Integral Light-Harvesting Proteins in the Dinoflagellate, *Symbiodinium* sp.

Thesis submitted by

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Statement on the Contribution of Others

Scientific Collaborations

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Abstract

Reef building (Scleractinian) corals are the major component of coral reefs and form an intimate symbiotic relationship with a single celled dinoflagellate (*Symbiodinium* sp.). It is a relationship that is highly sensitive to environmental changes, particularly when thermal stress is combined with a non-optimal light level. The success of the symbiosis is driven by the dinoflagellates' ability to harvest the energy of the sun through the process of photosynthesis and translocate reduced organic carbon to the coral host for calcification and growth. The process of photosynthesis is a complex and hazardous process involving a balance between solar energy harvesting and energy utilization, or dissipation. Our current understanding of eukaryotic photosynthetic gene expression is predominately derived from plant and green unicellular algae and observations at the level of gene transcription indicate distinct differences in how unicellular algae and higher plants acclimate their photosystems to environmental changes. Therefore the question arises; how do unicellular symbiotic dinoflagellates, such as *Symbiodinium*, respond to distinct environmental cues, do they mimic the response of other unicellular algae or that of higher plants. To determine whether *Symbiodinium* mimic the response of other unicellular algae or that of higher plants, a better understanding of the major light-harvesting protein complex (LHCs) utilized by *Symbiodinium* is required. In addition, knowledge of the LHC genes possessed by *Symbiodinium*, the expression of these genes under a variety of environmental cues, and an understanding of how translation and degradation combine to restructure the photosynthetic apparatus, is necessary.

Therefore, the aims of this research were to use expressed sequence tag data for *Symbiodinium* sp. sub-clade C3 to: sequence the chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC); to compare C3 acpPC with LHCs from other photosynthetic organisms; to determine whether C3 acpPCs were present in cultured *Symbiodinium* of varying clades and sub-clades; and to investigate acpPC gene expression patterns in response to varying light conditions. To achieve these aims an extensive sequencing project was performed and once complete sequences were obtained bioinformatics analysis, including analysis of conserved amino acid residues and homology of translated protein sequences with known light harvesting genes from symbiotic and free-living dinoflagellates, algae and higher plants, was performed. Phylogenetic comparison of translated protein sequences with light harvesting protein complexes from a variety of organisms was undertaken and the diversity of acpPCs within *Symbiodinium* from divergent lineages was investigated. Transcript sizes were determined using northern blots and Southern blots were used to determine acpPC gene copy numbers. A polyclonal antibody generated in rabbits to a synthesized peptide fragment based upon acpPC cDNA sequences was obtained and western blots used to investigate protein expression. Real-Time PCR, a relatively new tool to the study of *Symbiodinium*, was used for gene expression work following validation of normalization genes.

Phylogenetic analysis of 11 LHC sequences suggests the acpPC subfamily forms at least three clades within the Chl *a/c*-binding LHC family. The first clade includes rhodophyte, cryptophyte and peridinin binding dinoflagellate sequences; the second peridinin binding dinoflagellates only; while the third contains heterokonts, fucoxanthin and peridinin binding dinoflagellate sequences. *Symbiodinium* sp. C3 acpPC sequences generally contain three transmembrane helices and at least two proteins with LHC membrane-spanning helix duplication, deletion, and / or degeneration are evident. In addition, *Symbiodinium* encode transcripts containing one, two or three LHC polypeptides and the genome encodes single copy and as well as high copy acpPC genes. Analysis of *Symbiodinium* from divergent lineages demonstrates that C3 acpPC sequence information can be utilized to investigate closely related *Symbiodinium* at the transcript and protein level, in particular *Symbiodinium* C1, and findings suggest a number of C3 acpPC genes are common to *Symbiodinium* clades. β-actin and proliferating cell nuclear antigen (PCNA) can be used in controlled light experiments to investigate acpPC expression having been validated as normalization genes. The use of a polyclonal antibody for acpPC suggests protein expression varies between *Symbiodinium* sub-clades. Nevertheless, changes at the transcript level are relatively small when *Symbiodinium* are exposed to different light environments suggesting acpPC genes are possibly subject to translational control mechanism or post-translational controls.

With *Symbiodinium* genome information becoming publicly available insight into the complex genetic composition of these unique dinoflagellates will be possible. Until readily available, the information resulting from this research provides: a basis for future investigations into the major light-harvesting protein complexes utilized by *Symbiodinium*, specifically clade C *Symbiodinium*; a means to compare whether the acpPC subfamily differs between stress tolerant and susceptible *Symbiodinium* species; a methodology to measure how environmental factors impact the major LHCs used by these ecologically important dinoflagellates; and a potentially significant breakthrough in our understanding of the diverse and complex acpPC subfamily.

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1.1 Introduction

Symbiodinium are ecologically important marine dinoflagellates. Free-living *Symbiodinium* are evident in benthic and plankton communities, however, the genus is primarily recognized for forming mutualistic relationships with a cnidarian host, in particular reef-building corals. *Symbiodinium* are capable of performing photosynthesis and are an important source of organic carbon for the host organism. To perform photosynthesis efficiently photosynthetic organisms require optimal light conditions. In the marine environment managing a varying light environment is challenging, particular for endosymbiotic organisms of a sessile host as is the case for many *Symbiodinium* species. For all organisms that perform photosynthesis, maintaining a balance between the capacity to capture or harvest light energy, utilize captured energy and / or dissipate excess energy is crucial. When light-harvesting exceeds the rate at which electron transport and / or carbon fixation occurs, the maximum light saturation point of photosynthesis is reached and photo-oxidative damage can result, which in turn can lead to a decrease in photosynthetic efficiency. The formation of reactive oxygen species (ROS) resulting from excess light energy being transferred to oxygen can cause cellular damage and mortality (Lesser, 1997, Niyogi, 1999, Tchernov et al., 2004, Suggett et al., 2008). To limit photo-oxidative damage photosynthetic organisms have a number of mechanisms that enable varying light levels to be better managed. Referred to as photo-acclimation (Falkowski and La Roche, 1991, Anderson et al., 1995, Durnford and Falkowski, 1997), these mechanisms occur as a response to variation in photon flux density (PFD) and spectral distribution (Falkowski and La Roche, 1991).

Photo-acclimatory mechanisms include physiological, biochemical and molecular processes that are reversible, compensatory in nature, and protect not only the processes of photosynthesis but overall cell viability (Durnford et al., 2003). Cell viability can be damaged by extreme light stresses, such as high light (HL) or low light (LL) conditions, and photo-acclimation can involve reductions or increases to the light-harvesting capacity of the cell, or changes to the ability of the cell to utilize adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) in carbon fixation (Huner et al., 1998). Photo-acclimatory mechanisms can be induced rapidly and within minutes of a variation in PFD, and involve mechanisms associated with mediating photosynthetic efficiency such as the xanthophyll cycle (Demming-

Adams and Adams, 1996) or the altering of the photosystem II absorption cross section via state transitions (Wollman, 2001). More structural changes require hours to complete and involve mechanisms associated with the restructuring of the photosynthetic apparatus and involve altering the size of the light-harvesting antennae (Sukenik et al., 1987, Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a) and / or the number of reaction centres (Sukenik et al., 1990).

Photo-acclimatory mechanisms are well documented in green algae and higher plants, genes encoding the proteins associated with photo-acclimation have been identified, and the response of these genes to a range of environmental parameters such as changing light energy, temperature and carbon dioxide (CO_2), investigated (Maxwell et al., 1995, Montane et al., 1999, Walters, 2005). The activation signal responsible for initiating photo-acclimatory mechanisms is unclear, although imbalances in photosynthetic electron transport have been suggested as a possible activation signal (Maxwell et al., 1995, Escoubas et al., 1995, Pfannschmidt et al., 1999), as have more complex strategies involving major structural and functional changes of one or both photosystems (Bailey et al., 2001). In contrast to higher plants, algae exhibit a higher physiological plasticity and this enables acclimation to wide and varied light environments and the ability to make more rapid adjustments (Durnford and Falkowski, 1997).

Descriptions of photo-acclimatory mechanisms in dinoflagellates, including the genus *Symbiodinium*, have tended to focus on physiological and photo-biological responses (for example, Iglesias-Prieto and Trench, 1997a, Robison and Warner, 2006, Warner et al., 2006, Hennige et al., 2009), but more recently molecular aspects have been explored (McGinley et al., 2012, McGinley et al., 2013). Genetic information for *Symbiodinium*, and dinoflagellates overall, is limited. Genes involved in *Symbiodinium* photo-acclimation have not been documented nor has the link between gene expression patterns and physiological responses to changing environment cues. Identifying the process of photo-acclimation in *Symbiodinium*, and identifying key genes associated with these processes, is of particular interest to scientists investigating the association between reef-building corals and *Symbiodinium* and how fluctuating environmental factors impact this ecologically important relationship.

The research presented in the following chapters provides new information on the major light-harvesting complex (LHC) chlorophyll *a*-chlorophyll c_2-peridinin protein complex (acpPC) isolated from *Symbiodinium* sp. C3. Phylogenetic analysis of the acpPC subfamily with LHCs from varying photosynthetic organisms such as higher plants, green and red algae, and other phytoplankton, and the comparison of molecular information, provides evidence of regions of homology, evolutionary relationships, important domains of individual sub-units, and potential structural and functional features. Not all *Symbiodinium* lineages encode the same sub-set of acpPC genes although a number appear common across many species. Furthermore,

variation in gene expression is minimal in response to changing light environments suggesting post-transcriptional mechanisms may regulate acpPC expression.

1.2 Dinoflagellates

Dinoflagellates are a successful group of eukaryotic algae that are a vital component in the aquatic food web. Dinoflagellates are environmentally and morphologically diverse, exhibit unusual genetics, cell biology and plastid acquisition (see review by Hackett et al., 2004a). Mixotrophy and heterotrophy is widespread amongst dinoflagellates, with approximately half the described species capable of performing photosynthesis (Keeling, 2009) while species that are heterotrophic utilize a range of feeding methods, including phagocytosis and extrusion structures (Taylor et al., 2008). Dinoflagellates exist in both freshwater and marine ecosystems; of the approximate 2,000 extant species, it is estimated close to 1,700 are marine species (Taylor et al., 2008). As a major contributor to the primary productivity of water systems dinoflagellates can be free-living, attached and associated with the benthos, parasitic, or form mutualistic endosymbiotic relationships. Some species form toxic algal-blooms and seriously affect marine organisms, fisheries, aquaculture activities and humans. Other species are unique due to bioluminescence or the formation of significant biological associations, such as those between cnidarians and reef-building corals.

1.2.1 Morphology

Dinoflagellates exhibit many unusual and unique features. Although typically unicellular, some dinoflagellates form chains, colonies or can be multi-cellular (Taylor et al., 2008). Numerous cellular structures exist, and cell size and shape vary between orders and within genera, with cells ranging in size from 2 μm to 2 mm (Swadling et al., 2008). Motile dinoflagellates typically have two varying flagella that propel and turn the cell. Some cells are pelliculate or athecate (naked or unarmoured), others are thecate (armoured) and possess cellulose plates (thecal plates) which fit closely together. The patterns formed by the plates vary and provide a useful taxonomic tool (Taylor et al., 2008).

Photosynthetic dinoflagellates harbour a diversity of plastids. Plastids are organelles in the cytoplasm of photosynthetic cells; a chloroplast is a plastid, and a number of dinoflagellate lineages contain plastids derived from other algae through tertiary or serial secondary endosymbiotic events (Keeling, 2004). Plastids acquired through endosymbiotic events are generally enveloped by three membranes, although this can vary (Lukeš et al., 2009). The photosynthetic pigments utilized by dinoflagellates can provide insight into plastid acquisition and include chlorophylls *a* and c_2, and carotenoids β - carotene, fucoxanthin, peridinin, diadinoxanthin and dinoxanthin (Jeffrey et al., 1975, Venn et al., 2006). Peridinin is unique to dinoflagellate species while diadinoxanthin and dinoxanthin are involved in the dissipation of

excess light energy in a similar manner as xanthophylls are in higher plants and green algae (Arsalane et al., 1994, Ambarsari et al., 1997).

1.2.2 Biology

The dinoflagellate genes encoding proteins involved in photosynthesis are located in either the nucleus or plastid. The nucleus of most dinoflagellates lack nucleosomes, and histones are lacking or in very small quantities (Rizzo, 1991). Chromosomes remain condensed during interphase and mitosis (Spector et al., 1981) and dinoflagellates have specific transcription factors and promoters (Guillebault et al., 2002). Typically, dinoflagellates possess unusually large genomes (reviewed by Hackett et al., 2004a) but encode the smallest number of plastid genes of all photosynthetic eukaryotes (Hackett et al., 2004b). In relation to the human haploid genome, dinoflagellate haploid genomes are estimated to be 1-77 fold larger (Hou and Lin, 2009). Regression models project the dinoflagellate genome to contain between 40,000 and 90,000 protein coding genes, however, rather than representing a high functional diversity of the encoded proteome, this exceedingly high gene number for a unicellular organism is probably due in part to the high gene copy numbers detected in dinoflagellate (Hou and Lin, 2009). Evidence suggests the genus *Symbiodinium* may have one of the smallest dinoflagellate genomes (LaJeunesse et al., 2005), however smaller genomes may occur (Lin, 2006).

Non-symbiotic dinoflagellates can contain genomes possessing 3.6 – 225 pg $cell^{-1}$ of deoxyribonucleic acid (DNA) while endosymbiotic species are documented to contain much smaller genomes that possess DNA content between 1.5 – 4.8 pg $cell^{-1}$ (LaJeunesse et al., 2005). The dinoflagellate plastid genome is fragmented into minicircles of 2 – 10 kb in size and each minicircle contains between one to four genes (Nisbet et al., 2008) and a core region (Nisbet et al., 2004). In comparison, the plastid genome of typical green algae is between 100 - 200 kb in size and the red algae plastid genome can range between 150 – 192 kb (Barbrook et al., 2010). Currently only 16 genes on the dinoflagellate minicircles have been characterized which is much lower than the typical 100 – 135 genes on the green algae or 251 genes on the red algae plastid genome (Barbrook et al., 2010).

In dinoflagellates that utilize peridinin, it appears the remaining genes required for photosynthesis have been transferred to the nucleus (Bachvaroff et al., 2004, Hackett et al., 2004b). In fact, the vast majority of dinoflagellate genes required for the plastid are encoded in the nucleus (Bachvaroff et al., 2006) and this has implications for plastid protein import. Nuclear encoded, but plastid targeted proteins require additional mechanisms enabling them to be directed to and through the multi-layered membranes surrounding the plastid (Fast et al., 2001). For example, *Heterocapsa triquetra*, a non-symbiotic marine species, has been identified as containing at least two major classes of transit peptides which suggests two mechanisms may direct proteins to the plastid (Patron et al., 2005). As dinoflagellates, along with Euglenophyta,

are the only algae with three membranes surrounding the plastid (Cavalier-Smith, 1999a, Nassoury et al., 2003, Keeling, 2004, Patron et al., 2005), the presence of transit peptides in these organisms suggests that multiple endosymbiotic events have resulted in the LHCs being imported to the plastid as polyproteins (Durnford and Gray, 2006, Koziol and Durnford, 2008).

Dinoflagellate gene expression involves *trans*-splicing. The same 22 base pair (bp) 5' spliced leader (SL) sequence is added to all transcripts and this process may influence how gene expression is controlled in these organisms (Lin et al., 2002, Koziol and Durnford, 2008, Lukeš et al., 2009). In addition, a subset of dinoflagellate nuclear genes demonstrate the presence of polycistronic messages (Bachvaroff and Place, 2008) with multiple genes expressed on a single messenger ribonucleic acid (mRNA) (Lukeš et al., 2009). The polycistronic mRNA is broken into monocistronic mRNA and the 22-bp SL sequence is added to the 5'end of each monocistronic mRNA through *trans*-splicing. In dinoflagellates, the monocistronic mRNA appears to be repeats of the same gene (Bachvaroff and Place, 2008, Lukeš et al., 2009).

The unusual nuclear and plastid genome characteristics also extend to the mitochondrial genome. The dinoflagellate mitochondrial genome is: complex, highly reduced, standard start and stop codons are absent, gene fragments and non-coding regions repeat, genes occur in multiple copies, polycistronic transcripts are evident, and there is evidence of *trans*-splicing and extensive post-transcriptional RNA editing (Lin et al., 2002, Jackson et al., 2007, Nash et al., 2008). To further emphasize the unusual nature of these organisms, some phototrophic dinoflagellates possess a Form II ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) for CO_2 fixation rather than the common Form I RuBisCO (Morse et al., 1995). Previously only found in anaerobic non-sulphur purple bacteria, dinoflagellates are currently the only oxygenic photoautotrophs known to utilize the Form II RuBisCO isoform (Morse et al., 1995, Rowan et al., 1996, Whitney and Andrews, 1998).

1.2.3 Phylogeny

Dinoflagellates are one of the three major phyla that make up the superphylum Alveolata, a diverse lineage of eukaryotic microorganisms, which includes ciliates and apicomplexans. A fourth phylum has more recently been included in the Alveolata; this is the Chromerida (Moore et al., 2008). The three major phyla contain parasitic and predatory species, although ciliates are predominantly heterotrophic. Apicomplexans, such as *Plasmodium*, *Toxoplasma* and *Cryptosporidium*, are obligate parasites of animals and some are known to be important disease organisms in vertebrates (Lukeš et al., 2009). Most dinoflagellates are photoautotrophic, but some do not contain plastids and while many apicomplexans harbour apicoplasts (non-photosynthetic plastids), ciliates harbouring plastids have yet to be definitively demonstrated (Lukeš et al., 2009). Within the Alveolata, dinoflagellates and apicomplexans are sister clades (Fast et al., 2002) and the photosynthetic plastid harboured by chromerids, such as *Chromera*

velia, is related to the apicoplast of parasitic apicomplexans and the peridinin pigmented plastids of many photosynthetic dinoflagellates (Moore et al., 2008).

The alveolates are closely related to stramenophiles, a group frequently called heterokontophytes, and includes the diatoms, brown algae, chrysophytes and water molds. Alveolata and Heterokontophyta, together with Cryptophyta and Haptophyta, are the initial four monophyletic groups to comprise the "supergroup" Chromalveolata (Cavalier-Smith, 1999b). The chromalveolates have grown to include a number of smaller lineages and another supergroup, the Rhizaria (Reyes-Prieto et al., 2007). The Rhizaria includes amoeboflagellates such as chlorarachniophytes, euglyphids, foraminifera, and the radiolarian. Although the grouping of alveolates, heterokontophytes and Rhizaria is strongly supported (Hackett et al., 2007) and phylogenomic analysis supports monophyly of haptophytes and cryptophytes (Patron et al., 2007, Hackett et al., 2007), more recent analyses suggest that a monophyletic haptophyte-cryptophyte lineage is unlikely and that cryptophytes may potential branch with plants (Burki et al., 2012).

The term Chromalveolata was introduced to account for those photosynthetic Alveolata and Chromista members containing plastids of red algal origin. The term suggests that a single secondary endosymbiotic event with a red alga resulted in the plastid ancestor of all members of the supergroup (Cavalier-Smith, 1999b). Use of the term is contentious due to the diversity of eukaryotes encompassed by the supergroup and the potential that chromalveolates contain plastids obtained by serial endosymbioses. Nevertheless, the term continues to be used due to the available data and high support for the sister relationships of the different groups comprising the supergroup; though the monophyletic origin of the group is being strongly challenged (Kim and Graham, 2008). More recently constructed phylogenetic trees support the splitting of eukaryotes into two monophyletic megagroups with nearly all photosynthetic lineages linked and clustered together in one megagroup (Burki et al., 2008).

1.2.4 Plastid Evolution

Plastids evolved from the endosymbiotic uptake of a cyanobacterium and organelles present today have resulted from the progressive reduction and integration of these engulfed cells with the host cell (Delwiche, 1999, Keeling, 2004). Plastids were initially taken up by red algae, glaucophytes and land plants; a group that included green algae and land plants. Consequently, red and green algae contain a primary plastid bounded by a double membrane and which is thought to have originated from a single endosymbiotic event (McFadden, 2001). The uptake of a green alga or red alga by a second eukaryote resulted in secondarily acquired plastids. Independent uptake of green algae lead to the euglenoids and chlorarachniophyte, but it was the uptake of red algae, and the integration of the engulfed red alga plastid with the host cell, that lead to a much greater diversity of organisms (Keeling, 2009). Dinoflagellates, haptophytes,

heterokontophytes and cryptophytes contain red algal plastids acquired via secondary or tertiary endosymbiotic events. Organisms with a plastid of red algal origin may have acquired the plastid via a single endosymbiosis of a red alga or via several endosymbiotic events in which the organism engulfed another organism that had already integrated a plastid of red algal origin.

Multiple endosymbiotic events that have resulted in secondary or tertiary acquired plastids have also resulted in significant transfer of genomic information from the engulfed symbiont to the host cell. Secondary and tertiary plastids can be bound by three or four membranes and these additional membranes are attributed to multiple endosymbiotic events (Cavalier-Smith, 1999b, Cavalier-Smith, 2002). As a result protein import systems evolved that enable cytoplasmically synthesized proteins to target and transit the multiple membranes surrounding the plastid (Armbrust et al., 2004). An N-terminal signal peptide sequence directing proteins across the endoplasmic reticulum membrane and transit peptide sequences have been identified in a number of algae with plastids acquired through secondary and tertiary endosymbiosis (Patron et al., 2005, Patron and Waller, 2007). A transit peptide is required to transport proteins across the double membrane of primary plastids in green and red algae, while a bipartite targeting signal is required for proteins to cross the increased number of membranes surrounding secondary or tertiary plastids.

In addition to the varying evolution, the size and number of plastids within a cell can vary highly between species (Falkowski et al., 1985). For example, diatoms and chlorophytes contain relatively large numbers of plastids or the plastids account for a large area of the cell volume while dinoflagellates have few plastids (Falkowski et al., 1985). Approximately half of the known dinoflagellate taxa contain plastids and with at least five plastid types known in dinoflagellates, these algae are one of the most diverse plastid containing eukaryotes (Hackett et al., 2004a). In dinoflagellates a peridinin plastid originating from a red algal secondary endosymbiosis (Zhang et al., 1999) is the most widespread followed by a tertiary derived haptophyte plastid (Tengs et al., 2000) that is present in fucoxanthin containing dinoflagellates (Yoon et al., 2005). The *Symbiodinium* plastid primarily contains the pigments chlorophyll *a* and *c*, and the carotenoid peridinin. Unique to dinoflagellates, peridinin is associated with two light-harvesting protein complexes; the peridinin chlorophyll *a*-protein complexes (PCP) and the membrane bound chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC).

1.3 Genus *Symbiodinium*

Symbiodinium (Freudenthal, 1962) are members of the Suessiales order of dinoflagellates and are widely recognised for forming relationships with a diverse array of marine invertebrates and protists (Trench, 1993, Glynn, 1996, Rowan, 1998, Lobban et al., 2002), including species of molluscs (giant clams), cnidarians (corals, jellyfish, sea anemones), platyhelminthes (flatworms), radiolarians and foraminifera. To date, *Symbiodinium* research has predominantly

focused on documenting the physiological and genetic diversity of the genus with the aim to better understanding coral reef ecosystems (for example, LaJeunesse, 2002, Baker, 2003, Fabricius et al., 2004, Warner et al., 2006, Loram et al., 2007b). Frequently referred to as "zooxanthellae" due to the golden-brown pigmented colouring of the cells, *Symbiodinium* play a vital role in the development and survival of coral reef ecosystems.

1.3.1 Life Stages

Symbiodinium spp. reproduce asexually through mitosis although molecular evidence indicates recombination and sexual reproduction also occurs (LaJeunesse, 2001, LaJeunesse et al., 2005), but it is not yet well understood (Stat et al., 2006). The complete life stages of non-cultured *Symbiodinium* are unclear, but the life cycle of cultured (for free-living species refer to section 1.3.5) *Symbiodinium* exhibit coccoid non-motile and mastigote motile stages with doublets formed in the mitotic phase of cytokinesis, prior to cells becoming motile (Fitt and Trench, 1983). Following mitosis, which in culture varies depending on nutrient availability and *Symbiodinium* type, the two mastigotes produced are motile for a period and this also varies depending on *Symbiodinium* type (Fitt and Trench, 1983). Following the motile stage, cells transform to the coccoid non-motile stage. *Symbiodinium* are haploid but tetrads are evident and possibly a result of meiotic division (producing four haploid cells) or a product of two consecutive mitotic division (Fitt and Trench, 1983). Of the different life stages, the non-motile coccoid cells are most frequently described as they appear to be the dominant stage when in symbiosis with a host (Trench and Blank, 1987).

Symbiodinium cells demonstrate phase cell division (Fitt and Trench, 1983) and patterns of motility that are circadian; with cells motile during light phases and non-motile during dark phases (Fitt et al., 1981). Similar motility and division patterns are evident when cultured cells are grown at high and low irradiance levels, but the percentage of motile cells and growth rates are greater in cells at high irradiance levels than low irradiance (Fitt and Trench, 1983).

1.3.2 Genotypes

Symbiodinium were initially considered a single species and classified *Symbiodinium microadriaticum* Freudenthal (Freudenthal, 1962). Following a variety of physiological, morphological, biochemical and behavioural studies in the early 1980's (for example, Schoenberg and Trench, 1980a, Schoenberg and Trench, 1980b, Schoenberg and Trench, 1980c, Fitt et al., 1981, Chang and Trench, 1982, Chang et al., 1983, Fitt and Trench, 1983, Chang and Trench, 1984) three new species, *S. goreauii*, *S. kawagutii* and *S. pilosum* were introduced (Trench and Blank, 1987) and by the early 1990's 10 *Symbiodinium* species had been named (Trench, 1993). Fourteen species of *Symbiodinium* are currently named although only eight have been formally characterized (Guiry and Guiry, 2013). More recently, analyses of

Symbiodinium nuclear and chloroplast ribosomal genes have revealed variation in genotypes and the varying genotypes have been arbitrarily designated using an alpha-numeric system (Rowan and Powers, 1991a, Coffroth and Santos, 2005, Pochon et al., 2006). *Symbiodinium* spp. are (currently) divided into nine distinct and divergent phylogenetic lineages, referred to as phylotypes or clades, and these clades are denoted 'A – C' (Rowan and Powers, 1991a), 'D' (Carlos et al., 1999), 'E' (LaJeunesse, 2001), 'F-H' (Pochon et al., 2001, Pochon et al., 2004) and 'I' (Pochon and Gates, 2010). Of the nine distinct lineages, clade A is considered the ancestral group to all other *Symbiodinium* (Pochon et al., 2006).

The use of more variable markers, such as internal transcribed spacer regions (ITS-1 and ITS-2), is revealing that within each of the nine clades *Symbiodinium* can be classified further into functionally distinct evolutionary sub-phylotypes or sub-clades (Loh et al., 2001, Baker, 2003, LaJeunesse et al., 2004), and descriptions of new sub-clades continues to occur (LaJeunesse et al., 2010). An alternative method to differentiate sub-clades is to use the non-coding region present in dinoflagellate plastid genome mini-circles as a marker. This method is considered a plausible alternative due to the diversity of the region between and within clades (Barbrook et al., 2006) and use of the non-coding region of the plastid psbA minicircle is rapidly gaining popularity as a genetic marker for resolving *Symbiodinium* diversity (LaJeunesse and Thornhill, 2011).

It is not yet known which of the taxonomic units represent the species level, but there are indications that the nucleotide variation evident at the sub-clade level is equivalent to that seen between other dinoflagellate species (Pettay and LaJeunesse, 2007). The establishment of axenic mono-algal cultures and development of genetic markers has resulted in the naming of 14 *Symbiodinium* species, eight of which have been formally described (Guiry and Guiry, 2013), and many more are expected to exist. *Symbiodinium* genotypes demonstrate variation in photo-acclimation (Iglesias-Prieto and Trench, 1994), photo-physiology (Rowan, 2004, Loram et al., 2007b, Sampayo et al., 2008), thermal resistance (Warner et al., 1999) and coral bleaching susceptibility (Tchernov et al., 2004, Jones et al., 2008, Sampayo et al., 2008, Stat et al., 2009), but sequence data for the genes and proteins responsible for these variations is lacking.

ESTs and RNA transcript sequences are available for a number of dinoflagellate species. Within the Alveolata lineage, the most closely related phyla to dinoflagellates (refer to 1.2.3) with a sequenced genome are the parasitic apicomplexans such as *Plasmodium falciparium*. Much of the available sequence data for dinoflagellates stems from studies on *Amphidinium carterae* (Bachvaroff et al., 2004), *Heterocapsa triquetra* (Patron et al., 2005), *Karlodinium micrum* (Patron et al., 2006), *Karenia brevis* (Yoon et al., 2005), *Alexandrium tamarense* (Hackett et al., 2005) and *Lingulodinium polyedrum* (Bachvaroff et al., 2004, Tanikawa et al., 2004), all non-symbiotic dinoflagellates. Sequence information for symbiotic *Symbiodinium* is limited and is predominantly comprised of expressed sequence tags (ESTs) for symbiotic

Symbiodinium sp. sub-clade C3 (Leggat et al., 2007), cultured *Symbiodinium* sp. clade A (CassKB8) (Voolstra et al., 2009, Bayer et al., 2012) and clade B (Mf1.05b) (Bayer et al., 2012). Once available, the genome sequencing project for *Symbiodinium* being conducted at King Abdullah University of Science and Technology in Saudi Arabia (unpublished data) along with work recently published by the Okinawa Institute of Science and Technology in Japan (Shoguchi et al., 2013) will greatly add to the current publicly available datasets.

1.3.3 General Morphology and Physiology

Early studies on *Symbiodinium* demonstrated variations in morphology (Schoenberg and Trench, 1980b), host specificity (Schoenberg and Trench, 1980c), isoenzyme and soluble protein patterns (Schoenberg and Trench, 1980a), and photo-physiology (Chang et al., 1983). Taxonomic classification of *Symbiodinium* based on morphological variation has been, and continues to be, problematic because cells alternate between motile and non-motile morphologies (refer to 1.3.1) and descriptions of natural or cultured populations can be influenced by nutrient availability, level of irradiance, and growth phase at measurement (LaJeunesse, 2001).

Symbiodinium cells contain one or more plastids that occupy between 18 – 36 % of the cellular volume, thylakoids which are typically stacked in groups of three with parallel and / or peripheral arrangements, a pyrenoid, and one or more mitochondria (Trench and Blank, 1987). *Symbiodinium* cell size varies between clades (Schoenberg and Trench, 1980b, Trench and Blank, 1987, LaJeunesse, 2001) and growth phase and can range between 5 – 18 μm (Trench and Blank, 1987). Cladal variations can include the texture of the outer surface of the amphiesma (the outer region of the dinoflagellate cell) and presence or absence of an accumulation body or vacuole in the mastigote, which is evident in smaller cell lineages such as clade B (Schoenberg and Trench, 1980b).

Symbiodinium clades and sub-clades demonstrate different physiological responses to the environment and biochemistry (for example, Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b, Kinzie et al., 2001, Rowan, 2004, Tchernov et al., 2004, Sampayo et al., 2008, Hennige et al., 2009). Some *Symbiodinium* exhibit distinct host taxonomy, geographic and / or environmental distribution patterns (Iglesias-Prieto et al., 2004, Rowan et al., 1997, LaJeunesse et al., 2004, van Oppen et al., 2005), while others can be isolated from a variety of host species across widely distributed geographic regions (LaJeunesse, 2001). Many *Symbiodinium* symbiotic relationships appear stable over time, regardless of external environmental factors (for example, Goulet and Coffroth, 2003, Rodriguez-Lanetty et al., 2003: Iglesias-Prieto et al., 2004, LaJeunesse, 2005, Thornhill et al., 2006, Sampayo et al., 2008, Stat et al., 2009). Nevertheless, the varied responses to environmental cues or stress events suggests particular *Symbiodinium* may be more stress tolerant than others and should specific clades or

sub-clades demonstrate a greater stress tolerance, this may provide their host organism with a competitive advantage compared with hosts harbouring less tolerant *Symbiodinium* types. Therefore, the susceptibility of a host organism to particular environmental stressors may be related to the type of *Symbiodinium* clade or sub-clade harboured (Iglesias-Prieto and Trench, 1994, Warner et al., 1996, LaJeunesse et al., 2003).

1.3.4 Cultured *Symbiodinium* Cells

Morphological and physiological descriptions for *Symbiodinium* are primarily due to studies involving three cultured *Symbiodinium* species; *S. microadriaticum* (sub-clade A1), *S. pilosum* (sub-clade A2) and *S. kawagutii* (sub-clade F1) (for example, Schoenberg and Trench, 1980a, Schoenberg and Trench, 1980b, Schoenberg and Trench, 1980c, Trench and Blank, 1987, Iglesias-Prieto et al., 1991, Iglesias-Prieto et al., 1992, Iglesias-Prieto et al., 1993, Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b). A variety of clades and sub-clades have been successfully cultured (Santos et al., 2001, Coffroth et al., 2006), however, *Symbiodinium* demonstrate differential culturability. For example, clade A cells are generally quite easily cultured, but specific sub-clades, such as C3, have been challenging to culture suggesting particular clades or sub-clades may not be able to exist as free-living organisms outside their host (Pochon et al., 2010).

Cultured *Symbiodinium*, while representative of particular symbiotic genetic populations, may not be representative of the same genetic populations in symbiosis. Culturing of microbial cells is highly selective and can result in discrepancy and over representation of particular microorganisms or lineages (Amann et al., 1995, von Wintzingerode et al., 1997). In addition, the response of cultured cells to experimental parameters may not reflect that of cells in the environment because cells undergo morphological changes when placed in culture (Schoenberg and Trench, 1980b). Furthermore, an unlimited nutrient supply or controlled light conditions influence cell growth (Dixon and Syrett, 1988).

Variations between cultured cells and cells in symbiosis are clearly evident. Cultured *Symbiodinium* cells possess a prominent and thick pellicle which in non-cultured cells is distinctly reduced (Schoenberg and Trench, 1980b). The pellicle is a fibrous layer beneath the vesicles lining the inside of the cell membrane and in some athecate dinoflagellates the pellicle forms the primary strengthening layer of the amphiesma (Schoenberg and Trench, 1980b). In addition, factors such as light conditions and temperature influence cell growth rates. Cultured *Symbiodinium* exhibit a wide range of growth rates from 0.031 – 0.044 day $^{-1}$ (Karako-Lampert et al., 2005) to 0.33 – 0.48 day $^{-1}$ (Taguchi and R. A. Kinzie, 2001) with many variations within this range (Fitt and Trench, 1983, Chang et al., 1983, Robison and Warner, 2006, McBride et al., 2009). Cell doubling can range between 2.7 to 33 days, and temperature affects growth and doubling rates significantly (McBride et al., 2009). In comparison, doubling times for symbiotic

Symbiodinium cells can range between 53 – 73 days; this corresponds to a growth rate of 0.013 – 0.0094 day $^{-1}$ (Muscatine et al., 1984).

Research with symbiotic *Symbiodinium* poses difficulties not applicable to cultured *Symbiodinium*. The complex interactions between *Symbiodinium*, the host organism, and associated bacterial communities promotes studies on cultured cells, and while cultured *Symbiodinium* research is not without limitations, benefits include: the ability to directly compare specific genotypes, accurately measure growth parameters, and record responses to manipulated and controlled environmental factors without host and bacterial interference. The use of cultured *Symbiodinium* provides an unlimited supply of cells of a known molecular type from which information can be extracted. To date, cell descriptions, physiological, and molecular findings determined using cultured *Symbiodinium* have been central to differentiating *Symbiodinium* species. The major limitation of cultured cell work is that direct extrapolation of findings to cells in symbiosis, or even freshly isolated cells, is restricted for the same reason cultured research began: it does not account for the complex symbiont, host and bacterial associations.

1.3.5 Free-living *Symbiodinium*

Free-living *Symbiodinium* in the environment are important sources of symbionts for host organisms. The occurrence, dispersion and diversity of free-living *Symbiodinium* are less well researched or understood, but free-living *Symbiodinium* can be found in the water column, marine sediments (Coffroth et al., 2006, Pochon et al., 2010) and within macro-algal beds (Porto et al., 2008). The diversity of free-living species is somewhat inferred due to the diversity and geographic dispersion of hosts that acquire *Symbiodinium* symbionts directly from the surrounding marine environment (Baird et al., 2009).

Molecular markers commonly used in identifying symbiotic *Symbiodinium* diversity have previously lacked the specificity to detect free-living species from amongst the complex and diverse marine micro-eukaryotic communities present within water column samples (Pochon et al., 2010). More recent genetic studies on free-living *Symbiodinium* from the Hawaiian and Florida (Takabayashi et al., 2012) coastal regions suggest that although distribution patterns of free-living *Symbiodinium* clades are consistent with patterns demonstrated for symbiotic *Symbiodinium*, the types of free-living *Symbiodinium* evident in an area appears restricted in comparison to a regions symbiotic types (Takabayashi et al., 2012). Furthermore, some species of *Symbiodinium* are distinctly free-living (Hirose et al., 2008, Reimer et al., 2010, Yamashita and Koike, 2012, Jeong et al., 2014).

It has been suggested that environmental *Symbiodinium* can be divided into two groups; those that are exclusively free-living and those that are transiently free-living having been expelled from a host (Yamashita and Koike, 2012). Understanding the distribution, diversity

and characterization of free-living *Symbiodinium* spp., particularly transiently free-living species, is important in order to better understand horizontal *Symbiodinium* acquisition, which is the most common method of symbiont acquisition by a host organism (Baird et al., 2009). Evidence suggests that some clade A and clade B free-living *Symbiodinium* do not establish, or are not capable of establishing, symbiotic associations with a cnidarian host (Coffroth et al., 2006, Takabayashi et al., 2012, Yamashita and Koike, 2012) and remain exclusively free-living dinoflagellates. There is limited information available for clade E *Symbiodinium*, but it is possible this clade may be a specialized free-living form (Yamashita and Koike, 2012). A lack of evidence supporting the presence of dominant symbiotic *Symbiodinium* clade C as free-living species in the water column or sediments (Takabayashi et al., 2012) poses the question as to whether some dominant symbiotic *Symbiodinium* are capable of surviving outside their symbiont-host association.

1.3.6 Symbiotic *Symbiodinium*

Symbiodinium comprise the majority of symbiotic dinoflagellates (Rowan, 1998). Symbiotic cells receive and utilize metabolic and respiratory by products produced by the host such as inorganic nitrogen and phosphorous (Yellowlees et al., 2008) and which are limiting resources in tropical marine environments (Muscatine and Porter, 1977). The host provides protection from direct contact with the external environment (Titlyanov et al., 2002) and importantly, actively acquires CO_2 which is transported to the symbionts (Furla et al., 2000b, Furla et al., 2000a, Leggat et al., 2002). Active acquisition of CO_2 is common in plants and necessary for successful photosynthesis, but very unusual in an animal as animals generally expel CO_2. In return, *Symbiodinium* supplies the host organism with photosynthates and essential nutrients (reviewed by Yellowlees et al., 2008). Translocated photosynthates provide a substantial energy source and in excess of 90 % of the host organism's energy requirement can be provided by *Symbiodinium* in this manner (Muscatine, 1967, Muscatine and Porter, 1977).

The density of *Symbiodinium* in host tissues can vary considerably and fluctuate due to variations in temperature, nutrient availability, and irradiance level. Cell densities of a million cells per square centimetre are not uncommon in host tissue. *Symbiodinium* can be either intra- or intercellular. In cnidarian hosts, such as coral and sea anemones, *Symbiodinium* are typically intracellular and located within the endodermal tissue, but in molluscs, such as clams, *Symbiodinium* are intercellular and located within tubules originating from the stomach (Leggat et al., 2002). Acquisition of *Symbiodinium* by the host animal occurs by two methods: vertical transmission where the symbiont is inherited directly from the parent animal, or horizontal transmission, where symbionts are acquired by asymbiotic gamates and larvae directly from the environment. Following acquisition from the environment, the symbiont enters the host cell through phagocytosis where it resides and reproduces. Each *Symbiodinium* becomes surrounded

by a membrane-bound vacuole called a symbiosome which originates from the host organisms plasma membrane during phagocytosis and appears to function as an interface between symbiont and host interactions (Peng et al., 2010). Aposymbiotic hosts are likely to attract motile *Symbiodinium* by releasing a substance or substances, such as ammonia (Fitt, 1984).

Symbiodinium hosts are found over a wide range of light habitats and this has implications for *Symbiodinium* photo-acclimation and photosynthetic capacity (Anthony and Hoegh-Guldberg, 2003). Some host species can harbour a single type of *Symbiodinium* (Rowan and Powers, 1991b, Stochaj and Grossman, 1997) while others harbour mixed populations (Rowan and Knowlton, 1995, Baker, 2003, Mieog et al., 2007, Ulstrup and Van Oppen, 2003). Many coral hosts harbour multiple *Symbiodinium* clades and the dominance or distribution of the different clades can vary within the same coral species depending on whether it is located in shallow waters or deep waters. For example, *Orbicella annularis* and *O. faveolata* are two dominant western Atlantic coral species and both species harbour clade A, B and C *Symbiodinium*; clade C is more prominent in *O. annularis* and *O. faveolata* in deep water where the irradiance level is low, and clades A and B are more common in shallow water *O. annularis* and *O. faveolata* where the irradiance level is considerably higher (Rowan et al., 1997). The ability of *Symbiodinium* communities to shift from one type to another may be restricted to specific host organisms and is not a universal feature (Stat et al., 2009). The significance of these varying features and the ability of specific *Symbiodinium* to adjust their photosynthetic apparatus under varying light regimes have ecological implications for the survival of *Symbiodinium*-host associations.

1.3.7 *Symbiodinium* and Coral Reefs

The association of *Symbiodinium* with scleractinian corals is important to coral reef ecosystems (for example, Rowan, 1998, Hoegh-Guldberg, 1999). As highly productive, biologically diverse and important marine ecosystems, coral reefs provide at least 100 million people worldwide with food resources, support large fishing and tourism industries, contribute significantly to the economic wealth of countries with coral reefs, and protect coastlines from destructive storm surges, erosion and flooding (Hoegh-Guldberg, 1999, Hughes et al., 2003, Lesser, 2004). Unfortunately worldwide coral reefs are experiencing a decline in health and the threats to habitat structure, abundance and diversity are increasing due to anthropogenic induced stresses such as pollution, over-fishing and coastal development (Jackson et al., 2001, Pandolfi et al., 2003, Hughes et al., 2003, Lesser, 2004).

Warming sea temperatures due to climate changes have been clearly linked with the increased prevalence of coral diseases (Harvell et al., 2002) and the phenomenon termed ‘coral bleaching’, which is one major problem facing thermally stressed coral reefs (Hoegh-Guldberg and Smith, 1989, Hoegh-Guldberg, 1999). Coral bleaching can occur when there is a loss of

symbiotic *Symbiodinium* and / or the loss of dinoflagellate pigments (Hoegh-Guldberg and Smith, 1989), and can be severe enough to effect coral health, even causing mortality. A number of environmental factors, or stresses, are known to affect coral reef health and induce bleaching; including elevated temperature, reduced salinity, and extreme light intensities (Hoegh-Guldberg, 1999).

Understanding how coral reefs respond to stress events is made more challenging due to the complexity of studying a biological structure that involves an animal (cnidarian host), algae (symbiotic dinoflagellates) and complex bacterial communities all residing together on an aragonite scaffold. For example, the coral aragonite skeleton can enhance the light field and increase light absorption which can negatively affect an organism already exposed to stressful conditions (Enriquez et al., 2005). Furthermore, contamination from host and bacterial genes, or any other organism associated with the *Symbiodinium*-host holobiont at the time of collection, can affect genomic and proteomic studies and make differentiating between host and symbiont genes challenging, particularly for genes highly conserved between eukaryotic organisms.

