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Light-Related Photosynthetic Gene Expression and Enzyme Activity in the Heterokont Alga *Vaucheria litorea* and Its Symbiotic Partner the Sacoglossan Mollusc *Elysia chlorotica*

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LIGHT-REGULATED PHOTOSYNTHETIC GENE EXPRESSION AND ENZYME ACTIVITY IN THE HETEROKONT ALGA *VAUCHERIA LITOREA* AND ITS SYMBIOTIC PARTNER THE SACOGLOSSAN MOLLUSC *ELYSIA CHLOROTICA*

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An Abstract of the Thesis Presented
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Photosynthesis is comprised of tightly coupled reactions and therefore requires strict matrices of regulation, particularly involving alterations in gene expression and enzyme activity within the nucleus and plastid. Extensive research has been carried out on these light-regulated mechanisms in plants and green algae, however, much less is known in the red algal lineage, including heterokonts. The goal of this study was to investigate the influence of light on photosynthetic gene expression and select enzyme activity in the heterokont alga *Vaucheria litorea* and its symbiotic partner *Elysia chlorotica*, a photosynthetic

sacoglossan mollusc (sea slug). *Elysia chlorotica* harbors *V. litorea* plastids intracellularly in its digestive epithelia. The plastids remain intact and carry out photosynthesis for several months despite the absence of any algal nuclei in the sea slug. This is remarkable considering that greater than ninety percent of the plastid proteome, the metabolic and regulatory components of the plastid, is encoded by the nuclear genome. In this study, the nuclear-encoded plastid-localized Calvin cycle enzyme phosphoribulokinase (PRK) was targeted for characterization because of the evidence supporting horizontal gene transfer of *prk* from the alga to the sea slug. PRK was demonstrated, *in vivo*, to react differently to redox conditions in crude extracts from light- *vs.* dark-treated *V. litorea* but not in *E. chlorotica*. However, redox regulation of PRK, *in vitro,* was observed in crude extracts of both organisms following treatment with various oxidizing and reducing agents.

This study additionally characterized light regulation of gene expression in *V. litorea* and *E. chlorotica* through the quantitative analysis of nuclear and plastid gene expression. In *V. litorea,* nuclear (*prk*) and plastid (*psa*A, *rbc*L, *psb*A) transcript levels were quantified over a 12 hr light:12 hr dark photoperiod. Maximal plastid- and nuclear-encoded transcript levels were observed in response to light, 2 hr and 6 hr post-illumination, respectively. Nuclear (*prk*) and plastid (*psa*A, *rbc*L) genes in *E. chlorotica* were quantified 1 hr before and 1 hr after illumination. In contrast to *V. litorea*, expression of the nuclear gene *prk* was

down-regulated in *E. chlorotica* in response to illumination. Plastid gene transcript levels, however, were similarly up-regulated by light in the sea slug and alga, but on a much smaller scale in the sea slug.

Overall these results imply that the nuclear-encoded factors responsible for redox regulation of nuclear-encoded proteins, such as PRK, in *V. litorea* are probably not present in the sea slug. This could be due to the absence of homologous proteins encoded by the sea slug and/or the lack of horizontal gene transfer from the alga. The factors regulating chloroplast gene expression, however, appear to be present and functional in the sea slug. These findings are discussed relative to the differences reported for light regulation of PRK gene expression and activity in the green vs. red lineages of algae and in the context of the sea slug-algal plastid symbiotic association. Additionally, these studies represent the first molecular analysis of photosynthetic gene expression and regulation in a photosynthetic mollusc.

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Chapter 1

INTRODUCTION

1.1. Endosymbiotic Theory and Evolution.

The endosymbiotic theory explains the origin of both mitochondria and plastids through the separate and sequential engulfment of a prokaryote by a free-living organism. The origin of the mitochondria traces specifically to the enslavement of an α-proteobacterium followed by gene transfer and loss leading to the evolution of more complex heterotrophic eukaryotes (Roger, 1999). Subsequently, the plastid evolved in a separate single primary endosymbiotic event in which a photosynthetic cyanobacterium was engulfed by a heterotrophic mitochondrion-possessing eukaryote (Margulis, 1981; Bhattacharya and Medlin, 1995; Palmer, 2003; Weber et al., 2006). Similar to the evolution of mitochondria, the primary cyanobacterial endosymbiont underwent varying structural and genomic changes giving rise to the permanent plastid.

Organelle biogenesis required that the endosymbiont and the host work effectively and communicate in an efficient manner (Martin and Herrmann, 1998; Martin et al., 2002). Major steps in this process included endosymbiotic gene transfer (EGT) from the endosymbiont (pre-mitochondrion and pre-plastid) to the host cell's nuclear genome and the reduction of the endosymbiont's genome. However, before reduction of the endosymbiont's genome could occur, the genes

transferred to the host nucleus had to first be successfully expressed and retargeted back to the endosymbiont (Stegemann and Bock, 2006). Expression of the transferred genes required that the genes be integrated in close proximity to a downstream promoter. Retargeting, however, is a bit more complicated. For proteins to be targeted back to the organelles, genes first need to acquire an upstream targeting peptide sequence and a mechanism for the protein to enter the organelle (reviewed by Cavalier-Smith, 2002). Targeting peptides may originate through genetic mutations (Bock and Timmis, 2008), but the entry of proteins back into the plastid would require translocons in the outer and inner membranes. These may have originated from already existing mitochondrial or endomembrane translocators (Weber et al., 2006).

Following this extensive gene transfer and loss, the original free-living cyanobacterium evolved into a semi-autonomous organelle highly dependent on the cell's nuclear genome. Subsequent evolution of this autotrophic organism led to the emergence of three evolutionary lineages: the glaucophytes (dead-end lineage including the cyanelles), the chloroplastida or Viridiplantae (green algae and land plants) and the rhodophytes (red algae) (Bhattacharya and Medlin, 1995; Falkowski et al., 2004; Adl et al., 2005).

The glaucophytes retained a more primitive form of plastid, a cyanelle. Cyanelles have retained the original cyanobacterial peptidoglycan cell wall and light harvesting pigments (phycobilisomes and chlorophyll *a*) (Keeling, 2004).

The plastids in the rhodophytes also utilize phycobilisomes and chlorophyll *a* to harvest light energy, but differ from glaucophytes in having lost their peptidoglycan layer and possessing additional light harvesting accessory pigments (i.e. carotenoids). Plastids of the chloroplastida are the most diverged of the three primary lineages and lack the peptidoglycan layer and phycobilisomes. Instead, they contain chlorophylls *a* and *b* and additional accessory pigments.

Following primary endosymbiosis, the green and red algal lineages underwent secondary endosymbioses in which an entire single celled alga was engulfed by a heterotrophic eukaryote (McFadden, 1999). As a result of these additional endosymbiotic events the green and red algal phyla further diverged into their respective lines, accounting for the majority of the diversity in plastid-containing eukaryotes (Keeling, 2004). More specifically, the green line underwent two independent secondary events giving rise to the euglenids and the chlorarachniophytes. The red lineage underwent a single secondary event diverging into the Chromalveolates (cryptophytes, haptophytes, heterokonts and alveolates) (Archibald and Keeling, 2002). However, the exact number of secondary events involved in creating the Chromalveolate supergroup is still controversial (Sanchez-Puerta and Delwiche, 2008; Archibald, 2009).

During the early biogenesis of secondarily-derived plastids similar changes occurred as detailed for the primary plastids. Genes required for plastid functioning were transferred from the secondary endosymbiont's nucleus to the

host nucleus. Similar to the primary endosymbiotic relationship, this genetic transfer left the plastids dependent on the host's nuclear genome for thousands of proteins necessary for function and regulation (Martin and Herrmann, 1998). However, in the special case of the chlorarachniophytes and cryptophytes, the secondary endosymbiont nucleus was retained in a highly reduced state referred to as a nucleomorph (Gilson and McFadden, 1997; Douglas et al., 2001).

Subsequent tertiary and serial secondary endosymbiotic events involving secondarily derived endosymbionts have additionally been documented (Keeling, 2004; Yoon et al., 2005; Lane and Archibald, 2008; Sanchez-Puerta and Delwiche, 2008). Serial secondary endosymbiosis occurs when the primary plastid in a host is lost and successively replaced with a different primary plastid. Tertiary endosymbiosis, on the other hand, occurs when a heterotrophic or autotrophic eukaryote engulfs and maintains a secondarily-derived plastid (Bhattacharya and Nosenko, 2008). In organisms that evolved through serial secondary endosymbiosis, the transferred nuclear genes should already be present from the previous endosymbiotic event. In the case of tertiary endosymbiotic organisms, the genes required for plastid functioning may be present in the host from a previous endosymbiotic partner, or they may also be transferred from the newest endosymbiont's nuclear genome to the host's nuclear genome via horizontal gene transfer (HGT) (Lane and Archibald, 2008). HGT differs from EGT in that the transfer of the genes begins prior to, or independent of, the

establishment of a permanent endosymbiosis, and it is presumed to occur between unrelated organisms (Lane and Archibald, 2008).

1.2. *Elysia chlorotica* and *Vaucheria litorea.*

In this study the focus was on the tertiary endosymbiotic association formed between the photosynthetic sacoglossan mollusc (sea slug) *Elysia chlorotica* and plastids from its algal prey, *Vaucheria litorea. Vaucheria litorea* is a secondary, red algal-derived, heterokont alga. The four membranes surrounding its plastids reflect this secondary evolutionary history (Mujer et al., 1996; Rumpho et al., 2001). As expected, the plastid genome of *V. litorea* is in a reduced state compared to the ancestral cyanobacteria (Rumpho et al., 2008); consequently, the organelle is dependent on the algal nuclear genome to provide the majority of proteins essential for photosynthetic processes and regulatory mechanisms.

Elysia chlorotica is found in brackish marshes and frequently in association with filaments of *V. litorea.* Juvenile sea slugs feed on *V. litorea* during the course of development, sucking out the cellular contents of the filaments. Phagocytosis of the intact algal plastids follows with incorporation into the cytosol of the digestive epithelia (Trench, 1975; Graves et al., 1979). These stolen plastids, referred to as "kleptoplasts," remain functional in the cytoplasm of the sea slug for several months despite the absence of the algal nucleus (Trench,

1975; Mujer et al., 1996; Green et al., 2000). However, to maintain a fully functional plastid may require as many as 1000 to 5000 nuclear-encoded plastid-targeted (NEPT) proteins (Martin et al., 2002; Richly and Leister, 2004; Bock and Timmis, 2008). Regulation of the Calvin cycle enzymes in photosynthetic plastids requires NEPT factors as well as protein-protein interactions, most likely to ensure efficient light/dark metabolic coupling in order to prevent futile cycling of carbon and energy. This brings into question the source of essential nuclear-encoded plastid-proteins in the sea slug. In the alga, many of these proteins typically turnover rapidly due to damage from constant light exposure and are replaced by importing newly synthesized proteins from the nucleo-cytosol. In the absence of any algal nuclei in the sea slug, it is possible that the essential proteins are generated by: i) redirection from other biochemical processes, ii) unusual stability of the proteins from the original host, and/or iii) horizontal transfer of algal nuclear genes to the sea slug followed by expression and targeting to the kleptoplasts. Recent research by the Rumpho lab (Rumpho et al., 2008 and 2009) and Pierce lab (Pierce et al., 2007) has suggested that the HGT theory is a very likely source of at least some of the nuclear encoded plastid metabolic proteins in the sea slug. These conclusions are suggested by the findings of the algal nuclear encoded photosynthetic genes phosphoribulokinase (*prk*; Rumpho et al., 2009), the Mn-stabilizing protein of photosystem II (*psb*O; Rumpho et al., 2008), and three light harvesting proteins (*fcp*, *lhcv*1 and *lhcv*2; Pierce et al., 2007), within

the sea slug. This research does not exclude the other possibilities from our hypotheses due to the fact that five genes cannot fulfill the role of 1000 to 5000 NEPT proteins (Martin et al., 2002; Richly and Leister, 2004; Bock and Timmis, 2008) required to sustain plastid functioning and regulatory mechanisms within the sea slug.

1.3. Regulation of Plastid Activity.

Plastids are not only dependent on the nuclear genome for encoding proteins to sustain major metabolic processes, but they are also dependent on nuclear-encoded factors to regulate plastid functioning in response to various biotic and abiotic stimuli. This regulation of plastid functioning is essential in modulating photosynthetic activity to changes in the environment, and is of great significance when trying to understand plant physiology. Of the changing environment, light is a well known stimulus that is constantly changing and is therefore a primary concern for any photosynthetic system. Photosynthetic organisms need to regulate plastid and photosynthetic activity to these changes in light intensity and duration to avoid the damaging bi-products produced during photosynthetic activity. These light regulatory mechanisms may involve nuclear-encoded proteins involved in circadian, photoreceptor or various redox signaling pathways that ultimately alter photosynthetic activity at the transcriptional and post-translational levels.

Circadian rhythms are internal biological oscillators in eukaryotic organisms that are entrained by environmental signals to maintain metabolic and physiological processes during particular phases of the 24 hr day. These rhythms involve cellular feedback loops of particular nuclear-encoded gene products that alter the transcription of many target genes (reviewed in Dunlap, 1999). Of particular interest in plant systems is the effect of light on the entrainment of circadian feedback loops that alter photosynthetic gene transcription in the nucleus and in the plastid (McClung, 2001; Young and McKay, 2001). This form of circadian control has been documented in cyanobacteria (Stockel et al., 2008), green algae (Lemaire et al., 1999), land plants (Taylor, 1989; Millar and Kay, 1996), and red algae (Jacobsen et al., 2003; Goulard et al., 2004) throughout the 24 hr light/dark photoperiod. Such widespread findings of circadian control over photosynthetic gene transcription suggest that it may be a universal mode of light regulation.

Photoreceptors are known to affect nuclear photosynthetic gene expression and entrain these circadian rhythms in response to light (Millar and Kay, 1996). In land plants there are three accepted types of photoreceptors: 1) phototropins, 2) cryptochromes and 3) phytochromes (reviewed in Fankhauser and Staiger, 2002). The latter two are associated with entrainment of circadian rhythms and nuclear photosynthetic gene expression via blue- and red-light induction, respectively (Devlin and Kay, 2001; Quail, 2002). Phototropins also perceive blue light

wavelengths but are primarily involved with phototropic post-translational alterations (Briggs and Huala, 1999). In addition to the primary roles of the individual photoreceptors in land plants, significant cross-talk between the photoreceptors occurs (Folta and Spalding, 2001; Ma et al., 2001). Studies in heterokonts (i.e. *Vaucheria*) have alternatively focused primarily on blue light regulation based on the predominance of blue light in marine environments and the reduced ability of the shorter red light wavelengths to penetrate the thick water layers (Kirk et al., 1994). Takahashi et al. (2007) discovered a blue-light photoreceptor, aureochrome, in the heterokont algae *Vaucheria frigida* and *Fucus distichus*. Aureochrome was found to be related to the phototropin-type photoreceptors in land plants; however, unlike phototropins, aureochrome was able to directly bind to bZIP transcription domains of nuclear genes (Takahashi et al., 2007). This suggests that aureochrome may be acting within *Vaucheria* to regulate nuclear gene transcription based on light availability.

Additionally, it has been suggested that the circadian feedback loops that control photosynthetic gene transcription are affected by internal redox signals. These redox signals are generated by photosynthetic activity within plastids and occur in response to changes in light intensity, of which short- and long-term modulating processes in land plants and green algae (collectively known as chloroplastida or Viridiplantae) have been documented extensively (reviewed in Woodson and Chory, 2008). The short-term approach to changes in light intensity

involves internal plastid signals that influence post-translational processes that alter the action of specific proteins in order to maintain a balance of activity among the many coupled biochemical pathways within the plastid (Allen, 2003; Puthiyaveetil and Allen, 2008), whereas the long-term approach involves communication between the nucleus and the plastid via anterograde and retrograde signaling networks (depicted in Fig. 1.1). Anterograde signals are specifically transmitted from the nucleus to the plastid and affect photosynthetic functions at the transcriptional (Maliga, 1998; Allen and Pfannschmidt, 2000; Wagner and Pfannschmidt, 2006), post-transcriptional (Zoschke et al., 2007; Wobbe et al., 2008), and post-translational (Barneche et al., 2006; Marin-Navarro et al., 2007) levels. In contrast, retrograde signals involve chemical signals (e.g., H_2O_2) that are transported from the plastid to the nucleus effecting nuclear photosynthetic gene expression (Pfannschmidt et al., 2001, 2005, 2009; Chen et al., 2004; Kanamaru and Tanaka, 2004; Marri et al., 2005a; Von Gromoff et al., 2006; Kousseitzky et al., 2007).

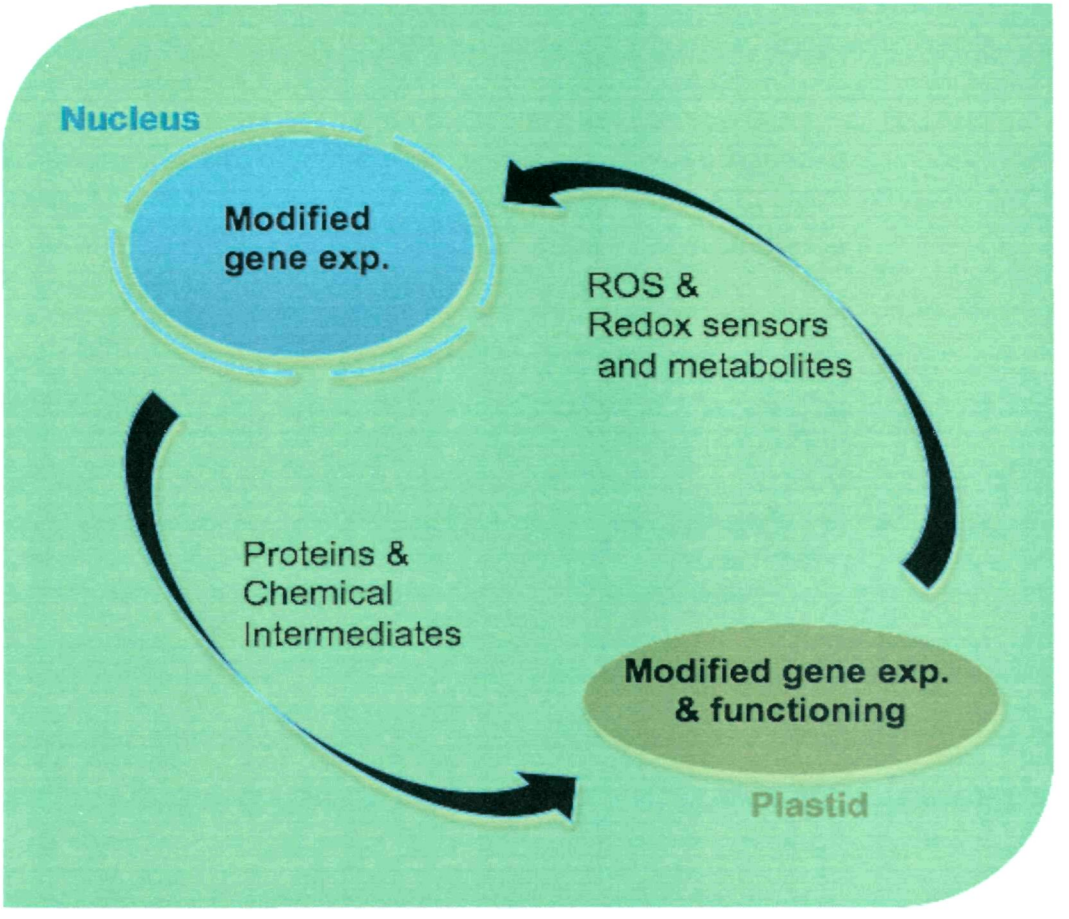

Figure 1.1. Obligate Intra-Organelle Signaling Network Between the Nucleus and the Plastid.

Despite the differences between the short- and long-term approaches to light acclimation, they both are regulated by unknown signaling pathways involving the redox state of proteins within the thylakoid membrane's photosynthetic apparatus. These proteins include plastoquinone (PQ) and photosystem I (PSI) within the apparatus (Escoubas et al., 1995; Pfannschmidt et

al., 1999); however, other major nuclear-encoded factors involved with these short- and long-term approaches may include sigma factors (Kanamaru et al., 2001; Ishizaki et al., 2005), thioredoxin and ferrodoxin (Howard et al., 2008; Marri et al., 2009) and various transcription factors (Wagner and Pfannschmidt, 2006; Krause and Krupinska, 2009; Waters et al., 2009).

Although the short- and long-term approaches to light acclimation originate from the same source they involve different signaling cascades. As stated previously, the long-term approach involves the action of numerous proteins in intricate signaling pathways, whereas the short-term approach involves the modifications of proteins within the immediate area (i.e. stromal proteins).

The main connection between PQ and long-term acclimation to changes in light intensity suggestively involves the two-component clock system in cyanobacteria (Imamura et al., 1998; Iwasaki et al., 2000; Hwang et al., 2002). This two-component system works through the use of a sensor kinase and a response regulator factor (RRF) (reviewed in Ashby and Houmard, 2006 and Puthiyaveetil and Allen, 2009). The sensor kinase is the first to act in this system and does so in response to a specific signal. Once the signal is recognized the sensor kinase then undergoes ATP-dependent autophosphorylation of a specific histidine residue. This phosphate group is then transferred to an aspartate residue on one or more corresponding RRF. Phosphorylation of the RRF produces an appropriate response to the environmental change that stimulates the cascade.

The connection between the two-component cyanobacterial system and the modern plastid's long-term acclamation involving the redox state of PQ is based upon the light harvesting complex II (LHCPII) kinases STN7/8 and Stt7/11 in land plants and green algae, respectively. The LHCPII kinases have been suggested to act as the sensor kinases that sense the change in the redox state of PQ (as depicted in Fig. 1.2; Depege et al., 2003; Bellafiore et al., 2005; Bonardi et al., 2005; reviewed in Rochaix JD, 2007). Once the LHCPII kinases receive this signal the histidine kinase cascade is initiated and, as proposed by Doran and Cattolico (1997), this cascade eventually ends with the RRF regulating plastid gene transcription. Additionally, these LHCPII kinases are suggestively involved with the initiation of a phosphorylation cascade that crosses the cytosol to alter nuclear photosynthetic gene expression (as depicted in Fig. 1.2; Bonardi et al., 2005; reviewed in Rochaix et al., 2007). Little is known about the pathway in which this response occurs; however, it is known that it involves a positive feedback loop that creates an amplified response based on the fact that the components control the operon in which they are encoded (Allen, 1995). This is seemingly characteristic of circadian rhythms and suggests that the plastid-encoded two-component system may be acting as an endogenous oscillator of photosynthetic gene transcription and potentially causes a signaling cascade in the nucleus to alter nuclear photosynthetic gene expression.

