This allows examination of both sequence conservation and structural conservation of the protein. The multiple sequence alignment was also carried out using a much recent and faster tool TM-Aligner (Bhat et al. 2017) but the topologies predicted by TM-Aligner was very different from that of many other tools making the transmembrane protein prediction

unsound (data not shown). Therefore, the alignment generated by TM-coffee was used for analyzing sequence conservation in the cross-species homologs.

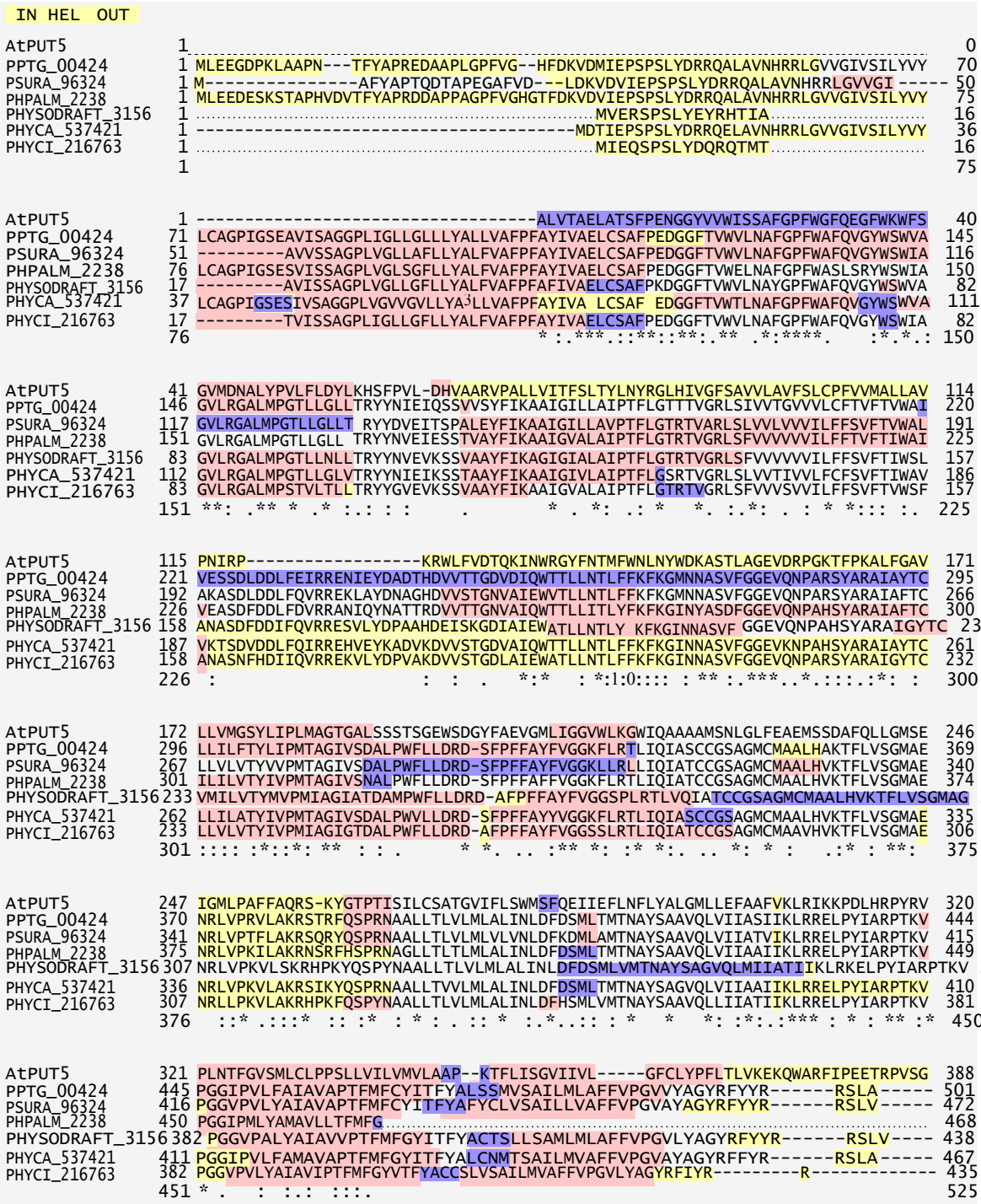

Fig. 13. Multiple sequence alignment of PPTG_00424 and its homologs from *P. ramorum*, *P. palmivora*, *P. capsici*, *P. cinnamomi* and *P. sojae*. Protein sequences were obtained from FungiDB and were aligned using TM-Coffee. Amino acids highlighted in pink indicate transmembrane domains while blue and yellow represent outside and inside of the plasma

membrane, respectively. Fully conserved residues are marked by an asterisk (*), highly conserved residues are marked by a colon and weak conservation is shown by a period.

According to the multiple sequence alignment, higher sequence conservation was observed near the third transmembrane domain across all six *Phytophthora* species and Arabidopsis (Fig. 13). Despite the sequence conservation, the membrane topologies predicted by TM coffee were highly variable across different *Phytophthora* members. In the last three transmembrane regions of the analyzed membrane proteins, the sequence conservation was poor but the membrane topology was conserved across all six *Phytophthora* species. However, the topologies were more variable through the first seven transmembrane domains. The predicted membrane topologies could be different in spite of the amino acid conservation as the prediction tools consider not only the hydropathic properties but also other factors such as overall charge distribution to predict the location of amino acids stretches (Van Geest and Lolkema, 2000). Therefore, the regions that have a higher sequence conservation do not necessarily show the same topology in different *Phytophthora* species. High variation of the structures of these cross-species homologs may indicate that these putative membrane proteins have been diversified after speciation and may differ in their transport properties largely.

2.5 Conclusions

As for most eukaryotes, polyamines are not a major source of C and N, but are required for various known biological processes in plant pathogens (Valdés-Santiago et al. 2012). Bioinformatic evidence suggests that oomycetes synthesize Put and other PAs via the ODC route similar to most eukaryotes. Some genes involved in the ADC pathway have been lost from the oomycete genomes due to their parasitic behavior. However, this gene loss is limited by the fact

that certain key biosynthetic enzymes must still function as oomycetes need to biosynthesize essential PAs that are required for survival outside the host.

A comparative genomics approach was utilized to identify PPTG_00424 as a putative PA transporter expressed in the zoospore stage of *P. parasitica*. According to the bioinformatic analyses, it can be hypothesized that PPTG_00424 is a membrane transporter that belongs to PUT family and it is most likely involved in the PA transport in *P. parasitica*. *PPTG_00424* is hereafter designated as PUT1 in the subsequent chapters. PUT1 protein shares 31% homology to Arabidopsis PUT5. The significant upregulation of *PUT1* in the zoospore stage indicates that it may play an important role in zoospore survival and pathogenicity. A functional characterization is required to determine the precise function of PUT1.

Bioinformatic analyses identified 45 members that are homologous to PUT1 from other *Phytophthora* genomes and they appear to be members of a large family. The PUT family of transporters in oomycetes is larger and more diverse than that of plants. The expansion of PUT orthologs in oomycetes relative to plant genomes was unexpected, given that there are fewer development stages in oomycetes relative to plant genomes. Furthermore, it can be hypothesized that expansion of the PUT family of transporters might have occurred in the last common ancestor of *Phytophthora* species.

According to the membrane topology predicted by multiple prediction tools, PUT1 has 12 transmembrane domains and few other common features shared by PA transporters that have been previously identified in Arabidopsis and rice. The membrane topologies of cross-species homologs of PUT1 are more variable compared to that of their Arabidopsis counterparts suggesting these *Phytophthora* proteins might deliver diverse functions and/or have different substrate specificities. Functional characterization of these putative PA transporters either by

heterologous expression and/or loss-of-function mutants would help determine the exact role of these putative PA transporters in the PA biology of *Phytophthora*.

CHAPTER 3. SUBCELLULAR LOCALIZATION OF THE PUTATIVE POLYAMINE TRANSPORTER PUT1 USING *P. SOJAE* AS A MODEL SYSTEM

3.1 Introduction: Oomycete transformation protocols

Despite the significant economic threat posed by the oomycete pathogens, much less attention has been given to their molecular studies, until recently. Genetic manipulation of oomycetes remains difficult due to their complex genomic nature. However, with expansion of the genome and transcriptome sequencing, development of reporter genes and novel techniques for gene silencing and gene editing have improved the functional analyses of oomycete genes in the recent years (Fang and Tyler, 2016; Fang et al. 2017 and 2020; McGowan and Fitzpatrick 2017; Lamour and Kamoun, 2007). One of the limitations that still exists for genetic manipulation of some oomycete pathogens such as *Phytophthora* species is the inefficiency of techniques available for transient and stable transformation (Lamour and Kamoun, 2007; Mcleod et al. 2008). Several methods have been used to deliver foreign DNA into oomycete cells. They include polyethyleneglycol (PEG)-mediated transformation, electroporation, microprojectile bombardment and *Agrobacterium tumefaciens*-mediated gene delivery (Judelson and Ah-Fong, 2009). Electroporation of either zoospores or protoplasts and *Agrobacterium*-mediated transformation were ineffective in producing homokaryotic stable transformants (Mcleod et al. 2008). The use of microprojectile bombardment is also restricted because it requires specialized equipment (Wu et al. 2016). The protoplast-mediated transformation technique developed by Judelson et al (1991) for *P. infestans* was a significant advancement in this field. PEG-mediated transformation of protoplasts has been the most widely used method to transform several *Phytophthora* members including *P. sojae* (Fang et al. 2017), *P. palmivora* (van West et al. 1999), *P. parasitica* (Meng et al. 2014), *P. capsici* (Dunn et al. 2013) and *P. cactorum* (Chen et

al. 2016). Fang and Tyler (2016) used CRISPR/Cas9- mediated gene disruption for the first time in *P. sojae* to edit an effector gene and they optimized the PEG-mediated method of protoplast transformation to deliver the Cas9 and guide RNA constructs. This method of transformation has been subjected to multiple modifications because the conventional method sometimes results in low protoplast regeneration rate needing large amount of starting material. Various constructs such as reporter gene constructs and gene-silencing vectors (Pan and Gao, 2018) have been introduced into *Phytophthora* genomes using optimized versions of PEG-mediated method. The only limitation of PEG-mediated method is the integration of the target gene into multiple locations in the genome. Although multi-copy integration allows higher gene expression levels, it can occasionally complicate functional analyses due to random disruption of multiple genes to varying degrees among transformants.

3.1.1 Vector systems for transgene expression in oomycetes

The vector systems used for other eukaryotes such as fungi do not suit *Phytophthora* species as the transcriptional and translational machineries have different sequence requirements (Kamoun, 2003). Various sequence elements from animals, plants and fungi are currently being used for expressing transgenes in many organisms, but such elements from non-oomycete organisms usually operate poorly in *Phytophthora* (Judelson et al. 1992). For example, many oomycete promoters lack the classic TATA sequence, but contain other oomycete-specific motifs (Judelson et al. 1992).