1.4 Photosynthesis

Photosynthesis provides the foundation of most life on Earth (Kiang et al., 2007) and carries out two globally important reactions: the photo-generation of molecular oxygen (O_2) from water, and energy production through the reduction of CO_2 (Heathcote et al., 2003). The process of photosynthesis efficiently converts light energy to chemical energy through a series of oxidation/reduction reactions (redox reactions). These reactions can be broken down into the light reactions and light independent reactions, or dark reactions. The light reactions are dependent on light and involve the conversion of light energy into chemical energy, while the dark reactions utilize the chemical energy to fix inorganic carbon into organic compounds. The light reactions are based around two photosystems, photosystem I (PSI) and photosystem II (PSII).

Photosystems utilize light energy to extract electrons from water, reduce the electron carrier $NADP^+$ to NADPH, and generate a pH gradient across the thylakoid membrane from which ATP can be derived. NADPH and ATP are then utilized by the Calvin-Benson Cycle and with the use of the enzyme RuBisCO, CO_2 is reduced to organic carbon (Larkum and Howe, 1997). An alternative form of photosynthesis can be performed by organisms such as Proteobacteria (purple sulphur bacteria) under anaerobic / anoxic conditions, however, hydrogen sulfide is used as the reducing agent rather than water and the process only involves one photosystem rather than two. Furthermore, anaerobic photosynthesis does not produce oxygen as a by-product.

The primary photochemistry performed by the photosystems involves the absorption of specific spectral light energies by pigments and the transfer of energy to the photosystems reaction centres, where charge separation of electrons occurs (Huner et al., 1998, Kiang et al.,

2007). To achieve this, organisms that perform oxygenic photosynthesis utilize antenna systems which can vary in structure and pigment composition.

1.4.1 Light Harvesting Complexes

The first antenna system was formed by phycobilisomes and this protein complex continues to be used by organisms such as cyanobacteria, glaucophytes and red algae (Neilson and Durnford, 2010). The antenna system now utilized by the majority of photosynthetic organisms comprises a peripheral antenna complex and a core antenna, which together form the light-harvesting complex (LHC) (Maxwell et al., 1995, Kiang et al., 2007).

LHCs are associated with both PSII and PSI, are located in the thylakoid membranes of plastids and contain chlorophyll (Chl) pigments. Chl *a* is a key component of the photosystem reaction centres and acts as the primary electron donor while other Chls, such as Chl *b*/*c*/*d*, are involved with light-harvesting (Heathcote et al., 2003). Although pigments associated with LHCs are synthesized in the chloroplast, the apoproteins are encoded in the nucleus, synthesized as larger precursors in the cytoplasm then imported into the chloroplast (Falkowski and La Roche, 1991). Once in the chloroplast transit and signal peptides are cleaved, the pigments bind to the mature proteins and the complex is inserted into the thylakoid membrane (Falkowski and La Roche, 1991). In addition to the chlorophyll pigments, LHCs bind carotenoids. These pigments are important for light-harvesting as well as photo-protective mechanisms (refer to 1.4.3) such as the dissipation of excess energy (Demming-Adams and Adams, 1996, Larkum and Howe, 1997), ROS quenching, and the direct stabilization of thylakoid membranes against lipid peroxidation (Havaux, 1993). Carotenoids known as xanthophylls are essential for rapid energy dissipation (seconds to minutes) and cycle between epoxilated and de-epoxilated states (violaxanthin, antheraxanthin and zeaxanthin in higher plants and green algae (Demming-Adams and Adams, 1996, Larkum and Howe, 1997); diadinoxanthin and diatoxanthin in brown algae and dinoflagellates (Larkum and Howe, 1997, Horton and Ruban, 2005)). Although the primary function of LHCs is to harvest light energy, it has been proposed that the original role of light-harvesting complex proteins may actually have been to protect reaction centres with the light-harvesting function a more recent addition (Montane and Kloppstech, 2000).

The LHC superfamily exhibits a high degree of sequence diversity and includes subfamilies such as the Chl *a* and *b* binding proteins (Cab), the Chl *a* and *c* binding proteins (Cac), the Chl *a* and phycobilin binding proteins (LhcaR, Lhc), and fucoxanthin-Chl *a* and *c* binding proteins (Fcp) and those associated with protective mechanisms and stress responses such as the LI818 and LI818-like proteins, PSII subunit S (PsbS), and early light inducible proteins (ELIP) (Heddad and Adamska, 2002).

1.4.1.1 Higher Plants and Green Algae LHC Proteins

Higher plants, green algae, euglenophytes and chlorarachniophytes contain Chl *a/b* binding proteins which also bind lutein. The two major LHCs utilized by organisms containing Chl *a/b* proteins are LHCI and LHCII. In higher plants, the genes, protein composition, and complex structure of the light-harvesting antenna, display a highly conserved complexity (Kühlbrandt et al., 1994, Jansson, 1999, Liu et al., 2004). The specific protein composition of membrane bound antenna determines the relative ability to harvest light through the expression of LHCII proteins (Maxwell et al., 1995, Montane et al., 1997, Thomas, 1997) and quench energy through the expression of xanthophyll binding ELIPs (Green and Durnford, 1996, Rossel et al., 2002, Murchie et al., 2005).

LHCII is primarily associated with PSII; comprised of at least six polypeptides assembled into monomeric and trimeric protein complexes, the major complex is encoded by the *Lhcb1-3* genes while *Lhcb4-6* encode the minor complexes (CP29, CP26 and CP24). The *Lhcb1-3* genes form trimers of monomeric protein complexes, and the *Lhcb4-6* genes form monomers. CP26, CP29 and another LHCII protein Lhcbm5, which were initially thought to be exclusively PSII associated proteins, now appear to shuttle between PSII and PSI during state transitions and provide docking sites for the trimeric LHCII proteins (Takahashi et al., 2006). The structure of LHCII in higher plants and green algae has revealed that the protein contains three transmembrane helices and a short amphipathic helix exposed to the luminal side of the thylakoid membrane (Kühlbrandt et al., 1994). More recent work has elucidated the presence of a second short amphipathic helix between the first and second helices, also on the luminal side of the thylakoid membrane. While the CP26 and CP29 complexes are each encoded by a single gene the LHCII, and LHCI, proteins of higher plants and green algae are encoded by a multigene family (Elrad and Grossman, 2004).

LHCI proteins are primarily associated with PSI and in higher plants encoded by six genes (*Lhca1 – Lhca6*) (Jansson, 1999). In higher plants PSI is monomeric, consists of at least 15 subunits (Jensen et al., 2007, Amunts et al., 2007) and contains a belt of four LHCI peripheral antenna complexes that assemble into two separate dimmers (Ben-Shem et al., 2003). LHCI of the green alga *Chlamydomonas reinhardtii* is larger than that evident in higher plants, is encoded by nine genes (*Lhca1 – Lhca9*) and the structure may modulate in response to environmental conditions (Neilson and Durnford, 2010).

1.4.1.2 Red Alga Chl a and Phycobilin Proteins

Limited data are available in relation to the photosynthetic apparatus of red algae even though the genome for the red alga *Galdieria sulphuraria* has been sequenced (Weber et al., 2004, Barbier et al., 2005) and genes for PSI analysed (Vanselow et al., 2009). Red algae possess phycobilisomes as the major antenna system for PSII (Wolfe et al., 1994) and a Chl *a*,

binding complex (LhcaR) associates specifically with PSI. Phycobilisomes are unlike most thylakoid membrane integral LHCs in that they are soluble and attach directly to the photosynthetic membrane (Gantt, 1981). Phycobilisomes are composed primarily of phycobiliproteins and extend the light absorbing range of red algae, extant cyanobacteria (Gantt, 1981) and glaucophytes (Neilson and Durnford, 2010).

In red algae, PSI is suggested to be chimeric in nature, containing elements of both cyanobacteria and higher plants, and may represent an evolutionarily more ancient PSI compared with higher plants, green algae and the current cyanobacteria (Vanselow et al., 2009). The PSI of cyanobacteria assembles as a trimer with each monomer consisting of 12 subunits (Melkozernov et al., 2006) while a model of PSI from the red alga *G. sulphuraria* suggests PSI is a monomer consisting of 11 or 12 subunits and may possibly contain a belt of LHCI peripheral antenna complexes (Vanselow et al., 2009). Furthermore, public genome and EST database searches of LHC-like sequences have revealed the possibility of a new red lineage Chl *a/b* like protein (Red CAP) specific to the red algal lineage (Engelken et al., 2010).

1.4.1.3 Heterokont, Haptophyte and Cryptophyte LHC Proteins

The heterokonts, haptophytes and cryptophytes are three of the four major Chl *c* containing algae lineages. Phylogenetic reconstructions of LHCs from Chl *c* containing algae indicate that individual algal lineages possess proteins from multiple LHC subfamilies, but the major LHC utilized by these algae is the fucoxanthin-Chl *a/c* proteins (Fcp).

Fcps are a LHC subfamily of chlorophyll *c*-binding light-harvesting proteins present in heterokontophytes (for example, diatoms and brown seaweeds) and haptophytes (for example, coccolithophores). Fcps are proposed to play dual roles in photosynthesis; as light-harvesting proteins and photo-protective proteins (Wolfe et al., 1994, Green and Kühlbrandt, 1995, Becker and Rhiel, 2006). Fcp cDNA can encode polyproteins and exhibit similarity to the Chl *a/b* binding proteins of green algae and higher plants even though these lineages diverged prior to the separation of the Chl *a/b* binding LHCI and LHCII sequences of green algae and higher plants (Durnford et al., 1996). Fcps bind different pigments than Chl *a/b* and Chl *a/c* proteins; binding fucoxanthin, chlorophyll *c* and chlorophyll *a*, but many of the amino acids identified as key chlorophyll binding and stabilization factors in the LHCII complex are conserved in Fcps.

Heterokontophytes, which include diatoms such as *Thalassiosira pseudonana, Odontella sinensis,* and *P. tricornutum* and raphidophytes such as *Heterosigma akashiwo* and *Heterosigma carterae*, differ from green and red algae in that they are believed to be derived from a secondary endosymbiotic event (Delwiche, 1999). Diatoms can contain species-specific multi-copy gene families and single-copy genes (Bowler et al., 2008) and the LHC family in diatoms can include at least 30 Fcps (Armbrust et al., 2004). Although the presence of genes of red algal origin supports the theory that the diatom plastid is of red algal origin (Bowler et al., 2008), no

red algal phycobiliprotein genes have been detected in diatoms (Armbrust et al., 2004), and neither have the PsbS proteins essential for the photo-protective xanthophylls cycle in higher plant, the minor complexes CP26-29, or ELIPs (Armbrust et al., 2004). Nevertheless, diatoms do utilize xanthophylls to dissipate excess light energy and the diatom xanthophylls cycle involves the interconversion of the carotenoids diadinoxanthin and diatoxanthin (Armbrust et al., 2004, Lavaud and Kroth, 2006, Gundermann and Büchel, 2008).

It has been unclear whether Fcps are associated specifically with either PSII or PSI or function as a single antenna system for both photosystems. Data for the diatom *Cyclotella meneghiniana* show Fcp polypeptides associated with the different photosystem fractions suggesting *C. meneghiniana* possesses two different light-harvesting antenna systems for PSI and PSII (Veith et al., 2009). Gene sequences for Fcps of diatoms (*fcp* 1-7 and 12 of *C. cryptic*; *fcp* A-F of *Phaedactylum tricornutum*) possess high sequence similarities and variation in molecular weights are minimal (17 – 19 kDa) (Veith et al., 2009).

Haptophytes, such as the coccolithophorid *Emiliania huxleyi* and chrysophyte *Isochrysis galbana*, also have Chl *c* containing plastids and encode Fcp (La Roche et al., 1994). Of the heterkont Fcp proteins, Fcp6, Fcp7 and Fcp12 share the highest homology to the haptophyte Fcp (Eppard et al., 2000). As with the heterokontophytes, haptophyte plastids derive from a red alga and are an important primary producer in aquatic environments (Andersen, 2004). Another commonality between heterokontophytes and haptophytes is the importance of the LI818 proteins (Dittami et al., 2010) a LHC subfamily suggested to be involved in photo-protection (Richard et al., 2000).

As with haptophytes and heterokontophytes (and also dinoflagellates), there is strong evidence that the plastids of cryptophytes, such as *Guillardia theta* and Rhodomonas CS24, evolved from a red algal-like ancestor (Douglas, 1998, Durnford et al., 1999, Reyes-Prieto et al., 2007, Keeling, 2009). Of the chromalveolate lineage, cryptophytes are the only group to retain a relict nucleus of the red algal symbiont, termed a nucleomorph (Archibald, 2007) and unlike the heterokontophytes, haptophytes and dinoflagellates; cryptophytes utilize phycobiliproteins, such as phycoerythrin and phycocyanin, which are present in red algae (Gould et al., 2007). In the cryptomonad *G. theta* two gene families are evident, one encoding the phycoerythrin alpha (α) subunits and one encoding the LHC proteins (Gould et al., 2007). Unlike red algae, the phycobiliproteins of cryptophytes do not assemble into phycobilisomes and are located not on the stromal side but within the thylakoid lumen (Gould et al., 2007). Rhodomonas CS24 utilize at least three different types of supercomplexes of PSI and peripheral Chl a/c_2 proteins, and two different types of supercomplexes of PSII and peripheral Chl a/c_2 proteins (Kereïche et al., 2008). The peripheral antenna in these supercomplexes shows no obvious similarity with that of green plant or green alga PSI-LHCI supercomplexes (Kereïche et al., 2008).

1.4.1.4 Dinoflagellate LHC Proteins

Dinoflagellates are the fourth major Chl *c* containing algae lineage in addition to those mentioned above in section 1.4.1.3. Dinoflagellates contain carotenoid – Chl *a/c* proteins similar to the Fcps, but the principal carotenoid of dinoflagellates is peridinin rather than fucoxanthin. A number of dinoflagellates, including *Karenia brevis* and *Karlodinium micrum,* bind fucoxanthin which may relate to the acquisition of a tertiary plastid of haptophyte origin (Tengs et al., 2000, Nosenko and Bhattacharya, 2007), however *Symbiodinium* bind peridinin. *Symbiodinium* have two major light-harvesting complexes that regulate the absorption of light energy and bind peridinin; the water-soluble protein peridinin-chlorophyll *a* proteins (PCP) that binds to the outside (luminal side) of the thylakoid membrane; and the water-insoluble chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC) integral to the thylakoid membranes.

The majority of photosynthetic pigments are associated with acpPC (Hiller et al., 1993, Iglesias-Prieto et al., 1993, Iglesias-Prieto and Trench, 1997a). Earlier studies determined that acpPC exist as a polypeptide of approximately 19 kDa (Hiller et al., 1995). It is now evident acpPC is considerably diverse and monomeric, dimeric and trimeric forms exist (Boldt et al., 2012). PCP is a unique protein and can exist in a monomeric form of 15 kDa or a dimeric form of 30 to 35 kDa (Prézelin and Haxo, 1976, Iglesias-Prieto et al., 1991, Norris and Miller, 1994, Sharples et al., 1996, Hiller et al., 2001, Weis et al., 2002); different cultured *Symbiodinium* species have been reported to possess both the monomeric and dimeric forms (*S. microadriaticum*), or either the monomeric (*S. kawagutii*) or dimeric (*S. pilosum*) form (Iglesias-Prieto et al., 1991).

Apart from containing the unique carotenoid peridinin, PCPs are unrelated to all known proteins (Norris and Miller, 1994, Weis et al., 2002) while acpPCs are homologous to the Chl *a/b* proteins of higher plants and green algae (ten Lohuis and Miller, 1998). In comparison to PCP, acpPC is relatively unstudied and limited information is available regarding the expression profiles of acpPCs under changing light energies or excitation pressure driven by environmental variables such as temperature and CO_2 availability. As with Fcps, it has been unclear whether acpPCs are associated specifically with either PSII or PSI, or function as a single antenna system for both photosystems, however, evidence suggests acpPC acts as an important quencher, particularly under high light (Kanazawa et al., 2014), and it has been speculated that membrane-bound acpPC may undergo a state transition response to reduce PSII over-excitation; dissociating from PSII and redistributing towards PSI (Hill et al., 2012).

A number of dinoflagellate studies have focused on *Amphidinium carterae*, a non-symbiotic dinoflagellate, and findings indicate photo-acclimation involves changes in the transcription of genes encoding acpPC (ten Lohuis and Miller, 1998). In *A. carterae* acpPCs are nuclear encoded genes and acpPC mRNA encodes a polyprotein, which is subsequently processed into

individual protein subunits (ten Lohuis and Miller, 1998). Although acpPCs are present in multiple forms the precise function of each of these forms is as yet unknown. Despite limited knowledge of the membrane bound protein complexes, their homology with Chl *a/b* proteins suggests that acpPCs are likely to be differentially expressed in response to environmental changes, and the ability to handle new environments will to some extent depend on the variety of acpPCs the dinoflagellate are able to express (ten Lohuis and Miller, 1998).

While the primary role of acpPCs is to harvest and pass on light energy to the reaction centres, the secondary role relates to the ability of these proteins to bind carotenoids (such as xanthophylls) that act as intermediaries in the transfer of excitation energy from chlorophyll to heat dissipation. As with diatoms, dinoflagellate xanthophylls include diadinoxanthin and diatoxanthin and are proposed as having a potential photo-protective function (Ambarsari et al., 1997) similar to that documented in higher plants (Demming-Adams and Adams, 1996). The xanthophylls are believed to bind to specific acpPCs (Iglesias-Prieto and Trench, 1997a) therefore, it seems reasonable to assume that the observed fluctuation concomitant with changes in light dosages correspond to an underlying change in the abundance of one or more specific acpPCs. In higher plants and algae, the presence of proteins that bind xanthophylls are predominantly determined at the level of mRNA transcription (Maxwell et al., 1995, Montane et al., 1999) and therefore it is tempting to assume that this will also be true of dinoflagellates.

1.4.2 Stress Response Proteins

A variety of stress response proteins have been identified in photosynthetic organisms, such as the PsbS protein of PSII (Kim et al., 1992, Li et al., 2000) and the LHC-like family of proteins which includes the ELIPs (early light-induced proteins) (Green and Kühlbrandt, 1995, Montane and Kloppstech, 2000), Seps (stress-enhanced proteins) (Heddad and Adamska, 2000), and OHPs (one-helix proteins) (Jansson et al., 2000, Andersson et al., 2003). These protein families share homology with the highly conserved polypeptide sequence that encodes the three membrane-spanning α-helices (Kühlbrandt et al., 1994) of the Chl *a/b*-binding LHCs, however, these distant relatives of the Chl *a/b*-binding LHCs contain between one and four transmembrane helices. For example, the PsbS family consists of four helix proteins (Jansson, 1999), while ELIPs are three-helix proteins (Montane and Kloppstech, 2000), and Seps are two-helix proteins (Heddad and Adamska, 2000). The one-helix proteins are homologous to the high light-induced proteins (HLIPs) of cyanobacteria and red algae and similar to the LHCs, ELIPs and PsbS proteins (Jansson et al., 1999, Jansson et al., 2000). Furthermore, an OHP (named Ohp2) associated with PSI has been identified and isolated (Andersson et al., 2003) which differs from all previously reported stress-induced proteins associated with PSII.

Under high light stress conditions, Seps and the HLIPs perform a photo-protective function (Montane and Kloppstech, 2000) while ELIPs, which are enriched in xanthophyll cycle

carotenoids, are selectively induced during extended exposure to light stress (Horton et al., 1996). Previously ELIPs were considered to have evolved following a deletion event involving a PsbS ancestor (Green and Durnford, 1996), but more recent proposals suggest ELIPS evolved independently in the green lineage and are not ancestral to LHC proteins or to PsbS (Engelken et al., 2010). HLIPs were also proposed as a candidate for the origin of LHC proteins although a more suitable candidate may be the two-helix Seps which are present in higher plants, green and red algae, glaucophytes (in which the LHC subfamilies are absent) as well as chromophytes such as diatoms (Engelken et al., 2010).

The LI818 and LI818-like proteins are another LHC subfamily identified as environmental stress-induced proteins (Richard et al., 2000, Koziol et al., 2007) and in green algae LI818 transcripts accumulate in response to environmental stresses such as high light (Savage et al., 2002). The LI818 subfamily is well represented within the Chl *a/c* containing algae, less so within the green lineage, and to date, LI818 proteins have not been detected within red algae (Dittami et al., 2010). Although LI818 polypeptides are predicted to possess three transmembrane-spanning helices, seen also in Chl *a/b* binding proteins, LI818 polypeptides group separately from the Chl *a/b* family (Richard et al., 2000). Furthermore, gene expression is not coordinated with that of PSI and PSII *cab* genes, is regulated differently by light, and peaks much earlier than Chl *a/b* mRNA (Richard et al., 2000).

1.4.3 Photo-damage and Photo-protection

Extreme light intensities, along with additional environmental factors such as elevated temperature, are known to stress and imbalance aspects of the light utilization, enzymatic and carbon fixation processes of photosynthesis (Coles and Jokiel, 1977, Hoegh-Guldberg and Smith, 1989, Adir et al., 1990, Heckathorn et al., 1997, Al-Khatib and Paulsen, 1999, Hoegh-Guldberg, 1999, Demmig-Adams and Adams, 2000). Photoautotrophs regularly experience daily and seasonal fluctuations in environmental conditions and stress responses induced by changing environmental cues can activate phenotypic changes and result in readjustments of an organism's tolerance level (Coles and Brown, 2003). Imbalances from stress events can result in PSII excitation pressure and affect an organism's photochemistry (Huner et al., 1998). To manage stress events, photosynthesizing organisms have photo-protective mechanisms to balance energy capture, dissipation and utilization.

Photo-protection mechanisms for the dissipation of excess energy absorbed by chlorophyll as heat can involve varying pathways, xanthophyll pigments, changes in the thylakoid pH gradient and specific pigment-binding proteins such as the PSII subunit PsbS (CP22) (Demmig-Adams and Adams, 2006). The xanthophyll cycle is an example of a reversible light-dependent photo-protective mechanism that is associated with LHCII and LHCI (Demmig-Adams and Adams, 2000, Demmig-Adams, 2003). Other photo-protective mechanisms can involve adjustments to

light-harvesting protein complexes, reaction centres, thylakoid membrane synthesis, electron transport pathways, and molecules and enzymes present to deal with heat dissipation and the generation of reactive molecules (Larkum and Howe, 1997, Niyogi, 1999). Activation of these processes in response to varying environmental factors can occur on varying time scales ranging from seconds or hours (for functional adjustments to individual proteins within the photosynthetic apparatus) to weeks or months (for developmental adjustments to the whole or parts of the organism) (Walters, 2005). Between these short and long term changes is a dynamic process termed photo-acclimation.

1.4.4 Photo-acclimation

Photo-acclimation involves reversible phenotypic changes that occur to compensate a change in spectral light (Falkowski and La Roche, 1991). The process involves adjustments to components of the photosynthetic apparatus (Walters, 2005), the expression and co-ordination of genes encoded in the nucleus and chloroplast (Durnford et al., 2003), and includes physiological, biochemical, and morphological modifications that allow for efficient utilization of available light energy (Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b). These changes can include the differential expression of proteins involved in the light-harvesting, photo-protection, charge separation, and carbon fixation processes of photosynthesis (Iglesias-Prieto and Trench, 1997a).

For example, photo-acclimation of green plants grown in high-light conditions can involve increases in levels of PSII proteins and pigments, the cytochrome b_6/f complex, components of the Calvin-Benson cycle, particularly RuBisCO, ATP synthase, and decreases in the level of chlorophyll binding LHCs associated with PSII (Walters, 2005). Such responses not only effect an organisms capacity to process captured light photo-chemically (Horton et al., 1996), but also to dissipate excess heat (Niyogi, 1999).

Observations at the level of gene transcription indicate distinct differences in how unicellular algae and higher plants acclimate their photosystems to environmental changes and that in comparison to higher plants, algae have a much greater ability to photo-acclimate (Falkowski and La Roche, 1991). For higher plants to complete photo-acclimate a change in the light field in addition to a change in excitation pressure between PSII and PSI is required, and cell to cell interactions are all important (Montane et al., 1999, Walters, 2005). However, in unicellular algae photo-acclimation is similar for an array of environmental cues and channelled via increases in excitation pressure regardless of the light field (Maxwell et al., 1995).

The question then arises; how do unicellular symbiotic dinoflagellates respond to distinct environmental cues, particularly given algal-cnidarian cell interactions have obviously played a prominent role in the evolution of the symbiotic association? To answer such a question requires a better understanding of symbiotic dinoflagellate photosynthetic apparatus, including

the major light-harvesting protein complexes and determining the role the complexes play in protecting the photosynthetic apparatus.

1.5 Project Aims, Methodology and Research Outcomes

The *Symbiodinium*-coral relationship is a key component of coral reef ecosystems. Nevertheless, the relationship is highly sensitive to environmental changes, and rising sea surface temperatures threaten this relationship (Glynn, 1996, Brown et al., 1999, Hughes et al., 2003) and are particularly problematic in summer when thermal stress is combined with high-light conditions. When the *Symbiodinium*-coral holobiont's ability to mediate or cope with stress events is exceeded, *Symbiodinium* can be expelled from coral host tissues and / or the *Symbiodinium* photosynthetic pigments can be reduced. Expulsion of *Symbiodinium* from the coral tissue or loss of photosynthetic pigments can lead to bleaching events, or ultimately the degradation and mortality of the coral host (Hoegh-Guldberg, 1999). Stress events can also impact and reduce the ability of *Symbiodinium* to perform photosynthesis efficiently and coral bleaching is predicted to be related to photosynthetic apparatus damage (Iglesias-Prieto et al., 1992, Warner et al., 1996, Jones et al., 2000, Fitt and Cook, 2001, Bhagooli and Hidaka, 2004, Hill and Ralph, 2006).

Current understanding of eukaryotic photosynthetic gene expression is predominately derived from plant and green unicellular algae such as *Arabidopsis* species and *Chlorella* species. To answer the questions: how do *Symbiodinium* respond to distinct environmental cues, do specific components of the photosynthetic apparatus respond to the cues, and do *Symbiodinium* mimic the response of other unicellular algae or that of higher plants, a better understanding of the major light-harvesting protein complex utilized by *Symbiodinium* is important. Furthermore, knowledge of the LHC genes possessed by *Symbiodinium*, the expression of these genes under a variety of environmental cues, and an understanding of how translation and degradation combine to restructure the photosynthetic apparatus, is necessary. Only a few genes associated with dinoflagellate photosynthesis have been described; and most of these genes have been characterized from non-symbiotic dinoflagellates. It is plausible to suggest that the response of symbiotic dinoflagellates to environmental cues will subtly vary from those organisms already characterized purely based on the unique nature of *Symbiodinium*.

The primary objectives of this research were to utilize the only available expressed sequence tag data for symbiotic *Symbiodinium* sp. sub-clade C3 to: obtain complete sequences for the chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC) present in *Symbiodinium* sp. C3; to compare *Symbiodinium* sp. C3 acpPC with LHCs from other photosynthetic organisms; to determine whether *Symbiodinium* sp. C3 acpPC were present in cultured *Symbiodinium* of varying clades and sub-clades; and to use the sequenced data to investigate acpPC gene expression patterns in response to varying light conditions.

To achieve these aims an extensive sequencing project was performed and once complete sequences were obtained bioinformatics analysis, including analysis of conserved amino acid residues and homology of translated protein sequences with known light harvesting genes from symbiotic and free-living dinoflagellates, algae and higher plants, was performed. Phylogenetic comparison of translated protein sequences with light harvesting protein complexes from a variety of organisms was undertaken as was the diversity of acpPCs within *Symbiodinium* from divergent lineages. Transcript sizes were determined using northern blots and Southern blots were used to determine acpPC gene copy numbers. A polyclonal antibody generated in rabbits to a synthesized peptide fragment based upon acpPC cDNA sequences was obtained and western blots used to investigate protein expression. Real-Time PCR, a tool relatively new to the study of *Symbiodinium*, was used for gene expression work following validation of normalization genes.

Phylogenetic analysis of 11 LHC sequences suggests the acpPC subfamily forms at least three clades within the Chl *a/c*-binding LHC family. The first clade includes rhodophyte, cryptophyte and peridinin binding dinoflagellate sequences; the second peridinin binding dinoflagellates only; while the third contains heterokonts, fucoxanthin and peridinin binding dinoflagellate sequences. *Symbiodinium* sp. C3 acpPC sequences generally contain three transmembrane helices and at least two proteins with LHC membrane-spanning helix duplication, deletion, and / or degeneration are evident. In addition, *Symbiodinium* encode transcripts containing one, two or three LHC polypeptides and the genome encodes single copy and as well as high copy acpPC genes. Analysis of *Symbiodinium* from divergent lineages demonstrates that C3 acpPC sequence information can be utilized to investigate closely related *Symbiodinium* at the transcript and protein level, in particular *Symbiodinium* C1. β-actin and proliferating cell nuclear antigen (PCNA) can be used in controlled light experiments to investigate acpPC expression having been validated as normalization genes. The use of a polyclonal antibody for acpPC suggests protein expression varies between *Symbiodinium* sub-clades. Nevertheless, changes at the transcript level are relatively small when *Symbiodinium* are exposed to different light environments suggesting acpPC genes are possibly subject to translational control mechanism or post-translational controls.

CHAPTER 2

2 General Methods and Materials

2.1 Sample Collection and Storage

Coral branches of *Acropora aspera* blue morph were collected from the reef flat opposite Heron Island Research Station (HIRS) (23°33'S, 151°54'E) at low tide. Branch tips (3-4 cm long) were removed using a pair of wire cutters or gloved hands and transported to seawater aquarium at HIRS in plastic bags containing seawater from the collection site. Either whole branches were frozen in liquid nitrogen or *Symbiodinium* cells were removed using an oral irrigator (WaterPik™) in 70 mL of 0.45 μm filtered seawater (FSW). The resulting homogenate was centrifuged for 2 min at 5,000 x *g*, the supernatant discarded, white coral mucus layer gently removed using a sterile pipette and pellet resuspended and washed in 5 mL of FSW. This protocol was repeated a second time before the pellet was transferred to a clean microcentrifuge tube and frozen in liquid nitrogen then transported back to the laboratory in a full charged nitrogen "dry shipper" and stored at -80°C.

2.2 Crushing Coral Branches

Intact coral nubbins were frozen in liquid nitrogen and stored at -80°C prior to crushing. All equipment was treated with RNAseZap® (Ambion Inc, USA), spatulas, tweezers, mortar and pestles were pre-cooled with liquid nitrogen until use and collection microcentrifuge tubes chilled on dry ice. Small segments of coral were crushed twice in a pre-chilled steel mortar and pestle using a hydraulic bench press. Crushed nubbins were transferred to a second mortar chilled with liquid nitrogen and ground to a fine powder with a pestle. Coral powder was transferred using a chilled spatula into microcentrifuge tubes on dry ice and stored at -80°C or used immediately to extract *Symbiodinium* RNA.

2.3 Cultured *Symbiodinium* sp.

Symbiodinium C1 (CCMP2466) was purchased from The Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences, USA (formally Center for Culture of Marine Phytoplankton)). Grown and provided by CCMP in L1 medium, cells were centrifuged on arrival for 1 min at 5000 x *g*, the L1 medium discarded and replaced with 50 mL of ASP-8A medium (Blank, 1987) (Appendices Tables 1 - 3) and treated with antibiotics (Polne-Fuller, 1991) (Appendices Table 4). Following antibiotic treatment cells were centrifuged for 1 min at 5,000 x *g*, the antibiotic containing medium discarded and cells transferred to 75 cm^2 Tissue Culture flasks with PE vented caps (Sarstedt, NC, USA) containing

100 mL of ASP-8A medium to recover and grow for six weeks with minimal disturbance. Nine *Symbiodinium* cultures obtained from The Santos Laboratory, Auburn University, Alabama USA in f/2 medium were treated as per CCMP2466 cells. For a complete list of cultured *Symbiodinium* refer to Appendices Table 5.

2.3.1 Maintenance

Symbiodinium cell cultures were grown and maintained in a light and temperature controlled refrigerated incubator TRISL-495-1-SD (Thermoline Scientific, Australia). The incubator was programmed to provide a 12 h:12 h (light:dark) photoperiod with the temperature set at 25^{o}C. Cultures were visually checked daily and media changed weekly. *Symbiodinium* cultures were grown in ASP-8A medium (Appendices Tables 1 - 3) prepared with Milli-Q water. Aliquots of ASP-8A media were prepared weekly in 2 L Duran® glass bottles (Schott Inc, NY, USA), pH adjusted to 8.5 with 32 % concentrated hydrochloric acid, autoclaved and allowed to cool to room temperature (23 – 26^{o}C) overnight. Prior to use the following day, filtered and aluminium foil covered 8A vitamin mix and vitamin B12 were added. Stock solutions for the media ingredients were prepared in specifically designated 50 – 200 mL Duran® glass bottles (Schott Inc, NY, USA), kept at 4^{o}C and regularly replaced to minimize contamination.

Alternative media trialled to determine ideal growth and maintenance for *Symbiodinium* cells included f/2 (Guillard and Ryther, 1962, Guillard, 1975) (Appendices Tables 6 - 8), f (2 x f/2), L1 (Guillard and Ryther, 1962, Guillard, 1975, Guillard and Hargraves, 1993) (Appendices Tables 9 - 11) and K (Guillard and Ryther, 1962, Guillard, 1975, Keller et al., 1987) (Appendices Tables 12 - 14). All *Symbiodinium* cultures received fresh media weekly, although longer periods between media changes of two to three weeks did not negatively affect *Symbiodinium*. The removal of old media and addition of fresh media was conducted in a laminar flow cabinet with culture tissue flasks, lids and media bottles flamed on opening and closing to minimize contamination. A single flask was opened at one time to minimize risk of cross contamination between *Symbiodinium* strains. The opened flask was flamed prior to the old medium being gently poured off into a large collection container, the flask flamed again prior to addition of 100 mL of ASP-8A medium, then flamed a third time prior to closing and placement back in the incubator.

Every four to six months *Symbiodinium* cells were transferred to new tissue culture flasks due to salt precipitation on the PE vented cap membrane and flask neck. This was conducted following the weekly media change allowing cells to be gently resuspended with disposable sterile cell scrappers (Sarstedt, NC, USA) in fresh media and poured directly into a fresh flask. Used flasks and cell scrappers were not recycled to avoid cross contamination problems.

New culture stocks, cells being treated with antibiotics and experimental control flasks were placed on shelves receiving optimal light intensity, while replicates of the culture collection were acclimated and maintained under both high light and low light conditions (Section 2.3.2).

2.3.2 Light Levels

Lighting on each of the four shelves within the incubator was supplied by four 18 W cool white fluorescent tubes (Polylux XLR™, GE Lighting). Light intensity on each shelf was varied by wrapping 50 – 70 % shade cloth around the shelf and lights until the desired light level was achieved then secured with cable ties. The top shelf was organized to receive 250 – 350 µmol quanta m^{-2} s^{-1} (high light shelf), the middle two shelves adjusted to 80 - 100 µmol quanta m^{-2} s^{-1} (medium light shelves) and the bottom shelf adjusted to 10 – 15 µmol quanta m^{-2} s^{-1} (low light shelf). Variation in light level across a shelf depended on positioning; the middle of each shelf received the highest light intensity while the sides the lowest. The use of shade cloth did not interfere with the temperature control within the incubator and light levels were regularly monitored using a LI-193SA Underwater Spherical Quantum Sensor with a LI-250A Light Meter (LI-COR® Biosciences, NE, USA)

2.3.3 Antibiotic Treatment

Antibiotic treatment (Polne-Fuller, 1991) was performed on cells received from CCMP and The Santos Lab. Following this initial treatment cells were checked every six months and treated based on contamination level. Axenic cultures were not achieved nor required, but bacterial and protozoan contamination was minimized with use of antibiotic treatment. Individual antibiotics were prepared with Milli-Q water and filter sterilized (0.22 µm) before use and/or storage according to manufacturer's protocol.

To treat cells aliquots were removed from culture flasks, centrifuged for 1 min at 5,000 x *g*, the old media discarded and cells resuspended in 40 – 50 mL of freshly prepared ASP-8A medium. The antibiotic stock mix was prepared in 100 mL of Milli-Q water. A diluted volume of the stock mix (1:10) (Appendices Table 4) was added to the small aliquots of cells and cells placed on a medium light shelf in the incubator. After 3 – 5 days treated cells were centrifuged for 1 min at 5,000 x *g*, the old media containing antibiotics discarded and cells resuspended in fresh ASP-8A medium. Cells were checked under a microscope prior to transfer into 25 cm^2 Tissue Culture flasks with PE vented caps (Sarstedt, NC, USA) containing 20 - 30 mL ASP-8A medium to determine effectiveness of antibiotic treatment. If bacterial and protozoan contamination had not decreased, antibiotic treatment was repeated. Once treatment was completed cells were left to recover for two to four weeks on a medium light shelf in the incubator. When cell density increased, cells were resuspended using disposable sterile cell

scrappers (Sarstedt, NC, USA) and transferred to 75 cm^2 Tissue Culture flasks with PE vented caps (Sarstedt, NC, USA) containing 100 mL ASP-8A medium.

2.4 Genotyping of *Symbiodinium*

2.4.1 Internal Transcribed Spacer 2 Region

The genotype of cultured and freshly isolated *Symbiodinium* was performed at six month intervals. The internal transcribed spacer 2 region (ITS 2) was used to classify genetically distinct *Symbiodinium* based on current clade designation. Genomic DNA was extracted from freshly isolated and cultured *Symbiodinium* as per Sections 2.5.4.1 and 2.5.4.2 respectively. The ITS 2 region was amplified using the forward primer ITSintfor2 (5'GAA TTG CAG AAC TCC GTG-3') (LaJeunesse and Trench, 2000) and reverse primer ITS2-Reverse (5'GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3') (Coleman et al., 1994) following the protocol of LaJeunesse (2002) (LaJeunesse, 2002). Purified reaction product (Section 2.5.8) was directly ligated into pGEM-T Vector (Promega, USA) and transformed into NM522 cells (Section 2.6.). Resulting bacterial colonies were collected by centrifugation (1 min at 10,000 x *g*) and following plasmid purification (Section 2.6.7) multiple clones were sequenced at the Australian Genome Research Facility using the forward primer M13F (5'GTT TTC CCA GTC ACG AC-3') and reverse primer M13R (5'CAG GAA ACA GCT ATG AC-3').

2.4.2 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) of the small subunit RNA (ssRNA) gene was used to determine diversity between *Symbiodinium* cultures prior to ITS 2 genotyping. Nuclear ssRNA genes were amplified with 'universal eukaryotic' primers ss5 (5'GGT TGA TCC TGC CAG TAG TCA TAT GCT TG-3') and ss3 (5'GAT CCT TCC GCA GGT TCA CCT ACG GAA ACC-3') (Rowan and Powers, 1992) and *Symbiodinium* specific primers ss5Z (an equimolar mixture of 5'GCA GTT ATA ATT TAT TTG ATG GTC ACT GCT AC-3' and 5'GCA GTT ATA GTT TAT TTG ATG GTT GCT GCT AC-3') and ss3Z (5'AGC ACT GCG TCA GTC CGA ATA ATT CAC CGG-3') (Rowan and Powers, 1991b). Amplified DNA fragments were digested with the restriction enzymes Taq I and Dpn II (New England BioLabs® Inc, USA) according to Rowan and Knowlton (1995) (Rowan and Knowlton, 1995) and electrophoresed in 1 % agarose gels stained with GelRed® Nucleic Acid Stain, 10,000X in dimethyl sulfoxide (DMSO) (Biotium Inc, USA).

2.5 General Nucleic Acid Methods

2.5.1 Isolation of Total RNA

All equipment used for RNA extractions was treated with RNAseZap® (Ambion Inc, USA), and any plasticware and reagents were RNAse/DNAse-free. RNA specific pipettes were used in

conjunction with filtered pipette tips in an RNA designated work area. Total RNA was quantified using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA) (Section 2.5.10) and integrity assessed by formaldehyde agarose gel (Section 2.5.11).

2.5.1.1 Freshly Isolated Cells

Total RNA was isolated using a RNeasy Plant Mini Kit (Qiagen, USA). *Symbiodinium* isolated from coral skeletons using an oral irrigator (WaterPik™) (Section 2.1), frozen in liquid nitrogen and stored at -80^{o}C were ground to a fine powder under liquid nitrogen in a mortar with a pestle. A maximum of 100 mg of disrupted cells were transferred to a cooled RNase-free microcentrifuge tube containing 450 µL RLT buffer (Qiagen, USA) and RNA extracted according to manufacturer's protocol, including the additional centrifuge and elution step. Twenty-five µL was used at each elution step resulting in a final volume of 50 µL. Samples exceeding 100 mg were processed in multiple tubes and RNA pooled once extracted. Total RNA was placed and stored at -80^{o}C or used immediately to synthesis complementary DNA (cDNA).

2.5.1.2 Cultured Cells

Total RNA from cultured *Symbiodinium* was isolated using a RNeasy Plant Mini Kit (Qiagen, Valencia USA). The required numbers of *Symbiodinium* cells were harvested from culture flasks, centrifuged for 2 min at 5,000 x *g* and 25^{o}C, the culture medium discarded and cells resuspended in 450 µL RLT buffer (Qiagen kit buffer). Cells and buffer were transferred to a Lysing Matrix D tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate including cell debris was transferred to a QIAshredder spin column and RNA extracted according to manufacturer's protocol, including the additional centrifuge and elution step. Twenty-five µL was used at each elution step resulting in a final volume of 50 µL. A maximum of 10 x 10^{6} cells were lysed per tube. Samples exceeding 10 x 10^{6} cells were processed in multiple tubes and RNA pooled once extracted.

Samples collected for RNA extraction at a later date were transferred to Lysing Matrix D tubes without RLT buffer, frozen in liquid nitrogen and stored at -80^{o}C. When processed, 450 µL of chilled RLT buffer was added to each sample immediately upon removal from the -80^{o}C freezer and extracted as per fresh RNA samples above.

2.5.2 Precipitation of RNA

RNA was concentrated by the addition of 2.5 v/v of 100 % absolute ethanol, 1/10 v/v of RNase-free 3 M sodium acetate pH 5.2 and placed at -80^{o}C overnight. Samples were centrifuged for 1 h at 37,000 x *g* and 2^{o}C in an Optima™ TLX Ultracentrifuge (Beckman Coulter, Inc, USA). The supernatant was discarded, pellet washed with 500 µL of 75 % ethanol

and centrifuged for 15 min at 37,000 x *g* and 2°C. RNA was air dried then dissolved in an appropriate volume of RNase free water. Samples were stored at -80°C and heated for 2 – 5 min at 55°C to redissolve RNA pellet prior to use.

2.5.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) amplification and genomic DNA (gDNA) elimination was performed with QuantiTect Reverse Transcription® Kit (Qiagen, USA) in a 20 µL reaction using 500 ng of total RNA as template and a RT primer mix consisting of random primers and oligo-dT. Negative controls prepared without total RNA as template were included for each series of reactions.

2.5.4 Isolation of Genomic DNA

2.5.4.1 Freshly Isolated Cells

Genomic DNA (gDNA) was isolated using a DNeasy Plant Mini Kit (Qiagen, USA). *Symbiodinium* isolated from coral skeletons using an oral irrigator (WaterPik™) (Section 2.1), frozen in liquid nitrogen and stored at -80°C were ground to a fine powder under liquid nitrogen in a mortar with a pestle. A maximum of 100 mg of disrupted cells were transferred to a cooled RNase-free microcentrifuge tube containing 400 µL buffer AP1 with 4 µL RNase A stock solution and gDNA extracted according to manufacturer's protocol. Elution with 50 µL of buffer AE was performed rather than 100 µL to increase gDNA concentration. If the volume disrupted exceeded 100 mg the sample was split between multiple microcentrifuge tubes each containing 400 µL buffer AP1 with 4 µL RNase A stock solution to avoid overloading the QIAshredder Mini spin column and gDNA pooled once extracted.