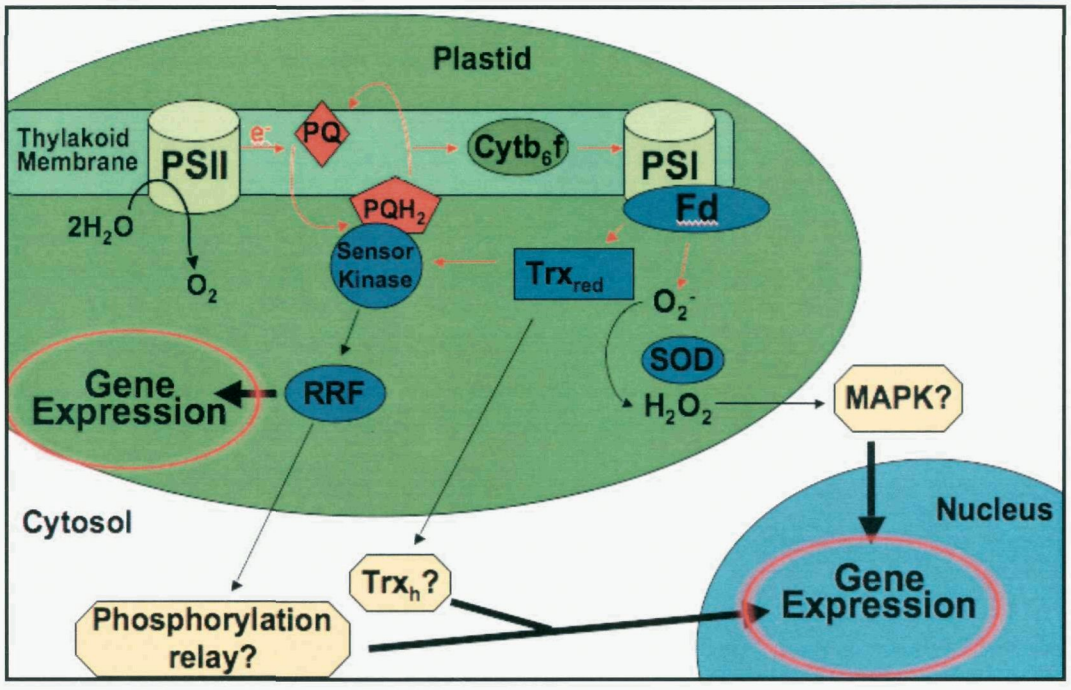

Figure 1.2. A Model Depicting the Long-Term Light Modulating Mechanisms Involving Proteins Associated with the Photosynthetic Apparatus. In the plastid the sensor kinase selectively alters gene expression through interactions with a response regulatory factor (RRF) in response to changes in the redox state of plastoquinone (PQ) and possibly plastidal thioredoxin (Trx_{red}). Additionally, these signaling pathways transduce across the cytosol in undifferentiated pathways (shown in yellow) to the nucleus, altering nuclear photosynthetic gene expression. The nuclear-encoded components within the plastid are shown in blue and the red lines associated with the thylakoid membrane depict electron flow. Abbreviations: cytochrome b_6f ($Cytb_6f$), cytosolic thioredoxin (Trx_h), ferredoxin (Fd), mitogen-activated protein kinase (MAPK), photosystem II (PSII), photosystem I (PSI) and superoxide dismutase (SOD).

The connection between PSI and the long-term modulating mechanisms to changes in light is suggested to occur through the redox mediator ferrodoxin (Fd). Ferredoxin is another key enzyme involved in the regulation of the redox state of the plastid, particularly when transitioning from dark to light. In the light, ferredoxin distributes high-energy electrons from PSI reducing various plastid components leading to changes in nuclear photosynthetic gene expression, most notably the reduction of oxygen and the plastidal thioredoxin (Trx_{red}) (Piippo et al., 2006; reviewed in Oelze et al., 2008). The reduction of oxygen by ferredoxin produces singlet oxygen (O_2^-), which is rapidly converted to H_2O_2 by a copper-zinc superoxide dismutase (SOD) (Munekage et al., 2002). Hydrogen peroxide is considered one of the longer lived and less toxic reactive oxygen species (ROS) produced by photosynthetic processes (Vranova et al., 2002). As a result, H_2O_2 has been implicated to have a role in signaling and has been demonstrated to interact with a mitogen-activated protein kinase (MAPK), acting in a retrograde signal transduction pathway that affects nuclear photosynthetic gene expression (Apel and Hirt, 2004). Plastid thioredoxin, on the other hand, alters the activity of the two-component sensor kinase involved with the modification of plastid photosynthetic gene expression (Rintamaki et al., 2000; reviewed in Rochaix et al., 2007) and potentially causes a signaling cascade in the cytosol, involving a cytosolic thioredoxin (Trx_h), to the nucleus that alters nuclear photosynthetic gene expression as well (Lemaire et al., 1999).

Short-term processes, as discussed previously, are also connected to the redox state of PQ and PSI and tend to occur within minutes of changes in light intensity. The redox state of PQ is well known to alter the location of light harvesting complex protein II (LHCPII) between PSII and PSI during state transitions when there are variations in light intensity. That is, when PQ is reduced during low light the system is said to be in state 2 (light favoring PSII activity). This reduced PQ then activates an LHCPII kinase (i.e. STN 7 in *Chlamydomonas reinhardtii)*, which then phosphorylates LHCPII causing it to move to from PSII to PSI to increase cyclic photosynthesis in order to promote ATP production even in low light conditions. When PQ is oxidized during high light, the system is in state 1 (light favoring PSI activity). When PQ is oxidized, the LHCPII kinase is deactivated, allowing a dephosphatase to cleave the phosphate from LHCPII causing it to move back to PSII to increase noncyclic photosynthesis to enhance ATP and NAPDH production during high light conditions.

The redox state of PSI is involved with various short-term modulating processes in response to light, similarly to the long-term responses, through the dissemination of the redox power via redox sensitive proteins such as ferrodoxin (Fd) and Trx_{red} (Fig. 1.3). Plastidal thioredoxin (Trx_{red}) has been shown to act in the Calvin cycle by activating key enzymes (i.e. fructose-1,6-bisphosphatase [FBPase], sedoheptulose-1,7-bisphosphatase [SBPase] and phosphoribulokinase

[PRK]) through the reduction of deactivating disulfide bridges. Additionally Trx_{red} acts to deactivate glucose-6-phopshate dehydrogenase (G6DPH) in the oxidative pentose phosphate pathway (OPPP) (Wenderoth et al., 1997). The OPPP and the Calvin cycle are coupled pathways and, as such, light deactivation of G6DPH prevents futile cycling.

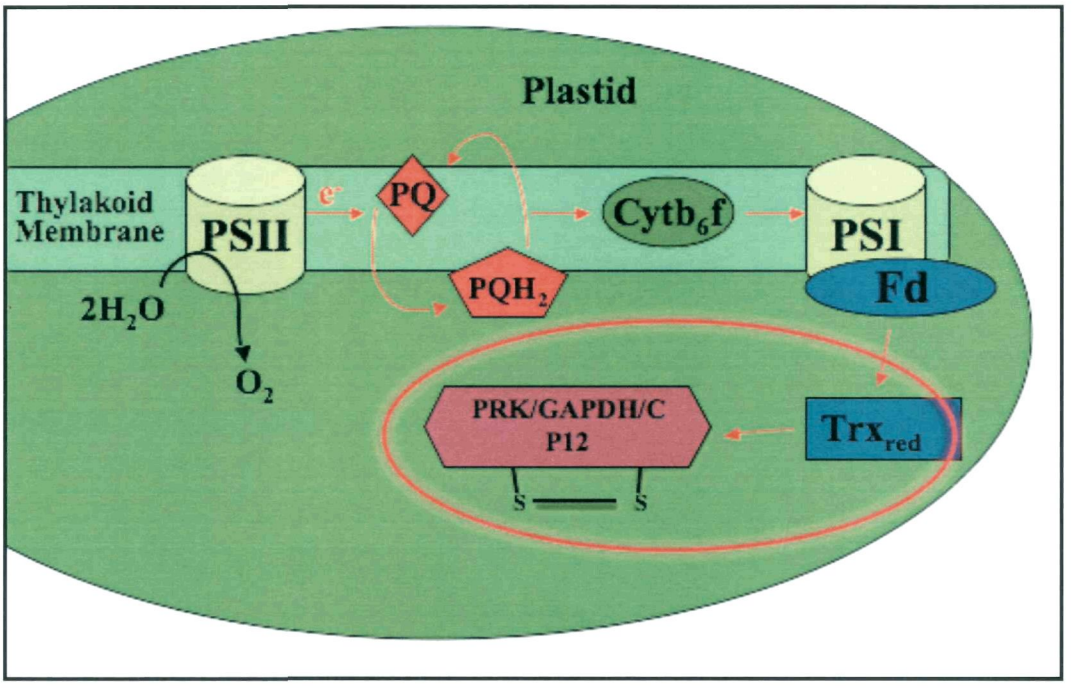

Figure 1.3. A Model Depicting the Short-Term Light Modulating Mechanisms Involving Proteins Associated with the Photosynthetic Apparatus. In the plastid photosystem I (PSI) dissipates redox energy via ferredoxin (Fd). Plastidal thioredoxin (Trx_{red}) is a redox-sensitive protein that reduces thiol-disulfide bonds after being reduced by Fd, particularly involving the phosphoribulokinase/ glyceraldehyd-3-phosphate dehydrogenase/CP12 (PRK/GAPDH/CP12) complex. The nuclear-encoded components within the plastid are shown in blue and the red lines associated with the thylakoid membrane depict electron flow. Abbreviations: cytochrome b_6f ($Cytb_6f$), cytosolic thioredoxin (Trx_h), ferredoxin (Fd), mitogen-activated protein kinase (MAPK), photosystem II (PSII), photosystem I (PSI) and superoxide dismutase (SOD).

As PRK is one of the few nuclear-encoded photosynthetic enzymes that can not be replaced by any other enzymatic reaction and that we can measure using biochemical techniques, therefore the interactions between Trx_{red} and PRK are of utmost importance to this study. More specifically, under light conditions the nuclear-encoded and plastid targeted thioredoxins are reduced within the plastid's stroma by electron flow associated with the light reactions of photosynthesis. Reduced plastidal thioredoxin serves to reduce the N-terminal Cys16 and Cys55 residues in the spinach PRK active site, thereby activating the enzyme by breaking the disulfide bond formed under oxidizing (dark) conditions (Porter and Hartman, 1988; Milanez et al., 1991; Brandes et al., 1996; Hirasawa et al., 1998; Geck and Hartmann, 2000). The corresponding Cys residues in *V. litorea* are Cys71 and Cys115 (Rumpho et al., 2009). A comparison of this conserved region among representative organisms throughout the Archaeplastida (glaucophytes, green and red algae, and land plants) reveals variability in the number of amino acids (aa) between the two regulatory Cys residues (Fig. 1.4).

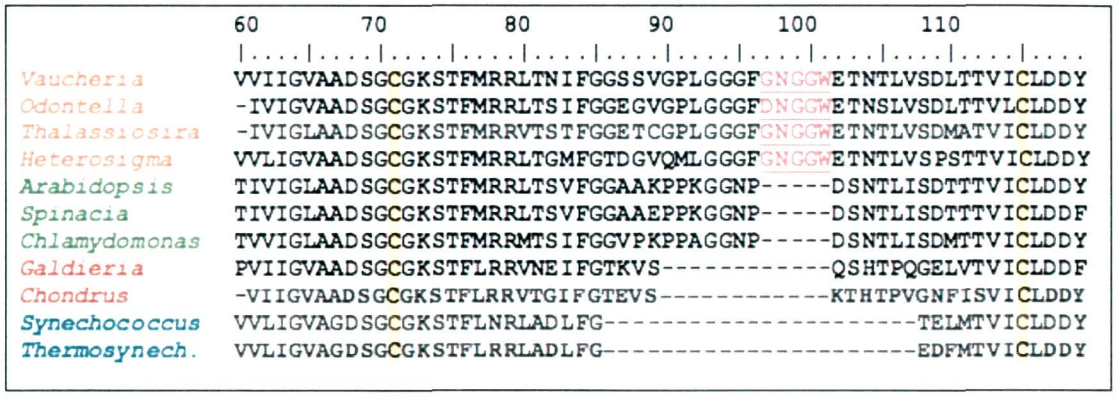

Figure 1.4. Alignment of Partial PRK Amino Acid Sequences. The two conserved regulatory Cys residues are highlighted in yellow and the five amino acid insertion between the Cys residues for *Vaucheria litorea* and three other chromalveolates is shown in red. The amino acids are numbered according to the PRK protein of *V. litorea*. Organisms include: chromalveolates (orange) *Vaucheria litorea* (AAK21910), *Odontella sinensis* (CAA69902), *Thalassiosira pseudonana* (XP002289281) and *Heterosigma carterae* (ABQ42600); chloroplastida (green) *Arabidopsis thaliana* (AAG50797), *Spinacia oleracea* (CAA30499) and *Chlamydomonas reinhardtii* (AAF36402); rhodophytes (red) *Galdieria sulphuraria* (CAC80070) and *Chondrus crispus* (AAX13965); and cyanobacteria (blue) *Synechococcus sp.* PCC 7942 (BAA96253) and *Thermosynechococcus elongatus* (BAC09466).

More specifically, 44 aa separate the regulatory Cys in land plants (streptophytes) and green algae (collectively known as chloroplastida), whereas in *V. litorea* and closely related heterokonts there are an additional 5 aa in this region. In contrast, the rhodophyte *G. sulphuraria* (Oesterhelt et al., 2007) exhibits a 12 aa deletion and the cyanobacteria *Synechococcus sp.* PCC 7942 (Tamoi et al., 1998; Kobayashi et al., 2003) and *Synechocystis sp.* PCC 6803 (Tamoi et al., 1998) have a 17 aa deletion. It has been suggested that these insertions and deletions (relative to chloroplastida) in the conserved region of

PRK from cyanobacteria, red algae and heterokonts impede the formation of the inhibiting disulfide bridge in oxidizing conditions, thereby affecting the redox sensitivity of PRK in these organisms (Tamoi et al., 1998; Kobayashi et al., 2003; Michels et al., 2005; Oesterhelt et al., 2007). However, the 5 aa insertion is found in the redox sensitive heterokont *H. carterae* (Cattolico et al., 1998), and also in the presumably non-redox sensitive heterokonts *O. sinensis* (Michels et al., 2005) and *A. formosa* (Boggetto et al., 2007). This inconsistency further supports the complex, multifaceted nature of redox regulation of PRK.

Such inconsistencies then raise the question of why PRK complexes with GAPDH and may suggest that *in vivo* this complex may act as more than a light/dark on/off switch (Howard et al., 2008). This is in agreement with the view that supermolecular complexes in plastids may offer a functional biological advantage and/or a regulatory role; channeling of intermediates between complexed proteins (Gontero et al., 1988; Sainis et al., 1989; Suss et al., 1993; Dani and Sainis, 2005; Anderson et al., 2006 and 2009) and conformational changes within individual proteins, have both been proposed (Lebreton et al., 1997; Lebreton and Gontero, 1999; Marri et al., 2005b; Erales et al., 2008).

Supermolecular complexes that channel intermediates frequently require that the enzymes involved catalyze consecutive metabolic reactions, but this is not the case for the PRK/GAPDH/CP12 complex. Nor is it the case for the other non-consecutive protein complexes that PRK forms with RUBISCO/GAPDH (Rault et

al., 1993) and GAPDH/CP12/aldolase (Erales et al., 2008). However, the PRK/GAPDH complex has alternatively been found to associate with consecutive proteins in free and thylakoid-bound complexes that link PRK and GAPDH intermediates in streptophytes (Gontero et al., 1988; Anderson et al., 1996; Babadzhanova et al., 2002; Anderson et al., 2009) and cyanobacteria (Dani et al., 2005). These findings overall suggest that PRK complexes with various proteins for efficiency and regulatory purposes, either within the stroma or attached to the thylakoid membrane.

A regulatory role must then be the primary role of the PRK/GAPDH/CP12 complex when it isn't complexed with proteins that catalyze consecutive reactions. The proposed regulatory roles of these complexes are based on the conformational changes that occur within individual proteins upon complex formation and for short periods after the complex dissociates (Avilan et al., 1997a and 1997b; Lebreton et al., 1997). More explicitly, a significant effect is seen in the catalytic functioning of the proteins undergoing these conformational changes upon complex formation. This effect is then maintained for short periods after dissociation due to 'information transfer' or 'imprinting' from other enzymes within the complex. The effects of such conformational changes have been studied extensively, particularly within the PRK/GAPDH/CP12 complex (Avilan et al., 1997a and 1997b; Lebreton et al., 1997; Lebreton and Gontero, 1999; Mouche et al., 2002; Graciet et al., 2004; Marri et al., 2009). Avilan et al. (1997a)

and Lebreton et al. (1997) found PRK primarily in three forms in the green alga *Chlamydomonas reinhardtii*: i) the stable reduced form, ii) the stable oxidized form, and iii) the metastable form. PRK is in its active stable form when it is reduced by the light reactions and uncomplexed. PRK then enters the stable, complexed oxidized form during dark conditions when the light reactions are no longer generating Trx_{red}. However, the instant the organism is introduced to light the reducing power generated by the light reactions produces Trx_{red} and the stable complexed form of PRK dissociates into the 'imprinted' metastable form. The stable uncomplexed form of PRK seems to act similarly in most photosynthetic organisms, where reducing conditions activate and oxidizing conditions inactivate the enzyme and allow it to complex with GAPDH/CP12. However, as discussed previously, PRK does not exhibit universal responses once complexed.

Lebreton et al. (1997) showed that in *C. reinhardtii* the oxidized stable uncomplexed PRK is inactive, but when oxidized PRK is complexed or in the metastable form, it gains catalytic competence. This suggests that formation of the complex may cause a conformational change in PRK that affects the ability of the inactivating disulfide bond to form. This inability of PRK to form an inactivating disulfide bond while in complex, is maintained after dissociation in the metastable form of PRK and credited, along with the increased flexibility of the free metastable PRK, with the significantly higher catalytic activity immediately after complex dissociation (Lebreton et al., 1997; Lebreton and

Gontero, 1999). These findings for *C. reinhardtii* (Lebreton et al., 1997), along with the similar findings in *Arabidopsis thaliana* (Marri et al., 2009), support the hypothesis that the PRK/GAPDH/CP12 complex maintains PRK in a state of super-reactivity that is promptly and easily activated upon complex dissociation. Such a state of super-reactivity has not been studied in cyanobacteria, red algae, or complex secondary red algal descendents. However, in these organisms, the variation in the number of amino acids separating the two redox regulatory Cys residues may affect the super-reactivity of PRK dissociating from the complex. This additionally suggests that the non-universal light/dark modulation of complexed PRK may be due to the amino acid insertions and deletions present in the regulatory region of PRK in cyanobacteria, red algae and heterokonts, relative to chloroplastida (Fig. 1.4).

Considerable research (as has just been discussed) pertaining to the light regulatory mechanisms within land plants and green algae has been carried out; however, much less is known for red algae and secondarily-derived organisms, despite the fact that they represent the vast majority of photosynthetic organisms in the world's oceans (Cattolico et al., 1998). This study not only adds to the small database of research pertaining to light regulatory mechanisms within the secondarily-derived red algal origins but also represents the first set of findings for light regulatory targets (i.e. transcripts and proteins) in the heterokont *V. litorea* and its symbiotic partner *E. chlorotica*. Furthermore, it is unclear which, if

any, plastid regulatory factors are present and/or functioning within *E. chlorotica*, particularly when considering the absence of the algal nucleus. The acquisition of *V. litorea* plastids by *E. chlorotica* introduces a new complexity for maintenance and regulatory processes within a photosynthetic organism and this study is the first attempt at understanding such processes supporting this unique symbiotic relationship.

1.4. Specific Objectives.

The overall goal of this study was to begin to understand the mechanisms of light regulation of plastid activity in *V. litorea* and compare these findings to kleptoplastic *E. chlorotica*. Understanding this will advance our knowledge of the role of the nucleo-cytosol in regulating plastid activity not only in the poorly understood heterokonts, but also in a totally foreign environment for plastids, a sacoglossan mollusc. The specific objectives included:

1. Measuring and comparing the enzymatic activity of the nuclear-encoded plastid-enzyme phosphoribulokinase (PRK) under various redox conditions in light- and dark-treated *Vaucheria litorea* and *Elysia chlorotica.*

2. Measuring select nuclear and plastid transcript levels by quantitative real time-polymerase chain reaction (qRT-PCR) in light- and dark-treated *Vaucheria litorea* and *Elysia chlorotica.*

Chapter 2

DIURNAL EXPRESSION OF NUCLEAR AND PLASTID PHOTOSYNTHESIS GENES IN THE HETEROKONT ALGA *VAUCHERIA LITOREA* AND ITS SYMBIOTIC PARTNER THE SACOGLOSSAN MOLLUSC *ELYSIA CHLOROTICA*

2.1. Introduction.

Photosynthesis is a complex physical and biochemical process that harvests the energy in sunlight and generates oxygen as a by-product, sustaining aerobic life on earth. The NADPH and ATP generated by the electron transfer system of the light reactions are tightly coupled to the temperature-dependent reactions of the Calvin cycle in which CO_2 is incorporated and reduced to sugars or lipids. Such coupling results in many of these reactions being highly regulated at the molecular level by various internal and external signals.

Redox internal signaling pathways are known to regulate photosynthetic activity in response to changing external signals (i.e. light); however, the signaling pathways remain elusive. Despite this, the integral photosynthetic protein plastoquinone (PQ) has been identified as a primary redox administrator. The redox state of the PQ pool affects photosynthetic activity at the transcriptional level potentially involving the two-component clock system in cyanobacteria (Imamura et al., 1998; Iwasaki et al., 2000; Hwang et al., 2002).

These components have been maintained in the plastids of non-green algae, but in chloroplastida and various secondary red algal descendents (i.e. *V. litorea*) the components have either been presumably transferred to their nuclear genomes or lost altogether (Puthiyaveetil et al., 2008; Rumpho et al., 2008; Puthiyaveetil and Allen, 2009). Nevertheless, Puthiyaveetil and Allen (2009) have recently proposed that the cyanobacterial two-component system is the link between the photosynthetic processes and the transcription of photosynthetic genes despite the location of the component genes.

Considerable research on such regulation of photosynthetic gene expression has been carried out in land plants and green algae; however, much less is known for red algae and secondarily-derived organisms, despite the fact that they represent the vast majority of photosynthetic organisms in the world's oceans (Cattolico et al., 1998). Considering that rhodophytes and chlorophytes diverged early in evolution, and that rhodophytes and some of their descendants have maintained the plastid two-component system, one might expect the regulation of photosynthesis in these organisms to differ considerably. To begin to address this, expression of both nuclear- and plastid-encoded photosynthetic genes were examined in the red alga-derived heterokont *Vaucheria litorea* and its symbiotic partner, the sacoglossan mollusc (sea slug) *Elysia chlorotica.*

Elysia chlorotica is of particular interest because of its ability to form a symbiotic (or kleptoplastic) association with plastids from *V. litorea.* This

symbiotic relationship is established during the course of development in which juvenile sea slugs feed on filaments of *V. litorea* and phagocytically incorporate the intact algal plastids into their own digestive cells (Trench, 1975; Graves et al., 1979). The stolen plastids, or "kleptoplasts," are then retained in a photosynthetically functional state in the cytoplasm of the sea slug cells throughout the entire life of the sea slug (about 10 to 12 months), despite the absence of any algal nuclei in the sea slug (Mujer et al., 1996; Green et al., 2000; Rumpho et al., 2001).

This absence of the algal nucleus creates a conundrum when considering the longevity of the plastids inside the sea slug due to the necessity for algal nuclear genes for plastid functioning and regulation. Recent published data support the horizontal gene transfer (HGT) of five algal nuclear genes to *E. chlorotica* from *V. litorea* encoding: the Calvin cycle enzyme phosphoribulokinase (*prk*) (Rumpho et al., 2009), the Mn-stabilizing protein of photosystem II (*psb*O) (Rumpho et al., 2008), and three light harvesting proteins (*fcp*, *lhcv*1 and *lhcv*2) (Pierce et al., 2007). Due to the estimated requirement of about 1000 to 5000 nuclear-encoded plastid proteins to fully sustain plastid functioning (Martin et al., 1998 and 2002), it is highly likely that more genes have been transferred from the alga (or other photoautotroph) to the sea slug and/or gene products are re-routed from biochemical processes that are already established within the sea slug.