3.1.2 Promoters and terminators

The promoters and terminators of two genes; *ham34* and *hsp70*, from the downy-mildew pathogen *Bremia lactucae* have been successfully used to obtain stable transformants of *P. infestans* (Judelson and Michelmore, 1991). This led to incorporation of these regulatory

elements into transformation vectors which were then used for transformation of oomycetes such as *Phytophthora* (Fong and Judelson, 2011; Wu et al. 2016) and *Pythium* (Lerksuthirat et al. 2015). In expression vectors that are used for oomycetes, the transgene is usually driven by one promoter and the selectable marker is driven by the other promoter to avoid trans-gene silencing which may occur by staking of promoters (Agapito-Tenfen et al. 2014). Furthermore, the use of heterologous promoters is recommended for a given oomycete species to prevent gene silencing which might occur due to the cross-talk between native genes and transgenes (Poidevin et al. 2015).

As for other oomycetes, promoters and terminators of *ham34* and *hsp70* genes have been a popular choice for expressing transgenes in *P. sojae* (Fang and Tyler, 2016; Fang et al. 2017 , 2020; Zhao et al. 2018). However, these regulatory sequences were initially optimized for *P. infestans* and they may not function equally well in other *Phytophthora* species including *P. sojae* owing to the interspecies genomic variation in *Phytophthora* genus (Judelson, 2012). *Bremia lactucae hsp70* and *ham34* promoters contain a motif that is characteristic to developmentally regulated oomycete genes (Poidevin et al. 2015; Roy et al. 2013). This motif is called INR-FPR (Initiator-like sequence and Flanking Promoter Region). In general, developmentally-regulated genes exhibit a restricted expression pattern at certain developmental timepoints. Due to the presence of such regulatory motifs, the *hsp70* and *ham34* promoters might be more prone to chromatin remodeling and to other mechanisms leading to low transcriptional activity (Poidevin et al. 2015). A new set of promoters have recently been identified from genes encoding *Phytophthora* ribosomal proteins and have been tested for driving transgenes. The authors observed stable and strong activity from the promoters of two *P. capsici* ribosomal proteins, *RPS9* and *RPL10* when expressed in *P. infestans*. These promoters lack the INR-FPR

motif, but instead, contain a 12-nt motif called PhRiboBox, which was predicted to have a minimal nucleosome occupancy leading to strong and constitutive promoter activity (Poidevin et al. 2015). The degree of expression stability of *RPS9* and *RPL10* was higher than that of *Bremia lactucae* promoters indicating that they may serve as effective candidates for transgene expression in *Phytophthora*.

3.1.3 Reporter genes

With the expansion of oomycete transformation techniques, various reporter genes have been developed for expression in *Phytophthora* species. Development of genetically-encoded reporters provided the advantage of visualizing target proteins with minimal disturbance to the cells, contrary to the classical immunolocalization techniques which involve specific antibodies and tedious fixation/permeabilization steps. Reporter genes have been used in oomycetes to examine functioning of promoters, to observe special morphological features, to quantify host's disease resistance and to observe disease progression in the host (Evangelisti et al. 2017; Judelson, 1997; Kamoun et al. 1998). Green fluorescent protein (GFP) and β-glucouronidase (GUS) are two such markers that have been vital for studying *Phytophthora* biology (Kamoun et al. 1998). These reporter genes not only permit visualization of different parameters of the transformed cells, but also analyzing the activity of regulatory elements such as promoters. van West (1998) studied the location and timing of expression of an *in planta*-induced gene (*ipiO*) in *P. infestans* by fusing the *ipiO* promoter with GUS reporter gene. The difficulty of detecting *in-vivo* gene expression was a limitation of GUS and non-destructive GUS assays have been developed as a solution (Gallagher, 2012). Firefly Luciferse (LUC) is another reporter gene that has been successfully used in plant system (Millar et al. 1992). van West (1998) expressed *LUC* as reporter gene in *P. infestans* but low percentage of transformants showed LUC activity which

was a limitation associated with this reporter. Bacterial luciferase gene, *LUX* has also been used in plant systems to evaluate the behavior of oomycete effectors (Fabro et al. 2011) but hasn't yet been used in oomycete systems.

The vector series developed by Fong and Judelson (2011) has been very useful for localization experiments in oomycetes. In their study, four spectrally distinct fluorescent proteins; cyan, green, yellow fluorescent proteins and mCherry were incorporated into vectors that can be fused with a desired protein of interest driven by promoters mentioned in 3.1.2. Fig. 14 shows two basic vectors that are designed by Ah-Fong and Judelson (2011) to express GFP and YFP either alone or fused to target proteins. The authors developed an additional set of vectors that contain fluorescent proteins targeted to five subcellular compartments namely nuclei, endoplasmic reticulum (ER), Golgi, mitochondria, and peroxisomes. These vectors were constructed by fusing fluorescent tags to *P. infestans* proteins that are targeted to different subcellular compartments. The functionality of above fluorescent markers was evaluated in *P. infestans*. However, certain optimizations have to be made when these reporters are used in other oomycetes to prevent possible constraints such as mislocalization and instability of transgene expression (Ah-Fong et al. 2018). These reporter constructs have been valuable in functional analyses of many oomycete proteins (Chen et al. 2019; Garavito et al. 2019).

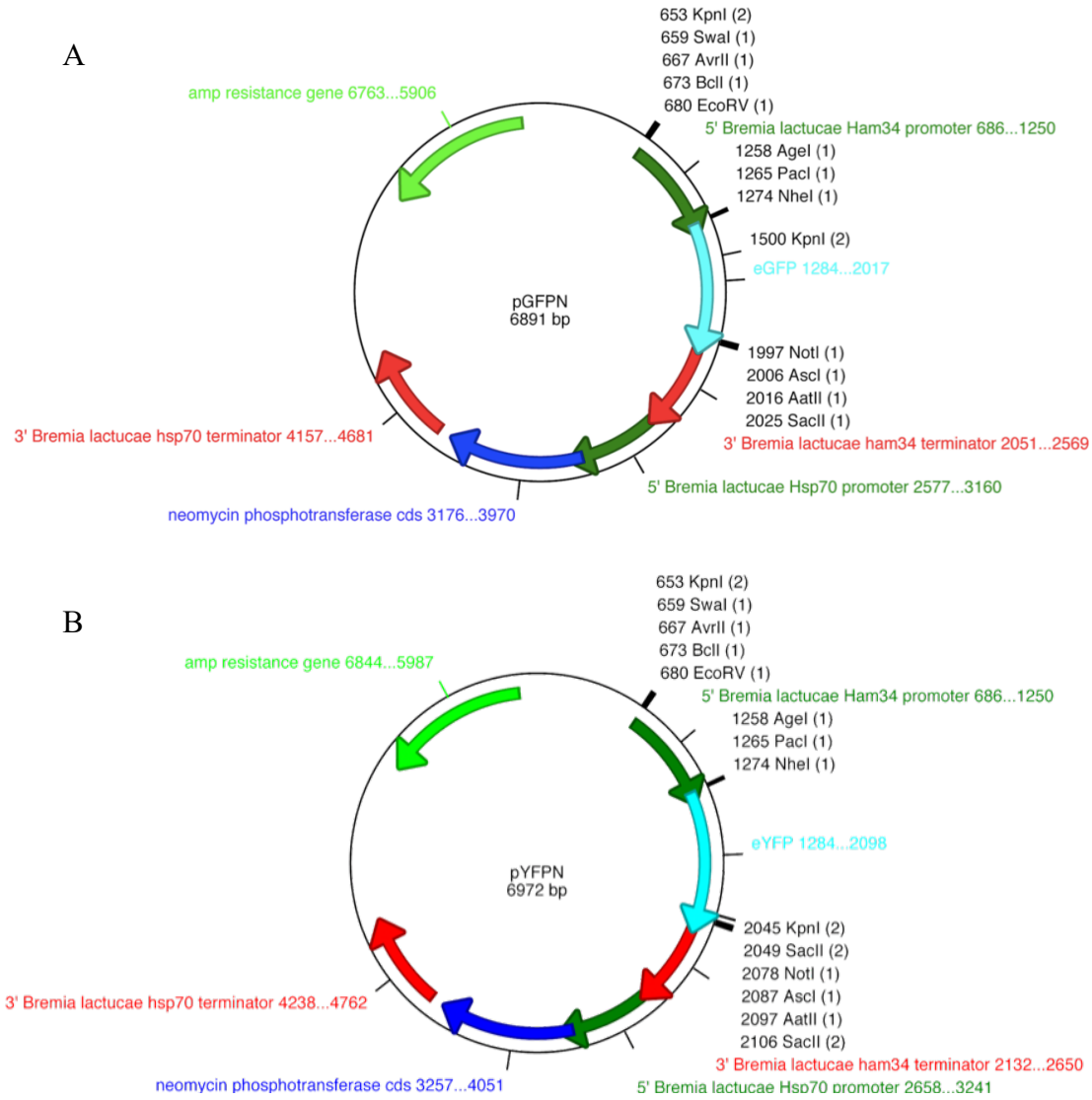

Fig. 14. Two basic vectors designed by Ah-Fong and Judelson (2011) for expression in oomycetes. A, pGFPN, a plasmid expressing GFP and B, a plasmid which expresses YFP. Both cassettes are in a backbone conferring geneticin (G418) resistance using *nptII* (neomycin phosphotransferase II). Both plasmids contain ampicillin resistance gene for bacterial selection. The fluorescent tag with/without the target gene was expressed under the control of promoter and terminator from ham34 whereas the *nptII* was driven by promoter and terminator from *hsp70*. The pGFPN was used for tagging *PUT1* with a GFP while pYFPN with a KDEL tag was used as an ER marker for localization experiments.

3.1.4 Selectable markers

A number of selectable markers have been validated and used in oomycete transformation protocols. The most widely used marker is the neomycin phosphotransferase II (*nptII*) which inactivates aminoglycoside antibiotics such as geneticin (G418) by phosphorylating hydroxyl groups (Shaw et al. 1993). Hygromycin phosphotransferase (*hpt*) has also been used in oomycetes such as *Saprolegnia* and *Phytophthora.* However, *nptII* has shown to be more effective in *Phytophthora* because more transformants were obtained compared with that of *hpt* (Judelson and Ah-Fong, 2009; Mort-Bontemps and Fèvre, 1997). Another disadvantage of hygromycin was its toxicity for humans use compared to G418. Streptomycin phosphotransferase (*spt*) is another selectable marker that was in use. However, *nptII* is preferred over *spt* as the latter gives rise to spontaneous antibiotic resistant populations (Judelson and Ah-Fong, 2009). Application of a N-acetyltransferase, a gentamicin-based selection marker has also recently been demonstrated in selecting *P. palmivora* and *P. infestans* transformants (Evangelisti et al. 2019). The concentration of any drug must be carefully optimized to allow recovery of true transformants avoiding background contamination. The use of auxotrophic markers has been limited for oomycetes because such markers reduce the fitness of the pathogenic oomycetes upon selection and most oomycetes show poor growth on selective media (Judelson and Ah-Fong, 2009).

3.2 Hypothesis and research goals

In chapter 2, a putative polyamine (PA) transporter *PUT1* of the oomycete pathogen *P. parasitica* was identified using a comparative genomics approach. According to the predicted secondary structure, *PUT1* was identified as a membrane protein belonging to the polyamine uptake protein (PUT) family. Considering the subcellular localizations demonstrated by other

members in the plant PUT family, it is hypothesized that the *PUT1* protein localizes to either plasma membrane, endoplasmic reticulum (ER) or Golgi. Although the target gene was originally from *P. parasitica*, *P. sojae* was used as a model organism because a system for transgene expression has already been established for *P. sojae* at BGSU and the organism was freely available to use. Furthermore, *P. sojae* serves as an excellent model of this genus having robust genomics and transcriptomic resources in place (genetic map, EST libraries etc.). The major goal of this chapter is to determine the subcellular localization of *PUT1,* using the related species *P. sojae,* as a model organism. The long-term goal of this localization study is to aid in the functional analysis of this transporter.

