Roles of disproportionating enzymes in the moss *Physcomitrella patens*

by

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Declaration

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Abstract

Starch is a polyglucan made up of the two glucose polymers, amylose and amylopectin. Plants use starch to store excess carbohydrates from photosynthesis which get used for growth during the night. Starch metabolism is well undertood in higher plants such as *A. thaliana thaliana* and *Solanum tuberosum* with well-established pathways worked out for the enzymes involved in its synthesis and degradation.

The bryophyte *Physcomitrella patens* has emerged as a popular choice for studying gene function in lower plants both because its genome has been sequenced and because of the ease of establishing knockout mutants via homologous recombination. Many metabolic functions have been studied in *P. patens* but, until now, little has been done in examining starch metabolism in moss.

This study focused on two enzymes that have been found to be involved in starch degradation in higher plants, Disproportionating enzyme 1 (DPE1) and Disproportionating enzyme 2 (DPE2)*.* DPE1 isoforms have been found to break down malto-oligosaccharides, which are products of starch degradation, into glucose within the chloroplast. On the other hand DPE2 catabolizes maltose to glucose in the cytosol. Higher plants that were silenced in these two genes were unable to degrade starch effectively, which lead to an increase in starch, malto-oligosaccharides or maltose and reduced growth.

Three orthologs were identified for *DPE1* in *P. patens* (*PpDPE1A*, *B* and *C*) and one for *DPE2* (*PpDPE2*). Only *PpDPE1B* and *PpDPE1C* were found to be expressed in *P. patens* at the beginning of the light period but further investigation would be necessary at different time points as these genes were shown to be optimally expressed at the end of the light period. Targeted gene knockouts were made for each in *P. patens* which showed a reduced growth phenotype for all, indicating that these genes do play a role in starch catabolism that influences growth. There was, however, no significant change in starch content between the mutant lines and wild type (Wt).

GFP fusion proteins showed PpDPE2 to be localized in cytosol, in close proximity to the chloroplast membrane. Similar findings have been found for *DPE2* in *A. thaliana* and *S. tuberosum*. We hypothesize that PpDPE2 may play a role in cold tolerance in moss as an increase in starch breakdown has been witnessed in cold treated moss as well as increased transcript levels of starch metabolism genes and a maltose transporter. This opens a door to the further study of these generated mutant lines under cold stress.

Opsomming

Stysel is 'n poliglukaan wat bestaan uit die twee glukose polimere: amilose en amilopektien. Plante gebruik stysel om oortollige koolhidrate van fotosintese wat vir groei gebruik word gedurende die nag te berg. Styselmetabolisme in hoër plante soos *A. thaliana thaliana* en *Solanum tuberosum* word goed verstaan, met gevestigde paaie uitgewerk vir die ensieme wat betrokke is by die sintese en afbreek daarvan.

Die briofiet *Physcomitrella patens* is 'n populêre keuse vir die bestudering van geenfunksie in laer plante, omdat die genoomvolgorde bepaal is en as gevolg van die gemak waarmee 'uitklop'-mutante via homoloë rekombinasie gevorm kan word. Baie metaboliese funksies is bestudeer in *P. patens* maar tot nou is min gedoen om die styselmetabolisme in mos te ondersoek.

Hierdie studie het gefokus op twee ensieme, DPE1 and DPE2, wat gevind is om betrokke is afbreek van stysel in hoër plante. Dit is voorheen bevind dat DPE1 isoforme malto-oligosakkariedes (wat produkte is van styselafbraak) afbreek na glukose in the chloroplast. Aan die ander kant kataboliseer DPE2 maltose na glukose in die sitosol. Hoër plante waarin hierdie gene stilgemaak is, is nie instaat daartoe om stysel effektief af te breek nie. Dit lei tot 'n verhoging in stysel, malto-oligosakkariede of maltose en verminderde groei.

Drie ortoloë is geïdentifiseer vir *DPE1* in *P. patens* (*PpDPE1A*, *B* en *C*) en een vir *DPE2* (*PpDPE2*). Slegs *PpDPE1B* en *PpDPE1C* word uitgedruk in *P. patens* aan die begin van die ligperiode, maar verder ondersoek sal nodig wees op verskillende tydpunte, omdat dit bewys is dat hierdie gene optimaal uitgedruk word tydens die einde van die ligperiode. Geteikende geen uiklop-mutante is gemaak vir elk in *P. patens* wat 'n verminderde-groei fenotipe vertoon het vir almal, wat aandui dat hierdie gene 'n rol speel in styselkatobolisme wat groei beïnvloed. Daar was egter geen beduidende verskil in styselinhoud van die mutante lyne en die wilde tipe nie.

GFP-fusieproteïne het gewys dat PpDPE2 gelokaliseer is in die sitosol, naby aan die chloroplast membraan. Soorgelyke bevindinge is ook gemaak in DPE2 in *A. thaliana* en *S. tuberosum*. Dit word gestel dat PpDPE2 moontlik 'n rol speel in kouetoleransie in moss, omdat 'n verhoging in styselafbraak opgemerk is in koue-behandelde moss sowel as verhoogde transkripsievlakke van styselmetabolisme gene en 'n maltose transporter. Dit maak 'n deur oop vir verdere studie van hierdie gegenereerde mutant-lyne onder kouestres.

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Chapter 1

Literature review

1.1 Starch

Two glucose polymers, amylose and amylopectin, form the polyglucan known as starch. This molecule is normally present as insoluble granules and is the main compound that most plants use to store carbohydrates. In leaves, carbon is fixed during photosynthesis and gets converted to soluble sugars and starch in specialized organelles known as chloroplasts. Starch formed here is often referred to as 'transitory starch' and has the important function of providing substrates for leaf respiration and to maintain sucrose synthesis during periods of darkness. As such its presence is of great importance for normal plant growth. For example, the *A. thaliana thaliana phosphoglucomutase* (*pgm)* mutant lacks starch in its leaves and accumulates large amounts of sugars during the light period which gets rapidly exported from the leaf during the night time (Caspar et al., 1985). Growth becomes inhibited in these mutants at night (Stitt and Zeeman, 2012) accompanied by a prominent decrease in endogenous sugar levels causing a highly modified expression of thousands of genes, highlighting the important role that sugars play in diurnal gene expression (Bläsing et al. 2005). It has recently been demonstrated that some of the genes which show decreased expression are involved in gibberellin biosynthesis and that this leads to reduced production of this phytohormone, linking decreased starch to reduced growth (Paparelli et al., 2013). In contrast to leaves, storage organs are often non-photosynthetic and contain specialized plastids known as amyloplasts. In these tissues sucrose, exported from the leaves, gets converted to storage starch which can be stored for extended periods before mobilisation. Such organs include seeds (e.g. from maize, barley, wheat, pea), tubers (e.g. from yams, potatoes, sweet potatoes) and roots (e.g. from cassava). Starch is often stored at very high levels in these organs and because they act as staple crops, makes up a large portion of calories in the human diet (Zeeman et. al., 2010).

Starch is not only useful as a source of energy for humans and farm animals, but is also used in a number of industrial processes. According to Röper (2002), 44.2 % of all starch and starch derivatives produced within Europe are used in non-food industries which take advantage of the adhesive and binding properties of starch. Table 1 gives an indication of the amount of starch that is utilized annually within the different non-food industries in Europe. Industries such as the paper,

cosmetic, pharmaceutical, detergent and biofuels, take advantage of its physicochemical properties which give it the capacity to bind water, act as an adhesive and increase viscosity of fluids. Figure 1 shows some of the many industrial uses that starch is put to. Native starches are often chemically or physically altered prior to use either by changing the size of the starch molecule or by the addition of substitutes such as phosphate, ester- or ether groups (Röper, 2002).

Table 1.1: Amount of starch used by industry per annum (Adapted from Röper, 2002).

Starch (t)	Non-food application
1,900,000	paper & corrugating
1,000,000	chemicals, fermentation
500,000	binders, adhesives
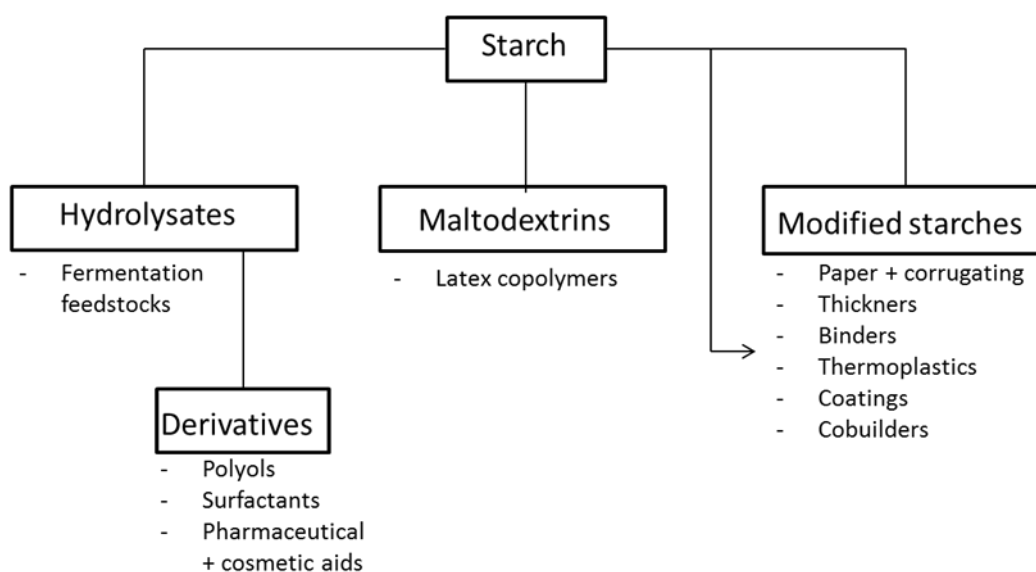
Figure 1.1: Uses of starch and starch derivatives in industry (adapted from Röper, 2002).

1.2 Starch structure

Starch is a polymer that is composed of glucose monomers which are differentially linked, yielding either the linear molecule amylose, or a bigger more branched molecule called amylopectin. Within the amylose molecule glucose moieties are bound to one another almost exclusively via α-1,4 bonds, whereas approximately 5% of the glucose units in amylopectin are joined by α-1,6 branchpoints (Jobling, 2004).