If this is true, then the nuclear-controlled regulatory processes exerted on the plastid in the native algal system may also be found in the non-native sea slug system. If not, photosynthetic gene expression would be expected to differ greatly between the two organisms in response to varying environmental conditions. Quantitative measurements of transcript levels of plastid- and nuclear-photosynthesis genes over a 24 hr light/dark photoperiod in *V. litorea* and *E. chlorotica* are presented.

2.2. Materials and Methods.

2.2.1. Algal and Sea Slug Culturing. Vaucheria *litorea* C. Agardh CCMP2940 cultures were maintained in a modified f/2 artificial seawater medium (ASW, 925 mosmol Instant Ocean, Aquarium Systems) at 18°C on a 12 hr photoperiod. Specimens of *Elysia chlorotica* Gould were collected from Martha's Vineyard, MA, and maintained without algae in aerated aquaria containing 925 mosmol ASW at 12°C on a 12 hr photoperiod for 2 weeks prior to sampling. For light-dark experiments, algal samples were collected every 4 hr over a 24-hr period beginning 2 hr after illumination at 7 am. For continuous dark experiments, algal samples were maintained in constant darkness for 2 days prior to collecting samples over an 18 hr period that began 1 hr prior and 1 hr post the "normal" onset of light and then every 4 hr for 16 hr. Due to limitations in the number of available sea slug specimens, fewer time-points could be collected and sampling

was limited to 1 hr pre- and 1 hr post-light exposure. All algal and sea slug samples were rinsed, blotted dry, weighed, frozen in liquid nitrogen (in matching dark or light conditions) and stored at -80°C until needed.

2.2.2. Primer Design. Primers for targeted genes (*psa*A, *psb*A, *rbc*L and *prk*) for quantitative real time-polymerase chain reactions (qRT-PCR) were generated using two computer-based programs: MIT Primer3 (www.genome.wi.mit.edu/cgibin/primer-primer) and IDT (www.idtdna.com) (Table 3.1). The primers were designed based on the following criteria: T_m = 55-60°C, 40-60% GC content, 100-160 bp length and 3′ clamp with a 50% GC content. The designed primers were synthesized by Integrated DNA Technologies INC. (Coralville, IA). All primers were tested for the generation of a single product and optimum T_m using a standard 40 cycle polymerase chain reaction (PCR) with an annealing gradient from 48°C to 60°C using *V. litorea* or *E. chlorotica* complementary DNA (cDNA) as template. Amplified products were subjected to electrophoresis in a 1% agarose gel and visualized by ethidium bromide (EtBr) staining to verify the single band products at their expected sizes. Once visually verified, the products were purified using the QIAquick PCR purification kit (Qiagen Inc.; Valencia, CA) and sequenced by the University of Maine DNA Sequencing Facility (Orono, ME) using an ABI 3730 capillary sequencer. Returned sequences were confirmed using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The respective PCR

products were then used in the preparation of plasmid standards for the absolute qRT-PCR experiments, as described below.

Table 2.1. Primers Used in the qRT-PCR Analysis of *V. litorea* and *E. chlorotica* Transcripts

Gene	Primer	Sequences 5′ to 3′	Amplicon size (bp)
*psa*A	psaA.for psaA.rev	TTCCTTGTGATGGTCCTGGT AGGTGTTACAGAGCCCCAAA	155
*psb*A	psbA.for psbA.rev	GGTGTTGCTGGTGTATTTGGTG ACCATGTGCAGCTACGATGT	159
*rbc*L	rbcL.s for rbcL.s rev	CCTGCTTGGATACCATCTGG TGGATTGGGCAGCATTACG	151
prk	prk.s2 for prk.s2 rev	TGACGTTGTCTTGCAGGTTT GTGCTCCCTTCATCGAACAAATA	135

2.2.3. Plasmid Preparation for qRT-PCR. Purified PCR products of the respective gene targets were ligated into pGEM-T Easy vector (Promega; Madison, WI) using T4 ligase and then transformed into NEB 5-alpha competent *E. coli* cells (New England Biolabs; Ipswich, MA). Transformed cells were incubated in super-optimal broth with catabolite repression (SOC) media (New England Biolabs; Ipswich, MA) for 60 min at 37°C and plated on Luria-Bertani (LB) agarose plates containing 50 μg mL^{-1} ampicillin and 40 μg mL^{-1} Xgal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). Plates were incubated for 12 hr at 37°C and then stored at 4°C. Colonies were subjected to standard PCR with

T7 and Sp6 plasmid-specific primers and visualized in a 1% agarose gel with EtBr staining. Colonies containing an insert matching the expected sizes of the respective target genes were grown in LB broth (50 mg mL^{-1} ampicillin) overnight at 37°C. Plasmids were isolated from the inoculated broth using a QIAprep Spin Miniprep Kit (Qiagen Inc.; Valencia, CA), sequenced to verify inserts and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.; Waltham, MA). Copy number was determined by dividing Avogadro's number by the molecular weight (mw = total number of nucleotides [bp] x 660 g/mol) of the plasmid. Plasmids were then used as standards in the qRT-PCR experiments.

2.2.4. RNA Isolation and cDNA Synthesis. Total RNA was isolated from frozen samples following the respective manufacturer's protocol as follows. For *V. litorea*, a Qiagen RNeasy mini kit was used and for *E. chlorotica,* a combination of the Trizol RNA reagent (Invitrogen Co.; Carlsbad, CA) and the Qiagen RNeasy mini kit were used, consecutively. For both extraction procedures, 1% (v/v) β-mercapethanol was added to increase RNA extraction efficiency. The *V. litorea* and *E. chlorotica* extracts were treated with RNase-free DNase (Qiagen) on-column and in solution to remove contaminating DNA. The quality of each extract was analyzed by agarose gel electrophoresis and nucleic acid levels quantified using the NanoDrop spectrophotometer.

First-strand cDNA was synthesized using Quanta BioScience's qScript Flex cDNA Synthesis Kit (Quanta Biosciences; Gaithersburg, MD) in a 20 μL reaction mixture containing1 μg of total RNA and following the 2007 manufacturer's protocol. To verify the presence of the target genes in the first-strand cDNA, each sample was subjected to a standard PCR amplification with the specific primer sets (Table 2.1). The primers for the nuclear gene *prk* were designed to flank an intron-exon boundary thereby facilitating the detection of any contaminating genomic DNA (note that plastid genes in *V. litorea* do not contain introns except for *trn*L). PCR products were then separated in a 1% agarose gel and visualized by EtBr staining to verify the amplification of a single product at the expected sizes.

2.2.5. Absolute qRT-PCR. The iCycler iQ Detection System (Bio-Rad Laboratories; Hercules, CA) was used to carry out and measure fluorescence for the qRT-PCR experiments. Plates were set-up with the 9-fold (10^1 to 10^9) plasmid standards in duplicate, and the cDNA experimental samples in triplicate, following Quanta's PerfeCTa™ SYBR® Green FastMix™ for iQ protocol. The 10 μL reactions contained 2.5 μL of the respective cDNA reaction (1:5 dilution), 10 μL 2x PerfeCTa™ SYBR® Green FastMix™ for iQ (Quanta Biosciences; Gaithersburg, MD), and 250 nM of each gene specific primer. The cycling parameters were 95°C 30s, followed by 40 cycles at 95°C 1sec, 52°C 30 sec, and 68°C 30 sec. Measurements of fluorescence were recorded every cycle at the

68°C elongation step. To confirm the amplification of a single product during each qRT-PCR experiment a dissociation curve (i.e. melting curve) was run after cycle 40 by heating from 55°C to 95°C with a ramp speed of 0.5°C per second. The results obtained from the fluorescence readings were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. The standard curve was generated from the plasmid C_T readings and used to estimate the absolute mRNA copy number (antilog(C_T-b)/m). The calculation of fold-change was made relative to the pre-light incubation control sample (5:00 am or 2 hr pre-light in *V. litorea* and 6:00 am or 1 hr pre-light in *E. chlorotica*). More specifically, the fold-increase was calculated as $(T_G)/(C_G)$ where T_G is the treated sample with the target gene and C_G is the control group with the target gene.

2.3. Results.

2.3.1. Differential Light Regulation of Nuclear and Plastid Transcript Levels.

Absolute mRNA transcript levels were quantified in *V. litorea* over a 12L:12D photoperiod and in *E. chlorotica* 1 hr pre- and post-illumination using nuclear- and plastid-gene specific primers in qRT-PCR measurements. The nuclear gene *prk*, which encodes the plastid Calvin cycle enzyme phosphoribulokinase, was differentially expressed after the onset of light in *V. litorea* and *E. chlorotica.* In *V. litorea*, *prk* transcript levels increased in response to illumination with maximal levels measured 6 hr post-illumination (1pm) (Fig.

2.1A). This represented a 78.6-fold increase over the transcript level at 2 hr pre-illumination (basis for all comparisons for *V. litorea*). A second, smaller increase (31.2-fold) in *prk* transcript level was seen 2 hr post-dark treatment. In contrast, *prk* transcript levels for *E. chlorotica* decreased in response to illumination (Fig. 2.1B) with an 11.6-fold decrease was measured at1 hr post-illumination (8 am) compared to *prk* transcript levels at 1 hr pre-illumination (6 am). This differential expression pattern of *prk* transcript levels upon illumination seen between the alga and the sea slug suggests that the nuclear factors that regulate algal *prk* transcription in response to light are lacking or non-responsive in the sea slug.

Slight discrepancies were also noted for the expression of plastid genes between the alga and the sea slug. Maximum transcript levels for *V. litorea* plastid genes *rbc*L, *psa*A, and *psb*A were observed at 2 hr post-illumination (9 am), with a second, smaller increase at 2 hr post-dark (9 pm) (Figs. 2.2A, 2.3A, and 2.4). The 8.4- and 5.4-fold increases in *rbc*L transcript levels at 9 am and 9 pm, respectively (Fig. 2.2A), were the smallest increases observed for all three plastid genes in *V. litorea*. The corresponding increases in transcript levels for the photosystem I gene *psa*A were 280.5- and 145.7-fold, and for the photosystem II gene *psb*A the increases were 652.2- and 480.1-fold. This is most likely due to the higher turnover rate of the photosystem proteins compared to the highly stable stromal ribulose-1,5- bisphosphate carboxylase/oxygenase (RUBISCO).

Transcript levels for the two plastid genes measured in *E. chlorotica*, *rbc*L and *psa*A, increased 8.2-fold and 4.8-fold, respectively, in response to 1 hr of illumination (Figs. 2.2B and 2.3B). This is in stark contrast to the 11.6-fold reduction noted for the *E. chlorotica* nuclear gene *prk* following illumination (Fig. 2.1B), but similar to the light induction seen for the *V. litorea* plastid transcripts. The maximal absolute changes in transcript levels for *prk* and *psa*A were significantly less in *E. chlorotica* compared to *V. litorea*, but comparable for *rbc*L. This similarity of plastid transcript levels upon illumination between the alga and the sea slug suggests that the sea slug may have acquired algal regulatory proteins or re-routed mitochondrial regulatory proteins that modify plastid gene transcription in response to light.

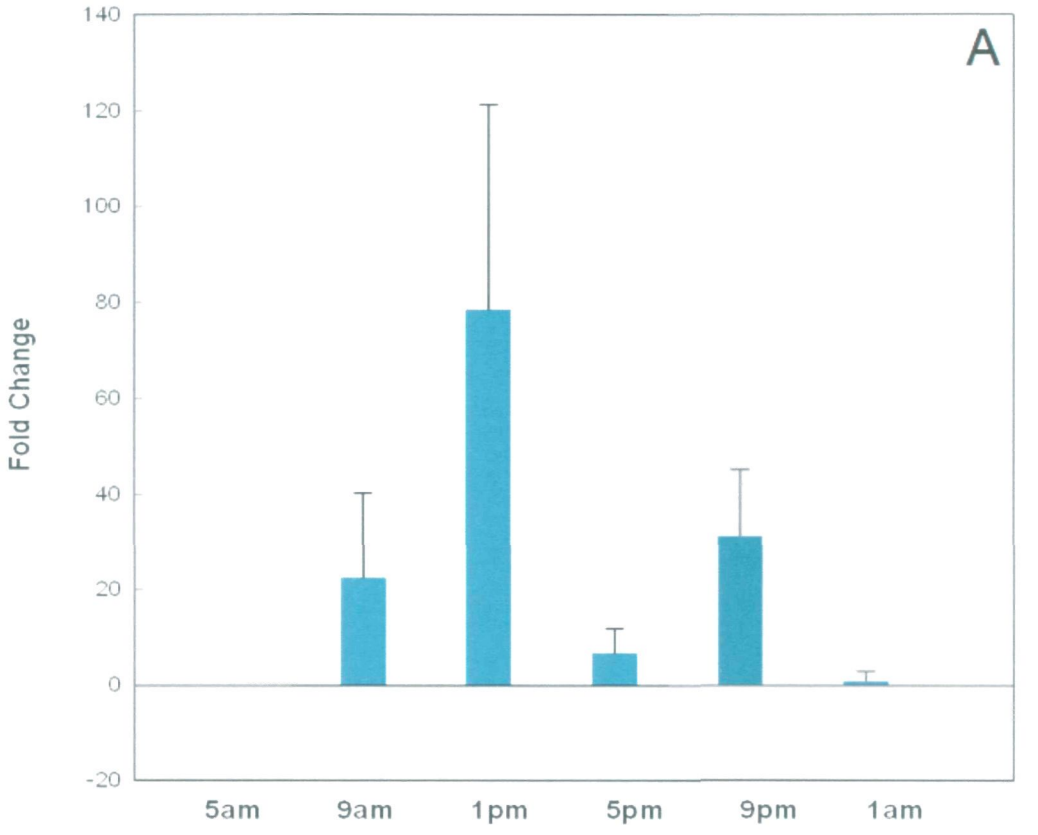

Figure 2.1. Fold-Change in Absolute Nuclear *prk* Transcript Levels. (A) *prk* transcript levels in *V. litorea* over a 12L:12D photoperiod. The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 4.

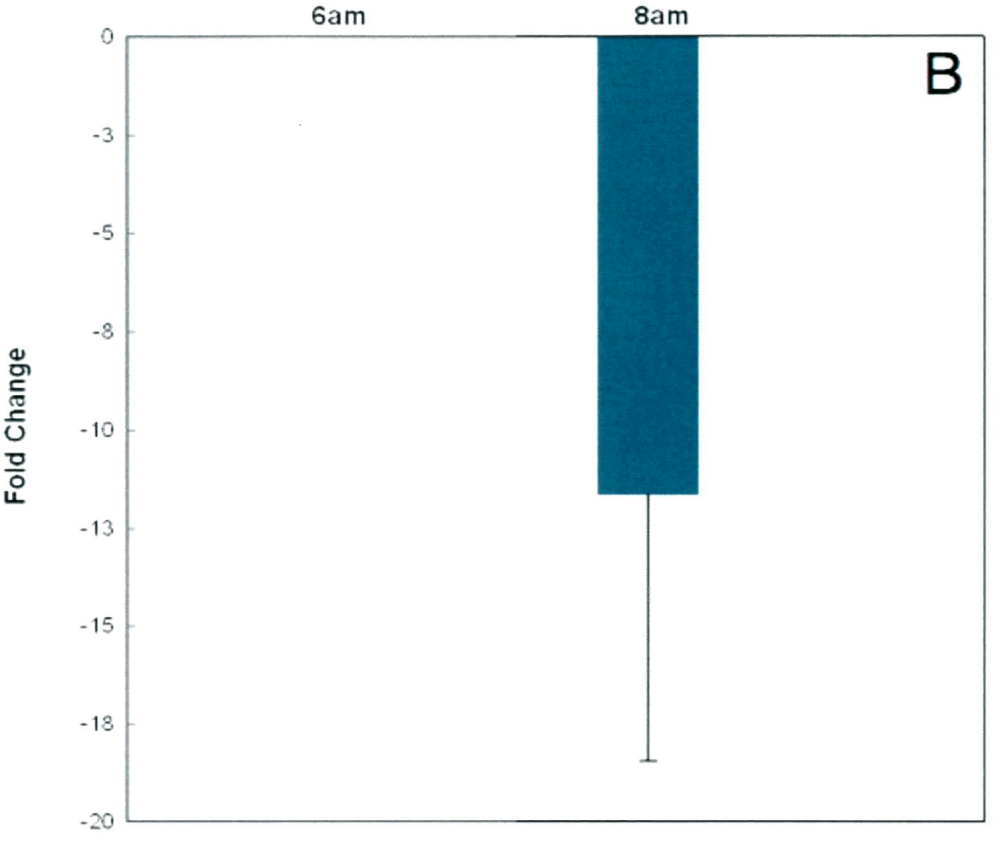

Figure 2.1. Continued. (B) *prk* transcript levels in *E. chlorotica* 1 hr post-illumination. The 6 am time point (1 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 3.

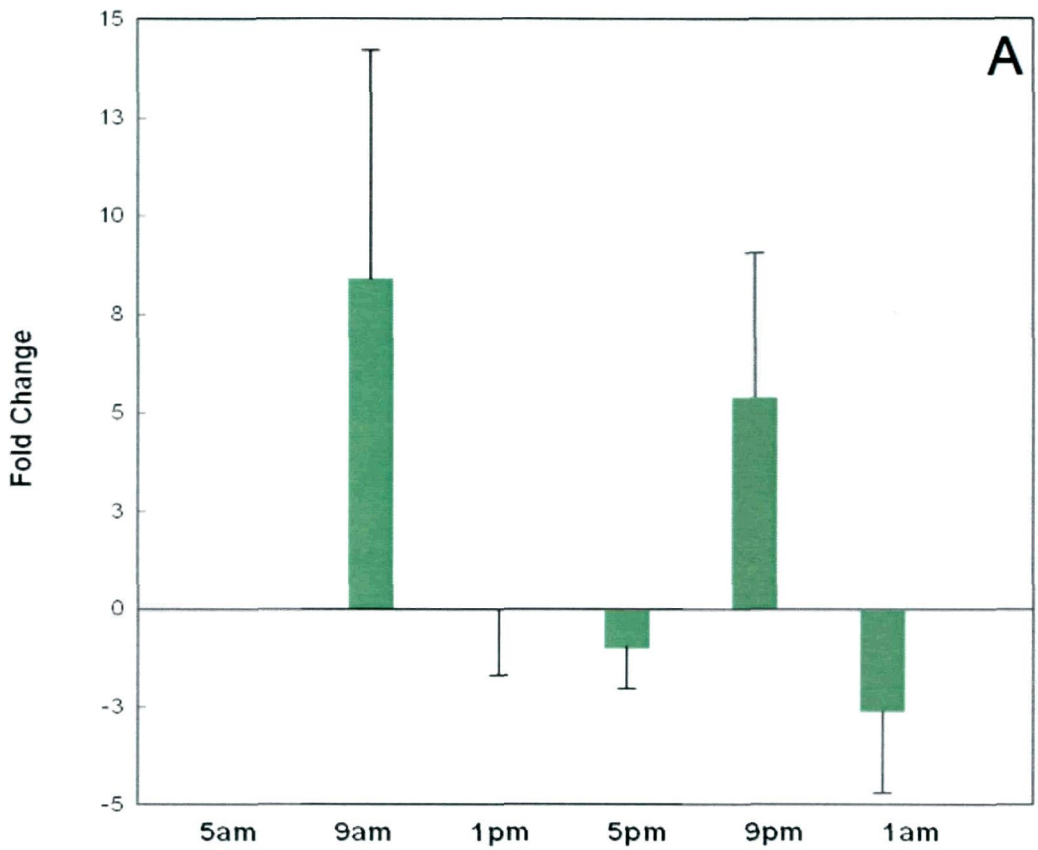

Figure 2.2. Fold-Change in Absolute Plastid *rbc*L Transcript Levels. (A) *rbcL* transcript levels in *V. litorea* over a 12L:12D Photoperiod. The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 5.

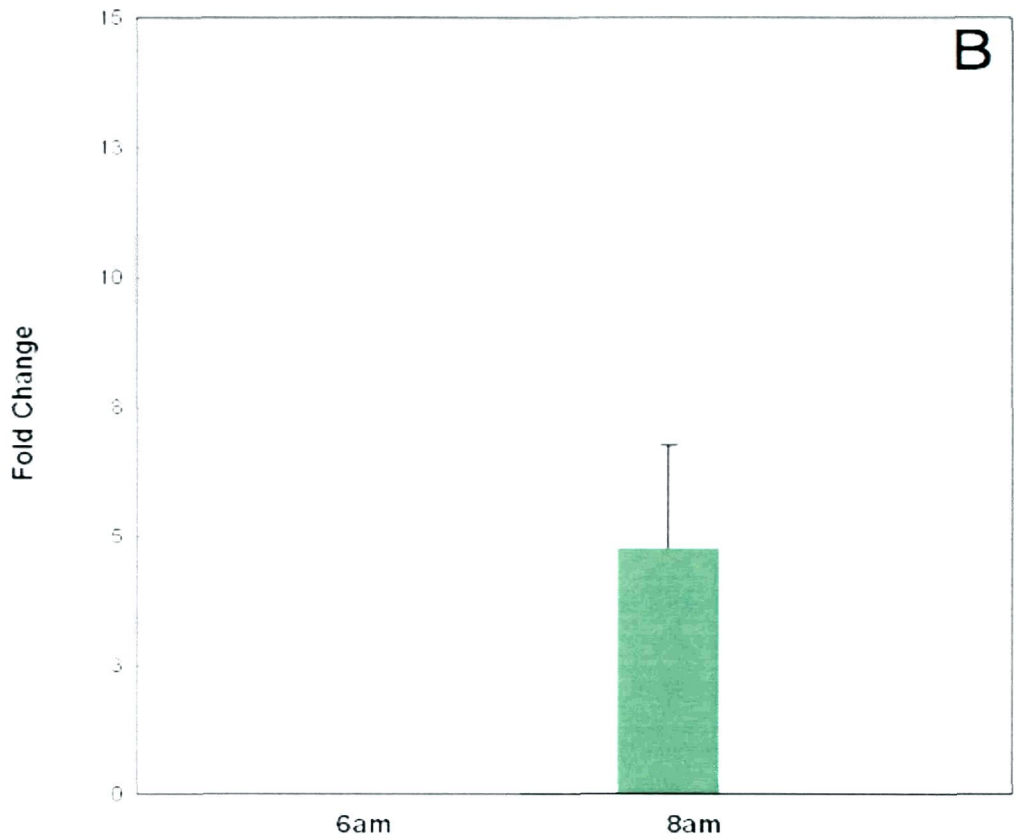

Figure 2.2. Continued. (B) *rbc*L transcript levels in *E. chlorotica* 1 hr post-illumination. The 6 am time point (1 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 3.