3.3 Materials and methods

3.3.1 Sources of the plasmids used in this study

To explore the subcellular localization of this hypothesized membrane protein PUT1, a vector that contains GFP fluorescent tag and *Bremia lactucae* regulatory elements (Fig. 14A) was used in the current study. The expression plasmid pGFPN contains the *nptII* gene as the selectable marker that is expressed under *hsp70* promoter and terminator (Fig. 14a). The target gene was fused to GFP at *NheI* site and was expressed under *ham34* promoter and terminator. A second plasmid, pCal-YFPN-KDEL was used as an endoplasmic reticulum (ER)-localized marker. This plasmid carries a YFP tag with a C-terminal ER-retention signal "KDEL" ligated at NotI/AatII site and a N-terminal signal peptide from a *P. infestans* calreticulin gene ligated at AgeI/NheI (Ah-Fong and Judelson, 2011). The YFP was expressed under the same regulatory elements as in pGFPN (Fig. 14b). Both pGFPN and pCal-YFPN-KDEL plasmids were gifted by Dr. Howard S. Judelson from University of California-Riverside. The use of these two constructs allowed the determination of sub-localization of PUT1 in *Phytophthora*.

3.3.2 Plasmid construction

The full-length protein sequence and the corresponding cDNA sequence of *PUT1* was retrieved from FungiDB. The full-length gene was synthesized by GeneScript (Genscript Corp., NJ) and was obtained in a pUC57 vector. The full *PUT1* gene was PCR amplified using primers 5'-ACC GGT CTT AAT TAA GGC TAG CAC CAT G-3' and 5'-GCC CTT GCC CAT GGT GCT AGC AGC CAA A-3'. The PCR conditions were; initial denaturation at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 51°C for 30 sec and 72°C for 30-45 sec followed by a final extension step of 72°C for 5 min. All PCR reactions were performed using Phusion High-fidelity DNA polymerase (New England Biolabs, UK) in a benchtop thermocycler (Bio-Rad, CA). Next, the PCR fragment was inserted into a linearized pGFPN vector (Fig. 14a). The pGFPN plasmid was first linearized by digesting with NheI and the PCR amplified target gene was inserted using In-Fusion cloning (Takara-bio, Japan) following the manufacturer's instructions. The insert was verified by Sanger sequencing (Genomics facility, University of Chicago, IL). The recombinant plasmid was designated as pGFPN- *PUT1*.

3.3.3 Transformation of *P. sojae*

Polyethyleneglycol (PEG) -mediated transformation was performed to transfer the pGFPN- *PUT1* and pCal-YFPN-KDEL constructs into *P. sojae* protoplasts according to the protocol described by Fang et al (2017). As a control, the empty pGFPN plasmid was also transformed into *P. sojae* protoplasts to test the expression of the construct. Briefly, three-day old *P. sojae* mycelium grown in nutrient pea broth was used for generation of protoplasts. The mycelium was rinsed with sterile distilled water and 0.8 M mannitol and incubated in 0.8 M mannitol for 10 mins. Then the plasmolyzed mycelia were subjected to digestion with gentle shaking in *Trichoderma*-derived cellulase for 50 min. Digestion products were collected using a

70 µM cell strainer and pelleted by centrifugation at 1200 × *g* for 1-2 mins. The pellet was washed with W5 solution (5 mM KCl, 125 mM $CaCl_2.2H_2O$, 154 mM NaCl, 177 mM Glucose) centrifuged again at the same speed for 2 min. The cell pellet was resuspended in 10 ml of W5 solution and concentration was adjusted to 2×10^6 - 2×10^7/ml. After incubating the protoplasts on ice for ~20 mins, the solution was centrifuged and the cell pellet was resuspended in 1 ml of MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7). Fifteen µg of each of the plasmid DNA in < 20µl was combined with 1 ml of the protoplasts in a 50 ml Falcon tube gently by tapping followed by incubation on ice for ~ 15 min. 1.74 ml of freshly prepared PEG-calcium solution was added in 3 aliquots while gently rotating the tube. Ten ml of cold regeneration medium (nutrient pea broth) was added to the transformation mixture and poured into a tall Petri dish that contained 10 ml of cold regeneration medium and ampicillin (50µg/ml). The transformation mixture was incubated without shaking for 12-18 hrs at room temperature in dark. Regeneration of protoplasts was confirmed by microscopic observation, following which the regenerated mycelia were harvested by centrifuging at 2000 × *g* and the supernatant was discarded until ~3 ml medium remains. The pellet was resuspended using a pipette and was evenly divided to three 50 ml Falcon tubes. Forty five ml of liquid (exactly at 42°C) regeneration medium containing 1% agar and 50 µg/ml G418 was added to each Falcon tube, and the tubes were inverted to mix. The mixtures were immediately plated on to the petri-dishes containing V8 media with 50 µg/ml G418 added for selection of the transformant. The petri dishes were incubated for 2-5 days at room temperature in the dark. Transformants carrying both the pGFPN-*424* and the pCal-YFPN-KDEL plasmids should have G418-resistance, GFP signal and YFP signal. The mycelial colonies that appeared on the selection plates were sub-cultured onto nutrient pea agar that contains 50 µg/ml G418 until further use.

3.3.4 Confirmation of transformants using PCR

To confirm the successful insertion of the *PUT1* gene in *P. sojae* by PCR, total genomic DNA was extracted from the transformant hyphae and PCR amplified a partial region (442 bp) of the *PUT1* gene using primers: 5'-CGC ATC TGT ATT TGG TGG TG-3' and 5'-TGC AGC ACT ATA AGC GTT GG-3' following the PCR conditions described in 3.3.2.

3.3.5 Confocal microscopic analysis

To confirm the successful expression of the transgene *PUT1* in *P. sojae*, confocal microscopy was used to detect the fluorescent signals. Transformants were grown on clarified 15% V8 juice agar with 50 μg/ml G418 added. Thin hyphal sections scraped out of the agar plate were used to prepare wet mounts on a glass slide. Confocal images of the hyphal sections were obtained using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) with the Leica Application Suite Advanced Fluorescence (LASAF) program. This microscope facility was situated at the advanced microscopy and imaging center in the Health Science campus at University of Toledo, OH. Images were captured using 63X oil objective (NA 1.40) in steps of 0.5 μm in the XYZ plane using sequential scan mode to avoid any spectral overlap in the individual fluorophores. Both GFP and YFP were excited at 488 nm. GFP signal was detected at 510 nm while YFP signal was detected at 550 nm. Background fluorescence from untransformed hyphae of *P. sojae* at similar settings was subtracted from images to determine fluorescence generated by tagged proteins. Images were merged using Adobe Photoshop 7.0. Images were acquired from multiple hyphal segments from one transformant.

3.4 Results and discussion

3.4.1 Transformation of *PUT1* gene in *P. sojae*

In this study, *P. sojae* protoplasts were co-transformed with the two plasmids pGFPN-*PUT1* and pCal-YFPN-KDEL. One transformant was obtained out of five experimental runs. The transformant was subcultured three times on to V8 agar plates containing 50 μg/ml G418 to confirm the stable transformation. The PCR amplification of the transformant displayed an amplicon of the expected size (442 bp) indicating the presence of *_PUT1* gene in its genome (Fig. 15A). Both plasmids were successfully integrated, as confirmed by confocal microscopy. Surprisingly, the transformant hyphae had a very slow growth rate in the presence of the selection marker G418 (Geneticin) compared to wild type hyphae (Fig. 15B).

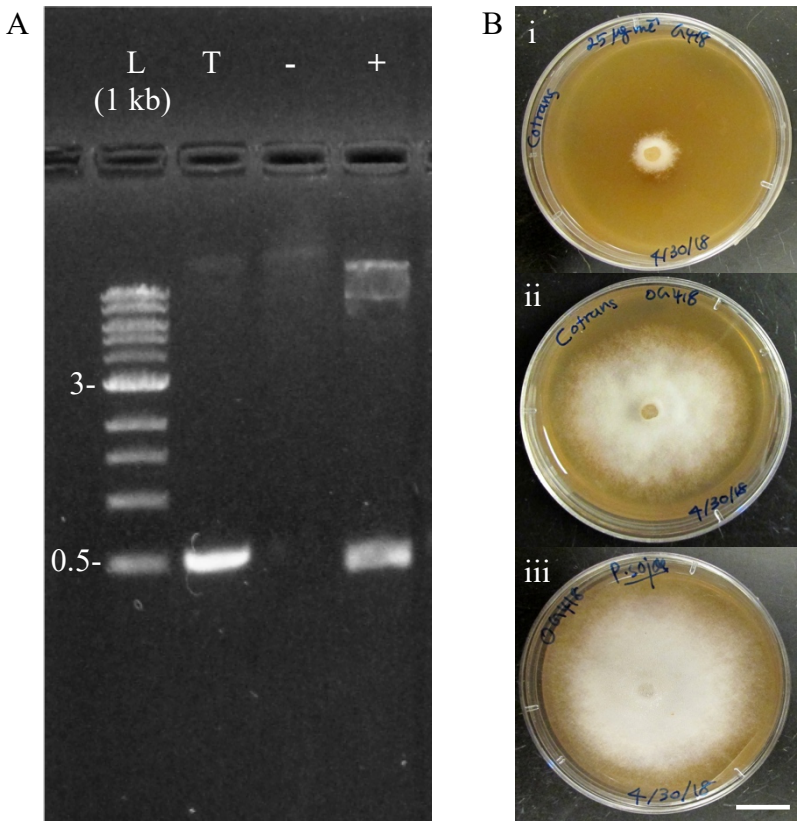

Fig. 15. A, PCR amplification of the partial fragment of *PUT1* gene (442 bp) from the *P. sojae* transformant expressing *PUT1* gene. L = 1 kb marker (New England Biolabs, MA), T = transformant hyphae, - = wild type hyphae, + = pGFPN-*PUT1* plasmid DNA. B, i) Transformant hyphae in the presence of 25 µg G418, ii) transformant hyphae in the absence of G418, iii) wild type hyphae in the absence of G418. Scale bar represents 20 mm.

The slow growth phenotype is most likely due to the incompatibility of the *hsp70* promoter that drove the antibiotic selection marker *nptII* (G418 selection) in *P. sojae* system. *hsp70* was proven to show strong expression in *P. infestans* (Judelson et al. 1991). However, *hsp70* has exhibited poor functioning in *P. sojae* (Personal communication, Yufeng Fang, 22 June 2018). Possible reasons for poor activity of *hsp70* in *P. sojae* may include either epigenetic processes (insertion into heterochromatic regions) or homology-based silencing

(Judelson and Whittaker 1995; Matzke and Matzke 1998). A second transformant expressing only pGFPN was also produced and it also showed a similar slower growth (data not shown). This partially confirmed the weakness of the *hsp70* promoter in expressing the *nptII* marker in *P. sojae*. However, promoters of some ribosomal genes from *P. infestans*, *P. capsici* (Poidevin et al. 2015) and *P. sojae* (Dou et al. 2008) have been tested to confer stable activity for transgene expression in *Phytophthora*. These ribosomal gene promoters contain a novel motif called PhRiboBox which is characteristic to the genes associated with essential cellular processes such as DNA replication, transcription and tRNA biogenesis (Poidevin et al. 2015). Therefore, these ribosomal gene promoters would serve as good candidates for expressing transgenes in *Phytophthora*. A promoter derived from *P. sojae* ribosomal protein gene *RPL41* have been successfully used and showed strong constitutive expression in the transformation assays in *P. sojae* (Fang et al. 2017). We propose that *RPL41* could be useful to drive the expression of *nptII* marker in a construct to express *PUT1* gene in *P. sojae*.