The chains within amylopectin are characterized as three types (Figure 1.2). (A) chains are present in clusters on the outside of the molecule and they form glycosidic bonds through C6 residues with inner (B) chains which, in turn, carry other (A) chains. There is only one (C) chain present per molecule and this contains the only reducing end. Amylopectin consists of short chains which contain the outside (A) chains and inside (B) chains as well as longer (B) chains. A backbone of long chains support clusters consisting of bunches of short chains (Figure 1.2; Buléon et al., 1998). Neighbouring short chains within the clusters of amylopectin form double helices which, because of their ordered nature, contribute to the crystallinity of lamellae within the starch granule. Amylose forms single helices which contribute to amorphous lamellae of the granule (Zeeman et al., 2010). These alternating crystalline and amorphous lamellae form growth rings within the starch granule (Ridout et al., 2003) which can be observed using light or electron microscopy of an acid or enzyme pre-treated starch granule (Figure 1.3; Buttrose, 1960; Pilling and Smith, 2003). In plants starch is stored it the form of semi-crystalline starch granules. The double helices within the clusters that contribute to the crystallinity are approximately 9nm in length and interspersed with amylose containing amorphous zones (Jenkins and Donald, 1995).
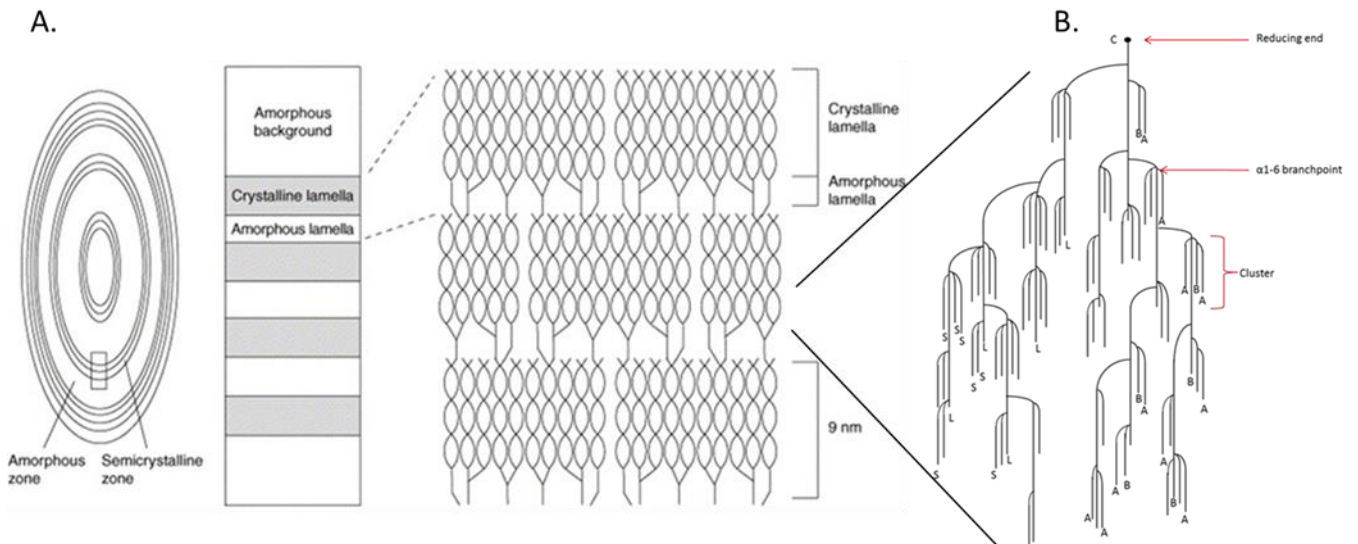
Figure 1.2: A) The starch granule is made up of alternating amorphous and crystalline zones with the crystalline parts composed of helical amylopectin bundles containing both amylopectin and amylose. **B)** The amylopectin molecule consists of outside (A) chains and inside (B) chains both the product of α1-6 branch points. The sole (C) contains the only reducing end. Short chains (S) consist of (A) and (B) chains and long chains (L) are made up solely from (B) chains (adapted from Jobling, 2004).
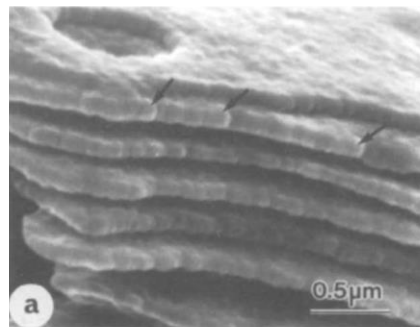
Figure 1.3: Scanning electron micrograph of wheat starch partially digested with α-amylase displaying the alternating crystalline lamellae (adapted from Gallant et. al., 1997).

1.3 Starch synthesis

Much light has recently been shed on the many types of enzymes involved in plant starch metabolism in higher plants (Figure 1.4; Sonnewald and Kossman, 2013). This introduces the possibility for producing 'designer starches' that contain specific desired properties for particular industries without the need of introducing this via chemical-, enzymatic- or physical manipulations that would increase cost (Jobling, 2004).

Glucose chains in amylose and amylopectin are generated from the precursor molecule, ADP-glucose, which gets polymerised by enzymes known as starch synthases. There are five genes that encode different starch synthase (ADP-glucose:(1→4)-α-D-glucan 4-α-D-glucosyltransferase; EC: 2.4.1.21) isoforms in *A. thaliana* (Table 2); Granule-bound starch synthase (GBSS) is found only within the starch granule (Ball & Morell, 2003). Transgenic and mutant plants lacking GBSS contain amylose free starch, demonstrating that this isoform is involved solely in amylose production (Visser et al. 1991; Kuipers et al., 1994) and such starches have great commercial use as a thickener and stabilizer in food (Jobling, 2004). The four remaining soluble isoforms; SSI, SSII, SSIII and SSIV are responsible for generating linear chains within amylopectin (Ball and Morell, 2003).

The first plants lacking soluble starch synthases were isolated in the mid-1990s and demonstrated that these enzymes were involved in amylopectin synthesis. Mutant and transgenic plants lacking these isoforms produce amylopectin with altered internal chain lengths (Abel et al. 1996; Craig et al, 1998; Lloyd et al., 1999; Edwards et al., 1999; Marshall et al, 1996). Since then many plants have been isolated and studied where different isoforms of these genes have been removed in both storage organs as well as leaf material.

A. thaliana plants lacking *SSI* due to T-DNA insertions within the *AtSS1* locus showed a compromised form of amylopectin structure containing fewer short B chains, indicating a prominent role of this enzyme within amylopectin synthesis specifically in cluster synthesis (Delvallé et al., 2005). On the other hand those lacking *SSII* contained amylopectin with increased short chains (Zhang et al., 2008), while plants lacking *SSIII* contained amylopectin with a higher frequency of linear chains. Alterations in starch synthase activity in storage organs have many effects. When *SSII* or *SSIII* was inhibited in potato tubers, an increase in the production of short chains of amylopectin existed (Abel et al. 1996; Edwards et al., 1999; Marshall et al., 1996). When *SSII* and *SSIII* were simultaneously inhibited, the starch took on a distorted shape with amylopectin having increased amounts of longer chains (Edwards et al.; 1999; Lloyd et al., 1999). This indicates that the different

isoforms interact with each other to produce the complex nature of the branching found in amylopectin (Zhang et al., 2008).

Taken together, these data indicate that SS1, SSII and SSIII are all involved in determining the chain lengths within the amylopectin fraction. More recently it has been shown that SSIII and SSIV are involved in starch granule initiation in *A. thaliana*. *Atss4* mutants accumulate single large starch granules in their chloroplasts (Roldán, 2007) while *Atss3/Atss4* double mutants are essentially starchless (Szydlowski, 2009). The precise roles of the different isoforms are, however, likely to differ between species, for example in rice it appears that starch granule initiation is performed by a combination of *SS1* and *SS3a* as double mutants lacking them also accumulate almost no starch in endosperm tissue (Fujita et al., 2011).

Chain elongation proceeds simultaneously with branching, a process that is catalysed by a group of enzymes known as starch branching enzymes ((1→4)-α-D-glucan:(1→4)-α-D-glucan 6-α-D-[(1→4)-α-D-glucano]-transferase; EC: 2.4.1.18). These cut pre-existing α-1,4-glucan chains and transfer them to the C6 position of a glucose unit either on the same or a different glucan chain (Zeeman et al., 2010). Plants contain two classes of *SBE*; *SBEI* and *SBEII*. The *SBEII* class in monocots is represented by two sets of genes referred to as *SBEIIa* and *SBEIIb*. Expression profiles of these genes are very species- and tissue specific. For example, in maize *SBEIIb* is only expressed in the endosperm while *SBEIIa* gets expressed to a higher extent in leaves. In other species, however, such as pea, both *SBEI* and *SBEII* are expressed in equal amounts in embryos (Ball & Morell, 2003). The amount of activity provided by each isoform is not necessarily indicative of their importance in determining starch structure. For example, Safford (1998) showed that silencing more than 90% of total *SBE* activity through use of an antisense construct designed to repress *SBEI* did not affect the ratio of amylose to amylopectin in potato tubers. On the other hand, silencing of the minor *SBEII* did lead to an increase in amylose (Jobling, 1999) with this phenotype being amplified when both *SBEI* and *SBEII* were silenced simultaneously (Schwall, 2000).

Another important group of amylopectin-synthesizing enzymes are the debranching enzymes (DBE) that are responsible for cleaving the α-1,6 branches within amylopectin, previously created by the SBEs. These enzymes are important in determining the structure of amylopectin as plants lacking them accumulate phytoglycogen, a highly branched water-soluble polysaccharide, instead of starch. It has been proposed that phytoglycogen is a pre-cursor of amylopectin formed on the surface of the starch granule which is converted to amylopectin by the action of DBEs (Ball & Morell, 2003). Two forms of these enzymes exist in plants; isoamylases, of which there are normally three isoforms,

(ISAI, ISAII and ISAIII; glycogen α-1,6-glucanohydrolase, EC: 3.2.1.68) and limit dextrinase (LDA; amylopectin-1,6-glucosidase; EC: 3.2.1.142) (Zeeman et al., 2010).

LDA and *ISA3* seem to be mainly involved in starch degradation (Delatte, 2006), whereas *ISA1* and *ISA2* are involved in converting phytoglycogen to amylopectin. In potato and *A. thaliana*, *ISA1* and *ISA2* gene products form a heteromultimeric protein, whereas in rice ISA1 can exist as a homomultimer or heteromultimer with ISA2. It was found in numerous species, such as in cereal endosperms, green algae, *A. thaliana* leaves and potato tubers, that when *ISA1* is silenced granular starch is significantly reduced and replaced with water-soluble phytoglycogen (Zeeman et al., 2010) and this phenotype is also found in *A. thaliana* and potato when *ISA2* is silenced (Wattebled et al., 2005; Bustos et al., 2004). *LDA* has also been implicated in this process in maize, but only when *ISA1* has been mutated as well (Dinges et al. 2003).

1.4 Starch degradation

During the night, starch stored within the chloroplast gets degraded to a transportable sugar (normally in the form of sucrose) to provide energy for the non-photosynthetic parts of the plant. *A. thaliana* mutants defective in starch degradation show reduced growth rates under a 12h-photoperiod (Caspar et al., 1991). Within the chloroplast, starch degradation is dependent on the phosphorylation of glucans at the surface of the granule which solubilizes the outer chains, lending access to glucan hydrolysing enzymes (Edner et al., 2007; Zeeman et al., 2010). This reversible phosphorylation is accomplished by two related enzymes. The first is known as glucan, water dikinase (*GWD*; EC: 2.7.9.4) and is responsible for phosphorylating glucose moieties within starch on the C6-position using ATP as a phosphate donor (Ritte et al., 2002). The necessity of starch phosphorylation by GWD during starch degradation has been shown in plants mutated within the *GWD* gene which ultimately leads to a starch excess (SEX) phenotypes after a long dark incubation period (Lorberth et al. 1998; Yu et al., 2001; Nashilevitz et al. 2009). The second starch phosphorylating enzyme, the phosphoglucan, water dikinase (PWD; EC: 2.7.9.5) phosphorylates the C3 position on the glucose moiety and is dependent on starch being pre-phosphorylated by GWD (Baunsgaard et al., 2005; Kötting et al., 2005).

Subsequent dephosphorylation has also been shown to be crucial in starch degradation as phosphate groups obstruct the activity of hydrolysing enzymes, such as β-amylases, that are unable to act once they have come into contact with a phosphate group (Kötting, 2009; Takeda & Hizukuri,

1981). Starch Excess 4 (SEX4) and Like Starch Excess Four (LSF2) are phosphoglucan phosphatases that remove groups previously incorporated by GWD and PWD (Kötting et al., 2009; Santelia et al., 2011). Mutations in either enzyme inhibit starch degradation and lead to the accumulation of phosphorylated malto-oligosaccharides, one of the main breakdown products of starch (Kötting et al., 2009; Santelia et al., 2011).