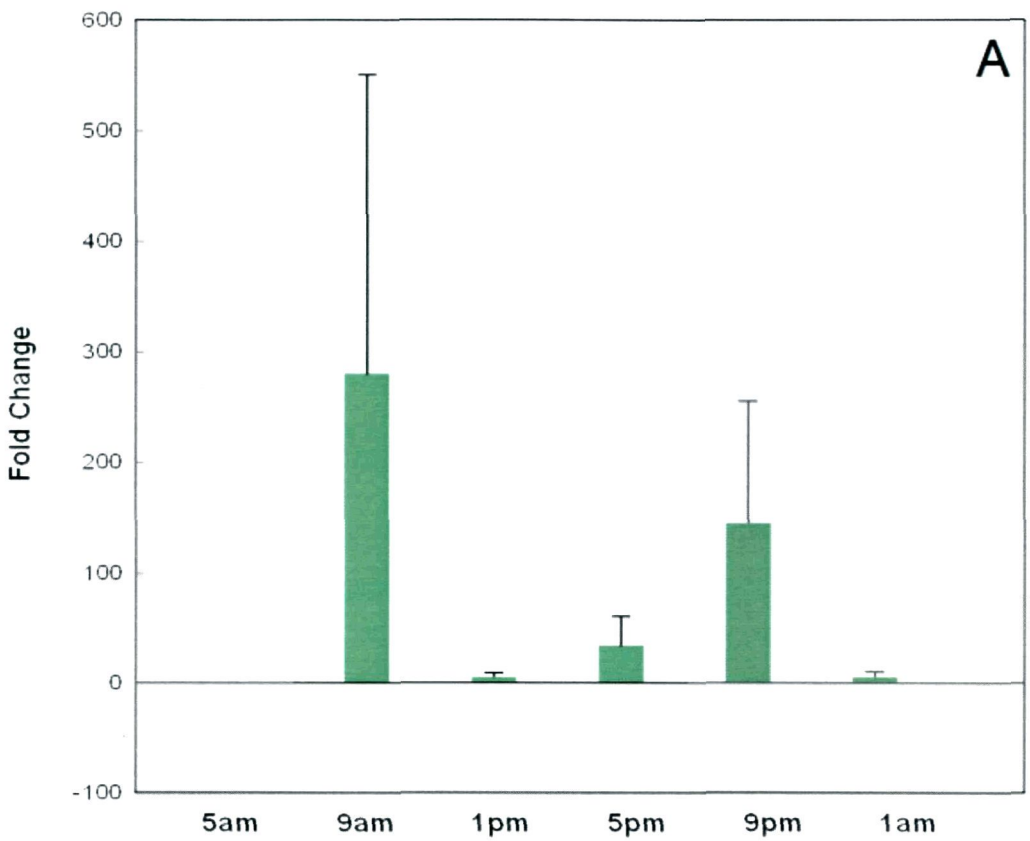

Figure 2.3. Fold-Change in Absolute Plastid *psa*A Transcript Levels. (A) *psaA* transcript levels in *V. litorea* over a 12L:12D photoperiod. The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 5.

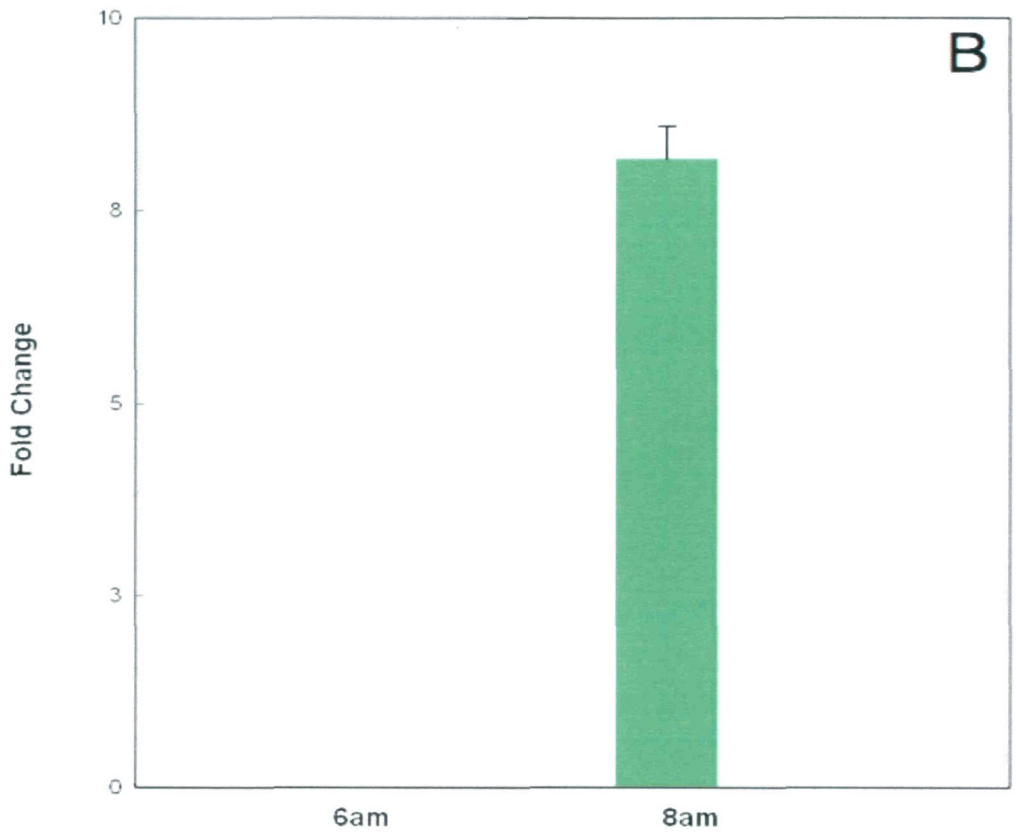

Figure 2.3. Continued. (B) *psa*A transcript levels in *E. chlorotica*1 hr post-illumination. The 6 am time point (1 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 3.

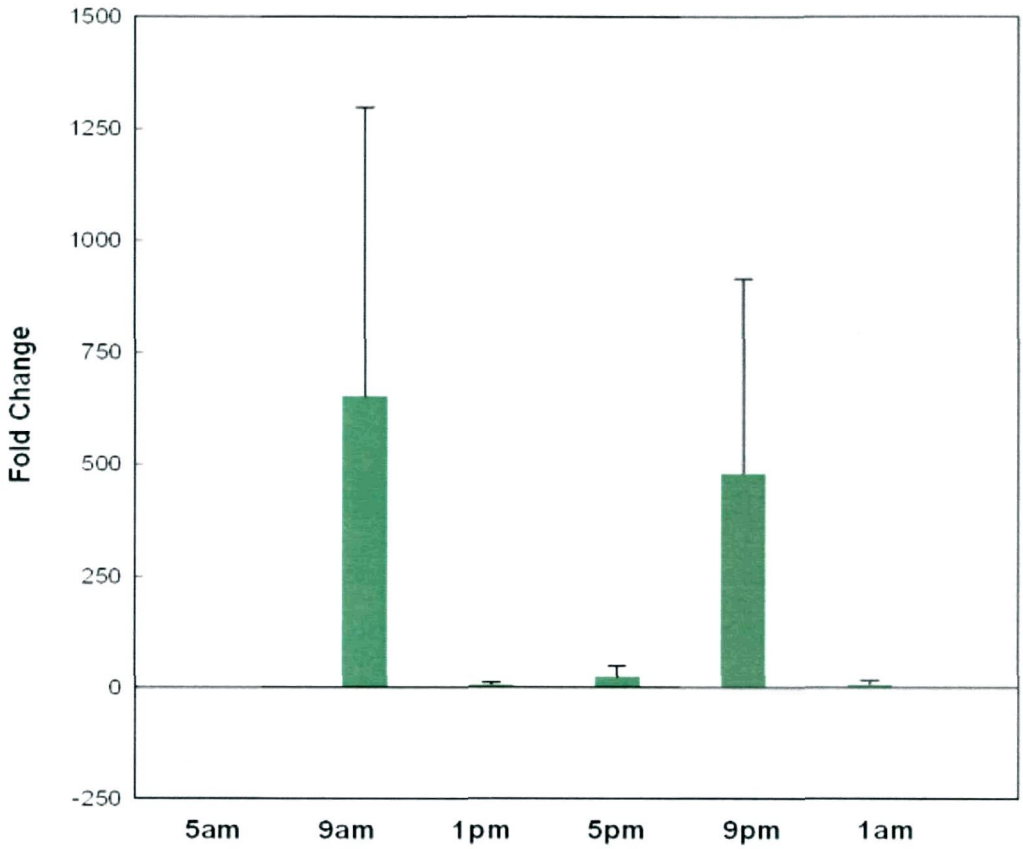

Figure 2.4. Fold-Change in Absolute Plastid *psb*A Transcript Levels in *V. litorea* Over a 12L:12D Photoperiod. The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent the mean ± SEM; n = 5.

2.3.2. Expression Pattern of Nuclear and Plastid Photosynthetic Genes in a 12 hr/12 hr dark Photoperiod. Absolute mRNA transcript levels were also quantified in *V. litorea* over a 12D:12D (DD) photoperiod after a 48 hr dark acclimation period. The pattern of transcript accumulation in *V. litorea* over the DD photoperiod was similar for nuclear (Fig. 2.5) and plastid (Figs. 2.6 and 2.7) gene targets, but considerably different from the patterns observed for the LD photoperiod (see Figs. 2.1-3A and 2.4). Transcript levels for *prk* (nuclear) and *psa*A and *psb*A (plastid) were at minimal levels at 1 hr (8 am), 5 hr (12 pm) and 13 hr (8 pm) into the DD photoperiod. More specifically, *prk* was found to be down-regulated by 122.8-, 102.1- and 111.6-fold; *psa*A by 10.4-, 13.7- and 16.0-fold, and *psb*A by 100.4-, 90.4- and 103.6-fold at 8 am, 12 pm and 8 pm, respectively. This down-regulation is opposite of the response observed in the LD photoperiod. However, the down-regulation was found to occur on a similar scale for *prk* transcripts in DD *vs.* the up-regulation seen in LD conditions, whereas *psa*A and *psb*A changes occurred on a much smaller scale in DD *vs.* LD conditions. This variation between the responses of algal plastid and nuclear gene transcripts from LD to DD conditions supports that the up-regulation of these transcripts under the LD conditions is due to the onset of light.

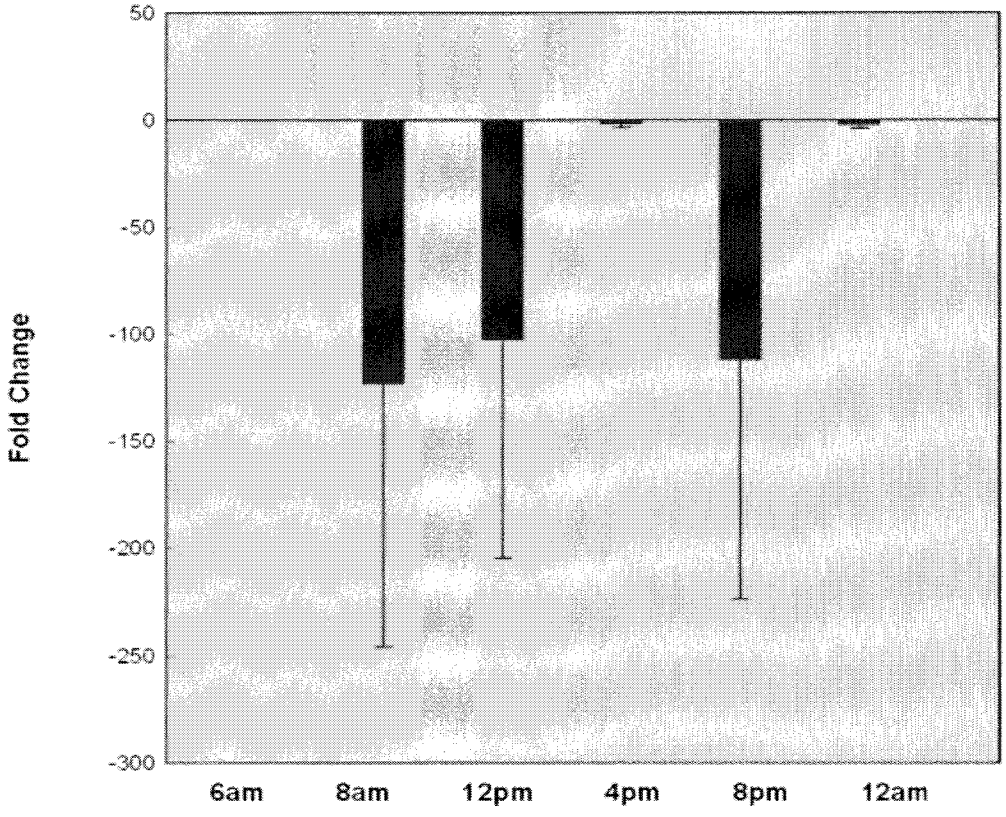

Figure 2.5. Fold-change in absolute nuclear *prk* transcript levels in *V. litorea* over a 12D:12D photoperiod. The 6 am time point was used as the baseline in calculating fold-change. The gray shading indicates the 24 hr dark period. Values represent the mean ± SEM; n = 2.

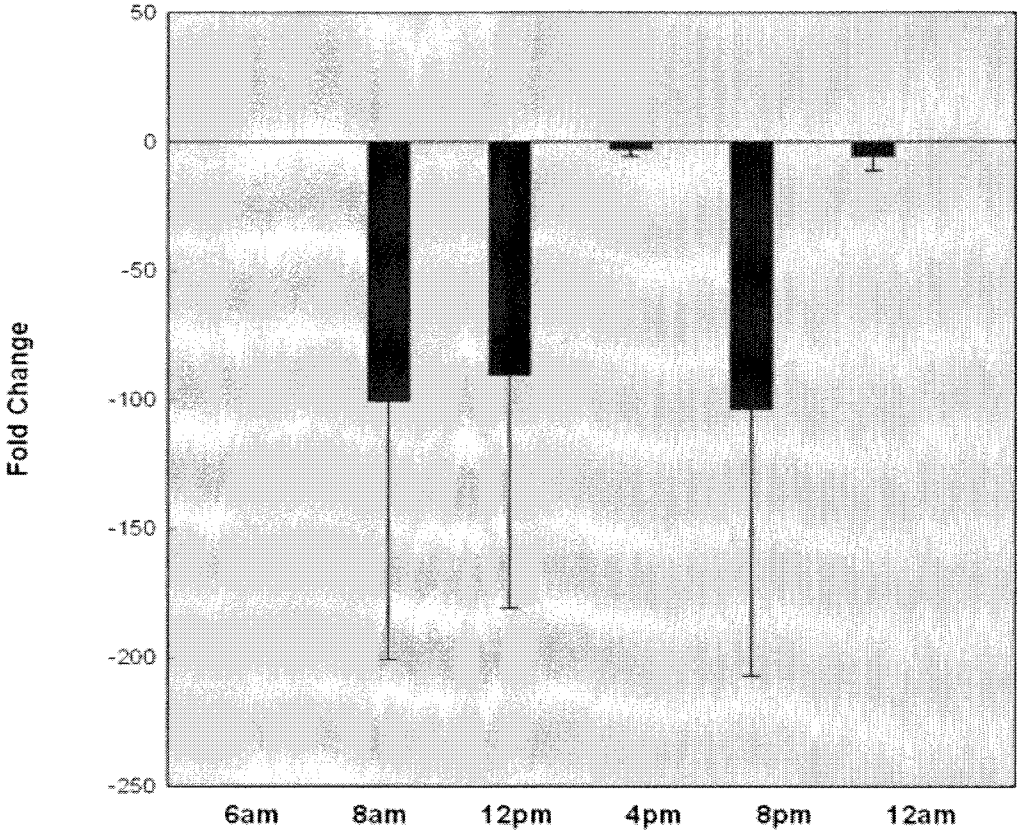

Figure 2.6. Fold-change in absolute plastid *psaA* transcript levels in *V. litorea* over a 12D:12D photoperiod. The 6 am time point was used as the baseline in calculating fold-change. The gray shading indicates the 24 hr dark period. Values represent the mean ± SEM; n = 2.

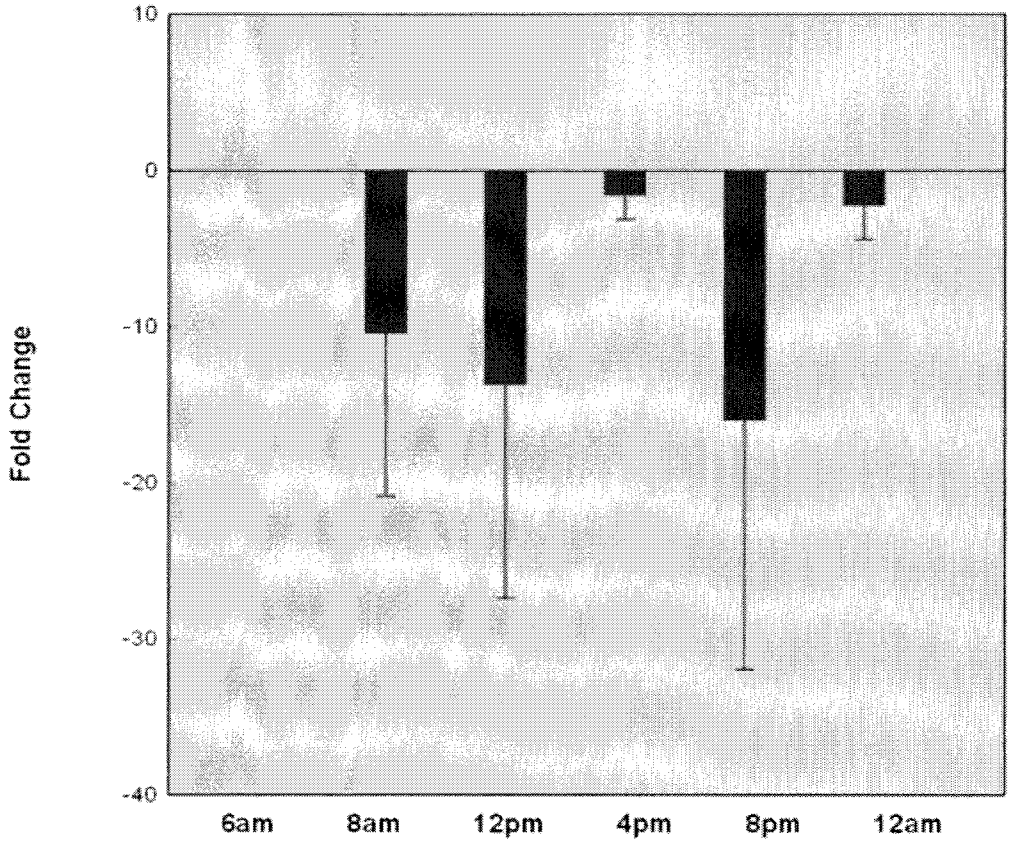

Figure 2.7. Fold-Change in Absolute Plastid *psb*A Transcript Levels in *V. litorea* Over a 12D:12D Photoperiod. The 6 am time point was used as the baseline in calculating fold-change. The gray shading indicates the 24 hr dark period. Values represent the mean ± SEM; n = 2.

2.4. Discussion.

The objective of this study was to examine how light influences gene expression profiles of particular nuclear and plastid genes in the heterokont alga *Vaucheria litorea*, and the *V. litorea* plastid-containing mollusc *Elysia chlorotica*. Nuclear and plastid genes were found to be differentially expressed in response to light conditions within both *V. litorea* and *E. chlorotica*. When comparing between the two organisms, differences in expression were only noted for the nuclear gene studied, *prk*. In this case, illumination resulted in a decrease in transcript level for the sea slug, but an increase for the alga. In contrast, the two plastid genes studied in both organisms, *rbc*L and *psa*A, were up-regulated after the transition from dark to light in both the sea slug and alga. This suggests that the nuclear factors necessary to control expression of algal-acquired nuclear photosynthetic genes (i.e. *prk*) may not be present or regulated within *E. chlorotica*, whereas the nuclear-encoded factors regulating plastid transcription may have either been re-routed from mitochondrial processes already present in *E. chlorotica* or horizontally transferred from *V. litorea* to *E. chlorotica*.

The up-regulation of *prk* expression after the onset of light, and to maximal levels in the middle of the light period in *V. litorea* (Fig. 2.1A), is similar to that seen in *Chlamydomas reinhardtii* in which *prk* transcription was reported to be controlled by light and circadian regulatory mechanisms (Lemaire et al., 1999). More specifically, Lemaire et al. (1999) demonstrated an 80%

reduction in *prk* transcript levels in the light when *C. reinhardtii* was exposed to DCMU (3-[3,4-dichlorophenyl]-1,1-dimethyl-urea), an agent that blocks electron transfer from photosystem II to PQ in the light reactions of photosynthesis. This reduction in *prk* transcript levels suggests that *prk* might be additionally controlled by the redox state of the PQ pool. This is congruent with the understanding that the state of PQ controls photosynthesis stoichiometry via the long-term response that counteracts the redox imbalances between photosystems II and I through altering photosynthetic gene expression in the nucleus (Escoubas et al., 1995; Pfannschmidt et al., 2001). The exact signal transduction pathway remains elusive; however, analyses have demonstrated the involvement of the LHCPII (light harvesting complex II) kinases STN7/8 and Stt7/11 in land plants and green algae, respectively (Depege et al., 2003; Bellafiore et al., 2005; Bonardi et al., 2005; reviewed in Rochaix JD, 2007).

Lemaire et al. (1999) additionally found that the quantities of *prk* transcripts in *C. reinhardtii* continued a cyclical pattern from a 12L:12D (LD) entrainment through a 48 hr period of continuous exposure to light or dark (LL or DD experiments). The cyclical patterning of transcript accumulation is based on the circadian negative feedback loops associated with the light entrainment of 'clock' gene expression, possibly induced by photoreceptor signaling networks at the onset of light (reviewed in Fankhauser and Staiger, 2002; Salome and McClung, 2004). The LL and DD treatments in *C. reinhardtii* occurred

consecutively to their respective LD treatments and were both expected to maintain the cyclical pattern of transcript accumulation for up to 48 hr. The DD measurements presented for *prk* transcripts in *V. litorea* (Fig. 2.5) were measured after a 48 hr acclimation period to dark conditions and were found to differ from the levels seen in the LD treatment (Figs. 2.1A). The 48 hr acclimation period of these samples prohibits us from making any circadian references to the data presented; however, these findings do support the involvement of light regulatory mechanisms on the pattern of *prk* transcript accumulation in *V. litorea* over the LD photoperiod. The specific role of PQ, ferredoxin, or photoreceptors in this regulation is yet to be determined and will require further research.

In contrast to what was observed for *V. litorea prk* transcript levels, *E. chlorotica prk* transcript levels were down-regulated after the onset of light (Fig. 2.1B). Such an opposite response suggests that light regulation of *prk* expression observed in the alga does not occur in the sea slug. This is not necessarily surprising, as light regulation of nuclear photosynthesis genes requires nuclear-encoded transcription factors that perceive signals from the plastids and photoreceptors, as discussed previously. The presence of these nuclear-encoded factors are not expected in *E. chlorotica*, but it is possible that the nuclear genes responsible for light and circadian regulation of nuclear photosynthesis genes have been transferred to the sea slug as recent evidence supports HGT of nuclear

genes from *V. litorea* to *E. chlorotica* (Pierce et al., 2007; Rumpho et al., 2008 and 2009).