2.5.4.2 Cultured Cells

Genomic DNA (gDNA) from cultured *Symbiodinium* was isolated using a DNeasy Plant Mini Kit (Qiagen, Valencia USA). The required number of *Symbiodinium* cells were harvested from culture flasks, centrifuged for 2 min at 5,000 x *g* and 25°C, the culture medium discarded and cells resuspended in 400 µL buffer AP1 with 4 µL RNase A stock solution. Cells and buffer were transferred to a Lysing Matrix A tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate including cell debris was transferred to a 1.5 µL microcentrifuge tube and gDNA extracted according to manufacturer's protocol. Elution with 50 µL of buffer AE was performed rather than 100 µL to increase gDNA concentration. A maximum of 10 x 10^6 cells were lysed per tube. Samples exceeding 10 x 10^6 cells were processed in multiple tubes and gDNA pooled once extracted.

Cells collected for gDNA extraction at a later date were transferred to Lysing Matrix A tubes without buffer and RNase A stock solution, frozen in liquid nitrogen and stored at -80°C. When processed, 400 µL of buffer AP1 and 4 µL RNase A stock solution was added to each sample

immediately upon removal from the -80°C freezer and extracted as per fresh gDNA samples above.

2.5.5 Restriction Enzyme Digestion

Restriction enzyme digestions were performed using 3.0 µg of gDNA extracted from *Symbiodinium* isolated from coral skeletons using an oral irrigator (WaterPik™) or cells harvested from culture flasks (Section 2.5.4). Standard digestions included 10 units of restriction enzyme, 2 µL of recommended 10X buffer, 100X BSA (10 mg mL^{-1}; 0.2 µL) if required and Milli-Q water in 20 µL reactions. Digestion reactions were incubated for 8 - 24 h at 37°C according to manufacturer's protocol. Restriction enzymes were purchased from New England BioLabs or Pharmacia Biotech.

2.5.6 DNA Ligation

Typical DNA ligations were performed using pGEM®-T Vector System (Promega, USA). Purified DNA fragments were incubated with vector DNA (insert molar ratio of 1:1 or 3:1 to vector DNA), 2X rapid ligation buffer (5 µL), T4 DNA ligase (3 Weiss units/ µL; 1 µL) and distilled water (10 µL final volume) overnight at 16°C. Variations to typical DNA ligations are detailed in subsequent chapters.

2.5.7 Polymerase Chain Reaction (PCR)

Standard amplification of DNA was performed using a MultiGene Gradient Thermal Cycler (Labnet International, Inc, USA) with Platinum® Taq DNA polymerase (Invitrogen, USA) using the following conditions: an initial cycle of 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 56°C and 2 min at 72°C, with a final 10 min at 72°C and a holding temperature of 4°C. Variations to the standard thermocycler program or PCR component mixtures are detailed in subsequent chapters.

Table 2.1 Standard components of PCR reaction mixtures used for amplification of DNA with Platinum® Taq DNA polymerase and a final volume of 30 µL.

Components	Volume (µL)	[Final]
dNTPs (10 mM each)	0.6	0.2 mM
$MgCl_2$ (50 mM)	0.9	1.5 mM
10X Buffer[a] minus Mg	3.0	1X
Primer 1 (10 µM)	1.2	0.4 µM
Primer 2 (10 µM)	1.2	0.4 µM
DNA template	3.0	variable

Platinum® Taq DNA polymerase (5U/µL)	0.1 – 0.2	0.5 – 1U
ddH_2O	20	-

[a] 20 mM Tris-HCL (pH8.0), 40 mM NaCl, 2 mM sodium phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50 % (v/v) glycerol

2.5.8 Quantitative Polymerase Chain Reaction (qPCR)

To optimize quantification accuracy template dilution series were prepared. After 4 fold dilution with ddH_2O, 3 µl of diluted template was analysed using the Rotor-Gene™ 6000 (Corbett Life Science, Australia). PCR was performed with 7.5 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corp, Carlsbad, USA) and gene specific primers (200 nM). The standard protocol was 95°C for 2 min, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C with the temperature increasing 1°C every 5 s from 66°C to 95°C in a final melt stage.

2.5.8.1 Quantitative Polymerase Chain Reaction (qPCR) Primer Design

Normalization primers for β-actin, PCNA and 18S rRNA were designed using combinations of MacVector Inc (USA) and DNASTAR Lasergene Primer Select (USA) based on alignments of multiple *Symbiodinium* cDNA sequences obtained from the NCBI GenBank database (www.ncbi.nlm.nih.gov).

Table 2.2 Normalization genes tested in qRT-PCR assays

Gene	Primers	Product size
β-actin	F1: TGG ACA ACG GAA GCG GAA TG B1: GCC AAC AAT GGA TGG GAA AAC T	80 bp
PCNA	F1: GAG TTT CAG AAG ATT TGC CGA GAT B1: ACA TTG CCA CTG CCG AGG TC	113 bp
18S rRNA	F3: GTC TAA CGC AAG GAA GTT TGAG B3: CAG GAC ATC TAA GGG CATC A	57 bp

2.5.9 Purification of PCR Products

Amplified DNA fragments were purified prior to use in downstream applications such as radio-labelled probes for northern and Southern blot analyses or insertion into plasmids with QIAquick PCR Purification Kit (Qiagen, USA) according to the manufacturer's protocol.

2.5.10 Quantification and Purity of Nucleic Acids

Nucleic acid quantification and purity was determined following each total RNA (tRNA) and genomic DNA (gDNA) extraction using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA). The absorbance of tRNA and gDNA solutions was measured at 260 and 280 nm with the ratio of absorbance (A_{260}/A_{280}) providing an estimation of nucleic acid purity. Absorbance ratios of ≥1.8 and ≥1.9 for gDNA and tRNA respectively were considered pure enough to use for Southern blot, northern blot and quantitative RT-PCR applications.

2.5.11 Agarose Gel Electrophoresis

Total RNA (tRNA), genomic DNA (gDNA) and PCR products were analysed using a Bio-Rad sub cell™ electrophoresis system. Ultrapure™ agarose was dissolved in tris-borate-ethylenediaminetetraacetic acid (TBE) (89 mM Tris, 2 mM Na_2EDTA, 89 mM boric acid, pH 8.3) (Appendices Table 15) to a final concentration of 0.9 – 3.0 %, depending on the expect DNA fragment size. GelRed® Nucleic Acid Stain, 10,000X in dimethyl sulfoxide (DMSO) (Biotium Inc, USA) was added (1 µL 10 mL^{-1}) and the agarose mixture cast in casting trays with appropriately sized combs. Samples (typically 10 µL) were mixed with 2 µL of 5x Green GoTaq (Promega, USA) and loaded into separate lanes and separated by electrophoresis (30 – 60 min, 80 – 100 V). The sizes of DNA fragments were estimated based on the migration of a standard 1 kb Plus or 25bp DNA Ladder (Invitrogen, USA).

Total RNA was prepared and analysed using 0.9 – 1.5 % agarose gels containing 2.2 M formaldehyde following the protocol of Sambrook and Russell (2001) (Sambrook and Russell, 2001) and stained with ethidium bromide (200 µg mL^{-1}). The sizes of RNA fragments were estimated based on the migration of a standard 0.5 – 10 Kb RNA Ladder (Invitrogen, USA).

2.5.12 Northern Blot Analysis

Two and one half µg of total RNA was separated on a 1.2 % formaldehyde agarose gels at 50 V for 4 - 6 h and subsequently transferred to Amersham Hybond™-N+ (GE Healthcare Life Science, UK) membranes according to Sambrook and Russell (2001) (Sambrook and Russell, 2001). Membranes were crossed linked for 90 s in a microwave (1100 watts) and prehybridized for 2 – 6 h at 58 – 60°C in Denhardt's buffer (5X SSC, 5X Denhardt's solution, 0.5 % w/v SDS) (Appendices Tables 16 and 17). Probes were labelled using dATP 5' – [α-^{32}P] (PerkenElmer, USA) and Prime-A-Gene® Labelling System (Promega Corporation, Madison WI, USA). After hybridization for 20 h at 58 – 60°C membranes were washed for 5 min in 2x SSC/0.1 % SDS at room temperature, 15 min in 1x SSC/0.1 % SDS at room temperature then 10 min in 1x SSC/0.1 % SDS at 55°C and air-dried on blotting paper. Saran wrapped membranes were exposed to a storage phosphor screen (Molecular Dynamics, GE Healthcare Life Sciences, UK)

for 48 - 96 h and visualized using a PhosphorImager Storm 860 by Molecular Dynamics (GE Healthcare Life Science, UK).

2.5.13 Southern Blot Analysis

Digested gDNA was visualized on a 0.8 % agarose gel run at 80 V for 6 -8 h or overnight at 20 V and subsequently transferred to Amersham Hybond^{TM}-N+ (GE Healthcare Life Science, UK) membranes according to Sambrook and Russell (2001) (Sambrook and Russell, 2001) . Membranes were crossed linked for 70 s in a microwave (1100 watts) and prehybridized for 2 – 3 h at 58 - 60^{o}C in aqueous buffer (6x SSC, 5x Denhardt's reagent, 0.5 % w/v SDS, 100 µg mL^{-1} salmon sperm DNA) (Appendices Tables 16 and 17). The same probes and labelling system used for northern blots was used for Southern blots. After hybridization for 20 – 24 h at 58 - 60^{o}C membranes were washed for 5 min in 2x SSC/0.1 % SDS at room temperature, 15 min in 2x SSC/0.1 % SDS at room temperature then 30 min in 1x SSC/0.1 % SDS at 58^{o}C and air-dried on blotting paper. Saran wrapped membranes were exposed to a storage phosphor screen (Molecular Dynamics, GE Healthcare Life Sciences, UK) for 72 - 96 h and visualized using a PhosphorImager Storm 860 by Molecular Dynamics (GE Healthcare Life Science, UK).

2.5.14 DNA Sequencing

All cDNA and gDNA sequencing was performed at the Australian Genome Research Facility. Resulting nucleotide sequences were trimmed and assembled using Lasergene® v8.0 (DNAStar Inc, USA) and identified based on transcript (blastN) and protein (blastX) similarity searches using NCBI databases. Sequence alignments were constructed in BioEdit Sequence Alignment Editor (Hall, 1999) using ClustalW Multiple Alignment (Thompson et al., 1994) default settings. Manual alignment refinement was performed using Jalview v2.4 (Clamp et al., 2004, Waterhouse et al., 2009).

2.6 General Protein Methods

2.6.1 Protein Extraction

Protein was extracted from *Symbiodinium* cells using Lysing Matrix C tubes (MP Biomedicals Australia) in 200 µL of cold tris-borate (TB) buffer (100 mM Tris-borate pH 8.0, 2 mM $MgCl_2$, 2 mM Na_2EDTA, 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF)) (Appendices Tables 18 and 19) (Owens and Wold, 1986) with 0.1 % v/v Triton® X-100 (Sigma-Aldrich, Australia). Samples containing 10 x 10^6 cells were lysed three times for 40 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia) and centrifuged for 10 min at 8,500 x *g* to remove the pellet and large debris. The supernatant was transferred to a fresh 1.5 µL microcentrifuge tube. Protein concentration was determined using the Bradford Assay with

standard curves and concentrations measured using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA) with the absorbance measured at 595 nm.

2.6.2 SDS-PAGE Analysis

Sodium dodecyl sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) was used to analysis *Symbiodinium* protein extracts under denaturing conditions. Protein samples diluted with 1 – 1.5 (v/v) cracking buffer (0.01 % (w/v) bromophenol blue, 125 mM Tris pH 6.7, 2 % (w/v) (Appendices Table 20) SDS, 10 % (v/v) glycerol, 10 % (v/v) β-mercaptoethanol) and boiled for a minimum of 5 min at 100^{o}C were separated using a Bio-Rad Mini-PROTEAN® Tetra cell (Bio-Rad Laboratories Inc, USA) electrophoresis system in handcast 12 – 18 % polyacrylamide gels (Appendices Table 21). Proteins were separated (50 - 70 min, 150 - 200 V) in SDS-PAGE running buffer (0.1 % SDS, 25 mM Tris, 192 mM glycine) (Appendices Table 22) and visualized by Coomassie Brilliant Blue R-250. Protein molecular masses were estimated using SeeBlue® Plus2 Prestained (Invitrogen, USA) protein standards.

2.6.3 Coomassie Stain

Coomassie Brilliant Blue R-250 was used to stain proteins in SDS-PAGE gels. Gels were incubated in Coomassie stain (50 % methanol, 10 % glacial acetic acid, 0.125 % (w/v) Coomassie Brilliant Blue R-250) (Appendices Table 23) for 2 – 24h then transferred to a destaining solution (10% methanol, 10 % glacial acetic acid) (Appendices Table 24) for a period equal the incubation in Coomassie stain. Alternatively, gels were incubated in Coomassie stain for 45 s in a microwave (1100 watts), transferred to the destaining solution, microwaved for 45 s (1100 watts) and if required destained further for 2 – 12 h at room temperature while gently shaken.

2.6.4 Western Blot-Analysis

Symbiodinium proteins in unstained SDS-PAGE gels were transferred (90 min, 90 V) to Immobilon™-P^{SQ} transfer membranes (Millipore, Australia) in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) (Appendices Table 25) using a Bio-Rad Mini-PROTEAN® Tetra cell (Bio-Rad Laboratories Inc, USA) electrophoresis transfer system. Membranes were incubated and gently shaken in blocking solution PBS-T-BSA (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 0.1 % Tween 20, 1 % bovine serum albumin (BSA)) or TBS-T-BSA (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 % Tween 20, 1 % BSA) for 1 - 4 h then incubated overnight in various dilutions of primary antibody (1:10000; 1:5000) in PBST (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.1 % Tween 20) or TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Tween 20) (Appendices Tables 26 and 27). Membranes were washed (3 x 10 min in PBST or TBST) and incubated (1 - 2 h) in a polyclonal swine anti rabbit secondary antibody, conjugated to horseradish peroxidise (HRP), diluted in PBST or TBST (1:1000;

1:2000). Following washing, proteins were visualized on membranes using FAST™ 3, 3'-Diaminobenzidine (DAB) Tablets (Sigma, USA). One set of DAB tablets dissolved in 5 mL distilled water were used per membrane and membranes immersed in the solution and gently shaken until developed. Reaction development was stopped by rinsing the membrane in tap water. Membranes were air dried away from direct light. Variations to the standard western blot procedure for enhanced chemiluminescence detection are detailed in subsequent chapters.

2.6.5 Preparation of Competent Cells

Chemically competent NM522 *Escherichia coli* cells were prepared using calcium chloride ($CaCl_2$) and a glycerol stock of NM522 *E. coli* cells. Five mL of antibiotic free LB media (Appendices Table 28) in 15 mL falcon tubes were inoculated with NM522 *E. coli* cells and the cultures grown overnight at 37°C while shaking (260 rpm). Cells were transferred to 500 mL conical flasks with sterile LB media and shaken at 37°C for three hours then placed on ice and kept on ice for the remaining preparation steps. Chilled cells were centrifuged for 15 min at 14,000 x *g* (Avanti™ J-20 XP, Beckman Coulter), supernatant discarded and the process repeated until all cells were pelleted. Gently resuspended with 0.1 M $CaCl_2$ and 15 % glycerol, cells were rested for 1 h then centrifuged for 15 min at 14,000 x *g* and $CaCl_2$ / glycerol solution discarded. Aliquots of competent cells (200 µL) were quickly transferred to 1.5 µL centrifuge tubes that had been chilled overnight at -80°C, snap frozen in liquid nitrogen and stored at -80°C.

2.6.6 Transformation of Competent Cells

Plasmids were introduced into NM522 competent cells or One Shot® TOP10 Competent Cells (Invitrogen, USA). An aliquot (200 µL) of competent NM522 cells gently thawed on ice were transformed with 3-5 µL of the ligation mixture on ice and heat shocked at 42°C for 90 s and returned to ice prior to plating. One Shot® TOP10 Competent Cells (Invitrogen, USA) were used according to manufacturer's protocol. Selection for transformants where performed on LB/ampicillin/IPTG/X-Gal plates (Appendices Tables 29 and 30) with 100 µL of cells and incubated overnight at 37°C in sealed bags to prevent excessive drying. Sterile LB aliquots were inoculated with single bacterial colonies containing DNA inserts and the cultures grown overnight at 37°C while shaking (260 rpm). Cells were collected by centrifugation (1 min and 10,000 x *g*) and purified. Plasmids were purified using either UltraClean™ 6 Minute Mini Plasmid Prep Kit (Mo Bio Laboratories Inc), PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen, USA) or NucleoSpin® Plasmid (Macherey-Nagel, Germany) and multiple clones sequenced at the Australian Genome Research Facility.

CHAPTER 3

3 Hyperdiversity of Genes Encoding Integral Light-Harvesting Proteins in the Dinoflagellate *Symbiodinium* sp.

3.1 Statement of Purpose

The superfamily of light-harvesting complex (LHC) proteins is comprised of proteins with diverse functions in light-harvesting and photoprotection. LHC proteins bind chlorophyll (Chl) and carotenoids and include a family of LHCs that bind Chl *a* and *c*. Dinophytes (dinoflagellates) are predominantly Chl *c* binding algal taxa, bind peridinin or fucoxanthin as the primary carotenoid, and can possess a number of LHC subfamilies. Chapter 3 reports 11 LHC sequences for the chlorophyll *a*-chlorophyll c_2-peridinin protein complex (acpPC) subfamily isolated from *Symbiodinium* sp. C3, an ecologically important peridinin binding dinoflagellate taxa. Phylogenetic analysis of these proteins suggests the acpPC subfamily forms at least three clades within the Chl *a/c* binding LHC family; Clade 1 clusters with rhodophyte, cryptophyte and peridinin binding dinoflagellate sequences, Clade 2 with peridinin binding dinoflagellate sequences only and Clades 3 with heterokonts, fucoxanthin and peridinin binding dinoflagellate sequences.

3.2 Introduction

Light-harvesting complexes (LHC) of photosynthetic eukaryotes bind pigments essential for augmenting light capture and photoprotection. The LHC superfamily can be divided into three major groups based on the pigments bound to the protein complexes (Green and Durnford, 1996). The LHCs of green plants, euglenophytes and chlorophytes bind Chl *a* and *b* (Cab; *Lhca* and *Lhcb* genes); a second group containing Chl *a* and *c* (Cac) binding LHCs is found in chromalveolates and includes the fucoxanthin-Chl *a/c* proteins (Fcp; *Lhcf* genes); the third major group contains the Chl *a* and phycobilin binding proteins of cyanobacteria, rhodophytes (LhcaR; *Lhcr* genes) and cryptophytes (Lhc; *Lhcc* genes). Further groups within the LHC superfamily include the LI818 and LI818-like proteins (Gagné and Guertin, 1992) while more recent phylogenetic analysis have identified the Lhcz proteins of cryptophytes, haptophytes and heterokonts (Koziol et al., 2007) and a new family of red lineage chlorophyll *a/b*-like proteins (Engelken et al., 2010).

Plants and chlorophytes have been extensively studied and contain two major Chl *a/b* binding proteins, LHCI and LHCII. These are primarily associated with photosystem I (PSI) and photosystem II (PSII) respectively. The functional unit of the green plant LHCII is a trimer,

each monomer of which possesses three membrane-spanning α-helices with two short amphiphilic α-helices on the lumenal side; one at the C-terminal end (Kühlbrandt et al., 1994) and the second between helices I and II (Liu et al., 2004, Standfuss et al., 2005). In the green plant LHCII model a minimum of 14 Chls (eight Chl *a* and six Chl *b*) and four carotenoids bind to the polypeptide (Liu et al., 2004, Standfuss et al., 2005).

While Chl *a/b*, Chl *a/c*, and Chl *a* phycobilin binding proteins demonstrate considerable sequence divergence, the highly conserved LHCII polypeptide sequences of green plants share homology with LHCI of Chl *a* binding rhodophytes (Tan et al., 1997a, Tan et al., 1997b) and the major Chl *a/c* binding proteins of dinoflagellates (Hiller et al., 1993, Hiller et al., 1995). Given this, rhodophytes and chromalveolates might be expected to display a similar LHC structure to that of the green plant LHCII (Kühlbrandt et al., 1994, Green and Durnford, 1996).

To date, no rhodophyte LHCs associated with PSII have been identified, instead phycobilisomes are used as the major PSII antennae (Wolfe et al., 1994, Tan et al., 1995). Rhodophyte LHCI (Tan et al., 1997a, Tan et al., 1997b) as well as chromophyte and dinoflagellate LHCs (Grossman et al., 1990, Hiller et al., 1993, Green and Pichersky, 1994) contain the Chl-binding and stabilization residues identified in green plant LHCII and possess three membrane spanning helices (Green and Pichersky, 1994) but do not contain the short amphiphilic α-helix on the lumenal side identified by Kühlbrandt, Wang, and Fujiyoshi (Kühlbrandt et al., 1994) in green plants. The greatest variation between Chl *a/b*, Chl *a/c* and Chl *a* binding proteins is evident in helix II and the interhelical connectors; for example, in green plants seven residues separate the two Chl-binding residues (*Q* and *E*) in helix II (Kühlbrandt et al., 1994), while eight amino acids separate the binding residues in the Chl *a/c* proteins and those that bind phycobilin (Tan et al., 1997b).

The Chl *a/c* containing dinoflagellates are an ecologically important group of unicellular eukaryotes found in fresh and marine waters as free-living organisms, or in symbiosis with a variety of marine invertebrates and protists (Yellowlees et al., 2008). Studies of photoautotrophic dinoflagellates provide unique insights into plastid acquisition as they have undergone multiple chloroplastic acquisitions and subsequent gene transfer and genome reorganization, and have evolved unique protein import pathways for chloroplast-targeted proteins (Bachvaroff et al., 2004, Patron et al., 2005). Dinoflagellate chloroplasts are similar to those in Euglenophyta in that they are surrounded by at least three membranes. This multi-membrane arrangement has necessitated novel strategies for the importation of proteins into the chloroplast. For example, *Euglena gracilis* LHCs are initially expressed in the cytosol as polyproteins containing multiple LHC polypeptides. These are transported to the chloroplast where specific proteases cleave the polypeptide into individual mature LHC polypeptides (Houlné and Schantz, 1988, Koziol and Durnford, 2008). *E. gracilis* LHC polyproteins contain either nearly identical repeating polypeptides, pairs of divergent polypeptides, or complex

divergent polypeptides (Koziol and Durnford, 2008). Each LHC polypeptide is separated from the next by a decapeptide linker that contains the protease cleavage site (Koziol and Durnford, 2008). Similarly, the dinoflagellate *Amphidinium carterae* encodes integral peridinin light-harvesting proteins as polyproteins containing up to 10 different but closely related polypeptides (Hiller et al., 1995). Circumstantial evidence also exists for other nuclear encoded chloroplast proteins being synthesized as polyproteins, for example the nuclear encoded form II RuBisCO in *Symbiodinium* (Rowan et al., 1996). In addition, a subset of dinoflagellate nuclear genes can be arranged in tandem repeats and expressed on a single polycistronic mRNA, with a 5' splice leader (SL) sequence added by *trans*-splicing (Zhang et al., 2007, Slamovits and Keeling, 2008, Bachvaroff and Place, 2008). The process of SL *trans*-splicing converts polycistronic messages into individual monocistronic mRNAs before translation (Zhang et al., 2007) and in dinoflagellates polycistronic mRNA appears to contain repeated coding sequences for the same gene (Bachvaroff and Place, 2008). In euglenophytes and dinoflagellates the import of nuclear encoded proteins into the chloroplast requires mechanisms to navigate the three or more membranes surrounding the plastid (Kishore et al., 1993, Nassoury et al., 2003).

One group of dinoflagellates of particular ecological importance are those belonging to the genus *Symbiodinium*. These are symbiotic with reef-building corals and a variety of other marine invertebrates and encode LHCs that bind Chl *a/c* and peridinin as the primary carotenoid. Peridinin is unique to dinoflagellates (Larkum, 1996) and is associated with two unrelated LHCs in *Symbiodinium*; the peridinin-Chl *a* protein complexes (PCP) located on the periphery of the thylakoid membranes, and the integral Chl *a*-Chl c_2-peridinin protein complex (acpPC; previously ACP or *i*PCP; proposed *Lhcd* genes (Jansson et al., 1999)). PCP is a soluble protein complex with no sequence similarity to other known LHCs (Norris and Miller, 1994). There is a considerable body of literature on PCP (Haidak et al., 1966, Prézelin, 1976, Chang and Trench, 1982, Roman et al., 1988, Govind et al., 1990, Iglesias-Prieto et al., 1991, Hiller et al., 2001, Reichman et al., 2003, Jiang et al., 2012) and the x-ray structure has been determined (Hofmann et al., 1996). In comparison, the integral protein complex acpPC does have sequence similarity with known LHCs and is part of the Chl *a/c* subfamily of LHCs (Hiller et al., 1993).

Chapter 3 reports a number of LHC sequences from the dinoflagellate *Symbiodinium* sp. (C3 *sensu* (LaJeunesse et al., 2003)). Phylogenetic analysis and structural comparison of a putative acpPC subfamily of genes from *Symbiodinium* demonstrates the diversity of LHC proteins in this organism. Interestingly, *Symbiodinium* sequences similar to those of the Chl *a* binding rhodophytes and Fcp binding chromophytes are evident within the one organism, although none group with the LI818 or LI818-like sequences. In addition, the results demonstrate a subset of *Symbiodinium* cDNA sequences encoding polypeptides suggesting *Symbiodinium*, like *A. carterae* and *E. gracilis,* either translates LHC genes as polyproteins or alternatively, repeats of specific LHC genes are expressed on a single polycistronic mRNA transcript.

3.3 Materials and Methods

3.3.1 EST Sequencing

Partial dinoflagellate gene sequences were obtained from an Expressed Sequence Tag (EST) library of *Symbiodinium* sp. C3 (Leggat et al., 2007). Specific primers (Sigma Genosys, Australia) were designed to putative acpPC sequences and DNA amplification was performed with Platinum® Taq DNA polymerase (Invitrogen, USA) as per Section 2.5.7. Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, USA) prior to ligation into pGEM-T Vector System (Promega, USA) and used to transform One Shot® TOP10 Competent Cells (Invitrogen, USA). Cells were grown, collected and plasmid purified according to Section 2.6.6.

Direct sequencing of EST library glycerol stocks of single bacterial colonies containing acpPC sequence inserts was also performed to confirm full insert coverage. Glycerol stocks were streaked onto LB agar with ampicillin and grown following the procedure outlined above. All clones were sequenced at the Australian Genome Research Facility and resulting nucleotide sequences trimmed and assembled using Lasergene® v8.0 (DNAStar Inc, Madison, USA). *Symbiodinium* acpPC sequences were identified based on transcript (blastN) and protein (blastX) similarity searches using NCBI databases and gene specific primers designed to the resulting consensus sequences using DNAStar Lasergene® v8.0 software PrimerSelect (USA).

3.3.2 5' Sequencing

To obtain 5' regions for the acpPC sequences amplified from the C3 EST library a 22 nucleotide 5' *trans*-spliced leader (SL) sequence (Zhang et al., 2007, Slamovits and Keeling, 2008) was used in combination with multiple gene specific primers and cDNA template transcribed from freshly extracted *Symbiodinium* sp. C3 RNA. RNA extraction and cDNA synthesis was performed on *Symbiodinium* as per Sections 2.5.1 and 2.5.3. *Symbiodinium* were removed from *Acropora aspera* branches collected from the reef flat at Heron Island (Great Barrier Reef ($23^{o}33$'S, $151^{o}54$'E) in April 2009 and frozen in liquid nitrogen.

Sequences were confirmed by amplification using high fidelity AccuPrime™ *Pfx* SuperMix (Invitrogen, USA) and 200 nM final concentration of each primer. The final 25 µL reaction mixture was amplified on a MultiGene Thermal Cycler (Labnet International Inc, NJ, USA) using the following conditions: an initial cycle of 5 min at 95 ^{0}C, followed by 35 cycles of 15 s at 95 ^{0}C, 30 s at 60 ^{0}C and 90 s at 68 ^{0}C, finally 10 min at 72 ^{0}C and a holding temperature of 4 ^{0}C. Zero Blunt® TOPO® PCR Cloning Kit for sequencing was used to insert amplified blunt-end products into the plasmid vector pCR®4Blunt-TOPO® and transformed into One Shot® TOP10 Cells (Invitrogen, USA) according to manufacturer's protocol. A second set of sequences were obtained following template purification using QIAquick PCR Purification Kit (Qiagen, USA), addition of dATP overhangs, ligation into pGEM-T Vector and transformation into NM522 cells. A third and final set of sequences obtained following template amplification with

Promega GoTaq® Flexi DNA polymerase (Promega Corporation, Madison, WI, USA) using the following conditions: an initial cycle of 2 min at 95 ^{0}C, followed by 35 cycles of 30 s at 95 ^{0}C, 30 s at 60 ^{0}C and 2 min at 72 ^{0}C, finally 10 min at 72 ^{0}C and a holding temperature of 4 ^{0}C. Purified PCR product (QIAquick PCR Purification Kit, Qiagen, USA) was directly ligated into pGEM-T Vector and transformed into NM522 cells. Each sequencing method either provided new sequence data or confirmed data already obtained. Following plasmid purification (PureLink™ HQ Mini Plasmid Purification Kit, Invitrogen, USA) multiple clones for each gene were sequenced at the Australian Genome Research Facility to confirm sequencing results.

3.3.3 Northern Blot Analysis for Symbiodinium acpPC

Transcript size of five different acpPC genes (acpPCSym_1, _4, _8, _13 and _15) was determined using northern blot analysis and the procedure in Section 2.5.12. Total RNA from *Symbiodinium* spp. C3 and C1 were extracted from 100 mg of crushed *A. aspera* skeleton (C3 cells) or 6 – 10 x 10^6 cultured C1 cells using a RNeasy Plant Mini Kit (Qiagen, USA). Cultured C1 *Symbiodinium* (CCMP 2466, USA) were lysed according to the protocol in Section 2.5.1.2 and RNA precipitations performed as per Section 2.5.2.

3.3.4 Phylogenetic Analysis

LHC sequences for 35 species belonging to 33 genera of green plants, chlorophytes, chlorarachniophytes, prasinophytes, heterokontophytes, a rhodophyte, euglenophyte, cryptophyte, haptophyte and dinophytes (Table 3.1) were aligned with acpPC sequences from *Symbiodinium* sp. C3. *Symbiodinium* sequences encoding multiple copies of the membrane spanning helices (acpPCSym_1, _5, _8, _10, _12, _13 and _17) were divided into individual units comprised of helices I and II, and helices III and IV when present. Alignments of protein sequences were constructed in BioEdit Sequence Alignment Editor (Hall, 1999) using ClustalW Multiple Alignment (Thompson et al., 1994) using default settings. Alignment refinement was manually performed using Jalview v2.4 (Clamp et al., 2004) based on the highly conserved Chl-binding residues and stroma localized motifs proposed by Kühlbrandt, Wang, and Fujiyoshi (Kühlbrandt et al., 1994) and Tan et al. (Tan et al., 1997b) as guides to helix location. Regions of high divergence were excluded from the analysis because of the ambiguity required to align these regions with only the first three helices, and short α-helix when present, used in analysis to maximize position homology. Phylogenetic analysis was performed using PhyML for maximum likelihood (Guindon and Gascuel, 2003) and MrBayes for Bayesian inference (Huelsenbeck and Ronquist, 2001). Using ProtTest v2.1 (Abascal et al., 2005), the LG + Alpha + Proportion Invariant + Frequencies (LG+I+G+F) model of protein evolution was determined as the best fit model for the final alignment data. LG (Le and Gascuel, 2008), a relatively new amino acid replacement matrix, is not a candidate model to determine Bayesian inference thus the WAG

matrix (Whelan and Goldman, 2001) was selected for MrBayes analysis. The most likely topology was calculated based on Sh-like branch support. For the Bayesian inference, two runs with 10 million generations each were calculated with topologies saved at each 1,000 generations. One fourth of the 10,000 topologies were discarded as burnin, and the remaining used to calculate the posterior probability.

Table 3.1 Light harvesting complex sequences from GenBank, NCBI, Swiss-Prot, PIR and EST databases

Molecular Database	Organism and Sequence	Accession Number
GenBank	*Solanum lycopersicum* Lhca1, Lhca2, Lhca3	AAA34140, CAA32197, CAA33330
	Solanum lyocpersicum Lhcb1*1, Lhcb5, Lhcb6a	AAA34147, CAA43590, AAA34143
	Polystichum munitum Lhcb	AAA68425
	Chlamydomonas moewusii Lhcb	CAA38635
	Zea mays Lhcb4	CAA90681
	Pinus sylvestris Lhcb1*2	CAA32658
	Pisum sativum Lhcb1	CAA40365
	Lemna gibba Lhcb2	AAA33396
	Chlamydomonas reinhardtii Lhcb	AAO16493
	Dunaliella salina Lhcb	AAA33278
	Arabidopsis thaliana Lhcb4	CAA50712
	Volvox carteri Lhca	AAB40979
	Chlamydobotrys stellata Lhca	CAA50763
	Chlamydomonas reinhardtii Lhca I20	CAA46235
	Mantoniella squamata Lhc	CAA49271
	Euglena gracilis Lhca1, Lhca2, Lhca5	DAA05888, DAA05887, ABW06947
	Guillardia theta Lhc10, Lhc13	CAM33413, AAF81522
	Phaedactylum tricornutum FcpB	CAA80897
	Porphyridium cruentum Lhca1, Lhca2	AAB39488, AAB39489
	Laminaria saccharina Fcp	AAG13008
	Saccharina japonica Fcp	ACE80197
	Heterosigma carterae Fcp1	CAA68028
	Macrocystis pyrifera Fcp3	AAC49017
	Pyrocystis lunula Cac	AF508261
	Karlodinium micrum Cac1, Cac2	ABI14390, ABA55565
	Heterocapsa triquetra Cac1, Cac2, Cac3, Cac5:2	AAW79361, AAW79364, AAW79365, AAW79366
	Amphidinium carterae Cac	CAA08771
	Bigelowiella natans LI818, LI818*1	DAA05890, AAP79202
	Mesostigma viride LI818	DAA05932
	Micromonas pusilla LI818	EEH60415
	Gymnochlora stellate LI818	ACF24539
NCBI	*Thalassiosira pseudonana* LI818, LI818*1	XP_002295258, XP_002287075
	Zea mays Lhcb6, Lhcb5	NP_001105375, NP_001105698
Swiss-Prot	*Odontella sinensis* Fcp	Q42395
	Isochrysis galbana Fcp	Q39709
	Dunaliella tertiolecta Lhcb1	P27517
PIR	*Giraudyopsis stellifer* Cac	S60048
EST	*Symbiodinium* sp. KB8 A1.1_155, _467	FE539302, FE539303, FE538572

Hydrophobicity plots generated with DNASTAR Lasergene® v8.0 Protean program (USA), using a Kyte-Doolittle scale of 20 residues, in conjunction with the hidden Markov model-based program HMM (Krogh et al., 2001), PolyPhobius (Käll et al., 2005), ChloroP (Emanuelsson et al., 2000) and NetPhos (Blom et al., 1990) were used to identify potential membrane-spanning

regions, signal peptides, chloroplast transit peptides and phosphorylation sites in acpPC sequences. Sequence logos of helices I - IV were generated using WebLogo (Crooks et al., 2004) from the sequence data used to generate phylogenetic analysis.

3.3.5 Accession Numbers

Sequence data for this chapter can be found in the EMBL/GenBank data libraries under the accession numbers FN646412-FN646425.

3.4 Results

3.4.1 Phylogenetic Analysis

Expressed sequence tags (ESTs) for *Symbiodinium* C3 isolated from *Acropora aspera* (Leggat et al., 2007) were used to obtain partial sequences for putative acpPC genes. ESTs for 33 clones were sequenced and assembled based on a minimum match percentage of 98 %. High fidelity amplification of the resulting 14 acpPC sequences provided 5' regions for nine, with the remaining five sequences extended but lacking full sequence coverage (Table 3.2). These sequences were named acpPCSym_1, 3-5, 8-15, 17 and 18. *Symbiodinium* acpPC sequences, 54 LHC protein sequences for green plants, chlorophytes, chlorarachniophytes, prasinophytes, heterokontophytes, a euglenophyte, rhodophyte, cryptophyte, haptophyte and dinophytes including EST sequence data for *Symbiodinium* genetic clade A1.1 (Voolstra et al., 2009) were aligned and the membrane-spanning sequences used for phylogenetic analysis (Figure 3.1).

Table 3.2 *Symbiodinium* sp. C3 chlorophyll *a*-chlorophyll c_2-peridinin characteristics

Clade C3 *Symbiodinium* Integral LHC Name	Complete Sequence Coverage	cDNA Size (bp)	Protein Size (aa) Protein Mass (kDa)	Polypeptide Number[a] Polypeptide Size - Mass (aa - kDa)	SPLR Motif	Predicted Chloroplast Transit Peptide/Signal Peptide/Trans-membrane Helices	Membrane Spanning Helix Organization
acpPCSym_15	yes	932 bp	276 aa 28842 kDa	1	no	no / no / yes	H1-H2[b]-H3
acpPCSym_18	no	785 bp	231 aa 24007 kDa	1	no	yes / no / yes	H1-H2[b]-H3
acpPCSym_1	yes	1376 bp	440 aa 45941 kDa	1	no	yes / yes / yes	H1-H3-H1-H3
acpPCSym_4	yes	1131 bp	292 aa 28979 kDa	1	no	yes / yes / yes	H1-H2[b]-H3
acpPCSym_5	no	1836 bp	592 aa 65482 kDa	1	no	no / no / yes	H1-H2[b]-H3-H1-H2-H3-H1-H2-H3
acpPCSym_8	yes	1403 bp	433 aa 44849 kDa	2 173 aa - 18357 kDa	yes x2	yes / yes / yes	H1-H2-H3-H1-H2-H3
acpPCSym_10	yes	1520 bp	468 aa 49364 kDa	2 173 aa - 18451 kDa	yes x2	yes / no / yes	H1-H2-H3-H1-H2-H3
acpPCSym_11	yes	888 bp	259 aa 26742 kDa	1 173 aa - 18424 kDa	yes x1	yes / yes / no	H1-H2-H3
acpPCSym_12	yes	1371 bp	441 aa 45922 kDa	2 173 aa - 18439 kDa 173 aa - 18496 kDa	yes x2	yes / yes / yes	H1-H2-H3-H1-H2-H3
acpPCSym_13	yes	2097 bp	681 aa 73911 kDa	3 192 aa - 21273 kDa 178 aa - 19587 kDa 210 aa - 22949 kDa	yes x2	yes / yes / yes	H1-H2[b]-H3-H1-H2-H3-H1-H2-H3
acpPCSym_17	yes	1545 bp	494 aa 53819 kDa	1	no	yes / yes / yes	H1-H2-H3-H4-H1-H2[b]-H3
acpPCSym_3[c]	no	844 bp	273 aa 29889 kDa	2 gene incomplete	yes x2	no / no / yes	H3-H1-H2-H3
acpPCSym_9[c]	no	1039 bp	319 aa 35093 kDa	1	no	no / no / yes	H3-H2-H3-H1
acpPCSym_14[c]	no	1570 bp	523 aa 56719 kDa	2 gene incomplete	no	no / no / yes	H2-H3-H1-H2-H3-H1-H3

[a] Sequences marked as containing one polypeptide are monomeric proteins while acpPCSym_8, _10, and _12 are bipartite polypeptides. The sequence for acpPCSym_13 encodes a tripartite polypeptide and the polypeptide at the 3' end lacks the C-terminal SPLR motif present in the first two polypeptides.

[b] Variation to Chl-binding residues in helix II from residues (*Q* and *E*) identified in the green plant LHCII three transmembrane and amphiphilic α-helix protein present.

[c] Sequences were not included in phylogenetic analysis.

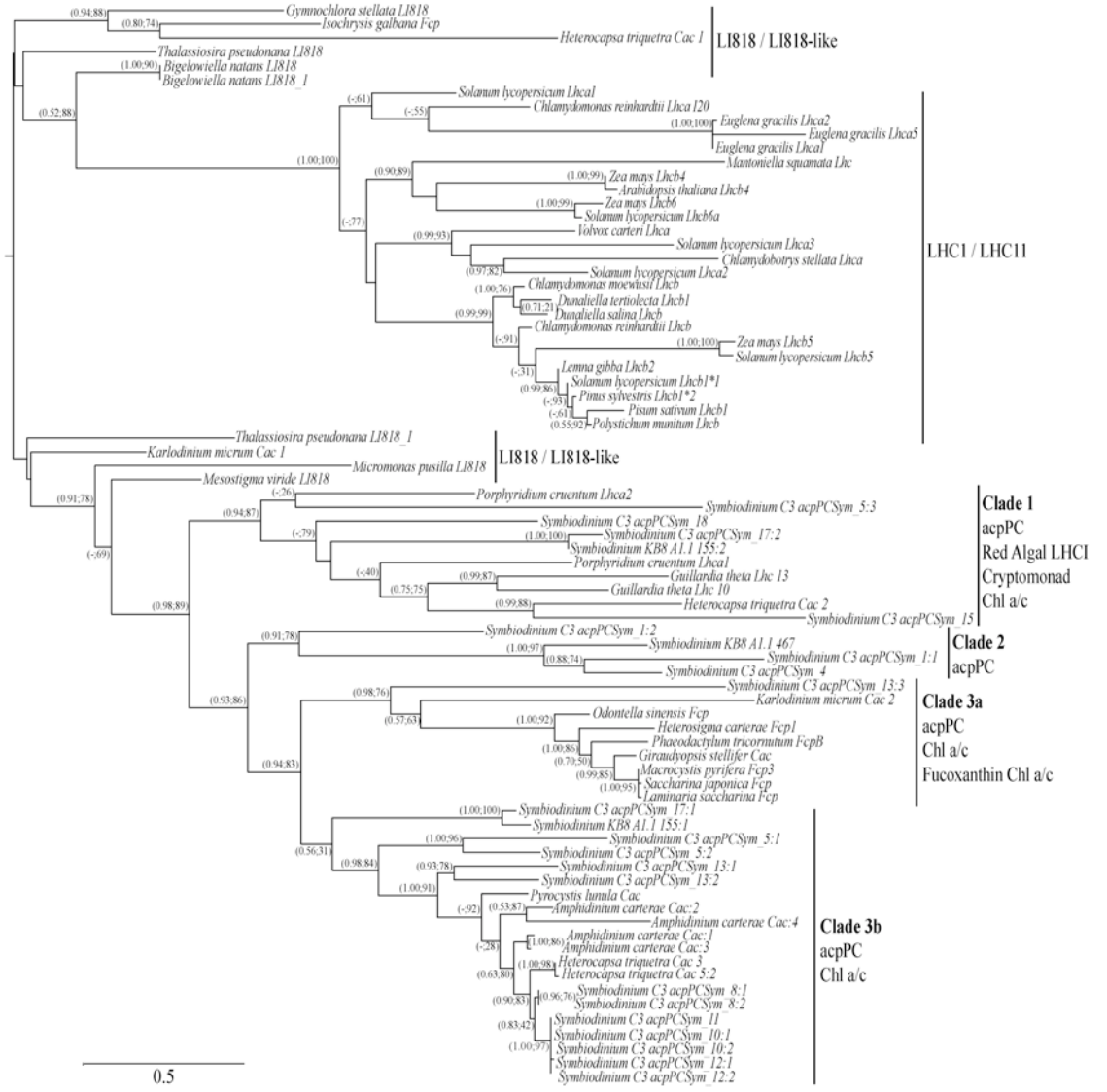


Figure 3.1 Phylogenetic analysis of *Symbiodinium* sp. C3 acpPC sequences with LHCs from green plants, chlorophytes, chlorarachniophytes, prasinophytes, heterokontophytes, a rhodophyte, euglenophyte, cryptophyte, haptophyte and dinophytes. A maximum likelihood tree rooted at the mid-point is shown with the support values for MrBayes (posterior probability) followed by PHYML included at the nodes. A total of 78 sequences with 97 amino acid sites (including gaps) were analysed. The Chl *a/b* binding protein complexes associated with PSI (*Lhca*) and PSII (*Lhcb*) in green plants, chlorophytes and euglenophytes cluster together while the Chl *a/c* binding protein complexes form a second cluster which includes the Fcp sequences of heterokontophytes, *Lhca*s of a rhodophyte and LHCs of a cryptophyte. The LI818 and LI818-like sequences group separately but include a haptophyte Fcp sequence and two dinophyte Chl *a/c* sequences. Within the Chl *a/c* cluster are three distinct clades: Clade 1 containing four *Symbiodinium* sp. C3 acpPC sequences and LHCs from a rhodophyte,

cryptophyte, and two peridinin-binding dinophytes, *Heterocapsa triquetra* and *Symbiodinium* type A1.1; Clade 2 contains *Symbiodinium* sequences; Clade 3 is divided into two clusters, a Chl *a/c* and Fcp cluster (3a), and a *Symbiodinium* sp. C3 acpPC and Chl *a/c* cluster (3b). Sequence data obtained from multiple databases is available in Table 3.1.