3.4.2 Confocal microscopy

Confocal microscopy images indicated that co-transformation was successful with the integration of both pGFPN-*PUT1* and pCal-YFPN-KDEL marker in the same transformant (Fig. 15). The control plasmid was also integrated as expected (data not shown). The Arabidopsis homolog of PUT1, AtPUT5 is reported to be localized to the ER when expressed in tobacco leaves and Arabidopsis protoplasts (Ahmed et al. 2017; Li et al. 2013). This led to the use of an ER marker for the current colocalization experiments.

According to the confocal images, the GFP signal was not observed along the cell membrane, but instead, throughout the cytoplasm (Fig. 16). This suggests that PPTG_00424 protein is not localized to the cell membrane. Interestingly, co-localization of GFP signal with

the YFP signal of the ER marker indicated that PUT1 is expressed in the ER which exists throughout the cytoplasm in hyphae (Fig. 16). The YFP marker used in this study has the C-terminal tag, KDEL, which targets the YFP protein mainly to ER and Golgi membranes to a lesser extent (Ah-Fong and Judelson, 2011; Napier et al. 1992). Both YFP and GFP signals had a similar pattern of subcellular localization in the hyphae as well as in sporangia (Fig. 16).

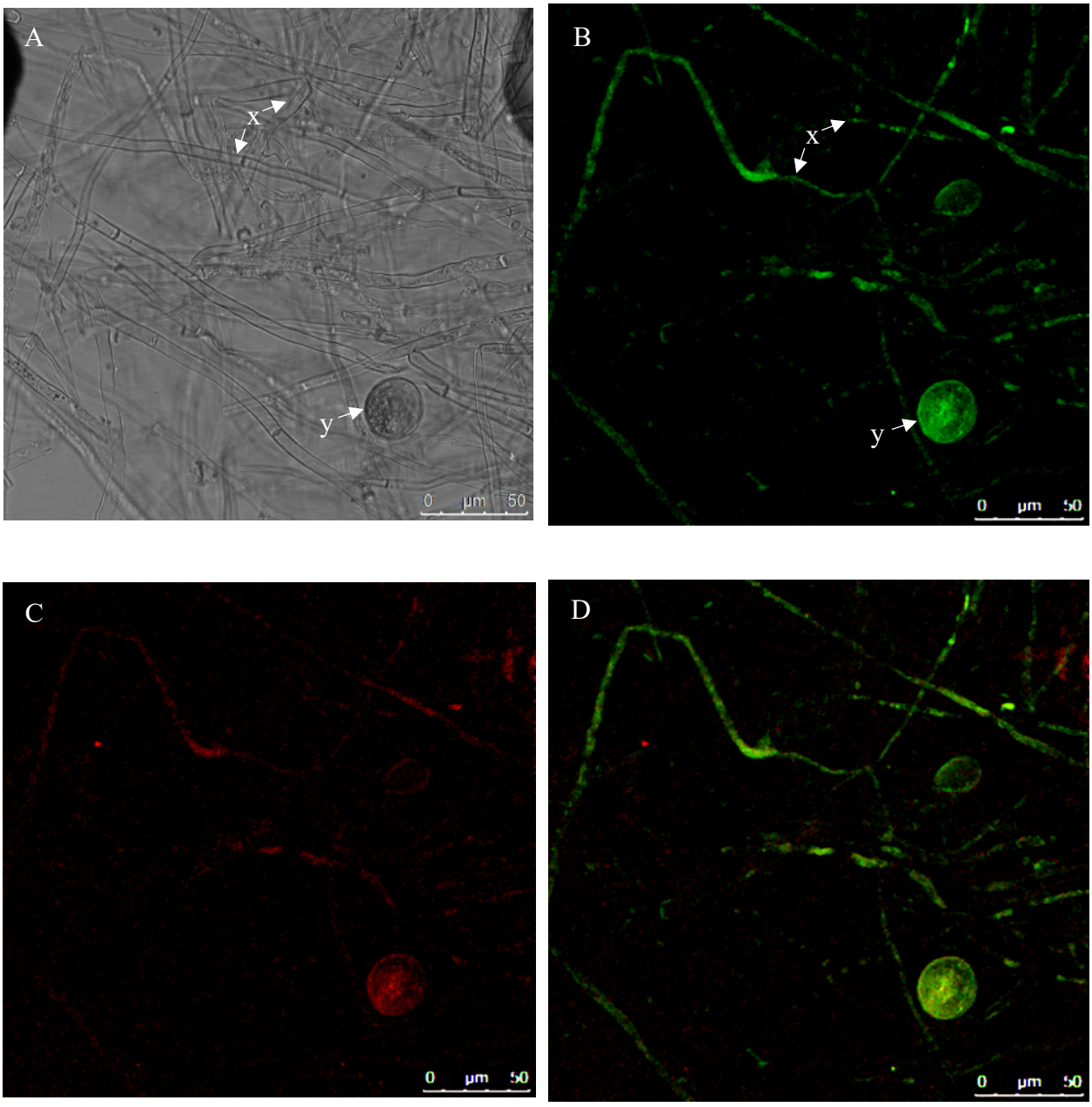

Fig. 16. Confocal images showing subcellular localization of PUT1 in *P. sojae* hyphae expressing pGFPN-*PUT1* (shown in a green signal) and pCal-YFPN-KDEL ER marker (shown in red signal). A = Brightfield, B = GFP wavelength, C = YFP wavelength, D = GFP and YFP merged. The scale bar is shown. Hyphae and a sporangium are labeled as x and y, respectively.

This observation was not surprising as the corresponding Arabidopsis protein reported to have a similar ER-targeting when it is transiently expressed in Arabidopsis protoplasts and *N. benthamiana* leaves according to the localization experiments done by Ahmed et al (2017) and Li et al (2013). Ahmed et al (2017) also show that the rice PUT, OsPUT1 localizes to the ER in a similar fashion. Being targeted to the ER network, two predictions can be made regarding cellular function of PUT1 in *P. parasitica*. It can be either involved in the sequestration of PAs from cytoplasm or in the export of PAs to the external environment through transport vesicles. The functionality of the Arabidopsis PUT5 in the ER has not yet been demonstrated. However, Ahmed et al (2017) hypothesized that AtPUT5 might function to sequester Spd from the cytoplasm based on the phenotypes of AtPUT5 Arabidopsis mutants. In their study, AtPUT5 mutant showed slightly early flowering, smaller leaves and thin stems compared to wild type plants. There are five members of PUT family in Arabidopsis that have been identified so far and localizations of four of them have been demonstrated. Despite the high degree of sequence similarity, these Arabidopsis PUTs are localized to different compartments of plant cell. AtLAT3/AtPUT1 was localized to the ER in Arabidopsis protoplasts whereas PAR1/AtPUT2 was localized to Golgi cisternae in Arabidopsis and rice protoplasts. The third protein, RMV1/AtPUT3, was plasma-membrane localized in onion epidermal cells and Arabidopsis root cells (Fujita et al. 2012; Li et al. 2013). The different subcellular localizations indicate that these Arabidopsis proteins are likely to be involved in distinct cellular functions (Fujita and Shinozaki, 2014). Furthermore, these transporters may localize to more than one cell compartment under different conditions as polyamines play a complex role in biotic and abiotic stress responses (Alcázar et al. 2010). In oomycetes, information is scarce regarding PA metabolism. The study by Mulangi et al (2012b) showed some bioinformatic evidence for the presence of PA

transporters in *P. sojae* and there is a lack of research that focuses specifically on oomycete PA transporters.

According to the phylogenetic analysis performed in chapter 2 (section 2.4.3), PUT1 belongs to a diverse family of transporters in *P. parasitica.* Taking the differential expression pattern of PUT1 family into consideration (phylogenetic tree and heat map in 2.4.3), it will be interesting to find out their subcellular targeting in the mycelium and zoospores of *P. parasitica*. The fluorescent fusion protein constructs developed by Ah-Fong and Judelson (2011) were useful in examining subcellular locations of several oomycete proteins. Out of this vector series, mitochondrial-targeted GFPN and mCherryN markers were used to reveal the locations of some glycolytic enzymes and serine and pyrimidine biosynthetic enzymes in *P. infestans* (Abrahamian et al. 2017; Garavito et al. 2019). Similarly, the GFP marker alone (localized to cytoplasm) and peroxisome-targeted CFP marker developed by Ah-Fong and Judelson (2011) have been used for histological characterization of *P. palmivora* during infection process (Ochoa et al. 2019). In their study, propidium iodide (PI) staining was used in combination with GFP tagging of *P. palmivora* to evaluate host cell death response in palm leaf tissues. The fluorescent markers developed by Ah-Fong and Judelson (2011) can only be used for protoplast transformation or electroporation. These vectors cannot be used for *Agrobacterium* mediated transformation since the vectors lack left and right T-DNA borders (Ochoa et al. 2019). However, it is not a major drawback of these vectors as *Agrobacterium* mediated transformation is not commonly utilized for oomycete transformation.

The phylogenetic analysis (2.4.3) suggests that the PUT family of *Phytophthora* is more diverse and has more PUT members in a single *Phytophthora* genome than its plant counterparts. Although *Phytophthora* pathogens can synthesize most essential PAs inside the cells, they also

take up PAs from the external environment and translocate PAs between different cellular compartments. This is supported by a study done by Ah-fong et al (2017) that reports *Phytophthora* pathogens express ~410 transporters for acquiring nutrients throughout their life cycle. These organisms should also be able to export PAs out of the cells in cases of hyperaccumulation in the cells. Since PAs are vital for many cellular functions, it is reasonable to hypothesize that *Phytophthora* pathogens, as a facultatively parasitic oomycete genus, may express multiple PA transporters in different locations of the cell. They might be involved in both import and export in different locations of the hyphae. However, it is surprising that *Phytophthora* genomes contain more copies of PUT genes than plants unless they function in different locations of the cell, have diverse substrate specificities or they function as subunits with each other.

3.5 Conclusions

PUT1, a putative PA transporter expressed by the oomycete pathogen *P. parasitica* was identified using a bioinformatic approach. The expression of *PUT1* with a GFP tag revealed a dispersed localization within the cytoplasm. Co-localization of PUT1 with an ER marker pCal-YFPN-KDEL indicated that *PUT1* encodes a membrane protein that localizes to the ER of *P. sojae*. The slow growth of the *P. sojae* transformants in the presence of G418 antibiotic could be due to weak promoter activity of *hsp70* in expressing the *nptII* marker. The use of *RPL41* promoter in expressing transgenes in *P. sojae* is suggested for future experiments. According to previous studies, the known plant PUTs localizes to different locations of the cell, such as plasma membrane, ER and Golgi and they are predicted to be involved in distinct functions. The two Arabidopsis proteins, AtPUT5 and AtPUT1, that share 31% and 29% homology, respectively, were demonstrated to have an ER-localization (Ahmed et al. 2017; Li et

al. 2013). This is the first study to explore the localization of a PA transporter in *Phytophthora*. Taking the ER-targeting into account, it can be hypothesized that PUT1 might be involved either in vesicle-mediated export of PAs or sequestration of PAs from the cytoplasm. However, the ER-localization of PUT1 must be independently verified by immuno-localization experiments.