Liberated glucan chains are hydrolysed by β-amylases (EC: 3.2.1.2) at their exposed, non-reducing ends (Baba & Kainuma, 1986; Scheidig et al. 2002; Kaplan & Guy 2004; Fulton, 2008), however, these enzymes cannot cleave α-1,6-branch points and require the additional contribution of ISA3 for complete amylopectin hydrolysis (Delatte, 2006). β-amylases produce maltose, but are unable to degrade chains shorter than three glucose residues, therefore maltose and maltotriose accumulate within the chloroplast. Maltotriose is further metabolized by the plastidial, disproportionating enzyme 1 (DPE 1; EC:2.4.1.25) which generates both glucose and longer glucan chains which can be acted on again by β-amylases. Therefore maltose and small amounts of glucose are the products of the combined action from β-amylases and DPE1 (Chia et al., 2004). These can be transported from the chloroplast into the cytosol by specific glucose (Weber, 2000) and maltose (Niittylä et al., 2004) transporters. Once in the cytosol, glucose can be metabolised directly, but maltose is first acted on by a transglucosidase (DPE2) which transfers a glucose unit from maltose and adds it onto a branched glucan chain, leaving behind the other free glucose molecule (Chia et al., 2004; Lloyd et al., 2004). The main route of export from the chloroplast appears to be maltose rather than glucose, as disruption of this pathway leads to a far more severe block on starch degradation (Lu & Sharkey, 2004; Chia et al., 2004; Lloyd et al., 2004; Niittylä et al. 2004) than when DPE1 is repressed (Critchley et al., 2001; Lloyd et al. 2004). However, this has only been studied in detail in *A. thaliana* and potato leaves.
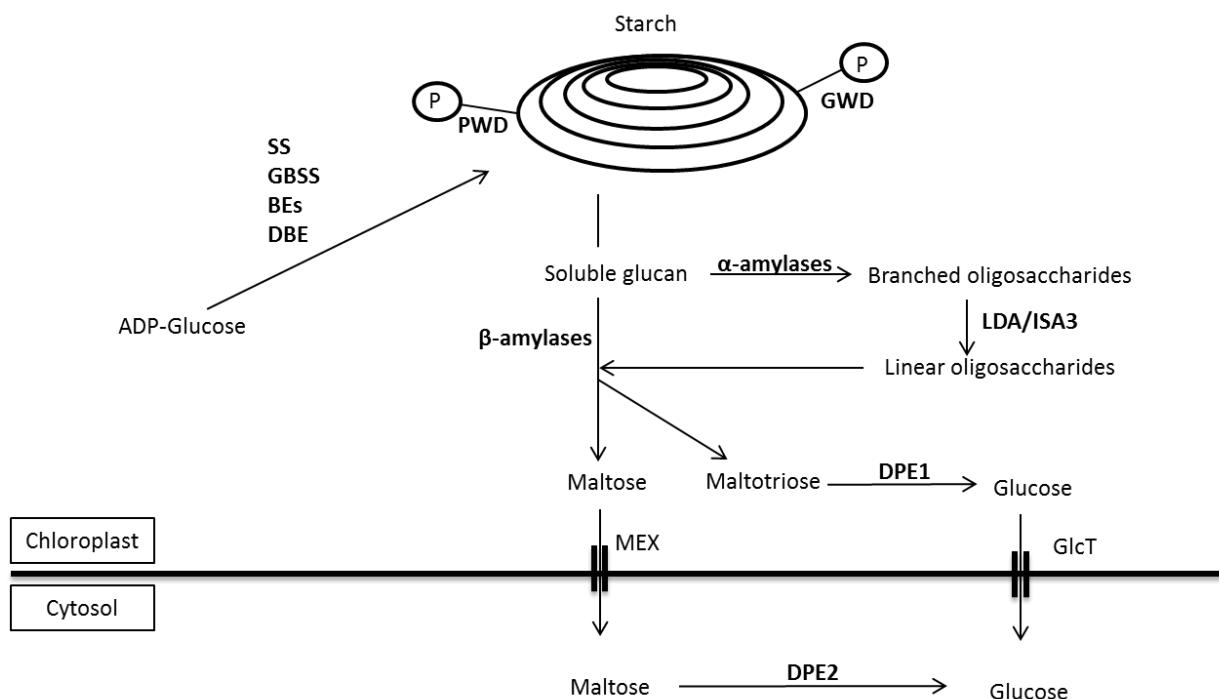
Figure 1.4: Starch turnover in chloroplasts. ADP-Glucose is polymerised to form starch by starch synthesizing enzymes (SS, GBSS, BEs and DBE). During the night, phosphoglucan, water dikinase (PWD) and glucan, water dikinase (GWD) phosphorylate the starch granule making the starch polymer soluble and accessible for further degradation by α-amylases and β-amylases. The linear glucans; maltose and maltotriose are eventually formed with maltose being able to exit the chloroplast by a maltose transporter (MEX). Maltose gets further degraded to glucose by the cytosolic localized disproportionating enzyme 2 (DPE2). Maltotriose gets converted to glucose in the chloroplast by the plastid localized disproportionating enzyme 1 (DPE1). Glucose can then be transported out of the chloroplast by a glucose transporter (GlcT) for further metabolism.

Just before the turn of the last century, a role for DPE1 outside of starch degradation was proposed based on data produced in the monocellular green alga *Chlamydomonas reinhardtii*. In this species, DPE1 activity was shown to be involved in amylopectin synthesis rather that starch degradation. A mutation within the *STA11* gene, encoding a DPE1 ortholog, led to a reduction in starch and modifications within the amylopectin structure (Colleoni et al., 1999a). Enzymatic studies of this D-enzyme indicated that it was involved in the transfer of gulcans with the consequential release of glucose and showed to transfer these glucans to the outer chains of amylopectin where these polysaccharide chains act as donors and acceptors. The enzyme was also able to transfer malto-oligosaccharides to glycogen and amylopectin outer chains (Colleoni, et al. 1999b). It may be that the role for DPE1 in starch synthesis is specific to *Chlamydomonas*, or that it has altered through

evolutionary time. No studies have examined this in species other than the three mentioned. One way that this could be examined is through producing knockout mutants in another non-vasular plant, such as *Physcomitrella patens*, a species amenable to genome alteration through homologous recombination (Schaefer & Zrÿd, 1997).

1.5 Moss

The moss *Physcomitrella patens* falls within the phylum Bryophyta (Beckert et al., 1999) which last shared a common ancestor with flowering plants approximately 200-400 million years ago (Cove, 2005). Through transcriptomic analysis it was found that more than 66% of *A. thaliana* genes have homologues in *P. patens* (Nishiyama et al., 2003). Its genome, which was sequenced in 2008 (*Physcomitrella* Genome Sequencing Consortium, 2008) is approximately 500Mbp and consists of 27 chromosomes.

The life cycle of *P. patens* consists of two phases; a haploid (where gametes are produced) and a diploid (where haploid spores are generated via mitosis (Fig. 1.5.). In moss, the haploid phase is dominant. Protonemal tissue, consisting of filaments that divide from the apical cells, generate from germinating spores. The first protonemal tissue type to develop from the germinating spore is named chloronemata. Cells from this tissue are densely packed and contain large chloroplasts. Caulonemal tissue develops from some of the chloronemal apical cells. Caulonemal cells have fewer, smaller chloroplasts. Gametophores start to develop from some of the side branches of the caulonemal tissue. It is on these 'leafy shoots' that gametangia will eventually develop. Within the antheridia the male spermatozoids are produced, which are motile gametes containing flagella. Within the archegonia that are present on the same shoot as the antheridia, female gametes will be produced (Cove, 2005).
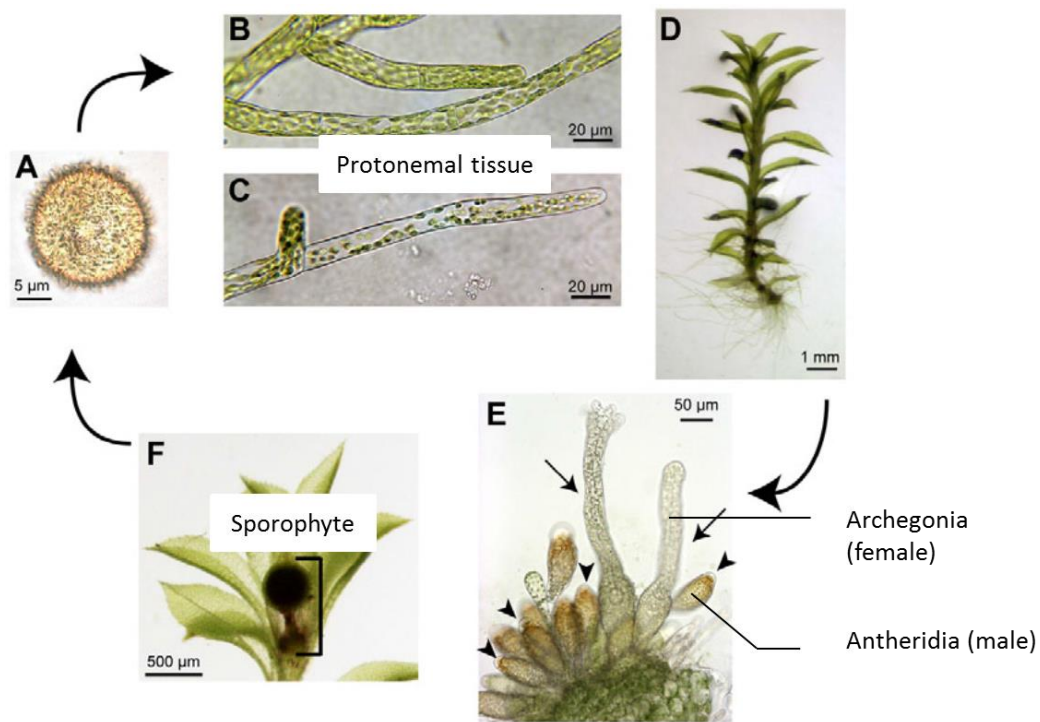
Figure 1.5: The life cycle of *P. patens*. Cloronemal cells (**B**) differentiate from haploid spores (**A**) and further develop into caulonemal cells (**C**). Gametophores (**D**) develop from the protonemal tissue (**B** + **C**) from which both archegonia and antheridia develop in the apex (**E**). The egg gets fertilized by a motile sperm which produces the sporophyte within the apex (**F**) (Adapted from Prigge and Bezanilla, 2010).

P. patens has become an attractive model system for studying gene function within plants through approaches revolving around targeted gene knockouts. This technique was first establish in yeast (Struhl, 1983) and then later transferred to mouse embryonic cells (Capecchi, 1989) as well as *Physcomitrella patens* (Schaefer & Zrÿd, 1997). It relies on homologous recombination, where a targeted gene becomes disrupted and the organism is studied in order to determine gene function (Reski, 1998).The ease of producing mutants in *Physcomitrella* is the most important reason for using *P. patens* as a model system in studying plant gene function (Kamisugi et al., 2005). Another reason is due to the simple way in which its vegetative tissue can be propagated, as any mechanically disrupted tissue modifies into chloronemal tissue which in turn generates new networks of filamentous growth (Prigge & Bezanilla, 2010). *P. patens* can also be easily transformed by an established poly-ethylene-glycol (PEG)-mediated uptake of DNA by protoplasts (Schaefer et al., 1991). Selection of stable transformants, where foreign DNA was successfully integrated within the genome, can be established within 4-6 weeks (Prigge & Bezanilla, 2010).

Mosses and higher plants have been shown to share similar responses when it comes to reacting to environmental stresses as well as to hormones (Knight et al., 1995; Werner et al., 1991). Few studies

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have been performed on starch metabolism within *Physcomitrella*, but it has been shown to contain starch granules (Thornton et al., 2005) and degradation of starch has been linked to freezing tolerance (Nagao et al. 2005). Orthologs to all *A. thaliana* genes known to be involved in starch metabolism can be found in *Physcomitrella* (Table 1.2), however, there appear to be multiple homologs of most genes (Table 1.2) consistent with the proposed genome duplication within the species revealed through the genome sequence. This was proposed to be an adaption to the movement to land, and was particularly noticeable in genes involved in carbon metabolism (*Physcomitrella* Genome Sequencing Consortium, 2008).

Table 1.2: **Orthologs of *A. thaliana* starch metabolising genes found in *P. patens* by searching the Phytozome database (http://www.phytozome.net).** The chromosomal locations of putative orthologous genes in *Physcomitrella* are indicated as well as the Identity and E value that was found when these protein sequences were blasted against each *A. thaliana* protein sequence using the BLAST-P algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC =blasthome).