Symbiodinium acpPC sequences encoding repeats of the three transmembrane helices in single LHC protein were treated as multiple sequences and numbered individually using a single colon to separate the sequence name from the transmembrane helices group in order from the 5' end (for example, acpPCSym_5:1, _5:2 and _5:3, each contain helices I, II and III) (Figures 3.1 and 3.2a). These results demonstrate *Symbiodinium* sp. C3 encode a diversity of integral LHC proteins ranging in size from 18 - 65 kDa and importantly, LHC sequences similar to those of the Chl *a* binding rhodophytes and Fcp binding heterokontophytes are evident within this one species of dinoflagellate (Figure 3.1).

Phylogenetic analysis of the sequences for the LHC membrane-spanning helices demonstrates the distinct separation of Chl *a/b* and Chl *a/c* binding protein lineages (Figure 3.1). The Chl *a/b* lineage comprises LHCII and LHCI sequences from green plants, chlorophytes, a prasinophyte and euglenophyte. The Chl *a/c* lineage includes Chl *a/c* and Fcp binding proteins from heterokontophytes and dinophytes, a rhodophyte and cryptophyte and the putative dinoflagellate acpPC family of sequences. This separation of LHC proteins into two distinct lineages corresponds with the presence or absence of chlorophyll *b* in light harvesting and has previously been well documented (Green and Pichersky, 1994, Durnford et al., 1999).

Within the lineage of Chl *a/c* binding proteins, three distinct clades can be found (Figure 3.1). Clade 1 includes the rhodophyte and cryptophyte sequences, two dinoflagellate Chl *a/c* sequences and four acpPC sequences (acpPCSym_5:3, _17:2, _15 and _18). Clade 2, contains three *Symbiodinium* sequences. acpPCSym_1 and _4 sequences encode monomers of 46 kDa and 29 kDa respectively with chloroplastic leader sequences and the N-terminal four-residue phenylalanine based motif features in the transit peptide.

The final cluster within the Chl *a/c* lineage, Clade 3, comprises two clusters; Clade 3a includes heterokontophyte Fcp sequences, Chl *a/c* sequences and one acpPC sequence, while Clade 3b is a dinoflagellate specific clade and contains sequences from four genera of dinoflagellate, including twelve acpPC sequences. Each dinoflagellate genera in Clade 3b encode LHCs with a C-terminal SPLR motif after helix III (Figure 3.3b). In *A. carterae* the Chl *a/c* polyprotein contains a protease cleavage site at the C-terminal arginine residue of the SPLR motif which generates at least five separate mature LHC polypeptides (Hiller et al., 1995). Four of the *Symbiodinium* acpPC sequences are potentially polyproteins containing multiple LHC polypeptides and C-terminal SPLR motifs (acpPCSym_8, _10, _12, _13). Three acpPC sequences acpPCSym_8, _10, and _12 encode bipartite polypeptides each of 18.4 kDa while

acpPCSym_13 encodes tripartite polypeptides of 21 kDa, 19.5 kDa and 23 kDa (Table 3.2). The two 5' end polypeptides in acpPCSym_13 (_13:1 and _13:2) contain the C-terminal SPLR motif while the third (_13:3) does not. A fifth sequence (acpPCSym_11) contains the C-terminal SPLR motif, but encodes only one LHC polypeptide of 18.4 kDa. This monomer has high identity at the protein level (93-95 %) and cDNA level (79-98 %) with polypeptides from acpPCSym_8, _10 and _12 (Table 3.3). In comparison, the two polypeptides containing SPLR within acpPCSym_13 have only 57 % identity at the protein level with acpPCSym_11, and polypeptide three is only 23 % (Table 3.3).

Two transcripts (acpPCSym_5 and _17) do not contain the SPLR motif but encode multiple copies of the three transmembrane helices found in single LHC proteins. The transcript for acpPCSym_5 encodes a 65.5 kDa protein with nine membrane-spanning regions, but lacks full coverage in the 5' region. The complete sequence for acpPCSym_17 encodes a 54 kDa protein with seven membrane-spanning regions and includes a chloroplastic leader sequence (Table 3.2) containing a transit peptide with a N-terminal four-residue phenylalanine based motif (Patron et al., 2005). The lack of a canonical C-terminal SPLR like motif in acpPCSym_5 and _17 suggests that the mature protein is either a monomer or has a proteolytic cleavage site different from the *A. carterae*, *Pyrocystis lunula*, *Heterocapsa triquetra* and acpPC sequences containing SPLR.

Table 3.3 Comparison of the cDNA and amino acid sequences in the *Symbiodinium* sp. C3 chlorophyll *a*-chlorophyll c_2-peridinin light harvesting complex[a]

LHC Name	acpPC Sym_ 11	acpPC Sym_ 8:1	acpPC Sym_ 8:2	acpPC Sym_ 10:1	acpPC Sym_ 10:2	acpPC Sym_ 12:1	acpPC Sym_ 12:2	acpPC Sym_ 13:1	acpPC Sym_ 13:2	acpPC Sym_ 13:3
acpPCSym	23	24	24	23	23	23	23	21	23	100
_13:3	23	36	36	37	37	38	38	36	35	100
acpPCSym	56	57	57	57	57	57	55	54	100	
_13:2	37	58	57	59	58	58	57	57	100	
acpPCSym	57	55	55	57	57	57	56	100		
_13:1	36	58	57	58	58	58	58	100		
acpPCSym	97	90	91	95	95	97	100			
_12:2	96	79	80	94	92	96	100			
acpPCSym	99	92	93	97	97	100				
_12:1	98	79	80	93	92	100				
acpPCSym	97	93	94	100	100					
_10:2	93	79	79	97	100					
acpPCSym	97	93	94	100						
_10:1	95	80	80	100						
acpPCSym	93	99	100							
_8:2	80	97	100							
acpPCSym	93	100								
_8:1	79	100								
acpPCSym	100									
_11	100									

[a] Comparisons are made at the percent identity level. The upper number refers to protein identity while the percent identity in the cDNA is beneath. acpPCSym_11 encodes a monomeric protein, acpPCSym_8, _10 and _12 encode bipartite polypeptides. Individual polypeptides in the polyproteins are numerically identified for example acpPCSym_13:1 is the polypeptide at the N-terminal end of the tripartite polyprotein. Each polypeptide has the C-terminus motif SPLR with the exception of acpPCSym_13:3.

3.4.2 Transmembrane Topology

Similar to rhodophyte, cryptophyte and heterokontophyte LHCs, acpPC sequences generally contain three transmembrane helices (Helix I, Helix II and Helix III; Figure 3.2a). In comparison, green plant and chlorophyte LHCs generally contain three transmembrane helices and a fourth amphiphilic α-helix. In *Symbiodinium* sp. C3 acpPC sequences the structural features and Chl-binding residues identified in the green plant LHCII model are generally present (Figures 3.2a and 3.2b). Twelve of the acpPC sequences code for eight amino acid residues between the *Q* and *E* Chl-binding residues in helix II while six lack one of these

residues (Table 3.2 and Figure 3.2a). Five proteins have a non-conservative substitution of the *Q* residue (Figure 3.3a), similar non-conservative substitutions can be found in the dinoflagellates *Symbiodinium* KB8 A1.1 and *Karlodinium micrum* and the green alga *Chlamydobotrys stellata*, while acpPCSym_13 has a conservative substitution in the second Chl-binding residue with *D* replacing *E* (Figure 3.3b). The conserved residue substitution in acpPCSym_13 is only evident in the first group of membrane-spanning helices (acpPCSym_13:1), the second (_13:2) and third (_13:3) group contain *Q* and *E* separated by eight residues. A similar substitution pattern is evident in the acpPCSym_5 monomer (Table 3.2).

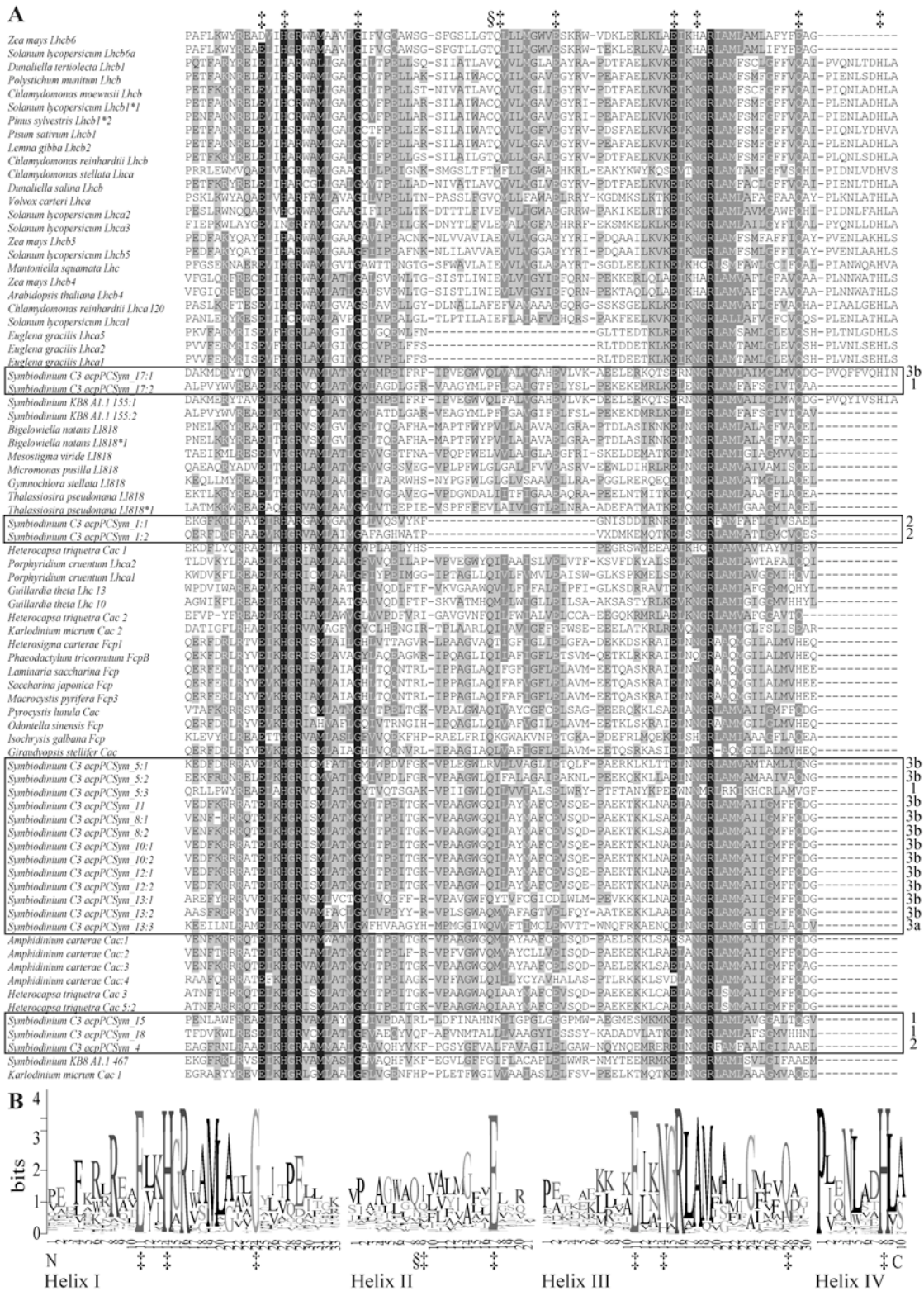


Figure 3.2 Alignment of *Symbiodinium* sp. C3 LHC with other LHC representatives. (a) Amino acid alignment of membrane-spanning helices for 78 sequences used to determine *Symbiodinium* sp. C3 integral LHC phylogeny. Black shading represents 99 % conservation, dark grey 70 % and light grey 35 %. *Symbiodinium* sp. C3 acpPC sequences are boxed and the side numbering (1, 2, 3a and 3b) refers to the three distinct clades within the Chl *a/c* binding

cluster. Clade 1 acpPC sequences cluster with a rhodophyte, cryptophyte, and dinophytes possessing three transmembrane helices. Clade 2 is a dinophyte cluster and includes acpPCSym_1 which lacks helix II. Clade 3a is a Chl *a/c* and Fcp cluster while Clade 3b is a second cluster of dinophytes possessing three transmembrane helices with the exception of acpPCSym_17, which has a fourth amphiphilic α-helix. (b) Amino acid sequence logo plot of LHC membrane-spanning helices I – IV. Position 1 denotes the start of a helix and the higher the bits score the greater the consensus across the data. In both the alignment and logo plot a manually inserted gap separates each helix and the nine proposed Chl-binding residues, identified in the green plant LHCII model (Kühlbrandt et al., 1994), are listed and marked (‡): Helix I: E-11, H-14, G-24; Helix II: Q-8, Q-9 E-17; Helix III: E-11, N-14, Q-28; Helix IV: H-8. In helix II, *Q* at position 8 (§) and 9 (‡) demonstrates the varied spacing between the two Chl-binding residues, with Chl *a/b* binding proteins separated by seven residues and Chl *a/c* binding proteins separated by eight. Two arginine residues (Helix I: R-16; Helix III: R-16) are proposed to play a stabilisation role in helices I and III.

In addition to variations of specific Chl-binding residues there are examples of significant divergence in the secondary structure of *Symbiodinium* LHCs. acpPCSym_1 is noticeably different from the other acpPC sequences in that it lacks helix II (Table 3.2 and Figure 3.2a) and encodes membrane-spanning helices (acpPCSym_1:1, _1:2) with the highly conserved helices I and III repeated. The absence of helix II sequence also occurs in *H. triquetra* Cac_1 that clusters with LI818 and LI818-like genes (Figures 3.1 and 3.2a), and in some *E. gracilis* membrane-spanning helices (Figure 3.2a). Within Clade 3b (Figure 3.1), acpPCSym_17:1 exhibits the canonical structure of a three transmembrane helix protein with an amphiphilic α-helix (Figures 3.2a and 3.3c) common amongst Chl *a/b* lineage LHCs rather than only three transmembrane helix protein common within the Chl *a/c* binding lineage. The presence of helix IV is not evident in proteins within the Chl *a/c* lineage with the exception of *Symbiodinium* KB8 A1.1_155:1.

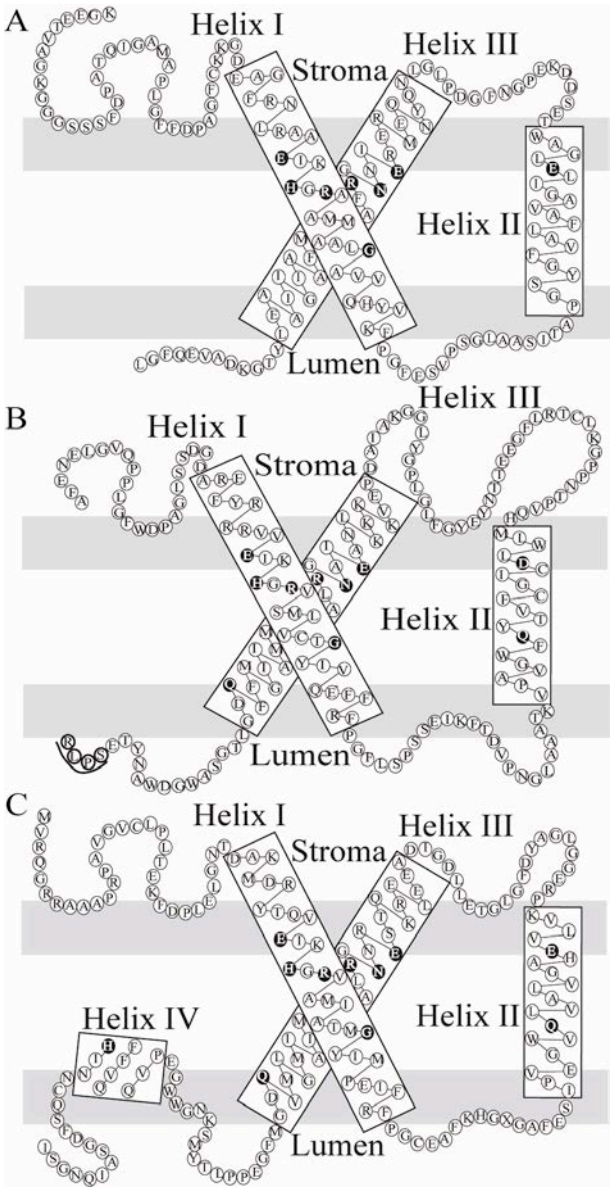


Figure 3.3 Schematic representations of the different *Symbiodinium* sp. C3 transmembrane domains. (a) Schematic of the three transmembrane polypeptide encoded by acpPCSym_4 in *Symbiodinium* sp. C3. acpPCSym_4 clusters with dinophyte LHCs and helix II and helix III contain a variation to one Chl-binding residue proposed in the Kühlbrandt, Wang, and Fujiyoshi (Kühlbrandt et al., 1994) model for green plants. (b) Schematic of the three transmembrane polypeptide acpPCSym_13:1 in *Symbiodinium* sp. C3. acpPCSym_13:1 clusters with Chl *a/c* LHCs and encodes a proposed proteolytic cleavage site for mature polypeptides (SPLR) on the lumenal side and C-terminal to helix III. (c) Schematic of the three transmembrane and fourth amphiphilic α-helix polypeptide encoded by acpPCSym_17:1 in *Symbiodinium* sp. C3. acpPCSym_17:1 cluster with Chl *a/c* LHCs, includes the short amphiphilic α-helix (helix IV), however helix II has eight amino acid residues between the two Chl-binding residues rather than the seven evident in helix II of green plants. acpPCSym_17:2 encodes a second set of helices I, II and III but not helix IV. Schematics are based on the LHCII

model of Kühlbrandt, Wang, and Fujiyoshi (Kühlbrandt et al., 1994) and residues identified as Chl-binding or involved in helix stabilisation are shown with a black background.

3.4.3 Polycistronic Transcript Variations

Symbiodinium sp. C3 encodes transcripts containing one, two or three LHC polypeptides. To determine if larger transcripts are present in *Symbiodinium*, LHC transcript size was examined by northern blot analysis on RNA from freshly isolated *Symbiodinium* sp. C3 (Figure 3.4) and the closely related cultured *Symbiodinium* sp. C1 (Figure 3.5). Probes used represented the diversity of LHC transcripts including the Chl *a/c* and Fcp-like acpPCSym_8 and _13 (Clade 3b), which contain multiple polypeptides, and acpPCSym_1, _4 (Clade 2) and _15 (Clade 1), which represent the LHC group with transcripts containing monomers. The probes hybridized strongly with cultured C1 RNA but there was weak hybridization of the same probes to C3, possibly due to coral RNA contribution within the sample. Probes for acpPCSym_1, acpPCSym_4 and acpPCSym_15 hybridized to RNA of 1.4 kb, 1.2 kb and 938 bp corresponding to the size expected for a monocistronic polypeptide transcript (Table 3.2). In contrast, probes for acpPCSym_8 hybridized to RNA of 3.1 kb, 4.6 kb, 6.1 kb and 7.6 kb while the acpPCSym_13 probe hybridized to RNA of 2.1 kb and 4.2 kb. Based on acpPC polypeptide size (Table 3.2), acpPCSym_8 mRNA comprises between four and ten 519 bp repeats (173 amino acid polypeptides) and acpPSym_13 comprises three to six 576/534/630 bp repeats (192/178/210 amino acid polypeptides).

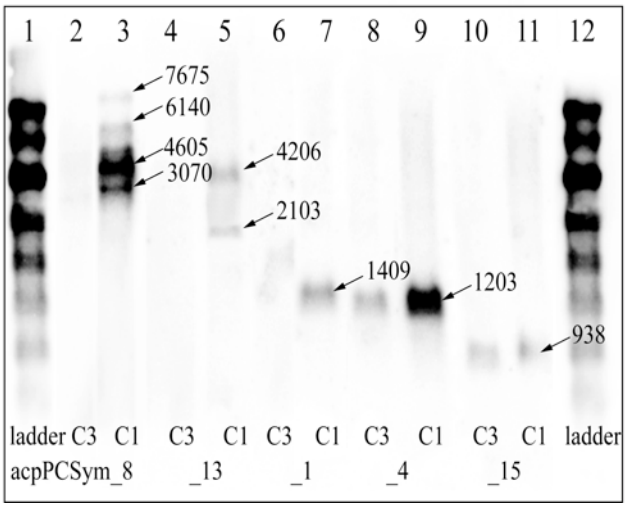


Figure 3.4 Northern blot analysis of freshly isolated *Symbiodinium* sp. C3 and cultured *Symbiodinium* sp. C1 RNA. The blot was probed with sequence specific PCR product for acpPCSym_8 (lane 2 and 3), acpPCSym_13 (lane 4 and 5), acpPCSym_1 (lane 6 and 7), acpPCSym_4 (lane 8 and 9) and acpPCSym_15 (lane 10 and 11) labelled using dATP 5' – [α-

^{32}P]. Equal quantities (2.5 µg) of RNA from *Symbiodinium* sp. C3 and C1 were applied. Lanes 1 and 12 contain RNA standard probed with lambda DNA.

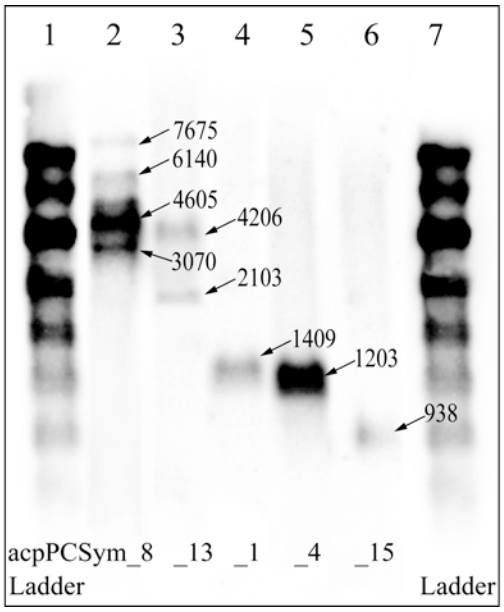


Figure 3.5 Northern blot analysis of cultured *Symbiodinium* C1 RNA. The blot was probed with sequence specific PCR product for acpPCSym_8 (lane 2), acpPCSym_13 (lane 3), acpPCSym_1 (lane 4), acpPCSym_4 (lane 5) and acpPCSym_15 (lane 6) labelled using dATP 5' – [α-^{32}P]. Equal quantities (2.5 µg) of RNA from *Symbiodinium* sp. C1 were applied. Lanes 1 and 7 contain RNA standard probed with lambda DNA.

3.5 Discussion

The *Symbiodinium* sp. C3 acpPC sequences reported in Chapter 3 encode a diversity of light-harvesting proteins within the Chl *a/c* binding family of LHCs (Figure 3.2) that exhibit similarities with both the rhodophyte Chl *a* binding proteins and the fucoxanthin Chl *a/c* binding proteins, but not the LI818 or LI818-like proteins. *Symbiodinium* sp. C3 acpPC sequences may share similar structural features and Chl-binding residues as those demonstrated in the LHCII sequences of Chl *a/b* binding proteins, but the position of the sequences within the Chl *a/c*, Fcp and Chl *a* grouping is strongly supported. The diversity evident in *Symbiodinium* LHCs suggests the acpPC subfamily may contain functionally distinct LHCs that enable these ecologically important dinoflagellates to efficiently utilize the varied light conditions experienced in the marine environment.

The major divisions within the phylogenetic reconstruction of LHCs in this study were similar to previously published results focusing on chlorophyte (Koziol et al., 2007) and stress-induced (Dittami et al., 2010) LHCs. The division of sequences into cryptomonad / red algal, Fcp / Chl *a/c*, LI818 family, and LHCI / LHCII subgroups remained clear (Figure 3.1), and the diversity of LHCs demonstrated in the one species of *Symbiodinium*, presented in this chapter,

reflects findings that Chl *a/c* containing algae possess LHCs from different subfamilies and lineages. Plastid genome sequencing of *Emiliania huxleyi* indicates the organelles of haptophytes, heterokontophytes and cryptophytes are closely related (Sanchez-Puerta et al., 2005), and genes from both red and green algal sources are evident in the heterokontophytes *Thalassiosira* and *Phaeodactylum* (Moustafa et al., 2009). Fcp sequences from *Cyclotella cryptic* share similarity with LI818 proteins of two *Chlamydomonas* spp. and the haptophyte *Isochrysis galbana*, and one *C. cryptic* Fcp sequence shows similarity to the red algal *Porphyridium cruentum* LHCI sequences *Lhca1* and *Lhca2* (Eppard et al., 2000).

The fucoxanthin-binding dinoflagellate *K. micrum* also exhibits diversity in LHCs (Figure 3.1, LI818/LI818-like and Clade 3a) and this may relate to the origin of plastids derived from haptophytes (Tengs et al., 2000). The tertiary derived plastids contain fucoxanthin-binding proteins in addition to those proteins remaining from the secondary dinoflagellate plastid that possess peridinin-binding proteins (Patron et al., 2006) and may be the case with acpPCSym_13:3 which groups with *K. micrum* and other fucoxanthin – binding algae (Clade 3a) rather than acpPCSym_13:1 and _13:2 (Clade 3b). The presence of LHC sequences in Clades 1, 2 and 3 of other dinoflagellate ESTs supports the hypothesis that this characteristic may be widespread in these algae. Whether the degree of expression diversity seen here in *Symbiodinium* sp. C3 is partially a factor of the diverse array of experimental conditions to which the algae were exposed (Leggat et al., 2007) is unknown.

Given the series of plastid acquisition and genome rearrangements in dinoflagellates (Zhang et al., 2007, Slamovits and Keeling, 2008), and the evidence for lateral gene transfer (Nosenko and Bhattacharya, 2007) it is not surprising dinoflagellates encode multiple LHC genes. Many dinoflagellate genes have very high copy numbers encoding similar proteins; for example the *A. carterae* actin copy number is at least 113 (Bachvaroff and Place, 2008) and the *Lingulodinium polyedrum* luciferin-binding protein gene has approximately 1,000 copies (Lee et al., 1993). While those *Symbiodinium* LHCs that are closely related may be functionally equivalent, the diversity in sequences raises the possibility that some may be functionally different and involved in distinct light-harvesting and photo-protection processes. The photo-protective strategy utilized by *Symbiodinium* remains unresolved, however LHC disassociation from PSII under high light conditions (Reynolds et al., 2008), with redistribution to PSI to minimise PSII overexcitation, (Hill et al., 2012) has been speculated. *Symbiodinium* acpPC bind the carotenoid pigments diadinoxanthin (Dn) and diatoxanthin (Dt) (Ambarsari et al., 1997), which are functionally equivalent to the xanthophyll pigments central to terrestrial plants photo-protective mechanisms (Demming-Adams and Adams, 1996). Recent findings suggests high light conditions activate xanthophyll quenching mechanisms associated with acpPC (Kanazawa et al., 2014), and xanthophyll cycling, along with LHC dissociation, potentially protects PSII from photodamage (Hill et al., 2012). Whether all acpPC isoforms are involved in photo-protective

mechanisms is unknown. With respect to this, the lack of similarity of *Symbiodinium* LHC sequences presented here with LI818 or LI818-like proteins is interesting. The two subgroups of LI818 and LI8180-like sequences in this chapter each contain one dinoflagellate representative: *H. triquetra* and *K. micrum* (Figure 3.1). Many proteins from the LI818 subfamily have a role in stress response and recent phylogenetic analysis suggests LI818 proteins originated in an ancestral Chl *a/c* containing organism (Dittami et al., 2010). In addition, the number of proteins from the LI818 family present in haptophytes and heterokontophytes suggests this family of proteins is important in the marine environment (Dittami et al., 2010).

3.5.1 LHCs Synthesized as Polyproteins

The use of polyproteins as a method for the importation of nuclear encoded Chl-binding proteins into algal chloroplasts has been reported previously for *Euglena* (Chl *a/b* binding protein) and the dinoflagellate *A. carterae* (Chl *a/c* binding protein): these have three or four and three chloroplast membranes respectively. Four *Symbiodinium* LHC proteins (acpPCSym_8, _10, _12 and _13) share identity with the Clade 3 LHCs and encode polypeptides within a single transcript. The SPLR motif was present in all *Symbiodinium* transcripts encoding polypeptides (acpPCSym_8, _10, _12 and _13). This is consistent with the mature LHCs being generated by cleavage at the arginine in this C-terminal motif (Hiller et al., 1995). The SPLR motif was also present at the C-terminal end of acpPCSym_11, which encodes a monomer. Its presence in this transcript and identity at the cDNA level of acpPCSym_11 with the polypeptides of acpPCP_8 (79 % and 80 %), _10 (95 % and 93 %) and _12 (98 % and 96 %) (Table 3.3) raises the possibility that acpPCSym_11 represents a component of the original gene, which following gene duplication and fusion, has given rise to polyprotein transcripts. This contrasts with the tripartite polypeptides of acpPCSym_13 that have significantly less identity and may represent an older series of gene duplication/fusion events (Table 3.3). Four other dinoflagellates, *A. carterae* (Hiller et al., 1995), *P. lunula* (Okamoto and Hastings, 2003a), *H. triquetra* (Patron et al., 2005) and *Amphidinium tamarense* (Kobiyama et al., 2005), also encode LHCs containing the motif SPLR.

Gene duplication is a well-documented feature in dinoflagellates (Le et al., 1997, Zhang et al., 2006) which presumably has given rise to polyproteins in addition to increasing the copy number of highly expressed genes (Bachvaroff and Place, 2008). Information on the organisation of dinoflagellates genomes is limited, but there is evidence that subsets of genes are arranged in repeated tandem arrays and result in polycistronic messages carrying multiple copies of a single gene (Bachvaroff and Place, 2008). Analysis of 15 highly expressed *A. carterae* genes showed that 14 were arranged in repeated tandem arrays while only two moderately expressed genes had a similar arrangement (Bachvaroff and Place, 2008). In addition *A. carterae* has been shown to encode up to ten LHC polypeptides within the one

transcript (Hiller et al., 1995). While transcripts encoding up to three LHC polypeptides were found in the screen of the *Symbiodinium* cDNA library, northern blot analysis indicates that *Symbiodinium* expresses larger transcripts. For example a probe using a portion of acpPCSym_8 that encodes two LHC polypeptides with a size of 1.54 kb hybridizes to transcripts of 3.1 kb, 4.6 kb, 6.1kb and 7.6 kb (Figure 3.5), corresponding to four, six, eight and ten LHC polypeptides.

The presence of LHC polyproteins in *E. gracilis* and dinoflagellates, which have acquired plastids from different algal lineages, probably represents the convergent evolution of an efficient strategy to import proteins through multiple chloroplastic membranes into secondary chloroplasts. The presence of multiple membranes around the plastid necessitates the presence of an N-terminal transit peptide for nuclear encoded chloroplast targeted proteins. In dinoflagellates these proteins possess presequences containing a signal peptide and a transit peptide of which there are two major classes, both possessing a four-residue phenylalanine based motif (Patron et al., 2005). The 5' regions of *Symbiodinium* sp. C3 acpPC sequences, for which I have full sequence coverage, generally contain these dinoflagellate presequence features.

3.5.2 *Symbiodinium* C3 Membrane-Spanning Helix Organization

It has been hypothesized the Chl *a/b* and Chl *a/c* binding proteins evolved from a gene encoding four membrane-spanning helices (Green and Pichersky, 1994). It is proposed that the gene for the four membrane-spanning helices arose from gene duplication of membrane-spanning helices I and III with helix IV subsequently undergoing degeneration (Green and Pichersky, 1994). The conservation of helices I and III in a diverse range of organisms, including *Symbiodinium*, supports this hypothesis and that helices I and III and the associated Chl-binding residues represent the core of the light-harvesting complex (Kühlbrandt et al., 1994), although alternative evolutionary scenarios have been suggested (Engelken et al., 2010).

This study also demonstrates that *Symbiodinium* encodes at least two proteins with LHC membrane-spanning helix duplication, deletion, and/or degeneration; a feature more common in the Chl *a/b* binding proteins of green plants, chlorophytes and euglenophytes rather than Chl *a/c* and Fcp proteins. Comparison of the *Symbiodinium* acpPC sequences with LHCII genes from green plants (Kühlbrandt et al., 1994) and LHCI genes in *Porphyridium cruentum* (Tan et al., 1997b) demonstrates the similarity between them, particularly with respect to the identified Chl-binding residues. *Symbiodinium* has eight amino acids separating the two Chl-binding residues in helix II as documented in rhodophytes and chromophytes (Tan et al., 1997b), rather than seven as in green plants (Kühlbrandt et al., 1994). While related to Chl *a/c* binding proteins, acpPCSym_17 and acpPCSym_KB8 A1.1_155 are particularly interesting and appear to have evolved independently from the polyproteins and Fcp binding proteins. Exhibiting 80 % identity at the protein level, these two proteins are from different genetic strains of *Symbiodinium*

(LaJeunesse, 2001, LaJeunesse et al., 2003) and contain all the structural features and Chl-binding residues identified in the original green plant LHCII model (Kühlbrandt et al., 1994). Both encode three transmembrane and an amphiphilic α-helix protein, although helix II has eight amino acids between *Q* and *E* (Figure 3.3c).

The green plant LHCII sequence and structure (Kühlbrandt et al., 1994, Liu et al., 2004, Standfuss et al., 2005) shares significant homology with LHC sequences from rhodophytes, cryptophytes, chromophytes and dinophytes. Superimposing rhodophyte and chromalveolate sequences over the green plant LHCII model enables initial structural and Chl-binding site comparisons between and within different Chl-carotenoid binding protein groups, although definitive conclusions from such comparisons require caution. Superimposing the putative family of acpPC sequences onto the green plant LHCII model highlights the extent of sequence identity and suggests *Symbiodinium* may have a similar LHC structure to LHCII in green plants. The presence of *H. triquetra* and *K. micrum* sequences in the LI818 and LI818-like group and within the Chl *a/c* clades (Clade 1 and Clade 3a respectively) indicates the diversity shown within *Symbiodinium* is unlikely to have resulted from sample contamination during collection and extraction processes. The diversity of *Symbiodinium* LHC proteins poses the question of how the structure of the PSI and PSII supercomplexes is formed given such a range of LHCs in the *Symbiodinium* genome. In green plants, the PSI supercomplex is composed of two heterodimers consisting of *Lhca1-4*, with lower concentrations of *Lhca5-6*; in *Arabidopsis* these are each encoded by a single gene copy (Jansson, 1999). PSI in *Chlamydomonas* is more complex with up to 18 LHC isoforms being present, although all of these are homologous to the six LHC proteins found in green plants with the diversity arising from multiple genes for some of the LHC classes being present in the genome. The green plant PSII also contains six LHC proteins that form either trimers or monomers. Given the small number of genes encoding LHC in the green lineage the question arises as to how the dinoflagellate photosystem supercomplexes are formed and regulated with such a diversity of LHC isoforms being expressed? Does PSI contain only acpPC proteins from Clade 1, as is the case with red algae Clade 1, while acpPCs in Clade 3 associate with PSII? What role do acpPC isoforms with non-canonical Chl-binding residues play - are they still capable of chlorophyll binding or do they play an adapted role in photo-protection? These questions require further research. However, whatever their function, the range and complexity of *Symbiodinium* integral LHC proteins presented in this chapter, provides further evidence that the organization of dinoflagellate light-harvesting systems is unique (Hiller et al., 1993)

3.6 Chapter 3 Summary Points

- Phylogenetic analysis of the 11 LHC sequences for the chlorophyll *a*-chlorophyll c_2-peridinin protein complex (acpPC) subfamily suggests the acpPC subfamily forms at

least three clades within the Chl *a/c* binding LHC family;

- Clade 1 clusters with rhodophyte, cryptophyte and peridinin binding dinoflagellate sequences,
- Clade 2 with peridinin binding dinoflagellate sequences only and
- Clades 3 with heterokonts, fucoxanthin and peridinin binding dinoflagellate sequences.

- *Symbiodinium* sp. C3 acpPC sequences generally contain three transmembrane helices;
- Encode at least two proteins with LHC membrane-spanning helix duplication, deletion, and/or degeneration; and
- Encode transcripts containing one, two or three LHC polypeptides.

CHAPTER 4

4 Measuring *Symbiodinium* sp. Gene Expression Patterns with Quantitative Real-Time PCR

4.1 Statement of Purpose

Quantitative real-time PCR is a popular method for measuring gene expression in many biological organisms. The use of quantitative real-time PCR to measure gene expression levels of *Symbiodinium* has been limited by the lack of validated normalization genes and the inability to purify mRNA from the alga without significant host contamination. Normalization genes to correct for errors inherent to this technique are essential and as yet no validated normalization genes have been identified for use in *Symbiodinium* gene expression studies. Chapter 4 reports the testing of three commonly used normalization genes, β-actin, proliferating cell nuclear antigen (PCNA) and 18S rRNA, for use as internal standards in light manipulation experiments of acpPC.

4.2 Introduction

The use of reverse transcription in conjunction with quantitative real-time polymerase chain reaction (qRT-PCR) is now a fundamental technique for measuring transcript levels of genes in a wide variety of organisms. The most commonly used approach involves the quantitation of transcript levels in relation to the level of an experimentally stable internal reference or normalization gene. Alternatively absolute copy numbers of the transcript of interest can be calculated from a standard curve. qRT-PCR, while relatively straight forward to perform, has technical problems. These include RNA variability, variability in extraction protocols and different reverse transcription and PCR efficiencies (Bustin and Nolan, 2004), while analysis of results is complicated by the method used for normalization (Dheda et al., 2004). Appropriate selection of normalization genes is important in controlling for these inherent problems, but the selection of an unstable gene may statistically influence results by either masking the detection of small changes or providing an incorrect result (Dheda et al., 2005). While growing numbers of studies test the potential of normalization genes for use with organisms such as plants (for example, Giorio et al., 2007, Jurca et al., 2008, Olbrich et al., 2008), Chromalveolata (Kobiyama et al., 2005, Siaut et al., 2007) and Cnidarians (Deboer et al., 2007, Rodriguez-Lanetty et al., 2008), the vast majority continue to use plasmid DNA or cell number (Bowers et al., 2000, Ulstrup and Van Oppen, 2003, Galluzzi et al., 2004, Zhang and Lin, 2005, Moorthi et al., 2006, Dyhrman et al., 2006, Handy et al., 2006, Park et al., 2007, Loram et al., 2007a, John

et al., 2007, Handy et al., 2008, Demir et al., 2008) to quantify absolute transcript levels (Table 4.1). Use of qRT-PCR to measure gene expression where both partners are symbiotic eukaryotes is significantly more complicated than studies with single organisms or symbioses between eukaryote and prokaryote partners. The greatest challenge with symbioses between two eukaryote partners involves differentiating RNA contribution of the host organism from the symbiont and determining variability in the RNA contribution between samples.

In dinoflagellate symbioses studies qRT-PCR is primarily used to determine or compare the presence of different algal populations (Loram et al., 2007a, Ulstrup et al., 2007, Mieog et al., 2007) within scleractinian corals. In these studies use of normalization genes or absolute transcript copy numbers is not necessary. Future approaches to eukaryotic symbioses studies are expected to incorporate exogenous RNA spikes. This enables quantification of symbiont contribution to RNA extracts, as was elegantly demonstrated for anthozoans harbouring endosymbiotic dinoflagellates (Mayfield et al., 2009). While utilization of qRT-PCR to measure gene expression of coral or their single-celled phototrophic dinoflagellate of the genus *Symbiodinium* is still in the initial stages, it will become an important tool for elucidating the effects of increasing anthropogenic and environmental stresses on this important symbiotic relationship.

Here validation of three commonly used normalization genes, 18S rRNA, β-actin and proliferating cell nuclear antigen (PCNA) are tested for stability under varying light conditions for future gene expression experiments with *Symbiodinium* sp. light harvesting chlorophyll *a* chlorophyll c_2 peridinin protein complex (acpPC) genes.

Table 4.1 Common normalization methods used in plant, algae, symbiosis and bacteria.

Normalization Standard	Organism
β-actin	Cnidaria (Reynolds et al., 2000, Mitchelmore et al., 2002, Deboer et al., 2007) Dinoflagellate (Kobiyama et al., 2005) Plant (Jurca et al., 2008)
18S rRNA	Plant (Giorio et al., 2007)
GAPDH	Plant (Jurca et al., 2008)
Plasmid DNA or cell numbers	Dinoflagellates (Handy et al., 2008, Demir et al., 2008, Miyaguchi et al., 2008, Park et al., 2007, Moorthi et al., 2006, Dyhrman et al., 2006, Zhang and Lin, 2005, Galluzzi et al., 2004, Bowers et al., 2000, Ulstrup and Van Oppen, 2003, Loram et al., 2007a) Raphidophytes (Handy et al., 2008, Handy et al., 2006)

	Pelagophytes and haptophytes (John et al., 2007) Cyanobacteria (John et al., 2007)

4.3 Material and Methods

4.3.1 *Symbiodinium* Samples

Coral branches from three colonies of *Acropora aspera* hosting *Symbiodinium* (clade C3) were collected from the reef flat opposite Heron Island Research Station (HIRS) (23°33'S, 151°54'E) shortly after low tide in the early afternoon of 21 June 2005 and placed in four flow through aquaria. Light levels in two aquaria were reduced by shading (average daily light dosage 0.7 mol quanta m^{-2} d^{-1}), while the two remaining tanks were left exposed (average daily light dosage of 17.4 mol quanta m^{-2} d^{-1}). Over the course of the experiment the maximum irradiance recorded in the shaded tanks ranged between 27.3 and 98.9 μmol quanta m^{-2} s^{-1}, while the exposed tanks ranged from 573 to 1540 μmol quanta m^{-2} s^{-1}. One sample from each colony was collected from each tank at 1300 h on days 1, 3, 5, 7 and 9 of the experiment for RNA extraction.

4.3.2 RNA Isolation

Symbiodinium were removed from the coral skeleton using an oral irrigator (WaterPik™) and the resulting algal pellet frozen in liquid nitrogen and stored at -80°C. (Section 2.1) prior to being ground and RNA extracted. Frozen algal pellets were ground under liquid nitrogen in a mortar with a pestle and total RNA isolated using a RNeasy Plant Mini Kit (Qiagen, USA) (Section 2.5.1 and 2.5.1.1). RNA quality was assessed using a 0.9% formaldehyde agarose gel (Section 2.5.11) and then quantified (Section 2.5.10).

4.3.3 Quantitative Real-Time PCR (qRT- PCR) Analysis

Reverse transcription and genomic DNA elimination was performed with QuantiTect Reverse Transcription kit (Section 2.5.3). The standard qRT-PCR protocol (Section 2.5.8) was used with gene specific primers for β-actin, PCNA, 18S rRNA and three acpPC genes (acpPCSym_1, _9 and _10) (Table 4.2). Each GeneDisc™-100 (Corbett Life Sciences, Australia) included reactions for one normalization and one *Symbiodinium* acpPC gene, with samples run in triplicate and non-template controls in duplicate. Standard curves for each normalization and *Symbiodinium* acpPC gene were generated on every run with five duplicates using a single template sample. An additional PCR efficiency check using four different RNA templates was conducted to ensure inter-disc comparability.

Primers designed for β-actin, PCNA and 18S rRNA (Section 2.5.8.1) were aligned and checked for specificity against nucleotide expressed sequence tags in NCBI GenBank database

(Section 2.5.14). Primer specificity was checked using coral cDNA extracted from *Acropora millepora* at both the prawn chip and donut developmental stages and *Symbiodinium* clade C3 cDNA extracted from *A. aspera*. *Symbiodinium* C3 acpPC primers were designed to an expressed sequence tag library (Leggat et al., 2007). acpPC primers were designed and checked as per normalization genes.