Plastid-encoded transcripts, on the other hand, were up-regulated in both the alga (Figs. 2.2-3A and 2.4) and the sea slug (Figs. 2.2-3B), but did not occur in algal samples acclimated to DD conditions (Figs. 2.6-7). This suggests that the regulation may likely come from inside the plastid via light regulatory mechanisms, possibly associated with the cyanobacterial or a homologous two-component system. The redox regulation of plastid gene transcription involves the redox state of PQ (Pfannschmidt et al., 1999) and an LHCPII kinase (e.g. STN7) (Bonardi et al., 2005), as similarly discussed with nuclear gene expression. Regulation of plastid genes occurs in the stroma near the thylakoid membrane-bound-PQ, whereas the signaling networks that affect nuclear gene expression must transduce out of the plastid and across the cytoplasm to the nucleus. The closer proximity of plastid genes (DNA) offers a bioenergetic advantage as is suggested by the CORR hypothesis (CO-location for Redox Regulation). The CORR hypothesis states that genes have been maintained in organelles for more direct and unconditional redox regulation of their expression (Allen, 1993; Puthiyaveetil et al., 2008). This hypothesis supports the suggestion that the cyanobacterial two-component system has been maintained in the plastids of non-green algae (Duplessis et al., 2007; reviewed in Puthiyaveetil and Allen, 2009). The plastid genes responsible for this two-component system are, however, not

present in the completed plastid genome of *V. litorea* (Rumpho et al., 2008). Future sequencing projects may reveal their presence in the nuclear genome of *V. litorea*, as already found for the closely related diatoms *Phaeodactyulum tricornutum* and *Thalassiosira pseudonana* (Puthiyaveetil et al., 2008).

Doran and Cattolico (1997) demonstrated this two-component endogenous rhythm and suggested that the rhythm of plastid transcript accumulation was driven by the activity of the photosynthetic electron transport chain in the heterokont *Heterosigma carterae.* They found the plastid transcripts in *H. carterae* increased at the onset of light to a maximum level early in the light period and then progressively decreased to minimum levels at the onset of the dark period. *V. litorea* plastid transcript levels for *rbc*L (Fig. 2.2A), *psa*A (Fig. 2.3A) and *psb*A (Fig. 2.4) were similarly observed to be maximal soon after the onset of light (+2 hr light). The *rbc*L gene encodes the large subunit of RUBISCO, a very stable protein in the Calvin cycle, whereas the *psa*A and *psb*A genes encode major core proteins in photosystems I and II, respectively. The *rbc*L transcripts increased 8.4–fold by 2 hr post-illumination, whereas *psa*A and *psb*A transcripts increased 280.5– and 652.2–fold in *V. litorea*, respectively. This can be attributed to the higher turnover rate of photosystem proteins that are easily light-damaged during photosynthesis. Additionally, in *V. litorea* plastid transcript levels were found to increase when transitioned to dark conditions (Figs. 2.2A, 2.3A and 2.4). This increase is most likely due to lower rates of plastid

translation in the dark; a process that has also been found to be light regulated through the redox potential of PQ and THX (Danon and Mayfield, 1994a and b). In *E. chlorotica*, *rbc*L (Fig. 2.2B) and *psa*A (Fig. 2.3B) transcript levels were similarly found to increase after the onset of light by 4.8- and 8.2-fold, respectively. The sample timing and/or the requirement of nuclear-encoded factors may explain the smaller absolute increase in *psa*A transcript levels in *E. chlorotica* compared to that seen in *V. litorea*.

In summary, this study presents the first molecular evidence to characterize light regulation of nuclear and plastid gene expression in the heterokont alga *Vaucheria litorea* and its symbiotic partner, the sacoglossan mollusc *Elysia chlorotica*. Expression of the nuclear gene *prk* was found to respond differently upon illumination between *V. litorea* and *E. chlorotica*, whereas plastid genes were similarly up-regulated upon illumination in both organisms. Furthermore, expression of algal nuclear- *vs.* plastid-encoded transcripts differed under continuous dark conditions compared to a typical 12 hr light/dark cycle in *V. litorea*. These results suggest that expression of nuclear- and plastid-photosynthesis genes in *V. litorea* are light modulated at the transcriptional level possibly involving the redox state of plastid- and nuclear-encoded factors. Additionally, the similar light responses observed for the alga and sea slug may suggest that nuclear-encoded factors (i.e. cyanobacterial two-component proteins) responsible for the primary control of plastid gene expression may have been

acquired by the sea slug either through HGT or the re-routing of proteins already encoded by the sea slug. The suggested redox regulation acquired by *E. chlorotica* may then assist in the maintenance and upkeep of the plastids for the 10 month life span of the sea slugs.

Chapter 3

DIFFERENTIAL REGULATION OF PHOSPHORIBULOKINASE ACTIVITY IN THE HETEROKONT ALGA *VAUCHERIA LITOREA* AND ITS SYMBIOTIC PARTNER THE SACOGLOSSAN MOLLUSC *ELYSIA CHLOROTICA*

3.1. Introduction.

Phosphoribulokinase (PRK; EC 2.7.1.19) is an essential nuclear-encoded plastid enzyme of the photosynthetic carbon reduction pathway (Calvin cycle). PRK catalyzes the regeneration of ribulose-1,5-bisphosphate (RuBP) from ribulose-5-phosphate (Ru5P) in an ATP-dependent manner (Miziorko, 2000). RuBP is the substrate for the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO; EC 4.1.1.39) in which atmospheric CO_2 is incorporated and reduced to sugars or lipids in photoautotrophic organisms. These reduced carbon products are necessary for energizing plant metabolic functions.

It is widely accepted that plastids evolved following the primary endosymbiotic engulfment of a cyanobacterium by a eukaryotic host as expounded by Margulis (1981). This event was followed by endosymbiotic gene transfer (EGT) from the cyanobacterium to the host nuclear genome (Martin et al., 1998 and 2002) and included the transfer of the PRK gene as well as the majority of the genes required for organellar functioning and division. This gene transfer

to the host nuclear genome left the reduced organelle dependent upon the nucleus for all its metabolic functions. The established primary photoautotrophic organism diverged into three phyla, including the glaucophytes (presumably a dead-end group), the Chloroplastida or Viridiplantae (green algae and land plants), and the rhodophytes (red algae) (Weber et al., 2006).

Some members of the green and red algae subsequently participated in secondary endosymbiotic events in which a heterotrophic eukaryote host engulfed an entire unicellular green or red alga (McFadden, 1999). In these cases, the uptake of the whole primary symbiont led to gene transfers from the symbiont nucleus, plastids and mitochondria to the host nucleus. This transfer of genes again left the organelle (or endosymbiont) dependent upon the host's nuclear genome. This secondary uptake of the green algae led to the euglenids and the chlorarachniophytes (Keeling, 2004; Rogers et al., 2007); whereas the red algae gave rise to the Chromalveolate supergroup containing cryptophytes, haptophytes, heterokonts and alveolates (Cavalier-Smith, 1998; Fast et al., 2001; Yoon et al., 2002; Harper and Keeling, 2003; Keeling, 2004). The chromalveolates have additionally been documented to undergo tertiary endosymbioses where EGT and horizontal gene transfer (HGT) similarly left the newly acquired plastids dependent upon the host's nuclear genome (Bodyl, 2005; Bodyl and Moszcynski, 2006; Sanchez-Puerta and Delwiche, 2008). Of particular interest to the study here is the unique tertiary endosymbiotic relationship between the sacoglossan

mollusc (sea slug) *Elysia chlorotica* and the plastids of the secondary-derived heterokont alga *Vaucheria litorea.*

Early in the developmental life cycle of *E. chlorotica*, the sea slug feeds on filaments of *V. litorea* where only the plastids are incorporated within the cells lining the digestive epithelia. Once incorporated the plastids are retained and continue to carry out photosynthesis for the 10- to 12-month life span of the sea slug (Graves et al., 1979; Green et al., 2000; Rumpho et al., 2001). However, molecular data (Green et al., 2000) and microscopical observations (Mujer et al., 1996; Green et al., 2000) support the absence of algal nuclei and algal mitochondria within the sea slug, presumably due to degradation in the digestive lumen and excretion from the sea slug. Consideration of this fact then raises the question of the source of the nuclear-encoded plastid-targeted (NEPT) proteins and regulatory factors necessary for plastid function.

Recently, evidence supporting HGT of five nuclear genes: *prk* (Rumpho et al., 2009), *psb*O (Rumpho et al., 2008), *fcp*, *lhcv*1 and *lhcv*2 (Pierce et al., 2007) from *V. litorea* to *E. chlorotica*, has been published. These findings support the hypothesis that essential nuclear genes in *V. litorea* have been transferred to *E. chlorotica*, possibly helping to sustain the observed photosynthetic activity in the mollusc. This also supports the possibility that *V. litorea* NEPT regulatory factors necessary for plastid functioning may also be found and expressed in *E. chlorotica.*

In land plants (Buchannan, 1991; Wedel et al., 1997; Marri et al., 2005b) and green algae (Tamoi et al., 1998; Wedel and Soll, 1998), PRK has specifically been shown to be redox regulated by both NEPT factors and protein-protein interactions. Redox regulation of PRK occurs through thioredoxin (Trx), one of the best-studied NEPT redox modulators (Geck and Hartman, 2000; Marri et al., 2009). Thioredoxin activates PRK through thiol/disulfide reduction (as depicted in Fig. 3.4), more specifically by reducing Cys residues (Cys^{16} and Cys^{55} as numbered for spinach; [Porter et al., 1988]) located in the active site of PRK (Hirasawa et al., 1998).

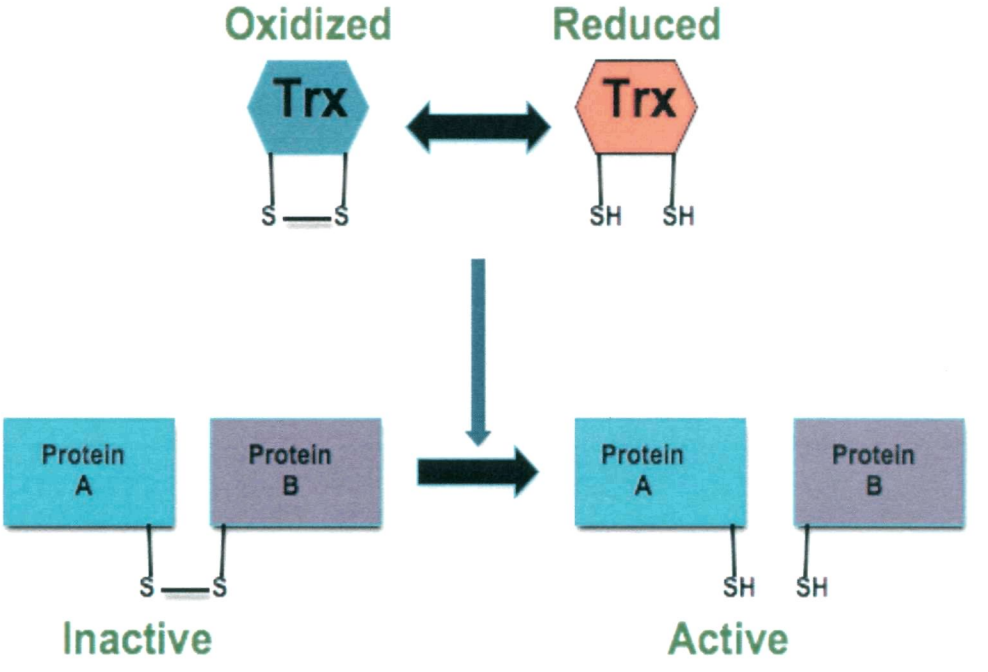

Figure 3.1. General Mechanism of Reduced Thioredoxins (Trx) on Protein Complexes Inactivated by Oxidized Disulfides.

PRK is also redox regulated through the formation of a supermolecular complex with the Calvin cycle enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.13) and frequently, the relatively small (8.5 to 15 kDa) CP12 peptide (as depicted in Fig. 3.5). CP12 has been found in this complex in cyanobacteria (Wedel and Soll, 1998; Tamoi et al., 2005), green algae (Wedel et al., 1997; Graciet et al., 2003), land plants (Wedel et al., 1997; Scheibe et al., 2002; Marri et al., 2005b), red algae (Oesterhelt et al., 2007) and heterokonts (Boggetto et al., 2007; Kroth et al., 2008). The PRK/GAPDH/CP12 supermolecular complex forms when the light reactions have slowed the production of reducing equivalents (i.e. NADPH and Trx_{red}) and all of the components are fully oxidized. The formation of the complex under oxidizing conditions in the dark has been shown to inhibit PRK and GAPDH activities in green algae (Avilan et al., 1997) to prevent futile cycling of carbon use between the coupled reductive Calvin cycle and the oxidative pentose phosphate pathway (OPPP) (Michels et al., 2005; Gruber et al., 2003).

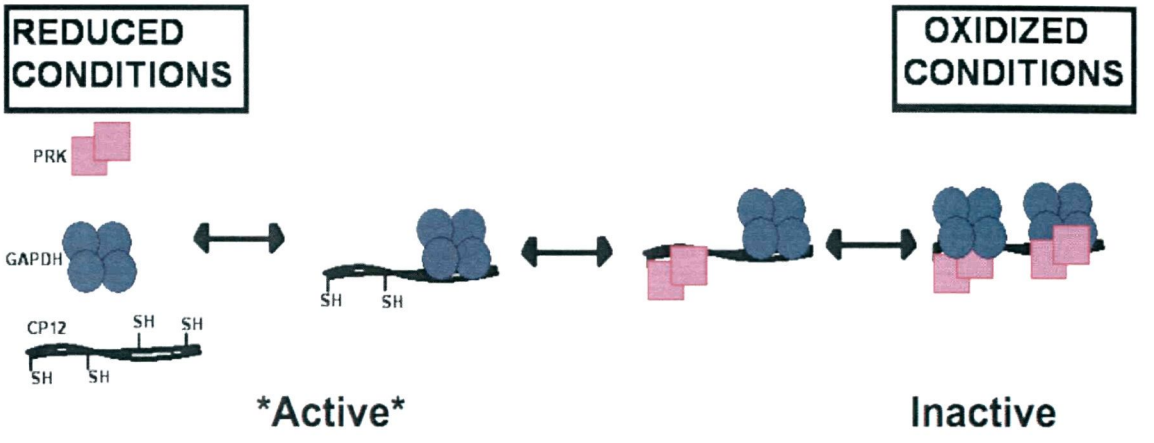

Figure 3.2. PRK/GAPDH/CP12 Complex Formation.

Although the significance of the PRK/GAPDH/CP12 complex in regulating photosynthetic functioning is fairly well established in green algae and land plants, little is known about the regulation of PRK in red algae and red algal descendents (i.e. heterokonts) (Cattolico et al., 1998; Michels et al., 2005; Boggetto et al., 2007; Oesterhelt et al., 2007). Here, PRK activity and its regulation by redox changes was characterized under light and dark conditions in the heterokont alga *V. litorea*. In turn, the findings are compared with those observed for PRK in its predator and symbiotic partner, the sacoglossan mollusc *E. chlorotica*, in which algal nuclear regulation is absent.

3.2. Materials and Methods.

3.2.1. Algal and Sea Slug Culturing. *Vaucheria litorea* C. Agardh CCMP2940 cultures were maintained in a modified f/2 artificial seawater medium (ASW, 925 mosmol Instant Ocean, Aquarium Systems) at 18°C on a 12 hr photoperiod. Specimens of *Elysia chlorotica* Gould were collected from Martha's Vineyard, MA. The sea slugs were maintained for 2 weeks (Time 0), 3, 4 and 6 months without algae (starved) in aerated aquaria containing 925 mosmol ASW at 12°C on a 12 hr photoperiod before collection. All algal and sea slug samples were collected either 5 to 7 hr into the light period (as noted in each experiment) or 6 hr into the dark period, blotted dry, weighed, frozen with liquid nitrogen and stored at -80°C until needed.

3.2.2. Protein Extraction. Crude protein extracts were prepared by homogenizing frozen *V. litorea* and *E. chlorotica* samples in liquid nitrogen at a ratio of 2:1 or 1:1 (fresh weight: extract volume), in 100 mM Tris – 10 mM $MgCl_2$ buffer (pH 8) with or without 5 mM reduced dithiothrietol (DTTr). The homogenate was centrifuged at 13,000*g* for 20 min at 4°C and then used in the PRK assay (typically 5 to 30 μL mL^{-1} reaction volume). Total protein was quantified using the Bradford protein assay (Bradford, 1976). For de-salting, crude extracts were centrifuged three times at 13,000*g* for 30 min at 4°C using Vivaspin 5,000 MWCO Centrifugal Concentrators (Sartorius AG, Goettingen, Germany). For *in vitro* oxidation/reduction experiments, crude extracts were

incubated for 20 min at 25°C with one of the following: 50 mM DTTr, 150 mM oxidized DTT (DTTo), 1 mM NADPH or 0.8 mM NADPH + 40 mM DTTr. Complete details are provided with each experiment.

3.2.3. Phosphoribulokinase Assay. PRK activity was measured via a coupled reaction involving the conversion of phosphoenolpyruvate (PEP) to lactate and the ATP dependent formation of Ru5P by PRK (Cattolico et al., 1998; as depicted in Fig. 3.6). The 1 mL reaction mixture contained: 100 mM Tris (pH 8), 3 mM $MgCl_2$, 5 mM DTTr, 2 mM PEP, 0.2 mM NADH, 2 mM ATP, 4 mM ribose 5-phosphate (Ri5P), 14 U pyruvate kinase, 10.5 U lactate dehydrogenase, 2 U phosphoriboisomerase and 5 to 30 μL crude extract. PRK activity was measured at 25°C as a change in NADH (A_{340}) per unit time (min) after the addition of Ri5P. The basal/control activity was measured for 2 min prior to the addition of Ri5P and subtracted from the measured PRK activity. One unit of activity is defined as the amount of enzyme necessary to catalyze the oxidation of 1 μmol of NADH per min with an extinction coefficient for NADH of 6220 $L/cm^{-1} \cdot mol^{-1}$.

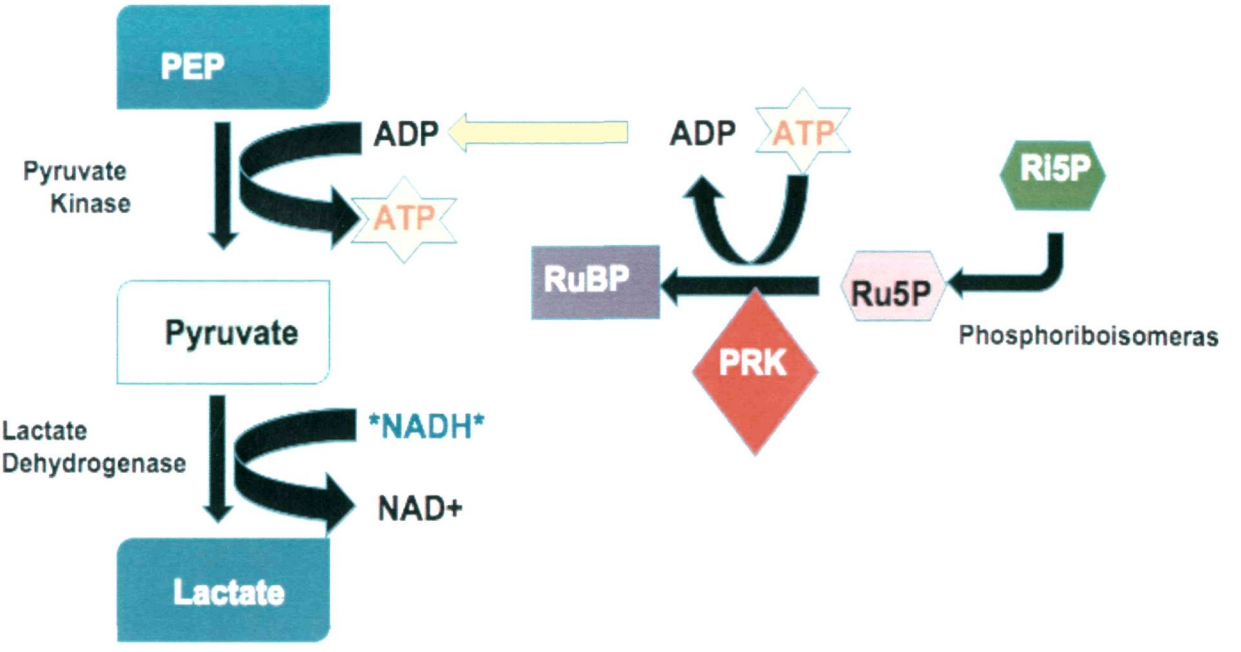

Figure 3.3. PRK Enzyme Assay. Reduction in NADH was recorded at A_{340} and used to calculate the activity of PRK.

3.2.4. Statistical Analysis. Analyses of variance (ANOVA) were carried out using SYSTAT© Software (Systat Software, Inc. Chicago) to determine the statistical differences among varying enzymatic conditions followed by a Tukey test. All values are given as the mean ± SEM (standard error of mean); differences were considered statistically significant at $p \leq 0.05$. The detailed analyses are presented in Appendix A, Tables 1-5.

3.3. Results.

3.3.1. Effect of Including Reduced DTT in the Extraction Media on PRK Activity.

PRK activity was measured in extracts from light- and dark-adapted algal and sea slug samples prepared with (+DTTr) or without (-DTTr) reducing agent in the extraction media. PRK activity in dark-adapted *V. litorea* extracts significantly increased by a factor of 2.9 ($p \leq 0.001$) when extracts were prepared with DTTr compared to without (Fig. 3.4A). However, no significant difference was noted in PRK activity within light-adapted *V. litorea* extracts whether prepared with or without reducing agent (Fig. 3.4A). Suggesting that there is a physical difference in PRK from light to dark conditions, congruent with the presence of the PRK/GAPDH/CP12 complex.

No significant change in PRK activity was observed in crude-extracts of light- or dark-treated *E. chlorotica* whether prepared with or without reducing agent (Fig. 3.4B). Suggesting no physical difference in PRK from light to dark conditions. Additionally, enzyme activity in general decreased with increasing starvation time (see data for 4 and 6 months-starved sea slugs), and may be suggestive of senescence.

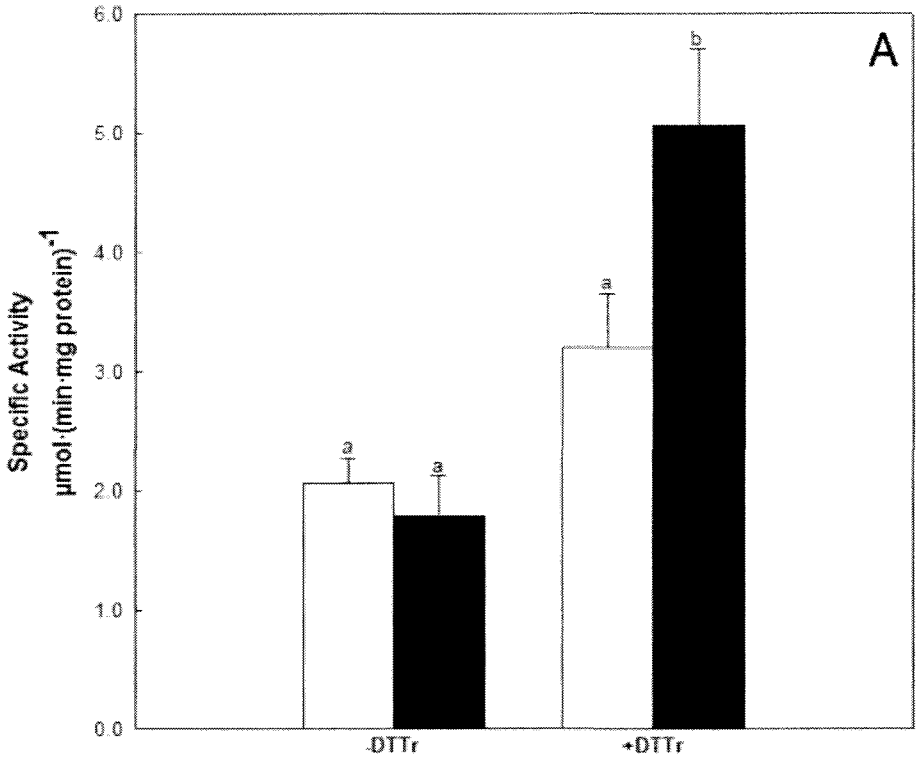

Figure 3.4. Effect of Reducing Conditions in the Extraction Media. (A) PRK Activity in *V. litorea*. Algal filaments were collected after 7 hr in the light □ or dark ■. Samples were extracted in the presence (+DTTr) or absence (-DTTr) of reducing agent and then all samples were assayed with DTTr in the assay media. Values represent the mean ± SEM; light (n = 6) and dark (n = 5). Letters above the bars reflect significant differences at the $p \leq 0.05$ level; statistics are summarized in Appendix A, Table 1.