A. thaliana gene	*P. patens* ortholog	Chromosome	Identity (%)	E value
Starch Synthases				
GBSS1 (AT1G32900)	Pp1s33_144V6	18	0	0
	Pp1S27_45V6	21	68	0
SS1 (AT5G24300)	Pp1s404_28V6	12	69	0
	Pp1s404_31V6	12	69	0
SS2 (AT3G01180)	Pp1s234_74V6	19	71	0
	Pp1s27_365V6	21	62	0
	Pp1s12_341V6	22	63	0
SS4 (AT4G18240)	Pp1s302_43V6	9	51	0
	Pp1s38_153V6	1	55	0

	Pp1s22_124V6	2	47	0
	Pp1s302_51V6	9	52	0
	Pp1s2_195V6	7	50	0
	Pp1s34_269V6	14	39	2e-143
Starch Branching Enzymes				
BE2 (AT2G36390)	Pp1s54_189V6	27	60	0
	Pp1s64_78V6	5	58	0
	Pp1s15_181V6	16	61	0
Isoamylases				
ISA1 (AT2G39930)	Pp1s36_254V6	14	64	0
ISA2 (AT1G03310)	Pp1s62_201V6	8	41	0
ISA3 (AT4G09020)	Pp1s25_63V6	3	62	0
Starch Phosphorylases				
α - glucan phosphorylase 1 (AT3G29320)	Pp1s195_41V6	6	67	0
	Pp1s53_212	6	63	0
	Pp1s86_213V6	1	64	0
	Pp1s30_182V6	2	50	0
	Pp1s180_124V6	6	49	0
α - glucan phosphorylase 2 (AT3G46970)	Pp1s195_41V6	6	60	0
	Pp1s86_213V6	1	58	0
	Pp1s30_182V6	2	52	0

	Pp1s180_124V6	6	51	0
Limit dextrinase				
LDA (AT5G04360)	Pp1s142_79V6	13	58	0
α - amylases				
AMY 1 (AT4g25000)	Pp1s223_59V6	13	52	7e-148
	Pp1s100_191V6	22	46	3e-114
	Phpat.017G004300.1	17	51	3e-133
	Phpat.004G058900.1	4	52	5e-129
AMY 2 (At1g76130)	Pp1s7_147V6	2	62	0
	Pp1s223_59V6	13	48	7e-135
AMY 3 (At1g69830)	Pp1s342_20V6	21	63	0
	Pp1s100_191V6	22	61	5e-178
	Pp1s223_59V6	13	49	6e-133
	Pp1s7_147V6	2	47	1e-130
	Pp1s526_11V6	11	46	8e-122
	Phpat.017G004300.1	17	68	5e-110

	Phpat.004G058900.1	4	47	7e-109
β - amylases				
BAM1 (AT3G23920)	Pp1s317_42V6	6	67	0
	Pp1s23_21V6	5	62	0
	Pp1s233_4V6	25	67	0
	Pp1s106_57V6	16	66	0
BAM2 (AT4G00490)	Pp1s121_168V6	22	70	0
	Pp1s16_320V6	24	62	0
	Pp1s9_318V6	20	59	0
BAM3 (AT4G17090)	Pp1s106_57V6	16	60	0
	Pp1s317_42V6	6	63	0
	Pp1s23_21V6	5	68	0
	Pp1s233_4V6	25	63	0
BAM4 (At5g55700) (Inactive,chloroplastic)	-			
BAM5 (At4g15210)	-			
BAM6 (At2g32290)	-			
BAM7 (At2g45880)	Pp1s121_168V6	22	61	0
	Pp1s16_320V6	24	57	0
BAM8 (At5g45300)	-			
BAM9 (At5g18670)	-			
GWD and PWD				
GWD (At1G10760)	Pp1s8_70V6	8	58	0

	Pp1s74_185V6	3	56	0
PWD (At5G26570)	Pp1s3_320V6	18	55	0
	Pp1s34_54V6	14	50	0
	Pp1s65_119V6	17	41	9e-151
SEX4 like (At3g52180)	Pp1s144_24V6	16	54	5e-114
	Pp1s14_180V6	6	52	2e-107
LSF1 (At3G01510)	Pp1s341_30V6	16	48	2e-159
LSF2 (At3G10940)	Pp1s31_1V6	11	35	2e-19
	Pp1s130_137V6	7	36	4e-19
Disproportionating enzymes				
DPE1 (At5G64860)	Pp1s8_30V	8	56	0
	Pp1s44_268v6	20	59	0
	Pp1s117_77V6	6	56	0
DPE2 (At2G40840)	Pp1s253_27V6	15	55	0
Maltose transporter				
Maltose excess protein 1 (At5G17520)	Pp1s14_134V6	6	53	4e-112
	Pp1s379_16V6	23	55	9e-106
	Pp1s268_82V6	24	52	1e-51
	Pp1s235_82V6	21	28	7e-19

Glucose transporter				
Plastidic glucose transporter (At5G16150)	Pp1s129_118V6	25	0	8.1e-13

1.6 Aims and objectives

This project is part of a larger one within the University of Stellenbosch examining whether the pathway of starch metabolism is conserved in *Physcomitrella patens*. The aim of this project is to examine the roles of three *DPE1* and one *DPE2* orthologs found in *Physcomitrella patens* (Table 1.2) by:

a) Production of *P. patens* lines where *DPE1* and *DPE2* ortohologs have been knocked out.
b) Establishing protein assays for *P. patens* DPE1A, 1B, 1C and DPE2 and measuring activity in the mutants.
c) Measurement of starch degradation rates in the mutants.
d) Examination of the subcellular localisation of the different proteins by expressing genes fused to the green fluorescence protein in *P. patens.*
e) Examination of expression of the different genes using semi-quantitative RT-PCR.

Chapter 2
Materials and Methods

2.1 Plant material, chloronemal generation and maintenance

 P. patens ssp. patens ecotype Gransden (Engel, 1968) protonemal tissue was maintained as described by Cove et al*.* 2009. The equivalent of one cell culture dish (100 mm x 20 mm) of protonemal tissue was ground with an IKA Ultra Turrax® Tube drive (Monmouth Scientific Limited, Somerset, U.K.). Following this 2-3 mL of homogenate was inoculated onto cellulose disks (80 mm diameter; A.A. packaging Limited, Preston, U.K.) overlaid on solid PPNH4 medium (Cove et al., 2009). Plates were sealed with 1,25 cm X 9,1 cm Micropore™ tape and incubated for 6 weeks at 24 ⁰C with a 16 h light/ 8 h dark regime which was kept with cool white fluorescent lights (50 µM photons m-2 s-1; OSRAM L58W/640, Germany) before sub culturing.

2.2 *P. patens* gDNA extraction

50 – 100 mg *P. patens* tissue was added to 500 µL extraction buffer (200 mM Tris-HCl [pH 7.5]; 250 mM NaCl; 25 mM Ethylenediaminetetraacetic acid (EDTA); 0.5 % [w/v] SDS) and ground in a 1.5 mL microcentrifuge tube with two glass beads by vortexing until a homogenous suspension was achieved. The supernatant was recovered by centrifugation at 16 060 *g* for 10 min before 450 µl was transferred to a fresh 1.5 mL microcentrifuge tube containing 450 µL isopropanol. This was inverted and incubated at -20 ⁰C for 30 min. Precipitated nucleic acid was recovered by centrifugation at 16 060 *g* at 4 ⁰C for 15 min and washed with 500 µL 70 % (v/v) ethanol. The supernatant was aspirated and the pellet was allowed to air dry. This was re-suspended in 50 µL TE solution (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA) and the supernatant collected by centrifugation at 16 060 *g* for 5 min before being transferred to a fresh microcentrifuge tube and stored at -20⁰C.

2.3 RNA extraction and cDNA synthesis

RNA was extracted from *P. patens* chloronemal tissue with the Qiagen RNeasy Plant Mini Kit (Qiagen) and was subsequently used to make single stranded cDNA with the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific).

2.4 Gene knockout plasmid construction

Amplicons encoding partial fragments of three putative *P. patens DPE1* (*PpDPE1A* = 1665 bp*, PpDPE1B* = 2314 bp *and PpDPE1C* = 2365 bp) paralogs and one *DPE2* homolog (2012 bp) were produced from *P. patens* gDNA by PCR using the primers displayed in Table 2.1. The PCR products were subsequently ligated into the pJET1.2/blunt cloning vector using a commercially available Kit (Thermo Scientific). The hygromycin resistance cassette (*HygR*) was restricted from pMBLH8a vector (Knight et al., 2009) by *BamHI* or *EcoRV*. *DPE1A, 1B* and *DPE2* were restricted with *BamHI* prior to ligation, while *DPE1C* was restricted with *MscI*. The *BamHI* fragment containing the hygromycin resistance cassette was ligated into the *DPE1A*, *DPE1B* and *DPE2* genomic fragments, while the *EcoRV* fragment was ligated into the *DPE1C* amplicon. Genes were disrupted leaving a flanking region of approximately 1 kb on either side of the antibiotic resistance marker. Linear DNA containing the flanking regions and selection cassette were amplified by PCR with the same primers used to amplify the gene fragments, prior to being transformed into *P. patens* protoplasts.

2.5 Isolation and transformation of *P. patens* protoplasts

Protoplasts were isolated and transformed as described by Cove et al. (2009). One week old caulonemal tissue was digested with 2% (w/v) Driselase (Sigma) before isolated protoplasts were filtered through a sterile 100 µm nylon cell strainer (BD Falcon, NJ, USA) and harvested by centrifugation at room temperature at 250 *g.*

60 µg of PCR product, 600 µL protoplast suspension containing approximately 960 000 protoplasts and 700 µL PEG solution (7 % [w/v] Mannitol, 100 mM $CaNO_3$, 10 mM Tris-HCl [pH7.2] and 40% [w/v] PEG 4000) were combined before the protoplasts were transformed by heat shock at 45ºC for 5 min. After plating out the transformed protoplasts, plates were incubated for 7 – 10 days at 24 ºC with a 16 h light/ 8 h dark regime.

2.6 Selection of stable transformants

Regenerating protoplasts were grown for 10 days on solid PPNH4 media supplemented with 25 µg/mL hygromycin B (Sigma). Cellophane disks containing regenerated protoplasts were then transferred to solid PPNH4 media without antibiotics and allowed to grow for 7 days before being transferred again to solid PPNH4 media containing 25 µg/mL hygromycin and incubated for 7 days. Surviving colonies were screened with the Phire Plant Direct PCR Kit (Thermo Scientific) with primer

pairs that bind upstream of the insert site and within the hygromycin selection cassette (PpDPE1A genomic + Hyg F, PpDPE1B genomic + Hyg F, PpDPE1C + Hyg F, PpDPE2 + Hyg R; Table 2.1).

2.7 GFP constructs

Coding sequences for *PpDPE1A – PpDPE1C* (which contain no introns) were amplified from *P. patens* gDNA while the coding sequence for *PpDPE2* was amplified from *P. patens* cDNA using primers described in table 2.1. PCR products were cloned into pENTR entry vectors with the pENTR™ D-TOPO® Cloning Kit (Invitrogen, Life Technologies) as described in the manufacturer's instructions.

Entry vectors subsequently underwent LR clonase reactions, transferring the coding sequences into pMDC85 GFP fusion vectors (https://www.A. thaliana.org/servlets/TairObject?type=vector&id=501100112).

Table 2.1: List of primers used

Primer	Sequence	Amplifies
PpDPE gene primers		
DPE 1A forward	5´-GGCCATGGTTGCCAACGGGCAGGG-3´	*DPE 1A*
DPE 1A reverse	5´-GGCCTCATTCTCTCTGCGCTGCTTCTGC-3´	
DPE 1B forward	5'-GTGTCAGCCGTGAGAATCGA-3'	*DPE 1B*
DPE 1B reverse	5'-GAGCACCTTTCCTTACAGGA-3'	
DPE 1C forward	5'-CTACAAACGCAAACTACTTT-3'	*DPE 1C*
DPE 1C reverse	5'-ATATTGTCAAAAAACCCATGT-3'	
DPE 2 forward	5´-CCGGGGATCTAGGTGGACGAACAAAC-3´	*DPE 2*
DPE 2 reverse	5´-CCGGGCAATGAGACTTAAGAATACGAAGACA-3´	
GFP primers		
DPE 1A gfp forward	5´-CACCATGGTTGCCAACGGGCAGGG-3´	*DPE 1A* GFP
DPE 1A gfp reverse	5´-TTCTCTCTGCGCTGCTTCTGCTA-3´	

DPE 1B gfp forward	5´-CACCATGACTACTTCCGTTGTTTG-3´	*DPE 1B* GFP
DPE 1B gfp reverse	5´-TGCTGTAGGAGTTGATTCG-3´	
DPE 1C gfp forward	5´-CACCATGGAGTTGTGTTCGACAAA-3´	*DPE 1C* GFP
DPE 1C gfp reverse	5´-GGGTTTGTCCTTGGGTTTC-3´	
DPE 2 gfp forward	5´-CACCATGGGAAGCGGCGGGGATCT-3´	*DPE 2* GFP
DPE 2 gfp reverse	5´-TGCTGTGACAATAGTATTAAAAT-3´	
Semi quantitative RT-PCR primers		
DPE 1A sqRT-PCR F	5'-CCATTCGGCAAAGTTGATTT-3'	DPE 1A sqRT-PCR
DPE 1A sqRT-PCR R	5'-ATACTGCCTCACGTGGTTCC-3'	
DPE 1B sqRT-PCR F	5'-CGAGCTTTACGACGAGTTCC-3'	DPE 1B sqRT-PCR
DPE 1B sqRT-PCR R	5'-TTCTCCGTCTCCAGCTCATT-3'	
DPE 1C sqRT-PCR F	5'-TTGTGTTCGACAAACCCGTA-3'	DPE 1C sqRT-PCR
DPE 1C sqRT-PCR R	5'-GTTCCCGGAGGAACTAAAGG-3'	
DPE 2 sqRT-PCR F	5'-GAGGCTGCGATTGCTTTATC-3'	DPE 2 sqRT-PCR
DPE 2 sqRT-PCR R	5'-TGCCCGTTCATGTCAAAATA-3'	
PpActin3U1	5'-CGGAGAGGAAGTACAGTGTGTGGA-3'	*P. patens actin* (accession no. AW698983
PpActin3D1	5'-ACCAGCCGTTAGAATTGAGCCCAG-3'	
Screening for *P. patens* mutants		
Hyg F	5'-AACTCCCCAATGTCAAGCAC-3'	