An additional primer check and negative control to determine the effect of coral contamination on *Symbiodinium* relative expression was performed. A total of 30 ng of template was used with decreasing amounts of *Symbiodinium* cDNA template (30 ng to 0 ng) mixed with increasing concentrations of coral cDNA from the donut developmental stage (0 ng to 30 ng of coral). qPCR was performed according to the above protocol using β-actin, PCNA, acpPCSym_1 and acpPCSym_10 primers and relative gene expression determined using the $\triangle\triangle C_t$ method (Livak and Schmittgen, 2001).

Table 4.2 Normalization genes and acpPC genes tested in qRT-PCR assays

Gene	Primers	Product size
β-actin	F1: TGG ACA ACG GAA GCG GAA TG B1: GCC AAC AAT GGA TGG GAA AAC T	80 bp
PCNA	F1: GAG TTT CAG AAG ATT TGC CGA GAT B1: ACA TTG CCA CTG CCG AGG TC	113 bp
18S rRNA	F3: GTC TAA CGC AAG GAA GTT TGAG B3: CAG GAC ATC TAA GGG CATC A	57 bp
acpPCSym_10	F1: TTC GCC GAT GTG CCT AAT GG B1: TTC CTG GGA GAC TTC GCA GAA A	102 bp
acpPCSym_1	F1: AGT GGA GTG AAC CAG GAA GCA A B1: AAC CAA TCG CAC CGA CCA AGA G	54 bp
acpPCSym_9	F1: CGA ATG GAA GTT GGT GGT AAC B2: GTG CTC AAC CCA CTG TCT TTT	51 bp

4.3.4 Statistical Analysis

Results were analysed using the geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) statistical algorithm software to calculate gene expression stability (*M*) for the three normalization genes tested. The gene or genes with the lowest *M* value is considered the most stably expressed (Vandesompele et al., 2002). All target samples collected on Day 7, which were determined to be the most stably expressed, and normalized to the two highest ranking normalization genes were subsequently analysed using geNorm to identify a calibration sample.

The three *Symbiodinium* acpPC gene expression profiles were analysed in the relative expression software REST© (http://www.gene-quantification.de/rest-2005.html) (Pfaffl et al., 2002). PCR efficiencies were calculated for each standard curve generated and threshold fixed at 0.0859 for all C_t calculations. This threshold value represented the average detection threshold across the 15 runs with the upper and lower bounds used to scan for an optimal threshold set between 1 and a value necessary to exclude background noise. Melting curve analysis was performed for each assay to check reaction specificity.

4.4 Results

4.4.1 Primer Specificity and Coral Contamination

Specificity of primer design for *Symbiodinium* sp. normalization genes was examined using coral cDNA isolated and transcribed from prawn chip and donut developmental stages, both early post fertilization aposymbiotic stages. qPCR primers designed for clade C3 *Symbiodinium* and amplified according to either standard PCR conditions or qPCR conditions, failed to amplify coral cDNA. Glyceraldehye-3-phosphate dehydrogenase (GAPDH) qRT-PCR primers designed for *A. millepora* were included as a positive control with pawn chip cDNA. When tested with C3 *Symbiodinium* cDNA, single amplicons of expected size were detected (data not shown).

A possible confounding factor in using qPCR to quantify symbiont gene expression is the unknown contribution of host RNA. To test for this the relative expression of acpPCSym_1 and acpPCSym_10 was examined with increasing concentrations of coral cDNA. The concentration of *Symbiodinium* RNA (which would probably include approximately 10-20% host contamination (Leggat et al., 2007)) used in the assay was decreased from 100% to 0.5%, while the coral cDNA was increased. The expression of acpPCSym_1 and acpPCSym_10 was then compared across this concentration range. A variation from a relative expression of 1, when compared to a sample with 100% *Symbiodinium* cDNA (30 ng) would indicate an effect of coral contamination. Only when *Symbiodinium* cDNA composed 0.5% (0.15 ng) of the total cDNA was there a significant deviation ($p = 0.02$) from a relative expression value of 1 (Figure 4.1). No fluorescence was recorded in samples without *Symbiodinium* template (30 ng of coral cDNA only).

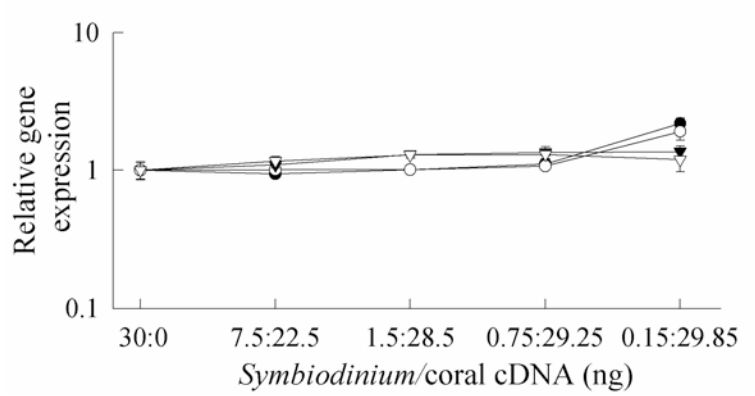


Figure 4.1 Effect of increasing the proportional coral cDNA concentration on relative expression of acpPCSym_1 normalized to β-actin (-●-) and PCNA (-o-) and acpPCSym_10 normalized to β-actin (-▼-) and PCNA (-▽-). Total template concentration is 30 ng for each sample and error bars indicating the standard error.

4.4.2 qRT-PCR Efficiency

Amplification plots were analysed for the three normalization and three *Symbiodinium* acpPC genes. Assay validations performed confirmed PCR efficiency and optimization of procedure. Samples from Day 5 were removed from all expression profile analyses due to three samples being compromised prior to RNA extraction. Comparison of C_t values for each of the six genes on all sample days, including four additional templates run across multiple discs to ensure inter-disc comparability, confirmed minimal variation between PCR efficiencies (Table 4.3), permitting comparison of genes and samples performed on different GeneDisc™-100 discs.

Table 4.3 PCR efficiencies for normalization genes and acpPC genes

Sample Day	Normalization Genes	PCR Efficiency	*Symbiodinium* Genes	PCR Efficiency
Day 1	18S ribosomal RNA	1.01	acpPCSym_10	0.99
	β-actin	0.98	acpPCSym_1	1.00
	PCNA	0.98	acpPCSym_9	1.07
Day 3	18S ribosomal RNA	1.01	acpPCSym_10	0.90
	β-actin	0.98	acpPCSym_1	0.94
	PCNA	0.95	acpPCSym_9	0.97
Day 7	18S ribosomal RNA	1.01	acpPCSym_10	0.96
	β-actin	0.95	acpPCSym_1	0.95
	PCNA	1.00	acpPCSym_9	1.05
Day 9	18S ribosomal RNA	1.03	acpPCSym_10	0.90

	β-actin	1.00	acpPCSym_1	0.97
	PCNA	1.00	acpPCSym_9	1.02

C_t values for 18S rRNA were considerably lower than those of β-actin, PCNA and the three *Symbiodinium* acpPC genes across all sampling days (Figure 4.2) indicating high transcript levels and the need for further optimization of this gene.

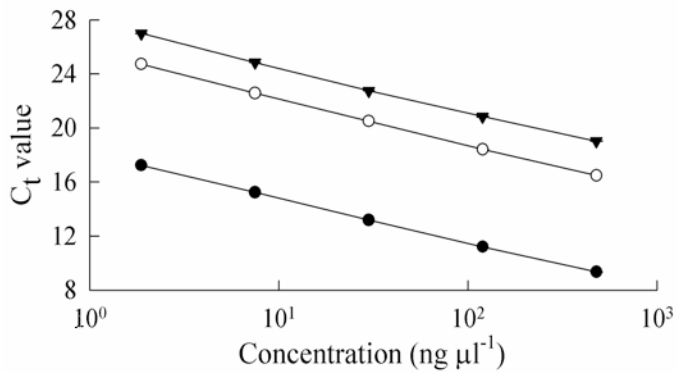


Figure 4.2 β-actin (-o-), PCNA (-▼-) and 18S rRNA (-●-) standard curves generated from amplification plots of serial four-fold dilution series. The plotted C_t is the average of triplicate samples and error bars (hidden by symbols) indicating the standard error.

4.4.3 Normalization Gene Stability

β-actin (*M* = 0.467) was marginally more stable than PCNA (*M* = 0.498) with 18S rRNA the least stable (*M* = 0.687) under the specific experimental conditions used here. Target samples normalized to β-actin and PCNA from Day 7 of the experiment expressed the greatest stability. The most stably expressed target sample from Day 7 was used as the calibration sample and PCR efficiencies for each of the six genes from Day 7 were used to qualitatively compare the expression of the three acpPC genes subjected to varying light levels.

Relative gene expression levels for the three *Symbiodinium* light harvesting genes were calculated using the REST© method (Pfaffl et al., 2002) (Fig. 4.3). The profile of all three genes was similar when normalized to β-actin (Figures 4.3a, c, e) with those samples shaded from light showing increased expression compared with light exposed samples. Light exposed samples exhibited minimal expression change across the nine day experiment and these results were replicated when acpPC genes were normalized to PCNA (Figures 4.3b, d, f). acpPCSym_1 normalized to PCNA (Figure 4.3f) were the only shaded samples not to increase expression.

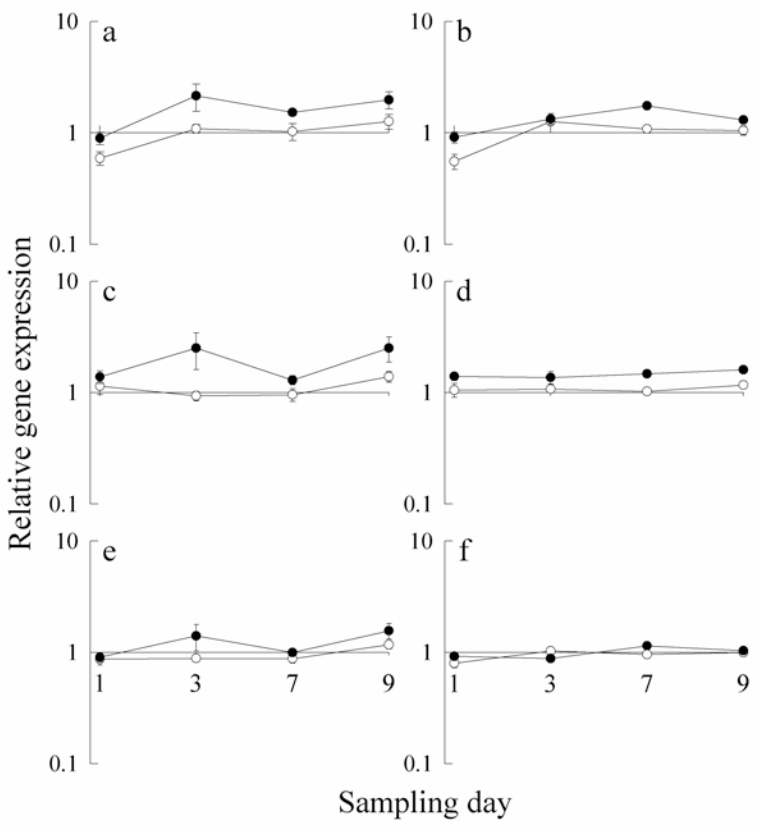


Figure 4.3 Expression profiles of *Symbiodinium* gene acpPCSym_10 (a, b), acpPCSym_9 (c, d) and acpPCSym_1 (e, f) normalized to β-actin (a, c, e) and PCNA (b, d, f) exposed to light (-o-) or shaded from sunlight (-●-). Error bars represent the standard error for the averaged C_t values used to calculate relative expression for six biological replicates.

4.5 Discussion

Three normalization genes, β-actin, PCNA and 18S rRNA, were tested for stability in light manipulation experiments with *Symbiodinium* major light harvesting protein complexes (acpPC). Using the statistical algorithm software geNorm, β-actin was found to be marginally more stable than PCNA, and gene expression profiles for three acpPC genes normalized to β-actin and PCNA exhibited similar trends (Figure 4.3).

Limited plant, alga or symbiotic studies have used quantitative analysis (Table 4.1) and those that have generally normalize data to β-actin (Reynolds et al., 2000, Mitchelmore et al., 2002, Deboer et al., 2007, Jurca et al., 2008), 18S ribosomal RNA (Giorio et al., 2007) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Jurca et al., 2008). PCNA was tested here rather than the more commonly used GAPDH to complement the use of β- actin and because synthesis of chloroplastic GAPDH is regulated over a diel cycle and exhibits greater than 50 % identity in the region of overlap with the cytosolic isoform (Fagan et al., 1999).

PCNA has been used as a normalization gene in a range of animal species and humans (Schiller et al., 2003), is present in plant genomes (Suzuka et al., 1989) and has been isolated

from a dinoflagellate where it was found not to significantly alter expression levels during cell cycle (Zhang et al., 2006), although this differs from other algal studies (Lin et al., 1994, Lin et al., 1995, Cheng et al., 1997, Wei et al., 2004).

Although using total RNA for normalization is not always reflective of the mRNA fraction, it does enable the testing of 18S rRNA stability. In this study 18S rRNA was found to be unsuitable and would require additional optimization. Compared with target mRNA transcripts the abundance of 18S rRNA is much higher (C_t value of approximately 10 compared to β-actin 17 and PCNA 19 indicating a 128-512 fold greater representation) (Figure 4.2) and this causes difficulties when analysing data and determining the background baseline to subtract from the data (Vandesompele et al., 2002).

Normalization of gene expression levels is required in order to control for experimental errors during the process of qRT-PCR. While a number of strategies can be applied including normalization to a sample size, total RNA and the use of an internal reference gene (Huggett et al., 2005), applying these strategies to two eukaryotic organisms in symbiosis is challenging as each organism contributes to the extracted RNA pool. The overwhelming issue when using qRT-PCR with organisms such as *Symbiodinium* isolated from coral is whether varying RNA contamination influences the relative expression of the target genes. Results here suggest coral contamination is not a major factor and does not influence relative gene expression of *Symbiodinium* acpPC genes (Figure 4.1) until transcript levels become very low, at which stage the variation between replicates increases. This suggests if valid normalization genes can be identified for specific experimental conditions, the use of mixed RNA populations will not confound results.

Microarray studies to date suggest dinoflagellate genes express small changes and that photosynthetic genes may only vary 2 – 3.4 fold (Van Dolah et al., 2007, Okamoto and Hastings, 2003b). The identification of suitable normalization genes is important if such minor changes in expression are to be detected and quantified. This work determined the stability of two normalization genes and is an initial step towards validating a suite of genes with potential for use in *Symbiodinium*-coral gene expression work.

4.6 Chapter 4 Summary Points

- In order to use quantitative real-time PCR to measure *Symbiodinium* acpPC gene expression levels, potential normalization genes were validated;
- Using the algorithm software program geNorm, β-actin and PCNA were found to be more stably expressed than the more highly expressed 18S rRNA genes;
- β-actin and PCNA resulted in similar gene expression profiles when used to normalize gene expression data for the three acpPC genes tested, acpPCSym_1, _9 and _10 genes;

- As a result, β-actin and PCNA can be utilized in controlled light experiments to investigate expression patterns in cultured *Symbiodinium* sp. clade C1.

CHAPTER 5

5 Identifying Chlorophyll *a*-Chlorophyll c_2-Peridinin Protein Complexes in Cultured *Symbiodinium* sp.

5.1 Statement of Purpose

Physiological and photo-biological responses of *Symbiodinium* light-harvesting complexes (LHCs) have been the focus of a number of studies (for example, Iglesias-Prieto and Trench, 1997a, Robison and Warner, 2006, Warner et al., 2006, Hennige et al., 2009). Linking these physiological changes to concomitant changes in LHC genes has not. Identifying *Symbiodinium* photosynthetic genes and investigating whether diverse *Symbiodinium* lineages encode the same genes or a sub-set of similar genes will enhance our understanding of the light-harvesting and photo-protective mechanisms utilized by these unique dinoflagellates. Sequence data for acpPC from *Symbiodinium* C3 enables comparative expression studies in cultured *Symbiodinium* clades and sub-clades from the nine diverse lineages. Chapter 5 reports whether C3 acpPC primer sets and polyclonal antibody return positive results when used to amplify complementary DNA (cDNA), genomic DNA (gDNA) and protein extracted from eight *Symbiodinium* clades and sub-clades (A1, A1.1, A2, B, B1,C1, F1 and F2) acclimated to different light conditions.

5.2 Introduction

The mutualistic relationship between reef-building corals and dinoflagellates of the genus *Symbiodinium* is sensitive to environmental stresses including thermal stress and extreme light levels (for example, Coles and Jokiel, 1978, Hoegh-Guldberg and Smith, 1989, Hoegh-Guldberg, 1999, Fitt et al., 2001, Hughes et al., 2003, Stat et al., 2009). Determining sea-water temperature or irradiance level is relatively straight forward, but measuring the light intensity directly 'experienced' by a single endosymbiotic cell poses greater challenges. Multiple factors alter the wavelength of light and / or light energy absorbed and 'experienced' by *Symbiodinium in hospite* (living in a host cell) including symbiont self-shading, whether the host is located in shallow or deep-waters, the position of the symbiont within a host, and the host's intricate aragonite skeleton which is a highly reflective environment capable of increasing the light energy captured by a single photon 2.5 times (Enriquez et al., 2005).

Use of cultured isolates eliminates natural environmental fluctuations as well as host interactions that must be considered when working with *Symbiodinium in hospite*. Valuable morphological, biochemical, and physiological information has been determined for *Symbiodinium* from different clades as a result of studies using cultured cells (Schoenberg and

Trench, 1980a, Schoenberg and Trench, 1980b, Schoenberg and Trench, 1980c, Fitt et al., 1981, Chang et al., 1983, Trench and Blank, 1987, Govind et al., 1990, Markell and Trench, 1993, Trench, 1993, Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, Banaszak et al., 2000). The use of cultured cells is not without limitations and caution is required when extrapolating results from isolates to those *in hospite*. Cultured cells are known to develop morphological differences; for example, the cell membrane of *Symbiodinium* in culture is thicker than membranes of freshly isolated cells (Schoenberg and Trench, 1980b). Consequently, findings from experiments with cultured cells may not be applicable to cells *in hospite* or to the coral-symbiont holobiont as they do not account for host-symbiont interactions and the role these interactions have on how *Symbiodinium* respond to environmental cues. Host-symbiont interactions are not well understood and an area requiring further research in order to fill gaps in our understanding of this important relationship. Nevertheless, use of cultured isolates does enable controlled manipulations otherwise not possible and therefore, are a valuable tool for investigating photosynthetic and photo-protective mechanisms used by *Symbiodinium*.

Symbiodinium utilize two light-harvesting complexes (LHC) to regulate the absorption of light energy; the peripheral peridinin-chlorophyll *a* proteins (PCP) and the membrane-bound chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC). Initially acpPC was thought to be encoded by a polypeptide of approximately 19 kDa (Hiller et al., 1993, Hiller et al., 1995), however the 11 acpPC sequences isolated from *Symbiodinium* C3 (extracted from a coral host) demonstrates that a greater diversity is evident within the acpPC LHC subfamily (Chapter 3) (Boldt et al., 2012). Monomeric, dimeric and trimeric acpPC isoforms encoded by polypeptides ranging from approximately 18.4 to 65.5 kDa (Chapter 3) (Boldt et al., 2012) are evident in *Symbiodinium* as well as PCP which exists in a monomeric 15 kDa form or dimeric 30 to 35 kDa form (Prézelin and Haxo, 1976, Iglesias-Prieto et al., 1991, Norris and Miller, 1994, Sharples et al., 1996, Hiller et al., 2001, Weis et al., 2002). *Symbiodinium* of varying sub-clades have been reported to possess both monomeric and dimeric forms of PCP (*S. microadriaticum*; A1), or either the monomeric (*S. kawagutii*; F1) or dimeric (*S. pilosum*; A2) form (Iglesias-Prieto et al., 1991). It has unknown whether varying *Symbiodinium* clades in culture and / or *in hospite* encode the same or a similar diversity of acpPC as that evident within *Symbiodinium* C3, however, such questions may potentially be answered by using C3 sequence data.

PCP is unrelated to acpPC and has been well documented (for example, Haidak et al., 1966, Prézelin, 1976, Chang and Trench, 1982, Roman et al., 1988, Govind et al., 1990, Iglesias-Prieto et al., 1991, Norris and Miller, 1994, Hofmann et al., 1996, Hiller et al., 2001, Reichman et al., 2003, Lee et al., 2005, Schulte et al., 2010, Jiang et al., 2012). The majority of photosynthetic pigments associated with *Symbiodinium* LHCs, including the photo-protective xanthophylls (Ambarsari et al., 1997), associate with acpPC rather than PCP (Hiller et al., 1993,

Iglesias-Prieto et al., 1993). The role of these LHCs is important for photosynthesis but the harvesting of excessive light energy can result in imbalances that potentially cause photo-oxidative damage. Exposure to low-light (LL; sub-saturating levels) or high-light (HL; super-saturating levels) can potentially reduce photosynthetic efficiency and when coupled with an additional stress increase the rate of photo-oxidative damage (Lesser and Shick, 1989b, Lesser, 1997) reviewed by (Ledford and Niyogi, 2005). In response to variations in light energy, and to protect photosynthetic processes and limit cellular damage, photosynthetic organisms utilize photo-acclimatory mechanisms (Falkowski and La Roche, 1991, Anderson et al., 1995, Durnford and Falkowski, 1997). It has not yet been determined exactly how *Symbiodinium* utilize photo-acclimatory mechanisms or whether mechanisms vary between *Symbiodinium* species.

Initially recognised as a single species, fourteen species of *Symbiodinium* are currently named with eight of these formally characterized (Guiry and Guiry, 2013). Use of molecular markers continues to provide information on diversity and ecology within the genus (Trench, 1993, Rowan, 1998, LaJeunesse, 2001, Baker, 2003, Coffroth and Santos, 2005) and many more species are expected to exist in addition to those currently named and characterized. Sequencing of internal transcribed spacer (ITS) regions and small ribosomal subunits is the common method of separating *Symbiodinium* clades. The current nine distinct and divergent lineages, or clades, are distinguished using alphabetical letters (A – I) (Rowan and Powers, 1991a, Carlos et al., 1999, LaJeunesse, 2001, Pochon et al., 2001, Pochon et al., 2004, Pochon and Gates, 2010). Within each clade are numerous taxonomically specific sub-clades and these are differentiated using alpha-numeric and lower case alpha designations (for example, A1, B2, C1 or C3u) (Rowan and Powers, 1991a, Carlos et al., 1999, LaJeunesse, 2001, Pochon et al., 2004, Coffroth and Santos, 2005).

Symbiodinium clades and sub-clades exhibit variations in photosynthetic pigment composition, geographic distribution, host associations, thermal tolerance and fluorescence patterns (Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, Rowan et al., 1997, Warner et al., 1999, LaJeunesse, 2002, LaJeunesse et al., 2003, Baker, 2003, LaJeunesse et al., 2004, Reynolds et al., 2008) and a number of *Symbiodinium* are categorised as thermally sensitive (for example, sub-clades A1.1 and B1) or thermally tolerant (for example F2) (Robison and Warner, 2006). Thermal stresses affect *Symbiodinium* LHC expression and photosynthetic processes (Robison and Warner, 2006, Takahashi et al., 2009, McGinley et al., 2012), and sub-clades more sensitive to thermal stress express lower levels of both acpPC and PCP (Takahashi et al., 2008). Sub-clades grown in culture and acclimated to different light levels demonstrate variations in cellular concentration of photosynthetic pigments associated with the LHCs, the relative abundance of LHCs, distribution of chlorophyll in the LHCs, and

arrangement of the photosynthetic apparatus (Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a).

Variations in LHCs are evident in *S. microadriaticum* Freudenthal (sub-clade A1) and *S. pilosum* Trench and Blank (sub-clade A2); both increase the xanthophylls content in acpPC under HL conditions (Iglesias-Prieto and Trench, 1997a), while *S. kawagutii* Trench and Blank (sub-clade F1) demonstrates limited changes in acpPC under HL or LL conditions (Iglesias-Prieto and Trench, 1997a). Such variations to changing environmental factors suggest *Symbiodinium* utilize mechanisms capable of managing stress events, such as fluctuating light conditions or elevated temperature. Comparative analyses of *Symbiodinium* LHC genes, protein expression, and mRNA synthesis have been limited. Expressed Sequence Tag (EST) datasets are available for two divergent clades of *Symbiodinium*, clade C (sub-clade C3) (Leggat et al., 2005) and clade A (sub-clade A1.1) (Voolstra et al., 2009), but the majority of publicly available dinoflagellate transcript sequences and ESTs are for none symbiotic dinoflagellates.

The ability to compare acpPC from diverse *Symbiodinium* clades, particular species recognised as having varied stress tolerance levels has potential to further advance our understanding of molecular and photo-biological processes utilized by these dinoflagellates. Chapter 5 incorporates sequence information for *Symbiodinium* C3 to report the presence or absence of acpPC in eight *Symbiodinium* spp. from four distinct lineages. The objective of this study was to determine whether C3 sequence data can be utilized to investigate acpPC from *Symbiodinium* for which there is limited or no available sequence data.

5.3 Materials and Methods

5.3.1 Algal strains and culture conditions

Symbiodinium goreauii Trench and Blank, sub-clade C1 (CCMP2466 strain, The Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences, USA (formally Center for Culture of Marine Phytoplankton)), and *Symbiodinium* spp. clade B and sub-clades A1, A1.1, A2, B1, F1 and F2 (The Santos Laboratory, Auburn University, Alabama USA) (Table 5.1) were cultivated in 75 cm^2 Tissue Culture flasks with PE vented caps (Sarstedt, NC, USA) containing ~100 mL of ASP-8A medium (Blank, 1987). Cell cultures were maintained within a light and temperature controlled refrigerated incubator TRISL-495-1-SD (Thermoline Scientific, Australia) and grown at 25 ^{0}C using the lighting system and light levels described in Section 2.3. Replicate flasks for each of the eight *Symbiodinium* type were grown and maintained at three light levels, high light (HL; ~250 – 350 µmol quanta m^{-2} s^{-1}), medium light (ML; ~80 µmol - 100 µmol quanta m^{-2} s^{-1}) and low light (LL; ~10 – 15 µmol quanta m^{-2} s^{-1}). Cells were regularly divided into fresh replicate flasks based on cell counts with cell numbers per flask determined using haemocytometer counts.

Cultures were visually checked daily and the ASP-8A media (Appendices Table 1) changed weekly.

Table 5.1 Cultured *Symbiodinium* spp. and the invertebrate host from which the algae were originally isolated, including geographic origins and ITS2 type

Culture ID	*Symbiodinium* sp.	Invertebrate Host	Geographic origin	*Symbiodinium* ITS2 type
CCMP2466	*Symbiodinium goreaui*	*Discosoma sancti-thomae*	Jamaica, Caribbean	C1
708	*Symbiodinium cariborum*	*Plexaura kuna*	San Blas, Caribbean	A1.1
Cx	*Symbiodinium microadriaticum*	*Cassiopea xamachana*	Jamaica, Caribbean	A1
Zs	*Symbiodinium pilosum*	*Zoanthus sociatus*	Jamaica, Caribbean	A2
702 (Pk702)	*Symbiodinium* sp.	*Plexaura kuna*	San Blas, Caribbean	B
707	*Symbiodinium pulchrorum or S. bermudense*	*Plexaura kuna*	San Blas, Panama, Caribbean	B1
Mv	*Symbiodinium kawagutii*	*Montipora verrucosa*	Kaneohe Bay, Hawaii, C. Pacific	F1
Sin	*Symbiodinium* sp.	*Sinularia sp.*	Guam, W. Pacific	F2

Restriction fragment length polymorphism (RFLP) of the small subunit RNA (ssRNA) gene (Section 2.4.2) and sequencing of the internal transcribed spacer 2 region (ITS 2) (Section 2.4.1) were used to determine the diversity and genetic classification of the cells based on current clade designations. Purified reaction product was directly ligated into pGEM-T Vector (Promega, USA) (Section 2.5.6) and transformed into NM522 cells (Section 2.6.6). Multiple clones were sequenced at the Australian Genome Research Facility using the forward primer M13F (5'GTTTTCCCAGTC ACG AC-3') and reverse primer M13R (5'CAGGAAACAGCTATGAC-3') (Section 2.5.14).

5.3.2 RNA and genomic DNA extractions

Symbiodinium C3 isolated from the blue morph (Dove, 2004) of the coral *Acropora aspera* collected from the reef flat at Heron Island (Great Barrier Reef (23^{o}33'S, 151^{o}54'E) (Section 2.1) in April 2009 were used to extract RNA and synthesize cDNA (Section 2.5.1.1 and 2.5.3). Genomic DNA was isolated from C3 cells ground to a fine powder under liquid nitrogen in a mortar with a pestle, using a DNeasy Plant Mini Kit (Qiagen, USA) (Section 2.5.4.1).

Total RNA from cultured *Symbiodinium* was isolated using the RNeasy Plant Mini extraction kit (Qiagen, Valencia USA) (Section 2.5.1.2). Complementary DNA (cDNA) amplification and genomic DNA (gDNA) elimination was performed with QuantiTect Reverse Transcription® Kit (Qiagen, USA) (Section 2.5.3) and total RNA analysed using 0.9 – 1.5 % agarose gels containing 2.2 M formaldehyde following the protocol of Sambrook and Russell (Sambrook and Russell, 2001)(Section 2.5.11). Genomic DNA from cultured *Symbiodinium* was isolated using the DNeasy Plant Mini extraction kit (Qiagen, Valencia USA) (Section 2.5.4.2) including the elution with 50 µL of buffer AE rather than 100 µL to increase DNA concentration. Amplification of cDNA and gDNA was performed using the standard C3 amplification protocol (Section 2.5.7) and reaction components (Table 2.1).

5.3.3 Southern Blot Analysis for *Symbiodinium* acpPC

Gene copy number for five acpPC genes (acpPCSym_1, _4, _8, _13 and _15) was investigated using Southern blot analysis. Ten µg of gDNA harvested from cultured C1 cells was digested with restriction endonucleases *BamH*I, *Hind*III, *Pvu*II, *Rsa*I and *Nco*I. Digestions included 20 units of restriction enzyme, 5 µL of recommended 10X buffer, 100X BSA (10 mg mL^{-1}; 0.5 µL) if required, and Milli-Q water in 50 µL reactions. Digestion reactions were incubated for 24 h at 37^{o}C according to the manufacturer's protocol and gDNA separated on 0.8 % agarose gel run at 20 V for 13.5 h then 80 V for 4.5 h. Genomic DNA was transferred and hybridized according to Section 2.5.13. Southern blots were probed with sequence specific PCR product for acpPCSym_1, _4, _8, _13 and _15 (Table 5.2), labelled using dATP 5' – [α-^{32}P] and visualized using a PhosphorImager Storm 860 by Molecular Dynamics (GE Healthcare Life Science, UK).

5.3.4 Protein extractions and analysis

Protein was extracted from C3 and cultured *Symbiodinium* cells using Lysing Matrix C tubes (MP Biomedicals Australia) (Section 2.6.1). A volume equivalent to 4 µg of protein was added to PBS buffer and 4x Laemmli buffer (Laemmli 1970) (Section 2.6.2) and proteins separated on 12.5 % polyacrylamide gel and transferred by electrophoresis to Immobilon™-P^{SQ} transfer membranes (Millipore, Australia) (Section 2.6.4). Immunodetection was performed using ECL Advance Western Blotting Detection Kit (Amersham, GE Healthcare, Life Sciences, UK)

according to the manufacturer's instructions and with the use of an antibody directed against the polypeptide encoding C3 acpPCSym_8, _10, _11, _12 and _13. The acpPC peptide antigen was designed, synthesized and conjugated by GenScript Corporation, USA.

Membranes were blocked with ECL Advance Western Blotting Detection Kit powder (Amersham, GE Healthcare, Life Sciences, UK) in phosphate-buffered saline (PBS) with Tween according to manufacturer's protocol for 2-3 h. Membranes were then incubated overnight with primary antibody diluted 1:1000 as described in the detection kit protocol. The primary antibody was detected by incubating membranes for 2 h in horse radish peroxidise (HRP) conjugated polyclonal swine anti-rabbit secondary antibody (DakoCytomation, Denmark) diluted 1:2000 according to the ECL protocol and detected with ECL detection reagents substrate (GE Healthcare, Life Sciences, UK) or using FAST™ 3,3'-Diaminobenzidine (DAB) Tablets (Sigma, USA). Membranes detected with ECL substrate were scanned using a Molecular Imager ChemiDoc XRS+ system (Bio-Rad Laboratories Inc, USA) for 10 min with images recorded every 30 s following the addition of the detection substrate.

5.3.5 Sequencing

Complementary DNA isolated from C1, A1, F1 and F2 isolates acclimated to ML conditions was amplified using the protocol documented in Section 2.5.7 with primers (Sigma Genosys, Australia) designed to bind either side of conserved transmembrane regions in C3 acpPCSym_1 and acpPCSym_4 (Table 5.2). Sequence data for acpPCSym_1 and acpPCSym_4 were selected based on positive cDNA amplification results (Table 5.3). Amplified cDNA was purified using QIAquick PCR Purification Kit (Qiagen, USA) (Section 2.5.9), ligated into pGEM-T Vector System (Promega, USA) (Section 2.5.6) and transformed into NM522 cells (Section 2.6.6). Cells were grown overnight at 37 ^{0}C on LB agar treated with ampicillin and sterile LB aliquots inoculated with single bacterial colonies containing acpPC sequence inserts. Cultures were grown overnight at 37 ^{0}C while shaking (260 rpm), cells collected by centrifugation (1 min at 10,000 x *g*), and plasmids purified using UltraClean™ 6 Minute Mini Plasmid Kit (Mo Bio Laboratories, Inc, USA). Four separate clones of sub-clades C1, A1, F1 and F2 were sequenced at the Australian Genome Research Facility and resulting nucleotide sequences trimmed, assembled and aligned (Section 2.5.14).

Table 5.2 *Symbiodinium* sp. C3 acpPC primers used to: amplify cDNA and gDNA extracted from cultured cells, sequencing of acpPCSym_1 and acpPCSym_4, and Southern blot probes.

Gene	Primer Name and Sequence	Product size
acpPCSym_1	F1: TCC CAC TCA GCG CCC AAG ACT	427 bp
	R1_rt: GAG AAA GGC CAA TGT CAA AGC AAC C	

acpPCSym_4	F1: GCT ATG GCT CCT TTG GGC TTC TTT	319 bp
	R1: GGT TCA GGC CAA GGG GGT CAC	
acpPCSym_8	F1: TGA CTG GAA GTG CTT GGG GTG AC	395 bp
	R2: TCC TGC TGC AGC TGG GGT TC	
acpPCSym_10	F1: TGG GCC AAC TAC ACA GCA TCT CC	438 bp
	R1_rt: TTC GGC ATT GAG CTT CTT GGT TTT C	
acpPCSym_13	F1_rt: GGC CGC CGG GTA TCA CAT CA	490 bp
	3utr: GCC ATC CGC CAT CCC ATC T	
acpPCSym_15	F1: CAT GAC GTG GTG AAG GGA CTG C	672 bp
	R1: CTG AGG AAT GAA TGG GAA GTG GTT	
acpPCSym_17	F1: CGG CAG CAC TTT GGG CGT TAT C	1165 bp
	R1: CAT CTC CTT CTC CTT CTC GGG TTC C	
acpPCSym_18	F1: CCT GGG CTT CTC TGA TAC CTT TGA T	373 bp
	R1: GGC CAG CCG ACC ATT ATT CA	

5.3.6 Accession Numbers

Sequence data for *Symbiodinium* sp. C3 acpPC referred to in Chapter 5 is available in the EMBL/GenBank data libraries under accession numbers FN646412, FN646414 - FN646418, FN646421 - FN646425 and discussed in detail in Chapter 3.

5.4 Results

5.4.1 Amplification of *Symbiodinium* sp. C3 acpPC complementary DNA sequences in cultured *Symbiodinium*

Using a standard C3 cDNA amplification protocol, primers for acpPCSym_4 returned a positive PCR result in 69 % of the cultured *Symbiodinium* while primers for acpPCSym_1 and _8 returned positive results in 56 % and 52 % of the cultured *Symbiodinium* respectively (Table 5.3, Figure 5.1). Results for five other acpPC genes were 39 % (acpPCSym_10), 22 % (acpPCSym_15), 17 % (acpPCSym_13) and 13 % (acpPCSym_17 and _18) (Table 5.3). Of the eight *Symbiodinium* types tested C1 returned a positive PCR result with each C3 primer set (Table 5.3, Figures 5.1a and 5.1b). Primers for acpPCSym_8 and _10 amplified products of two sizes, which were expected because acpPCSym_8 and _10 are bipartite polypeptides, have 79 % to 80 % identity at the cDNA level and the primers used bind within both polypeptides (Chapter 3; Table 3.3). acpPCSym_11 and _12 primers were not tested due to the high identity at the cDNA level of the acpPCSym_11 monomer and the two polypeptides from acpPCSym_12 (96 - 98 %) (Chapter 3; Table 3.3); differentiation of acpPCSym_11 and _12 is based on sequence data in the 3’ and 5’ regions.

C3 acpPCSym_4 primers amplified template from all cells except A2. Positive PCR results were returned for acpPCSym_4 with template from clade B and sub-clade F2 cells acclimated to HL, ML and LL; B1 cells acclimated to ML and LL conditions; A1.1 and F1 cells acclimated to HL and ML; and A1 cells from the ML conditions (Table 5.3, Figure 5.1c). Similar to acpPCSym_4, primers for acpPCSym_1 did not amplify A2 template from HL, ML or LL conditions; positive results were obtained with A1.1 and F2 cells from each light level; A1 cells acclimated to ML; B cells to LL; and F1 cells acclimated to HL and ML conditions (Table 5.3, Figure 5.1b). Apart from the C1 cells, acpPCSym_17 and _18 did not return a positive PCR result and acpPCSym_13 and _15 only returned positive results with template from clade F isolates. The percentage of positive PCR results with acpPC_8 primers was similar to acpPC_1 however the results were achieved with a greater diversity of *Symbiodinium*. Furthermore, acpPCSym_8 primers primarily amplified template extracted from ML or HL acclimated cells, except F2. In comparison C3 acpPCSym_10 primers generally amplified template from cells acclimated to LL.

The variability in results for *Symbiodinium* isolates from lineages other than clade C could be due to a number of factors. It is plausible that the different *Symbiodinium* lineages and sub-clades within a lineage express different acpPC genes and that expression of these genes may vary depending on the light environment. In addition the genes encoding the LHC proteins may vary sufficiently that the C3 primers used did to anneal efficiently to result in a positive PCR result. With this study, it is also probable that the amplification protocol, along with the primers, which were optimized for C3 isolates extracted from a coral host, requires clade specific optimization. Additional factors that may have contributed to the varying results include the use of ASP-8A media. C1 cells were provided in L1 medium with all other cell cultures were obtained in f/2 medium. ASP-8A media was selected in order to compare results with previous studies (Blank, 1987, Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a), and this was the justification for the three light levels also.

Sustaining sufficient cell numbers at each light level and for all eight *Symbiodinium* types was problematic and affected a number of results. The LL condition negatively affected growth of C1 and B cells but was most detrimental to F1 cells. The inconsistent and very slow growth of C1, B and F1 cells under LL conditions prevented sufficient cell numbers for replicate comparisons, while the HL condition negatively affected *Symbiodinium* A1.1 growth.

Table 5.3 cDNA amplification results using C3 acpPC primers with template from eight *Symbiodinium* spp. acclimated to low light (LL), medium light (ML) and high light (HL) conditions

	C1			A1.1			A1			A2			B			B1			F1[a]			F2	
LHC Name	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	HL	ML	LL
acpPCSym_1	✓	✓	✓	✓	✓	✓	×	✓	×	×	×	×	×	×	✓	×	×	×	✓	✓	✓	✓	✓
acpPCSym_4	✓	✓	✓	✓	✓	×	×	✓	×	×	×	×	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓
acpPCSym_8	✓	✓	✓	×	✓	×	✓	✓	×	✓	✓	×	✓	✓	×	×	✓	×	✓	×	×	×	×
acpPCSym_10	✓	✓	✓	×	×	×	×	✓	✓	×	×	✓	×	×	✓	×	×	✓	×	×	×	×	✓
acpPCSym_13	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	✓	×	×	×	×
acpPCSym_15	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	✓	×	✓	×
acpPCSym_17	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
acpPCSym_18	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×

[a]F1 cells could not be grown successfully under low light (LL) conditions

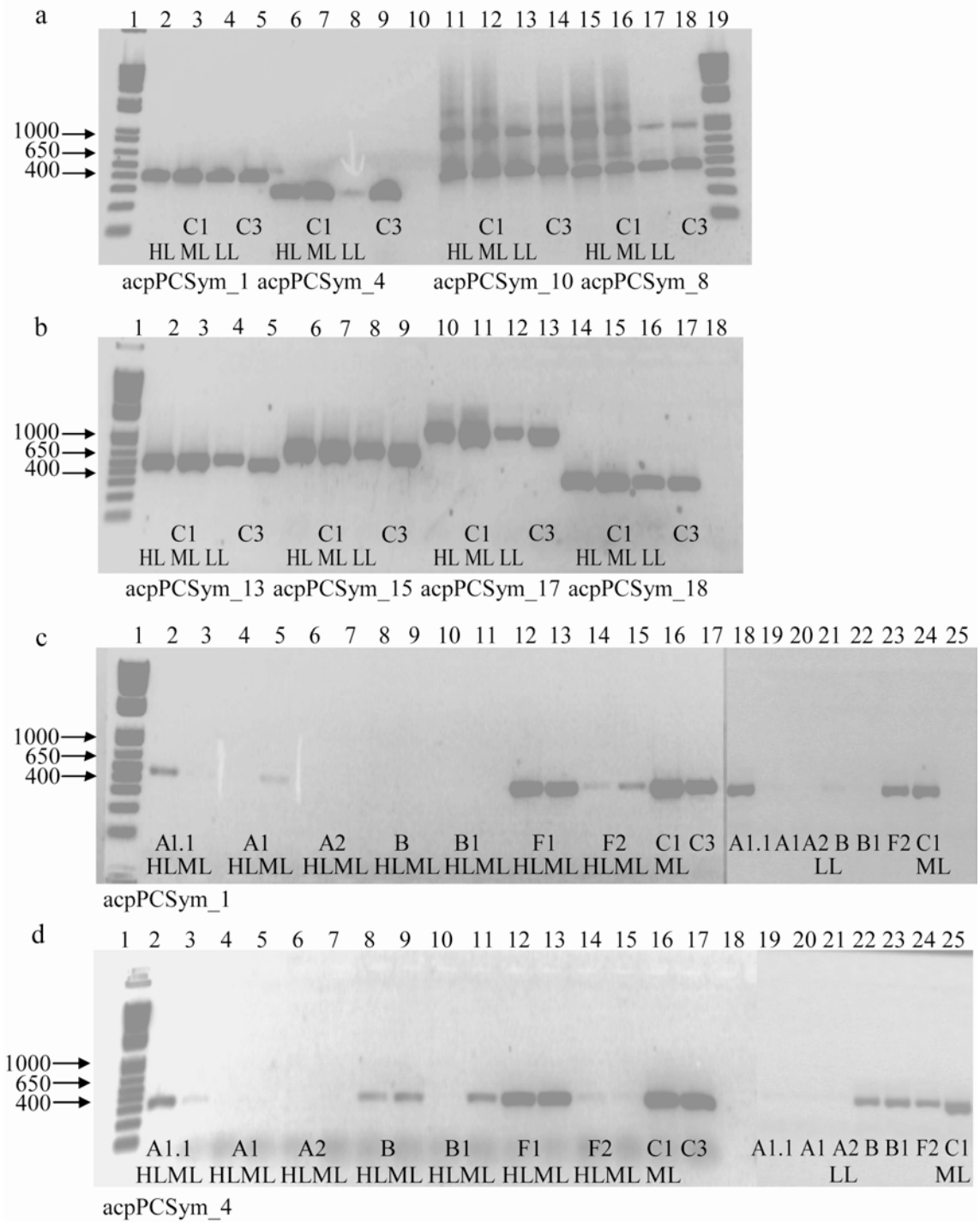


Figure 5.1 Complementary DNA template extracted from *Symbiodinium* cells acclimated to three light levels; low-light (LL; 10 – 15 µmol quanta m^{-2} s^{-1}), medium-light (ML; 80 µmol - 100 µmol quanta m^{-2} s^{-1}) and high-light (HL; 250 – 350 µmol quanta m^{-2} s^{-1}) and amplified with primers designed specifically to *Symbiodinium* C3 cDNA sequence. (a) Amplified cDNA extracted from *Symbiodinium* C1 and C3 using acpPCSym_1 (lanes 2 – 5), _4 (lanes 6 – 9), _10 (lanes 11 – 14), _8 (lanes 15 – 18) primers designed to *Symbiodinium* C3 cDNA. Lane 10 is blank; lanes 1 and 19 are a 1 Kb Plus DNA Ladder with molecular markers

to the left in base pairs. (b) Amplified cDNA extracted from *Symbiodinium* C1 and C3 using acpPCSym_13 (lanes 2 – 5), _15 (lanes 6 – 9), _17 (lanes 10 - 13) and _18 (lanes 14 - 17) primers designed to *Symbiodinium* C3 cDNA. Lane 1 is a 1 Kb Plus DNA Ladder with molecular markers to the left in base pairs and lane 18 is a negative control for (a) and (b). (c) Amplified cDNA extracted from *Symbiodinium* A1.1 (lanes 2, 3, 18), A1 (lanes 4, 5, 19), A2 (lanes 6, 7, 20), B (lanes 8, 9, 21), B1 (lanes 10, 11, 22), F1 (lanes 12, 13) and F2 (lanes 14, 15, 23) using acpPCSym_1 primers designed to *Symbiodinium* C3 cDNA. *Symbiodinium* F1 cells acclimated to LL conditions were not included due to poor growth. *Symbiodinium* C1 cells acclimated to ML conditions (lanes 16, 24) and *Symbiodinium* C3 (lane 17) were used as positive controls. Lanes 25 is a negative control and lane 1 is a 1 Kb Plus DNA Ladder with molecular markers to the left in base pairs. (d) Amplified cDNA extracted from *Symbiodinium* A1.1 (lanes 2, 3, 19), A1 (lanes 4, 5, 20), A2 (lanes 6, 7, 21), B (lanes 8, 9, 22), B1 (lanes 10, 11, 23), F1 (lanes 12, 13) and F2 (lanes 14, 15, 24) using acpPCSym_4 primers designed to *Symbiodinium* C3 cDNA. *Symbiodinium* C1 acclimated to ML conditions (lanes 16, 25) and *Symbiodinium* C3 (lane 17) were used as positive controls. Lane 18 is a negative control and lane 1 is a 1 Kb Plus DNA Ladder with molecular markers to the left in base pairs.