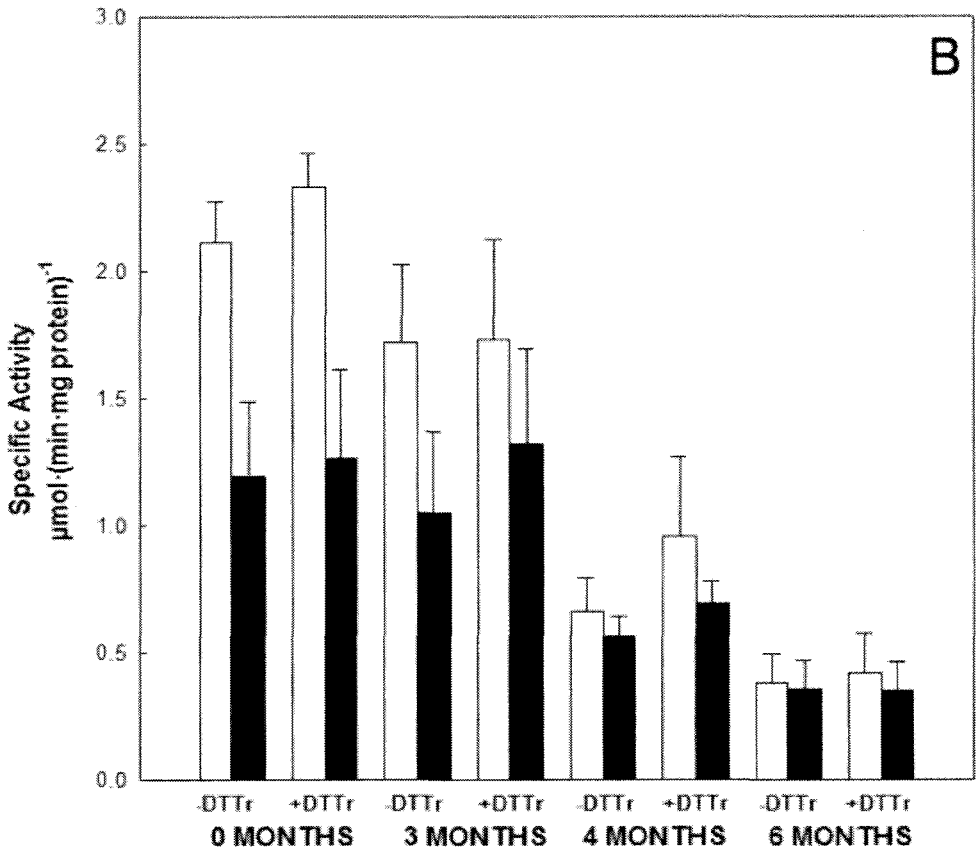

Figure 3.4. Continued. (B) PRK Activity in *E. chlorotica*. Sea slugs were collected after 5 or 6 hr in the light □ or dark ■ at 0, 3, 4 or 6 months starvation time. Samples were extracted in the presence (+DTTr) or absence (-DTTr) of reducing agent and then all samples were assayed with DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 1.

3.3.2. Effect of Excluding Reduced DTT in the Extraction and Assay Media on PRK Activity. To further explore redox effects on PRK activity in *V. litorea* and *E. chlorotica* (starved 4 mo.), crude extracts from light- and dark-adapted samples were prepared without any reducing or oxidizing agents and PRK activity was initially assayed in the absence of any redox agents. These extracts were then subsequently exposed to oxidizing (DTTo) or reducing (DTTr, NADPH/DTTr, NADPH) conditions, assayed for PRK activity, and then re-exposed to opposing redox conditions for further activity measurements.

The activity of PRK in crude extracts (-DTT) of dark-adapted *V. litorea* and *E. chlorotica* were 1.9- and 1.6-times higher, respectively, than that for light-adapted specimens when assayed without DTTr in the assay media (Figs. 3.2A *and* 3.2B). However, this was not statistically significant at the $p \leq 0.05$ level. This observed general increase in activity in extracts from dark-adapted organisms was consistent throughout the oxidizing and reducing conditions for both *V. litorea* (Figs. 3.2A *and* 3.3A-C) and *E. chlorotica* (Figs. 3.2B and 3.3D).

Incubation of *V. litorea* extracts with the oxidizing agent DTTo resulted in a 14- and 16.3-fold reduction in PRK activity for light and dark-adapted *V. litorea*, respectively (Fig. 3.5A). Subsequent incubation with DTTr resulted in a full recovery of activity for PRK in light-adapted extracts and a 92% recovery for dark-adapted algal extracts. PRK from *E. chlorotica* was similarly affected for extracts from 4 month starved sea slugs when incubated under the same oxidizing

and subsequent reducing conditions (Fig. 3.5B). In particular, treatment of the light and dark-adapted extracts with DTTo resulted in a 4.6- and 3.8-fold reduction in PRK activity, respectively, which was partially recovered with subsequent treatment with DTTr. Overall, this suggests that for both the alga and sea slug, PRK activity is inhibited *in vitro* under oxidizing conditions and reactivated by reducing conditions.

Incubation with reducing agents and subsequent oxidizing reagents affected PRK activity differently between *V. litorea* and *E. chlorotica* crude extracts. PRK activity in light-adapted *V. litorea* extracts was not significantly affected by sequential incubation with any of the reducing agents (+DTTr, +NADPH/DTTr, +NADPH) (Figs. 3.6A-C) compared to the –DTT control. Similarly, sequential incubation with NADPH/DTTr (Fig. 3.6B) or NADPH (Fig. 3.6C) did not significantly affect PRK activity in dark-adapted *V. litorea* extracts. However, unlike the light-adapted *V. litorea* extracts, the dark-adapted extracts incubated with DTTr exhibited a highly significant ($p \leq 0.001$) increase in PRK activity (Fig. 3.6A). Further, subsequent incubation with DTTo significantly ($p \leq 0.05$) reduced PRK activity in the light- and dark-adapted extracts reduced with DTTr (Fig. 3.6A) and NADPH/DTTr (Fig. 3.6B). The 8.1- and 4.5-fold reduction seen after DTTo incubation of the NADPH light and dark-adapted extracts, on the other hand, was not statistically significant ($p \leq 0.8$).

In contrast to *V. litorea,* PRK activity in *E. chlorotica* light- and dark-adapted extracts prepared and assayed without reducing agent, was not significantly affected by subsequent incubation with reducing agents (DTTr, NADPH/DTTr, NADPH) (Fig. 3.6D). Whereas subsequent incubation under oxidizing conditions (+DTTo) similarly inhibited PRK activity in both light- and dark-adapted extracts ($p \leq 0.05$).). These findings further support the previous suggestion that there is a physical difference in PRK in light *vs.* dark conditions within the alga but not in the sea slug.

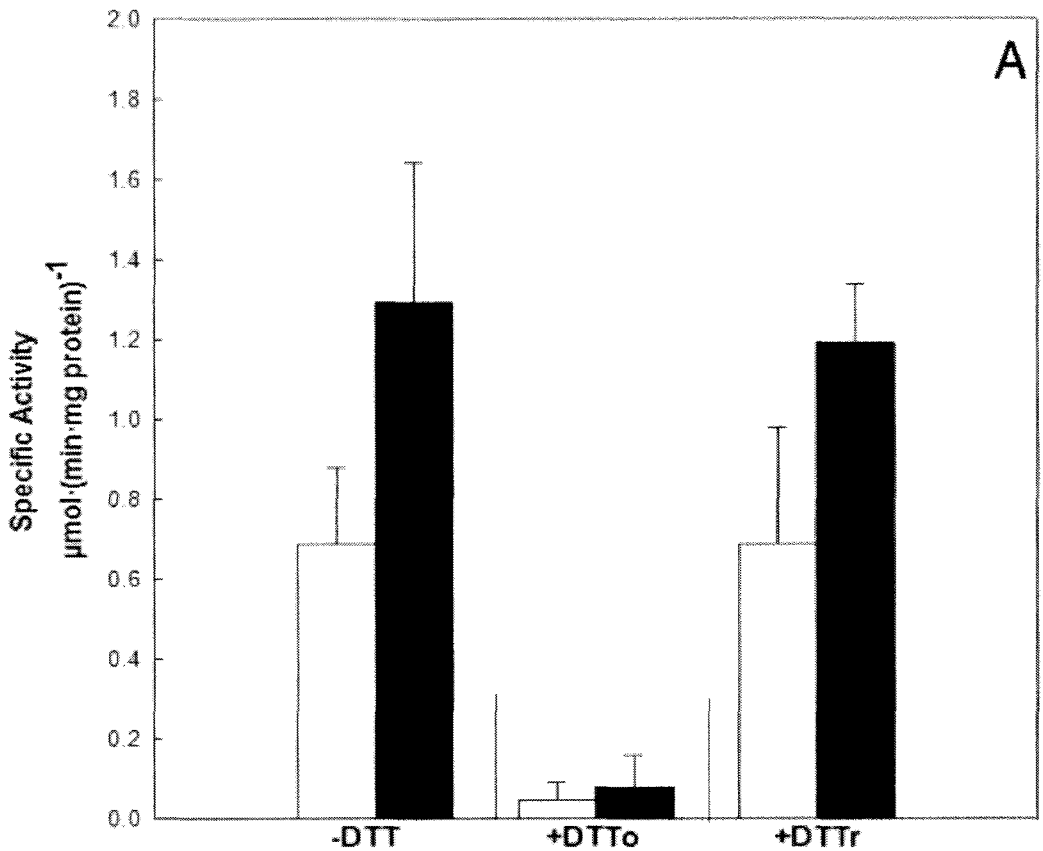

Figure 3.5. Effect of Oxidizing and Subsequent Reducing Conditions. (A) PRK Activity in *V.* litorea. Samples were collected after 6 hr in the light □ or dark ■, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with oxidizing (+DTTo) and subsequent reducing agents (+DTTr), and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 2.

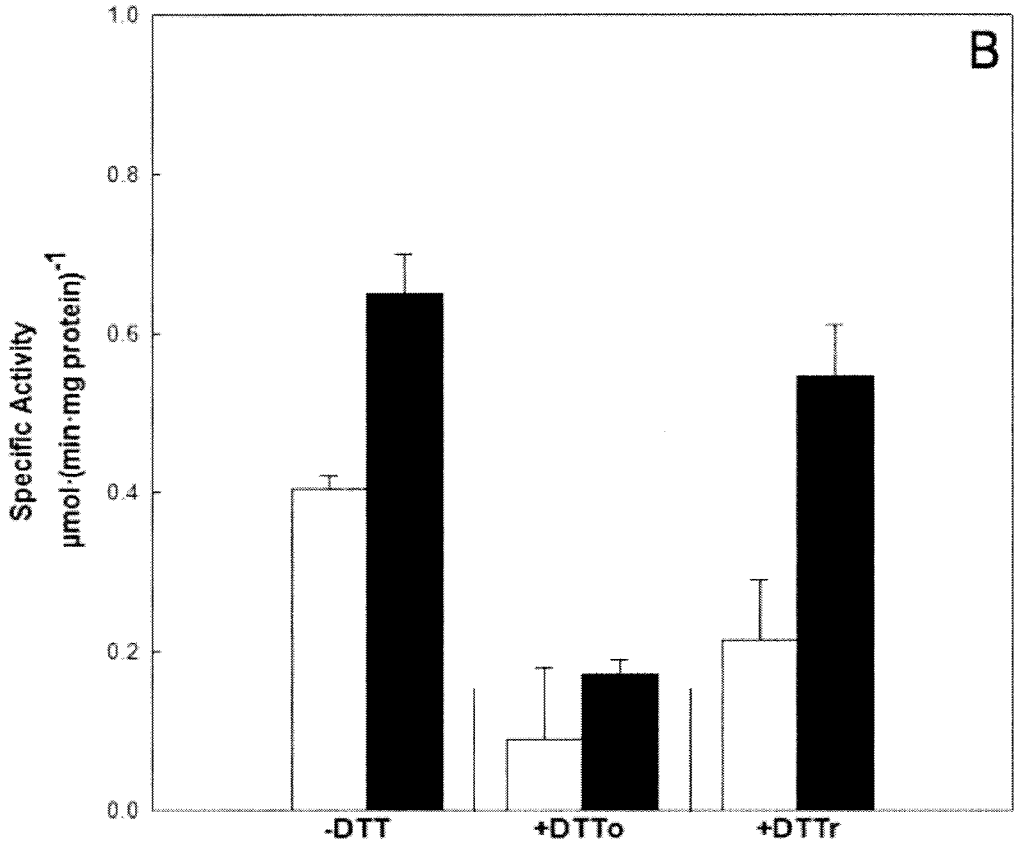

Figure 3.5. Continued. (B) PRK Activity in *E. chlorotica* (starved 4 months). Samples were collected after 6 hr in the light □ or dark ■, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with oxidizing (+DTTo) and subsequent reducing agents (+DTTr), and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 3.

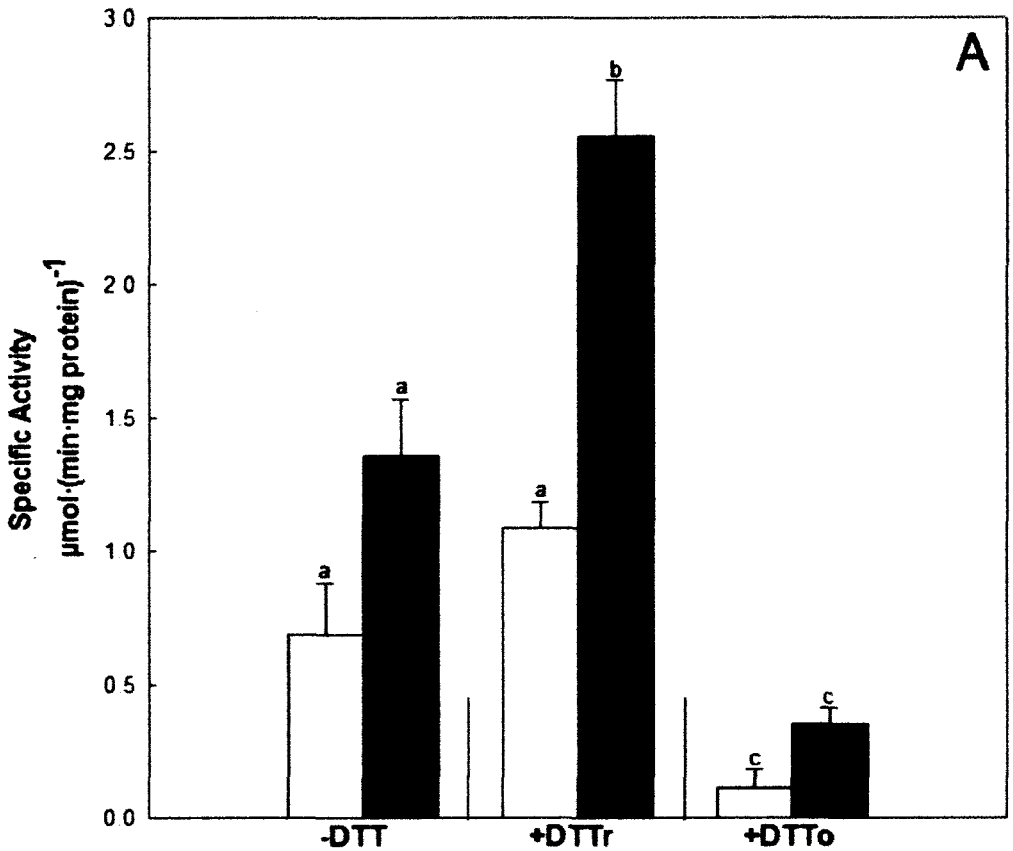

Figure 3.6. Effect of Reducing and Subsequent Oxidizing Conditions. (A) PRK Activity in *V. litorea*. Samples were collected after 6 hr in the light □ or dark ■ and extracted in the absence of redox reagents and assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with DTTr and subsequent oxidizing (+DTTo) reagents before assaying without DTTr in the assay media. Values represent the mean ± SEM. Letters above the bars in Fig. 3A reflect significant differences at the $p \leq 0.05$ level; sample sizes and statistics are summarized in Appendix A, Table 2.

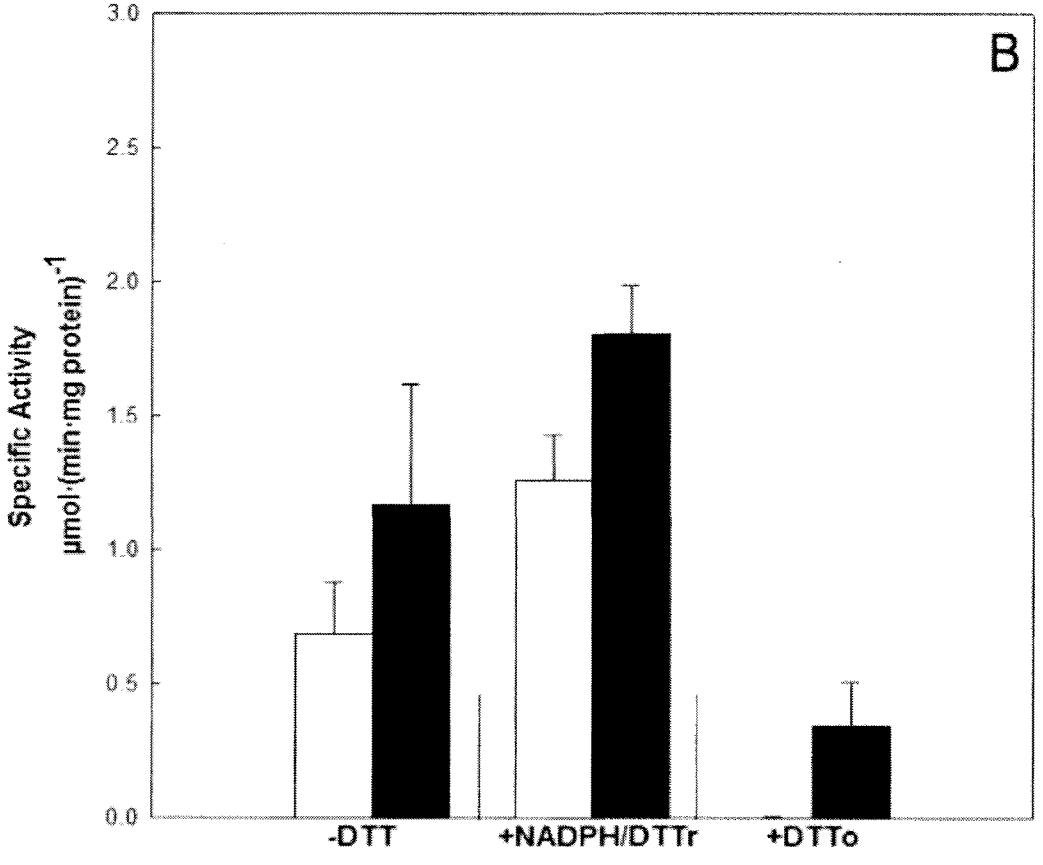

Figure 3.6. Continued. (B) PRK Activity in *V. litorea*. Samples were collected after 6 hr in the light □ or dark ■ and extracted in the absence of redox reagents and assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with NADPH/DTTr and subsequent oxidizing (+DTTo) reagents before assaying without DTTr in the assay media. Values represent the mean ± SEM. Letters above the bars in Fig. 3A reflect significant differences at the $p \leq 0.05$ level; sample sizes and statistics are summarized in Appendix A, Table 2.

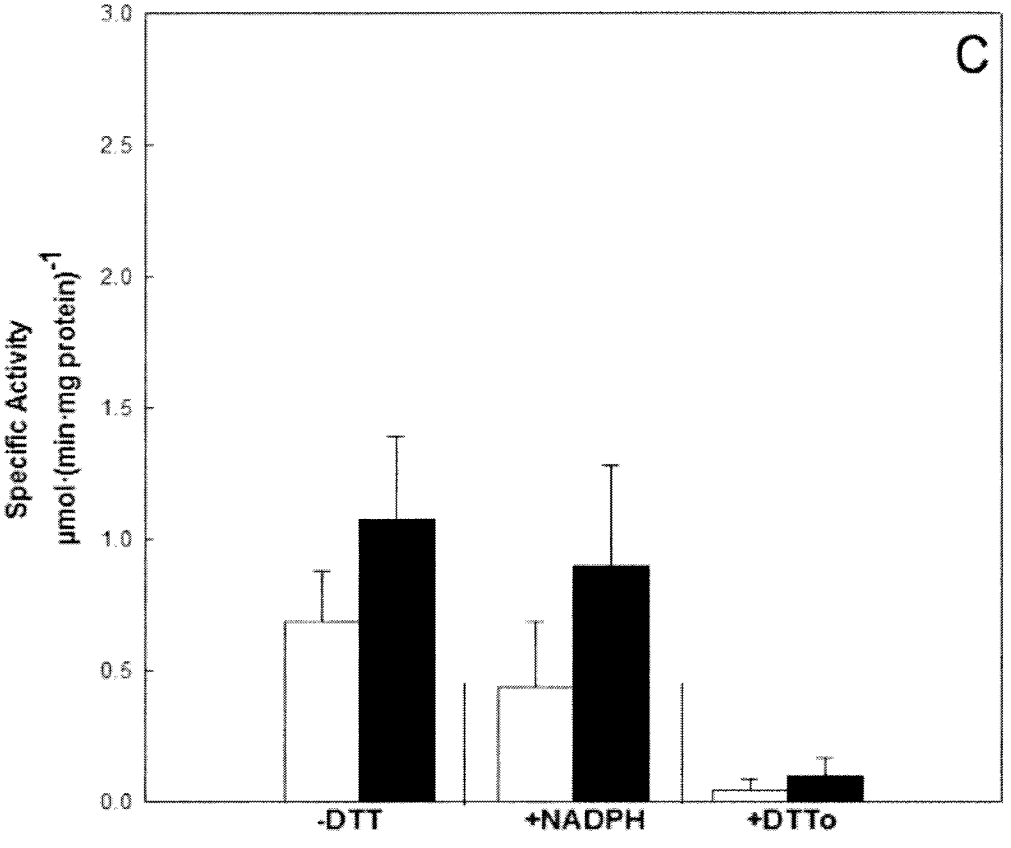

Figure 3.6. Continued. (C) PRK Activity in *V. litorea*. Samples were collected after 6 hr in the light □ or dark ■ and extracted in the absence of redox reagents and assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with NADPH reducing agents and subsequent oxidizing (+DTTo) reagents before assaying without DTTr in the assay media. Values represent the mean ± SEM. Letters above the bars in Fig. 3A reflect significant differences at the $p \leq 0.05$ level; sample sizes and statistics are summarized in Appendix A, Table 2.