Hyg R	5'-CTATCAGAGCTTGGTTGACGG-3'	Screen for integration of hygromycin cassette in *P. patens* genome
PpDPE1A genomic	5'-GGCCATGGTTGCCAACGGGCAGGG-3'	
PpDPE1B genomic	5'-CCGGATGACTACTTCCGTTGTTTG-3'	
PpDPE1C genomic	5'-CCGGATGTCTTGTTGCAAGAGCAAC-3'	
PpDPE2 genomic	5'-ATACATTTGGCACCCTGCTC-3'	

2.8 Subcellular localization

GFP constructs were transformed into *P. patens* protoplasts. Protoplasts were then left to incubate in the dark for 48 hrs before being visualized by a confocal microscope 780 LSM with an Alpha Plan-Apochromat 100x/1.46 oil DIC M27 Elyra objective. The two lasers that were used were the 488 laser at laser power of 6% (emission wavelength 490-569) and 405 laser at 1.8% (emission wavelength 668-735). The master gain was set to 800 for the 488 laser and 600 for the 405 laser. Beam splitters used were the MBS 488/561/633 for 488 laser and MBS 405 for 405 laser. Images were captured with a 2.0 zoom with Z-stacks taken every 1.842 micrometres.

2.9 Starch Determination

Approximately 100 mg fresh weight protonemal tissue was collected from plates that were incubated for 2 weeks at 24 ºC with a 16 h light/ 8 h dark regime. Samples were taken at the end of the light period. 1 mL of 80% (v/v) ethanol was added to each sample and incubated at 80 ºC for 1 hour to remove sugars. The ethanol was then removed and samples were washed again with 1 ml 80 % (v/v) ethanol. Once the ethanol was removed, 0.4 ml of 0.2 M KOH was added and samples were left to incubate for 1 hour at 95 ºC. Samples were neutralized by adding 70 µL of 1 M acetic acid.

10 µL of solubilized starch was mixed with 10 µL of 50 mM NaAC (pH 4.5) containing 10 U/ml amyloglucosidase and incubation at 37 ºC for 2 h. 250 µL of glucose assay buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 U/mL hexokinase) was added. The OD was measure at 340 nm and as soon as readings stabilized 1 U/mL glucose 6-phosphate dehydrogenase was added. Once absorbance stopped increasing, the change in OD_{340} was used to determine glucose equivalents in each sample. Six biological repeats were used in all starch measurements with three technical repeats.

2.10 1-Way ANOVA

Values were tested for equal variance by a modified version of Levene's test as described by Glantz & Slinker (1990).

2.11 Protein extraction

100 mg fresh tissue was ground with a mortar in 1.2 mL extraction buffer (100 mM 4-Morpholinepropanesulfonic acid (MOPS) [pH 7.0], 10% [v/v] glycerol and 1 mM DL-Dithiothreitol (DTT), 50 mg Poly(vinylpolypyrrolidone)). Homogenates were then centrifuged at 15 000 g for 10 min. The supernatants were desalted on a Sephadex G25 resin (Sigma) before being used in assays.

2.12 DPE 1 activity assay

50 – 100 µg of desalted protein extract was added to 125 µL of assay buffer (50 mM MOPS [pH 6.8], 60 mM maltotriose (Sigma)). Samples were incubated for 30 min at room temperature and reactions were terminated by heating at 100 ºC for 5 min. Blanks for the assay consisted of reactions being immediately terminated at 100 ºC for 5 min upon addition of protein to the assay buffer. Glucose was then measured by incubating 50 µL of DPE 1 assay product with 250 µL assay buffer (50 mM Tris-HCl, [pH 6.9], 5 mM $MgCl_2$, 1 mM ATP, 1 mM NAD, 1 U/mL Glucose 6-phosphate dehydrogenase from *Leuconostoc* (Megazyme, glucan, Wicklow, Ireland). Reactions were started upon addition of 1 U/ml Hexokinase and the increase in absorbance at 340 nm recorded.

2.13 Semi-quantitative RT-PCR

Three week old chloronemal and leafy shoot material was harvested at the end of the dark period and flash frozen in liquid nitrogen. Total RNA was extracted from each tissue type with the Qiagen RNeasy Plant Mini Kit (Qiagen) of which 1 µg of total RNA was used to make cDNA using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific). Of the generated cDNA, 2 µL was used in semi-quantitative RT-PCR using the following primers: DPE 1A sqRT-PCR F +R, DPE 1B sqRT-PCR F+R, DPE 1C sqRT-PCR F+R, DPE 2 sqRT-PCR F+R (Table 2.1) and for *P. patens actin*; PpActin3U1 and PpActin3D1 (Table 2.1, Aoki et al., 2004). It was established that the optimum number of cycles to use was 25 cycles, which was used for all PCR reactions. PCR products were visualized on a 1% agarose gel.

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Chapter 3
# Results & Discussion

3.1 Phylogeny of PpDPE sequences

The *Physcomitrella patens* genome present in the Phytozome database (www.phytozome.com) was searched (BLASTx algorithm) using *A. thaliana* coding sequences for *DPE1* (At5G64860) and *DPE2* (At2G40840). Three orthologs for *DPE1* (*PpDPE1A, 1B* and *1C*) and one for *DPE2* (*PpDPE2*; Table 1.2) were identified. These sequences were aligned, with those form other *DPEs* from a number of species using the ClustalW program in BioEdit. The 3'- and 5' ends of the genes were excluded as these regions were too different to align which is most likely due to the assumption that they are not part of the active sites of the enzymes and, therefore, are not under evolutionary constraint.

To examine the evolutionary relationship between the DPEs a phylogenetic tree was constructed using the Bayesian method (Bay) for both *DPE1* and *DPE2* using MrBayes V3.2.4. Both tree's showed high Bayesian posterior probabilities as support with all estimated sample sizes (ESS) above 2000. The likelihood value for DPE1 was -16065.49 while for DPE2 it was -17245.9612. For each tree 5 million generations were utilized with burn in values of 1.25 million. Each of the codons for both DPE1 and DPE2 showed independent rates of evolution with the first codon position of DPE1 at 0.686, second position at 0.399 and the third position at 1.914. For DPE2, the rate of evolution for the first codon position stood at 0.508, for the second codon position at 0.306 and for the third codon position at 2.186. This agreed with the assumption that third codon positions evolve the quickest and that second codon positions evolve the slowest. All potential scale reduction factor (PSRF) values for both DPE1 and DPE2 were as close to 1 as possible with a few values within a 1000th of 1.

PpDPE1s are shown to group together with *DPE1s* from vascular species, having split from the green alga, *Ostreococcus tauri*. PpDPE1A and PpDPE1B seem to have split from PpDPE1C which could be explained as a duplication event that occurred, leading to two new paralogous genes evolving due to selective pressure. The grouping of DPE1s from the vascular plants (*Silaginella moellendorffii, Amborella trichopoda, A. thaliana, S. tuberosum, Zea mays and Oryza sativa*) is to be expected (Fig. 3.1A) based on their known evolutionary relationships.

PpDPE2 clusters together with *S. moellendorffii*, having split from the other vascular plants whose *DPE2s* cluster together (Fig. 3.1; B). This makes sense when one takes into account that *Selaginella,* which forms part of the lycophyte clade, is the oldest lineage of vascular plants with fossil record estimating that this clade diverged from other vascular plants around 400 m.y.a (Weng et. al., 2005) just after the colonization of land by the Bryophytes (Cove, 2005).
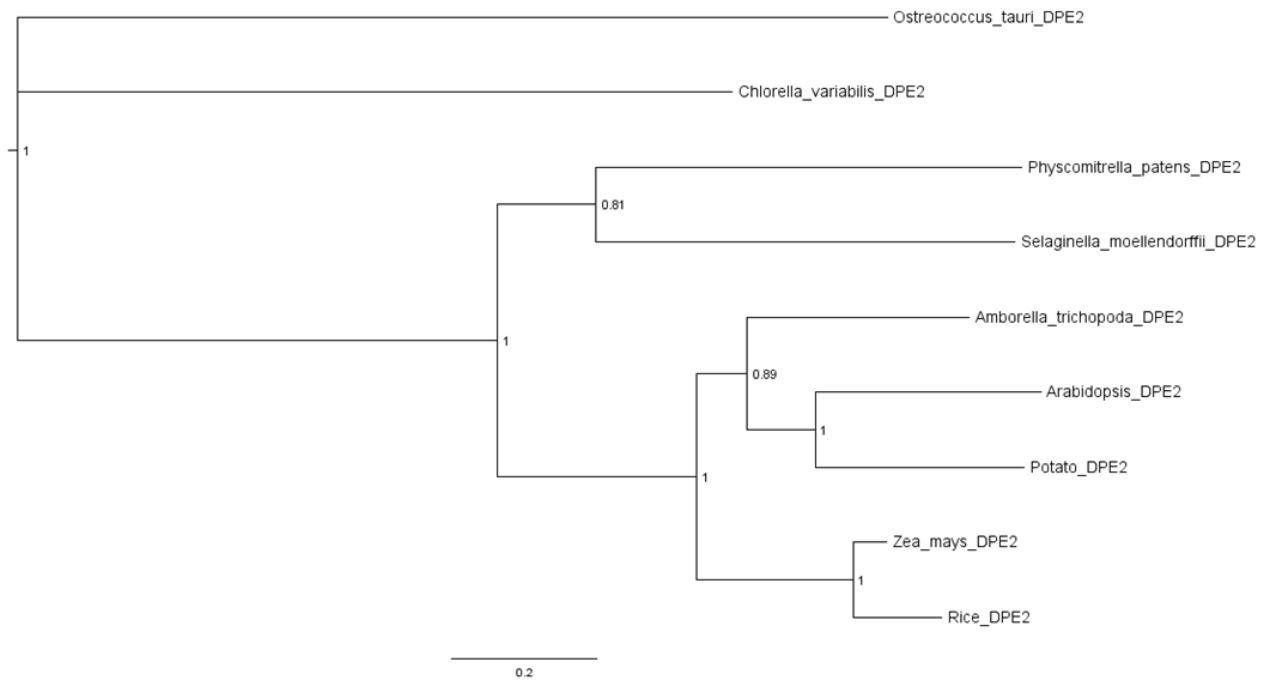
A.
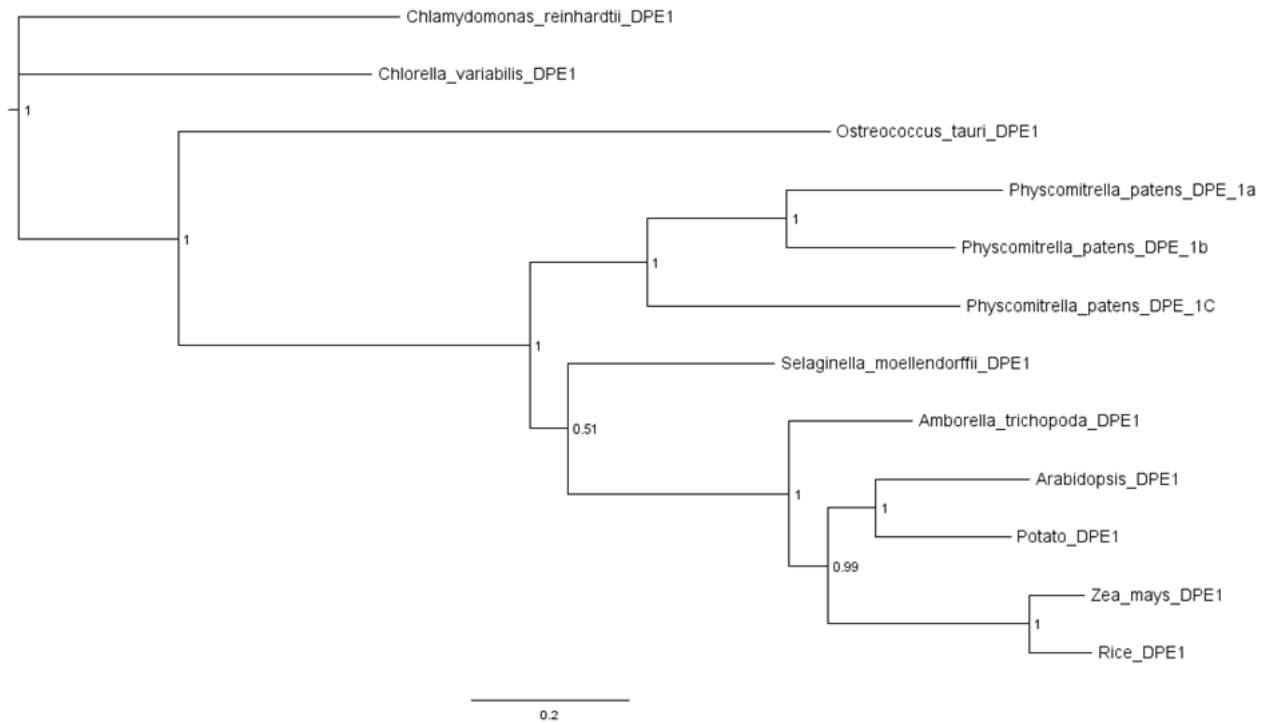
B.