5.4.2 Amplification of *Symbiodinium* sp. C3 acpPC genomic DNA sequences in cultured *Symbiodinium*

As an explorative test eight primer sets (Table 5.2) designed to amplify C3 acpPC cDNA were used with gDNA extracted from *Symbiodinium* acclimated to varying light conditions. PCR results for gDNA (Table 5.4) varied considerably from cDNA results (Table 5.3) as expected. Positive PCR results were returned with 100 % of the C1 cDNA samples but only acpPCSym_1, _4, _8, _10 and _15 returned positive PCR results with gDNA from C1 cells acclimated to HL and ML conditions (Table 5.4). These same primer sets returned positive results with gDNA from A1.1 cells acclimated to HL, ML and LL conditions. Overall PCR products were detected in 83 % of samples using C3 acpPCSym_8 primers; 74 % with acpPCSym_4; and 65 % of samples with acpPCSym_10 and _15 primers. Of the remaining primer sets, acpPCSym_1 amplified gDNA from 39 % of samples; acpPCSym_17 in 17 %; and acpPCSym_13 and _18 in 9 % of samples (Table 5.4).

Table 5.4 gDNA amplification results using C3 acpPC primers with template from eight *Symbiodinium* spp. acclimated to low light (LL), medium light (ML) and high light (HL) conditions

	C1			A1.1			A1			A2			B			B1			F1[a]			F2	
LHC Name	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	LL	HL	ML	HL	ML	LL
acpPCSym_1	✓	✓	×	✓	✓	✓	✓	×	×	×	×	×	×	×	×	✓	×	×	✓	✓	×	×	×
acpPCSym_4	✓	✓	×	✓	✓	✓	×	×	×	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×
acpPCSym_8	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	×
acpPCSym_10	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	✓	✓	✓	✓	×	×	×	×	×
acpPCSym_13	×	×	×	×	×	×	×	×	×	×	×	×	×	✓	✓	×	×	×	×	×	×	×	×
acpPCSym_15	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	×	×	×	×	×	✓	✓	×
acpPCSym_17	×	×	×	×	×	×	✓	✓	✓	×	×	×	×	×	✓	×	×	×	×	×	×	×	×
acpPCSym_18	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	✓	✓	×	×	×

[a]F1 cells could not be grown successfully under low light (LL) conditions

5.4.3 Gene Copy Variation

Genomic DNA isolated from cultured *Symbiodinium* sp. C1 cells and digested with restriction endonucleases was used to investigate acpPC copy numbers. With the exception of acpPCSym_8 which was cut in three places, restriction endonucleases used to digest C1 gDNA possessed single cut sites within each protein. Sequence specific PCR products used to probe northern blots (Chapter 3; Figures 3.4 and 3.5) were used to probe Southern blots (Table 5.2). Results suggest the *Symbiodinium* C1 genome contains a single copy of the acpPCSym_1 gene (Figure 5.2; lane 3) and multiple copies of the acpPCSym_4 (Figure 5.2; lane 5), _8 (Figure 5.2; lanes 9 and 10) and _15 genes (Figure 5.2; lane 7). The acpPCSym_15 probe contained a single enzyme cutting site within the probe; therefore the actual gene copy number for acpPCSym_15 may be at least half that revealed in the Southern blot. Identifying and selecting optimal restriction endonucleases proved challenging for acpPCSym_13 which encodes a tripartite polypeptide, and acpPCSym_8 which is a bipartite polypeptide. Even with restriction enzymes cutting acpPCSym_8 at three sites, and within the probe, results suggest the existence of many acpPCSym_8 copies within the genome. Alternatively, the multiple hybridization bands could have been the result of additional enzyme cutting sites located in the intron regions of acpPC genes and the single band for acpPCSym_1 could potentially result from the enzyme not cutting the DNA. The acpPCSym_13 control probe produced a strong hybridization result although probing of digested gDNA was less conclusive with no clear hybridization bands identified (Figure 5.2, lane 12). The restriction enzyme used for acpPCSym_13 cut at a single site close to the 5’ end of the acpPCSym_13 probe and this may have affected the results. In the absence of *Symbiodinium* genomic data, verification of potential cut sites within intron regions and identifying alternative cutting enzymes is not yet possible for these genes. Consequently, results suggested from this study require further investigation.

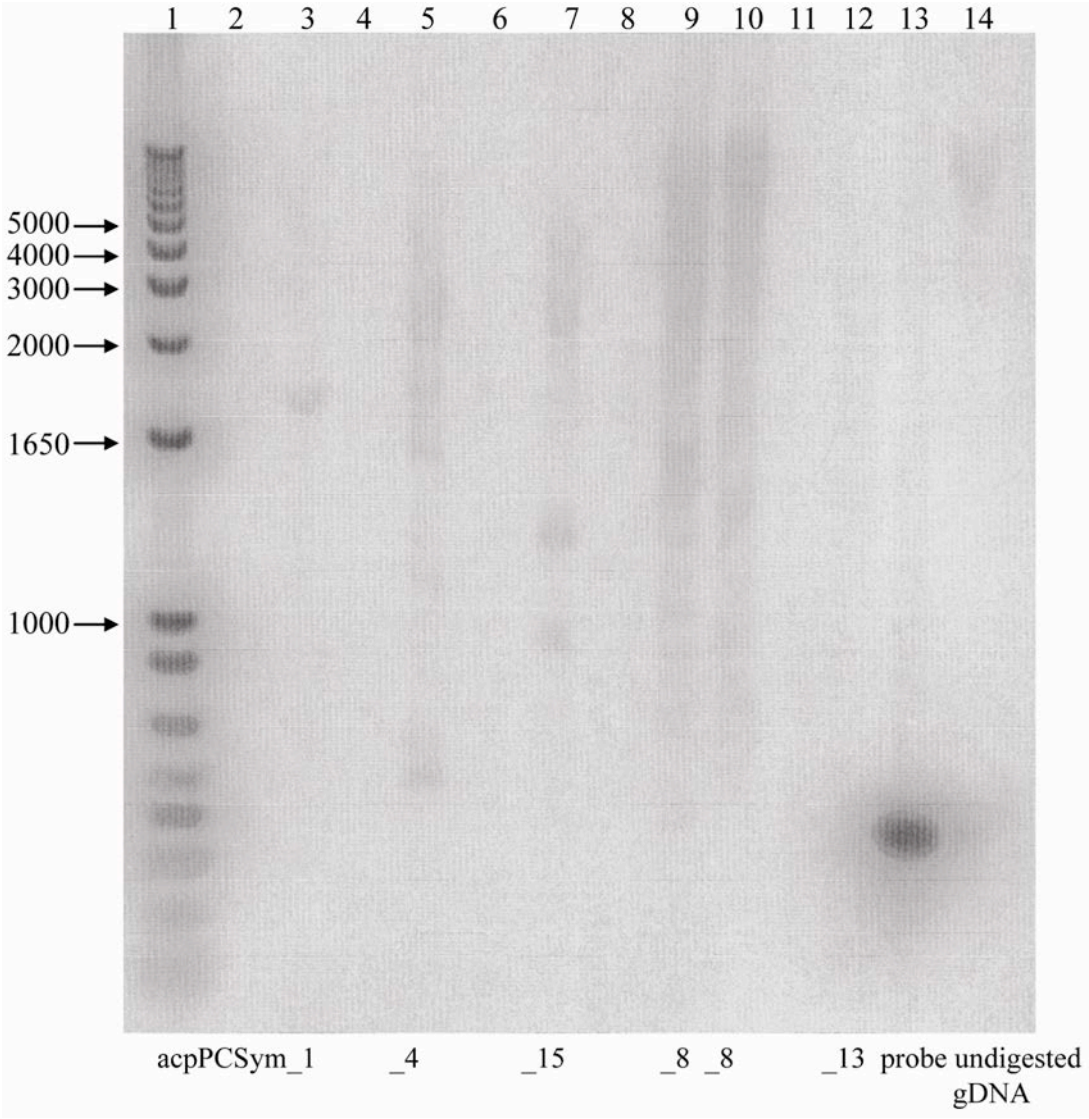


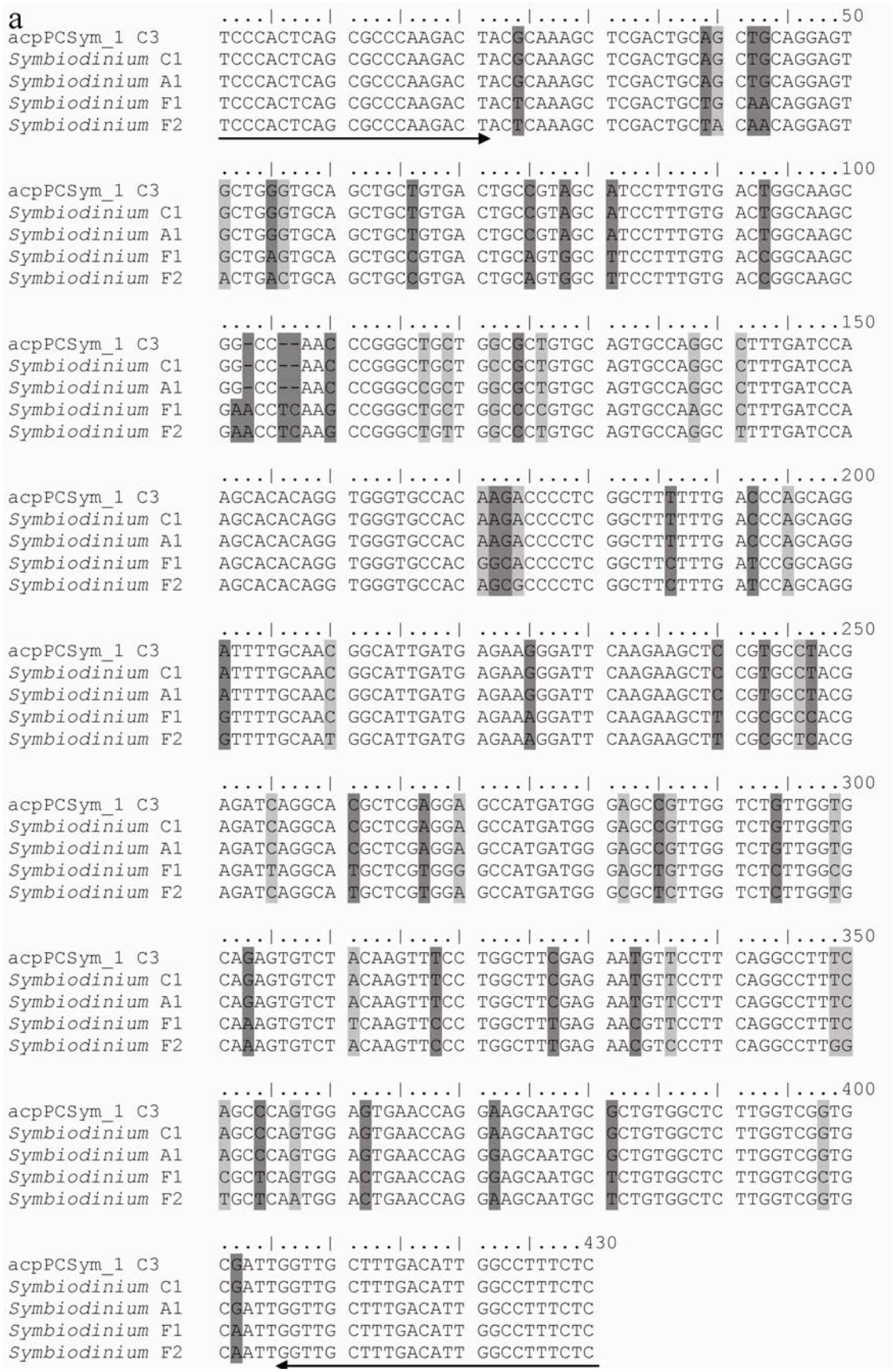
Figure 5.2 Southern blot analysis of *Symbiodinium* sp. C1 gDNA. Southern blot probed with sequence specific PCR product for *Symbiodinium* acpPCSym_1 (lane 3), acpPCSym_4 (lane 5), acpPCSym_15 (lane 7), acpPCSym_8 (lanes 9 and 10), acpPCSym_13 (lane 12) labelled using dATP 5' – [α-^{32}P]. Equal quantities (10 µg) of gDNA from *Symbiodinium* sp. C1 were applied. Lanes 2, 4, 6, 8, and 11 are blanks. Lane 1 is a 1 Kb Plus DNA Ladder with molecular markers to the left in base pairs. Lane 13 is the acpPCSym_13 probe control and lane 14 undigested gDNA.

5.4.4 cDNA sequence comparison

Sequence data for C1, A1, F1 and F2 *Symbiodinium* acclimated to ML conditions (~80 µmol - 100 µmol quanta m^{-2} s^{-1}) was aligned with C3 acpPCSym_1 (Figure 5.3a) and acpPCSym_4 (Figure 5.3b). Alignments demonstrated minimal sequence variation between the four cultured sub-clades and C3 extracted from a coral host. Sequence data for F1 and F2 encode three nucleotide insertions (Figure 5.3a; base pair 103, 106 and 107) not evident in acpPCSym_1 from C3, C1 and A1 (Figure 5.3a). Sequence identity for C3 and C1 acpPCSym_1 and _4

differed by a single nucleotide (Figure 5.3a base pair 122 and Figure 5.3b base pair 105 respectively). Furthermore, C3 and A1 acpPCSym_1 differed by a single nucleotide (Figure 5.3a; base pair 117) while four nucleotides differed between C3 and A1 acpPCSym _4 (Figure 5.3b; base pairs 105, 144, 186 and 263). Sequence data for F1 and F2 acpPCSym_1 demonstrated 89 % and 88 % identity with C3; representing a difference of 44 and 47 nucleotides respectively. Both F1 and F2 varied from C3 acpPCSym_4 by 21 nucleotides (93 % identity).

High sequence similarity between sub-clades C3 and cultured C1, Al, F1 and F2 cells suggests *Symbiodinium* from diverse lineages encode similar light-harvesting genes. The *Symbiodinium* C3 acpPCSym_1 primers amplify a section of the first conserved transmembrane helix while acpPCSym_4 primers amplify sequence for the first and second transmembrane helices. In comparison, primer sets designed to C3 acpPCSym_8, _10, _13, _15, 17 and _18 amplify regions within the third transmembrane helix, or non-conserved regions between the transmembrane regions, or 3' regions of C3 acpPC sequences. These findings indicate that it is possible to use *Symbiodinium* C3 acpPC sequence data to extract information from clades and sub-clades for which no sequence data is publicly available with primers designed to regions encoding the first and second conserved transmembrane helices.


a
....|....| ....|....| ....|....| ....|....| ....|....50
acpPCSym_1 C3 TCCCACTCAG CGCCCAAGAC TACGCAAAGC TCGACTGCAG CTGCAGGAGT
Symbiodinium C1 TCCCACTCAG CGCCCAAGAC TACGCAAAGC TCGACTGCAG CTGCAGGAGT
Symbiodinium A1 TCCCACTCAG CGCCCAAGAC TACGCAAAGC TCGACTGCAG CTGCAGGAGT
Symbiodinium F1 TCCCACTCAG CGCCCAAGAC TACTCAAAGC TCGACTGCTG CAACAGGAGT
Symbiodinium F2 TCCCACTCAG CGCCCAAGAC TACTCAAAGC TCGACTGCTA CAACAGGAGT
....|....| ....|....| ....|....| ....|....| ....|....100
acpPCSym_1 C3 GCTGGGTGCA GCTGCTGTGA CTGCCGTAGC ATCCTTTGTG ACTGGCAAGC
Symbiodinium C1 GCTGGGTGCA GCTGCTGTGA CTGCCGTAGC ATCCTTTGTG ACTGGCAAGC
Symbiodinium A1 GCTGGGTGCA GCTGCTGTGA CTGCCGTAGC ATCCTTTGTG ACTGGCAAGC
Symbiodinium F1 GCTGAGTGCA GCTGCCGTGA CTGCAGTGGC TTCCTTTGTG ACCGGCAAGC
Symbiodinium F2 ACTGACTGCA GCTGCCGTGA CTGCAGTGGC TTCCTTTGTG ACCGGCAAGC
....|....| ....|....| ....|....| ....|....| ....|....150
acpPCSym_1 C3 GG-CC--AAC CCGGGCTGCT GGCGCTGTGC AGTGCCAGGC CTTTGATCCA
Symbiodinium C1 GG-CC--AAC CCGGGCTGCT GCCGCTGTGC AGTGCCAGGC CTTTGATCCA
Symbiodinium A1 GG-CC--AAC CCGGGCCGCT GGCGCTGTGC AGTGCCAGGC CTTTGATCCA
Symbiodinium F1 GAACCTCAAG CCGGGCTGCT GGCCCCGTGC AGTGCCAAGC CTTTGATCCA
Symbiodinium F2 GAACCTCAAG CCGGGCTGTT GGCCCTGTGC AGTGCCAGGC TTTTGATCCA
....|....| ....|....| ....|....| ....|....| ....|....200
acpPCSym_1 C3 AGCACACAGG TGGGTGCCAC AAGACCCCTC GGCTTTTTTG ACCCAGCAGG
Symbiodinium C1 AGCACACAGG TGGGTGCCAC AAGACCCCTC GGCTTTTTTG ACCCAGCAGG
Symbiodinium A1 AGCACACAGG TGGGTGCCAC AAGACCCCTC GGCTTTTTTG ACCCAGCAGG
Symbiodinium F1 AGCACACAGG TGGGTGCCAC GGCACCCCTC GGCTTCTTTG ATCCGGCAGG
Symbiodinium F2 AGCACACAGG TGGGTGCCAC AGCGCCCCTC GGCTTGTTTG ATCCAGCAGG
....|....| ....|....| ....|....| ....|....| ....|....250
acpPCSym_1 C3 ATTTTGCAAC GGCATTGATG AGAAGGGATT CAAGAAGCTC CGTGCCTACG
Symbiodinium C1 ATTTTGCAAC GGCATTGATG AGAAGGGATT CAAGAAGCTC CGTGCCTACG
Symbiodinium A1 ATTTTGCAAC GGCATTGATG AGAAGGGATT CAAGAAGCTC CGTGCCTACG
Symbiodinium F1 GTTTTGCAAC GGCATTGATG AGAAAGGATT CAAGAAGCTT CGCGCCCACG
Symbiodinium F2 GTTTTGCAAT GGCATTGATG AGAAAGGATT CAAGAAGCTT CGCGCTCACG
....|....| ....|....| ....|....| ....|....| ....|....300
acpPCSym_1 C3 AGATCAGGCA CGCTCGAGGA GCCATGATGG GAGCCGTTGG TCTGTTGGTG
Symbiodinium C1 AGATCAGGCA CGCTCGAGGA GCCATGATGG GAGCCGTTGG TCTGTTGGTG
Symbiodinium A1 AGATCAGGCA CGCTCGAGGA GCCATGATGG GAGCCGTTGG TCTGTTGGTG
Symbiodinium F1 AGATTAGGCA TGCTCGTGGG GCCATGATGG GAGCTGTTGG TCTCTTGGCG
Symbiodinium F2 AGATCAGGCA TGCTCGTGGA GCCATGATGG GCGCTCTTGG TCTCTTGGTG
....|....| ....|....| ....|....| ....|....| ....|....350
acpPCSym_1 C3 CAGAGTGTCT ACAAGTTTCC TGGCTTCGAG AATGTTCCTT CAGGCCTTTC
Symbiodinium C1 CAGAGTGTCT ACAAGTTTCC TGGCTTCGAG AATGTTCCTT CAGGCCTTTC
Symbiodinium A1 CAGAGTGTCT ACAAGTTTCC TGGCTTCGAG AATGTTCCTT CAGGCCTTTC
Symbiodinium F1 CAAAGTGTCT TCAAGTTCCC TGGCTTTGAG AACGTTCCTT CAGGCCTTTC
Symbiodinium F2 CAAAGTGTCT ACAAGTTCCC TGGCTTTGAG AACGTCCCTT CAGGCCTTGG
....|....| ....|....| ....|....| ....|....| ....|....400
acpPCSym_1 C3 AGCCCAGTGG AGTGAACCAG GAAGCAATGC GCTGTGGCTC TTGGTCGGTG
Symbiodinium C1 AGCCCAGTGG AGTGAACCAG GAAGCAATGC GCTGTGGCTC TTGGTCGGTG
Symbiodinium A1 AGCCCAGTGG AGTGAACCAG GGAGCAATGC GCTGTGGCTC TTGGTCGGTG
Symbiodinium F1 CGCTCAGTGG ACTGAACCAG GGAGCAATGC TCTGTGGCTC TTGGTCGCTG
Symbiodinium F2 TGCTCAATGG ACTGAACCAG GAAGCAATGC TCTGTGGCTC TTGGTCGGTG
....|....| ....|....| ....|....430
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Symbiodinium C1 CGATTGGTTG CTTTGACATT GGCCTTTCTC
Symbiodinium A1 CGATTGGTTG CTTTGACATT GGCCTTTCTC
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Symbiodinium F2 CAATTGGTTG CTTTGACATT GGCCTTTCTC

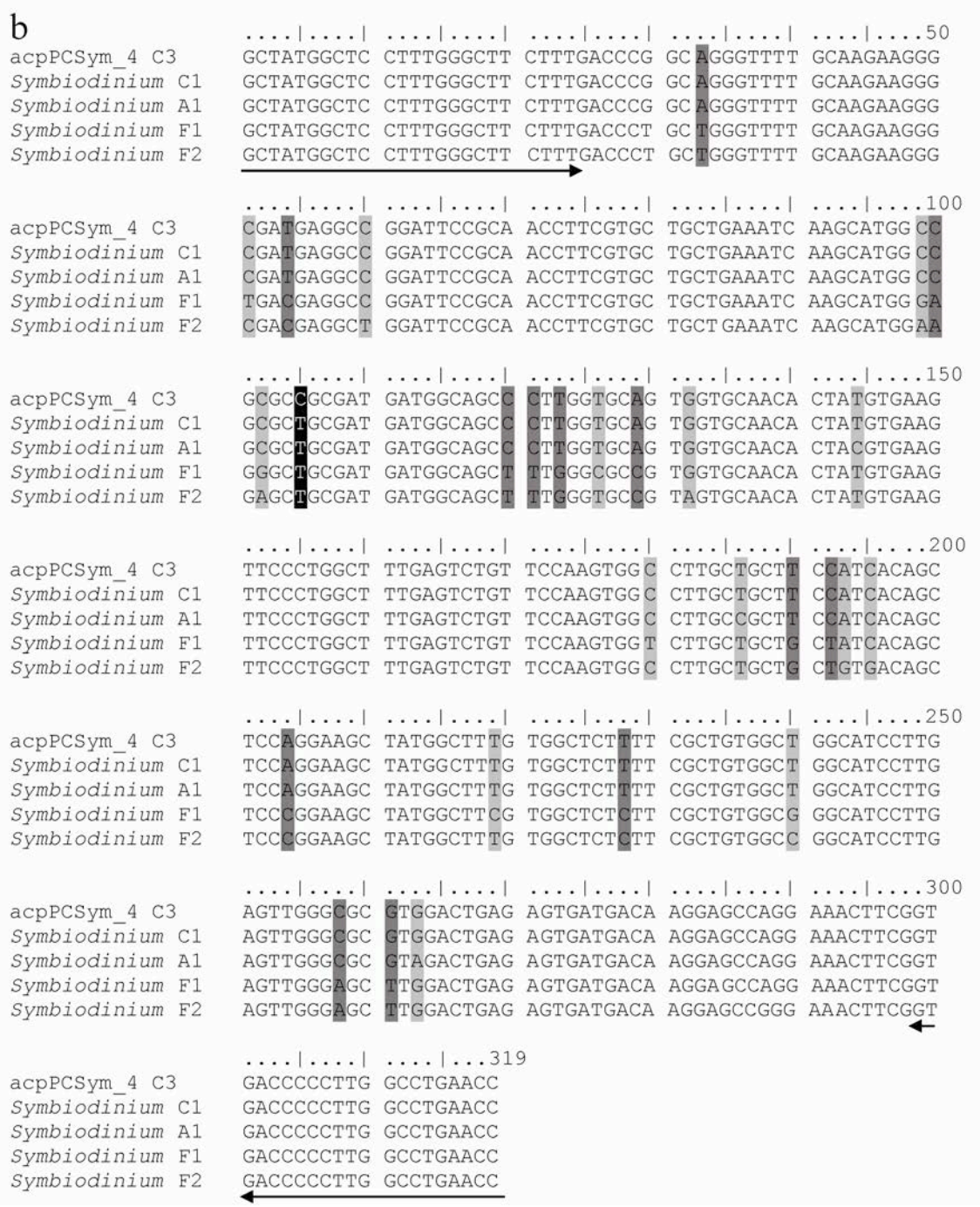

Figure 5.3 Comparison of cDNA nucleotide sequences amplified from *Symbiodinium* sub-clades C1, A1, F1 and F2 acclimated to a medium light level (ML; 80 µmol - 100 µmol quanta m^{-2} s^{-1}) using primers designed to *Symbiodinium* C3 and that amplify the integral light-harvesting proteins acpPCSym_1 (a) and acpPCSym_4 (b). Primers are indicated by arrows and shading highlights nucleotide variation. Light grey shading indicates a single nucleotide variation from acpPC C3 cDNA sequence in one *Symbiodinium* sequence, dark grey indicates a single nucleotide variation in two *Symbiodinium* sequences, and black indicates all four *Symbiodinium* sequences vary from the *Symbiodinium* C3 sequence.

5.4.5 Protein comparison

C3 acpPC was detected in a subset of *Symbiodinium* using immunoblot analysis and an antibody specific to *Symbiodinium* sp. C3 acpPCSym_8, _10 and _13. SDS-PAGE fractionated protein visualized by Coomassie blue staining suggest *Symbiodinium* A1, A1.1, A2 and B1 acclimated to LL conditions express increased levels of acpPC compared with cells acclimated to HL or ML conditions (Figure 5.4a and 5.4b). A prominent acpPC band at ~ 19.5 kDa was clearly visible in the LL A1, A2 (Figure 5.4a; lanes 4 and 10 respectively), and B1 (Figure 5.4b; lane 7) samples and detectable in HL and ML A1, A2, B, B1, C1 and F2 cells (Figure 5.4a – c). Two additional acpPC bands at ~ 18.4 and 21.2 kDa were detected in SDS-PAGE fractionations of A1 and A2 (Figure 5.4a) and immunoblots of clade B (Figure 5.4d). Three bands at ~ 18.4, ~ 19.5 and 21.2 kDa correspond with peptide sizes for C3 acpPCSym_8, _10, _11, _12 and _13 and although a prominent ~ 19.5 kDa band was clearly detected in *Symbiodinium* C1 two faint bands below and above ~ 19.5 kDa were evident also (Figure 5.4e).

The ~ 19.5 kDa acpPC band evident in this study and documented previously in acpPC expression studies (Takahashi et al., 2008), was visible in all sub-clades and each experimental light condition with the exception of A1.1 and F1 cells. No acpPC band was detected in HL A1.1 (Figure 5.4a; lane 5) and F1 cells (Figure 5.4c; land 2) however results could not be verified with replicate extractions due to experiment light conditions limiting cell growth. A very faint band corresponding in size to acpPC was detected A1.1, B1, F1 and F2 acclimated to ML, and B1 and C1 acclimated to HL. The prominent band at ~ 15 kDa in LL sub-clades A1, A1.1, A2, B1 and F2 potentially represents the monomeric form of PCP, the minor LHC utilized by *Symbiodinium*.

Problems encountered sustaining sufficient cell numbers for all *Symbiodinium* types and light conditions, and the harvesting of cells for cDNA and gDNA extraction prior to protein extractions, prevented replicate protein extractions for a number of *Symbiodinium* types and light conditions. For this reason the result for F1 and A1.1 isolates from HL conditions, clade B and sub-clade C1 isolates from LL conditions were excluded from ECL analyses.

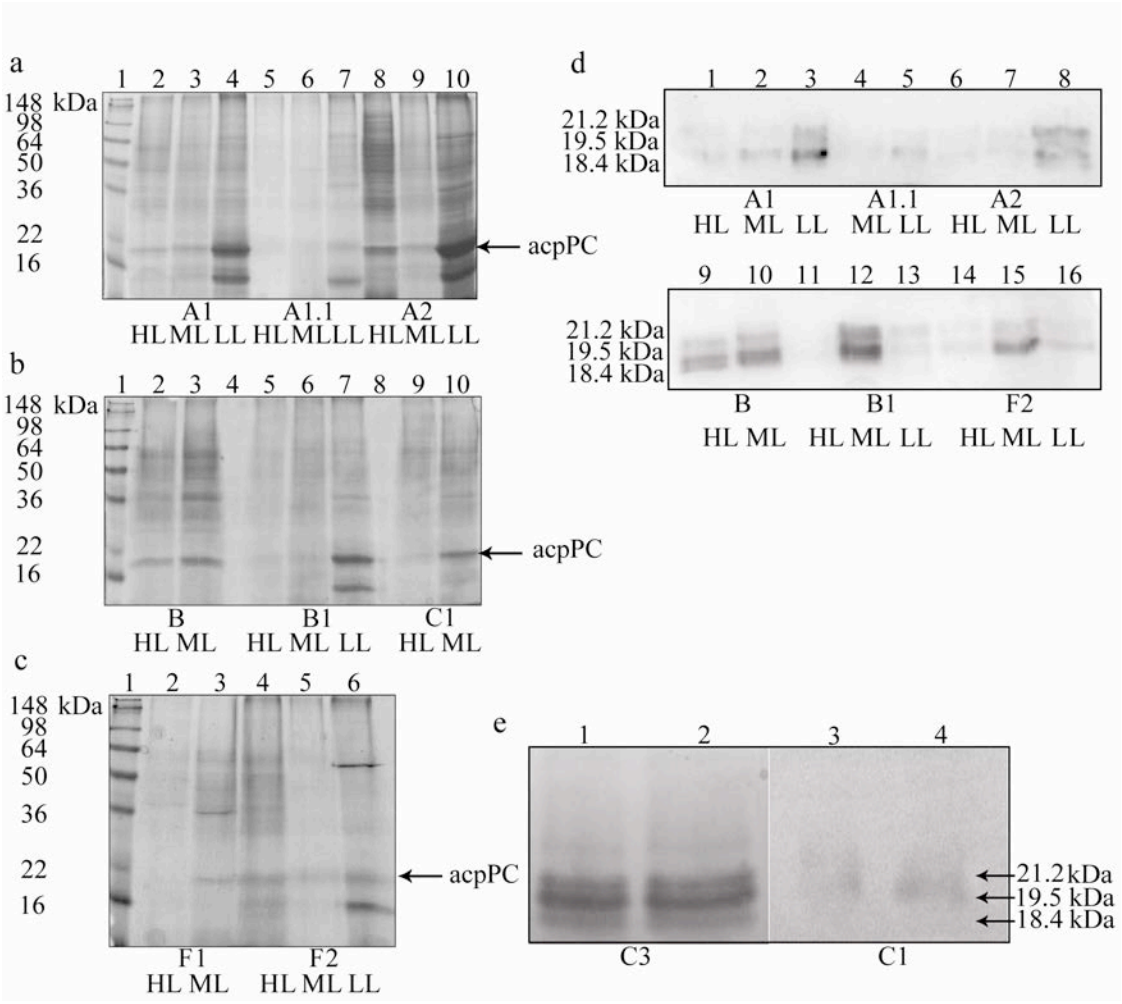

Figure 5.4 Protein extracts from *Symbiodinium* cells acclimated to three light levels; low light (LL; 10 – 15 µmol quanta m^{-2} s^{-1}), medium light (ML; 80 µmol - 100 µmol quanta m^{-2} s^{-1}) and high light (HL; 250 – 350 µmol quanta m^{-2} s^{-1}). Protein separated by SDS-PAGE and visualized by Coomassie blue staining, or transferred onto polyvinylidene fluoride membranes for immunoblot analysis. (a) Protein samples extracted from sub-clades A1 (lanes 2 – 4), A1.1 (lanes 5 – 7) and A2 (lanes 8 – 10) acclimated to HL, ML and LL conditions, (b) Clade B acclimated to HL and ML (lanes 2 and 3), sub-clade B1 acclimated to HL, ML and LL conditions (lanes 5 – 7) and sub-clade C1 acclimated to HL and ML (lanes 9 and 10). Lanes 4 and 8 were left blank. (c) *Symbiodinium* sub-clades F1 acclimated to HL and ML (lanes 2 and 3) and F2 cells acclimated to HL, ML and LL conditions (lanes 4 – 6). Molecular weight marker protein sizes are indicated beside Lanes 1 (a, b and c) and the position of acpPC isoforms indicated by an arrow (←acpPC). (d) Enhanced chemiluminescence detection of acpPC extracted (equivalent to 4 µg of protein) from *Symbiodinium* A1, A1.1, A2, B, B1 and F2. acpPC detected using rabbit anti-acpPC diluted 1:1000 and horse radish peroxidase conjugated polyclonal swine anti-rabbit Ig diluted 1:2000. (e) Immunoblot analysis showing SDS-PAGE fractionated *Symbiodinium* C3 isolated from a coral host and cultured *Symbiodinium* C1 whole

cell extracts immunostained with the acpPC antibody. Three prominent bands at 18.4, 19.5 and 21.2 kDa correspond with peptides for C3 acpPCSym_8, _10, _11, _12 and _13.

Table 5.5 Prominent peptides detected by *Symbiodinium* C3 acpPC specific antibody

Peptide Sequence (acpPC antibody)	LHC Name	Polypeptide Mass (kDa)	Polypeptide Size (aa)
LTGSAWGDWANY	acpPCSym_8	18.3	173
	acpPCSym_11	18.4	173
	acpPCSym_10	18.4	173
	acpPCSym_12	18.4	173
	acpPCSym_13	21.2	192
		19.5	178

Enhanced chemiluminescence (ECL) was used to detect acpPC in *Symbiodinium* using an acpPC antibody recognizing three *Symbiodinium* C3 peptides (~ 18.4, 19.5 and 21.2 k Da) (Table 5.5, Figure 5.4e). Results suggest acpPC expression is increased in sub-clades A1, A1.1 and A2 acclimated to LL conditions (Figure 5.4d; lanes 3, 5 and 8), and B, B1 and F2 cells acclimated to ML (Figure 5.4d; lanes 10, 12 and 15). Three acpPC peptides detectable in C3 cells (Figure 5.4e) were not clearly differentiated in cultured cells other than clade B cells (Figure 5.4d; lanes 9 and 10). The ~18.4 and 19.5 kDa peptides generally appeared as a single band in immunoblots and attempts to fractionate proteins using polyacrylamide gels > 12.5 % did not separate bands further (data not shown). Although acpPC expression was not quantified, results confirm the presence of C3 acpPC in *Symbiodinium* from at least three lineages (clade A, B and F), in addition to clade C. Furthermore, results suggest acpPC expression varies between clades and sub-clades acclimated to different light conditions. It is well recognised that cells acclimated to LL have significantly higher cellular concentrations of photosynthetic pigments than cells exposed to HL conditions (Chang et al., 1983, Iglesias-Prieto and Trench, 1994). Chlorophyll concentrations were not determined during this study however observable variations in cell colouration were evident between the light levels (Figure 5.5).

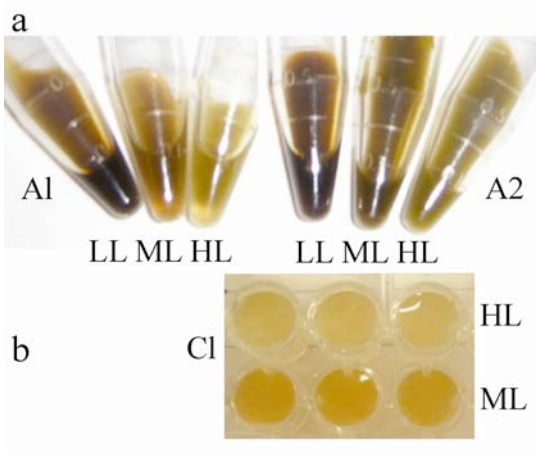

Figure 5.5 *Symbiodinium* A1, A2 and C1 cells acclimated to varying light conditions. (a) Total protein extracts from *Symbiodinium* A1 and A2 cells (~ 10 x 10^6 cells) acclimated to low-light (LL; 10 – 15 µmol quanta m^{-2} s^{-1}), medium-light (ML; 80 µmol - 100 µmol quanta m^{-2} s^{-1}) and high-light (HL; 250 – 350 µmol quanta m^{-2} s^{-1}) from which 4 µg was used for SDS-PAGE gels, western blot and enhanced chemiluminescence detection. (b) Medium-light (ML; 80 µmol - 100 µmol quanta m^{-2} s^{-1}) and high-light (HL; 250 – 350 µmol quanta m^{-2} s^{-1}) C1 cells (~ 4.5 x 10^5) harvested for chlorophyll *a* fluorescence measurements (Chapter 6).

5.5 Discussion

Symbiodinium from different lineages potentially encode and express a similar diversity of light-harvesting proteins as that evident in *Symbiodinium* sp. C3. Using sequence data for C3 chlorophyll *a*-chlorophyll c_2-peridinin complexes (acpPC) comparative analyses of *Symbiodinium* lineages is possible, although results are influenced by light and the region of the transcript amplified by the C3 primers. C3 and C1 are ancestral symbionts of the highly diverse clade C *Symbiodinium* group (LaJeunesse et al., 2010). C3 and C1 are considered generalist symbionts, associate with a variety of host organisms, and are widely distributed geographically (LaJeunesse, 2005). The success at amplifying C1 cDNA using primers designed to C3 sequences is therefore not surprising. The results provide confidence for future research endeavours using cultured C1 cells and information available from the C3 EST library (Leggat et al., 2007), particularly as C3 symbionts are not readily available in culture. An attempt at culturing C3 cells freshly extracted from *Acropora aspera* during this study was unsuccessful and a standard antibiotic treatment protocol (Section 2.3.3) used with established cultures caused high cell mortality (data not included).

The detection of a greater number of C3 acpPC genes in clade F cells (F1 and F2) was anticipated due to lineage relatedness (LaJeunesse, 2001), but the variation between sub-clades A1, A1.1 and A2 was not predicted; particularly as *Symbiodinium* A1 and A1.1 are closely related based on ITS 2 phylogenetic reconstructions (LaJeunesse, 2002). The percent of positive PCR results for acpPCSym_1 (56 %) and _4 (69 %) suggest a number of acpPC genes present

in C3 *Symbiodinium* are potentially core LHC genes common to *Symbiodinium* types and lineages. High cDNA sequence identity of acpPC_1 and _4 transcripts suggests sub-clades C3, C1, F1, F2 and A1 express similar, if not the same, acpPC genes (Figures 5.3a and 5.3b) even when extremely low PCR product is amplified (Figures 5.1c and 5.1d, lanes 5 and 15).

Results indicating the C1 *Symbiodinium* genome encodes highly expressed genes that are likely arranged in repeat tandem arrays (acpPCSym_8), as well as single copy genes (acpPCSym_1) and lesser expressed genes (acpPCSym_4 and acpPCSym_15) is not unusual for dinoflagellates (Bachvaroff and Place, 2008, Lee et al., 1993). *Amphidinium carterae* genomes contain both highly expressed tandem repeat genes and less expressed genes that contain significantly higher intron density (Bachvaroff and Place, 2008), the *Lingulodinium polyedrum* genome encodes approximately 5,000 identical copies of the PCP gene (Le et al., 1997) while *Symbiodinium* PCP is encoded by a much lower copy number (Reichman et al., 2003).

The highly expressed acpPCSym_8 gene encodes a bipartite polypeptide within a single transcript, and transcripts corresponding to four, six, eight and ten LHC polypeptides are evident (Chapter 3, Figure 3.4 and 3.5). In comparison, acpPCSym_1, _4 and _15 encode single peptides. While the acpPCSym_15 sequence clusters with those of rhodophytes, cryptophytes, dinoflagellate Chl *a/c* and *Symbiodinium* acpPC sequences, acpPCSym_1 and _4 cluster together with other *Symbiodinium* acpPC sequences (Chapter 3, Figure 3.1). Whether the function of the single acpPCSym_1 gene differs from closely related *Symbiodinium* LHCs with multiple copy numbers is yet to be determined, but acpPCSym_1 encodes repeats of the highly conserved membrane-spanning helices I and III and lacks helix II (Chapter 3; Table 3.2 and Figure 3.2a), a variation also evident in a *Heterocapsa triquetra* CAC sequence that clusters with the stress response LI818 and LI818-like genes (Chapter 3; Table 3.2 and Figure 3.2a).