CHAPTER 4. HETEROLOGOUS EXPRESSION OF A POLYAMINE TRANSPORTER FROM *PHYTOPHTHORA PARASITICA*, *PUT1* IN YEAST

4.1 Introduction: Heterologous expression of membrane proteins

Membrane proteins are an indispensable class of proteins that perform many biological functions such as transport of specific substances necessary for cellular metabolism and cellular communication. About 25% of the prokaryotic and eukaryotic protein-coding genes code for membrane proteins (Wagner et al. 2006) and they are continuously being studied for assigning specific functions. Heterologous expression can be defined as overexpression of the target protein in a particular host that is used as a model organism. This has revolutionized the opportunities for detailed investigation of membrane proteins and their isolation for unraveling their 3-dimensional (3D) structures. Heterologous expression of proteins could be unsuccessful for a few reasons. First, the protein could be expressed at very low levels limiting the detection of it. Secondly, the expressed protein could be toxic to the host. The third possibility is improper folding of the expressed protein leading to an inactive form (Miroux and Walker, 1996). The process of molecular characterization of a protein includes cloning of the protein coding gene in a vector, expression of the protein in the host followed by detection and biochemical analysis of the protein. Traditional restriction enzyme and ligase-based techniques that are being used for generation of expression constructs are time consuming and labor-intensive. The development of rapid cloning techniques such as Gateway technology (Invitrogen, CA), TA or TA/TOPO cloning (Invitrogen, CA), Infusion cloning (Clonetech, CA), Gibson assembly (Bassler, 2019) and Golden Gate cloning (Marillonnet and Grützner, 2020) is a significant breakthrough in the recombinant DNA technology. Gateway cloning is a universal recombination-based method which is used to transfer a gene from one plasmid to another. This method is based on the

specific integration and excision sites called "*att*" sites of λ phage and the two enzyme mixes called BP clonase and LR clonase. In Gateway BP reaction, the PCR amplified gene with flanking *att*B sites is mixed with a donor vector containing *att*P sites to generate an entry clone which contains *att*L sites flanking the target gene. In the Gateway LR reaction, the entry clone is mixed with a destination vector containing the *att*R sites, tags and other regulatory elements necessary for expression of the target gene resulting in an expression clone (Reece-Hoyes and Walhout, 2018). Many gateway plasmids are available with various regulatory elements and tags for cloning purposes. TOPO cloning utilizes the enzyme topoisomerase I which functions as a restriction enzyme as well as a ligase (ThermoFisher Scientific, 2015). Gibson assembly is a technique that allows ligating up to 12 overlapping fragments under isothermal conditions using combined action of 5' exonuclease, DNA polymerase and ligase (Bassler, 2009). This method doesn't work very well if the fragment size is less than 200 bp because they will be completely chewed back by the activity of 5' exonuclease. Golden Gate cloning allows simultaneous assembly of multiple DNA fragments using T4 DNA ligase and type IIS restriction enzymes in one reaction (Engler et al. 2008). A drawback of this method is that type IIS restriction sites are common in DNA. Therefore, the fragments should be carefully checked before assembling.

4.1.1 Bacterial systems

Escherichia coli is the most popular bacterial host for producing membrane proteins. It is widely used for recombinant expression of proteins due to low cost, convenience of use and short generation time (Sahdev et al. 2008). To monitor the protein production in bacterial hosts, C-terminal drug-resistance markers, polyhistidine tags and GFP fusion are used (Schlegel et al. 2014). Some examples of functional expression of eukaryotic membrane proteins in *E. coli* include K^+ transporters of Arabidopsis and Eucalyptus and ATP/ADP transporters of

Arabidopsis (Tjaden et al. 1998; Uozumi et al. 1998). Eukaryotic membrane proteins are usually inserted in the bacterial plasma membrane because bacteria have no subcellular membranes. However, overexpression of eukaryotic membrane proteins using *E. coli* system can be problematic sometimes as the bacteria lack the eukaryotic chaperons, post-translational modifications and other molecular mechanisms for membrane insertion, folding and correct function. Overexpressed eukaryotic membrane proteins could become toxic to the bacterial host. Scientists attempted to overcome these limitations by codon-optimization, expressing different protein tags and co-expressing post-translational machineries to promote proper folding and functioning (reviewed in Wang and Yan, 2014). Due to above mentioned limitations, *E. coli* system has not been a popular choice for expressing eukaryotic membrane proteins.

4.1.2 Yeast systems

Being single celled and having genetic and biochemical properties similar to those of higher eukaryotes, yeast cells are useful for heterologous analyses of eukaryotic proteins. *Saccharomyces cerevisiae, Schizosaccharomyces pombe and Pichia pastoris* are some yeast species that have been genetically characterized. Because of the convenience of use and short generation times, these yeast species have been utilized to overexpress eukaryotic membrane proteins (Bornert et al. 2012; Dreyer et al. 1999; Mulangi et al. 2012a). Many plant membrane proteins have been characterized using yeast system (Locascio et al. 2019; Remy et al. 2017). Plant sugar transporters and auxin transporters are two such examples (Huang et al. 2009). Membrane proteins that transport primary metabolites can be identified by functional complementation of yeast mutants (Larsen et al. 2017; Tal et al. 2016).

The functional complementation is achieved by using a mutant yeast strain that is dependent on the activity of a target protein for its growth. The complemented function could be

an import of a certain substrate that is essential for growth. The complemented function could also be export or sequestration of a toxic analog from the cytoplasm (Geisler et al. 2005). Disadvantages of using yeast cells in transporter studies include incorrect localization of membrane proteins and hyperpolarization of cell membranes (Madrid et al. 1998).

4.1.3 Insect cells and baculovirus system

The baculovirus system in insect cells is a widely used method to express eukaryotic membrane proteins (Contreras-Gómez et al. 2014). This system was preferred for many eukaryotic proteins because it is a low-cost method and a good alternative for bacterial and mammalian systems (Bernaudat et al. 2011). This system provides a similar codon usage and post-translational modifications to that of the eukaryotic system making it a more compatible alternative for heterologous protein expression. In this method, a recombinant baculovirus is generated by site-specific transposition of an expression cassette into a bacmid (baculovirus shuttle vector) that is amplified in *E. coli*. This method has been modified by Invitrogen, CA and named as Bac-to-Bac system. Next, the insect cells are transfected with the recombinant virus encoding the target gene. *Spodoptera frugiperda* and *Trichoplusiani* are two popular insect cell lines that are widely being used (He et al. 2014).

4.1.4 Plant systems

Aarabidopsis thaliana is used as a popular model for plant genetics especially because its genome has been completely sequenced. A stable transformation technique was already practiced widely for transforming *A. thaliana,* which is the floral dip method of *Agrobacterium*-mediated transformation. However, the drawbacks of using *A. thaliana* include the long reproductive cycle for generation of seeds (~2 months) compared to prokaryotic and yeast systems (Bernaudat et al. 2011). *Nicotiana benthamiana* is another plant host that has been widely adopted for transient

expression of genes due to the convenience of efficient transformation and regeneration. *N. benthamiana* is commonly used for studying protein interactions, protein localizations, protein expression and subsequent purification (Bernaudat et al. 2011). Transgenes from higher plants can be rapidly evaluated using *Agrobacterium*-mediated transformation of leaf disks in the *N. benthamiana* system. This has been considered a simple but robust method to produce sufficient numbers of recombinant proteins (Vézina et al. 2009).

4.1.5 Heterologous expression of polyamine transporters

Polyamines (PAs) are vital polycations for all living organisms. All cell types have the ability to both biosynthesize and transport PAs. Polyamine transporters have been well studied in bacteria and yeasts (Kashiwagi and Igarashi, 2011). Some solute-carrier (SLC) type proteins in human cells were also studied for their transport activities (Abdulhussein ad Wallace, 2014). Mulangi et al (2012a) and Fujita et al. (2012) have characterized plant PA transporters using phenotype-driven screening and function-driven screening. The function-driven screening is achieved through a successful heterologous expression system. Mulangi et al. (2012a and 2012b) identified three rice PA transporters and three Arabidopsis transporters both of which belong to polyamine uptake transporter (PUT) family based on sequence homology to known PA transporters encoded by two protist genomes. The authors introduced these PUT genes to a *S. cerevisiae* strain that is deficient in spermidine (Spd) uptake and performed a yeast complementation assay by exposing the cells to different PA concentrations. Their study showed that expression of the *PUT* genes partially complemented the uptake mutant making it more sensitive to high PA concentrations. This observation indicated that the identified plant *PUT*s are involved in PA uptake (Mulangi et al. 2012b). Fujita et al (2012) demonstrated an *in-planta* phenotype-driven screening of Arabidopsis *PUT3*/*RMV1*/*AtLAT1*. According to their findings,

Arabidopsis plants overexpressing *RMV1* were more sensitive to Put, Spd and Spm indicating the PA uptake by this protein. Functional and kinetic studies of mammalian PA transporters that belong to SLC family have been carried out in specific cell types such as human embryonic cells, in animal model systems such as mice model and in membrane vesicles generated from either bacterial cells or Chinese hamster ovary cells (Ariyaratne et al. 2019; Uemura and Gerner et al. 2011; Winter et al. 2011).

4.2 Hypothesis and research goals

Bioinformatic evidence and analyses suggest that *Phytophthora* genus expresses a diverse family of transporters that are likely involved in PA transport. These evidence support the hypothesis that *PUT1,* a putative transporter expressed in *P. parasitica* zoospores is involved in PA transport. Presumably, no previous research has functionally characterized PA transporters in oomycetes. Chibucos and Morris (2006) showed that zoospores take up PAs from the environment and presented evidence for the existence of PA uptake transporters in *P. sojae*. Some bioinformatic evidence also suggests the presence of PA transporters in *P. sojae* (Mulangi et al. 2012b; Valdés-Santiago et al. 2012). However, functional studies of oomycete PA transporters are lacking mainly because not many research groups are studying oomycete-PA biology. This study aims to explore the function of *PUT1,* a putative PA transporter expressed in *P. parasitica* zoospores, using *S. cerevisiae* as a system for heterologous expression. The long-term goal of this work would be to provide insights into targeting PA transporters for controlling oomycete diseases.

4.3 Materials and methods

4.3.1 Plasmid construction

The full-length protein sequence and the corresponding cDNA sequence of *PUT1* gene were retrieved from FungiDB. The full-length gene was codon optimized by GeneScript (Genscript Corp., NJ) and was obtained in a pUC57 vector. The full-length gene was amplified using the primers 5'-CACCATGGGAATGTTGGAAGAAG-3' and 5' AGCCAAACTTCTTCTGTAATAAAATCTG 3'. The PCR conditions were; initial denaturation at 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 51°C for 30 sec and 72°C for 30-45 sec followed by a final extension step of 72°C for 5 min. All PCR reactions were performed using Phusion High-fidelity DNA polymerase (New England Biolabs, MA) on a thermocycler (Bio-Rad, CA). The PCR fragment was cloned into pENTR/SD/D-TOPO cloning vector (Thermo Fisher Scientific, MA) according to the supplier's instructions. Next, PPTG_00424 gene was transferred to an expression vector, pYES-DEST52 (Fig. 17), via a recombination reaction (Gateway LR clonase, Thermo Fisher Scientific, MA) where the target gene is driven by GAL1 promoter (Fig. 6). pYES-DEST52 uses the gene *URA3* necessary for uracil biosynthesis as the selectable marker in yeast. This plasmid construct was designated as pYES-DEST52-*PUT1* and was used for heterologous expression in yeast.