Figure 3.1: DPE1 and DPE2 Phylogenetic tree's displaying the relationship of PpDPE's with other DPE's of other higher plants. Trees were constructed using the Bayesian method (Bay). Node values display Bayesian posterior probabilities. *Chlamydomonas reinhardtii* and *Chlorella variabilis* DPE1s were used as out groups for the DPE1 tree (A) with an effective sample size (ESS) of 1248.2084. *Ostreococcus tauri* and *Chlorella variabilis* were used as out groups for the construction of the DPE2 phylogenetic tree (B) with an effective sample size (ESS) of 306.1234.

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3.2 Subcellular localization of DPE proteins

The subcellular localizations of the PpDPE1s and PpDPE2 were examined by manufacturing constructs were the full coding sequence of each isoform were fused in frame at the 3' end with a gene encoding the green fluorescence protein (GFP) under control of a constitutive cauliflower mosaic virus 35S promotor in the vector pMDC85 (Curtis and Grossniklaus, 2003). Constructs were introduced into *P. patens* protoplasts via PEG-mediated DNA uptake and evaluated after 3 days with a confocal fluorescence microscope (Fig. 3.2). Untransformed protoplasts were used as a negative control (Fig. 3.2D, E and F). The localization of all the PpDPE1 constructs was irreproducible and so the data is not shown. PpDPE2 on the other hand showed a consistent pattern of GFP distribution (Fig. 3.2A+C) and appeared to be present in the cytosol but in close proximity to/in contact with the chloroplasts.

Fig. 3.2A shows GFP localization in areas where no chlorophyll is present, indicating that PpDPE2 is localized in the cytosol, associated with the chloroplast envelope. This supports what was found in *A. thaliana* and potato, where DPE2 was localized to the cytosol (Chia et al., 2004, Lütken et al., 2010). DPE2 could, in principle, be localised either inside or outside the chloroplast and still function in starch degradation because it liberates glucose from the maltose that is produced in the stroma by β-amylases. Genes encoding proteins similar to higher plant chloroplastidial glucose and maltose transporters are present in the *P. patens* genome (Table 1.2) meaning that both sugars could be transported from the stroma to the cytosol. Maltose could be present, therefore, either within or outside of the chloroplast. Interestingly a recent transcriptomic analysis of cold-stressed *P. patens* colonies (Beike et al., 2015), conditions under which starch is actively degraded, demonstrated that expression of a putative maltose transporter was rapidly up-regulated, followed later by transcripts from a putative glucose transporter. This indicates that maltose export form the chloroplast during times of starch degradation is likely, consistent with the extra-plastidial localisation of DPE2 in this study.

The glucose that DPE2 produces would have to be phosphorylated by hexokinase isoforms before being able to enter metabolism (Fig. 3.3). Interestingly *P. patens*, unlike higher plants, contains a hexokinase isoform within the chloroplast stroma (Olsson et al., 2003). This particular protein catalyses the majority of total hexokinase activity in *P. patens* cells, with the remainder being made up of other isoforms. Those localise to a number of subcellular locations including the cytosol, mitochondrial matrix or attached to the chloroplast membrane (Nilsson et al. 2011), similar to the localisation of hexokinases found in vascular plants (Kandel-Kfir et al., 2006; Cho et al., 2006; Karve et al., 2008). A *P. patens* knockout mutant lacking the plastidial hexokinase is unable to respond to the presence of glucose in the growth medium in terms of its known stimulation of caulonemal development (Olsson et al. 2003). In that paper it was hypothesised that this isoform phosphorylated glucose produced as a product of starch degradation and that this flux would be important for

caulonemal development, but no data was presented to demonstrate this *in vivo*. Further work will be needed to show this through examining lines where starch degradation is impaired. Clearly the mutants produced in this study could be used to examine that process, however, the subcellular location of the different DPE isoforms would influence their potential roles. As PpDPE2 is located in the cytosol it would not be expected to influence glucose production within the chloroplast, however, the assumption would be that the DPE1s would be present there and would produce glucose within that compartment. As such, mutants lacking one or more of the DPE1 isoforms could be used to examine this hypothesis.
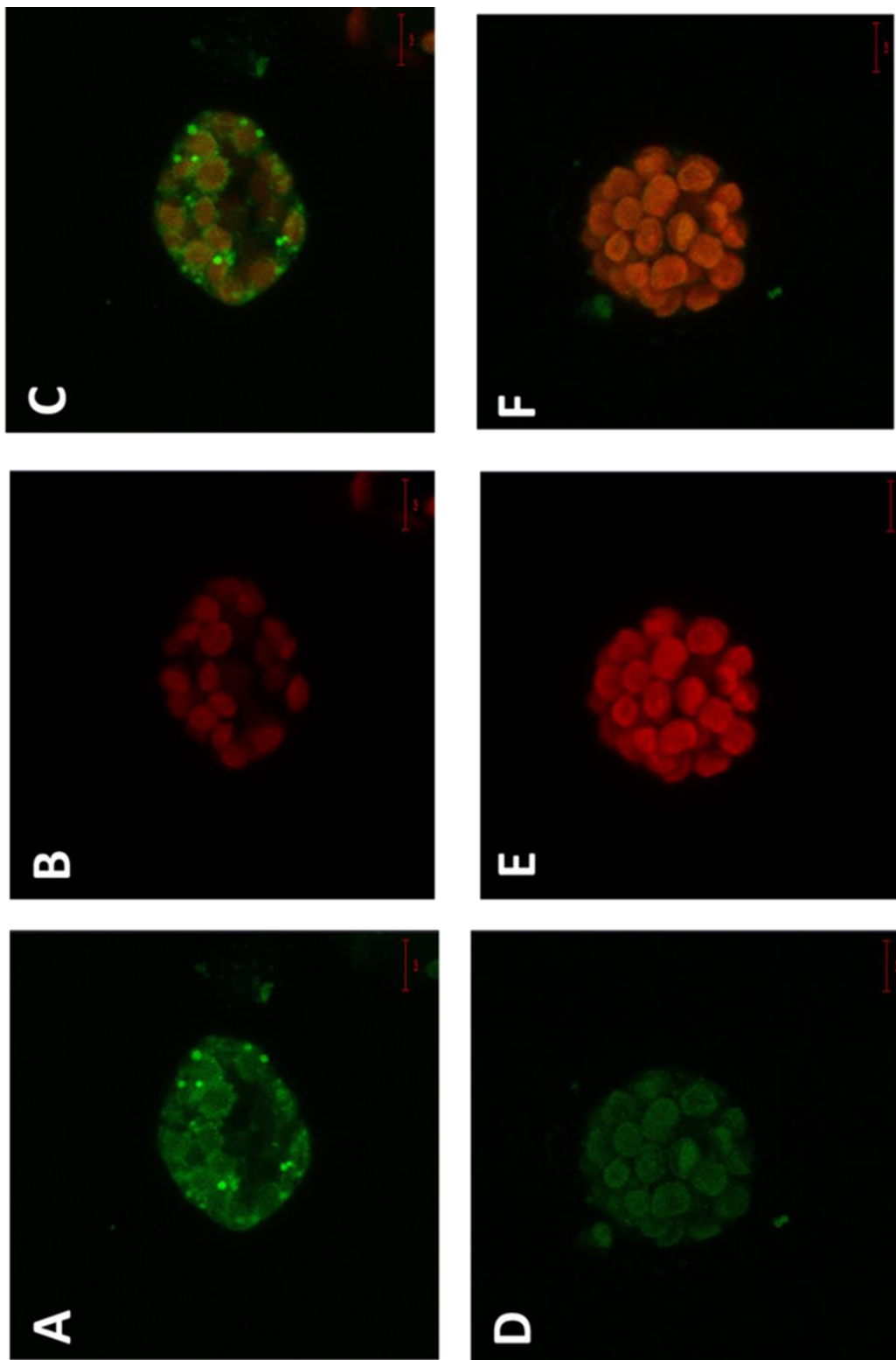
Figure 3.2: Localization of PpDPE2-GFP fusion protein. *P. patens* chloroplasts were transformed with constructs allowing transient expression of PpDPE2 (A, B and C) or were left untransformed (D, E and F) as a negative control. Fluorescence of GFP signals in green (A and D), chlorophyll auto fluorescence in red (B and E), GFP and chlorophyll auto fluorescence overlaid to distinguish the two (C and F).
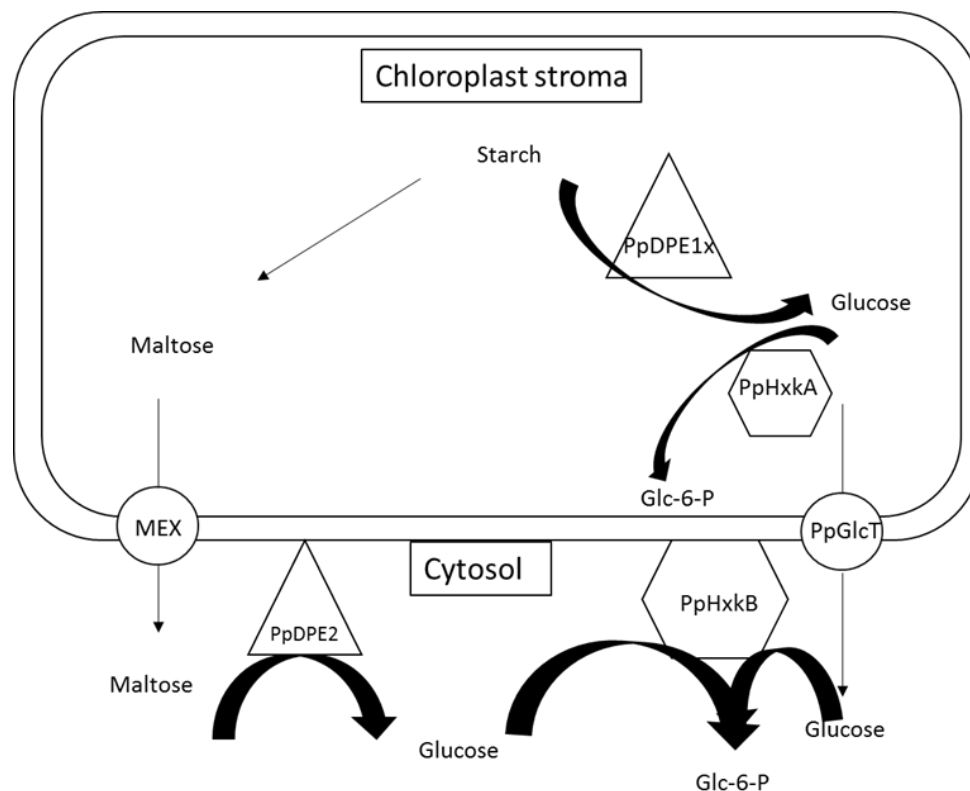
Figure 3.3: Presumed localization of DPE1s, DPE2 and type A and B hexokinases in *P. patens.*
DPE1 paralogs are thought to be localized to the stroma, if the model is followed which has been worked out in *A. thaliana* (Figure, 1.4), were they break down malto-oligosaccharides to glucose which can then be phosphorylated by the plastid localized type A hexokinase (PpHxkA, Olsson et al., 2003) or exported by the plastidial glucose transporter (PpGlcT). PpDPE2, on the other hand, was shown to be localized to the cytosol, in close proximity to the chloroplast envelope. Here it can break down maltose, which is exported out of the chloroplast via the MEX transporter, to glucose. Glucose can then be phosphorylated in the cytosol by the type B hexokinase (PpHxkB) which is attached to the chloroplast membrane (Nilsson et al., 2011).