The negative PCR results for A1.1 cells acclimated to LL with both acpPCSym_8 and _10 (Table 5.3) but detection of acpPC in immunoblot analyses of the same cells (Figure 5.4d, lane 5) suggests the C3 antibody detected acpPCSym_11 or _12; two genes not amplified using C3 primers due to high identity at the cDNA level. Similar results were noted for F2 cells acclimated to HL and ML conditions (Table 5.3; Figure 5.4d, lanes 14 and 15). Protein bands at ~ 18.4, ~ 19.5 and ~ 21.2 kDa correspond with C3 acpPCSym_8, _10, _11, _12 and two _13 peptides (Table 3.2 Chapter 3). Detection of the same bands in cultured *Symbiodinium* cells of different clades and sub-clades demonstrates that a number of C3 acpPC isoforms are expressed in divergent *Symbiodinium* lineages and similar band sizes have been detected in two clade A *Symbiodinium* species using an antibody specific to acpPC from *Amphidinium carterae*, a non-symbiotic dinoflagellate (Takahashi et al., 2008). Although protein expression was not quantified, expression varied between light levels and *Symbiodinium* types, and protein expression generally increased under LL or ML conditions compared with HL.

Non-optimal light conditions impact the energy capturing capabilities within a photosynthetic organism and LHCs and the pigments bound to the complexes vary when light conditions are not ideal. The absorption properties of acpPC from *Prorocentrum minimum* and *Heterocapsa pygmaea* (non-symbiotic dinoflagellates) grown under HL and LL conditions differ due to variations in cell pigmentation (Jovine et al., 1995), a variation also evident in PCP from *Symbiodinium* exposed to HL, ML or LL conditions (Iglesias-Prieto and Trench, 1997a). Under HL and LL light conditions the abundance of the two different PCP isoforms differs Iglesias-Prieto, 1997a} and species with apparent preferences for extreme light conditions or the ability to adapt to fluctuating light levels may possess only one of the isoforms, while other species may express both forms (Govind et al., 1990, Iglesias-Prieto and Trench, 1997a, Rowan and Powers, 1991a). Similar variations in acpPC isoform expression are anticipated and the validation that C3 sequence data can be used to identify acpPC from different clades and sub-clades enables more extensive investigation of *Symbiodinium* LHCs.

Research on plant and chlorophyte LHC gene expression and the impact of varying environmental conditions is significantly advanced compared to our current understanding of *Symbiodinium* LHCs. At present, *Symbiodinium* are known to optimize photosynthesis and adapt differently to varying environmental conditions (Chang et al., 1983, Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b, Banaszak and Trench, 1995, Lesser and Shick, 1989a). Physiological responses and photo-acclimatory capabilities of different *Symbiodinium* indicate that particular species photo-adapt to different light environments (Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a), and these varied responses to light conditions may be an important niche diversification characteristic for *Symbiodinium*-host associations (Iglesias-Prieto and Trench, 1994). Clade C symbionts form relationships with coral hosts across all depths (van Oppen et al., 2001), while in the Caribbean, clade A symbionts are particularly prevalent in shallow-water habitats (LaJeunesse, 2002) where light intensities are higher than those of deep-water habitats. Furthermore, clade A *Symbiodinium* are proposed to have enhanced capabilities which promote symbiont survival in HL environments in comparison to clades B, C, D and F (Reynolds et al., 2008), and particular clades play a role in temperature tolerance (Rowan, 2004), and susceptibility of the *Symbiodinium*-host symbiosis break-down (Sampayo et al., 2008).

Interpretations of the results from this study require caution due to the specific methodology used and results may actually under-represent acpPC from clades A, B and F. Variations between cDNA and gDNA template amplifications were likely due to primers crossing cDNA exon-exon boundaries while variation in amplicon size are predicted to be a result of introns. Without genomic sequence information for *Symbiodinium* C3, the identification of primer boundaries or presence of introns is hindered. Therefore, no conclusions have been postulated for the gDNA PCR results other than C3 primers designed to cDNA can provide positive PCR

results that may be of benefit to future studies and / or when genomic data for *Symbiodinium* becomes more readily available.

Whether symbiont genotype determines or influences *Symbiodinium* adaptability to particular light conditions is yet to be definitively answered. With genomic information just beginning to become publicly available and with limited transcriptomic data for *Symbiodinium,* determining whether sequence data from *Symbiodinium* sub-clade C3 can be used to identify acpPCs from *Symbiodinium* clades A- I, is a step closer to identifying and fully understanding how environmental cues impact photosynthetic processes in these ecologically important marine dinoflagellates.

5.6 Chapter 5 Summary Points

- Sub-clade C3 acpPC data can be utilized to investigate closely related *Symbiodinium* at the transcript and protein level, in particular *Symbiodinium* C1;
- Results suggest acpPCSym_1, _4 and _8, may potentially be core light-harvesting complex genes common to *Symbiodinium* types and lineages;
- Both low copy numbers and very high copy numbers of acpPC genes exist in the *Symbiodinium* genome; and
- *Symbiodinium* acclimated to varying light conditions demonstrate varied C3 acpPC expression at the protein level.

CHAPTER 6

6 Light-Harvesting Complex Gene Expression in *Symbiodinium* sp. in Response to Different Light Environments

6.1 Statement of Purpose

To answer the questions: how do *Symbiodinium* respond to distinct environmental cues, do specific components of the photosynthetic apparatus respond to the cues, do *Symbiodinium* mimic the response of other unicellular algae or that of higher plants, the expression of the major light-harvesting protein complex utilized by *Symbiodinium* was investigated under a variety of light conditions. Our current understanding of eukaryotic photosynthetic gene expression is predominately derived from plant and green unicellular algae such as *Arabidopsis* species and *Chlorella* species and although a few genes associated with dinoflagellate photosynthesis have been described; most of these genes have been characterized from non-symbiotic dinoflagellates. It is plausible to suggest that the response of symbiotic dinoflagellates to environmental cues will subtly vary from organisms already characterized considering the unique and diverse nature of these algae, but this has not been confirmed. In this study, quantitative real-time PCR (qRT-PCR) with normalization genes validated for *Symbiodinium* C3 (Chapter 4) was used to determine chlorophyll *a*-chlorophyll c_2-peridinin protein complex (acpPC) expression in *Symbiodinium* C1 cells acclimated to high and medium-light conditions prior to transfer to a different light environment for nine days. The efficiency of photosystem II (PSII) to perform photosynthesis was measured in order to determine whether acpPC mRNA transcripts correlated with variation in PSII efficiency during the experimental period.

6.2 Introduction

Dinoflagellates are a diverse group of eukaryotic algae that inhabit both marine and freshwater environments. Algae belonging to the genus *Symbiodinium* form important associations with a variety of marine organisms, including jellyfish, sea anemones, giant clams, flatworms, radiolarians and foraminifera (Trench, 1993, Glynn, 1996, Rowan, 1998, van Oppen et al., 2001, Lobban et al., 2002). *Symbiodinium* are best recognised for their mutualistic relationships with reef-building corals. Approximately half the describe dinoflagellate species are capable of performing photosynthesis (Keeling, 2009) and a key component of the *Symbiodinium*-coral relationship is the dinoflagellates' ability to perform photosynthesis. Dinoflagellates harbour a diversity of plastids; the organelles of photosynthetic cells, and a

number of dinoflagellate lineages contain plastids derived from other algae through tertiary or secondary endosymbiotic events (Keeling, 2004). Dinoflagellate plastids contain protein complexes that bind chlorophyll *a*, *c* and the carotenoid peridinin, and these pigments are associated with two unrelated light-harvesting protein complexes (LHCs); the water-soluble protein peridinin-chlorophyll *a* proteins (PCP) and the water-insoluble chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC). PCP has no sequence similarity to other known LHCs (Norris and Miller, 1994, Weis et al., 2002) and can exist in a monomeric or dimeric form (Prézelin and Haxo, 1976, Iglesias-Prieto et al., 1991, Norris and Miller, 1994, Sharples et al., 1996, Hiller et al., 2001, Weis et al., 2002). Conversely, acpPC demonstrates high sequence diversity and within *Symbiodinium* sp. C3, monomeric, dimeric and trimeric forms exist (Boldt et al., 2012). Furthermore, acpPCs exhibit similarities with Chl *a*-binding proteins of red algae and the fucoxanthin Chl *a/c*-binding proteins of haptophytes and heterokontophytes (Chapter 3) (Boldt et al., 2012).

To perform photosynthesis efficiently photoautotrophs require an optimal light environment. The process of photosynthesis converts light energy to chemical energy through a series of redox reactions that can be broken down into the light reactions and dark reactions. The light reactions are dependent on light and based around two photosystems, photosystem I (PSI) and photosystem II (PSII). PSI and PSII perform primary photochemistry involving the absorption of light energy by pigments bound to light-harvesting protein complexes and the transfer of energy to the photosystem reaction centre, where charge separation of electrons occurs (Huner et al., 1998, Kiang et al., 2007). In plants and chlorophytes the two major light-harvesting protein complexes are the Chl *a/b*-binding proteins, LHCI and LHCII, and these primarily associate with PSI and PSII respectively. The end products of photosynthesis are oxygen (O_2) and photosynthates, and these are an important source of organic compounds. Photosynthates are a vital component for the *Symbiodinium*-coral relationship; photosynthates provide the majority of the coral's metabolic energy requirements and in return *Symbiodinium* are provided with inorganic carbon and nutrients (Muscatine and Porter, 1977).

Light optimization can be challenging in the marine environment, particularly for *Symbiodinium* which are located within the endodermal tissue of the coral host (Muscatine et al., 1975). Photoautotrophs regularly experience daily and seasonal fluctuations in environmental conditions, however, when light is limited or in excess, imbalances can occur and result in PSII excitation pressure that affects an organism's photochemistry (Huner et al., 1998). When light energy absorption exceeds the capacity for carbon fixation, production and accumulation of reactive oxygen species (ROS) may damage cells and the PSII reaction centre (Niyogi, 1999, Tchernov et al., 2004, Suggett et al., 2008). Although damage to the reaction centre of PSII occurs under all conditions, the rate of damage is highest under excessive light conditions and is exacerbated with the addition of other stresses (Horton et al., 1996). The

balancing act required for efficient photosynthesis to occur suggests PSII excitation pressure, and thus the redox state of the electron transport system, which can be varied by changing a variety of environmental stresses including irradiance, temperature or nutrient availability, may be the driving factor behind gene regulation in photosynthetic organisms and represent a sensing or signalling system (Maxwell et al., 1995). To maintain efficient photosynthesis photoautotrophs must acclimate to changing light environments. *Symbiodinium* possess a range of photo-acclimation responses to light conditions (Tchernov et al., 2004, Robison and Warner, 2006, Suggett et al., 2008) including alterations to pigment concentrations and changes in the photosynthetic reaction centres (Porter et al., 1984, Iglesias-Prieto and Trench, 1994, Hennige et al., 2009). Low-light (LL) and high-light (HL) acclimated *Symbiodinium* demonstrate a decrease in PSII function when PSII repair is inhibited, however, the decrease in PSII function is greater in HL acclimated algae (Jeans et al., 2013). Furthermore, some species such as *S. microadriaticum* respond to environmental conditions by varying the rate of photosynthesis, respiration, and growth, as well as varying cellular chlorophyll concentrations (Karako-Lampert et al., 2005).

Activation of these mechanisms in response to varying environmental factors can occur on varying time scales ranging from seconds or hours (for functional adjustments to individual proteins within the photosynthetic apparatus) to weeks or months (for developmental adjustments to the whole or parts of the organism) (Walters, 2005). Acclimation studies involving green algae demonstrate how light level influences LHCII abundance through the transcription of *cab* mRNA and / or post-translationally through the control of chlorophyll synthesis (Falkowski and La Roche, 1991, La Roche et al., 1991, Maxwell et al., 1995, Escoubas et al., 1995). A shift from HL to LL can induce increases in cellular chlorophyll within 24 hours (Sukenik et al., 1990) and this correlates with changes in LHCII abundance (Sukenik et al., 1987) which are preceded by increases in *cab* mRNA levels (La Roche et al., 1991). In *Chlamydomonas reinhardtii*, transcriptional and post-transcriptional mechanisms control light-harvesting complex gene expression, therefore short-term changes occur in transcription and transcript stability, long-term changes primarily occur at the translational level (Durnford et al., 2003). When moved from LL to HL *C. reinhardtii* respond with a decrease in the amount of chlorophyll and LHC per cell, and although a reduction in *Lhc* transcript abundance occurs within 1-2 hours of the shift, mRNA levels recover to the LL concentrations within 6-8 h even if kept in HL (Durnford et al., 2003). This type of response is predicted to be due to a combination of high transcript turnover time and light mediated control of transcription (Durnford et al., 2003).

The distribution of chlorophyll among LHCs varies between *Symbiodinium* cells acclimated to LL and HL (Iglesias-Prieto and Trench, 1997a); LL acclimated cells demonstrate increased pigment concentrations (Falkowski and Dubinsky, 1981) and maximum quantum yield (Warner

et al., 1999) in comparison to HL acclimated cells. This strategy to increase light absorption could result from a combination of an increase in the number of chlorophyll-binding proteins of the photosystem antennae and / or an increase in number of the photosystems (Iglesias-Prieto and Trench, 1994). Conversely, HL acclimated cells demonstrate an increased dependence on photo-repair mechanisms (Jeans et al., 2013). The response to HL conditions can be species specific with some *Symbiodinium* able to induce an enrichment in photo-protective xanthophyll pigments, while other species show marginal capacity to incorporate photo-protective xanthophylls into the LHC proteins (Iglesias-Prieto and Trench, 1997a).

Various environmental factors affect the operation and efficiency of photosynthesis in *Symbiodinium*, including temperature, CO_2 concentration, and most importantly, light (Levy et al., 2004, Levy et al., 2006). Environmental factors, such as elevated temperature and extreme light intensities, are known to stress and imbalance aspects of the light utilization, enzymatic and carbon fixation processes of photosynthesis (Coles and Jokiel, 1977, Hoegh-Guldberg and Smith, 1989, Adir et al., 1990, Heckathorn et al., 1997, Al-Khatib and Paulsen, 1999, Hoegh-Guldberg, 1999, Demmig-Adams and Adams, 2000). Stress responses induced by changing environmental cues can activate phenotypic changes and result in readjustments of an organism's tolerance level (Coles and Brown, 2003). *Symbiodinium* therefore alter components of their photosynthetic apparatus to maximise the efficiency of light capture and hence primary productivity under varying environmental conditions (Iglesias-Prieto and Trench, 1994, Iglesias-Prieto and Trench, 1997a, MacIntyre et al., 2002).

Modifying the expression of specific genes involved in photosynthesis may be a subcellular means by which *Symbiodinium* can adjust to environmental change such as light intensities. The present study focused on how *Symbiodinium* C1 acclimated to a ML or HL environment responded following transition to a different light regime. Using measures for maximum quantum yield of PSII, which is an indicator of how efficient the PSII reaction centres can perform photosynthesis, and investigating the transcriptional response of four nuclear-encoded light-harvesting complex genes, this study sought to determine whether photosynthetic efficiency could be linked with changes in the expression for a key component of the photosynthetic process, the chlorophyll *a*-chlorophyll c_2-peridinin protein complexes (acpPC). The outcomes from this study provide further insight into the stability of nuclear-encoded mRNA under varying light environments and support the proposition that most changes in dinoflagellate protein expression occur post-transcriptionally (Morse et al., 1989, Fagan et al., 1999, Le et al., 2001, Leggat et al., 2011).

6.3 Materials and Methods

6.3.1 *Symbiodinium* Cultures

Symbiodinium C1 (CCMP2466) cells obtained from The Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences, USA (formally Center for Culture of Marine Phytoplankton), were grown in 75 cm^2 Tissue Culture flasks with PE vented caps (Sarstedt, NC, USA) containing 100 mL of ASP-8A medium (Blank 1987). Flasks were maintained in a light and temperature controlled refrigerated incubator TRISL-495-1-SD (Thermoline Scientific, Australia) programmed to provide a 12 h light:dark cycle photoperiod and temperature of 25^oC. *Symbiodinium* C1 were grown on shelves adjusted to 80 µmol - 100 µmol quanta m^{-2} s^{-1} provided by four 18 W cool white fluorescent tubes (Polylux XLR™, GE Lighting). Light levels were measured using a LI-193SA Underwater Spherical Quantum Sensor with a LI-250A Light Meter (LI-COR® Biosciences, NE, USA) and manipulated using shade cloth to achieve the desired light intensities.

Symbiodinium genotype was confirmed through sequencing of the internal transcribed spacer 2 region (ITS 2). Genomic DNA was isolated using a DNeasy Plant Mini kit (Qiagen, Valencia USA). Harvested cells resuspended in 400 µL buffer AP1 with 4 µL RNase A stock solution were transferred to a Lysing Matrix A tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate, including cell debris, was transferred to a 1.5 µL microcentrifuge tube and DNA extracted according to manufacturer's protocol. Elution with 50 µL of buffer AE was performed rather than 100 µL to increase DNA concentration. The ITS 2 region was amplified using the forward primer ITSintfor2 (5'GAATTGCAGAACTCCGTG-3') (LaJeunesse and Trench, 2000) and reverse primer ITS2-Reverse (5'GGGATCCATATGCTTAAGTTCAGCGGGT-3') (Coleman et al., 1994) following the protocol of LaJeunesse (LaJeunesse, 2002). Purified reaction product was directly ligated into pGEM-T Vector (Promega, USA) and transformed into NM522 cells and multiple clones sequenced at the Australian Genome Research Facility using the forward primer M13F (5'GTTTTCCCAGTCACGAC-3') and reverse primer M13R (5'CAGGAAACAGCTATGAC-3').

6.3.2 Experimental Protocol

Symbiodinium C1 were cultured on shelves adjusted to 80 µmol - 100 µmol quanta m^{-2} s^{-1} in replicate flasks (n=3). At week seven cells from the replicate flasks were sub-divided (n=12) and the new replicate flasks were evenly distributed (n=4) to shelves with varying light levels; high-light (HL, ~250 – 350 µmol quanta m^{-2} s^{-1}), medium-light (ML, ~80 µmol - 100 µmol quanta m^{-2} s^{-1}), and low-light (LL, ~10 – 15 µmol quanta m^{-2} s^{-1}). Replicates were maintained at these light levels for 25 weeks. During the 25 week acclimation period cells on the HL and ML shelves were regularly divided into fresh flasks containing 100 mL of ASP-8A media. LL

replicates received fresh media at the same intervals, but were not divided into new flasks due to limited cell growth. The resulting 18 flasks (HL, n=9; ML, n=9) were used in a 10 day light manipulation experiment.

Control measurements were recorded for HL and ML cells during the mid-point of the light cycle at time point zero (Day 0). During the mid-point of the dark cycle (Day 0) three flasks from the HL shelf were moved to the ML shelf (HL to ML), three to the LL shelf (HL to LL) and three remained on the HL shelf (HL control). The process was repeated with flasks from the ML shelf; three were moved to the HL shelf (ML to HL), three to the LL shelf (ML to LL) and three remained on the ML shelf (ML control). Cells for RNA extraction and fluorometry measurements were collected at 12 noon, the mid-point of the light cycle 12 h post transfer (Day 1), 36 h (Day 2), 60 h (Day 3) and 9 days (Day 9). At each time point 300 µL of cells were harvested from all experimental and control flasks for maximum quantum yield (F_v/F_m) measurements. F_v/F_m was measured on a Maxi-Imaging Pulse Amplitude Modulated Chlorophyll Fluorometer (Heinz Walz GmbH, Germany) after 15 min of dark adaptation and used as an indicator of PSII functionality for *Symbiodinium* C1 isolates.

6.3.3 RNA Extraction and cDNA Synthesis

All equipment used for RNA extractions was treated with RNAseZap® (Ambion Inc, USA), and any plasticware and reagents were RNAse/DNAse-free. Total RNA from cultured *Symbiodinium* was isolated using an RNeasy Plant Mini kit (Qiagen, Valencia USA). Cultures were harvested by centrifugation at 5000 x *g* for 2 min, the pellet resuspended in 450 µL RLT buffer (Qiagen kit buffer), transferred to a Lysing Matrix D tube (MP Biomedicals, Australia) and lysed twice for 20 s at 4.0 ms^{-1} on a FastPrep®-24 Instrument (MP Biomedicals, Australia). The lysate including cell debris was transferred to a QIAshredder spin column and RNA extracted according to manufacturer's protocol, including the additional centrifuge and elution step using the initial eluate to increase RNA concentration. Total RNA integrity was assessed by formaldehyde agarose gel following the protocol of Sambrook and Russell (Sambrook and Russell, 2001) and stained with ethidium bromide (200 µg mL^{-1}). RNA quantification and purity was determined using a NanoDrop-1000 (NanoDrop Technologies, Wilmington USA). Absorbance was measured at 260 and 280 nm with the ratio of absorbance (A_{260}/A_{280}) providing an estimation of nucleic acid purity. Absorbance ratios of ≥1.9 for RNA were considered pure enough to use for qRT-PCR applications.

cDNA amplification and genomic DNA elimination was performed with QuantiTect Reverse Transcription® Kit (Qiagen, USA) in a 20 µL reaction using 500 ng of total RNA as template and a RT primer mix consisting of random primers and oligo-dT. Negative controls prepared without total RNA as template were included for each series of reactions.

6.3.4 Primer Design

Normalization primers for β-actin and PCNA validated and tested for stability under varying light conditions with acpPC genes from *Symbiodinium* sp. clade C3 (Boldt et al., 2009) were used for qRT-PCR. Primers for acpPC designed to sequences obtained from an Expressed Sequence Tag (EST) library of *Symbiodinium* sp. C3 (Leggat et al., 2007) were tested for compatibility against cDNA isolated from cultured *Symbiodinium* clade C1. acpPC primers were designed and checked as per normalization genes. Primers used for qRT-PCR are listed in Table 6.1.

Table 6.1 *Symbiodinium* sp. C3 acpPC primers used for cultured *Symbiodinium* sp. C1 qRT-PCR assays

Gene	Primer Sequence	Product size
β-actin	F1: TGG ACA ACG GAA GCG GAA TG B1: GCC AAC AAT GGA TGG GAA AAC T	80 bp
PCNA	F: GAG TTT CAG AAG ATT TGC CGA GAT B1: ACA TTG CCA CTG CCG AGG TC	113 bp
acpPCSym_1	F1: AGT GGA GTG AAC CAG GAA GCA A B1: AAC CAA TCG CAC CGA CCA AGA G	54 bp
acpPCSym_10	F1: TTC GCC GAT GTG CCT AAT GG B1: TTC CTG GGA GAC TTC GCA GAA A	102 bp
acpPCSym_15	F4: GCT TCA TGG ATG GGG TAA AT R1: TCA ACT CAG CTT CAC GGA AC	92 bp
acpPCSym_17	F3: TTT CCA GGT GAC AAG TTC CA R1: ACC TTT GAA GAG GAG CCA GA	138 bp

6.3.5 Quantitative Real-Time PCR (qRT-PCR)

Reverse transcription and genomic DNA elimination was performed with QuantiTect Reverse Transcription kit (Section 2.5.3). To optimize quantification accuracy template dilution series were prepared. After 4 fold dilution with ddH_2O, 3 µl of diluted template was analysed using the Rotor-Gene™ 6000 (Corbett Life Science, Australia). The standard qRT-PCR protocol (Section 2.5.8) was used with gene specific primers for β-actin, PCNA and four acpPC genes (acpPCSym_1, _10, _15 and _17) (Table 6.1). GeneDisc™-100 (Corbett Life Sciences, Australia) included reactions for normalization and *Symbiodinium* acpPC genes run in triplicate and non-template controls in triplicate. Standard curves for the normalization and acpPC genes

were generated with four triplicates using template from ML and HL Day 0 control samples. These same controls were used as an additional PCR efficiency check and included on each GeneDisc™-100 to ensure inter-disc comparability.

6.3.6 Statistical Analysis

One-way ANOVA tests were used to identify differences between light treatments and control samples using SigmaPlot (Version 13). Tukey's *post hoc* comparisons were performed on data where significant differences were detected. A significance level of 0.05 was applied to these analyses and data were tested for assumptions of normality (Sharpiro-Wilk) and homogeneity of variance (Brown-Forsythe). The relative gene expression levels for four acpPC genes (acpPC_1, _10, _15 and _17) were analysed using the relative expression software tool, REST© 2008 v2.0.7 (http://www.rest.gene-quantification.info) (Pfaffl et al., 2002). REST estimates the up or down-regulation of gene expression normalized against multiple reference genes and provides 95% confidence intervals for expression ratios using a variety of bootstrapping techniques. acpPC genes were normalized against β-actin and PCNA and expressed relative to control samples (ML control; HL control). PCR efficiencies were calculated for each standard curve generated and threshold fixed at 0.09 for all C_t calculations. This threshold value was set in the exponential phase of the run and represented the average detection threshold across the control runs with the upper and lower bounds used to scan for an optimal threshold set between 1 and a value significantly above the level of background noise. Melting curve analysis was performed for each assay to check reaction specificity.

6.3.7 Accession Numbers

Sequence data for *Symbiodinium* sp. C3 acpPC referred to in Chapter 6 is available in the EMBL/GenBank data libraries under accession numbers FN646412, FN646414 - FN646418, FN646421 - FN646425 and discussed in detail in Chapter 3.

6.4 Results

6.4.1 Photosystem II (PSII) Efficiency

Maximum quantum yield measurements for *Symbiodinium* C1 acclimated to ML and transferred to HL or LL conditions, and HL acclimated cells transferred to ML or LL conditions, were determined for each sampling day. F_v/F_m results can act as an indicator of the efficiency of PSII reaction centres. Sampling on Day 0, the day prior to replicates being transitioned to a new light level, demonstrated that acclimation to ML (Figure 6.1a) or HL (Figure 6.1b) conditions significantly affected maximum photochemical efficiency ($p<0.001$). F_v/F_m for C1 isolates grown and held at 80 – 100 µmol quanta m^{-2} s^{-1} (ML controls) remained relatively stable for the duration of the experiment decreasing from 0.37 (Day 0) to 0.35 (Day 9;

p=0.381). F_v/F_m for ML to HL isolates significantly declined (p=0.014) over the nine experimental days while PSII efficiency of ML to LL isolates significantly increased (p=007; Figure 6.1a). In relation to ML controls, PSII efficiency significantly declined in ML to HL cells (p=0.008) and remained relatively unchanged in ML to LL (p=0.199), particularly during the initial three days of the experiment. By Day 9 PSII efficiency for the cells transferred from ML to LL had increased significantly to 0.47 (p=0.003) while the ML control cells remained stable at 0.35. ML to HL samples showed minimal signs of recovery at the end of the experimental period; following an initial drop from 0.37 to 0.26 on Day 1, F_v/F_m remained impaired at 0.28 on Day 9. Overall, the maximum quantum yield for ML C1 cells shifted to HL and LL differed significantly for the duration of experiment (p<0.001). A corresponding result was not evident in the HL cells transitioned to ML and LL conditions (p=0.625).

The maximum quantum yield for *Symbiodinium* C1 grown and maintained between 250 µmol - 350 µmol quanta m^{-2} s^{-1} (HL controls) did not vary significantly from Day 0 to Day 9 (p=0.238; Figure 6.1b). PSII efficiency decreased from 0.27 to 0.24 by Day 3 then remained unchanged. The opposite was evident in the HL to ML samples (p=0.086), with F_v/F_m remaining stable following an initial increase on Day 1. The transition from HL to LL had a significant effect (p=0.002) and results correlated with those observed in ML to LL (Figure 6.1a). By Day 9 PSII efficiency for cells transferred from HL to LL conditions increased to 0.44 while the HL to ML samples remained stable (~0.34) and relatively unchanged from Day 1. The Day 9 F_v/F_m values for both HL to LL and HL to ML were significantly higher (p=0<001, p=0.025 respectively) than Day 9 HL control samples which stabilized at 0.24. In relation to the HL controls, PSII efficiency was significantly higher in *Symbiodinium* C1 transferred from HL conditions to ML and LL (p=0.027, p=0.005 respectively).

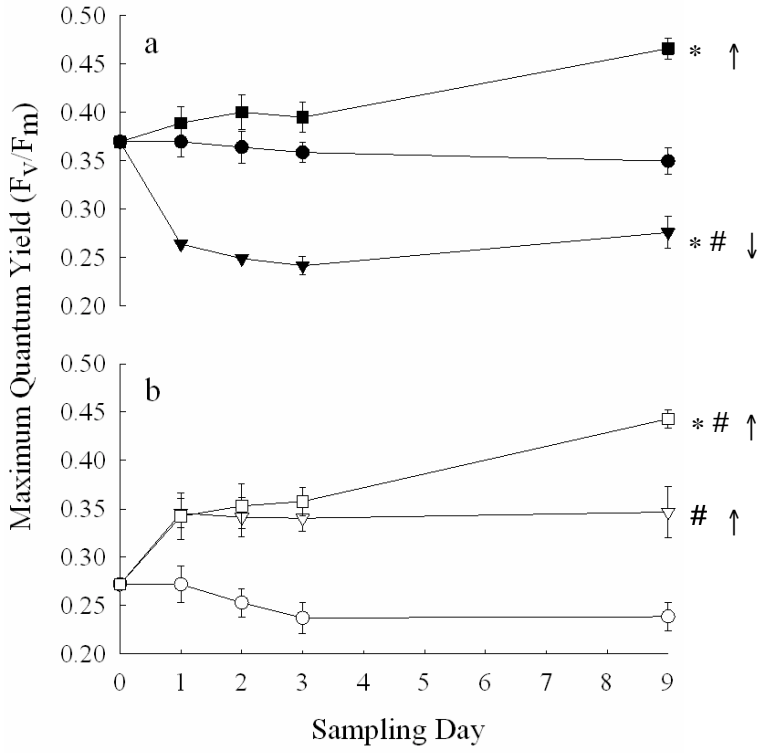

Figure 6.1 Photosystem II photochemical efficiency (F_v/F_m) of *Symbiodinium* sp. C1 acclimated to medium-light (-●-) or high-light (-○-) conditions prior to transfer and exposure to a varied light regime. (a) F_v/F_m for C1 cells acclimated to ML conditions (80 – 100 µmol quanta m^{-2} s^{-1}) then transitioned to HL (-▼-) (250 µmol - 350 µmol quanta m^{-2} s^{-1}) or LL (-■-) (10 – 15 µmol quanta m^{-2} s^{-1}) conditions. (b) F_v/F_m for C1 cells acclimated to HL conditions (250 µmol - 350 µmol quanta m^{-2} s^{-1}) then transitioned to ML (-▽-) (80 – 100 µmol quanta m^{-2} s^{-1}) or LL (-□-) (10 – 15 µmol quanta m^{-2} s^{-1}) conditions. Control samples acclimated to ML (-●-) and HL (-○-) were maintained at the same light condition for the duration of the experiment. Asterisk (∗) indicate statistically significant values from Day 0 to Day 9; hash (#) indicate treatments that differed significantly overall from the control; up arrows (↑) indicate where values on Day 9 were significantly higher than control values; down arrows (↓) indicate where values on Day 9 were significantly lower than control values. Error bars represent the standard error for expression values for three biological replicates.

6.4.2 Quantitative Real-Time PCR (qRT-PCR)

To examine the effect of varying light levels on *Symbiodinium* C1 isolates the expression of mRNAs encoding four acpPC genes was measured using qRT-PCR. *Symbiodinium* C1 acclimated to ML conditions and moved to HL or LL conditions exhibited similar relative expression patterns for acpPCSym_1, _4, _15 and _17 (Figure 6.2). HL acclimated cells transferred to ML or LL conditions also demonstrated similar acpPC expression patterns (Figure 6.3), although variation in expression levels were more stable than those observed in ML

acclimated cells. On Day 1 of the experiment minimal down-regulation was observed in acpPC genes moved from ML to either HL or LL conditions with the exception of acpPCSym_1 (Figure 6.2a) which remained unchanged. On Day 2 transcript levels for acpPCSym_1 (Figure 6.2a) and acpPCSym_10 (Figure 6.2b) significantly decreased ($p<0.05$, REST) in the ML to HL samples. This down-regulation equated to a 0.78 decline in acpPCSym_1 and 0.65 in acpPCSym_10 abundance. A slight decrease was observed for acpPCSym_15 (Figure 6.2c) while acpPCSym_17 expression remained stable (Figure 6.2d). In ML to LL cells, acpPCSym_10 was up-regulated 2.16 fold ($p=0.000$, REST) on Day 3 (Figure 6.2a) and a similar increase in expression was observed for acpPCSym_10 and _1 from ML to HL cells but less so for acpPCSym_15 and _17. Relative expression of acpPCSym_1, _10, _15 and _17 from ML to LL cells had returned to Day 0 or Day 1 levels by Day 9. With the exception of acpPCSym_1, the expression of acpPCSym_10, _15, and _17 from ML to HL continued to decline between Day 3 to Day 9.

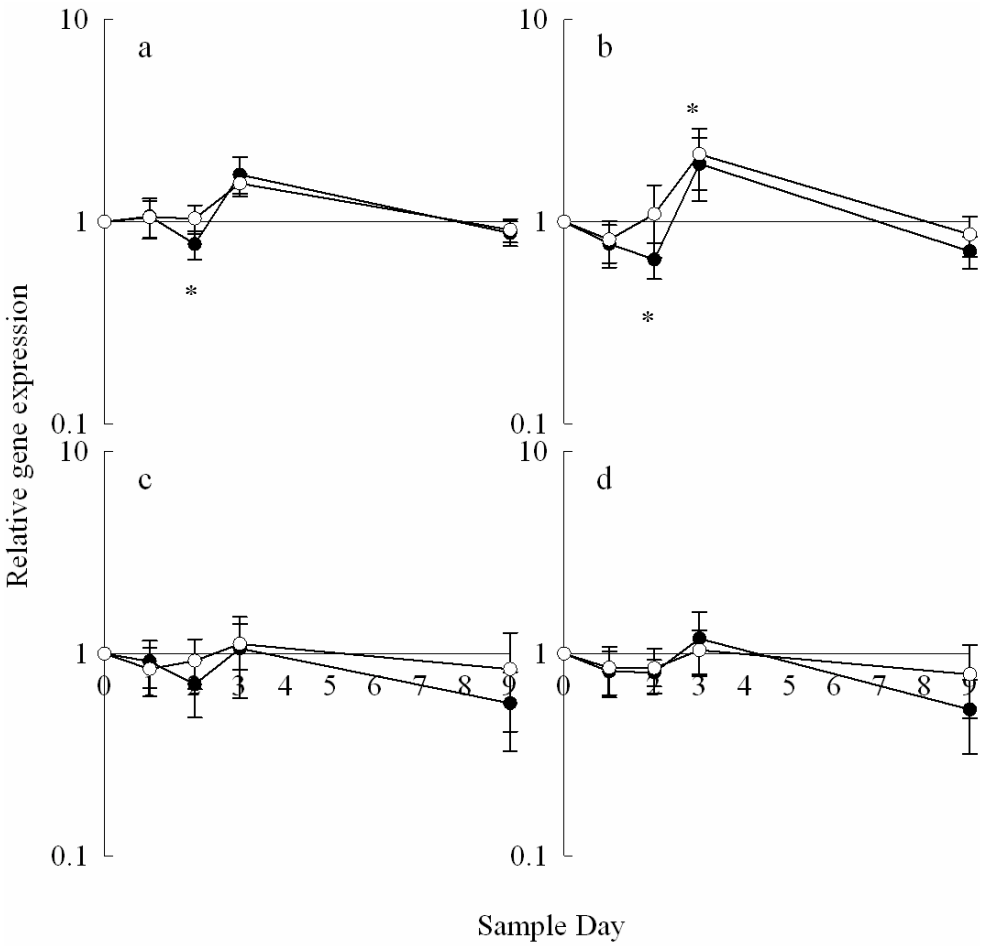

Figure 6.2 Expression profiles of *Symbiodinium* gene acpPCSym_1 (a), acpPCSym_10 (b), acpPC_15 (c) and acpPCSym_17 (d) normalized to β-actin and PCNA and acclimated to medium-light conditions prior to transitioning to high-light (-●-) or low-light conditions

(-o-). Asterisks (*) indicate expression levels that statistically differ to the control ($p<0.05$). Error bars represent the standard error for expression values for three biological replicates.

The relative expression level of acpPCSym_1, _10, _15, and _17 from *Symbiodinium* C1 acclimated to HL conditions was relatively stable across the nine day period (Figure 6.3). Minimal variation was evident between genes or light treatments. A down-regulation equating to a 0.85 decline in acpPC_1 abundance occurred in cells moved from HL to ML conditions ($p=0.000$, REST) on Day 9, but no further changes in mRNA transcript levels were noted for any of the genes. Similar expression patterns for acpPC_1 and _15 were noted (Figure 6.3a and c) and a slight increase evident on Day 2 in the HL to LL cells corresponded with a decrease in the HL to ML cells. By Day 3 acpPC expression levels had stabilised and remained constant for the following six days. As with acpPCSym_1 and _15, expression patterns for acpPCSym_10 and _17 were similar (Figure 6.3b and 6.3d); a slight decrease in expression on Day 2 was observed in the HL to ML samples, but by Day 3 acpPC expression in HL cells shifted to ML or LL conditions recovered and remained stable for the duration of the experiment.

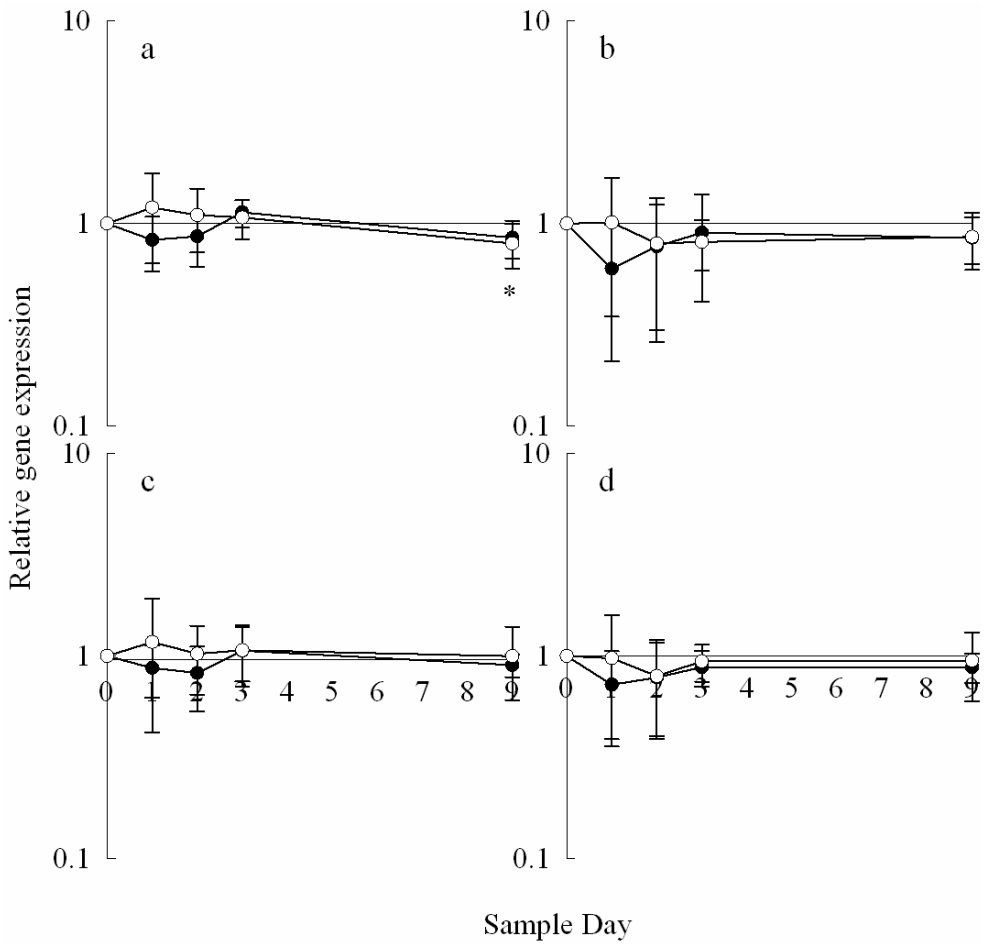

Figure 6.3 Expression profiles of *Symbiodinium* gene acpPCSym_1 (a), acpPCSym_10 (b), acpPC_15 (c) and acpPCSym_17 (d) normalized to β-actin and PCNA and acclimated

to high-light conditions prior to transitioning to medium-light (-●-) or low-light conditions (-o-). Asterisk (*) indicates expression levels that statistically differ to the control ($p<0.05$). Error bars represent the standard error for expression values for three biological replicates.

6.5 Discussion

Light is recognised as a major regulator of expression of photosynthetic proteins (Escoubas et al., 1995, Pfannschmidt et al., 1999, Murchie et al., 2005, Kobiyama et al., 2005, Pfannschmidt et al., 2009, Van Dolah et al., 2007) and photoautotrophs exposed to limiting light conditions demonstrate strategies that increase light absorption while organisms inhabiting HL environments demonstrate strategies that alleviate oxidative stress (Lesser and Shick, 1989a). The LHCII are one of the first detectable proteins to increase production when green algae are transferred from HL to LL conditions (La Roche et al., 1991) and with *Dunaliella tertiolecta*, a decrease in light level corresponds with a 4 fold increase in *cab* mRNA within the first 9 hours (La Roche et al., 1991). This initial increase is followed by a decrease in mRNA levels and a new stable state is achieved that is 2 fold higher than the *cab* mRNA levels of HL cells (La Roche et al., 1991). The new steady state is reached within the first 18 h of being shifted to the LL conditions and is maintained for at least another 18 h (La Roche et al., 1991).

Increases in *cab* mRNA are detectable within 1.5 h of the cells being moved from HL to LL and changes in *cab* mRNA abundance correlate with increases in the amount of chlorophyll per cell (La Roche et al., 1991). Although *Symbiodinium* C1 cells acclimated to HL demonstrate an increased potential to perform photosynthesis when moved to ML or LL conditions (Figure 6.1b), similar responses were not detected in *Symbiodinium* mRNA levels as those of *D. tertiolecta*. Transitioning *Symbiodinium* C1 from HL to ML conditions resulted in a minor decrease in transcript abundance but mRNA levels stabilized and returned to pre-transition levels within 60 h (Figure 6.3) while PSII efficiency stabilized at a new elevated steady state within 12 h (Figure 6.1b). Expression of acpPCSym_1 (Figures 6.3a) and acpPCSym_15 (Figure 6.3c) increased slightly in HL to LL cells during the first 12 h post transition then returned to starting levels within 36 h. In comparison, mRNA for acpPCSym_10 (Figure 6.3b) and _17 (Figure 6.3d) declined on Day 2 then recovered and remained stable. Between Day 3 (60 h post transition) and Day 9, no changes in expression were noted for acpPCSym_1, _10, _15 or _17 in either the HL to LL or HL to ML cells. As with HL to ML cells, photosynthetic efficiency of PSII initially increased in HL to LL cells, but rather than stabilizing, F_v/F_m values continued to increase slowly and by Day 9 were significantly higher than Day 0 values (Figure 6.1b). A similar response was observed for the PSII efficiency of ML to LL cells (Figures 6.1a).

Differences in mRNA transcript levels were more pronounced in *Symbiodinium* C1 acclimated to ML and then transferred to HL or LL conditions; there was a significant down-regulation of acpPCSym_1 and _10 expression 36 h post shift to HL (Figure 6.2a and 6.2b), but

only a slight decrease in acpPCSym_15 and _17. The initial down-regulation was followed by an up-regulation in acpPCSym_1 and _10, but any further fluctuations in expression, including those noted for acpPC_15 and _17, did not vary significantly from the control ML cells. By Day 9 the acpPC transcript levels had stabilized and expression levels were similar to those evident on Day 1 and / or Day 2 except for acpPCSym_17 which continued to experience a decline in mRNA levels (Figure 6.2). *Symbiodinium* moved from ML to LL conditions demonstrated similar expression levels to the ML to HL cells, and apart from a 2 fold up-regulation on Day 3 for acpPC_10 (Figure 6.2b), expression levels remained relatively stable. In contrast to mRNA levels, the photosynthetic efficiency of PSII for ML to HL cells did not recover but continued to decline during the first 60 h and only began to show slight signs of recovery on Day 9 (Figure 6.1a). The decline in PSII efficiency for cells moved to HL conditions corresponds with responses evident in higher plants and green algae which can acclimate to HL conditions by lowering quantum yield of PSII (Iwai et al., 2007).