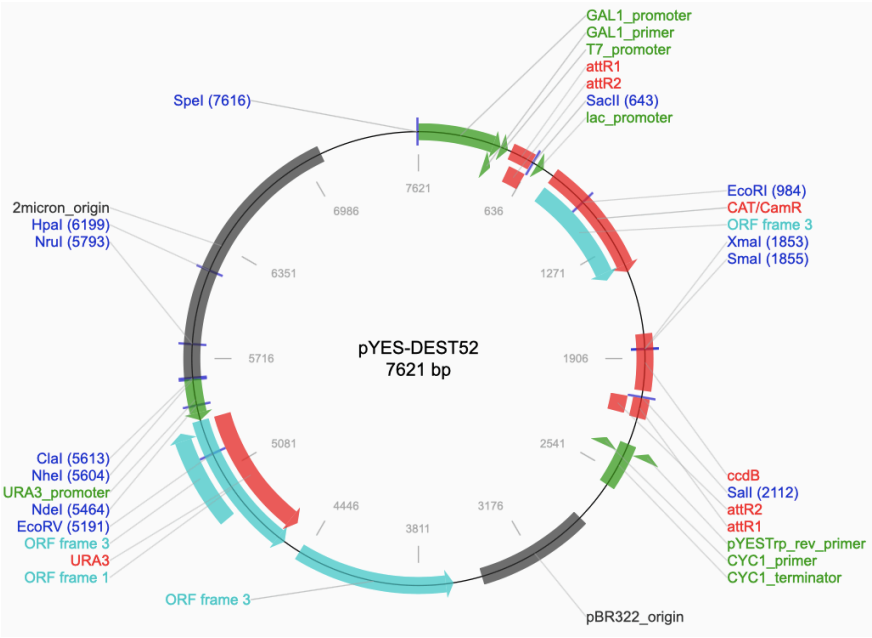

Fig. 17. Plasmid map of pYES DEST52, the vector used for heterologous expression of *PUT1* in yeast (http://www.addgene.org).

4.3.2 Transformation of yeast

The pYES-DEST52-*PUT1* construct was introduced into TPO5Δ (strain Δykl174c) (Tachihara et al. 2005) and wild type (strain BY4741) cells using lithium acetate method of transformation according to the instruction manual provided with pYES-DEST52 plasmid (Invitrogen, 2002). Few modifications were made to optimize the transformation efficiency. Briefly, the cells were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C to mid-log phase. The overnight culture was diluted with fresh YPD to an optical density at 600 nm (OD_{600}) of 0.4 and grown for additional ~2 hours until OD_{600} reaches 0.6. The cells were centrifuged (3000 rpm, 15 min), washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA), washed again with 5 ml of LiAc mixture (100 mM lithium acetate, 10 mM Tris-HCl, 1mM EDTA) and resuspended in 0.5 ml of the same LiAc mixture. After incubating the cells in LiAc mixture for 60 min at room temperature, 10 µl of 10 mg/ml boiled sheared salmon sperm DNA (Sigma-Aldrich, MO) and 1 µg of plasmid DNA (in ~10 µl) were

added followed by an incubation step at room temperature for 30 mins. Then, 1 ml of transformation mixture which contains 40% polyethyleneglycol 3500, 100 mM lithium acetate, 10 mM Tris-HCl and 1mM EDTA was added and incubated at 30ºC for another 30 mins with gentle vortexing every 10 mins. Next, 40 µl of dimethylsulfoxide was added to the mixture. The resulting mixture was incubated at 42ºC for 10 min and immediately chilled on ice for 3 mins. The cells were then pelleted at 1250 rpm for 5 mins and resuspended in TE buffer. The cells were plated on to compete supplement mixture lacking uracil (0.77 g CSM-Ura, 6.7 g yeast nitrogen base, 20 g dextrose, 20 g agar per liter). The plates were incubated at room temperature for 2 days until transformants appear. The transformant colonies were verified by PCR amplification of a partial region (442 bp) of the *PUT1* gene using primers: 5'-CGCATCTGTATTTGGTGGTG-3' and reverse 5'-TGCAGCACTATAAGCGTTGG-3' using PCR condition described in 3.3.2.

4.3.3 Yeast complementation assays

The yeast growth assays were conducted according to the protocol described by Aouida et al (2013). Transformant and non-transformant wild type (BY4741) and TPOΔ (Δykl174c) cells were grown overnight in 2 ml of YPG broth at 30°C until mid-log phase (OD_{600} = 0.5-0.8). Then, the cells were diluted down to an OD_{600} of 0.1 with fresh YPG broth. The cell suspensions were dispensed into a 24-well plate with either spermidine (sigma-aldrich) or putrescine dihydrochloride (sigma-aldrich) to the desired concentration maintaining the total volume constant (2 ml). The plate was covered with a lid and was incubated at 30°C while shaking at medium setting for 26 hours and the growth was measured at 540 nm every 30 mins using a Synergy HT microplate reader (BioTek, VT). The spermidine concentrations used were 0, 5. 7.5 and 12.5 mM while putrescine concentrations used were 0, 200, 250 and 300 mM. At least three

replicates were used for each polyamine concentration and cell strain and each experiment was repeated at least twice.

4.3.4 Yeast transport assays

4.3.4.1 Uptake assays

The uptake and export assays were carried out following the protocols described by Tachihara et al (2005) with some modifications. For time course PA uptake assays, transformant and non-transformant wild type (BY4741) and TPOΔ (Δykl174c) cells were grown overnight in YPG media (1% yeast extract, 2% peptone) in the presence of 2% galactose at 30°C. Cells at the mid-log phase were harvested, washed three times with the uptake buffer [20 mM Na-HEPES (pH 7.2), 2% galactose] and were resuspended in the same buffer at a concentration of 2000 μg/ml. Aliquots of 990 μl cell suspension were transferred to microcentrifuge tubes and incubated at 30°C for 5 mins. The polyamine uptake was initiated by addition of 10 μl of 0.5 mM spermidine labeled with ^{3}H Spermidine (4 ×10^{5} dpm/ml of cells) at 30°C. At selected time points (0, 5, 10, 15 min), 200 μl of cells was removed, filtered through 0.45 μm glass microfiber filters (Whatman 25 mm GF/A) and washed with 3 ml of ice-cold uptake buffer containing tenfold higher concentrations of spermidine to remove non-specific binding of labeled spermidine. The filters were then transferred to scintillation vials containing 10 ml of scintillation fluid Ecoscint A (National Diagnostics, GA). The radioactivity on the filters were determined in a Beckman LS-7000 scintillation counter (Beckman Coulter Inc., CA). Similarly, ^{3}H Spermidine was added to pre-chilled cells on ice and the radioactivity was measured after 15 mins as a control. Three replicates were used for each experiment.

4.3.4.2 Export assays

For the export assay, cells were grown overnight in YPG broth at 30°C. Cells at the mid-log phase were harvested, washed three times with the export buffer [20 mM Na-MES, 2% galactose (pH 5.5)] and were resuspended in the same buffer at a concentration of 2000 μg/ml. Aliquots of 990 μl cell suspension were incubated with 0.5 mM spermidine labeled with ^{3}H Spermidine (1×10^{6} dpm/ml of cells) for 90 mins. The cells were then filtered through 0.45 μm glass microfiber filters (Whatman 25 mm GF/A) and washed with 3 ml of ice-cold export buffer containing tenfold higher concentrations of spermidine to remove non-specific binding of labeled spermidine. The filter was transferred to a glass vial which contained 1 ml of export buffer at room temperature. At each time point (0, 5, 10, 15 min), 200 μl of cells was transferred to a fresh eppendorf tube on ice and immediately centrifuged at 10000 rpm for 1 min to separate cells from the supernatant. The supernatant was transferred to a scintillation vial containing 10 ml of scintillation fluid Ecoscint A (National Diagnostics, GA). The radioactivity of the supernatants was determined in a Beckman LS-7000 scintillation counter (Beckman Coulter Inc., CA). Pre-chilled cells were incubated in the export buffer for 15 mins on ice as a control. Three replicates were used for each experiment.

4.4 Results and discussion

This study aims to investigate the function of a putative PA transporter expressed by *P. parasitica* using yeast as a heterologous expression system. In order to confirm optimal expression in *S. cerevisiae,* the *PUT1* gene was codon-optimized by GeneScript (Genscript Corp., NJ). Next, the complete gene was cloned into an expression vector that contains a *URA3* marker that allows the growth of transformants in the absence of uracil in the medium. Complementation and transport experiments were carried out using an export-deficient yeast

mutant (TPO5Δ) because our previous localization data (described in chapter 3) showed that PUT1 is an ER-targeted membrane protein that may be involved in either export or sequestration of PAs. To compare the effect of the introduced gene with the generic yeast PA transport, we included a wild type *S. cerevisiae* strain (BY4741) in our experiments.

4.4.1 Yeast complementation assays

Three concentrations of Spd (5, 7.5 and 10 mM) were used for the complementation assays until the concentration at which growth inhibition could be observed. Sterile distilled water was added in place of Spd as control. The growth curves at 0 mM and 5 mM Spd are shown in Fig. 19. No growth was observed at 7.5 and 10 mM due to the oxic effect of Spd (data not shown).