3.3 Expression of DPE1 and 2 orthologs at the end of the dark period in *P. patens*

Due to the life cycle of *P. patens* and the diverse number of tissue types involved (protonema, caulonema, chloronema and gametophores) it was decided to test whether all these orthologs are expressed and, if so, in which tissue types. Semi-quantitative RT-PCR was therefore utilized to test the expression patterns using cDNA synthesised from RNA which was extracted either from protonema (consisting of a mixture of caulonema and chloronema tissue) or gametophores, referred to as 'leafy shoots' which resemble leaves. Tissue samples were collected at the end of the dark period. Actin was used as a housekeeping gene with primers described by Ichikawa et al. (2004).

All primer pairs were first tested on the corresponding genes ligated into pJET1.2 vectors to test their effectiveness in detection of the genes.

PpDPE1B and *PpDPE1C* were found to be the only genes expressed in both leafy shoots and in protonema at the end of the dark period (Fig. 3.4). The possibility does exist, however, that the genes that were not detected (*PpDPE1A* and *PpDPE2*) could be expressed at different time points, something that could be evaluated in the future. In *A. thaliana* a distinctive expression pattern of nine genes involved in starch degradation (including *DPE1* and *DPE2*) showed to slowly decrease during the dark period to their lowest level at the beginning of the light period, following which expression of the genes increased quickly (Smith et. al., 2004). For the semi-quantitative RT-PCR in this study, only one time point (end of the dark period) was evaluated, so it would be interesting to see whether these orthologs are differentially expressed at different times of the light cycle. Although only *PpDPE1b* and *1c* were detected in that experiment, it is evident that *PpDPE2* must also be expressed as a full length *PpDPE2* amplicon was amplified from cDNA synthesized from RNA extracted at mid-day that was used to manufacture a construct for subcellular localisation experiments (Materials and Methods, section 1.2.8).
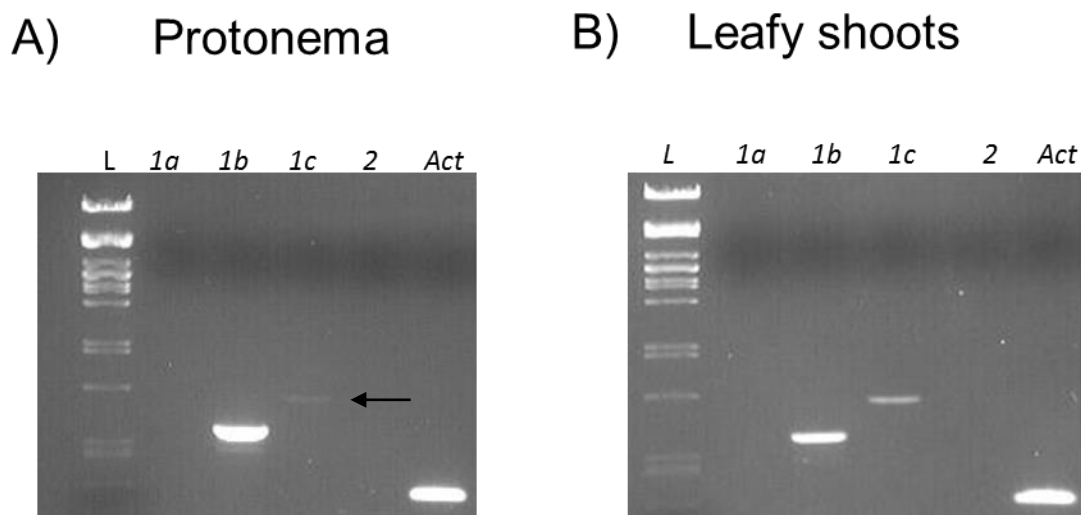

Figure 3.4: Semi quantitative RT-PCR of *P. patens* DPE1 and DPE2 orthologs in A) protonema and B) leafy shoots. Amplicons representing all four orthologs were produced after 25 cycles as described in the Materials and Methods with actin (Act) used as the positive control for the cDNA. For the negative control, no template was added for each of the primer sets (data not shown). The molecular marker is λ phage DNA digested with *PstI*.

3.4 Production of *P. patens DPE* mutants

To examine the roles of the different DPE isoforms in starch metabolism in *P. patens*, knockout mutants for each of these isoforms were manufactured. Approximately 1500-2000 bp of gDNA was amplified from each gene by PCR and ligated into a cloning vector. These were interrupted by digestion with restriction enzymes and subsequent ligation of a hygromcin resistance cassette into the amplicons leading to the production of the cassette flanked by approximately 1kb of gDNA on both sides. The disruption cassettes were amplified by PCR to linearize them prior to being transformed into *P. patens* protoplasts by PEG mediated transformation. These were plated onto medium containing hygromycin and colonies were allowed to develop. To distinguish individual plants which had undergone successful transformation and integration of constructs from those that were transiently expressing the hygromycin resistance gene, colonies were transferred to media containing no hygromycin for 7 days, before being put back on selection. Surviving colonies were then further analysed using PCR with one primer that binds within the hygromycin cassette and another immediately upstream of the amplified section (Table 2.1). Untransformed colonies were not expected to show amplification due to the absent hygromycin cassette sequence.

The expected sized fragment were obtained for all four mutants; 3080 bp for *PpDPE1A* (Fig. 3.5A, lane 1), 2444 bp for *PpDPE1B* (B, lane 1), 1570 bp for *PpDPE1C* (C, lane 1) and 1608 bp for *PpDPE2* (D, lane 1). For all four mutants the control Wt did not yield amplification with the same primers (Fig. 3.5A, B, C and D, lane 2). This indicated the successful integration of the hygromycin cassette, leading to the disruption of each gene.
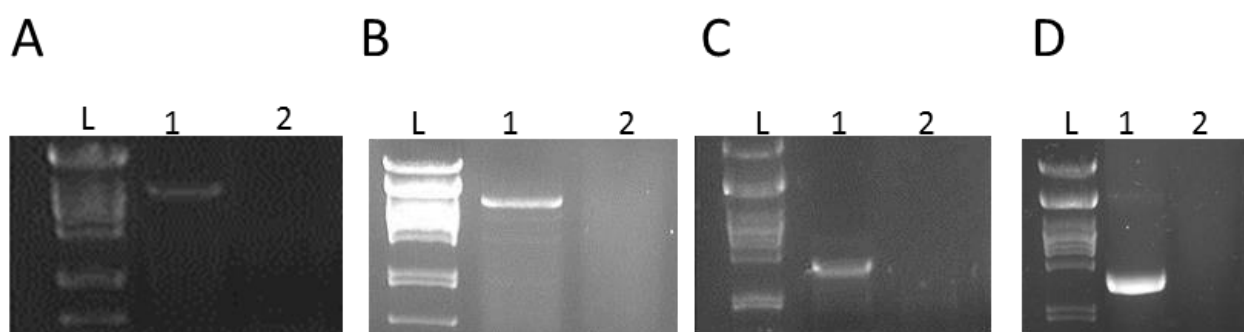

Figure 3.5: Genotyping of *P. patens* PpDPE mutants of colonies surviving antibiotic selection. The molecular marker (L) is λ phage DNA digested with *PstI*. Amplification was obtained in all of the mutants for *PpDPE1A* (A, lane 1), *PpDPE1B* (B, lane 1), *PpDPE1C* (C, lane 1) and *PpDPE2* (D, lane 1), yielding expected fragments sizes of 3080 bp (A), 2444 bp (B), 1570 bp (C) and 1608 bp (D) respectively. PpWt was used as a control in the all four PCR's yielding no product (lane's 2 in all gels).

3.5 Presence of DPE1 and DPE2 in WT *P. patens*

In order to test whether DPE1 activity was reduced in mutants by the disruption of the orthologs with the hygromycin cassette, a DPE1 assay was established for *P. patens*. DPE1 is the only higher plant enzyme known to metabolise maltotriose, releasing glucose (Critchley et al., 2001). Assuming that the *P. patens* orthologs demonstrate the same activity, maltotriose can be used as a specific substrate to determine activity. Extracted proteins were therefore incubated with maltotriose and the amount of released glucose determined spectrophotometrically

Glucose production could not be detected in crude protein extracts from *P. patens* Wt although the positive control using potato leaf material did demonstrate activity (Fig. 3.6). There could be a number of reasons for the lack of activity in extracts from *P. patens*. It is possible that there is a necessary co-factor for the enzymes from lower plants that is currently unknown and, therefore, not included in the assay mix. The lower plant enzymes may also use a different substrate than maltotriose, or may be active at a time point other than when the sample was taken for protein extraction, which was in the first few hours of the light period. The extracts also came from *P. patens* Wt grown on PPNH4, which was shown to lead to tissue containing less starch than when grown with a carbon source in the media (Fig. 3.8). Therefore, there may be more activity when a carbon source is used to supplement the growth medium. Finally, it is also possible that DPE1 is not important for starch metabolism in *P. patens,* with a route mainly utilizing DPE2 as a starch degradation pathway.

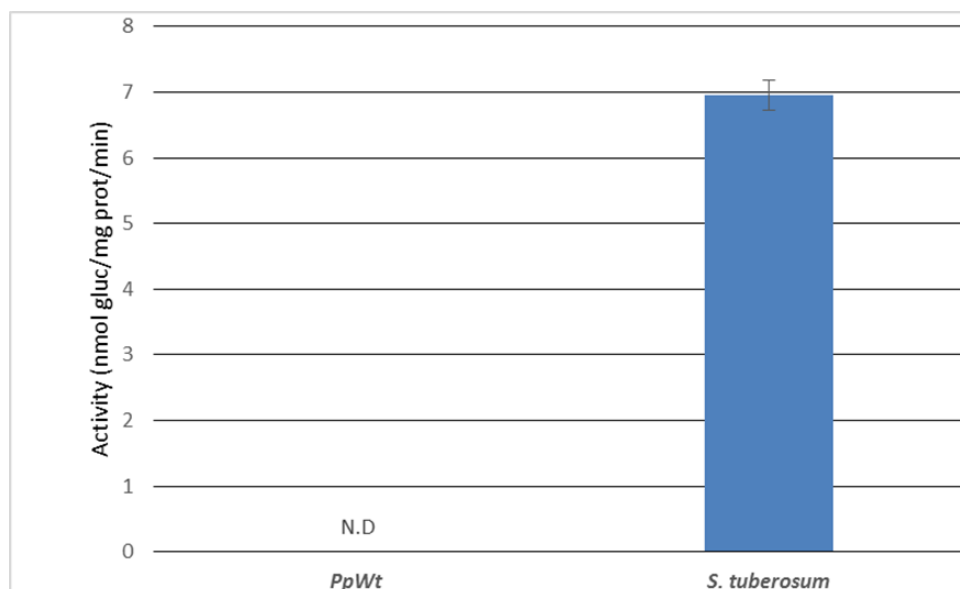

Figure 3.6: DPE1 activity in *P. patens* wild type (PpWt) and *S. tuberosum* leaf assayed by examining glucose released from maltotriose. Error bars represent the standard error of the mean with six biological repeats. N.D. = Not Detectable.

3.6 Growth of mutants

P. patens mutants and WT leafy shoot colonies were ground and plated onto NH4 media overlaid with cellophane disks to induce production of chloronema. All four mutants showed reduced tissue re-generation when compared to WT, with *PpDPE1B* and *PpDPE2* showing the most severe effects (Fig. 3.7).