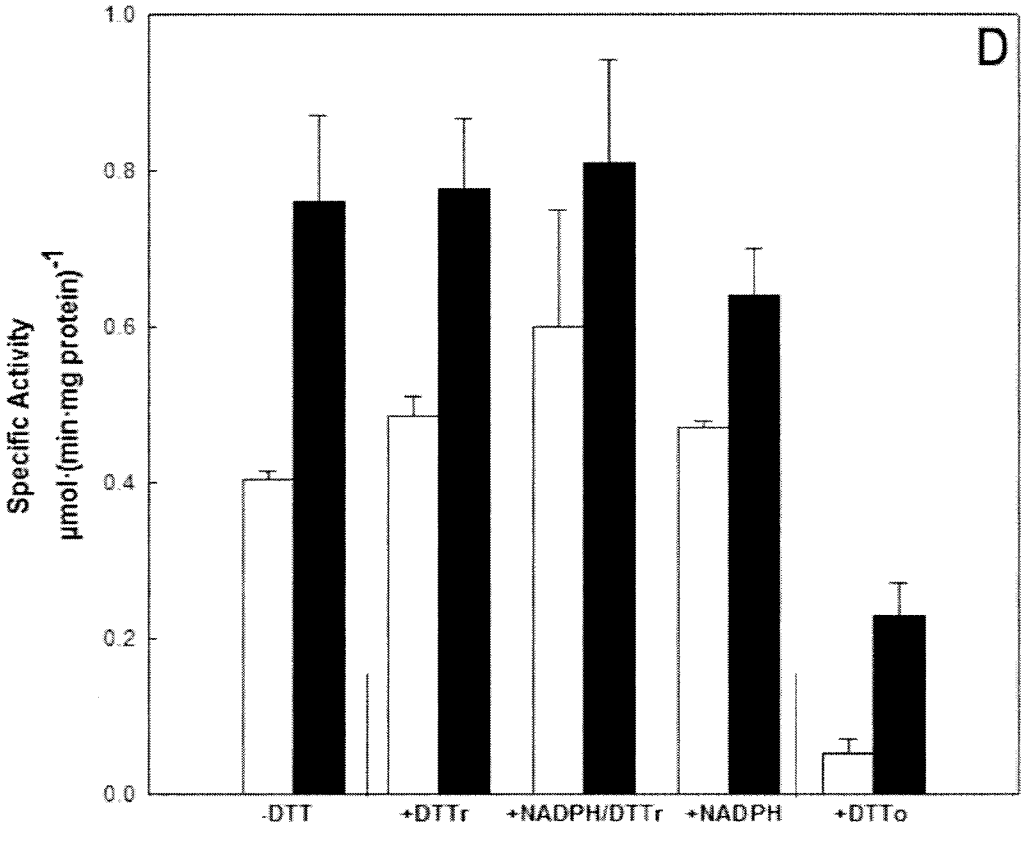

Figure 3.6. Continued. (D) PRK Activity in *E. chlorotica* (starved 4 months). Samples were collected after 6 hr in the light □ or dark ■ and extracted in the absence of redox reagents and assayed without DTTr in the assay media (-DTT). Extracts were then consecutively incubated with reducing (+DTTr, +NADPH/DTTr, +NADPH) agents and assayed again for activity without DTTr in the assay. Reduced samples were all subsequently oxidized (+DTTo) and assayed. Activity values from these subsequent oxidations were averaged and plotted here. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 3.

3.3.3. Effect of Desalting on Redox Regulation of PRK Activity.

Crude extracts from light-adapted *V. litorea* and *E. chlorotica* were desalted as indicated in the 'Materials and Methods,' to determine if the crude extracts contained factors that may affect PRK activity under varying redox conditions. The crude extracts were prepared without any reducing or oxidizing agents, desalted, and then assayed for PRK activity in the absence of any reducing or oxidizing agents. Extracts were subsequently exposed to oxidizing (DTTo) or reducing (DTTr, NADPH/DTTr) conditions, assayed for PRK activity, and then incubated in the opposing redox conditions for further activity measurements.

The effect of desalting on PRK activity is shown for *V. litorea* in Figs. 3.7A and B, and for *E. chlorotica* in Figs. 3.7C and D. The non-desalted control values in Figs. 3.7A-D originate from the data previously shown in Figs. 3.5A and 3.6A-C (*V. litorea*) and Figs. 3.5B and 3.6D (*E. chlorotica*). The results reported for both *V. litorea* (Figs. 3.7A and B) and *E. chlorotica* (Figs. 3.7C and D) depict that the PRK activity in desalted extracts were affected similarly to that of the non-desalted extracts when exposed to the various redox conditions. Though the apparent decreases in PRK activity in the desalted *vs.* non-desalted extracts were not significantly different at the $p \leq 0.05$ level under any of the redox conditions, possibly due to the longer processing times of the desalted extracts then the non-desalted extracts.

This suggests that the desalting process does not change the affects seen by the redox conditions on PRK activity within the alga or the sea slug and therefore will not be discussed further.

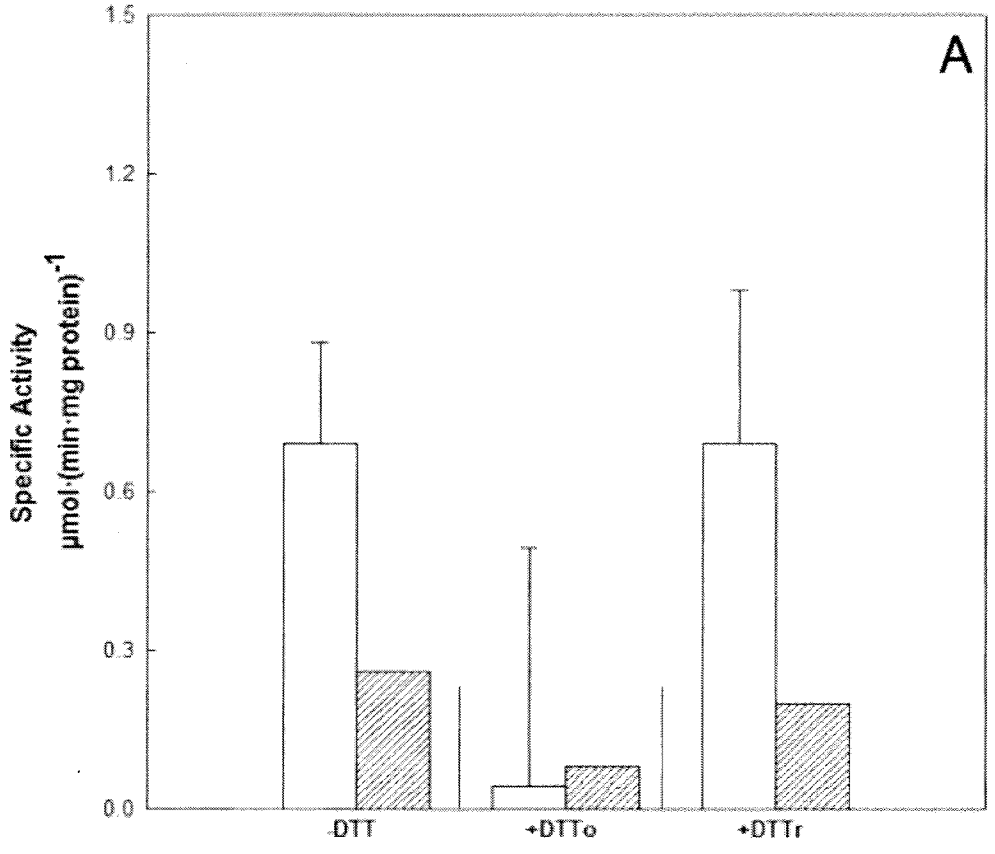

Figure 3.7. Comparison of the Oxidation/Reduction Effect on PRK Activity in Desalted and Non-Desalted Extracts. (A) PRK Activity in *V. litorea*. Samples were collected after 6 hr in the light, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT) either before (open bars) or after (hatched bars) desalting (see methods). Extracts were then consecutively incubated with oxidizing (+DTTo) and reducing (+DTTr) agents, and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 4.

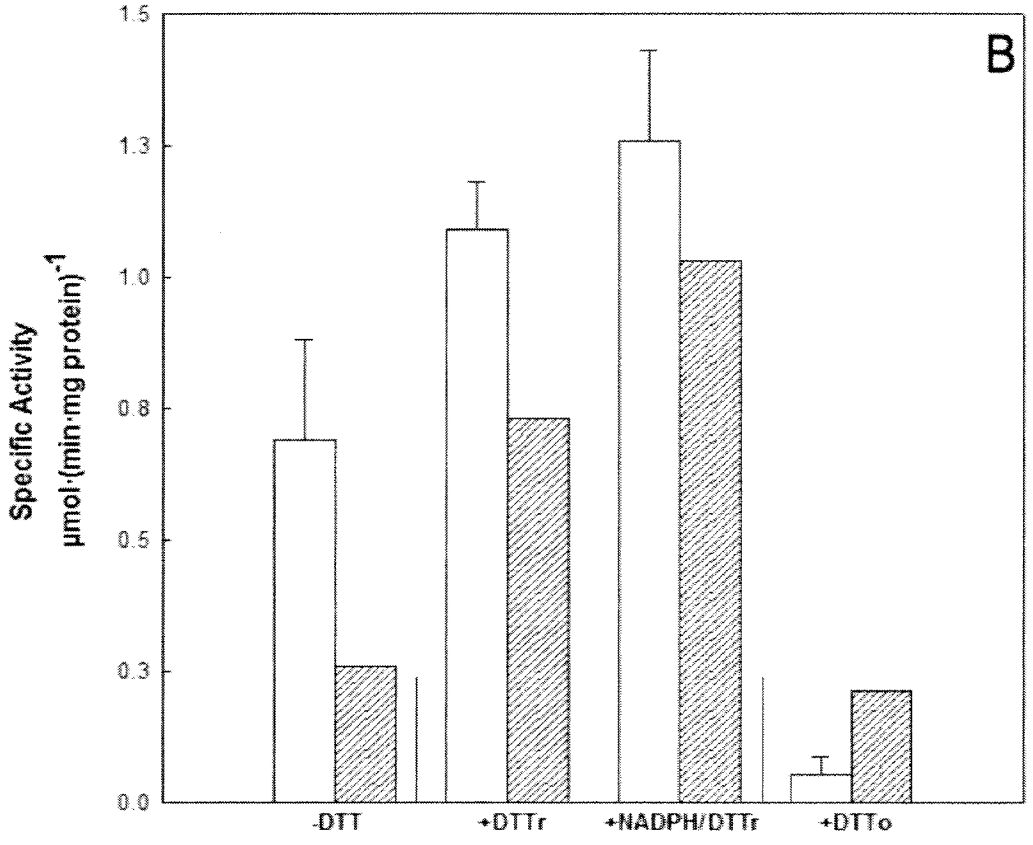

Figure 3.7. Continued. (B) PRK Activity in *V. litorea*. Samples were collected after 6 hr in the light, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT) either before (open bars) or after (hatched bars) desalting (see methods). Extracts were then consecutively incubated with reducing (+DTTr, +NADPH/DTTr, +NADPH) and oxidizing (+DTTo) agents, and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 4.

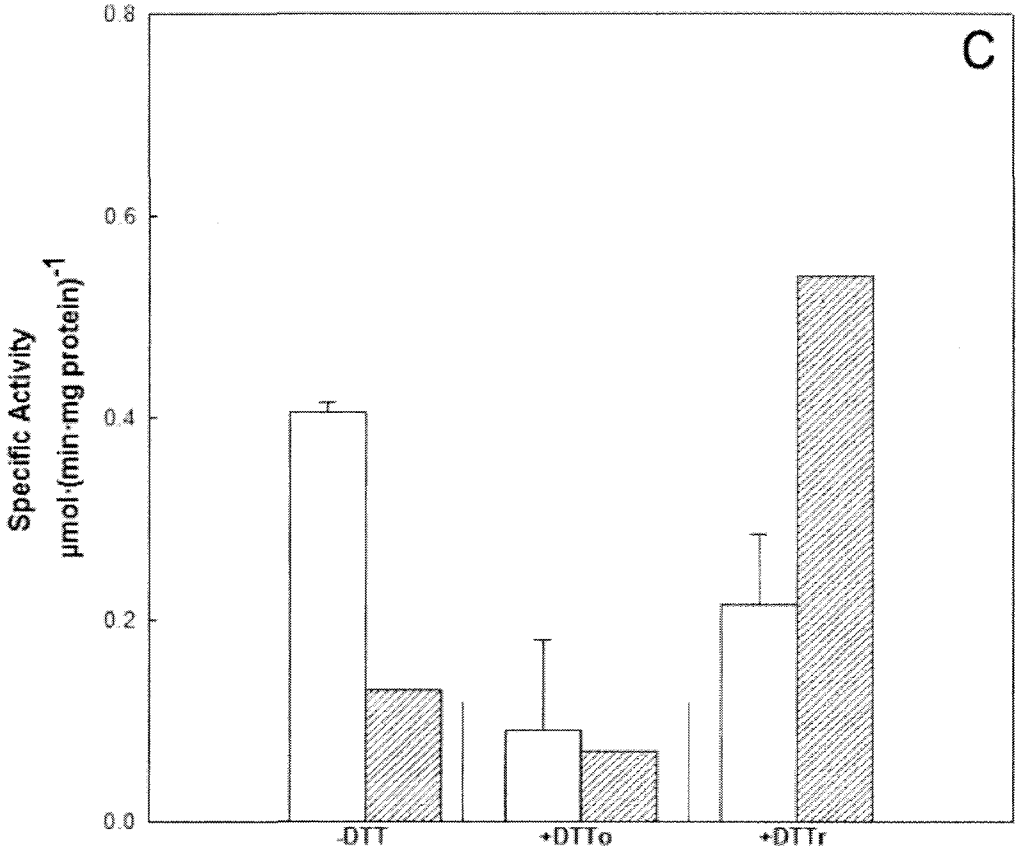

Figure 3.7. Continued. (C) PRK Activity in *E. chlorotica*. Samples were collected after 6 hr in the light, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT) either before (open bars) or after (hatched bars) desalting (see methods). Extracts were then consecutively incubated with oxidizing (+DTTo) and reducing (+DTTr) agents, and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 5.

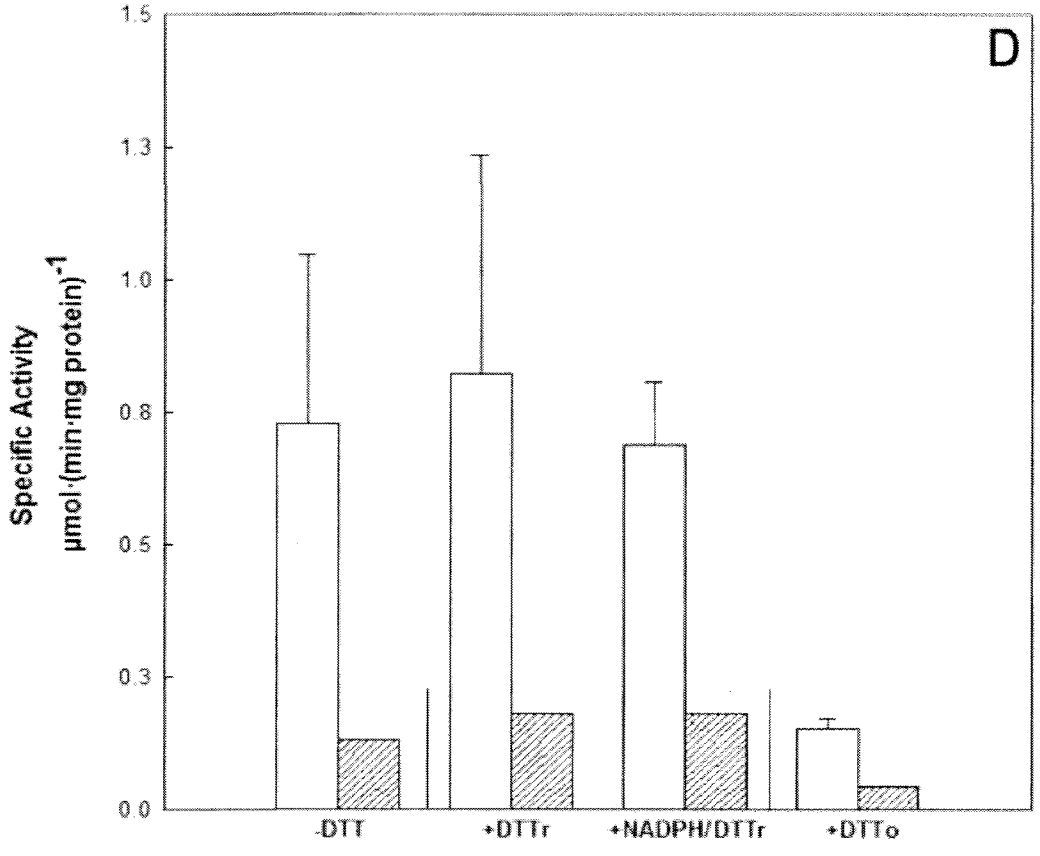

Figure 3.7. Continued. (D) PRK Activity in *E. chlorotica* Extracts. Samples were collected after 6 hr in the light, extracted in the absence of any redox agents, and then assayed without DTTr in the assay media (-DTT) either before (open bars) or after (hatched bars) desalting (see methods). Extracts were then consecutively incubated with reducing (+DTTr, +NADPH/DTTr, +NADPH) and oxidizing (+DTTo) agents, and assayed without DTTr in the assay media. Values represent the mean ± SEM; sample sizes and statistics are summarized in Appendix A, Table 5.

3.4. Discussion

One of the objectives of this study was to determine if light/dark, and indirectly redox, conditions differentially affected PRK activity in *Vaucheria litorea* compared to *Elysia chlorotica.* The main difference noted here was that PRK activity was found to be higher in extracts of dark-adapted *V. litorea* prepared in the presence of reducing agent, but the presence of reducing agent had no effect on PRK activity from dark-adapted *E. chlorotica.* This implies that the enzyme appears to be redox regulated *in vivo* only in *V. litorea.* Possibly suggesting that PRK is inactivated under dark/oxidizing conditions in the alga, but not in the sea slug. In contrast, no significant changes in PRK activity were observed between *V. litorea* and *E. chlorotica* when extracts were treated *in vitro* first to oxidizing conditions and then subsequent reducing conditions. These results suggest that, *in vitro,* PRK is similarly redox modulated in both *V. litorea* and *E. chlorotica* extracts.

The redox regulation of PRK observed for *V. litorea* is similar to that reported for the related heterokont *Heterosigma carterae* (Cattolico et al., 1998), as well as for land plants (Gontero et al., 1993; Marri et al., 2005b) and green algae (Lebreton et al., 1997; Lebreton and Gontero, 2000). Yet similar redox regulation is not seen in an ancestral red alga *Galdieria sulphuraria* (Oesterhelt et al., 2007) or the cyanobacterium *Synechococcus sp. PCC 7942* (Kobayashi et al., 2003), and possibly not in other heterokonts, i.e. the diatoms *Odontella sinensis*

(Michels et al., 2005) and *Asterionella formosa* (Boggetto et al., 2007). These findings are not surprising as it was suggested by Petersen et al. (2006) that the PRK found in chromalveolates (i.e. heterokonts) originated via horizontal transfer from a green alga, which was also confirmed by phylogenetic analysis for *V. litorea* PRK by Rumpho et al. (2009). This however does not explain the difference between PRK from *V. litorea* and the closely related diatoms, which may be explained by the loss of the oxidative pentose phosphate pathway within these diatoms as suggested by the authors (Michels et al., 2005; Boggetto et al., 2007).

Redox regulation of PRK has been shown to be mediated by thioredoxin (Trx_{red}) under light conditions. The regulation more explicitly occurs through the reduction of a disulfide bond between the Cys residues (i.e. Cys 71 and 115 in *V. litorea*) and through the formation of a supermolecular complex. This supermolecular complex forms between PRK, GAPDH and the small peptide CP12 linker under oxidizing (i.e. limited light, dark or stress) conditions in cyanobacteria (Wedel et al., 1998; Tamoi et al., 2005), green algae (Avilan et al., 1997a; Wedel et al., 1998), streptophytes (Pohlmeyer et al., 1996; Wedel et al., 1997; Marri et al., 2005b; Howard et al., 2008), red algae (Oesterhelt et al., 2007), and possibly in heterokonts (Boggetto et al., 2007). Although the presence of this complex in *V. litorea* has not been molecularly confirmed the biochemical data presented here (Figs. 3.4A and 3.6A) shows a difference between PRK from light-

and dark-adapted extracts under DTTr conditions, which is consistent with regulation by protein-protein interactions as described for this complex.

Such widespread findings suggest that this supermolecular complex may be evolutionarily conserved among photosynthetic organisms (Wedel and Soll, 1998); however, light regulation of PRK is not universal. In streptophytes and green algae, PRK activity is significantly inhibited by the formation of the complex in dark, oxidizing conditions. Yet, the results reported for the untreated crude extracts (-DTTr) from *V. litorea* (Figs. 3.4A, 3.5A and 3.6A-C) suggest that PRK activity is not fully inhibited in the dark. This is similar to reports for other heterokonts (Michels et al., 2005; Boggetto et al., 2007), red algae (Oesterhelt et al., 2007) and cyanobacteria (Tamoi et al., 1998), in which light/dark regulation is less efficient. This less efficient light/dark regulation of PRK is suggestively due to the variation among the number of amino acids separating the Cys residues within the N-terminal conserved redox region as compared to land plants and green algae (as depicted in Fig. 1.4). More specifically, the literature suggests that the insertions and deletions seen among these regions in PRK from heterokonts, red algae and cyanobacteria inhibit the full deactivation of PRK due to the inability of the disulfide bond to form properly between these Cys residues.

This non-universal light/dark complex control of PRK formation suggests that this complex *in vivo* is more than a light/dark on/off switch (Howard et al., 2008). This is in agreement with the view that supermolecular complexes in

plastids may offer a functional biological advantage and/or a regulatory role; the channeling of intermediates between the complexed proteins (Gontero et al., 1988; Sainis et al., 1989; Suss et al., 1993; Dani and Sainis, 2005; Anderson et al., 2006 and 2009) and the conformational changes within individual proteins, have both been proposed (Lebreton et al., 1997; Lebreton and Gontero, 1999; Marri et al., 2005b; Erales et al., 2008).

In addition, previous studies have shown that NADPH also mediates complex dissociation in streptophytes (Wedel et al., 1997; Scheibe et al., 2002; Marri et al., 2005b) and cyanobacteria (Tamoi et al., 2005). Conversely, the studies by Howard et al. (2008) on *Pisum sativum*, and the results reported here for *V. litorea* (Figs. 3.6B-C), suggest that NADPH has no direct effect on PRK activity in light- or dark-adapted extracts. This supports the conclusion that NADPH may only enhance dissociation once the complex has started to dissociate.