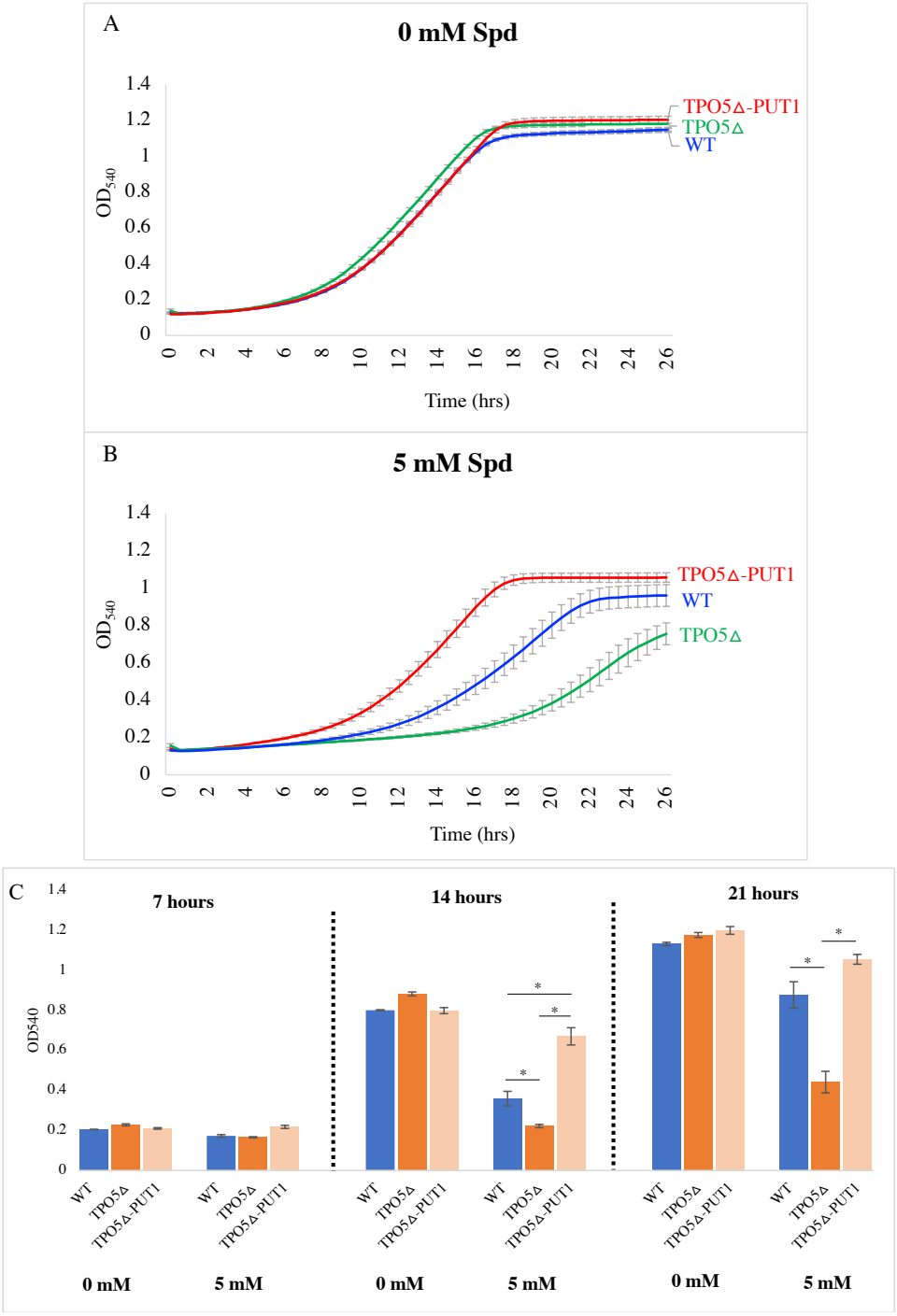

Fig. 18. Yeast complementation assay. A, in the absence of spermidine and B, in the presence of 5 mM spermidine. Growth curves of TPO5Δ cells and TPO5Δ cells expressing *PUT1* are shown in red whereas wild type cells are shown in blue. No growth was observed at 7.5 and 10 mM Spd (data not shown). C, The growth of each yeast strain measured at early log phase (~7 hours), mid

log phase (~14 hours) and stationary phase (~21 hours), with and without the presence of 5 mM Spd. Asterix represents statistical significance between means (*, $P < 0.05$, student's t-test). All cells were grown in a microplate at 30°C for 26 hours. Error bars represent standard error of the mean. n=6.

As shown in Fig. 18A and C, growth curves of all four cell types were almost similar in the absence of Spd. This observation indicates that expression of *PUT1* gene in the yeast did not impose any negative effects towards the growth rate in the absence of PAs. TPO5Δ mutant was sensitive to 5 mM Spd compared to both wild type and TPO5Δ-*PUT1* (Fig. 18B) as the lack of vesicle-mediated exporter encoded by *TPO5* resulted in accumulation of Spd that retarding its growth. This growth reduction led by excess Spd could be associated with inhibition of protein synthesis. It has been reported that, in the bacterial system, overaccumulation of Spd inhibited protein synthesis by excess spermidine binding to ribosomes (Raj et al. 2001). The growth curves in Fig. 18B and C show that TPO5Δ cells expressing *PUT1* significantly grew faster than the non-recombinant TPO5Δ cells in the presence of 5 mM Spd. This data indicates that expression of *PUT1* conferred protection to TPO5Δ cells against Spd toxicity by facilitating Spd export (Fig 18A and B). It is also noteworthy that the growth curve of TPO5Δ-*PUT1* reached the stationary phase faster than the wild type cells (Fig. 18B and C).

Tachihara et al (2005) reported that TPO5Δ cells grow faster than the wild type cells in the absence of PAs because existence of *TPO5* gene in wild type cells exerts a growth inhibition through excretion of PAs under normal conditions. In contrast to their report, we did not see an increased growth of TPO5Δ compared to wild type in the absence of exogenous Spd (Fig. 18B). This may be due to the differences in the experimental conditions such as growth media. It can also be hypothesized that apart from the export function, *TPO5* in the wild type cells might be

involved in regulating other genes associated with the PA homeostasis thereby arresting the cell cycle as an adaptation to high Spd exposure. A similar example is AGP2, a PA importer of *S. cerevisiae* that controls expression of several other PA transporters in addition to its import function (Aouida et al. 2013). This hypothesis is supported by the gene expression analysis performed by Chattopadhyay et al (2009) where the expression of *TPO5* showed an upregulation more than 2-fold after exposure to 10^{-3} mM of Spd. In our study, the faster growth of TPO5Δ-*PUT1* compared to wild type in the presence of 5 mM Spd suggests that *PUT1* gene does not seem to substitute the growth suppression mediated by *TPO5* gene in response to high Spd levels in the yeast system.

The growth experiments were performed using the same cell strains with Put as a substrate. When cells were exposed to varying concentrations of Put, *PUT1* did not confer protection to TPO5Δ cells at 200, 250 and 300 mM Put (data not shown). It is possible that PUT1 exports Spd more effectively than it does for Put. This prediction is supported by the fact that plant polyamine uptake proteins (PUTs) show Spd-preferential activity over Put. The homology of amino acid sequences between plant PUTs and *PUT1* is approximately 30%. Although *S. cerevisiae* is vastly utilized as a heterologous expression system in membrane protein research, the functionality of the protein is sometimes problematic when overexpressed in yeast, especially for larger proteins (>60 kDa) with many transmembrane domains (>6) (Ito et al. 2008). Therefore, a loss-of-function experiment in *P. parasitica* of *PUT1* would help draw a more informative conclusion regarding its role in transporting PAs.

4.4.2 Yeast transport assays

4.4.2.1 Uptake assay

In order to test the transport activity of the membrane protein encoded by *PUT1,* uptake experiments were conducted using ^{3}H labeled Spd in the PA sensitive mutant TPO5Δ and TPO5Δ cells expressing *PUT1*. Wild type *S. cerevisiae* cells were used as a control. The time course uptake of ^{3}H labeled Spd for TPO5Δ, TPO5Δ expressing *PUT1* and wild type is shown in Fig. 19.

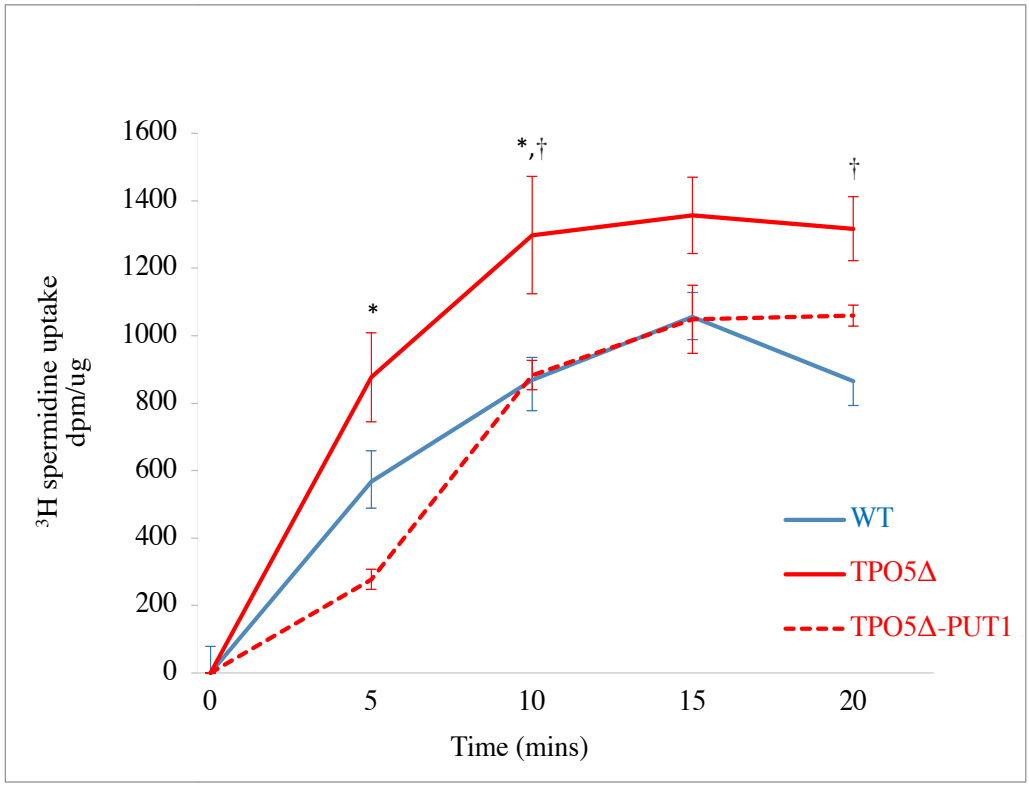

Fig. 19. Uptake of ^{3}H Spd measured every five minutes for 20 mins by TPO5Δ, TPO5Δ expressing *PUT1* and wild type. The net uptake was measured in the units of disintegrations per minute (dpm) per µg protein. Error bars show standard error of means. "*" represents statistically significant differences ($P < 0.05$) of TPO5Δ compared with the TPO5Δ- *PUT1*. "†" represent statistically significant differences ($P < 0.05$) between WT and TPO5Δ- *PUT1*. There were no significant differences between WT and TPO5Δ-*PUT1* at any time point. Comparisons were made using one-way ANOVA followed by Tukey's test. n = 4.

The net uptake of ^{3}H labeled Spd by TPO5Δ and TPO5Δ-*PUT1* increased during the first 10 mins and gradually levelled off by 20 mins. Wild type strain showed a similar increase and then decreased after 15 mins. The export-deficient mutant TPO5Δ cells internalized the highest amount of Spd compared to all other cell types. This observation is in agreement with the expected phenotype of TPO5Δ as these export-deficient cells must accumulate more PAs

(Tachihara et al. 2005). The Spd amount that TPO5Δ cells internalized was significantly higher through the first 10 mins compared to the TPO5Δ expressing *PUT1* gene. In other words, the amount of radioactivity TPO5Δ-*PUT1* cells imported was significantly lower than that of TPO5Δ cells indicating that the heterologously expressed PUT1 protein does not accumulate Spd. It is possible that the PUT1 protein is involved in the export of ^{3}H Spd in the yeast system. However, this prediction must be supported by data from a PA export assay. Furthermore, the net ^{3}H Spd uptake by *S. cerevisiae* cells in general was low throughout the experiment compared to the uptake data reported by previous studies (Aouida et al. 2005; Tachihara et al. 2005). The restricted use of ^{3}H Spd in the substrate (10^{-3} nmol) could account for low net uptake and higher background radiation in this study. A higher concentration of isotope-labeled polyamines could be used at picomolar levels to collect robust data in the uptake assay.