It has been shown that plants that are defective in degrading starch ultimately show a decrease in growth as starch cannot be effectively converted to sucrose during the dark period for growth (Chia et al., 2004; Yu et al. 2001; Takaha et al., 1998). This result therefore implies that these mutants are indeed impaired in starch degradation, showing that *PpDPE1A* and *PpDPE2* are indeed expressed even though they could not be detected with semi quantitative RT-PCR.
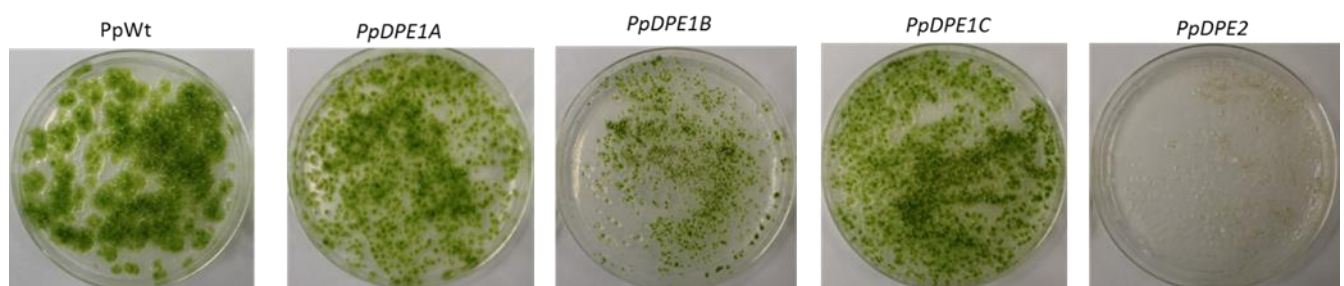

Figure 3.7: ***P. patens* WT and mutants grown on NH4 media**. All mutants showed a reduction in regeneration after being plated out onto NH4 media after being ground from healthy shoot material. The most severe phenotype was seen in mutants *PpDPE1b* and *PpDPE2.*

3.7 Examination of the effect of growth media on starch yield in *P. patens*

The starch content of *P. patens* Wt grown on NH4 media was assessed and it was found to contain very little (0.146 mg starch/ g fresh weight, Fig. 3.8). It was therefore decided to examine whether *P. patens* grown on NH4 media supplemented with sucrose could increase the yield of starch. *P. patens* has been shown to encode for type II (plasma membrane) and type III (vacuolar membrane) sucrose uptake transporters (Reinders et al., 2012) and is able to use sucrose to enhance growth (Frank et al., 2005). When this disaccharide is added under dark growth conditions, the development of caulonemal tissue is increased whilst chloronemal tissue development is supressed (Cove et al., 1978). Under short day length or low light intensities, the addition of sucrose leads to an increase in the growth rate of chloronema tissue (Cove et al., 1978).

Colonies were grown on NH4 media supplemented with either 0.1% (w/v) or 1% (w/v) sucrose. Either concentration increased the starch yield, with 1% (w/v) sucrose being the most effective, yielding 10.6 mg starch/ g fresh weight (Fig. 3.8) similar to published amounts found in *A. thaliana* leaves (Yu et al., 2001). This indicates therefore that the addition of an external carbon source is necessary to support photosynthesis in producing starch in *P. patens* grown under laboratory conditions.
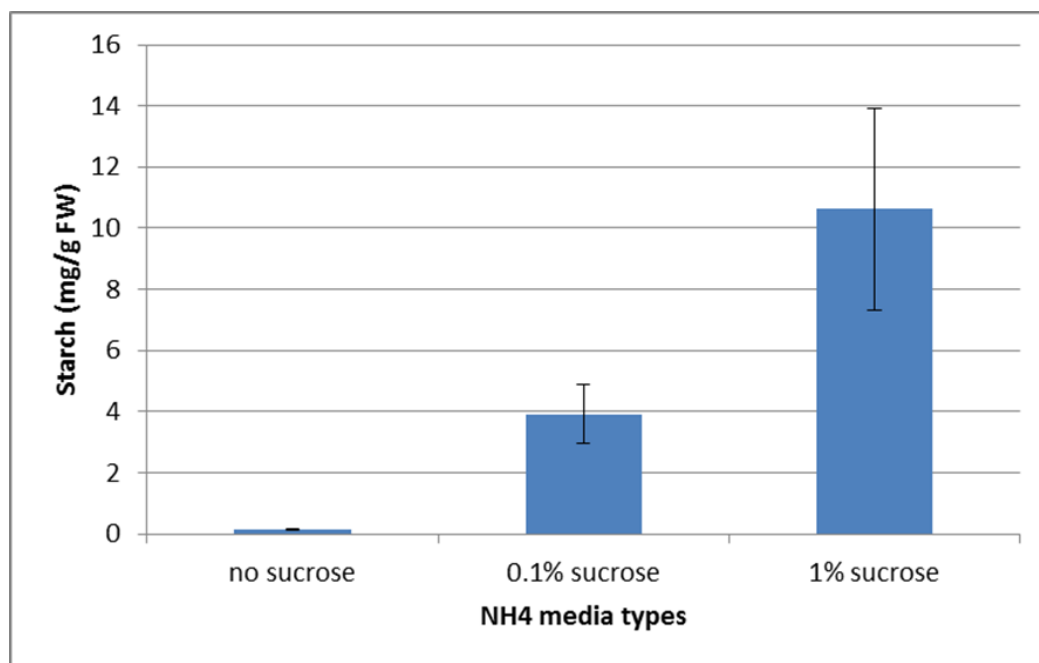

Figure 3.8: Starch contents of *P. patens* Wt grown on NH4 media supplemented with 0.1% (w/v) or 1% (w/v) sucrose. Error bars indicate the standard deviations with six biological repeats for each.

3.8 Measurement of starch in *P. patens DPE* mutants

To examine the effects of the mutations on starch metabolism, plants were grown on NH4 media containing 1% (w/v) sucrose and were subsequently transferred to the same media without sucrose and incubated in the dark for 48 hours. Starch was measured after 0, 24 and 48 hours of darkness (Fig. 3.9).

The only significant differences that could be identified, demonstrated an increase in starch between days 0 and 2 for the *PpDPE1B* mutant (P=0.015) and for PpWt (P=0.007). This is the opposite of what would be expected as starch degradation should be increased in the dark to provide sugar during periods where photosynthesis cannot take place. One possibility to explain this is that *P.*

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patens stored the exogenously supplied sucrose to an extent that it did not need to degrade starch during the time period of the experiment.

Alternatively, starch degradation could be less important for *P. patens* under the growth condition in this experiment than it has been shown to be when specific abiotic stress is applied (Nagao et al., 2005). That study found that cold stress and abscisic acid (ABA) leads to a reduction in the sizes of starch granules in *P. patens* protonemal cells. This most likely accounts for the significant increase of soluble sugars that serve as osmolytes to increase cold tolerance. It is known that starch degradation plays an important role in acclimatising *A. thaliana* to this stress through the production of maltose (Kaplan and Guy, 2004; Espinoza et. al. 2010). Although starch degradation has been observed to occur in cold stressed *P. patens* (Nagao et al., 2005), the enzymes involved in that process under those conditions have not been examined *in vivo*. Beike et al. (2015) analysed the whole-genome transcriptome of *P. patens* under cold stress and found upregulation of many genes that are involved in starch synthesis and degradation, including a β-amylase (Pp1s106_57V6.1) and a maltose transporter (Pp1s268_82V6.1). That implies that maltose will be produced under these circumstances in *P. patens* and be exported from the chloroplast into the cytosol, however, the role of these genes in diurnal starch turnover is unknown. My data indicate that starch does not get degraded during periods of darkness after being grown on media containing sucrose, but in some cases shows an increase in starch (*PpDPE1B* and PpWt), implying that starch synthesis rather takes place.

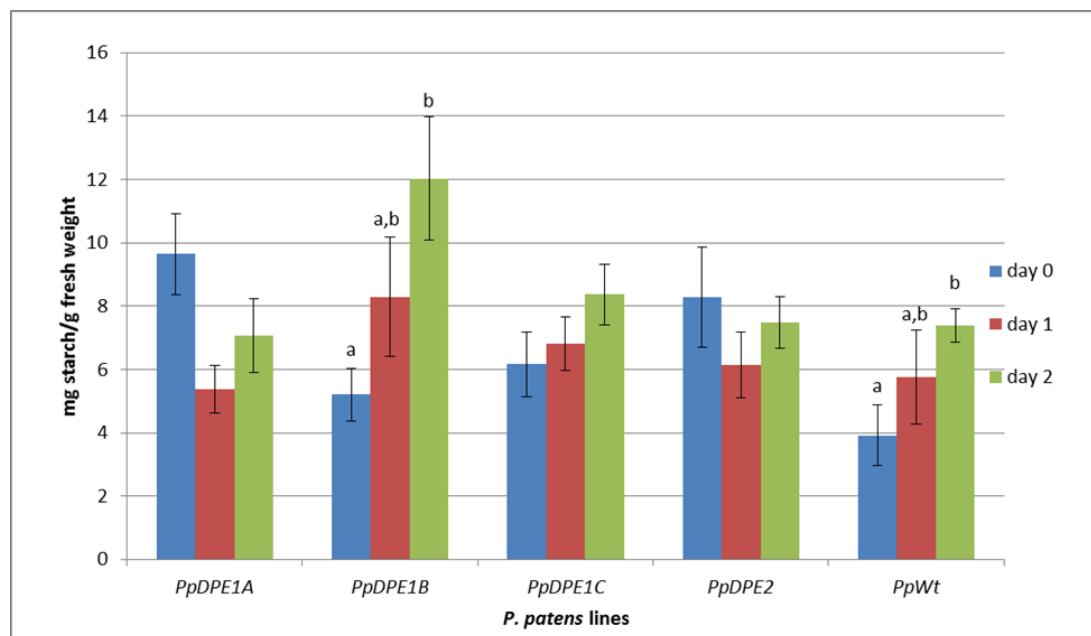**Figure 3.9: Starch measurements of *P. patens* mutants *PpDPE1A-B*, *PpDPE2* and PpWt**. Data represents means ± SEM of six independent replicates. Significant differences are only shown where they are found within genotypes as determined by *post-hoc* Bonferroni-Holm tests, with *PpDPE1B* and PpWt showing a significant increases in starch between days 0 and 2 (P=0.015 for *PpDPE1B* and P=0.007 for PpWt).

3.9 Conclusion

The aim of this study was to examine part of the pathway of starch catabolism that is catalysed by two disproportionating enzymes in *A. thaliana* to see if it is functionally similar in the *bryophyte P. patens.* To do that, genes encoding different isoforms of disproportionating enzyme from *P. patens* were amplified and used to try and understand what role(s) they play in turnover of starch during a day night cycle. Unfortunately it was not possible to demonstrate that they influence this pathway as mutants where the genes were knocked out individually showed no reduction in starch degradation (Fig. 3.9), which would be expected if they play the same role in *P. patens* as in *A. thaliana*. It should however be taken in to consideration that these mutants were grown on media containing sucrose which could have contributed to the lack of degradation witnessed. Otherwise this may be because there is a completely different pathway of starch catabolism in this lower plant than in *A. thaliana*. That seems unlikely as a recent screen in the unicellular green alga *Chlamydomonas reinhardtii* identified both a β-amylase and a plastidial maltose transporter as being involved in starch degradation in that species (Tunçay et al., 2013). This implies that an extra-plastidial DPE2-like protein must also be important as it would be needed to remove maltose in the cytosol that had been exported from the chloroplast. As *C. reinhardtii* is an even more primitive plant that *P. patens*, it seems likely that this pathway has been conserved over evolutionary time. A more likely alternative is that there is more functional redundancy in the pathway in this moss than in higher plants, which could be examined if double, triple and quadruple mutants were made lacking multiple DPE isoforms. Currently work is undergoing to examine this.

Clearly a more detailed examination of starch degradation in these mutants in needed before firm conclusions can be drawn. This work concentrated on that process in plants grown on media supplemented with sucrose, before being placed onto media with no external carbon source. As mutations in DPE isoforms have no effect here they could also be tested under other circumstances where starch is known to be degraded, such as cold stress (Nagao et al., 2005). Alternatively it may have been advantageous to use genes where there is less chance of functional redundancy to examine this process. For example, there are only two genes encoding glucan water dikinase-like proteins in *P. patens* (Table 1.2) and it may have been easier to target these two enzymes than the four disproportionating enzyme isoforms in this study.

Although this project was unable to answer the questions that it set out to in the time frame available, it remains the first study of its kind trying to examine the pathway of starch metabolism in moss. The lines generated as part of it will be used in future to examine the role of starch in this plant, which will provide novel information concerning the evolution of the pathway of starch metabolism.

Chapter 4

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