The response of *Symbiodinium* C1 moved to HL conditions does appear to mimic that of other unicellular algae, but the response time and degree of change differs. For example, *C. reinhardtii* alter the concentration of light-harvesting mRNAs significantly and rapidly and within 1-2 h of a shift to HL transcripts reach a minimum (Durnford et al., 2003). The abundance of mRNA encoding the PSII associated antennae proteins, LHCII and CP29 (a minor antennae complex), and mRNA encoding a LHCI complex, all decrease rapidly in response to HL conditions but recover to near starting abundance after approximately 8 h at HL (Durnford et al., 2003). Based on the findings present here, *Symbiodinium* C1 acpPC mRNA declines during the first 12 – 36 h of a shift to HL and start to recover approximately 60 h post shift (Figure 6.2). Considering circadian regulation of transcript levels of several photosynthetic genes in dinoflagellates, including Chl *a/c*-binding proteins, can increase transcript abundance approximately 2 h after exposure to light (Okamoto and Hastings, 2003b) the possibility that more rapid changes in *Symbiodinium* acpPC transcript levels occurs within the first few hours post transition cannot be ruled out.

The transition of algae from HL to LL diverts macromolecular biosynthesis to proteins rather than lipid and carbohydrates and within 24 h the light-harvesting protein complexes of cells can double (Sukenik et al., 1990). The reverse also occurs and *D. tertiolecta* shifted from LL to HL demonstrate increased carbon being used for carbohydrates and lipids rather than proteins (Sukenik et al., 1990). In addition, once changes in LHCII have occurred other components of the photosynthetic apparatus increase, balancing out the ratio of components irrespective of the light level, and within approximately 72 h photosynthetic components have reached a new steady state level (Sukenik et al., 1990). For all acpPC genes tested, and irrespective of whether cells were acclimated to HL then moved to ML or LL, approximately 60 h post shift a steady

state level had been reached which was generally the same, or marginally less, than the starting level suggesting similar balancing of components may occur within *Symbiodinium*.

Overall, relatively small changes were observed at the transcript level for *Symbiodinium* C1 suggesting post-translational factors may influence *Symbiodinium* acpPC expression. Minimal transcriptional changes in response to varying light levels have also been noted for acpPC from *Symbiodinium* C3 cells isolated from a coral host (Chapter 4) (Boldt et al., 2009). Furthermore, changes in the range of 1.8 to 5.4 for core photosystem genes (the plastid-encoded *psbA* and *psaA* genes which encode the D1 protein of PSII and P_{700} protein of PSI respectively) in *Symbiodinium* exposed to thermal stress have been documented (McGinley et al., 2012). In addition, gene expression varied between *Symbiodinium* types (clade and sub-clade; cultured isolates and *in hospite* populations) (McGinley et al., 2012). Small 2 – 4 fold difference in mRNA levels for dinoflagellate genes is not unusual (Okamoto and Hastings, 2003b, Van Dolah et al., 2007) and dinoflagellate genes are plausibly subject to translational or other controls that result in greater changes at the physiological level than those of the transcripts (Okamoto and Hastings, 2003b).

Translational control mechanisms of circadian clock genes such as bioluminescence (Morse et al., 1989), the nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fagan et al., 1999), and the water-soluble LHC protein PCP (Le et al., 2001), are evident in dinoflagellates, as are post-translational controls (Nassoury et al., 2001). Post-transcriptional controls are not specific to dinoflagellates or circadian clock genes. A protein pivotal in managing light responses in the diatom *Phaeodactylum tricornutum* is also induced at the protein level without changes in mRNA level being observed (Bailleul et al., 2010) and changes within *C. reinhardtii* occur prior to a HL induced reduction in LHC antennae polypeptides, suggesting additional translational controls may play a factor in long-term acclimation to HL environments (Durnford et al., 2003). Given the diversity of dinoflagellate LHC genes the question arises as to whether LHC isoforms are regulated by the same control mechanism(s) and on the same times scale – are rapid short-term changes controlled by transcriptional mechanisms and longer term changes at the translational level, and does this differ for isoforms with a photo-protective role?

Of the proteins involved in light-harvesting, the early light-inducible proteins (ELIPs), and HLIPs in cyanobacteria, appear induced by HL conditions; other LHCs are down-regulated under HL conditions, but accumulate under LL conditions (Montane and Kloppstech, 2000). ELIPs, photo-protective xanthophyll pigments, and their mRNA, accumulate in higher plants under increasing PSII excitation pressure irrespective of light (Montane et al., 1998). Therefore, light conditions alone may not account for increases in LHCs associated with PSII. Chlorophyll *a/b* mRNA accumulation in the green alga *D. salina,* can occur under conditions of high PSII excitation pressure and this supports the suggestion that the redox state of the electron transport

system may be a driving factor behind gene regulation in photosynthetic organisms (Maxwell et al., 1995). The findings of this study suggest acpPCSym_1, _10, _15 and _17 are not like the ELIPs in higher plants and are not induced under HL conditions nor do they accumulate under LL conditions. For these four acpPC isoforms, light alone is possibly not the only sensing or signalling mechanism and the response of acpPC from *Symbiodinium* exposed to conditions of high PSII excitation pressure, in combination with varying light environment, deserves further investigation.

6.6 Chapter 6 Summary Points

- Normalization genes validated in Chapter 4 were utilized in a controlled light experiment to investigate acpPC expression in HL and ML acclimated cells exposed to varying light environments;
- acpPC from *Symbiodinium* sp. C1 exhibit small changes at the transcript level which occur slower than responses evident in other unicellular algae;
- *Symbiodinium* C1 acclimated to ML then moved to HL conditions demonstrate decreases in mRNA levels while the reverse occurs when moved to LL conditions;
- Changes in the photosynthetic efficiency of PSII could not be clearly linked with up or down-regulation in acpPC expression;
- Translational or post-translational control mechanisms potentially influence *Symbiodinium* LHC protein expression.

CHAPTER 7

7 General Discussion and Summary

7.1 General Discussion

Coral reefs are highly productive, biologically diverse and important marine ecosystems. They provide at least 100 million people worldwide with food resources, support large fishing and tourism industries, contribute significantly to the economic wealth of countries with coral reefs, and protect coastlines from destructive storm surges, erosion and flooding (Hoegh-Guldberg, 1999, Hughes et al., 2003, Lesser, 2004). Unfortunately worldwide coral reefs are experiencing a decline in health and the threats to habitat structure, abundance and diversity are increasing due to anthropogenic induced stresses such as pollution, over-fishing and coastal development (Jackson et al., 2001, Hughes et al., 2003, Pandolfi et al., 2003, Lesser, 2004). Warming sea temperatures have been clearly linked with the phenomenon termed 'coral bleaching', which is one major problem facing thermally stressed coral reefs (Hoegh-Guldberg, 1999), and increased prevalence of coral diseases (Harvell et al., 2002). Coral bleaching can occur when there is a loss of symbiotic dinoflagellates and / or the loss of dinoflagellate pigments (Hoegh-Guldberg and Smith, 1989) and can be severe enough to effect coral health even causing mortality. A number of environmental factors, or stresses, are known to effect coral reef health and induce bleaching; including elevated temperature, reduced salinity and extreme light intensities, and reduced (Hoegh-Guldberg, 1999).

Understanding how coral reefs will respond to stress events resulting from anthropogenic influences requires a better understanding of how coral reefs work. This is a challenging task due to the complexity of studying a biological structure involving at least three different organisms; one an animal, one a plant, and the other a plethora of bacteria. The significance of this research project is that it provides new information at the gene and protein level (Chapter 3) for one of these organisms, the symbiotic dinoflagellate; it establishes a methodology that enables the analysis of *Symbiodinium* gene expression changes under varying light stresses (Chapter 4) and this advance has meant that researchers have been able to investigate the transcriptomic responses of *Symbiodinium* to varying stresses (Leggat et al., 2011, McGinley et al., 2012, Ogawa et al., 2013). Furthermore, this work demonstrates that genes encoding integral LHC proteins varies between *Symbiodinium* clades and subclades, and although light potentially influences protein expression (Chapter 5), variations in light levels result in small changes in transcript levels (Chapter 6).

Coral susceptibility to some environmental stressors may be related to the taxon of *Symbiodinium* harboured (Iglesias-Prieto and Trench, 1994, Warner et al., 1996, LaJeunesse et al., 2003). Given that light is a co-factor in coral bleaching the light acclimatory processes in different *Symbiodinium* strains has the potential to affect the bleaching susceptibility and survival of the entire coral holobiont (animal, plant and bacteria). In addition corals are found over a wide range of light habitats and experience varying light fields both of which have implications for photo-acclimation and photosynthetic capacity (Anthony and Hoegh-Guldberg, 2003). Added to this, multiple scattering by the coral skeleton can enhance the light field and increase light absorption which may have negative effects on the coral when exposed to stressful conditions; but positive effects when environmental light levels are low (Enriquez et al., 2005). It is this type of host interaction that makes studying *Symbiodinium in hospite* complex and promotes studies on cultured *Symbiodinium* such as the controlled light manipulation study documented in Chapter 6.

Under conditions when environmental parameters such as light, temperature and CO_2 are limiting or in excess, imbalances between energy absorbed and energy utilized occur. Photoautotrophs regularly experience daily and seasonal fluctuations in environmental conditions and the resulting imbalances can result in PSII excitation pressure and affect an organism's photochemistry (Huner et al., 1998). Stress responses induced by changing environmental cues can activate acclimation (Huner et al., 1998) involving phenotypic changes and result in readjustments of an organism's tolerance level (Coles and Brown, 2003). Photo-acclimation in photoautotrophs, including *Symbiodinium*, involves physiological, biochemical and morphological modifications that allow for efficient utilization of available light energy (Iglesias-Prieto and Trench, 1997a, Iglesias-Prieto and Trench, 1997b). These changes can include the differential expression of proteins involved in the light harvesting, photo-protection, charge separation and carbon fixation processes of photosynthesis (Iglesias-Prieto and Trench, 1997a). For example, plants (Zer and Ohad, 2003) and algae (Falkowski and La Roche, 1991) can adjust the structure and composition of their photosynthetic apparatus to protect PSII under light stress, varying the ratio of light harvesting chlorophylls and abundance of nuclear encoded PSII light harvesting protein complexes (LHCII). Such responses not only effect an organisms capacity to process captured light photo-chemically (Horton et al., 1996), but also to dissipate excess heat (Niyogi, 1999). In addition, photoautotroph acclimation may involve increases to the number of electron consuming sinks through elevation in the levels of Calvin-Benson cycle enzymes and thereby increase the capacity for CO_2 assimilation or photorespiration relative to electron transport (Huner et al., 1998). These types of mechanisms are generally invoked with fluctuating light periods when the energy absorbed by the antennae structure limits or exceeds the energy requirements for carbon fixation. With *Symbiodinium* there is now evidence that the Mehler reaction acts as a photoprotective mechanism under high light conditions (Roberty et al.,

2014), but whether *Symbiodinium* also adjust the structure of PSI and / or PSII under light stress is unknown. Given the diversity of LHCs in the *Symbiodinium* genome (Chapter 3) an understanding of what role the different acpPC isoforms play is required.

Observations at the level of gene transcription indicate distinct differences in how unicellular algae and higher plants acclimate their photosystems to environmental changes. In unicellular algae, photo-acclimation is similar for an array of environmental cues and channelled via increases in excitation pressure between PSII and PSI regardless of the light field (Maxwell et al., 1995). Higher plants require a change in the light field, in addition to a change in excitation pressure, to complete photo-acclimation and cell to cell interactions are all important (Montane et al., 1999, Walters, 2005). The question then arises; how do unicellular symbiotic dinoflagellates respond to distinct environmental cues, do they mimic the response of other unicellular algae or that of higher plants, particularly given host-dinoflagellate cell interactions have obviously played a prominent role in the evolution of this symbiosis? Findings from this research suggest *Symbiodinium* do respond to distinct environmental cues at the physiological level, as evident by changes in the potential of PSII to perform photosynthesis, but changes in the light field do not result in significant changes at the level of gene transcription (Chapter 6). The *Symbiodinium* genome evidently encodes a variety of acpPC genes (Chapter 3) in single and multiple copies (Chapter 5) that can be arranged in tandem arrays (Chapter 3), and although this can make working with transcripts challenging, it contributes substantially to our understanding of dinoflagellate and *Symbiodinium* genome organisation.

The insight into the relative expression of LHC genes encoded by *Symbiodinium* and the small changes that occur in mRNA levels suggest translational or post-translational control mechanisms potentially influence *Symbiodinium* LHC protein expression. Translational control mechanisms of circadian clock genes such as bioluminescence (Morse et al., 1989), the nuclear-encoded chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fagan et al., 1999), and the water-soluble LHC protein PCP (Le et al., 2001), are evident in dinoflagellates, as are post-translational controls (Nassoury et al., 2001). Biological clocks or circadian rhythms are known to exist in a variety of eukaryotic organisms and physiological and behavioural functions of marine phytoplankton are well recognised as being regulated by the photoperiod either as a direct response to light or through circadian rhythmicity (Van Dolah et al., 2007). Microarray analysis using *Pyrocystis lunula* has indicated a 2-fold diurnal variation in acpPC transcript level (Okamoto and Hastings, 2003b); a trend confirmed in *Amphidinium tamarense* (which shows a 7-fold increase in mRNA levels of acpPC) under light conditions in comparison to dark when measured over a 24-hour period (Kobiyama et al., 2005). A similar trend is yet to be confirmed in *Symbiodinium* and a pilot test undertaken during this research project was unsuccessful as primers designed to *Symbiodinium* C3 sequence data proved unsuitable. The comparative analysis performed following this confirmed that C3 acpPC data can be utilized to

investigate closely related *Symbiodinium*, but diversity of genes detected between and within clades is evident and primers are best designed to the highly conserved regions of the transmembrane helices (Chapter 5).

While the primary role of acpPCs is to harvest and pass on light energy to the reaction centres, the secondary role relates to the ability of these proteins to bind carotenoids (such as xanthophylls) that act as intermediaries in the transfer of excitation energy from chlorophyll to heat dissipation. Xanthophylls are proposed as having a potential photo-protective function in coral-dinoflagellate symbiosis (Ambarsari et al., 1997) similar to that documented in higher plants (Demming-Adams and Adams, 1996). As xanthophylls are believed to bind to specific acpPCs (Iglesias-Prieto and Trench, 1997a) it seems reasonable to assume that the observed fluctuation concomitant with changes in light dosages correspond to an underlying change in the abundance of one or more specific acpPCs. In higher plants and algae, the presence of proteins that bind xanthophylls are predominantly determined at the level of mRNA transcription (Maxwell et al., 1995, Montane et al., 1999) and therefore it is tempting to assume that this will also be true of dinoflagellates.

Time-resolved spectroscopic studies of acpPC from dinoflagellates, including *Symbiodinium*, reveal photo-protective capabilities of peridinin and diadinoxanthin in acpPC and demonstrate that the majority of carotenoids, and other accessory light absorbers such as chlorophyll c_2, are very well suited to support chlorophyll *a* in light harvesting (Niedzwiedzki et al., 2014). However, the carotenoids photo-protective performance in the acpPC is unusual and possibly explains the low resistance of the acpPC complex against photo-induced damage under even moderate light conditions (Niedzwiedzki et al., 2014). Variation in stoichiometry and number of bound pigments in acpPC from *Symbiodinium* (Niedzwiedzki et al., 2014) and *A. carterae* (Hiller et al., 1993) is interesting and it has been suggested may be how the hyperdiversity of *Symbiodinium* acpPC (Chapter 3) is manifested (Slouf et al., 2013). The carotenoid and chlorophyll content were not investigated as part of this research, nor was the chlorophyll binding capability of the different acpPC isoforms. Given changes in light level induce increases in cellular chlorophyll that correlate with changes in LHCII abundance with green algae (Sukenik et al., 1990) the effect of varying light on chlorophyll and carotenoid synthesis and / or content bound to different acpPC isoforms requires investigation.

In dinoflagellates, as with land plants and green algae, genes encoding light-harvesting complexes responsible for the capture and transfer of light energy to photosynthetic reaction centres for conversion to chemical energy, are transcribed in the nucleus, but the mature forms of the apoproteins are located within the chloroplast thylakoid membranes. The LHCs of land plants and green algae encoded by the Chl *a/b* polypeptides are nuclear encoded proteins, post-translationally imported into the chloroplast where the apoproteins are processed into their mature forms and inserted and assembled into the thylakoid membrane (Maxwell et al., 1995).

Four *Symbiodinium* LHC proteins (acpPCSym_8, _10, _12 and _13) encode polypeptides within a single transcript (Chapter 3) therefore it is expected that *Symbiodinium* LHCs are translated and post-translationally cleaved in a similar manner as the Chl *a/b* polypeptides of land plants and green algae.

Clade C *Symbiodinium*, which were the focus of this research, are very dominant and diverse across the Great Barrier Reef (Tonk et al., 2013) and throughout the Indo-Pacific region (LaJeunesse et al., 2003, LaJeunesse et al., 2004, LaJeunesse et al., 2010). The hosts of symbiotic dinoflagellates may harbour single algal genotypes (Rowan, 1991, Stochaj and Grossman, 1997) or a mixed population of varying genotypes (Rowan and Knowlton, 1995). The significance of this, when taken in context of the physiological and molecular capabilities of specific *Symbiodinium* to make adjustments to their photosynthetic apparatus depending on the light field, has ecologically relevant implications considering the predicted changes to marine environments resulting from global environmental stresses. *Symbiodinium* A1, A1.1, B1 and F2 have previously exhibited differential responses in PSII activity when photo-acclimated to different light levels and subsequently exposed to thermal stress (Robison and Warner, 2006). *Symbiodinium* A1.1 and B1 are suggested to be thermally susceptible sub-clades and photosynthetic function of these two *Symbiodinium* is exacerbated when acclimated to HL (Robison and Warner, 2006). In comparison sub-clade F2 has a greater capacity to photo-acclimate and grow under HL conditions (Robison and Warner, 2006). Maximum quantum yield of varying clades of *Symbiodinium* have been shown to be lower in cells grown in HL conditions compared with cells grown under LL conditions (Robison and Warner, 2006), a response also evident in *Symbiodinium* C1 isolates (Chapter 6).

It has been speculated that acpPC may demonstrate a state transition response with redistribution towards PSI to reduce PSII over-excitation under HL conditions (Hill et al., 2012), similar to the rearrangement and state transitions evident amongst the membrane-bound LHCs of higher plants and green algae. While this possible alternative form of photo-protection may be species-dependent and have implications for the coral hosts' susceptibility to environmental stresses (Hill et al., 2012), more recent work demonstrates that in a number of *Symbiodinium* strains, the Mehler reaction is the main site for light-dependent oxygen uptake and plays a photo-protective role under HL conditions (Roberty et al., 2014). Investigations of PCP from different *Symbiodinium* species suggest there is no relationship between symbiont genotype and photobiology (Iglesias-Prieto and Trench, 1997a). For example, the cellular concentrations of PCP, the antenna complex located on the periphery of the thylakoids, increase in LL cultures relative to HL cultures in *S. microadriaticum* (A1), *S. pilosum* (A2) and *S. kawagutii* (F1) (Iglesias-Prieto and Trench, 1997a) however, immunoblot analyses indicate that while both the monomeric and dimeric forms are present simultaneously in the three cultured *Symbiodinium* species, the monomeric form is more abundant in HL cultures while LL cultures

have a greater abundance of dimeric PCP (Iglesias-Prieto and Trench, 1997a). In addition, the different cultured dinoflagellates possess different PCP forms; *S. kawagutii* possesses the monomeric form, *S. pilosum* the dimeric form and *S. microadriaticum* both the monomeric and dimeric PCP forms (Iglesias-Prieto and Trench, 1997a). *S. microadriaticum* is adapted to fluctuating irradiance while *S. pilosum* is adapted to high irradiance (Iglesias-Prieto and Trench, 1997a) and both group in clade A (Rowan and Powers, 1991a). *S. pulchrorum* and *S. californium*, both clade B symbionts (Rowan and Powers, 1991a) also express different forms of PCP; *S. pulchrorum* expresses the monomeric form while *S. californium* expresses both the monomeric and dimeric forms (Govind et al., 1990, Stochaj and Grossman, 1997). Given this varying expression of PCP isoforms the question arises as to whether the different acpPC isoforms will be differentially expressed based on symbiont genotype.

The results from this research address a number of gaps in our knowledge of *Symbiodinium* LHCs, including methods to investigate relative expression of the LHC genes encoded by *Symbiodinium*. With only two EST studies on two different strains of *Symbiodinium* (Leggat et al., 2007, Voolstra et al., 2009) and a proteomic study comparing symbiotic and cultured *Symbiodinium* strains (Stochaj and Grossman, 1997), there is the need for more genomic, proteomic and transcriptomic research studies, and genome and transcriptome sequencing from different *Symbiodinium* strains both *in hospite* and in culture to continue to advance our knowledge and understanding. The data from a high-throughput sequencing effort occurring for *Symbiodinium* sp. sub-clade A1 at King Abdullah University of Science and Technology in Saudi Arabia (Voolstra et al. unpublished data) in addition to the *Symbiodinium minutum* genome information now available (Shoguchi et al., 2013) will enable further advances in the near future and help researchers address those areas still poorly understood in relation to *Symbiodinium*.

7.2 Thesis Summary

The results from this research provide a platform from where coral reef scientists can continue to improve our understanding as to why some coral-dinoflagellate combinations are able to survive in a variety of environmental habitats whilst others cannot and it helps in enabling more accurate projections of the effects of environmental stresses on coral reef systems. Experimental tools relatively new to the study of *Symbiodinium* and photosynthetic processes, such as Real-Time PCR, were used for gene expression work in tandem with more established molecular techniques and pulse amplitude modulated fluorescence. The outcomes from this project provide valuable information on how our currently changing environment affects the photosynthetic ability of this plant-animal (and bacteria) symbiosis which is fundamentally important to reef development and survival.

The membrane bound acpPC light harvesting genes of clade C3 *Symbiodinium* sp. were characterized at the nucleotide and protein level. Full-length sequencing of putative light harvesting acpPCs from established expressed sequence tags (ESTs) of C3 *Symbiodinium* isolated from the branching coral *Acropora aspera* (Leggat et al., 2007), and that had been subjected to a variety of stresses, was undertaken and specific primers designed for the putative light harvesting proteins were used to obtain full-length sequences. Once the complete sequences were obtained bioinformatics analysis, including analysis of conserved amino acid residues and homology of translated protein sequences with known light harvesting genes from symbiotic and free-living dinoflagellates, as well as red algae and higher plants, was performed.

The phylogenetic analysis suggests the acpPC subfamily forms at least three clades within the Chl *a/c*-binding LHC family. The first clade includes rhodophyte, cryptophyte and peridinin binding dinoflagellate sequences; the second peridinin binding dinoflagellates only; while the third contains heterokonts, fucoxanthin and peridinin binding dinoflagellate sequences. *Symbiodinium* sp. C3 acpPC sequences generally contain three transmembrane helices and at least two proteins with LHC membrane-spanning helix duplication, deletion, and / or degeneration are evident. Furthermore, *Symbiodinium* encode transcripts containing one, two or three LHC polypeptides. A comparative analysis of *Symbiodinium* from divergent lineages concluded that C3 acpPC data can be utilized to investigate closely related *Symbiodinium* at the transcript and protein level, specifically *Symbiodinium* C1. Findings suggest a number of C3 acpPC genes, in particular acpPCSym_1, _4 and _8, are potentially core light-harvesting complex genes common to *Symbiodinium* clades and the *Symbiodinium* genome encodes single copy and high copies of acpPC genes. β-actin and PCNA were validated as normalization genes and then used in a controlled light experiment investigating acpPC expression in cultured *Symbiodinium* sp. clade C1. The use of a polyclonal antibody for acpPC demonstrated that light affects acpPC protein expression however changes at the transcript level are relatively small. Finally, acpPC gene expression could not be linked to a physiological measurement for photosynthetic efficiency suggesting translational control mechanism or post-translational controls potentially influence *Symbiodinium* LHC protein expression

Australia's Great Barrier Reef is an important natural and economic resource, generating billions of dollars from tourism and fishing industries. Protecting and managing this World Heritage Area is becoming increasingly difficult due to increased anthropogenically induced stresses. Outcomes from this work provide coral reef researchers and photo-biologists with a better understanding of coral-dinoflagellate photosynthetic mechanisms and how light stresses may imbalance and damage this relationship. Specifically the important outcomes from this project can be incorporated into models to determine the interactions light has with other environmental factors, such as thermal stresses, and how these factors influence the health of corals, and determining whether specific symbiotic dinoflagellates are better suited to varying

environmental light conditions. Furthermore, as *Symbiodinium* genome data becomes readily available insight into the complex genetic composition of these unique dinoflagellates will be possible. Until this time, the information resulting from this research provides: a basis for future investigations into the major light-harvesting protein complexes utilized by *Symbiodinium*, specifically clade C *Symbiodinium*; a means to compare whether the acpPC subfamily differs between stress tolerant and susceptible *Symbiodinium* species; and a methodology to measure how environmental factors impact the major LHCs used by these ecologically important dinoflagellates. This information is crucial if coral biologists and reef managers are to understand why certain coral-dinoflagellate relationships are better able to survive in our changing climate while others cannot.

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APPENDICES

Table 1. ASP-8A Medium used for culturing of *Symbiodinium* cells

Components	Molecular Weight (g / mol)	Stock Solution	1 L
NaCl	58..44	-	25 g
KCl	74.55	35 g / 500 ml	10 ml
$MgSO_4.7H_2O$	246.47	450 g / L	20 ml
$CaCl_{2.}2H_2O$	147.02	55 g/ 500 ml	10 ml
$NaNO_3$	84.99	2.5 g / 50 ml	1 ml
KH_2PO_4	136.09	0.5 g / 50 ml	1 ml
Nitrilotriacetic acid (NTA)	191.14	1.5 g / 50 ml	1 ml
Tris Base (pH 9.0)	121.14	100 g / L	10 ml
PII Metal Mix	-	(separate)	10 ml
NH_4NO_3	80.04	0.05 g / 50 ml	1 ml
8A Vitamin Mix (x2)	-	(separate)	0.25 ml
Vitamin B_{12}	1355.38	10 ug/ 1 ml	0.1 ml

Prepare stock solutions in distilled water and store at 4 ^{O}C. Dissolve NaCl in 900 mL of distilled water, add all components except the 8A vitamin mix and B_{12}, pH to 8.5, bring to 1 L, autoclave medium at 121^{o}C for 22 min, allow to cool to room temperature before adding the 8A vitamin mix and B_{12}.

Table 2. 8A Vitamin Mix for use in the preparation of ASP-8A Medium for culturing *Symbiodinium* cells

Components	Molecular Weight (g / mol)	Stock Solution (1 L)
p-Aminobenzoic acid, Na salt	159.12	10 mg
Biotin (d-)(Vitamin H)	244.32	0.5 mg
B_{12} (cyanocobalamin)	1355.38	0.5 mg
Choline di H_2 citrate	295.29	500 mg
Folic acid	441.40	2.5 mg
Folinic acid, Ca salt	511.51	0.2 mg
Inositol (myo-)	180.16	1000 mg

Nicotinic acid (niacin)	123.11	100 mg
Orotic acid, mono Na salt	178.08	20 mg
D-Pantothenic, hemi-Ca salt (Vitamin B5)	238.27	100 mg
Pyridoxamine, di HCl	241.11	20 mg
Pyridoxine, HCl (Vitamin B6)	205.64	40 mg
Putrescine, di HCl	161.07	40 mg
Riboflavin (Vitamin B2)	376.36	5 mg
Thiamine, HCl (Vitamin B1)	337.27	200 mg
Thymine	126.11	800 mg

Prepare vitamin mix with distilled water, bring the volume to 1 L, filter sterilize the mix and store at 4^{o}C in 50 µL aliquots covered in aluminium foil.

Table 3. PII Trace Metal Mix for use in the preparation of ASP-8A Medium for culturing *Symbiodinium* cells

Components	Molecular Weight (g / mol)	Stock Solution (1 L)
$CoSO_4.7H_2O$	281.10	4.8 mg
EDTA.2Na	336.2	1000 mg
$FeCl_3.6H_2O$	270.29	49 mg
H_3BO_3	61.83	1140 mg
$MnSO_4.4H_2O$	223.06	164 mg
$ZnSO_4.7H_2O$	287.54	22 mg

Prepare PII trace metal mix with distilled water, bring the volume to 1 L, heat to dissolve components if required and store at 4^{o}C covered in aluminium foil.

Table 4. Antibiotic stock solution, 10X concentration, prepared in 100 mL of Milli-Q water and diluted 1:10 for use with cultured *Symbiodinium* cells.

Antibiotic	Stock Solution (100 ml)
Penicillin-G	1000 mg
Streptomycin	2000 mg
Kanamycin	1000 mg
Neomycin	200 mg
Nystatin	15 mg

Erythromycin	6 mg
Gentamycin	8 mg
Polymixin-B	16 mg
Tetracyclin	12 mg
Vancomycin	12 mg

Prepare stock solutions with distilled water, filter sterilize the mix and store at $4^{o}C$ or $-20^{o}C$ in 5 mL aliquots.

Table 5. Cultured *Symbiodinium* collection maintained and used to research *Symbiodinium* integral light harvesting protein complexes. Data includes details provided by the laboratories supplying the cells (Santos Lab and CCMP) with the addition of genotyping of the ITS2 regions and culture medium used on receivership of cells by the Leggat Lab

Culture ID	Invertebrate Host	*Symbiodinium* sp.	Geographic Location	Ocean	Isolated by	18S-rDNA RFLP (Santos Lab)	Cp23S-rDNA genotype (Santos Lab)	ITS2 Genotype (Leggat Lab)	Medium Used By Supplier	Medium Used By Leggat Lab	Cells Supplier
Sin	*Sinularia sp.*	-	Guam	W. Pacific	S.R. Santos	C	F179	F2	f/2	ASP-8A	Santos Lab
Mv	*Montipora verrucosa*	*S. kawagutii*	Kaneohe Bay, Hawaii	C. Pacific	R.A. Kinzie	C	F178	F1	f/2	ASP-8A	Santos Lab
Mf 8.5 Tb.2	*Orbicella faveolata*	-	Florida Keys	Caribbean	M.A. Coffroth	C	ND	F5.2b	f/2	ASP-8A	Santos Lab
Zs	*Zoanthus sociatus*	*S. pilosum*	Jamaica	Caribbean	R.A. Kinzie	A	A188	A2	f/2	ASP-8A	Santos Lab
707	*Plexaura kuna*	*S. pulchrorum or S. bermudense*	San Blas, Panama	Caribbean	M.A. Coffroth	B	B184	B1	f/2	ASP-8A	Santos Lab
Cx	*Cassiopea xamachana*	*S. microadriaticum*	Jamaica	Caribbean	R.A. Kinzie	A	A194	A1	f/2	ASP-8A	Santos Lab
708	*Plexaura kuna*	*S. cariborum*	San Blas	Caribbean	M.A. Coffroth	C	ND	A1.1	f/2	ASP-8A	Santos Lab
CassMJ300	*Cassiopea sp.*	-	Kaneohe Bay	C. Pacific	R.A. Kinzie	A	A198	A3	f/2	ASP-8A	Santos Lab
702 (Pk702)	*Plexaura kuna*	-	San Blas	Caribbean	M.A. Coffroth	B	B211	B	f/2	ASP-8A	Santos Lab
CCMP2466	*Discosoma sancti-thomae*	*S. goreaui*	Jamaica	Caribbean	S. Chang	-	-	C1	L1	ASP-8A	CCMP

Table 6. f/2 Medium used for culturing of *Symbiodinium* cells. To prepare 'f medium' double the quantity of all components as 'f/2 medium' is a reduced 'f medium' mix.

Components	Stock Solution	Quantity (1 L)
$NaH_2PO_4.H_2O$	5 g L^{-1}	1.0 mL
$Na_2SiO_3.9H_2O$	30 g L^{-1}	1.0 mL
$NaNO_3$	75 g L^{-1}	1.0 mL
f/2 Trace Metal Mix	(separate)	1.0 mL
f/2 Vitamin Mix	(separate)	0.5 mL

Prepare stock solutions with distilled water. For final f/2 medium add components except the vitamin mix to 900 mL of filtered seawater, bring the volume to 1 L, autoclave the medium at 121^{o}C for 22 min, allow medium to cool to room temperature before adding the vitamin mix.

Table 7. f/2 Vitamin Mix for use in the preparation of f/2 Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Quantity (1 L)
Biotin (d-)(Vitamin H)	1.0 g L^{-1}	1.0 mL
B_{12} (cyanocobalamin)	1.0 g L^{-1}	1.0 mL
Thiamine, HCl (Vitamin B1)	-	200 mg

Prepare stock solutions with distilled water. For final vitamin mix dissolve thiamine in 900 mL of distilled water, add 1 mL each of biotin and B_{12} stock solutions and bring the volume to 1 L. Filter sterilize the mix and store at 4^{o}C.

Table 8. Trace Metal Mix for use in the preparation of f/2 Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Stock Solution (1 L)
$CuSO_4.5H_2O$	9.8 g L^{-1}	1.0 mL
$Na_2MoO_4.2H_2O$	6.3 g L^{-1}	1.0 mL
$CoCl_2.6H_2O$	10.0 g L^{-1}	1.0 mL
$Na_2EDTA.2H_2O$	-	4.36 g
$FeCl_3.6H_2O$	-	3.15 g
$MnCl_2.4H_2O$	180.0 g L^{-1}	1.0 mL
$ZnSO_4.7H_2O$	22.0 g L^{-1}	1.0 mL

Prepare stock solutions with distilled water. For final metal mix dissolve $Na_2EDTA.2H_2O$ and $FeCl_3.6H_2O$ in 900 mL of distilled water, add 1 mL of each of the remaining components, bring the volume to 1 L and store at 4°C.

Table 9. L1 Medium used for culturing of *Symbiodinium* cells.

Components	Stock Solution	Quantity (1 L)
$NaH_2PO_4.H_2O$	5 g L^{-1}	1.0 mL
$NaNO_3$	75 g L^{-1}	1.0 mL
L1 Trace Metal Mix	(separate)	1.0 mL
L1 Vitamin Mix	(separate)	1.0 mL

Prepare stock solutions with distilled water. For final L1 medium add components except the vitamin mix to 900 mL of filtered seawater, bring the volume to 1 L, check pH is between 8.0 – 8.2, autoclave the medium at 121°C for 22 min, allow medium to cool to room temperature before adding the L1 vitamin mix.

Table 10. L1 Vitamin Mix for use in the preparation of L1 Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Quantity (1 L)
Biotin (d-)(Vitamin H)	0.5 g L^{-1}	1.0 mL
B_{12} (cyanocobalamin)	0.5 g L^{-1}	1.0 mL
Thiamine, HCl (Vitamin B1)	-	100 mg

Prepare stock solutions with distilled water. For final vitamin mix dissolve thiamine in 900 mL of distilled water, add 1 mL each of biotin and B_{12} stock solutions and bring the volume to 1 L. Filter sterilize the mix and store at 4°C.

Table 11. L1 Trace Metal Mix for use in the preparation of L1 Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Stock Solution (1 L)
$CuSO_4.5H_2O$	2.5 g L^{-1}	1.0 mL
$Na_2MoO_4.2H_2O$	19.9 g L^{-1}	1.0 mL
$CoCl_2.6H_2O$	11.90 g L^{-1}	1.0 mL
$Na_2EDTA.2H_2O$	-	4.36 g
$FeCl_3.6H_2O$	-	3.15 g

H_2SeO_3	1.29 g L^{-1}	1.0 mL
$NiSO_4.6H_2O$	2.63 g L^{-1}	1.0 mL
Na_3VO_4	1.84 g L^{-1}	1.0 mL
K_2CrO_4	1.94 g L^{-1}	1.0 mL
$MnCl_2.4H_2O$	178.10 g L^{-1}	1.0 mL
$ZnSO_4.7H_2O$	23.0 g L^{-1}	1.0 mL

Prepare stock solutions with distilled water. For final metal mix dissolve $Na_2EDTA.2H_2O$ and $FeCl_3.6H_2O$ in 900 mL of distilled water, add 1 mL of each of the remaining components, bring the volume to 1 L and store at 4°C.

Table 12. K Medium used for culturing of *Symbiodinium* cells.

Components	Stock Solution	Quantity (1 L)
NH_4Cl	2.67 g L^{-1}	1.0 mL
$NaNO_3$	75 g L^{-1}	1.0 mL
Na_2 β-glycerophosphate.$6H_2O$	2.16 g L^{-1}	1.0 mL
H_2SeO_3	1.29 g L^{-1}	1.0 mL
Tris-base pH 7.2	121.1 g L^{-1}	1.0 mL
K Trace Metal Mix	(separate)	1.0 mL
K Vitamin Mix	(separate)	0.5 mL

Prepare stock solutions with distilled water. For final K medium add components except the vitamin mix to 900 mL of filtered seawater, bring the volume to 1 L, autoclave the medium at 121°C for 22 min, allow medium to cool to room temperature before adding the K vitamin mix.

Table 13. K Vitamin Mix for use in the preparation of K Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Quantity (1 L)
Biotin (d-)(Vitamin H)	0.1 g L^{-1}	1.0 mL
B_{12} (cyanocobalamin)	1.0 g L^{-1}	1.0 mL
Thiamine, HCl (Vitamin B1)	-	200 mg

Prepare stock solutions with distilled water. For final vitamin mix dissolve thiamine in 900 mL of distilled water, add 1 mL each of biotin and B_{12} stock solutions and bring the volume to 1 L. Filter sterilize the mix and store at 4°C.

Table 14. K Trace Metal Mix for use in the preparation of K Medium for culturing *Symbiodinium* cells

Components	Stock Solution	Stock Solution (1 L)
$CuSO_4.5H_2O$	2.5 g L^{-1}	1.0 mL
$Na_2MoO_4.2H_2O$	7.26 g L^{-1}	1.0 mL
$Na_2EDTA.2H_2O$	-	37.220 g
$FeCl_3.6H_2O$	-	3.15 g
$FeNaEDTA.3H_2O$	-	4.930 g
$MnCl_2.4H_2O$	-	0.178 g
$ZnSO_4.7H_2O$	23.00 g L^{-1}	1.0 mL
$CoSO_4.7H_2O$	14.05 g L^{-1}	1.0 mL

Prepare stock solutions with distilled water. For final metal mix dissolve $Na_2EDTA.2H_2O$, $FeCl_3.6H_2O$, $FeNaEDTA.3H_2O$ and $MnCl_2.4H_2O$ in 900 mL of distilled water, add 1 mL of each of the remaining components, bring the volume to 1 L and store at 4^oC.

Table 15. 10X Tris-borate-EDTA buffer (TBE) for electrophoresis

Components	Quantity
Tris	108 g
Na^2EDTA	9.3 g
Boric acid	55 g

Dissolve in 900 mL of distilled water and pH to 8.3 the make buffer up to 1 L.

Table 16. Sodium chloride-sodium citrate buffer

Components	10x SSC	20x SSC
NaCl	87.66 g	175.32 g
Tri-sodium citrate	44.117 g	88.32 g
dH_2O	Final volume 1 L	Final volume 1 L

Check pH is 7 - 8 and store at room temperature for up to 3 months

Table 17. 100X Denhardt's solution for pre-hybridization and hybridization solutions

Components	Quantity
Polyvinilpyrolidone (PVP)	20 g
Ficoll	20 g

Bovine Serum Albumin fraction V (BSA)	20 g

Dissolve in 1 L of distilled water and store at -20^{o}C in 200 mL aliquots. Alternatively filter solution (0.2 μm filter) and store at 4^{o}C. Prior to use, warm solution to an appropriate temperature.

Table 18. Tris-borate-EDTA buffer (TBE) for protein extractions

Components	Concentration
Tris-borate pH 8.0	100 mM
$MgCl_2$	2 mM
Na^2EDTA	2 mM
Phenyl-methyl-sulphonyl-fluoride (PMSF)	1 mM

Add components and make up to 100 mL with distilled water and store at 4^{o}C.

Table 19. Tris-borate stock solution

Components	Quantity
Tris	24.228 g
Boric Acid	12.366 g

Dissolve components in 200 mL of distilled water on a heater/stirrer plate and pH to 8.0.

Table 20. Cracking buffer for SDS-PAGE

Components	Concentration
Bromophenol blue	0.01 % w/v
Tris pH 6.7	125 mM
SDS	2 % w/v
Glycerol	10 % v/v
β - mercaptoethanol	10 % v/v

Dissolve components in distilled water and store at room temperature.

Table 21. SDS-PAGE Gel composition

Components	10 %	12.5 %	15 %	18 %	4 % Stacking Gel
Tris-HCL 1.5M pH 8.8	2.5 mL	2.5 mL	2.5 mL	2.5 mL	-
Tris-HCL 0.5 M pH 6.8	-	-	-		2.5 mL
Acrylamide/bisacrylamide	2.5 mL	3.125 mL	3.75 mL	4.5 mL	1.33 mL

(29:1)					
dH_2O	4.8 mL	4.2 mL	3.5 mL	2.8 mL	6.1 mL
10 % SDS	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL
10 % Ammonium persulphate (APS)	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL
TEMED	0.01 mL	0.01 mL	0.01 mL	0.01 mL	0.01 mL

Table 22. Running buffer for SDS-PAGE

Components	Concentration
Tris	25 mM
SDS	0.1 % w/v
Glycine	192 mM

Dissolve components in distilled water and store at room temperature.

Table 23. Coomassie stain for SDS-PAGE

Components	Concentration
Methanol	50 %
Glacial acetic acid	10 %
Coomassie Brilliant Blue R-250	0.125 %

Dissolve Coomassie brilliant blue R-250 in distilled water then add methanol and acetic acid. Store at room temperature.

Table 24. Destain solution for SDS-PAGE

Components	Concentration
Methanol	10 %
Glacial acetic acid	10 %

Add components to distilled water and store at room temperature.

Table 25. Transfer buffer for SDS-PAGE

Components	Concentration
Tris	25 mM
Glycine	192 mM
Methanol	20 %

Add components to distilled water, adjust pH to 8.3 and store at room temperature.

Table 26. Phosphate buffered saline solution (PBS)

Components	Concentration
Phosphate buffer pH 7.4	10 mM
NaCl	150 mM

PBST: TBS + 0.1 % Tween 20

PBS – T – BSA: TBS + 0.1 % Tween 20 + 1% bovine serum albumin (BSA)

Table 27. Tris buffered saline solution (TBS)

Components	Concentration
Tris-HCL pH 7.5	10 mM
NaCl	150 mM

TBST: TBS + 0.1 % Tween 20

TBS – T – BSA: TBS + 0.1 % Tween 20 + 1% bovine serum albumin (BSA)

Table 28. Luria-Bertani (LB) media

Components	Quantity
Peptone, bacteriological grade	10 g
Yeast extract, bacteriological grade	5 g
NaCl	10 g
Tris 1 M, pH 7.5	10 mL

Dissolve components in 900 mL of distilled water and adjust pH to 7 – 7.5. Make up broth to 1 L, autoclave at 121^{o}C for 22 min and store sterile broth at 4^{o}C.

Table 29. Luria-Bertani Agar (LB agar)

Components	Quantity
Peptone, bacteriological grade	10 g
Yeast extract, bacteriological grade	5 g
NaCl	10 g
Agar	15 g
Tris 1 M, pH 7.5	10 mL

Dissolve components in 900 mL of distilled water and adjust pH to 7 – 7.5. Make up broth to 1 L, autoclave at 121^{o}C for 22 min, allow broth to cool to 55^{o}C prior to addition of antibiotics if required, plate immediately.

LB/ampicillin (LBA): LB agar with addition of 100 μg/mL ampicillin

Table 30. X-Gal/IPTG solution

Components	Quantity
X-Gal	200 mg
Isopropylthio-β-D-galactoside (IPTG)	58 mg

Dissolve in 10 mL of dimethylformamide and store covered in aluminium foil at -20^{o}C. To set LBA plates spread 50 μL of X-Gal/IPTG prior to addition of bacterial cells.