In contrast to *V. litorea* PRK, the enzyme in light- and dark-adapted *E. chlorotica* was found to act similarly to reducing conditions (Figs. 3.4B and 3.6D). As discussed above, differential light/dark regulation of PRK presumably involves protein-protein interactions that require algal nuclear-encoded proteins (i.e. GAPDH and CP12) and factors (i.e. Trx) and therefore, the results obtained are not surprising given that no algal nuclei have been found in the sea slug to date. Although data on the conserved Cys residues and the presence of Trx_{red} is

lacking for *E. chlorotica*, I would expect the Cys residues and region between them to be conserved between the alga and sea slug due to the high sequence similarity between the PRK gene in *V. litorea* and the partial sequence for *prk* detected in *E. chlorotica* (Rumpho et al., 2009), along with the similar *in vitro* responses of PRK to oxidation and subsequent reduction in both organisms.

In summary, this study presents the first comprehensive biochemical analysis of redox and light/dark regulation of PRK in the heterokont *V. litorea* and its symbiotic partner, the sacoglossan mollusc *E. chlorotica.* The results provide evidence supporting redox regulation of *V. litorea* PRK *in vivo* and *in vitro*, but only *in vitro* for *E. chlorotica.* These results may be explained by the conserved Cys residues in the algal and sea slug PRK protein and the formation of a PRK/GAPDH/CP12 complex in the alga, but not in the sea slug due to the absence of essential nuclear-encoded algal proteins in the mollusc.

Chapter 4

CONCLUSIONS, LIMITATIONS AND FUTURE SUGGESTIONS

4.1. Summary of Conclusions and Significance.

The aim of this study was to identify and compare possible redox regulated transcriptional and post-translational processes in two unrelated organisms connected through symbiosis. These partners, the heterokont alga *V. litorea* and the sacoglossan sea slug *E. chlorotica* are connected through the presence of *V. litorea* plastids in the digestive epithelia of the sea slug. The plastids remain functional and sustain the sea slug photoautotrophically even though there is no evidence for the presence of the algal nuclear genome in the sea slug (Mujer et al., 1996; Green et al., 2000; Rumpho et al., 2001), or an obvious source for the 1000 to 5000 nuclear-encoded proteins presumed necessary for efficient plastid functioning and regulation (Martin et al., 1998 and 2002).

Evidence supporting horizontal gene transfer (HGT) from the algal nuclear genome to the sea slug, and possibly supporting long-term plastid functioning in the sea slug, has been recently published (Pierce et al., 2007; Rumpho et al., 2008 and 2009). The putative HGT of the algal nuclear-encoded gene *prk* is particularly interesting as this gene encodes the Calvin cycle enzyme phosphoribulokinase; an enzyme essential for regenerating the starting product for

CO_2 fixation in photosynthetic organisms. PRK has been highly documented in land plants and green algae to be a target of redox regulation at the transcriptional and post-translational levels. Fewer studies have been carried out on red algae and secondarily-derived chromalveolates, including *V. litorea.* The results presented here support light and redox regulation of *prk* at the transcriptional and post-translational levels in *V. litorea*, possibly involving nuclear-encoded factors. Redox regulation of *E. chlorotica prk in vivo* was not observed, i.e. *prk* transcript levels did not increase in the animal in response to light as observed for *V. litorea;* rather, they decreased. Furthermore, extractable activity of PRK from dark-treated sea slugs showed no difference when extracted in the presence or absence of reducing agent. However, redox regulation of PRK activity was observed *in vitro* in the sea slugs when crude extracts were treated with various oxidizing and subsequent reducing agents. This implies that the nuclear-encoded factors responsible for redox regulation of PRK in *V. litorea* may not be present or regulated in the sea slug.

Conversely, redox regulation of plastid transcript expression was found to act similarly whether the plastids were housed in *V. litorea* or *E. chlorotica.* The similar up-regulation of *psa*A and *rbc*L plastid transcript levels at the onset of illumination suggests that plastid transcription is under the control of nuclear-encoded factors that have been transferred to the sea slug or have been rerouted from other biochemical processes within the sea slug. Quantitatively, the redox

response in *E. chlorotica* was much lower than in *V. litorea*. This most likely reflects the lower photosynthetic activity in the sea slug due to the absence of plastid division and renewal, and other essential nuclear-encoded factors for complete pathways in the animal.

Finally, the lack of the continued pattern of *prk*, *psa*A and *psb*A transcript accumulation from LD to DD conditions in *V. litorea* further supports the hypothesis that up-regulation of nuclear- and plastid-encoded transcripts is due to light regulatory mechanisms involving the redox state of plastoquinone and thioredoxin.

4.2. Limitations and Future Recommendations.

In this study, nuclear- and plastid-encoded transcripts were quantified using quantitative real time-PCR (qRT-PCR), a molecular tool that employs the use of a DNA binding fluorophore (i.e. SYBR Green). The fluorophore progressively emits more and more energy as it binds to the primer specific gene targets that are amplified by the Taq polymerase during the PCR thermo-cycling (Wittwer et al., 1997; Valsek and Repa, 2005). From these qRT-PCR experiments a role for light in regulating nuclear and plastid transcript expression was suggested; however, the involvement of PQ and Trx_{red} in this regulation, and the potential for circadian regulation, are still unknown. Further analysis to determine the involvement of PQ and Trx_{red} will require the use of DCMU and

genetic mutants as described by Lemaire et al. (1999) and Verdoucq et al. (1999), respectively. To determine the role of circadian regulation, transcripts will need to be measured over a 48 hr period of continuous light and dark conditions subsequent to LD treatment in the alga and the sea slug, as described by Lemaire et al. (1999).

Using qRT-PCR to quantify transcripts is a reliable and relatively rapid process that allows for the accurate profiling of the expression of specific genes, especially when biological materials are limiting (Fink et al., 1998; Siaut et al., 2007). Preliminary investigations prior to the use of qRT-PCR are required to ensure: i) the quality of RNA, ii) the efficiency of cDNA generation from RNA, iii) the lack of contaminating genomic DNA and iv) that the product being quantified is the specific gene of interest. These preliminary concerns were addressed here by: i) checking the quality of RNA through EtBr visualization in an agarose gel and a contamination check for proteins and organic compounds using 260/280 and 260/230 spectrophotometer ratios, respectively, ii) normalizing transcript readings to a constitutively-expressed housekeeping gene, iii) using a primer set that spans an intron-exon boundary of the gene of interest and iv) running a dissociation curve analysis post qRT-PCR for 40 cycles to determine the generation of a single product. All of these preliminary investigations were carried out for the studies reported here; however, attempts to identify an appropriate normalizer were unsuccessful.

Normalization is a well documented issue with qRT-PCR experiments because it requires that a gene be constitutively expressed at the same rate throughout the experimental treatments. 'Housekeeping' genes are commonly used as normalizers, but several researchers have pointed out that the use of these genes can be unreliable under various experimental conditions (Thellin et al., 1999; Suzuki et al., 2000; Lee et al., 2002; Dheda et al., 2005). Considering this unreliable nature of housekeeping genes, and the use of a wide array of regulatory mechanisms that act in response to changes in light and photosynthetic activity, it is not surprising that an appropriate normalizing gene was not found in our preliminary investigations.

The specific investigations employed in the search of an appropriate normalizing gene followed the same protocol as noted in '2.2. Materials and Methods' using the primers for *rrs* (the 16S plastid ribosomal subunit), *act* (a cytoskeletal protein) and *18S* (a nuclear ribosomal subunit) (Appendix B, Table 1). The findings from the preliminary investigations showed that *rrs* (Fig. 4.1.), *act* (Fig. 4.2.) and *18S* (Fig. 4.3.) were not constitutively expressed over the LD photoperiod and were inappropriate normalizing genes. Attempts were then made to PCR amplify elongation factor 1 alpha (EF1α) and an actin-related protein (ARP2.2) using degenerate versions of the primers listed in Le Bail et al. (2008) since they were found to be the best normalizing genes for diurnal treatment in *Ectocarpus siliculosus*. Using these degenerate primers and varying PCR

conditions, no successful amplification products were obtained for *V. litorea.*

An alternative to using a normalizing gene was proposed by Libus and Storchova (2006) involving quantifying and then adding "known" equal quantities of cDNA to the qRT-PCR reaction. This approach was tried using equalized *V. litorea* cDNA (based on NanoDrop spectrophotometer readings) as the template for *rrs* amplification by qRT-PCR. Similar results were obtained for the equalized and non-equalized data (Fig. 4.4). Thus, it was concluded that using equal RNA concentrations to prepare the final cDNA was acceptable. Following these less than successful attempts to identify a suitable normalizing gene or standardization approach, it was decided that the results of the studies would be presented as a relative change compared to a specific pre-illumination time-point. However, future experiments on other potential normalizing genes, or further analysis of the EF1α and ARP2.2 genes, could potentially assist in a more reliable means of normalization.

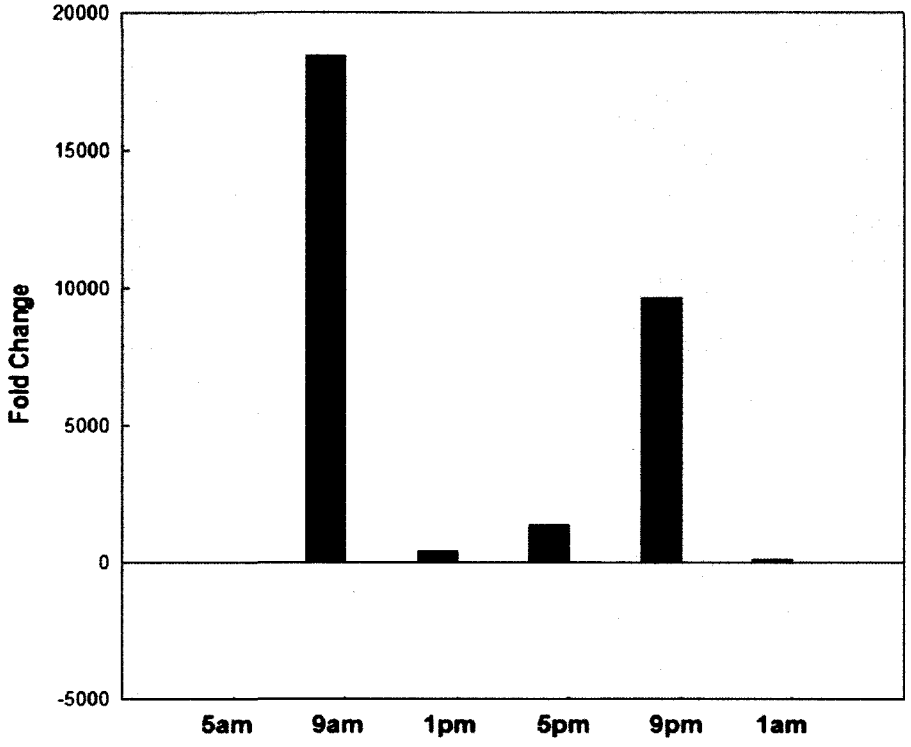

Figure 4.1. Variable Expression of Plastid-Encoded *rrs* (16S) Transcript Levels over a 12L:12D Photoperiod in *V. litorea.* The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent an n = 1.

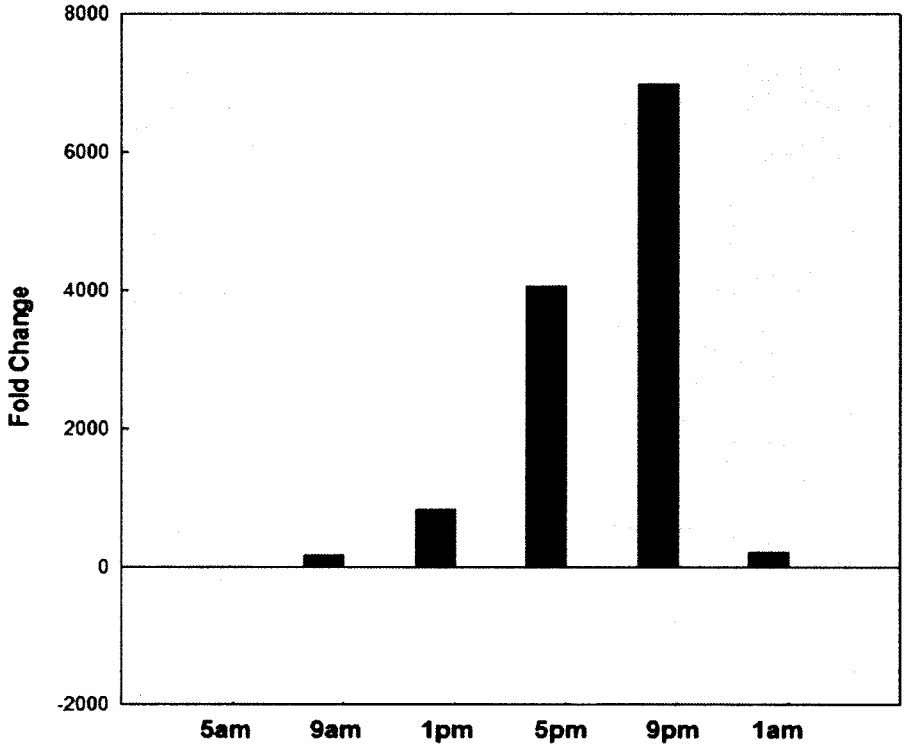

Figure 4.2. Variable Expression of Plastid-Encoded *act* Transcript Levels over a 12L:12D Photoperiod in *V. litorea.* The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent an n = 1.

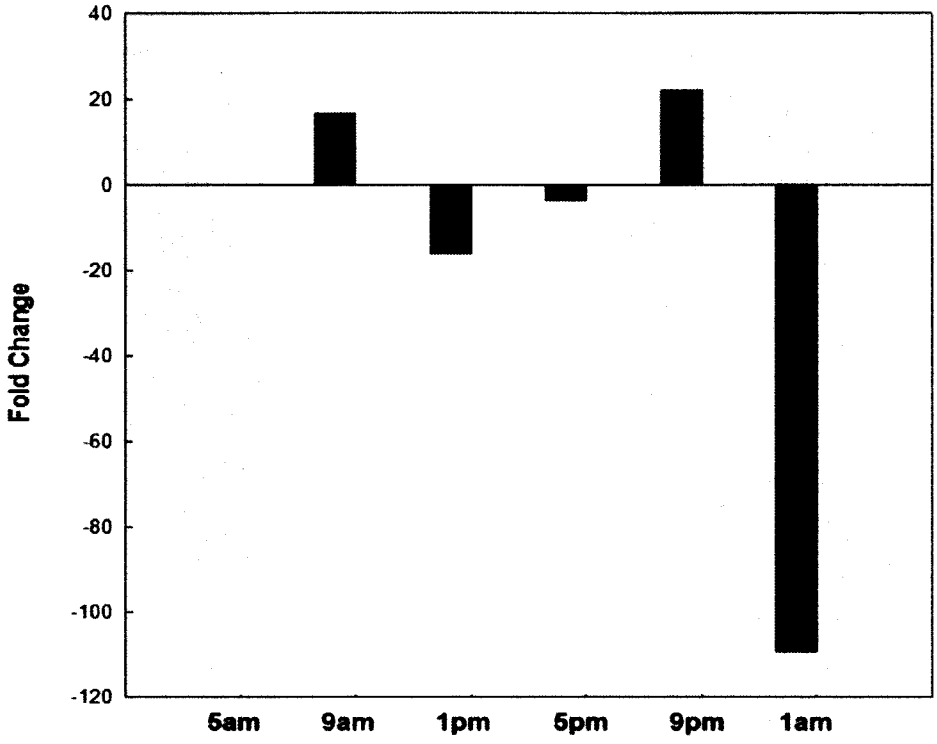

Figure 4.3. Variable Expression of Plastid-Encoded *18S* Transcript Levels over a 12L:12D Photoperiod in *V. litorea.* The 5 am time point (2 hr pre-illumination) was used as the baseline in calculating fold-change. The gray shading indicates the 12 hr dark period. Values represent an n = 1.

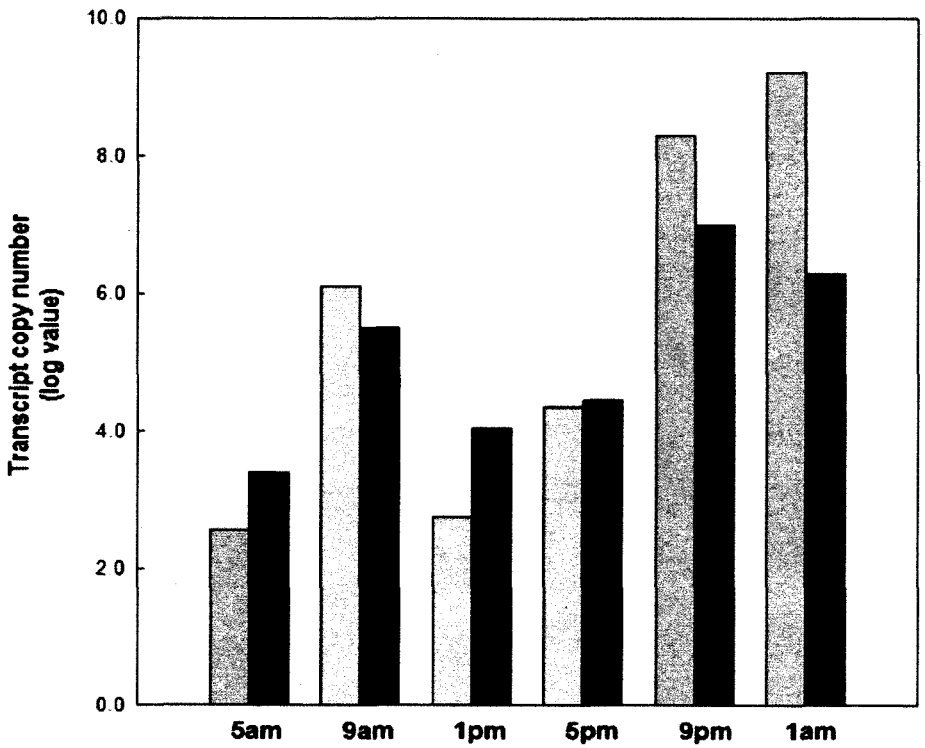

Figure 4.4. Similar Absolute *rrs* Transcript Levels in *V. litorea* Equalized (gray bars) and Non-Equalized (red bars) cDNA Samples over a 12L:12D Photoperiod. Transcript levels are represented as the log of the absolute copy number. The gray background shading indicates the 12 hr dark period. Values represent an n = 1.

The qRT-PCR experiments also presented some statistical limitations. Peccoud and Jacob (1996) suggested that a large number of replicates are required for the statistical analysis of qRT-PCR measurements. This was not possible in this study due to the expensive nature of qRT-PCR experiments and the limited supply of algal and sea slug samples. Based on the small sample sizes it was concluded that the ANOVA analysis is not necessarily the best way to evaluate the results. A more appropriate way would be to determine if each biological replicate experiment gave the same general pattern. Future experiments in which a large number of biological replicates could be obtained would aid in the statistical analysis of experiments following the same protocol as described in Chapter 2. Following this same approach, analyses could additionally be carried out on slugs starved for longer than two weeks to ensure that regulatory factors were not maintained within the plastids from the last feeding.

To analyze the effects of light on post-translational processes a coupled enzyme assay (as described in Chapter 3 and depicted in Fig. 3.3) was used to measure PRK activity under varying redox conditions in extracts of light- and dark-treated algal and sea slug specimens. These measurements allowed for the determination of the effects of light on PRK in the alga and sea slug; however, the involvement of the PRK/GAPDH/CP12 complex could only be alluded to. Therefore, further molecular analyses employing western blotting or gel filtration

techniques will be necessary to determine whether this complex is involved in the light regulation of PRK activity.

In spite of these limitations, light regulation was characterized at the transcriptional and post-translational levels within this unique symbiotic association to begin to better understand the unprecedented plastid photosynthetic activity within the foreign host.

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APPENDIX A

SUPPLEMENTARY INFORMATION RELATED TO CHAPTER 3

Tables A.1-A.5. Analysis of Variance (ANOVA) was carried out using SYSTAT© Software to determine the multi-variable p value followed by a Tukey test to determine the significant variables.

Table A.1. Statistical Summary for Figures 3.4A-B.

Sample	Sample size[1]	Statistical analysis	Two or three-way ANOVA	Significant variables $p \leq 0.05$
V. litorea	L: 5 D: 6	Two-way ANOVA	0.016	a < b
E. chlorotica	L: 3 D: 3	Three-way ANOVA	0.923	N/A

Table A.2. Statistical Summary for *V. litorea* Figures 3.5A and 3.6A-C.

Figure	Sample size[1]	Statistical analysis	Two or three-way ANOVA	Significant variables $p \leq 0.05$
Fig. 3.5A	L: 2 D: 4	Two-way ANOVA	0.832	N/A
Fig. 3.6A	L: 2 D: 3	Two-way ANOVA	0.018	c < a < b
Fig. 3.6B	L: 2 D: 3	Two-way ANOVA	0.692	N/A
Fig. 3.6C	L: 2 D: 4	Two-way ANOVA	0.800	N/A

[1] Refers to biological replicates in each experiment and figure.

Table A.3. Statistical Summary for *E. chlorotica* Figures 3.5B and 3.6D.

Figure	Sample size[1]	Statistical analysis	Two or three-way ANOVA	Significant variables $p \leq 0.05$
Fig. 3.5B	L: 2 D: 2	Two-way ANOVA	0.181	N/A
Fig. 3.6D	L: 2 D: 3	Two-way ANOVA	0.950	N/A

Table A.4. Statistical Summary for *V. litorea* Figures 3.7A and B.

Figure	Sample size[2]	Statistical analysis	Two or three-way ANOVA	Significant variables $p \leq 0.05$
Fig. 3.7A	Des: 1 nonDes: 2	Two-way ANOVA	0.417	N/A
Fig. 3.7B	Des: 1 nonDes: 3	Two-way ANOVA	0.527	N/A

Table A.5. Statistical Summary for *E. chlorotica* Figures 3.7C and D.

Figure	Sample size[1]	Statistical analysis	Two or three-way ANOVA	Significant variables $p \leq 0.05$
Fig. 3.7C	Des: 1 nonDes: 3	Two-way ANOVA	0.440	N/A
Fig. 3.7D	Des: 1 nonDes: 3	Two-way ANOVA	0.181	N/A

[2] Refers to biological replicates in each experiment and figure.

APPENDIX B

SUPPLEMENTARY INFORMATION RELATED TO CHAPTER 4

Table B.1. Primers used in the preliminary qRT-PCR analysis of potential normalizing genes in *V. litorea*.

Gene	Primer	Sequences 5′ to 3′	Amplicon size (bp)
rrs	rrs2.for rrs2.rev	AGGCGGGGAATAACTATTGG TACTGATCGTCGCCTTGG	139
act	VLactin.S2 for and rev	CCATCATCGGTAAGGAAGCC GTCATTCTGTCACCAATTCCAGG	148
18S	VL18S.s for and rev	GCAATGTACCCGTCGAGA CTGTCAATCCTCGCCATGT	169

BIOGRAPHY OF THE AUTHOR

Kara Marie Soule was born and raised in Waterville, Maine on December 30, 1985. She later moved to Belfast, Maine where she graduated from Belfast Area High School in June 2004. She attended the University of Maine and graduated in August 2007 with a Bachelor's degree in Biochemistry. She then entered the Biochemistry, Microbiology and Molecular Biology graduate program at the University of Maine in the fall of 2007. Kara is a candidate for the Master of Science degree in Biochemistry from the University of Maine in December, 2009.