4.4.2.2 Export assay

To test the hypothesis that *PUT1* is involved in the export of Spd, excretion of ^{3}H Spd was examined using cells preloaded with ^{3}H labeled Spd. The four yeast strains were first allowed to uptake ^{3}H Spd for 90 mins and then were filtered and transferred to an export buffer for time course measurement of ^{3}H Spd excretion. The export data is shown in Fig. 20.

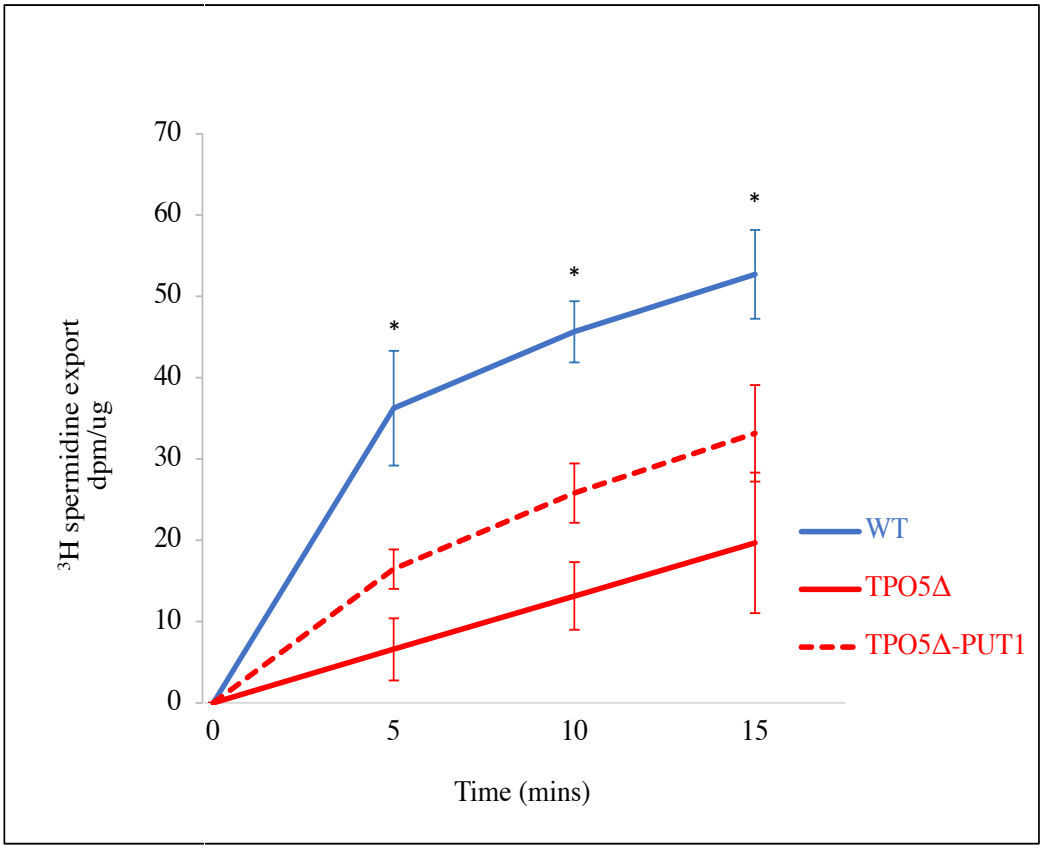

Fig. 20. Export of ^{3}H Spd measured every five minutes for 15 mins by TPO5Δ, TPO5Δ expressing *PUT1* and wild type. The exported ^{3}H Spd amounts were measured in the units of disintegrations per minute (dpm) per µg protein. Error bars show standard error of the means. "*" represents statistically significant differences ($P < 0.05$) of wild type compared with the TPO5Δ. There were no significant differences between either WT and TPO5Δ- *PUT1* or TPO5Δ and TPO5Δ-*PUT1* at any time point. Comparisons were made using one-way ANOVA followed by Tukey's test. n=3.

As shown in Fig. 20, the export of ^{3}H Spd by all cell types increased over time following the 90 min incubation. The TPO5Δ-*PUT1* showed higher excretion of ^{3}H Spd compared to TPO5Δ cells providing a sign that *PUT1* might be involved in Spd excretion. However, this

increase in export was not statistically significant compared to TPO5Δ. Wild type cells showed the highest level of excretion compared to both TPO5Δ and TPO5Δ expressing *PUT1* (Fig. 20).

When the export data (Fig. 20) is compared with the uptake data (Fig. 19), it is noticeable that the *S. cerevisiae* cells in general have exported only 1-4 % of the PAs that they had internalized. Previous studies that used the same TPO5Δ and wild type strains reported that the cells export approximately 10% of the imported polyamines (Tachihara et al. 2005). The lower uptake rate observed in the current study could be due too high background and insufficient incubation in the labeled PA. This export experiment could benefit by preloading the cells with at least 10-fold higher (~10^7 dpm/ml of cells) concentration of ^{3}H Spd. The export data was inadequate to quantify the precise export kinetics of *PUT1*, however, it provided a baseline to optimize the export assay to detect substantial levels of PAs using the given cell strains.

According to the literature, the uptake activity of plant PUTs was measured using a similar approach. Mulangi et al (2012b) analyzed the PA uptake of the rice transporter, *OsPUT1*, by measuring the amount of radiolabeled PAs that the protein imports when heterologously expressed in yeast cells. The inhibition of uptake by other substrates such as different PAs, amino acids and PA precursors could also be assessed by measuring the uptake in the presence of different unlabeled substrates. Fujita et al (2012) assessed the transport activity of Arabidopsis RMV1/AtPUT3 using paraquat, a structural analog of PAs. Paraquat and PAs share similar uptake mechanisms in both plant and animal systems (Hart et al. 1992). Fujita and his coworkers analyzed the paraquat uptake of AtPUT3 by measuring the radioactivity retained in Arabidopsis seedlings after incubating their roots in a ^{14}C labeled paraquat, both with and without competitive and inhibitory substrates. Concentration-dependent kinetics (K_{mv}values) of plant PA uptake proteins were also measured using a similar approach (Fujita et al. 2012; Mulangi et al.

2012b). The K_m values of plant PUTs were determined using yeast cells or plant roots expressing the transporter gene where they were exposed to increasing concentrations of radiolabeled PA or paraquat followed by estimation of intracellular radioactivity (Fujita et al. 2012; Mulangi et al. 2012b). Unlike import, estimation of cellular PA efflux is challenging as the techniques have to be fine-tuned to capture the minute amounts of labeled PAs that are being exported after preloading. Cellular efflux of auxin by a multiple drug resistance (MDR)-type plant transporter was determined using Arabidopsis mesophyll protoplasts (Geisler et al. 2005). In their method, the protoplasts expressing the export protein were first incubated in radiolabeled auxin for a given time period and the incubation was halted by rapid centrifugation through a silicon oil barrier. The amount of radioactivity that has been retained in the protoplast pellets and the amount exported into the aqueous phase was measured by scintillation counting. Furthermore, expression of eukaryotic transporters in *Xenopus laevis* oocytes and *Nicotiana benthamiana* are also popular tools for characterizing efflux and intracellular transport of biomolecules (reviewed in Larsen et al. 2017).

Collectively, the uptake and export experiments of the current study provide evidence for the role of *PUT1* in the cellular export of Spd. The amount of radioactivity effluxed by TPO5Δ-*PUT1* was greater than the TPO5Δ cells indicating that the heterologously expressed PUT1 protein exports Spd independently from the TPO5 protein. However, this needs to be confirmed by higher concentrations of preloaded ^{3}H Spd. The yeast complementation assays (Fig. 18) confirm that expression of *PUT1* gene partially rescued the mutant phenotype of TPO5Δ from the toxicity caused by 5 mM Spd.

4.5 Conclusions and future directions

To date, no PA transporters have been characterized in oomycetes, especially *Phytophthora* pathogens. In chapter 2, a comparative genomics approach and available transcriptome data were used to identify a putative PA transporter in *P. parasitica. PPTG_00424* (*PUT1*) was identified as a putative PA transporter in *P. parasitica* which belongs to the polyamine uptake transporter (PUT) family. PUT1 protein shares 31% homology to Arabidopsis PUT5. Chibucos and Morris (2006) had previously reported that oomycetes express PUTs that are homologous to plant PUTs. The expression of *PUT1* is highest in the zoospore stage compared to other life stages indicating that the biological function of this protein is associated with zoospore survival and subsequent pathogenicity. The phylogenetic analysis indicate that PUT family in *Phytophthora* is surprisingly diverse than plant PUTs. Our localization experiments described in chapter 3 confirmed that the GFP-tagged PUT1 protein localizes to the ER network of *P. sojae* hyphae.

The growth experiments of the current study support the hypothesis that the heterologously expressed PUT1 protein is involved in the export of Spd in *S. cerevisiae*. The uptake assay confirmed that this protein is not directly involved in the import of Spd into the *S. cerevisiae* cells. The export data provides evidence to classify PUT1 as a Spd exporter that functions independently from TPO5, however, this needs to be confirmed by a more robust export assay with more radiolabeled PAs preloaded and probably for a longer time. Future experiments could support the functionality of *PUT1* in the yeast expression system using either a reverse transcription PCR (RT-PCR) or a western blotting. To explore the affinity of *PUT1* to Spd, kinetic constants (K_m and V_{max}) could be determined by measuring radioactivity effluxed by yeast cells expressing *PUT1* upon exposure to increasing concentrations of ^{3}H Spd.

Although the function of *PUT1* could be predicted as Spd transport, the importance of Spd transport in zoospore is unclear. It has been reported that Spd regulates cell growth largely by modulating protein synthesis at translational level (Igarashi and Kashiwagi 2010). One example is modification of eukaryotic translational elongation initiation factor eIF5A (Saini et al. 2009). Chibucos and Morris (2006) detected both Put and Spd at nanomolar concentrations in *P. sojae* zoospores. They also demonstrated concentration-dependent uptake of Put and Spd by *P. sojae* zoospores predicting the existence of at least two high affinity PA transporters, a Spd preferential transporter and a Put specific/preferential transporter. However, transcriptome data indicate that *PUT1* is upregulated at the zoospore stage of *P. parasitica*. If *PUT1* functions as a Spd exporter in *P. parasitica*, upregulation of *PPTG_00424* in the zoospores indicate that export of Spd is as crucial as uptake. According to literature, spermidine content increases during conidiation and then decreases rapidly during germination of conidia in the fungal pathogen that causes rice blast (Choi et al. 1998). In the same fungal pathogen, exogenous addition of PAs including Spd has suppressed the appressoria formation. In light of this, it is possible that export of Spd occurs as a preparation for germination of *Phytophthora* zoospores in a later stage following zoospore emergence.

Notably, none of *PUT1* homologs in *P. parasitica* are significantly upregulated during infection. This would suggest that PUTs may not play a role in transport during infection. Mining the gene expression dataset of PUT genes of other oomycetes during infection may shed light on the role of PUTs in oomycete virulence.

A gene disruption study could answer several important questions regarding the function of *PUT1* gene. They include would the gene confer resistance to higher levels of Spd, and would it affect the normal growth and reproduction of *P. parasitica*. *PUT1*-membrane protein family

has more than six members in most *Phytophthora* species which makes a gene disruption analysis challenging. However, CRISPR gene editing technology has been already implemented for oomycetes (Fang et al. 2017). Therefore, different members of *PUT1* family could be knocked out to reveal their specific function. Functional characterization of *PUT1* family would provide novel targets for controlling *Phytophthora* diseases.

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